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Christian X. Figueroa

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Influence of mycorrhizal fungi on soil geochemistry and plant uptake of cadmium and zinc: greenhouse and soil studies of Palmerton Zinc Superfund Site, Pennsylvania, U.S.A.

Christian X. Figueroa

**Thesis submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Geology**

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Department of Geology and Geography

**Morgantown, West Virginia
2015**

Keywords: cadmium, *Populus*, *Paxillus involutus*, phytoremediation, sequential extraction, single-extraction methods, zinc

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ABSTRACT

Influence of mycorrhizal fungi on soil geochemistry and plant uptake of cadmium and zinc: greenhouse and soil studies of Palmerton Zinc Superfund Site, Pennsylvania, U.S.A.

Christian X. Figueroa

The application of bioremediation as a means to ameliorate potentially toxic metals from contaminated soils has been on the forefront of sustainable technologies in the field of environmental studies. Conventional methods available for soil remediation are employed in both in situ or ex situ and these are: soil flushing, solidification/stabilization, vitrification, electrokinetics, chemical reduction/oxidation, soil washing, and excavation. In general, these technologies are limited by their applicability to small areas and the high costs to execute. The use of biota, in particular fungi and trees, has been an emerging low-cost and “environmentally-friendly” alternative to conventional technologies. In this study, I evaluate four soils collected from the Lehigh Gap Wildlife Refuge and Nature Center adjacent to the Palmerton Zinc Superfund Site by using commonly used single extraction methods and the revised European Bureau of Common References (BCR) sequential extraction to determine availability of Cd and Zn. I also explore how the rhizosphere-based method (RBM) that mimics rhizosphere conditions compares to the liquid cultures of the ectomycorrhizal fungus *Paxillus involutus* to interpret change in metal availability and the potential for environmental risk associated with fungal inoculation of soils when used to aid remediation projects. Additionally, comparison between extractants (EDTA, DTPA, Mehlich-III, Fraction 1 and Pseudototal of the revised BCR sequential extraction method) that evaluate plant availability to determine the efficacy of the previous mentioned extractants. Findings were that both components of the BCR method grossly overestimates both Cd and Zn compared to aforementioned extractants. Additionally, fungal liquid performed similarly to the Mehlich-III as comparable extractant for determining Cd and Zn availability, where fungal liquid and RBM had similar extractability for Zn only.

Work was also done to investigate effects of *P. involutus* and a *Populus* hybrid on fractionation of metals in these soils by the revised BCR sequential extraction method. Findings suggest that availability and mobility of Cd and Zn in contaminated soils can be altered significantly by *P. involutus* and *Populus* and plant uptake of Cd is greater than Zn. With further

research with more varieties of hybrid poplars and mycorrhizal fungi more effective symbionts can be chosen to aid in remediating metal contaminated sites that may pose a risk to the environment and human health.

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List of Abbreviations

Al = Aluminum
AM = Arbuscular Mycorrhiza(e)
ATPase = Adenosine triphosphatase
BCR = Bureau of Common References
CaCl₂ = Calcium chloride
CaCO₃ = Calcium carbonate
Cd = Cadmium
Cu = Copper
Cys = Cysteine
DTPA = Diethylenetetraaminepentaacetic Acid
DW = Dry Weight
EDTA = Ethylenediaminetetraacetic Acid
Fe = Iron
Glu = Glutathione
Gly = Glycine
H⁺ = Hydrogen ion
K⁺ = Potassium ion
LGWR – Lehigh Gap Wildlife Refuge and Nature Center
LMWOA – Low molecular weight organic acids
M (mol/L) = Molarity
µg = microgram
mg/kg = milligram per kilogram
mM = millimolar
MMN – Modified Melin-Norkrans media
Mn = Manganese
Myc = Mycorrhizae
NTA = Nitrilotriacetic Acid
Pb = Lead
Pi/Pax = *Paxillus involutus*
Pop = Poplar/*Populus*
PSII = Photosystem II
RBM = Rhizosphere Based Method
ROD – Record of Decision
SD = Standard Deviation
SE = Standard Error
USEPA – United States Environmental Protection Agency
USFWS – United States Fish and Wildlife Service
UNEP – United Nations Environment Programme
Zn = Zinc

1 INTRODUCTION

Metals are ubiquitous within the environment, however due to anthropogenic activity, potentially toxic metals have become more commonplace and may affect biota and the environments in which they persist. Due to the bioavailability and potential for harm, metals have become a pollutant of concern where contaminated soils are prevalent. As a result of human activities such as mining and smelting of metalliferous ores, electroplating, fossil fuel exhaust, energy and fuel production, fertilizer and pesticide application, etc., metal pollution has become one of the most serious environmental problems today (Adriano, 2001; Alkorta et al., 2004). Metals exhibit a multiplicity of molecular-scale interactions with soil particulates or organic matter whereby they can be sequestered in the soil environment (Brown et al., 1999). Within an ecosystem, plants, soil microbes and fungi can transform metal solubility and availability extracellularly or may internally sequester metals and reduce their environmental burden (Kabata-Pendias, 2004; Bellion et al., 2006). Understanding these interactions between plants and soil microbes in contaminated soils may increase the capacity and appreciation for phytoremediation.

1.1 Plant-mycorrhizal interactions with toxic metals

Within the rhizosphere, most plants form symbiotic relationships with mycorrhizal fungi that enable availability of necessary nutrients as well increasing tolerances to environmental hazards. Mycorrhizal fungi have shown excellent efficacy in reducing metal toxicity to their host plants (Hartley et al., 1997; Godbold et al., 1998; Schützendübel and Polle, 2001) and these symbioses also assist in ameliorating toxicity of metals by transforming and sequestering these metals within the rhizosphere and intracellularly (Gadd, 2001; Hall, 2001; Meharg, 2003; Bellion et al., 2006). The performance of mycorrhizal fungi in ameliorating the effect of metals is dependent on species and isolate, metal used or encountered, and experimental conditions. Fungal isolates from heavy metal contaminated sites have shown better success at tolerance than those fungi isolated from non-contaminated sites (Galli et al., 1994). Mechanisms that enable fungi to sequester metals within the extracellular environment include chelation by extruded

organic compounds and binding to the cell-wall components (Gadd, 2001). Intracellularly, metals within the cytosol can be rendered inert by binding with a range of cytosolic ligands, increased efflux of heavy metal bound ligands out of the cytosol into the rhizosphere, and compartmentalization within sequestering organelles (Hartley et al., 1997).

Fungi have the capacity to limit intercellular passage by a variety of pathways. Exudation of different ligands under metal stress contributes to the overall reduction of influx of toxic metals into the cytosol. These ligands include low/high-molecular weight organic acids, siderophores, alcohols, proteins, polysaccharides, and other polymeric substances. Blaudez et al. (2000) observed that, in *Paxillus involutus* under cadmium stress, oxalic acid exudation reduced Cd uptake by 85%, compared to six other organic acids, suggesting oxalate exudation under Cd stress may relieve entry into the cell, decreasing Cd availability. Formina et al. (2006) found that pine seedlings infected with a Zn-resistant *P. involutus* strain accumulated less Zn in presence of Zn-phosphate sources and increased dissolution and uptake in low-phosphorus conditions. Increased solubilization of phosphates is stimulated by organic acid exudation in response to phosphate deficiency and other nutrient stress and deficiencies (Jones, 1998), where these compounds may chelate or precipitate metals within the rhizosphere.

Extracellularly, metals can actively adhere to negatively charged sites that comprise the cell wall, such as chitin, cellulose, cellulose derivatives, polysaccharides, and melanin (Gadd, 1993; Blaudez et al., 2000). Frey et al. (2000) found that, in symbionts *Picea abies*–*Hebeloma crustuliniforme*, Cd was localized in the cell walls of the Hartig net, whereas Zn was localized within the cell wall of the mantle hyphae, Hartig net hyphae, and in the cell walls of the cortical cells. This eluded that Cd was being confined within the apoplast of the Hartig net, where Zn was being extracellularly complexed and or sequestered within the cytosol. Jentschke and Godbold (2000) noted that the mechanisms of amelioration by fungi of metal uptake from the rhizosphere is an extremely dynamic and not well understood process, in that more sorption related studies and exploration of the hydrophobic tendencies of mycorrhiza need to be carried out to understand the complexity of extracellular binding.

Once metals have passed from the external environment into the cell there are several pathways in which metals can be translocated, sequestered, or discharged from the intracellular compartment to the exterior by efflux mechanisms or leakage. These mechanisms are enhanced by intracellular compartmentalization within organelles by complexation with sulfhydryl groups of metallothioneins and its derivatives (glutathione, γ -glutamylcysteine), which render these once toxic metals inert or by binding to polyphosphate granules (Galli et al., 1994; Meharg, 2003; Bellion et al., 2006). Metal-binding compounds include glutathione, metallothioneins, and phytochelatins and are produced in fungi during exposure to metals, whether in high or low concentrations (Courbot et al., 2004; Jaekel et al., 2005). These compounds have a role in both transport and storage of metals and may help in combating against oxidative stress (Hartley et al., 1997; Noctor et al., 1998). Metallothioneins are cysteine-rich low molecular weight proteins that are virtually found in every organism on earth to help combat against metal toxicity. Phytochelatins scavenge metals ions by thiolate coordination, where phytochelatins have the general structure of $[\gamma\text{-GluCys}]_n\text{-gly}$ ($n = 2$ to 11) that are enzymatically synthesized from glutathione, glycine, and cysteine amino acid residues. Polyphosphate granules located within vacuoles have also been found to be important in maintaining ionic compartmentation (Gadd, 1993; Galli et al., 1994) and that they have the capacity to bind most divalent metal cations, representing yet another means of intracellular metal detoxification.

1.2 Plant interactions with cadmium and zinc

Interactions between plant cells and potentially toxic elements are well documented in the literature (Marschner, 2002; Prasad, 2004). Both Zn and Cd show synergistic properties within plants due to their chemical behavior and likeness (Das et al., 1998). Where Zn is an essential element at appropriate concentrations, Cd is recognized to be lethal at extremely low concentrations. Due to the similarities between these ions, Cd is able to mimic the uptake properties of Zn and Zn may inhibit the uptake of Cd when at higher concentrations. When Zn is at low concentrations, plants will uptake more Cd than Zn (Kirkham, 2006). In soils the availability of both elements is strongly dependent on pH; both elements form inorganic and organic complexes, which widely affect availability and uptake (Bradl, 2004).

Cadmium is a non-essential element in plants and has no recognized physiological function. Cadmium has pronounced effects on plants at low concentrations. Natural environmental concentrations in non-polluted soils are 0.1 mg/kg and maximum concentration in plants is 0.2 mg/kg (Kirkham, 1977; Brooks, 1998). At higher exposures, adverse effects are the following: chlorosis, growth retardation, inhibition of photosynthesis, induction/inhibition of enzymes, altered stomatal function, water relations, interference with uptake and transport of macronutrients, and formation of free radicals (Prasad, 1995; Robinson et al., 2000). Non-hyperaccumulators have developed defense mechanisms to counteract high concentrations of Cd both inter-/extracellularly, including the accumulation of high concentrations of Cd in roots rather than transferring to the shoots, vacuolar sequestration, increased syntheses of phytochelatins and metallothioneins, and the upregulation of proteins that alleviate free radical oxidative damage (Das et al., 1998; Sanità di Toppi and Gabbrielli, 1999; Nan et al., 2002).

Zinc is a required element for the activity of many enzymes in plants, however Zn may be toxic in high concentrations (Nan et al., 2002). In plants, Zn is absorbed from the soil solution primarily as Zn^{2+} and then subsequently is translated to the shoots via the xylem (Broadley et al., 2007). As Zn is transferred from the rhizosphere, it enters via epidermal and cortical cells to the root xylem through a maze of cells linked by the plasmodesmata by symplastic or apoplastic pathways. A deficiency of Zn in plants leads to observed effects as chlorosis, necrosis, bronzing, rosetting, epinasty, and reduced growth (Marschner, 2002).

Zinc toxicity occurs in agricultural crops and native ecosystems as well as urban landscapes where anthropogenic inputs of Zn contaminate these soils. However, Zn is also available by the chemical and physical weathering of Zn-enriched bedrock and soils. These sources are especially available in low-pH soils (Broadley et al., 2007). Symptoms observed in plants subject to high concentrations of Zn are stunted growth, reduced yield, Fe-deficiency induced chlorosis, and interference of phosphorous uptake (Marschner, 2002). Important mechanisms in alleviating Zn toxicity in plants include increased efflux across the plasma membrane, vacuolar compartmentalization, an increase low molecular weight organic acid production, altered root-to-shoot translocation, and increased accumulation in older leaves (Hall, 2001; Qin et al., 2007). Todeschini et al. (2011) observed the effects of 300 mg/kg soil

concentration on the morphology and change in biochemistry in poplar leaves and noted the high alteration of leaf morphology, ultrastructure, and photosynthesis. This group also found that intercellular Zn was mostly found in the xylem and parenchyma cells, where an abundant increase of calcium oxalate crystals was observed in leaves of Zn treated plants, suggesting an increase in free Ca^{2+} ions following zinc accumulation to cell walls.

Both Cd and Zn have chemically similar behaviors in higher plants (Adriano, 2001). Cadmium is known to mimic the behavior of essential element Zn for its uptake, thus may affect physiological functions that Zn performs. Chaney et al. (1976) observed pronounced interactions between Cd and Zn in cadmium uptake and translocation in soybean, perhaps due to the non-selectivity of chelators that aid in these processes. Das et al. (1998) describes that the ratio of Cd to Zn in cellular tissues may induce and alter the binding characteristics of metallothionein affecting distribution and competition of other metalloenzymes essential in physiological and metabolic functions.

1.3 Interactions of cadmium and zinc in soils and the environment

Within terrestrial ecosystems fluxes of metals occur between four basic compartments including: atmospheric, organic, soil and parent material, and biological organisms (Adriano, 2001). Reactions and mechanisms controlling these exchanges between compartments include, but are not limited to: weathering, biological uptake, mineralization, uptake and release of gases, sorption, and aerosol transport and deposition. Biogeochemical cycling of nutrients and potential toxic elements follow various pathways between these compartments, and connect organisms to individual ecosystems to the entire biosphere.

Within the rhizosphere many complex interactions occur between soils and metals and these interactions control the fate and toxicity of metals in an ecosystem. Partitioning of metals in soil systems are controlled by an interactive processes that occur between soil constituents and biology (Bradl, 2004). The most available form of metals in soils exists within the soil solution, where metals are soluble free ions or complexed in pore water as inorganic or organic complexes. Here, movement of ions to plants for uptake occurs by diffusion or by mass flow

driven by transpiration. Primary and secondary minerals may not only act as sources of metals, but may also function as active absorbing sites through ion exchange onto clay minerals, which have high surface areas that may act as binding sites depending on physiochemical properties of the soil (Brown et al., 1999). Decomposing products of plant and animal tissues, the soil organic matter, may also act as sink and source of metals due to the properties of functional groups of organic residues. Soil biomass is another factor within the rhizosphere that will affect metal biogeochemistry and is the actual living component of this environment, the plant roots, invertebrates, bacteria, fungi, and algae that act as binding sites. The last component is strongly physiochemically dependent and, under certain conditions (pH and Eh driven), precipitates will form. These include precipitates of phosphate, carbonate, and sulfides. Interactions within any of these components will lead to alteration of bioavailability of metals within the biosphere.

