THE SHORT- AND LONG- TERM EFFECT OF ZINC AND COPPER CONTAMINATION ON SOIL MICROBIAL FUNCTIONS

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ABSTRACT

Soil microbial functions are vital for maintaining soil health and are therefore also essential for the provision of services required to support human and ecological health. The inadvertent release of trace metals into soil ecosystems through their extraction, processing, and use of metal ores has led to elevated soil trace metal concentrations worldwide. To preserve soil ecosystem functions and soil health, soil regulatory limits that define acceptable limits of trace metals in the environment need to be set. This is done to reduce the exposure of the soil microbial community to potentially hazardous and toxic substances. However, no single concentration of trace metals can be used to ensure the universal protection of all soil ecosystems. There is therefore a need to define the safe limits of exposure to trace metals in different land-uses. Considerable evidence shows soil microbial functions are affected by trace metal contamination in the short term but are restored over time. However, the long-term effect of trace metals on soil microbial functions is poorly understood. Much of the available data on adaptation is derived from studies focused on agricultural soils in Europe. This study aimed to examine soil enzyme activity across a range of different soil types and determine the long-term effects of zinc and copper contamination, focusing on artificially managed and natural systems, such as agricultural, urban, and anthropic soils, grassland, and forest soil. Initially this investigation validated new methods to determine soil enzyme activity. Standard methods of assessing soil enzyme activity require significant quantities of soil to be collected to then assess contaminated sites and determine toxicity. This experiment aimed to reduce the overall quantity of soil required by an order of magnitude. The assessment of soil nutrient cycling forms a part of all site investigations carried out by Canadian risk assessors. One of the key factors influencing the cost and therefore the scope and size of any potential investigation is the transportation of soils from a site to laboratories for experimentation. Therefore, a low soil requirement assay would significantly expand the potential for site investigations into nutrient cycling. This work demonstrates the validity of a low soil requirement nutrient cycling enzyme (phosphorus, sulfur, and carbon) assay and functional (nitrification) assay. These assays were applied to investigate different soil remediation treatments as part of a site investigation. Heterotrophic and autotrophic nitrification responded differently to lime addition over a decade and the assay demonstrated the effectiveness of different soil treatments (biochar, smectite) and hydro-seeding on soil enzyme activities. The central research of this work focused the soil enzyme activities of 18 soils from across Western Canada. Providing a clear demonstration of the capability of soil enzyme assays for terrestrial eco-toxicity assessment and how this method meets the requirements of the CCME for the assessment of contaminated sites and determining soil quality criteria. This experiment also demonstrated the importance of soil properties and land-use. The experiment provides a range of eco-toxicity data and the first steps towards making soil enzyme

assessment a part of the Canadian regulatory regime. This study used North American soils from a range of land-uses to identify differences in adaptation rate and sensitivity, and aimed to evaluate methods used to assess the hazards from trace metals using spiked soils. First, soils (n=18) were spiked with zinc (Zn) and copper (Cu) applied at eight nominal concentrations (0, 300, 1000, 2000, 3000, 5000, 10,000, and 20,000 mg kg^{-1}. Dose-response curves were then determined for enzyme activities (nitrification, dehydrogenase, arylsulphatase, and acid phosphatase). Land-use was shown to be a factor ($P < 0.01$) affecting the toxicity of zinc and copper. Boreal forest soil nitrification rates had the most sensitive end-point to zinc (EC_{50} = 202 $\pm$ 44 mg kg^{-1}) and copper (EC_{50} = 197 $\pm$ 23 mg kg^{-1}). Across all land-uses and soil types, a similar pattern of sensitivity was noted: potential nitrification > dehydrogenase > arylsulfatase > acid phosphatase. Soils were then monitored to identify adaptation of these microbial functions over 180 days. All soils enzyme activity responses showed adaptation to both zinc and copper over time. The relative sensitivity of each enzyme activity remained consistent after adaptation had occurred. Finally the long term consequences of the adaptation to trace metals by the soil microbial community were investigated. This study tested whether adaptation to a severe stress (trace metals) affects resistance or resilience to a subsequent mild stress. The resistance, resilience, and relative soil stability index (RSSI) of soils were determined for heat (60°C for 24h) and moisture stress, applied as short-term secondary stresses. Soils that had adapted to zinc and copper were not less resilient to additional stresses after adaption, than before recovery. The results of this experiment suggest that activity is a good indicator of soil microbial health but metal concentration is not. Metal speciation was determined using synchrotron-based X-ray Absorption Spectroscopy (XAS) at the Canadian Light Source Inc. Specifically Zn k-edge near-edge structures (XANES) data were collected at the HXMA beamline (06-ID1). Overall these studies provide new insights into the long-term consequences of Zn and Cu in soils. This study showed that soils dosed with zinc in the form of $ZnSO_4$ did not undergo the changes in metal speciation to become less available, which may have been expected. Soil zinc speciation remained consistent overtime and across soils either over time or soil in the most available form of zinc (aqueous zinc). In conclusion, detailed analysis of soils artificially contaminated with zinc and copper in this study allowed the long term consequences for soil microbial functioning to be studied in greater detailed than they have been before. This gave an improved understanding of the effects of zinc and copper to a range of soils. It also improved the understanding of how soils artificially contaminated with metals for hazard testing differ from field contaminated soils. This study provides an improvement in the understanding of how microbial data can be incorporated into soil quality guidelines for Canadian soils.