Cadmium in the environment, if not a product of anthropogenic activity, is largely associated with Zn, Zn-Pb, or Pb-Zn-Cu ores associated with parent materials. Once introduced into the rhizosphere, both in contaminated and native soils, Cd is relatively immobile (Adriano, 2001). This may only hold true in soils meeting certain physiochemical requirements. In low pH soils, Cd is quite mobile (Kirkham, 1977). General background concentrations of Cd in agricultural soils are 1 mg/kg, however when observed in higher concentrations than 1 mg/kg, these concentrations are derived from geologic formations and soils sourced from these formations, phosphate fertilizers and or atmospheric deposition (Koopmans et al., 2008; Sanità di Toppi and Gabbriellini, 1999). Major sites for Cd accumulation in soils have been associated with various forms of organic matter in both the solid and dissolved forms (Koeppel, 1977; Krishnamurti and Naidu, 2003; Akkajit and Tongcumpou, 2010). The functional groups of these materials are generally oxygen-containing functional groups, such as carboxylic acids and phenolic groups that form stable complexes with Cd (Krishnamurti and Naidu, 2003). Pueyo et al. (2003), using the modified BCR method, found that Cd was mostly bound to the exchangeable and weak acid soluble fraction, with extraction yields ranging between 25% to 75%, depending on the soil and concentration.

In the environment, the primary inputs of Zn into soils are from the chemical and physical weathering of parent rocks (Broadley et al., 2007). Zinc occurs in minerals from

nonspecific replacement of Mg and Fe with Zn. Zinc concentration in agricultural soils from around the world range 10 to 200 mg/kg soil (Adriano, 2001). Zinc is most bioavailable in the water soluble form, especially in contaminated environments and remains in the upper most portion of the soil column (Černík et al., 1994). Secondary inputs of Zn in the environment are generally from anthropogenic activity, generally a result of smelting and mining. The content and distribution of Zn are linked to various processes and factors, such as weathering, organic matter and clay content, and pH (Adriano, 2001). Due to Zn being an essential element in photosynthesis and respiration, Zn is generally depleted in the soil column and translocated via complexation with organic ligands to the shoots via the xylem (Todeschini et al., 2011). Interactions between Zn and Cd in soils are known, the usual geochemical ratio is 1 µg Cd: 100 µg Zn found in most co-pollution environments, where Zn reduces Cd uptake more than reciprocal (Souza and Rauser, 2003; Chaney et al., 2007).

1.4 Sequential extraction

Toxicity of metals within the environment strongly depends on the form/compartiment in which they occur. The use of sequential extraction has been paramount since its inception in the late 1970's (Tessier et al., 1979) in providing an indirect approach of describing how potential toxic metals are operationally defined by choice chemical extractants. Various instrumental techniques can be used to directly determine the content of contaminated soils, such as extended x-ray absorption fine structure, however these methods are not generally available and may have poor detection limits in soils containing trace contents of the metal of interest. The alternative is to use indirect methods that may be more laborious, but are more readily available for the scientific community. In sequential extraction, reagents used are applied to the same sample to sub-divide the total metal content based on its extraction response. Numerous sequential extraction methods exist, leading to confusion when comparing results between methods (Bacon and Davidson, 2009). More recently, European scientists, collectively through the Standards, Measurements and Testing Program, have improved and optimized the sequential extraction method from the original protocol (the revised Bureau of Common References, "BCR" procedure). The revised sequential extraction method was developed by agency experts and tested and scrutinized by interlaboratory studies (Rauret et al., 1999).

The revised sequential extraction protocol consists of three-steps of increasing extractability. Each step is associated with a nominal target phase, in which the reagent targets specific mineral phases. This protocol is outlined in **Table 1.1**. Step one includes the use of acetic acid to target exchangeable and water- and acid-soluble cations associated with carbonates or weakly bound to substrates. Step two targets the reducible fraction within the sample associated with Fe- and Mn-oxyhydroxides and this is accomplished using hydroxylamine hydrochloride buffered to a pH of 1.5. Step three uses hydrogen peroxide digestion at 85°C, followed by extraction with ammonium acetate that targets the oxidizable fraction associated with organic matter and sulfides. Residuum from previous steps is then subjected to aqua regia digestion, where this step has replaced previous methods of digestion of residuals using hydrofluoric acid. Each analyte of interest is summed from each step to determine total extracted, which is compared to accompanying, but separate, pseudo total extraction that is carried out by aqua regia digestion of the original sample.

Issues that surround sequential extraction may be as great as the information produced from these extractions. Many have concluded that the problems with sequential extraction are not only the difficulty with reproducibility, but also the non-selectivity of the extractants, the overwhelming potential for redistribution of analytes among phases during the extraction, incomplete extraction during steps, and the precipitation of new mineral phases during the extraction (Belzile et al., 1989; Bermond, 2001; Bacon and Davidson, 2009). Sequential extraction coupled with synchrotron analysis is the only means of interpreting whether a specific trace metal binds to a specific substrate (Tipping et al., 1985; Scheckel et al., 2003; Wan et al., 2006). Tipping et al. (1985) showed that Pb redistributed between Mn-oxides to Fe-oxides in natural sediments from a former lead mine when treated with hydroxylamine hydrochloride. Wan et al. (2006) showed that redistribution of Zn, Cd, Pb and Cu in fly ash strongly depended on geochemical nature of sediments studied. It has also been suggested that H^+ consumption and pH may affect metal distribution (Bermond, 2001). Most extractants used are buffered to a low starting pH, however by the time the reaction step is over, there is generally significant pH change. This evokes exchange reactions with soil components, dissolution of $CaCO_3$, and reduction of Mn- and Fe-oxyhydroxides. pH change during these reactions has led scientists to

believe that this phenomenon is indeed problematic, because such changes may cause these extractants to be non-selective and lead to high redistribution.

In plant and agricultural sciences, sequential extraction can be used as a proxy to measure what has been loosely defined as “bioavailable” to plants. The measurement of availability of either essential or toxic elements to plants through sequential extraction has been defined as the concentration determined for the exchangeable fraction (step 1) or the combination of steps 1 through 3 (Bacon and Davidson, 2009). Sequential extraction was mentioned in a review by Luster et al. (2008) as a qualifier to determine metal distribution and availability. Sequential extraction could be used as a comprehensive characterization of soil-bound elements that may show good correlation between extractability and plant uptake. Pueyo et al. (2003) used soil-to-plant transfer factors, determined from the ratio of trace element concentration in plants versus concentrations found in soils where the plant was grown. This method was carried out for step 1 and the combination of step 1 and 2 of the revised BCR method, in addition a comparison to 0.01 and 1 mol L⁻¹ CaCl₂ soil extractions. More recently, sequential extraction has been used to observe the effects of EDTA treatment “before” and “after” on uptake and fractionation by *Taraxacum officinale* (Massop et al., 2009). Massop et al. (2009) found that the sum of potential toxic elements in steps 1 through 3 poorly correlated to plant uptake, However, in regards to EDTA treatment, there was positive correlation between change in fractionation in the first step associated with soil washing process, in respect to the operationally defined fractions in the BCR protocol.

1.5 Single extraction methods to determine bioavailability

Using total concentration values in soil and water have proven misleading for determination of bioavailability, because only a small component of the total may really be available (Shan et al., 2003). The use of one-step single extraction methods for the determination of bioavailability has been popularly used to secure a measurement of the labile quantities of toxic and essential metals in soil samples. However, there is no universal single extraction method that is useful in determining bioavailability due to the heterogeneity and complexity of soils (Sauvé et al., 2000). Very commonly used single extractions use aminopolycarboxylic

acids, such as DTPA and EDTA (Lindsay and Norvell, 1978; Feng et al., 2005; Matúš, 2007). Both of these complexing agents show similar extraction efficiency, but may over-represent the amount of metal transferred from the soil to the plant depending on soil characteristics and properties. Feng et al. (2007) found in rhizosphere soils that both DTPA and EDTA were good indicators for root and shoot content of barley for Zn, but poor for Cd, where a cocktail of organic acids (acetic, lactic, citric, malic, formic acids), noted as the “rhizosphere-based method”, was a good predictor of Zn and Cd contents from soils transferred to roots and shoots of barley in four types of soils used.

The use of low molecular weight organic acids (LMWOAs) has proven to simulate, and be more effective indicators of, rhizosphere availability. The primary source of organic acids is roots, which exude a broad array of C-containing compounds, many of which are responsive to metals in the rhizosphere (Jones, 1998; Jones et al. 2003). Secondary sources of organic acids in the rhizosphere are produced from the excretion from microorganisms during breakdown of other C substrates (Jones, 1998). With this consideration, it is not out of the question to use single- or a cocktail of organic acids to measure labile concentrations of metals. Meers et al. (2007) found that 0.11 M acetic acid extracted the same concentration as the first-step of the revised BCR sequential extraction method, finding it more effective at determining relative Cd extractability in acidic, silty sandy soil types. Shan et al. (2003) suggested a similar method to that of Feng et al. (2005) (the rhizosphere-based method), using a cocktail of organic acids (>10 mM) to gain a more realistic conditions encountered in the rhizosphere.

1.6 Phytoremediation

Phytoremediation is a multifunctional approach to introduce plants into contaminated environments and allows plants to assimilate toxic substances into their biomass. There are three main strategies in phytoremediation: phytoextraction, rhizofiltration, and phytostabilization (McIntyre, 2003). McGrath et al. (2001) describe several approaches used in phytoremediation: (1) Use of hyperaccumulators that have tendencies to absorb higher than normal concentrations; (2) Use of high biomass crops that can be aided in extracting higher amounts of metals through the use of chemical extractants, such as EDTA; and (3) Use of fast growing trees that are easily

harvestable and maybe economically beneficial for alternative energies such as *Populus* and *Salix*.

Phytoextraction largely depends on the sequestration of toxic elements within the above ground portion of the accumulating plant per surface area. Hyperaccumulators (i.e., *Thlaspi caerulescens*) generally are not necessarily the best candidate for phytoextraction because of high metal concentration in plant is most often negatively correlated to plant growth (Lebeau, 2008). High biomass with significant concentrations per surface area with allowance for multiple harvests per year may serve as a better candidate for phytoextraction. Phytostabilization functions as a means to prevent further disruptive actions on the environment, and renders harmful toxins inert by changing their chemical speciation by sequestration through organic acid precipitation or other dissolved organic matter or substances exuded by plants. Plants grown under these conditions may not even remove much or any contamination, but aid in regenerating soils if they can tolerate and grow under these elevated conditions (McIntyre, 2003). A limitation of phytostabilization is the extensive use of fertilizers or extensive soil modification using amendments to help aid growth and stabilization capabilities (Gratão et al., 2005).

Chemical additions of synthetic amino polycarboxylic acids have been used in phytoremediation to promote uptake in plants (Cooper et al., 1999; Robinson et al., 2000; Grčman et al., 2001; Evangelou et al., 2007). Robinson et al. (2000) found that the addition of EDTA, DTPA, and NTA to poplar clones caused a temporary increase in uptake of cadmium, however the additions of 2000 mg/kg EDTA and 500 mg/kg NTA treatments resulted in significant growth reduction and abscission of leaves. Chelate assisted phytoremediation works best in conditions where metals of interest have low bioavailability, allowing the establishment of large biomass before the chemical is added (McGrath et al., 2002). The use of synthetic chelates has high potential risks associated with leaching, residence time in soil, and effects on soil biota (Gratão et al., 2005). A more effective alternative to chemical chelates may be the enhanced understanding of rhizosphere processes and use of microbes that work synergistically with the host to promote the removal and or sequestration of toxic metals (Donnelly and Entry, 1999; Meharg and Cairney, 2000; Wenzel, 2009).

1.7 Lehigh Gap Wildlife Refuge and Nature Center and Palmerton Zinc Superfund Site

The Lehigh Gap Wildlife Refuge and Nature Center location that was chosen for this study is located in the Borough of Palmerton, Carbon County, Pennsylvania. Approximately 30 kilometers northwest from Borough of Allentown, PA, the Lehigh Gap Wildlife Refuge and Nature Center is situated on the western side of Lehigh Water Gap on Blue Mountain at the confluence of the Lehigh River with Aquashicola Creek (**Figures 1.1-1.4**). Smelting of zinc ore had been the major industry within this valley from the early 1890's until its declaration as a Superfund site in the early 1980's. Two facilities deemed the West and East Plants once owned by New Jersey Zinc Company had produced zinc and other metals for machinery, pharmaceuticals, pigments and many other products. During the operation of these smelters and associated facilities, huge quantities of zinc, lead, cadmium, and sulfur dioxide had been aurally emitted, leading to the defoliation of nearly 2,000 acres on Blue Mountain (USEPA, 1987). The main objectives set by the USEPA to ameliorate the site are: (1) Minimize direct contact with contaminated soils; (2) Reduce runoff; (3) Reduce contamination in runoff; and (4) Mitigate environmental damage.

Since the original Record of Decision (ROD) by the USEPA (1987), extensive efforts have been made to use the best available technologies and practices to remediate the site (USEPA, 2002). These include application of biosolids to vegetate the north slope of Blue Mountain nearest to the East Plant, minimizing both runoff from the Cinder Bank adjacent to East Plant and off of Blue Mountain, controlling runoff and leachate formation from the Cinder Bank to protect surface- and ground-water, installation of a vegetative barrier on the Cinder Bank to reduce windborne contaminated emissions and erosion, water quality improvement by creating "metal removal zones" that are pits lined with iron rich materials to interact with incipient runoff, and the creation artificial wetlands to filter runoff from both the Cinder Bank and Blue Mountain. Control of runoff was deemed critical because of findings that most contamination exists within the top 6 to 10 inches and mostly bound to organic matter preventing the downward movement of metals. In the ROD from 1987, five samples sites on Blue Mountain were selected and analyzed for the metals mentioned above. In these samples, recorded levels of cadmium ranged from 364 to 1,300 mg/kg, lead from 1,200 to 6,475 mg/kg, and zinc from 13,000 to 35,000 mg/kg. All of these samples exceed background levels 100-to 1000-fold.

In early 2002, talks began to purchase the land that is now the Lehigh Gap Wildlife Refuge and Nature Center. By 2003, the three properties that encompass the 750-acre site had been purchased. Subsequently following the purchase, work began on habitat restoration and management of the ecological damaged portion of the refuge. Since efforts have been placed on remediating this area, tremendous headway has been made using native vegetation and sustainable practices in ameliorating soil conditions to promote revegetation that have produced good quality habitat on about 75 acres (Latham et al., 2007) by planting 56 one-acre plots by tractor and spreader that have since converged into one large plot. A USDA NRCS Soils Map with sampling locations is included in **Figure 1.4**. In the summer months of 2006, further work was done to seed the steep slopes up along the ridge by aerial application of fertilizer, lime, and seed. By July of 2006, the entire area once considered degraded had been reclaimed, placing the partnership between government agencies, private parties, and the organizers of the Refuge for recognition and holding up the accomplishments as a national example (Hoopes, 2007). With continued success since its inception in 2002, the site has completely metamorphosed from a barren wasteland into an interactive outdoor laboratory that services the community and draws continued interest from universities to state and federal government.

The Lehigh Gap Wildlife Refuge and Nature Center was chosen because of the unique opportunities for ecological restoration activities. Studies of heavy metal contamination in soil and plant tissues are of great interest due to contaminant transport within the biosphere. Soils from this site met the criteria for observing the effects of mycorrhizal inoculation and plant growth on soil chemistry and fractionation. Four locations from this site were used in this study and photography of sampling locations can be seen in **Figure 1.5**.

1.8 Purpose of this study

The first component of this study determined the extractable amount of both Cd and Zn in soil samples collected from Lehigh Gap Wildlife Refuge and Nature Center using common single extraction methods (i.e., RBM, EDTA, DTPA, Mehlich-III, H₂O) and compared these to extractability of collected fungal liquid media (modified Melin-Norkrans) grown with *Paxillus involutus*. Additionally, these single extraction methods were compared against the first fraction

of the modified BCR sequential extraction method, commonly used to determine the bioavailable component in soils of interest and the pseudototal, an estimate of the total content of the metal of interest. The modified BCR sequential extraction method was used to fully characterize the chemical fractionation of these soils. The goal was to characterize and identify the availability of Cd and Zn in these soils and assess the effectiveness of rhizosphere based method or synthetic amino polyaminecarboxylic acids as representative extractants relative to that of the collected fungal media grown with *Paxillus involutus*.

The second component of this study was to grow a *Populus* hybrid with and without *Paxillus involutus* inoculants in two Lehigh Gap Wildlife Refuge and Nature Center soils, “amended” and “non-amended” as part of reclamation activities. Along with plant growth, metal content and leachate measurements, chemical fractionation of these soils were determined to observe the difference from “before” and “after” growth. The purpose of this component is to observe the success of plant growth in these two different soils and the short-term change in chemistries of these soils due to the interactions with both fungi and *Populus* growing symbiotically. This may aid in decision making associated with future reforestation projects at the LGWR and its temporary effects on the availability of both cadmium and zinc.

1.9 References

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Table 1.1 Revised BCR Sequential Extraction Protocol

Fraction	Chemical	Fraction Label	Nominal Target
Step 1	0.11 M Acetic Acid	Exchangeable/Water and acid-soluble	Soluble and exchangeable cations and carbonates
Step 2	0.1 M Hydroxylamine Hydrochloride	Reducible	Fe- and Mn oxyhydroxides
Step 3	8.8 M Hydrogen Peroxide/1 M Ammonium Acetate	Oxidizable	Organic matter and sulfides
Step 4	Aqua Regia	Residual	

Table adapted from Bacon and Davidson (2009)

Figure 1.1 Satellite Imagery illustrating Palmerton Borough Vicinity and LGWR

Figure 1.2 USGS 1:100,000 Allentown Topographic Map illustrating Palmerton Borough and LGWR

Figure 1.3 Satellite Image Illustrating Sampling Locations at LGWR

Figure 1.4 USDA NRCS Soils Map with Sampling Locations at LGWR

Figure 1.5 Photography of Sampling Locations at Lehigh Gap Nature Center/Wildlife Refuge, Palmerton, PA, USA.

2 Cadmium and zinc partitioning in zinc smelter contaminated soils

2.1 INTRODUCTION

Contamination of soils by metals is a significant problem throughout the world. Agricultural, industrial, mining/smelting, and military activities increase the concentrations of zinc (Zn), cadmium (Cd), and other metals in soils (Pierzynsky and Schwab, 1993; Cunningham and Ow, 1996; Giller et al. 1998), which lead to broad ranging disruptions to plant community structure and function (Frey et al., 2006; Gallagher et al., 2008). At toxic concentrations, metals may eliminate a majority of the existing vegetation, leading to significant erosion of soil, loss of wildlife habitat, loss of carbon sequestration capacity, and loss of aesthetic appeal.

The evaluation of bioavailability of potentially toxic elements in environmental samples is of great interest to researchers and land managers in determining potential harm to humans and ecosystems. Although the total concentration of a metal in soils does not reflect its bioavailability, no universal method exists for predicting potential exposure and toxicity of metals in soils (Shan et al., 2003; Feng et al., 2005; Soriano-Disla et al., 2010). Traditional methods of testing soils include the use of single and sequential extraction procedures to assess availability to plants and potential for groundwater contamination. One-step extraction methods are frequently used to procure soil metal concentrations due to ease of operation and their procedural simplicity (Feng et al., 2005). These tests include the use of salts, organic acids, and/or synthetic organic chelates to promote the solubilization and mobility of metals within these samples for subsequent analyses.

Synthetic aminopolycarboxylic acids, such as diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA), have been employed in many studies to evaluate/interpret bioavailability of metals in environmental samples and enhanced uptake by plants (Lindsay and Norvell, 1979; Norvell, 1984; Heil et al., 1999; Feng et al., 2005; Evangelou et al., 2007; Leštan et al., 2008). The basis for the use of these synthetic chelates originates from the ease at which these compounds form soluble complexes with free metal ions, although many

factors including lability of metals in soil, extractant concentration, interactions between metals and other ions, pH, and soil matrix may affect extraction efficiency (Sun et al., 2001).

The activity plants and microbes residing on metal-contaminated soils influences metal mobility and partitioning in the rooting zone (Awad and Römheld, 2000; Degryse et al., 2008; Quartacci et al., 2009). Plants species and/or genotypes may differentially absorb and accumulate metals and may differentially release metal-chelating compounds into the rhizosphere (Hall, 2002; Panda and Matsumoto, 2007). Further, plants may preferentially form symbiotic associations with arbuscular mycorrhizal (AM) and/or ectomycorrhizal (ECM) fungi, which may produce a suite of novel compounds that also alter metal biogeochemistry (Wang et al., 2007; Cornejo et al., 2008; Johansson et al. 2008; Mrnka et al., 2012). These ubiquitous mycorrhizal associations may modify metal availability in soils due to biosorption to fungal hyphae, the production of metal-chelating fungal compounds, or altered uptake and cycling by host plants (Khan et al., 2000; Seguel et al., 2013). These changes will affect the availability and ecosystem flux of metals.

Using low molecular weight organic acids that are generally produced by plants and mycorrhizal fungi may better simulate conditions at the root-soil interface and provide an approach to determining metal bioavailability (Feng et al., 2005; Soriano-Disla et al., 2013). To our knowledge, evaluation of the influence of exudates produced by mycorrhizal fungi on metal availability or partitioning in soils has not been evaluated. In this study, we present the results from single-batch soil extractions to assess the bioavailability of Cd and Zn in soils collected from an abandoned zinc-smelter site. We compare Cd and Zn extractions using ECM fungal exudates from *Paxillus involutus* with those using EDTA, DTPA, the rhizosphere-based method (RBM), and Mehlich-III extraction methods in prediction of bioavailability. In conjunction, we also compare results from the European Commission, Community Bureau of Reference (BCR) sequential extraction procedure to the single-batch soil extraction methods.

2.2 MATERIALS AND METHODS

2.2.1 Soil samples

Soil samples were collected from the Lehigh Gap Wildlife Refuge and Nature Center (LGWR) located at the Palmerton Zinc Superfund Site in Palmerton, Pennsylvania, U.S.A. The site is located adjacent to a historical zinc smelter (s) that had been in operation for nearly a century. Activity at these smelters have led to the defoliation of approximately 8.1 km² (2000 acres) in the vicinity (USEPA, 1987) mostly by aerial deposition of toxic elements cadmium, lead, and zinc. The site is mostly a boulder field that developed in the last Pleistocene glaciations (Pazzaglia et al., 2006). A USDA NRCS Soils Map (<http://websoilsurvey.nrcs.usda.gov>) with sampling locations is included in **Figure 1.4**. Samples were gathered from the top 0-30 cm depth, each of which supported little or no plant life.

Four soil samples were collected from different locations within the LGWR in the spring of 2009. One sample was collected from one of several amended monitoring plots that were created in 2003 that have received various fertilizers, mushroom compost, and warm season grasses. The remaining samples were collected throughout the site where no observable amending has been done, these samples for remainder will be called “unamended” (**Table 2.1**). Samples were brought into the laboratory, dried at room temperature, homogenized with a cement mixer to insure homogeneity, then refrigerated until analysis at 4 °C.

2.2.2 General soil characterization

Soil pH was measured using the method established by Thomas (1996), where 1:1 soil slurry with deionized water and air-dried soil is measured in triplicate with a pH electrode (Beckman Φ 50 pH meter). Organic matter content was measured by loss in ignition at 500 °C. Percent moisture was determined by placing samples in a drying oven at 105 $\pm$ 5 °C until soils were at a constant weight.

Cation exchange capacity (CEC) was determined using the compulsive exchange reaction method of Hendershot and Duquette (1986). Samples (2-g) were weighed and placed into 50 mL

centrifuge tubes. A washing step with 20 mL of 100 mM BaCl₂ followed by three independent washing steps with 20 mL of 25 mM BaCl₂ was executed with a 2 h shaking time on the shaker table at 150 rpm. A single overnight mixing-step was used with 20 mL of 25 mM MgSO₄. The mixture was then centrifuged at 2500 x g for 10 min, followed by decanting and filtration of the supernatant with a 0.45-µm Whatman syringe filter, and stored at 4 °C until analysis. The cation exchange capacity was calculated as the difference in total magnesium added minus the magnesium in the final solution multiplied by the milliequivalents of the magnesium containing extracting solutions.

2.2.3 Fungal culture

The mycorrhizal fungus *Paxillus involutus* (isolate ATCC 200175) was grown on a petri dish in agar containing modified Melin-Norkrans (MMN) media. The MMN media contained the following: 10 g/L glucose, 2 mM (NH₄)₂HPO₄, 4 mM KH₂PO₄, 0.5 mM MgSO₄, 1 mM CaCl₂, 0.25 mM NaCl, 0.025 mM KCl, 0.0125 mM H₃BO₃, 0.001 mM MnSO₄*H₂O, 0.001 mM ZnSO₄*7H₂O, 0.00025 mM CuSO₄*5H₂O, 0.00025 mM Na₂MoO₄, 0.00025 mM CoCl₂*6H₂O, 0.01 mM Fe,Na-EDTA, 0.003 mM thiamine HCl, 0.002 mM pyridoxine HCl, 0.006 mM ascorbic acid, and 0.04 mM nicotinic acid. The pH was adjusted to 5 using 0.1 M HCl before autoclaving.

Following four weeks of incubation on solid MMN media, fungal colonies were transferred to a 250-mL conical flask containing ~50 mL of liquid MMN liquid media. Following four weeks of growth, cultures were filtered and culture media was then collected, following removal of fungal bodies. Liquid aliquots were vacuum filtered with a 0.45-µm Whatman paper using a Buchner funnel and stored at -20°C until use to minimize degradation.

2.2.4 Determination of total metal concentrations in soil

Determination of Cd and Zn was carried out after digestion with aqua regia of concentrated HNO₃ and HCl by microwave-assisted digestion by Geoscience Laboratories (Ontario, Canada) using the protocol established by

(http://www.mndm.gov.on.ca/mines/ogs/labs/default_e.asp). Samples were leached overnight with room temperature aqua regia then digested for 1 hr at 100°C. These concentrations are referred to as Total-Cd and Total-Zn.

Pseudototal concentrations of Cd and Zn were also determined in accordance with Rauret et al. (1999). Soils were air-dried, passed through a 2-mm sieve, and placed in closed Teflon digestion vessels and subjected to microwave-assisted digestion in a MARSXpress system (CEM USA, Matthews, NC). Samples (3 g) were digested with 28 mL of aqua regia (3:1 mixture of 37% HCl and 70% HNO₃) at room temperature for 16 hr. Subsequently, the samples were transferred to the microwave digester at 130 °C at reflux conditions for 2 hr. The supernatant was filtered through a 0.45-µm Whatman syringe filter and diluted to 100 mL with 0.5 M HNO₃ and stored in polyethylene bottles at 4 °C until analysis.

2.2.5 Sequential extraction

Air-dried soils were subjected to the revised (four-step) sequential extraction protocol (the “BCR” method) developed by the Bureau of Common References (Rauret et al., 1999). Each soil sample was extracted in triplicate. The pH of the extractant was measured at the beginning and end of the agitation of the sample in each step, excluding step 4. All the extractants from each step were passed through a 0.45-µm Whatman syringe filter and refrigerated at 4 °C until analysis.

Step 1 (*water/acid soluble and exchangeable fraction*): 1 g sample was treated with 40 mL of 0.11 M acetic acid solution and was agitated on an orbital mixer at 30 +/- 10 rpm for 16 hr at room temperature. Separation of the supernatant was accomplished by centrifugation at 3000 x *g* for 20 min. Following the removal of the supernatant, an additional washing step with 20 mL of deionized water for 15 min on the orbital mixer was used followed by centrifugation at 3000 x *g* for 20 min. The supernatant was then decanted and discarded.

Step 2 (*reducible fraction*): The solid residue from the previous step was subjected to 40 mL of 0.5 M hydroxylammonium chloride solution, adjusted to a pH of 1.5 with a fixed volume of 2 M HNO₃. The sample was agitated on an orbital mixer at 30 +/- 10 rpm for 16 hr at room temperature. The supernatant and residue was separated as above. The residue was washed in the same fashion as above.

Step 3 (*oxidizable fraction*): To the residue from step 2, 10 mL of 8.8 M H₂O₂ solution was added. The slurry was digested for 1 hr at room temperature and placed on a block digester for 1 hr at 85+/-5 °C, and the volume was reduced to less than 3 mL. An additional 10 mL of H₂O₂ solution was added, the sample was digested for 1 hr at 85+/-5 °C, and the volume was reduced to approximately 1 mL. The extraction was accomplished by the addition of 50 mL of 1 M ammonium acetate to the slurry, which was agitated on an orbital mixer at 30 +/- 10 rpm for 16 hr at room temperature. The supernatant and residue was separated similarly as the first step. The residue was washed in the same fashion as the first step.

Step 4 (*residual fraction*): The residue from the previous step was digested by aqua regia, however the volumes of HCl and HNO₃ used have been reduced to 7 mL and 2.3 mL, respectively. The mixture was digested similarly as the Pseudototal determination.

2.2.6 Single Extraction experiments

Leaching experiments were carried out to determine the bioavailability of Cd and Zn in soils collected from Palmerton. A variety of extractants were used to observe the effectiveness in extracting both analytes and to compare against fraction 1 and pseudototal of the sequential extraction protocol and are summarized in **Table 2.2**. Fungal media collected from MMN liquid cultures of *P. involutus* were also used to examine any similarity in extractability between the RBM and these solutions. Each sample was agitated on a windmill mixer, centrifuged as specified by each method, filtered with a 0.45-µm Whatman filter and stored at -20°C until analysis. Extra caution was used with organic-based extractants where each sample container was covered with aluminum foil to minimize photodegradation.

2.2.7 Analyses and quality control

All glassware and plasticware were washed with 0.2 M HCl and allowed to soak overnight and rinsed with deionized water. All reagents were of analytical grade. Concentrations of Cd, Zn, and Mg were determined using a Varian 220FS atomic absorption spectrometer (Varian Inc., Mulgrave, Vic., Australia). Blanks were included in microwave digestions and sequential extractions, and quality of analysis was analyzed by spike and recovery. BCR CRM 701 was analyzed by BCR procedure, to ensure proper extraction using the BCR revised method.

The recoveries of analytes (Cd and Zn) using spike and recovery in extracts and digests were $100\pm 20\%$. Recovery of analytes (Cd and Zn) from BCR CRM 701 from each batch sequential extraction was 100%. The recovery of analytes (Cd and Zn) from the BCR extraction procedure, where combining the individual steps and comparing amounts detected to the results of a separate aqua regia digest, ranged from 73% to 144%.

2.3 RESULTS

2.3.1 Soil characterization

All soils collected from the LGWR had mean concentrations of Cd and Zn that exceed the guidelines set by the USEPA (USEPA, 2001) (**Table 2.3**). Soil pH of the four soils were slightly acidic and typical of soils found within Blue Mountain province (USDA, 2011), with exception of the one sample that had a higher pH likely in response due to amendment. The two samples containing the highest metal concentration, both PLM-B1 and PLM-B3, had both higher TOC and CEC values compared to the two samples containing lower metal concentration.

2.3.2 Sequential extraction

The revised BCR sequential extraction data illustrating both Cd and Zn extractability for all four soils by fraction and extraction yield are on **Figure 2.1**. Actual values with standard deviation and means with statistical analysis using Tukey-Kramer Method for each soil sample

are located on **Table 2.4**. Overall trends using extraction yields to evaluate both Cd and Zn availability in samples are as follows: Cd reducibles>exchangeables>oxidizables>residuals; Zn exchangeables>residuals>reducibles>oxidizables. Pseudototals determined by summing all fractions of the sequential extraction show trends of the following: Cd PLM-B3 $\geq$ PLM-B1>PLM-B2>PLM-B4; Zn PLM-B3 $\geq$ PLM-B1>PLM-B2>PLM-B4.

In the first step of the extraction scheme, pH values of the acetic acid solution ranged from 3.40 to 3.75 with a Δ pH (Final pH – Beginning pH) ranging from 0.46 to 0.69 where the pH rose from original pH (the pH value of the 0.11 M acetic acid solution was 2.85). The range in extracted Zn from fraction 1 is 2887 to 5396 mg/kg dry weight. In soil sample PLM-B3, 5396 mg/kg equates to 51% of the total Zn within this sample, where in soil sample PLM-B4 nearly 62% of the total Zn was removed in the first step of this extraction protocol. The range in extracted Cd from fraction 1 is 14 to 89 mg/kg dry weight. The range of percent Cd extracted is from 20 to 36% of the total Cd within these samples. In all soil samples Zn is mostly bound to the exchangeable and weak-acid soluble phase. In soil sample PLM-B2, Cd is mostly operationally bound to fraction 1.

The second step of the extraction scheme, pH values of the extracting solution ranged from 1.62 to 1.91 while the Δ pH ranged from +0.06 to +0.24 (the pH value of the 0.5 M hydroxylammonium chloride solution was 1.56). The range in extracted Zn from fraction 2 is 203 to 3263 mg/kg dry weight. This relates to a 4 to 25% Zn extracted from the total available Zn in these samples. The range of extracted Cd from fraction 2 is 6 to 112 mg/kg dry weight. The range of percent Cd extracted is from 9 to 45% of the total Cd available. In both soil samples PLM-B1 and B3 Cd is mostly bound to reducible phase.

The third step of the BCR extraction scheme the pH of the extracting solutions range from 2.16 to 2.26 while the Δ pH ranged from +0.01 to +0.03 (the original pH value of the 1 M ammonium acetate solution was 2.09). The range in extracted Zn from this fraction is 355 to 1156 mg/kg dry weight. This relates to a 4 to 9% Zn extracted from the total available Zn in these samples. The range of extracted Cd from fraction 3 is 25 to 38 mg/kg dry weight. The

range of percent Cd extracted is from 9 to 46% of the total Cd available. In soil sample PLM-B4, the oxidizable fraction was the greatest sequester of Cd in all of the fractions for this sample.

The overall effectiveness of the sequential extraction protocol can be scrutinized by comparing the sum of all four fractions versus the pseudototal of the soils of interest. The percent Zn extracted for soils PLM-B1 through PLM-B4 are 83, 90, 99, and 105% respectively. The percent Cd extracted for soils PLM-B1 through PLM-B4 are 83, 95, 99 and 80% respectively.

2.3.3 Batch leaching extractions

Concentration averages for Cd and Zn extracted by the single batch extractions for all samples are illustrated in **Figure 2.2a**, respectively. In respect to common extractants used for determining plant availability, the general trend for extraction efficiency for Cd is $\text{EDTA} \cong \text{DTPA} > \text{Mehlich-III} > \text{RBM} > \text{H}_2\text{O}$. As for efficacy of removal of Cd from soils utilizing *P. involutus* (*Pax*) exudates, this solution only slightly underperformed compared to RBM and Mehlich-III combined. *Pax* solution effectively removed 64% and 27% more than the above mentioned extractants, respectively. This may reflect pH and buffering capacity of solutions pre- and post-extraction, where *Pax* and Mehlich-III have similar pH pre-extraction, where RBM is ~0.75 units higher. Whereas, post-extraction ΔpH for these three extractants are $\text{Pax} > \text{Mehlich-III} > \text{RBM}$, suggesting RBM is a more buffered solution than the others. Fraction 1 of the modified BCR sequential extraction method grossly overestimates Cd, even compared to EDTA and DTPA by a factor of 3. Average percentages of Cd extracted with the extraction methods used compared to total concentration are presented in **Figure 2.2b**. Statistically, as for percent Cd extracted compared to the pseudototal, *Pax* and Mehlich-III show no statistical difference, where the RBM shares statistical similarity to MMN.

Zinc extractability in soils by common extractants had a general trend for extractability for Zn are $\text{DTPA} \geq \text{EDTA} > \text{Mehlich-III} > \text{RBM} > \text{H}_2\text{O}$ as illustrated in **Figure 2.2c**. EDTA and DTPA extracted nearly twice as much in these samples than any other extractant used in this study, with no statistical difference between the extractants. The *Pax* extraction yielded statistically similar concentrations to Mehlich-III and the RBM. The statistically similarity of

Pax with RBM and Mehlich-III may foreshadow that fungal exudate solution as an extractant may be a good estimate of Zn availability, whereas, compared to EDTA and DTPA, it underestimates Zn concentration by a factor 1.5. As similar to Cd, Fraction 1 overestimates Zn extractability as an estimate of bioavailability compared to the other extractants used, minimally by a factor greater than 3. Average percentages of Zn extracted with the extraction methods used compared to total concentration are presented in **Figure 2.2d**. As for percent Zn extracted compared to the pseudototal, *Pax* shows no statistical difference between Mehlich-III, RBM and MMN.

2.4 DISCUSSION

Soils collected from smelter sites generally have a signature of extremely high contents of heavy metals (Grčman et al., 2001; Ettler et al., 2004). At the LGWR and within Palmerton vicinity, both Cd and Zn persist at levels that have led to defoliation of the Borough of Palmerton and the surrounding areas (USEPA, 2002). Elliott and Shastri (1999) reported concentrations of Cd and Zn at Palmerton to be 280 and 2700 mg/kg, respectively. Adriano (2001) shares that during the 100 years of operation, smelting operations by New Jersey Zinc Company in Palmerton, PA emitted an estimated total of 260,000 tonnes of Zn and 330 tonnes of Cd, relating to approximately 50 to 100 kg Zn/1 kg Cd emitted. Due to the size and heterogeneity of the site, these gross indices may greatly underestimate the extent of contamination that may be affected by the sampling size. Using single extraction methods to determine extractability of metals has been a convention used to simply and efficiently measure metals in soil samples. Conversely, the use of sequential extraction is a powerful indirect approach to determine how metals operationally act in soils of interest (Bacon and Davidson, 2009). When used together, these methods may give a more representative picture of how metals are interacting in different partitions and can be potentially used as a check and balance against the effectiveness of single extraction.

In the revised BCR sequential extraction protocol the pseudototal extraction, an aqua regia extraction, gives a preliminary understanding of the total concentration of the soils that are being explored. This can be used to compare against metal(s) total data received from an outside

source or other laboratories studying the same soils to ensure quality of extraction. The pseudototal can also be used to check against the efficacy of the four-step extraction process incorporated in the sequential extraction, where all four steps are summed than compared to the pseudototal. The efficiency of our extraction compared against an outside laboratory (Geoscience Laboratories, Ontario, Canada) for Cd and Zn in study for soils PLM-B1 through B4 are as followed, respectively: 109% and 95%, 106% and 101 %, 95% and 96%, 173% and 97%. The outlier, PLM-B4 for Cd, may be result of operator error or some other erroneous account, where the sum of the four steps for this soil compared to the pseudototal results in an extraction efficiency of 80%.

In general, all methods used for single extractions for Zn when compared to the first-step in the revised BCR sequential extraction protocol greatly underestimate “exchangeable” or “acid-soluble” content of these soils. Extracting solutions EDTA and DTPA remove approximately from 19 to 49% of Zn of what is considered exchangeable in the sequential extraction protocol. Whereas for Cd, these acids only removed approximately 28 to 57% of Cd in the four soils studied where compared to the first step of the sequential extraction protocol. This may be a factor of concentrations of the extractants used, where the acetic acid used in step 1 is a factor of 22 greater in concentration than the polyaminecarboxylic acids used. Due to the non-selectivity of H^+ ions in these solutions, the lower concentrated acids may see complete consumption of H^+ ions by reactions with calcareous materials, oxides, and may invoke exchange reactions with mono- and divalent ions (Bermond, 2001). Other possibilities are the differences in solution to soil ratio and leaching time for all extractants used compared to Step 1. The solution to soil ratio for all extraction with exception to water was 10, where in Steps 1 and 2 the ratio is 40. In the leaching process, due to solubility and distribution equilibrium, solution to soil ratio may affect the constituent release of ions and leaching parameters such as: pH, redox, dissolved organic carbon content, speciation, and reaction kinetics (Schuwirth and Hofmann, 2006).

Comparing sequential extraction data from other studies have shown to be difficult due to uniqueness of compared site, source of pollution, and variety materials and methods used. The classic Tessier et al. (1979) five-step sequential extraction method was the first method

developed to interpret fractionation in fluvial bottom sediments. Since the development of this method, the sequential extraction method has deviated into several other methods that have different procedures, number of extraction steps, reagents and optimized conditions, having led to increased difficulties in interpreting and comparing data from different sources (Bacon and Davidson, 2009). For the ease of comparing fractionation patterns from this study versus methods used in many others, combining the first two steps (Exchangeable/Bound to Carbonates) of Tessier et al. (1979) and the first three steps (Water Soluble/Exchangeable/Carbonate Bound) of Ma and Rao (1997) will suffice the comparison of Step 1 of the Revised BCR sequential extraction method (Rauret et al., 1999). In Elliott and Shastri (1999) study of a Palmerton soil found that approximately 40% of Cd and 29% of Zn is associated with the first two steps of Tessier method. As for Ma and Rao (1997) study of multiple soil types and sources, samples collected from smelting sites that had a concentration of $\text{Zn} > 1000 \text{ mg/kg}$ and $\text{Cd} > 50 \text{ mg/kg}$, that approximately 30% of Cd and 15% of Zn was associated with the first three steps of the sequential extraction scheme used. In this study of four soils from Palmerton, approximately 35% of Cd total and 48% of Zn total was determined to be associated with Step 1 of the revised BCR method. The concentrations found in this study slightly differentiate then the reported values of Elliott and Shastri (1999), however due to both physical and chemical changes weighted by time, these values correspond to rehabilitating soils that have seen fractionation change due to natural and human induced activities. These compounding variables have led to the gradual translation of fractionation of Cd and Zn in all samples, where both these elements have become more available over time, from residual to non-residual fractions. Interestingly, Cd remains strongly associated with Fe-Mn oxide and organically bound fractions, where Zn has maintained the highest availability in exchangeable and residual forms.

The use of fungal media as collected in the fashion of this study enabled a comparison between traditional extraction methods (i.e., EDTA, DTPA, etc.) and more modern methods for predicting bioavailability using a “cocktail” of organic acids approach, such as the *Rhizosphere Based Method*. Feng et al. (2007) argue that the RBM may be more representative of rhizosphere conditions due to the LMWOAs used that are exudates of plants, fungi and bacteria that may mimic more real world conditions found within the rhizosphere. In simple batch extractions, the single medium used had been fungi grown on liquid media to simulate these similar conditions

referred to by the RBM chemically, however by a living organism. In determining extractability of Cd in all four samples, the RBM and Mehlich-III were statistically significantly ($p < 0.05$) different to the *P. involutus* medium. The extractability of Zn by *P. involutus* medium proved that the Mehlich-III and RBM extractions were statistically similar. This may suggest that in using the RBM as a qualifier for predicting bioavailability of Cd and Zn as a viable tool smelter contaminated soils, however other methods (i.e. Mehlich-III) should be used to test the efficacy of this sensitive extraction. Additionally, a greater understanding of how fungal inoculation in remediation projects that aid host species establish in these metal laden environments may lead to subsequent high initial release of heavy metals into the environment by leaching action.

2.5 CONCLUSION

The influence of inoculants on heavy metal mobility has profound effects on biota in regards of increased potential for uptake and release into the surrounding environment. Alongside with synthetic organic acids used to increase uptake in plants for remedial purposes, much care must be taken to insure that these applications are effective yet safe. Several single extraction methods were used to evaluate the extractability of Cd and Zn from four Palmerton soils against a liquid fungal medium derived from *Paxillus involutus* liquid culture. We found that the liquid culture extracted Cd and Zn similarly as commonly-accepted extractants used to determine bioavailability. There is still considerable work that needs to be done in characterizing exudates produced in mycorrhizal fungi when exposed to elevated metal levels. These exudates have the ability to sequester and release heavy metals within the rhizosphere, so much consideration must be taken when using fungal inoculants in conjunction with symbiotic plant species.

The Rhizosphere-Based Method proved to be a good qualifier as for comparing to the single fungal medium used for determining availability of Zn, where Mehlich-III extraction had greater efficacy for determining both Cd and Zn availability. These tests can act in preliminary assessments in determining the potential for release of heavy metals associated with amendment additions, such as mushroom compost, when vegetation is being reintroduced to contaminated sites. The use of polyaminecarboxylic acids such as EDTA and DTPA may over estimate labile

heavy metal concentrations and yet may underestimate what is generally considered by many the “exchangeables” or “water- and acid-soluble” fractions of the revised BCR sequential extraction method.

2.6 REFERENCES

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Table 2.1 Plot descriptions of site and sample locations at LGWR, Palmerton, Pennsylvania, U.S.A.

Site/Sample	Longitude/Latitude (WGS84)		Elevation (m)	Management History	Vegetative Cover
PLM-B1	-75.6212	40.7904	248	Aerial Fertilizer/Seed Application	60% Grasses
PLM-B2	-75.6217	40.7912	218	Aerial Fertilizer/Seed Application	95% Grasses
PLM-B3	-75.6223	40.7912	218	Aerial Fertilizer/Seed Application	Existing Tree Grove (Red Oak, Sassafras)
PLM-B4	-75.6225	40.7432	166	Amended with Turkey Manure, Compost, Lime, warm season grasses	80% Grasses, revegetated by <i>Betula</i> spp.

Table 2.2 Summary of Single Extractions used in Batch Leaching

Extractants	Leaching Time	Concentration	¹ SSR mL/g	Reference
EDTA	1 hr	0.005 M	10	Matús et al. (2005)
DTPA	1 hr	0.005 M	10	Matús et al. (2005)
Rhizosphere Based Method	16 hrs	-	10	Feng et al. (2005)
Mehlich-III	5 min	-	10	Mehlich (1984)
H ₂ O	24 hrs	-	5	Kubová et al. (2005)
<i>Fungal Media</i>				
Modified Melin Norkins (MMN)	16 hrs	-	10	-
<i>Pax</i>	16 hrs	-	10	-

¹SSR – Soil to Solution Ratio

Table 2.3 Soil Characteristics of the LGWR, Palmerton, Pennsylvania, U.S.A.

Sample	Soil pH _{H2O}	TOC (%)	CEC (meq/100 g)	Cd _{Total} (mg/kg)	Zn _{Total} (mg/kg)	Pb _{Total} (mg/kg)	Al _{Total} (mg/kg)	Fe _{Total} (mg/kg)	Mn _{Total} (mg/kg)	Ca _{Total} (mg/kg)	K _{Total} (mg/kg)	Mg _{Total} (mg/kg)
PLM-B1 (Non-Amended) ¹	5.42	18.3	28.6	254	13503	1735	10802	33188	8305	4342	675	1134
PLM-B2 (Non-Amended) ¹	5.60	6.8	13.7	138	7909	795	11781	32842	4630	2789	901	1133
PLM-B3 (Non-Amended) ¹	5.44	21.5	29.6	262	11170	1947	9934	33104	9794	3608	625	1024
PLM-B4 (Amended)	6.36	2.8	8.7	40	4783	197	16381	33308	2834	1674	917	1248
Typical ²	-	-	-	0.01-2	1-900	-	-	-	-	-	-	-
USEPA ³	-	-	-	1.2	150	-	-	-	-	-	-	-
PA DCNR ⁴	-	-	-	293- 1750	11800- 135000	-	-	-	-	-	-	-

¹All element totals of field samples were determined by Geoscience Laboratories, Ontario, Canada.

Non-Amended – collected outside “test-plots” from a non-amended location; Amended – collected within “Test-Plots” and have received a regime of amendments (In methods).

²Typical soil concentrations found within the environment in non-polluted conditions are from *Trace Elements in Terrestrial Environments: Biogeochemistry, Bioavailability, and Risk of Metals*, 2nd Edition, D. Adriano, Springer, New York, 2001.

^{3/4}Both USEPA and PA DCNR soil concentrations are from Region III Ecological Guidelines and the Palmerton Zinc Superfund Site, Palmerton, PA, respectively.

These values are found in *Preassessment Screen Determination for the Palmerton Zinc Pile Superfund Site, Pennsylvania DCNR, Harrisburg, 2003*.

Table 2.4 Revised BCR Sequential Extraction Results for Palmerton Soils

Sample	Cd _{pseudototal} (mg/kg)	Zn _{pseudototal} (mg/kg)	Fraction 1		Fraction 2		Fraction 3		Fraction 4		Sum of Fractions	
			Cd (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Zn (mg/kg)
PLM-B1 (Non-Amended)	278 ±23a	12900 ±2630a	88 ±3a	3700 ±223b	112 ±5a	3260 ±242a	24 ±4b	1155 ±69a	6 ±2ab	2325 ±572a	231 ±50a	10490 ±1124a
PLM-B2 (Non-Amended)	147 ±5c	7990 ±1360b	58 ±5b	3430 ±242b	50 ±2b	1010 ±101c	25 ±6b	675 ±65b	5 ±1b	2077 ±116a	138 ±24b	7211 ±1245b
PLM-B3 (Non-Amended)	250 ±11b	10700 ±1210a	89 ±6a	5390 ±264a	112 ±3a	2270 ±223b	38 ±4a	466 ±94c	8 ±1a	2464 ±471a	247 ±47a	10649 ±2036a
PLM-B4 (Amended)	69 ±2d	4650 205±c	14 ±4c	2880 ±247c	5 ±2c	233 ±66d	32 ±2a	350 ±66d	3 ±0.3c	1431 ±108b	55 ±13c	4911 ±1229c

Bureau of Common References Revised (Four-Step) Sequential Extraction Protocol (Rauret and others, 1999) was used to determine fractionation/ pseudototal; Non-Amended – collected outside “test-plots” from a non-amended location; Amended – collected within “Test-Plots” and have received a regime of amendments; Values (n=3) are means ± SD. Means followed by the same letters within fractions are not significantly different (p<0.05) defined by Tukey Kramer Method

Figure 2.1 Revised BCR Sequential Extraction results from LGWR, Palmerton, PA soils. Results presented are means of replicate analysis (n=3).

Figure 2.2 Plant Availability by extractant, where a weighted average across all samples was utilized to determine the overall efficiency of each extractant to estimate plant nutrient availability (n=12). Means±SE followed by the same letters within rows are not significantly different (p<0.05).

3 Effect of *Paxillus involutus* on fractionation and uptake by *Populus* hybrid of zinc and cadmium in zinc-smelter contaminated soils

3.1 INTRODUCTION

Contamination of soils and water with heavy metals is a worldwide problem. Anthropogenically-released metals are of major concern because of their availability, toxicity, and persistence in ecosystems. Approximately 3,000 tons of cadmium (Cd) and 52,000 tons of zinc (Zn) are released every year in air and water by mining and smelting activities (UNEP, 2011). Both of these metals have the capacity for acute and long-term effects in humans including high reproductive and developmental implications, organ failure, and potential cancer initiators.

Active and past smelting of metalliferous ores has led to large accumulations of metals in surrounding landscapes due to atmospheric deposition. One such area found in east central Pennsylvania, USA, surrounding the New Jersey Zinc Smelter in Palmerton, PA. Although smelting ceased in the 1980's, extensive soil contamination eliminated vegetation from the surrounding mountains. Such "hot-spots" act as areas of nonpoint source pollution, where metal-contaminated soil particles may be mobilized through erosion processes or metals may leach to groundwater or adjacent to water bodies. Typical methods of ameliorating metal-contaminated soils include soil washing with chemical extractants, electromechanical stripping, excavation and removal, and capping (Adriano, 2001). Such engineering approaches to remediation are expensive, prone to mobilizing metals, and may be impractical in certain landscapes. Additional limitations of the mechanical/chemical amelioration approaches include the loss of soil nutrients, organic matter, and microbial communities in the soil that are critical for ecosystem function.

Plant-assisted bioremediation (phytoremediation) is a low cost, effective means for ameliorating metal-contaminated sites (McGrath et al., 2001; McIntyre, 2003; Khan, 2005). Phytostabilization and phytoextraction have been recognized as approaches to reduce metal mobility and toxicity in the environment. The establishment of vegetative cover on metal-contaminated sites will reduce wind and water erosion, reducing the flux of contaminant metals

to other ecosystems. Furthermore, phytoremediation creates possibilities for harvesting biomass and metals within the biomass and reduce site-specific metal loads (Chaney et al., 2007). In addition, the use of phytoremediation technologies may be more effective over extensive areas or in terrain that is unsuitable for standard engineering approaches. For example, in Germany, farmers used metal accumulating willow varieties to mitigate cadmium-contaminated farmlands, where soils were returned to vegetative production within a period of 6 years (Lebeau et al., 2008).

Gaining a greater understanding and exploiting the synergies that exist between plants and soil symbiotic microorganisms may prove effective in metal contaminated soil remediation efforts. Mycorrhizal fungi (both ectomycorrhizae and arbuscular mycorrhizae) have shown excellent efficacy in reducing metal toxicity to plant hosts (Hartley et al., 1997; Godbold et al., 1998). These symbionts assist in ameliorating toxicity of metals by transforming and sequestering these metals within the rhizosphere and intracellularly within fungal tissues (Gadd, 2001; Hall, 2002; Bellion et al., 2006; Meharg, 2003). Extracellularly, metal immobilization can be accomplished when metal ions bind to the complex macromolecular compounds that comprise the cell wall (biosorption). Precipitation and chelation of toxic metals may also occur by interactions with low-molecular-weight ligands produced by microbes as metal detoxification mechanism (Gadd, 1993; Johansson et al., 2008). Intracellular detoxification predominately relies on the internal sequestration of toxic metals by metal-induced molecules, such as metallothioneins and phytochelatins (Inouhe, 2005).

Paxillus involutus, a common ectomycorrhizal fungus, has been shown to ameliorate metal toxicity to its host species by alleviating metal stress through a myriad of pathways (Fomina et al., 2005; Denny and Wilkins, 1987; Ott et al., 2002; Hegedűs et al., 2007; Blaudez et al., 2000). Formation of thiol-containing compounds from cysteine and its derivatives (γ -glutamylcysteine, glutathione) and higher molecular mass compounds metallothioneins due to exposure with cadmium was observed in cytosolic fluids of *P. involutus* as a means to curb metal toxicity (Courbot et al., 2004). Schützendubel and Polle (2002) discuss how mycorrhizae protected pine roots against the negative effects of Cd, where no significant change in fresh

biomass due to Cd treatment was experienced, when seedlings were 80% associated with a wild strain of *P. involutus* isolated from metal contaminated site.

Accumulation of Cd and Zn is well documented in *Populus* spp. (Dos Santos Utmazian et al., 2007; Gadd, 2001). Tree species within the genus *Populus* are good candidates for rehabilitating contaminated sites due to their ability to accumulate relatively high concentrations of metals, easy propagation, deep root nature, adaptability to most soils, and high growth rates (Chaney et al., 2007). Although not recognized as a hyperaccumulator, hybrid poplar has increasingly become a model organism for phytoremediation (Wu et al., 2010). Phytostabilization of metals by poplar may occur through increases in soil organic matter, root exudation that may cause metal precipitation and toxicity/mobility, sorption to root surfaces, as well as uptake and intercellular sequestration (Wenzel et al., 2004; Wenzel, 2009; Gratião et al., 2005). Poplar grown in soils amended with metal-resistant mycorrhizae such as *P. involutus* may present a promising approach for metal stabilization in contaminated soils, reducing the potential for environmental harm while being economically feasible (Krpata et al., 2009; Sell et al., 2005).

Sequential extraction is a useful, indirect approach for identifying the behavior of potentially toxic metals in soils by operational speciation (Bacon and Davidson, 2009). Little work has been done utilizing sequential extraction in greenhouse or *in situ* experiments monitoring the “before” and “after” effects of trees or their symbiotic mycorrhizal fungi on metal fractionation in contaminated soils. Tao et al. (2005) recognized that the complexity of soil-plant interactions alter physiochemical parameters of the rhizosphere, which may lead to fractionation changes. Furthermore, chelate-assisted phytoextraction using a chelating agent (EDTA) has been used with *Taraxacum officinale* to increase in the proportion of analytes recovered from available fractions and increase the uptake of metals by the plant (Massop et al., 2009). No work has explored the effects of mycorrhizal relationships on fractionation changes of metals in soils. As the vast majority of higher plants are mycorrhizal, understanding the influence of mycorrhizae on metal fractionation is important in elucidating metal behavior in re-vegetating soils contaminated by metals.

In the present study, we present the application of the European Commission, Community Bureau of Reference (BCR) sequential extraction procedure to observe changes in fractionation in two contaminated soils from an abandoned zinc-smelter site. These soils were utilized in a greenhouse experiment with a *Populus* hybrid (*P. deltoides* x *P. trichocarpa*) and the ectomycorrhizal fungus *P. involutus*. During the greenhouse experiment, leachate was collected to monitor leaching of analytes of interest. The fractionation patterns of Cd and Zn before and after plant/fungal growth were assessed by the revised BCR three-step sequential extraction method to gain insight into how operational fractions were altered by poplar and its ectomycorrhizal symbiont.

3.2 MATERIALS AND METHODS

3.2.1 Soil samples

Soil samples were collected from the Lehigh Gap Wildlife Refuge and Nature Center (LGWR) located at the Palmerton Zinc Superfund Site in Palmerton, Pennsylvania, U.S.A. Four soil samples were collected from different locations within the LGWR. A sample was collected from a preexisting test plot and the others gathered from a non-amended area. The amended test plot is one of many plots on site that had been amended with various fertilizers, mushroom compost, and a warm season grass mix. The site is located adjacent to a historical zinc smelter(s) that had been in operation for nearly a century and has subsequently been turned into USEPA Superfund Site following the closure of both facilities. Activity at these smelters has led to the defoliation of approximately 8.1 km² (2000 acres) in the vicinity (USEPA, 1987) mostly due to aerial deposition of potential toxic elements cadmium, lead, and zinc. The landscape is mostly a boulder field developed in the last Pleistocene glaciations (Pazzaglia et al., 2006) where little to no soil has developed. Samples were gathered from the top 0-10 cm depth in between boulders.

3.2.2 General soil characterization

Soil pH was measured using a 1:1 soil slurry with deionized water and air-dried soil (Thomas, 1996). Organic matter content was measured by loss in ignition at 500 °C. Cation

exchange capacity was determined using the compulsive exchange reaction (Hendershot and Duquette, 1986). Soil samples (2 g) were weighed and placed into 50-mL centrifuge tubes. The first centrifuge washing step with 20 mL of 24 g/L $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ was followed by three independent washing steps with 20 mL of 6 g/L $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$, each executed for a 2 hr period on a shaker table at 150 rpm. Samples were then subjected to a single overnight mixing-step with 20 mL of 3 g/L MgSO_4 . The mixture was then centrifuged at $2500 \times g$ for 10 min, followed by decanting and filtration of the supernatant with a 0.45- μm Whatman syringe filter. Filtrates were stored at 4 °C until analysis.

Measurements of extractability of Cd and Zn were made using three single-batch extraction techniques to gather more understanding of bioavailability of these elements. Matúš et al. (2007) developed a simple method using EDTA and DTPA to determine phytoavailable concentrations of Al in environmental samples. We modified this technique to be utilized for determination of the Cd and Zn, where none of the experimental methods were changed. Mehlich-III extraction, a more commonly used method was also employed to evaluate levels of available Cd and Zn in soils and their potential for phytoavailability (Mehlich, 1984). All three extractions gave a proxy of the amount Cd and Zn that can be easily removed by relatively weak extractants.

3.2.3 Fungal culture

Paxillus involutus (ATCC 200175) was grown on a modification of modified Melin-Norkrans (MMN) agar media. The MMN media contained the following: 10 g/L glucose, 0.26 g/L $(\text{NH}_4)_2\text{HPO}_4$, 0.54 g/L KH_2PO_4 , 0.06 g/L MgSO_4 , 0.11 g/L CaCl_2 , 1.5 mg/L NaCl, 1.9 mg/L KCl, 0.77 mg/L H_3BO_3 , 0.17 mg/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.29 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.062 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.060 mg/L Na_2MoO_4 , 0.060 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 4.2 mg/L Fe, Na-EDTA, 1 mg/L thiamine HCl, 0.41 mg/L pyridoxine HCl, 1.1 mg/L ascorbic acid, and 4.9 mg/L nicotinic acid (Cumming et al., 2001). The pH of the media was adjusted to 5 using hydrochloric acid and 10 g/L of agar was added before autoclaving. Following four weeks of growth on solid media, fungal colonies were transferred to 250-mL conical flasks containing ~50 mL of liquid MMN

media with an additional 14 mg/L Zn treatment. These cultures were to generate fungal inocula for greenhouse experiment with *Populus*.

3.2.4 Growth experiment

Before planting, two field-collected soils that had been sun-dried were separately homogenized in a cement mixer and sieved with a 10-mm aperture wire mesh. Common unrooted hybrid poplar DTAC-7 (*Populus trichocarpa* Torr. and Gray $\times$ *Populus deltoides* Marsh.) were used in this experiment and purchased from hybrid poplar grower (Segal Ranch, Granview, WA). Plants were watered with a modified artificial rain solution (Lee and Weber, 1979) containing 1.6 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.85 g/L NaNO_3 , 1.6 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.40 g/L KNO_3 , 1.3 g/L $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, and 0.68 KH_2PO_4 . Watering of pots with artificial rain solution was conducted daily, where initially each received approximately 15 mL. Subsequently after placing pots beneath lamps, watering was conducted twice a day to encourage growth and minimize water loss due to transpiration.

The greenhouse experiment was conducted over an 8-week period in a climate-controlled glasshouse. Plants were grown in 5-cm diameter $\times$ 17.5 cm deep pots ('Cone-tainers' TM, Stuewe and Sons, Corvallis, OR) with fine mesh screening at the bottom to minimize loss of soil. Pots containing no poplar cuttings or mycorrhiza (untreated = u), containing mycorrhiza (myc), were established in triplicate (n=3). Pots containing poplar cuttings and lacking mycorrhiza (poplar = pop) and cutting with mycorrhiza (pop $\times$ myc) each had a sample size of n = 16. Cuttings approximately 15 cm in length and 5 mm in diameter were inserted into pots that were filled with approximately 0.035 kg of acid-washed coarse-grained sand to encourage proper drainage and filled to the rim with approximately 0.240 kg of soil. Pots with mycorrhiza treatment were first filled three-quarters full with soil then an aliquot of 40 mL of fungal slurry, containing both the fungal tissue plus MMN. In addition, five plugs (1-cm in diameter) were added to encourage colonization. Following fungal inoculation, the pots were filled to the rim and weighed. Cuttings were grown in a climate-controlled greenhouse with supplemental lighting providing a 14-h photoperiod (mixed metal halide source) and day and night temperatures of $24/19 \pm 3$ °C. Relative humidity fluctuated with temperature and day.

As leaves began to be lost, they were collected and retained for analysis. At the end of the greenhouse experiment, remaining leaves were removed and were amassed with fallen leaves per individual pot. Soil was washed from roots with deionized water by hand. Both the leaves and roots were oven dried at 60°C and stored until analysis, and soils were air-dried at room temperature.

3.2.5 Digestion of plant and soil

Plants analysis was accomplished by washing shoots and roots with deionized water, drying leaves and roots at 60 °C and grinding to pass through a 20-mesh sieve. Both shoots and roots were digested by microwave-assisted digestion. Plant material (0.1 g) was digested in 5-mL of 70% HNO₃ and 2 mL of 30% H₂O₂ and allowed to react overnight. The samples were then microwave digested at 200°C for 1.5 hr. The digested solutions were passed through a 0.45-μm Whatman syringe filter.

Pseudototal concentrations of soils were determined of field-collected soils and rhizosphere soils collected following the greenhouse experiment. Soils were air-dried, passed through a 2-mm sieve, placed in closed Teflon digestion vessels, and were subject to microwave-assisted digestion in a MARSXpress system (CEM USA, Matthews, NC). Samples (3 g) were digested with 28 mL of aqua regia (3:1 mixture of 37% HCl and 70% HNO₃) at room temperature for 16 hr. Subsequently, the samples were then transferred to the microwave digester at 130 °C at reflux conditions for 2 h. The supernatant was then filtered through a 0.45-μm Whatman syringe filter and diluted to 100 mL with 0.5 M HNO₃ and stored in polyethylene bottles at 4 °C until analysis.

3.2.6 Sequential extraction

Both native and pot experiment rhizosphere air-dried soils were subjected to the revised (four-step) sequential extraction protocol developed by the Bureau of Common References (Rauret et al., 1999). Each soil sample was extracted in triplicate. The pH of the extractant was

measured at the beginning and end of the agitation of the sample in each step, excluding step 4. All the extractants from each step were passed through a 0.45- μm Whatman syringe filter and refrigerated at 4 °C until analysis.

Step 1 (water/acid soluble and exchangeable fraction): 1 g sample was treated with 40 mL of 0.11 M HOAc solution and was agitated on an orbital mixer at 30 $\pm$ 10 rpm for 16 hr at room temperature. Separation of the supernatant was accomplished by centrifugation at 3000 X g for 20 min. Following the removal of the supernatant, a washing step with 20 mL of deionized water for 15 min on the orbital mixer and centrifuged at 3000 X g for 20 min. The supernatant was then decanted and discarded.

Step 2 (reducible fraction): The solid residue from the previous step was subjected to 40 mL of 0.5 M $\text{HONH}_2\cdot\text{HCl}$ solution, adjusted to a pH of 1.5 with a fixed volume of 2 M HNO_3 . The sample was agitated on an orbital mixer at 30 $\pm$ 10 rpm for 16 hr at room temperature. The supernatant and residue was separated similarly as the first step. The residue was washed in the same fashion as the previous step and the supernatant was decanted and discarded.

Step 3 (oxidizable fraction): The residue from step 2, 10 mL of 8.8 M H_2O_2 solution was added. The slurry was digested for 1 hr at room temperature and placed on a block digester for 1 hr at 85 $\pm$ 5 °C, and the volume was reduced to less than 3 mL. An additional 10 mL of H_2O_2 solution was added and was digested for 1 hr at 85 $\pm$ 5 °C, and the volume was reduced to approximately 1 mL. The extraction was accomplished by the addition of 50 mL of 1 M NH_4OAc to the slurry where it was agitated on an orbital mixer at 30 $\pm$ 10 rpm for 16 hr at room temperature. The supernatant and residue was separated similarly as the first step. The residue was washed in the same fashion as the previous step and the supernatant was decanted and discarded.

Step 4 (residual fraction): The residue from the previous step was digested by aqua regia, however the volumes of HCl and HNO_3 used have been reduced to 7 mL and 2.3 mL, respectively. The mixture was digested similarly as the Pseudototal determination.

3.2.7 Analyses and quality control

All glassware and plasticware were washed with 0.2 M HCl and allowed to soak overnight and rinsed with nanopure water. All reagents were of analytical grade. Concentrations of Cd, Zn, and Mg were determined using a Varian 220FS atomic absorption spectrometer (Varian Inc., Mulgrave, Vic., Australia). Blanks were included in microwave digestions and sequential extractions, and quality of analysis was analyzed by spike and recovery. BCR CRM 701 was analyzed by BCR procedure, to ensure proper extraction using the BCR revised method. NIST SRM 1515 was used to ensure proper digestion of leaves using microwave digestion. Comparison between summed fractions of the sequential extraction and the Pseudototal of a separate aqua regia digestion were compared to determine the effective extractability of the analytes of concern, as recommended by Rauret et al. (1999).

The Translocation Factor (TF) was calculated using the final root and shoot metal concentrations ($TF = [M]_{\text{Shoot}}/[M]_{\text{Root}}$). Biomass was calculated using the final root and shoot mass (Biomass = Root Mass + Shoot Mass). Shoot and root ratio was calculated using final root and shoot mass (Shoot:Root = Shoot Mass/Root Mass). Total metal accumulation was calculated for each treatment by multiplying the dry weight of shoots (kg) by the metal concentration in the shoots (mg/kg).

Statistical analysis used to determine the effects of mycorrhizae vs. non-mycorrhizae on plant mineral content were analyzed by two-way analysis of variance (ANOVA) separately per soil type, followed by Tukey-Kramer's HSD test (significance level of $P < 0.05$) to identify significant difference between plant content and response means. Statistical analysis was used also to determine the effects of mycorrhizae and poplar on soil mineral content using a two-way ANOVA soil type, followed by a Tukey Kramer's HSD test (significance level of $P < 0.05$) to identify significance between soil content and response means. Statistical analyses were carried out using SAS JMP 9.0 (SAS Institute, Cary, NC).

3.3 RESULTS

3.3.1 Soil characteristics

Soils collected from the LGWR were heavily contaminated with both Cd and Zn. Samples collected from various locations within the LGWR exceed typical concentrations of both Cd and Zn in instances where these levels exceed 15- and 130 –fold, respectively (**Table 3.1**). All soils exceed concentrations that warrant remedial action set by the USEPA (USEPA, 2001). Soil pH values of samples collected show mild acidity, a product of parent materials from which the soils originated (Web Soil Survey USDA NRCS). The two soils used PLM-B2 (non-amended) and PLM-B4 (amended) that proved to be less phytotoxic contained less TOC and had much less CEC than the two soils omitted (both non-amended), which displayed phytotoxicity to poplars grown in these soils. Common soil extraction methods were used to determine the bioavailability of both Cd and Zn. Both the EDTA and DTPA extractions tended to remove similar concentrations of each analyte from each substrate. Mehlich-III extraction showed to be similar to the other extraction values only for PLM-B4, where for the other soils this extraction varied greatly and underestimated bioavailability by 2- to 3-times less.

3.3.2 Quality control

The recoveries of analytes (Cd and Zn) using spike and recovery in extracts and digests were 100±20%. Recovery of analytes (Cd and Zn) from BCR CRM 701 from each batch sequential extraction was 100%. The recovery of analytes (Cd and Zn) from the BCR extraction procedure, where combining the individual steps and comparing amounts detected to the results of a separate aqua regia digest, ranged from 73% to 144%. The recovery of analytes (Cd and Zn) from NIST SRM 1515 apple leaves standard to determine the completeness of microwave digestion of plant materials was 90%.

3.3.3 Tree growth and plant metal concentrations

Tree growth in soils was limited to two soils (PLM-B2 and PLM-B4). The remainder of this discussion will only mention the results of these soils and will be referred to as non-amended and amended, respectively. **Table 3.2** contains all greenhouse experiment information regarding growth and concentrations of Zn and Cd in both roots and shoots. Biomass showed to be greatest in poplars grown in amended soils ($p < 0.005$). Mycorrhizal association proved to be effective in increasing biomass in both treatments ($p < 0.05$). Root to shoot ratios was higher in amended soils ($p < 0.05$), where root growth is higher in these soils however they showed no mycorrhizal effect.

Cadmium trends illustrate that treatment effects had greatest influence alleviating toxicity in poplars grown in amended soils. Mycorrhizal association in both treatments increased root and shoot Cd content. In Cd shoot content, all treatment effects were very significant ($p < 0.005$), where for root content soil type ($p < 0.005$) and mycorrhizal association ($p < 0.05$) were the causation for success. The total content of Cd was affected greatest by mycorrhizal treatment ($p < 0.01$) allowing for increased accumulation of Cd in shoots. The interactive effect of soil and mycorrhizal treatment ($p < 0.005$) followed by mycorrhizal treatment ($p < 0.01$) had the largest impact on the translocation of Cd.

Zinc in plants showed that amended soils had greater success than non-amended soils in the outcome of zinc distribution. Zinc in shoots was greatest in amended soil, where in roots, zinc was highest in non-amended soil. In zinc shoot content, it was observed that mycorrhizal effect is significant ($p < 0.05$) for the translocation of Zn to the aerial component of the host. The total content of Zn in the aboveground compartment was two-fold greater in amended versus non-amended soil. This trend is also observed in the translocation values for zinc, where amended soil plant treatments out compete non-amended soil treatments.

3.3.4 Pseudototal metal concentrations

Pseudototal (aqua-regia extractable) metal concentrations in soils used are tabulated in **Table 3.3**. *U* indicated untreated (receiving only artificial rain solution), *Myc* = addition of

mycorrhizae to soil, *Pop* = presence of Poplar, *Myc* x *Pop* = addition of mycorrhizae and presence of Poplar. All soils were above criteria set by all regulatory values presented in **Table 3.1** for both cadmium and zinc. However, these concentrations are less than reported by literature compiled from the USFWS report for Palmerton (USFWS, 2003). Results for *untreated* soil were consistent with the field sample exemplifying reproducibility of the aqua-regia extraction. Cadmium was highest in *U* from amended soil, where in non-amended soil, *Myc* contained highest concentration. Zinc was highest in *U* and *Pop* x *Myc* soils in both soil types. Overall reduction in concentration in both cadmium and zinc in both amended and non-amended soils was with *Pop* treatment. Soils were not appreciably deteriorated of both cadmium and zinc based on their pseudototal values, due to the small application of artificial rain solution to leach analytes and short growth period to allow significant root and shoot growth to deplete aqua-regia soluble soil metal content.

3.3.5 Sequential extraction of greenhouse soils

The Revised BCR Sequential Extraction scheme was used to determine how choice poplar and mycorrhizae could alter soils collected from the LGWR. **Figure 3.2** and **Figure 3.3** both illustrate fractionation (%) change between treatments compared to the field sample. Cd fractionation for non-amended soil demonstrates an increase in exchangeable, reducible, and residual fractions as compared to Field Sample soil. In amended soil, all fractions besides the oxidizable component were altered significantly. The pool from which other fractions gain greatest Cd content from is from the oxidizable fraction, where the Field Sample contains 58%, the other treatments range from 2-6%. Zn fractionation for non-amended soil exhibits a decrease in exchangeable and residual fractions, with a general increase in both reducible and oxidizable fractions as compared to Field Sample soil. As for amended soil, exchangeables are reduced in all treatments and decrease in residual fraction for both *Pop* and *Pop* x *Myc* treatments (~10% reduction). Reducible fraction was increased in all treatments with exception of *U*.

Cadmium fractionation patterns in non-amended soils are shown in **Figure 3.1**. In Fraction 1, all treatments maintain or fell below the original concentration of the Field Sample, with exception to *Myc* treatment. In the reducible Fraction 2, all treatments increase

concentration of Cd with exemption to *Pop* treatment that showed a decrease in reducible Cd. In Fraction 3, all treatments reduced Cd in the oxidizable form, with the greatest decrease of 97% in *Myc* treatment. In the remaining residual Fraction 4, all treatments with exception of *Pop* x *Myc* Treatment were greater than the original Field Sample, with maximum increases of a 100 %. The changes in Fractionation for amended soils are illustrated in **Figure 3.1**. As for Fraction 1, all treatments show an increase in Cd, with a 192 % increase in the greatest effected soil. In the reducible Fraction 2, all treatments have an increase in Cd, with a 333% increase in the *Pop* x *Myc* Treatment that had the largest increase in Cd in this fraction. Fractional change in oxidizable Fraction 3, all treatments fell way below the original concentration of the Field Sample with average reduction overall treatments of 91%. In Fraction 4, residual concentration increased significantly for treatments without poplar (Average over both non-poplar treatment of 316% increase in Cd), where both treatments containing poplar maintained relative concentrations similar to the original Field Sample Cd concentration.

The fractionation patterns for Zn in non-amended soils are shown in **Figure 3.2**. In Fraction 1, all treatments had been altered and reduced compared to the Field Sample. In the reducible fraction, there was an increase in all fractions with all means showing similarities with exception to the *U* treatment, with increases in Zn concentration up to 100%. Fractionation change in the oxidizable fraction increased in all fractions with the greatest at 42% increase in Zn. The residual fraction illustrates an overall decrease in Zn in all treatment with the greatest reduction of Zn in *Pop* x *Myc*. The fractionation patterns for Zn in amended soils are shown in **Figure 3.3**. Fraction 1 of the sequential extraction scheme exhibits an increase in all treatments with exception to the *Myc* treatment. In Fraction 2, all treatments with exception to the *U* treatment demonstrated an ~300 % increase in Zn compared to the Field Sample recorded value. In Fraction 3 the oxidizable component increased in all treatments, with greatest increase at ~200% increase in Zn as established from the original Field concentration. In the remaining residual fraction, there was a split in fractionation change compared to soils with poplar and without. Soils grown with poplar saw a 39% decrease in Zn, where soils grown without increased in Zn concentration.

3.3.6 Mobility of metals in soils

In non-amended soils, metal mobility from the cones was initially high and declined markedly over time (**Figure 3.4**). Average concentrations for Cd in non-amended soils in leachate in the first week were 0.05 mg/kg and declined to 0.006 mg/kg after 60 days across all treatments. In these soils, with addition of *P. involutus* in the very short term (<4 days) led to increased mobility, but then Cd export declined in these treatments for the rest of the experimental period. On a mass basis, Cd was most mobile from cones with *U* and *Pop* treatments and declined with the addition of *P. involutus* to either cone containing the *Myc* or *Pop* x *Myc* treatment. The sum total difference of Cd exported from cones due to the addition of *P. involutus* was 0.25 mg/kg from cones without poplar (*Myc*) and 0.31 mg/kg with poplar cuttings (*Pop* x *Myc*). The sum total difference of Cd exported from cones without inoculation was 0.26 mg/kg for both treatments. The addition of poplar cuttings alone across all treatments on average leached 11% more Cd than cones without any poplar cuttings addition.

The average Zn concentrations in leachate in the first week were 2.1 mg/kg and declined to 0.48 mg/kg after 60 days across all treatments. In these soils, with addition of *P. involutus* in the very short term (<7 days) led to increased mobility, but then Zn export declined in these treatments for the rest of the experimental period. On a mass basis, Zn was most mobile from cones with *U* and *Pop* treatments and declined with the addition of *P. involutus* to either cone containing the *Myc* or *Pop* x *Myc* treatment. The sum total difference of Zn exported from cones due to the addition of *P. involutus* was 19.7 mg/kg from cones without poplar (*Myc*) and 27.3 mg/kg with poplar cuttings (*Pop* x *Myc*). The sum total difference of Zn exported from cones without inoculation was 24.5 mg/kg (*U*) and 29.8 mg/kg (*Pop*). The addition of poplar cuttings alone across all treatments on average leached 22% more Zn than cones without any poplar cuttings addition.

Concentrations on average for Cd in amended soils for leachate in the first week were 0.0092 mg/kg and dropped to 0.0016 mg/kg after 60 days across all treatments. Same conditions persisted for Cd as did Zn in these soils, metal mobility due to addition of *P. involutus* inoculum

was significantly longer than non-amended soils (~25-30 days). The sum total difference of Cd exported from cones due to the addition of *P. involutus* was 0.05 mg/kg from cones without poplar (*Myc*) and 0.06 mg/kg with poplar cuttings (*Pop* x *Myc*). The sum total difference of Cd exported from cones without inoculation was 0.02 mg/kg (*U*) and 0.03 mg/kg (*Pop*). The addition of poplar cuttings alone across all treatments on average leached 19% more Cd than cones without any poplar cuttings addition.

As with amended soils, metal mobility was originally high with concentrations of Zn exceeding in the first week 0.84 mg/kg and declined to 0.094 mg/kg after 60 days across all treatments. However, in amended soils, the pattern of stimulated metal mobility due to addition of *P. involutus* inoculum was significantly longer than non-amended soils (**Figure 3.4**). Inoculation of soils alone, sustained Zn export for 15 days from cones containing *Pop* treatment, where in *Pop* x *Myc* treatment sustained Zn leaching occurred for duration of the experimental period. The sum total difference of Zn exported from cones due to the addition of *P. involutus* was 4.8 mg/kg from cones without poplar (*Myc*) and 7.08 mg/kg with poplar cuttings (*Pop* x *Myc*). The sum total difference of Zn exported from cones without inoculation was 3.85 mg/kg (*U*) and 2.37 mg/kg (*Pop*). The addition of poplar cuttings alone across all treatments on average leached 9% more Zn than cones without any poplar cuttings addition.

3.4 DISCUSSION

Soils collected from the LGWR are extremely contaminated with Cd and Zn reflecting approximately 100 years of smelting that has led to the complete defoliation of the areas surrounding the two smelting facilities and the surrounding vicinities, including the community of Palmerton, PA (USEPA, 1987). It has been estimated that over the 80 years of smelting in the borough of Palmerton, approximately 3740 tons of Cd and 286,000 tons of Zn had been emitted through aerial deposition (USFWS, 2003). Total Cd and Zn from soils collected determined by aqua regia digest ranged 40 to 262 mg/kg and 4783 to 13503 mg/kg, respectively. Based on batch extractions to determine extractability of Cd and Zn, the effectiveness shows similar extractability by both EDTA and DTPA for both of these analytes (Figueroa, 2015). The Mehlich-III extraction, a standard method developed to evaluate levels of available micro- and

macronutrients in soils in the southeastern USA (Mehlich, 1984), extracted nearly half than the other two extractants for Zn in non-amended soil PLM-B2, but removed equal amount of Zn in amended soil PLM-B4. Whereas for Cd, Mehlich III extraction removed approximately two-thirds less Cd than EDTA and DTPA in PLM-B2 soil and half the amount for PLM-B4 soil. The amended soil (PLM-B4) had been amended with agricultural lime, fertilizer, mushroom compost, and turkey manure as part of a 56 one-acre test plots remediation plan in 2003 to evaluate the success of planting warm-season grasses to revegetate hillslope at the site. Results from the test plots in 2003 indicated that where mushroom compost was applied 63% live plant cover existed and in 2004 over 81% coverage had been accomplished (Latham et al., 2007). In these soils it suggests that due to amending and plant growth the pool of Cd and Zn has been reduced by sequestration into vegetation and redistribution into less-labile forms as compared to soils collected outside of the test plots.

In two soils, PLM-B1 and B3, concentrations of heavy metals evidently exceeded levels to permit growth of poplar. These soils both contained the highest Cd and Zn concentrations across all soils collected (**Table 3.1**). In these soils, poplar cuttings initiated limited shoot elongation, but roots were unable to develop sufficiently to overcome the phytotoxicity of these soils and were not evaluated in the growth and ‘after’ soil experiments. When Zn and Cd is in large supply, inhibition of root elongation and chlorosis in young leaves are common (Marschner, 2002) and were observed in this study for poplar grown in these soils. In the remaining soils, PLM-B2 (*non-amended*) and B4 (*amended*), poplar cuttings became established and were aided by the application *P. involutus* inoculum and additionally influenced the uptake and mobility of metal in these soils (**Table 3.2** and **Figure 3.4**). Across all treatments, plant growth was 62% greater in amended soil as compared to non-amended soil, with most allocated to root growth. With the addition of *P. involutus* inoculum plant growth increase 27% across the two soils focused on in this study. The shoot:root ratios of Cd and Zn were below the threshold of 1 (**Table 3.2**) and metals were mostly sequestered in the roots, which is typical of non-hyperaccumulating plants (McGrath et al., 2001). The “bioavailable” pools of Cd and Zn in these soils were significantly higher than the soils not used (PLM-B1 and B3) as determined by the summation of the first three-steps of the sequential extraction protocol (Bacon and Davidson, 2009), however this interpretation is not straightforward. Pooling all samples R^2 values were

0.91 for Cd (both tissue types) and 0.69 for Zn (both tissue types); for non-mycorrhizal treatment, R^2 values were 0.95 for Cd and 0.57 for Zn; and for mycorrhizal treatment, R^2 values were 0.91 for Cd and 0.78 for Zn.

The differences observed in treatments were driven by differences in availability of Cd and Zn in these soils. Zinc concentrations of roots in amended soils were less than that of non-amended soils, yet shoot Zn concentration was higher in amended soils. This pattern may be a result of increased growth and soil exploration of roots with lesser extractable Zn, where enhanced shoot growth and leaf area would lead to greater transpirational accumulation of Zn in leaves. This phenomenon may reflect that different species have morphological features associated with leaves that may alter and affect evapotranspiration, translating that to metal uptake rates and patterns of movement to new shoots (Dos Santos Utmazian et al., 2007; Hall, 2002). In general, it is believed natural Zn tolerance is by either exclusion or compartmentalization (McIntyre, 2003; Hammer and Keller, 2002), by organic acid exudation, cell wall immobilization, or vacuolar sequestration (Ryan et al., 2001; Qin et al., 2007). Through mycorrhizal symbiosis, it has been observed that alteration may occur in root-to-shoot translocation or preferential change in localities where Zn is stored extra-/intracellularly both within the host and symbiont (Jones et al., 2003; Krpata et al., 2009).

In contrast to Zn, Cd concentrations in poplar roots and shoots were less in amended soils than in non-amended soils, suggesting that the treatment of soils with amendments warm-season grasses mentioned above may have altered the soil's structure and composition to improve the success of these pioneering species. The increasing of pH by liming, addition of organic matter from fertilizer and manure, and reintroduction of soil biota by inoculation with mushroom compost may have all led to the redistribution of heavy metals to a more favorable speciation increasing uptake by plants and soil biota or may have reduced the activity of metals by sequestration by complexation into in-/organic forms. Reclamation may have increased the bioavailability of Cd and its subsequent uptake and impact on poplar. This inference is based on increased TF values for both treatments, suggesting the increased translocation of Cd from the roots to shoots as compared to Zn (**Table 3.2**). This led to a 35% greater translocation of Cd overall treatments versus Zn in these soils. Inoculation of amended soils with *P. involutus* did

lead to increased foliar and root concentrations, which may reflect a change in mobility and bioavailability of Cd in this treatment.

The synergistic properties of Cd and Zn make these two elements association unique in which they may display similar attributes and properties in biological systems. As seen in this experiment and others (Wu et al., 2010; Koopmans et al., 2008), greater translocation of Cd may be due to the following: active transport, lack of absorption by chelators either fixed or soluble in the soil-root interface, or interaction and competition with other divalent ions for root uptake (Das et al., 1997; Lorenz et al., 1997). Other responses mechanisms of higher plants to Cd include mechanisms described as means for Zn tolerance but also include the formation of phytochelatins, metallothioneins, peroxidases, stress proteins, and ethylene production (Sanità di Toppi et al., 1999; Prasad, 1995). These mechanisms are enacted to effectively cope with Cd stress through exclusion, detoxification, and damage repair onset by Cd interaction.

The fractionation of metals from soils collected from LGWR was altered by the growth of poplar and the introduction of *P. involutus* in the soils (**Figure 3.1**). Difference between the “Before” treatment (field collected soil) and the four other treatments reflect changes that occur in metal biogeochemistry from the activities of native microbes (“U”) or introduced mycorrhizal fungi (“Myc” and ‘*Pop x Myc*’) or poplar (“Pop” and “*Pop x Myc*”). Bacon and Davidson (2009) address the usefulness and strength in sequential extraction when comparing ‘before and after’ application in monitoring general change in metal reactivity in operational fractions and to observe the effectiveness of the treatment used. In general, the use of soils in the greenhouse in any of the treatments led to decreases in extractable and residual Zn and increases in reducible and oxidizable Zn content. The increase of Zn in both reducible and oxidizable fractions (**Figures 3.2 and 3.3**) may reflect fluxes from the exchangeable and residual pools induced by biological activity and uptake. In contrast, Cd displayed an increase in the exchangeable, reducible, and residual fractions and a decrease in the oxidizable fraction. pH and organic matter are two of the most important factors in controlling Cd availability (Jastrow and Koeppel, 1980; Bradl, 2004; Adriano, 2001). Due to the properties of materials associated with the oxidizable fraction (organic matter), during plant growth this pool can be easily depleted due to high instability due to pH and biogeochemical cycling of carbon (Haung et al., 2005). In Canadian

forest soils Kirkham (2006) reviews that the quality of soil organic matter may be the limiting factor on the ability to bind and accumulate Cd. Due to the lack of vegetative cover and its persistence over time due to metallurgic smelting adjacent to the LGWR, this may be an explanation for the huge depletion of Cd seen in both amended and non-amended soils from this fraction.

3.5 CONCLUSION

The revised BCR sequential extraction method proved to be a valuable tool in assessing how poplar and *Paxillus involutus* can alter zinc smelter contaminated soils, increasing the likeliness of remediating sites similar to the one studied in this research. Fractionation patterns for Zn in non-amended soil showed an overall decrease in residual pool and increase in the residual pool for all treatments in comparison to the original field soil. Where in non-amended soils, fractionation patterns showed Cd was redistributed to the oxidizable pool and an increase in the reducible and residual pools when compared to the original field soil. In amended soil, the fractionation patterns for Zn had reductions in the exchangeable and reducible pools mostly in the *Pop* and *Pop* x *Myc* treatments. Cadmium was redistributed in amended soil the greatest, with a majority of the oxidizable pool being transferred to the reducible and exchangeable pools. This reflects the transfer of cadmium from soil to shoot and roots of the poplar used in this study. Leachate Cd and Zn concentrations released in this study from pots was overall insignificant in its effect on fractionation; however the concentrations released can be considered an environmental hazard.

Phytoremediation potential using poplar hybrids with desired symbiotic mycorrhizal fungi was achieved in this experiment, where amended soil from Palmerton allowed the highest translocation of metals, biomass, and root to shoot ratio. Treatment effects exhibited that the combination of poplar hybrid and *Paxillus involutus* used in this study had demonstrated greater efficacy in removing Cd from soils than Zn. The mycorrhizal treatment in this study had little or no influence in translocating Zn from soil to shoots, but rather sequestered Zn within the roots of the poplar used, more so in non-amended soils than amended soils. Using either the concentrations determined by the first step or the sum of the first three steps of the sequential

extraction method to determine the relationship with plant uptake that is used loosely in many studies to determining bioavailability should be used cautiously, however using this method showed to be significant in this soil-plant system studied.

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Table 3.1 Soil Characteristics and Metal Availability for four soils collected from LGWR in Palmerton, Pennsylvania

Sample	Soil pH _{H2O}	TOC (%)	CEC (meq/100 g)	Cd _{Total} (mg/kg) ¹	Zn _{Total} (mg/kg) ¹	0.005 M EDTA ²		0.005 M DPTA ²		Mehlich-III ²	
						Cd (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Zn (mg/kg)
PLM-B1 (Non-Amended)	5.42	18.3	28.6	254	13503	31	1835	26	1659	9	614
PLM-B2 (Non-Amended)	5.60	6.8	13.7	138	7909	23	982	17	1018	10	594
PLM-B3 (Non-Amended)	5.44	21.5	29.6	262	11170	28	1444	25	1771	9	854
PLM-B4 (Amended)	6.36	2.8	8.7	40	4783	8	542	5	605	3	575
Typical ³	-	-	-	0.01-2	1-900	-	-	-	-	-	-
USEPA ⁴	-	-	-	1.2	150	-	-	-	-	-	-
PA DCNR ⁵	-	-	-	293- 1750	11800- 135000	-	-	-	-	-	-

¹Both Zn and Cd totals of field samples were determined by Geoscience Laboratories, Ontario, Canada. Non-Amended – collected outside “test-plots” from a non-amended location; Amended – collected within “Test-Plots” and have received a regime of amendments (In methods).

²EDTA and DPTA extraction methods from Matúš and others (2005); Mehlich-III extraction method from Mehlich (1984). Results are from Figueroa (2015)

³Typical soil concentrations are from *Trace Elements in Terrestrial Environments: Biogeochemistry, Bioavailability, and Risk of Metals 2nd Edition*, D. Adriano, Springer, New York, 2001.

^{4/5}Both USEPA and PA DCNR soil concentrations are from Region III Ecological Guidelines and the Palmerton Zinc Superfund Site, Palmerton, PA, respectively. These values are found in *Preassessment Screen Determination for the Palmerton Zinc Pile Superfund Site, Pennsylvania DCNR, Harrisburg, 2003* (USFWS, 2003).

Table 3.2 Summary statistics for Populus hybrid growth response to Cd, Zn-contaminated soils and modification by inoculation with *P. involutus*

Treatment		Biomass (mg)	Root:Shoot Ratio	Cadmium Plant Information				Zinc Plant Information			
Soil	Myc			Cd _{Shoot} (mg/kg) dry weight	Cd _{Root} (mg/kg) dry weight	Cd _{Total Content} (mg/plant)	TF	Zn _{Shoot} (mg/kg) dry weight	Zn _{Root} (mg/kg) dry weight	Zn _{Total Content} (mg/plant)	TF
Non-Amended	Non-Myc	1100±689b	0.28±0.18b	24±2.69a	85.9±9.97a	0.025±0.016ab	0.28±0.056b	724±170bc	5065±670a	0.76±0.51b	0.15±0.036b
	Myc	1540±581ab	0.35±0.27ab	24.1±3.56a	98.5±4.79a	0.030±0.015a	0.24±0.036b	600±81c	5423±670a	0.77±0.41b	0.11±0.025b
Amended	Non-Myc	1960±611a	0.45±0.13a	9.7±0.72c	41.1±7.99b	0.014±0.0047b	0.24±0.030b	918±108a	3606±500b	1.27±0.31a	0.26±0.055a
	Myc	2330±680a	0.42±0.31ab	16.6±2.39b	44±6.62b	0.028±0.0089a	0.38±0.065a	842±172ab	3396±160b	1.46±0.55a	0.21±0.057a
<i>Treatment Effects</i>											
Soil		**	*	***	***	***	-	*	***	***	***
Myc		*	-	*	***	*	**	**	*	-	-
Soil x Myc		-	-	-	***	-	-	***	-	-	-

Non-Amended sample refers to PLM-B2 and *Amended* sample refers to PLM-B4

Biomass = Root Wt. + Shoot Wt.; Translocation Factor (TF) = $\frac{[M]_{\text{Shoot}}}{[M]_{\text{Root}}}$; $[M]_{\text{Total Content}} \text{ (mg plant}^{-1}\text{)} = \text{Avg. Shoot Wt.} \times [M]_{\text{Shoot}}$

Statistical Significance: * = p<0.05; ** = p<0.01; *** = p<0.001

Values (n=4) are means ±SD.

Table 3.3 Pseudototal (aqua-regia soluble) of Cadmium and Zinc in LGWR soils following 8-week growth with Populus and/or P. involutus (mg/kg)

Soil	Metal	Field Sample	U	Myc	Pop	Myc x Pop
Non-Amended	Cd	147±5	135±9.73ab	151±7.60a	124±18.1b	139±14.6ab
	Zn	7990±1360	7920±281a	7690±624a	7040±548a	7760±800a
Amended	Cd	69±2	65±2.91a	58±2.88a	40±12.9b	41±11.8b
	Zn	4650±1205	4790±146a	4550±474a	4440±305a	4750±240a

Results presented are means±SD of replicate analysis (n=4) besides “Field Sample” (n=3). Means followed by the same letters within rows are not significantly different (p<0.05).

Non-Amended sample refers to PLM-B2 and *Amended* sample refers to PLM-B4

U = untreated, *Myc* = mycorrhizae present, *Pop* = poplar present, *Myc x Pop* = Both mycorrhizae and poplar present

Figure 3.1 Revised BCR Sequential Extraction Fractionation Data for LGWR soils following 8-weeks of growth with *Populus* and/or *P. involutus*. Non-amended refers to sample PLM-B2 and amended refers to sample PLM-B4. Field = original sample; U = untreated; Myc = mycorrhizae added; Pop = with poplar; Pop x Myc = with poplar and mycorrhizae. Results presented are mean of replicate analysis (n=4) besides "Field" Sample (n=3).

Figure 3.2 Non-amended sample data from the Revised BCR Sequential Extraction for LGWR soils following 8-weeks of growth with *Populus* and/or *P. involutus*. Original field soil concentration illustrated by cross-lines. Values (n=4) are means $\pm$ SD. Differing letters indicate significant differences among planting treatments by Tukey Kramer HSD $\alpha = 0.05$. U = untreated; Myc = mycorrhizae added; Pop = with poplar; Pop x Myc = with poplar and mycorrhizae.

Figure 3.3 Amended sample data from the Revised BCR Sequential Extraction for LGWR soils following 8-weeks of growth with *Populus* and/or *P. involutus*. Original field soil concentration illustrated by cross-lines. Values (n=4) are means $\pm$ SD. Differing letters indicate significant differences among planting treatments by Tukey Kramer HSD $\alpha = 0.05$. U = untreated; Myc = mycorrhizae added; Pop = with poplar; Pop x Myc = with poplar and mycorrhizae.

Figure 3.4 Leachate Depletion Curves for greenhouse experiment for LGWR soils following 8-weeks of growth with *Populus* and/or *P. involutus*. U = untreated; Myc = mycorrhizae added; Pop = with poplar; Pop x Myc = with poplar and mycorrhizae.

4 CONCLUSIONS

In this study we observed how contaminated soils from a derelict zinc-smelter site can be highly available when exposed to treatments, both biological and chemical. With the aid of commonly-used single-extractions (e.g., EDTA, DTPA, Mehlich-III) we were able to establish the availability of both Cd and Zn in four soil samples collected, based on traditional soil extraction techniques to determine plant availability. Along with these common extractions, we confirmed methods that mimic rhizosphere conditions (RBM) with fungal exudates produced along with liquid fungal cultures are useful in interpreting availability and these treatment effects significantly influence availability. The use of the revised BCR Sequential Extraction method elucidated conditions that are operationally defined by extractant, but gave insight to availability defined in the first-step to compare against these common extraction methods to determine availability in environmental samples. The short-term release of Cd and Zn was also determined to show the potential for environmental risk associated with inoculation of soils when used to aid remediation projects. Along with background work associated with metal availability studies, we observed the “before” and “after” effects on two soils collected that overall demonstrated that with introduction of biology (e.g., fungi and poplar) the chemical fractionation determined by sequential extraction changed significantly. Specifically, the *Populus* hybrid along with *Paxillus involutus* used in this study had great influence on soil chemistry, but also sequestered Cd with significant effects. This affirms that this system can be used to ameliorate contaminated sites affected by Cd exposure and with further research more varieties of hybrid poplars and mycorrhizal fungi can be tested to be effective on both metals studied along with many other heavy metals that pose a risk to the environment and human health.

5 APPENDIX

Table A.1 Significance of treatment effects for soils exposed to mycorrhizal, poplar, and mycorrhizal x poplar treatments on non-amended and amended Palmerton (LGWR) soils in 8-week growth study

Non-Amended	MYC					Pop					Pop*Myo				
	df	ss	F	P		df	ss	F	P		df	ss	F	P	
F1_Zn	1	0.00500	1.36	0.268		1	0.000740	0.202	0.662		1	0.00498	1.36	0.269	
F2_Zn	1	0.112	6.24	0.0296		1	0.270	15.1	0.00250		1	0.0721	4.03	0.0700	
F3_Zn	1	0.00327	0.252	0.626		1	0.156	12.0	0.00530		1	0.0290	2.23	0.164	
F4_Zn	1	0.0190	0.579	0.463		1	0.912	28.7	0.000200		1	0.0850	2.68	0.130	
SUM_Zn	1	0.000318	0.0507	0.826		1	0.00947	1.51	0.245		1	0.00205	0.327	0.579	
Pseudototal_Zn	1	0.00593	0.945	0.202		1	0.00845	1.34	0.271		1	0.0116	1.84	0.202	
F1_Cd	1	0.0100	2.49	0.143		1	2.67E-06	0.000700	0.980		1	0.0260	6.49	0.0271	
F2_Cd	1	0.0325	2.50	0.143		1	0.0622	4.77	0.0150		1	0.000204	0.0156	0.903	
F3_Cd	1	1.76	251	<0.0001		1	0.710	101	<0.0001		1	2.08	297	<0.0001	
F4_Cd	1	0.0125	0.684	0.426		1	1.47	80.6	<0.0001		1	0.0175	0.960	0.348	
Sum_Cd	1	0.00640	3.69	0.0809		1	0.0242	13.9	0.00330		1	0.000422	0.244	0.631	
Pseudototal_Cd	1	0.0645	6.43	0.0276		1	0.0182	1.81	0.206		1	0.000544	0.0542	0.820	

Amended	MYC					Pop					Pop*Myo				
	df	ss	F	P		df	ss	F	P		df	ss	F	P	
F1_Zn	1	0.0190	5.99	0.0324		1	0.000181	0.0570	0.816		1	0.0110	3.45	0.0901	
F2_Zn	1	2.03	126	<0.0001		1	2.24	139	<0.0001		1	2.22	138	<0.0001	
F3_Zn	1	0.647	1.58	0.235		1	0.684	1.67	0.235		1	0.0708	0.173	0.685	
F4_Zn	1	0.0235	2.03	0.182		1	0.748	64.6	<0.0001		1	0.0276	2.39	0.151	
SUM_Zn	1	0.0000201	0.00420	0.949		1	0.0328	6.92	0.0234		1	0.000560	0.118	0.738	
Pseudototal_Zn	1	0.00000833	0.00150	0.970		1	0.00160	0.295	0.598		1	0.0162	2.98	0.112	
F1_Cd	1	0.0235	3.11	0.106		1	0.0217	2.87	0.118		1	0.00933	1.24	0.290	
F2_Cd	1	0.0156	2.18	0.168		1	0.363	50.6	<0.0001		1	0.00445	0.620	0.448	
F3_Cd	1	1.52	206	<0.0001		1	0.177	23.9	0.0005		1	1.62	220	<0.0001	
F4_Cd	1	0.300	8.43	0.0143		1	5.24	147	<0.0001		1	0.00767	0.216	0.651	
Sum_Cd	1	0.0461	8.29	0.0150		1	0.108	19.4	0.00110		1	0.0884	15.9	0.00210	
Pseudototal_Cd	1	0.00721	0.144	0.711		1	0.796	15.9	0.00210		1	0.0138	0.276	0.610	

Table A.2 Significance of treatment effects on *Populus* hybrid exposed to amended soils and mycorrhizal fungi (*P. involutus*) in 8-week growth study

Measurement	Amended				Myc				Amended*Myc			
	df	ss	F	P	df	ss	F	P	df	ss	F	P
Shoot Cd	1	4.89	207	<0.0001	1	0.884	37.5	<0.0001	1	0.871	36.9	<0.0001
Root Cd	1	7.14	349	<0.0001	1	0.125	6.10	0.0175	1	0.0151	0.739	0.395
Shoot Zn	1	1.00	24.7	<0.0001	1	0.225	5.55	0.0230	1	0.0312	0.769	0.385
Root Zn	1	1.96	121	<0.0001	1	0.000213	0.0131	0.909	1	0.0493	3.04	0.0880
Root Weight	1	6.95	54.9	<0.0001	1	1.23	9.74	0.00320	1	0.595	4.69	0.0358
Shoot Weight	1	2.05	8.17	0.0065	1	0.615	2.46	0.124	1	0.0270	0.108	0.744
Biomass	1	2.95	18.3	0.0001	1	0.790	4.90	0.0321	1	0.0834	0.517	0.476
Root:Shoot Ratio	1	1.46	6.84	0.0122	1	0.107	0.502	0.482	1	0.368	1.73	0.195
Translocation Factor Cd	1	0.213	5.14	0.0284	1	0.345	8.30	0.00610	1	1.12	26.9	<0.0001
Translocation Factor Zn	1	5.76	92.1	<0.0001	1	0.239	3.82	0.0570	1	0.159	2.54	0.118
Total Content Cd	1	0.608	2.30	0.137	1	2.974	11.2	0.00170	1	0.592	2.23	0.142
Total Content Zn	1	5.91	21.7	<0.0001	1	0.0963	0.354	0.555	1	0.000149	0.000500	0.981