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DEDICATION

I dedicate this work to my partner Bethany Thiessen and our dog Cooper. It is your love and support which made this possible.

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ABBREVIATIONS

AOA . . . . . Ammonia Oxidizing Archaea

AOB . . . . . Ammonia Oxidizing Bacteria

AOM . . . . . Ammonia Monooxygenase

C . . . . . Carbon

CEC . . . . . Cation Exchange Capacity

CEPA . . . . . Canadian Environmental Protection Act

CCME . . . . . Canadian Council of Ministers of the Environment

CLS . . . . . Canadian Light Source

Cu . . . . . Copper

d . . . . . Day

EC . . . . . European Commission

EC_{50} . . . . . Median Effective Concentration

EPA . . . . . Environmental Protection Agency

Eqn . . . . . Equation

EU . . . . . European Union

EXAFS . . . . . Extended X-ray Absorption Fine Structure

FCSAP . . . . . Federal Contaminated Site Action Plan

GDP . . . . . Gross Domestic Product

h . . . . . Hour

HNA . . . . . Heterotrophic Nitrification Activity

HXMA . . . . . Hard X-ray Micro-Analysis

HOHM . . . . . Soils with high organic matter content and high metal content from sites in Flin Flon, Manitoba

INTF . . . . . Iodonitrotetrazolium Formazan

ISO . . . . . International Standards Organization

LOHM . . . . . Soils with low organic matter content and high metal content from sites in Flin Flon, Manitoba

min . . . . . Minute

N . . . . . Nitrogen

NCSRP . . . . . National Contaminated Site Remediation Program

NEPC . . . . . National Environmental Protection Council

OECD . . . . . Organization for Economic Cooperation and Development

P . . . . . Phosphorus

PICT . . . . . Pollution Induced Community Tolerance

PNR . . . . . Potential Nitrification Rate

REACH . . . . . Registration, Evaluation, Authorization, and Restriction of Chemicals.

RSSI . . . . . Relative Soil Stability Index

S . . . . . Sulfur

SQG . . . . . Soil Quality Guidelines.

SSSA . . . . . Soil Science Society of America

WHC . . . . . Water Holding Capacity

XAS . . . . . X-ray Absorption Spectroscopy

XANES . . . . . X-ray Absorption Near Edge Structures

Zn . . . . . Zinc

1 General Introduction

Contaminated soils pose a significant hazard to human and ecological health worldwide, and Canada is no exception. There are 12,000 contaminated sites under administration by the Government of Canada and an estimated 20,000 - 30,000 contaminated sites in total across Canada (De Sousa, 2001; Government of Canada, 2016). Contaminated sites have been created around the world from the release of organic and inorganic chemicals into ecosystems. Global chemical usage has increased since the 1970s due to industrialization and urbanization. Mining and smelting, pesticide use, vehicle emissions, and manufacturing have all led to the inadvertent release of chemicals into the wider environment (Kuppusamy et al., 2016). There are some hotspots of chemical pollution worldwide, with areas of oil production, mining, and ore processing associated with the largest areas of chemical releases (Kuppusamy et al., 2016). The costs associated with the clean-up and remediation of contaminated sites have been estimated in the billions of dollars (Government of Canada, 2016). Setting appropriate regulatory limits for the clean-up of contaminated sites requires detailed knowledge of the effects chemicals have on organisms.

Trace metals are naturally and ubiquitously present on the Earth's surface and their distribution is governed by geological processes acting over time. This natural distribution has, however, been altered, through the extraction, processing, refining, and use of trace metals and has led to an increase in surface trace metal concentrations around the globe. Trace metals pose a particular problem due to their persistence in the environment and the potential toxic effects they can cause. Trace metals are well known to be toxic to most organisms when present in excessive concentrations (Adriano, 1986). The negative effects of trace metals to microorganisms in soils were first reported by Lipman and Burgess (1914), who observed lower bacteria count and

nitrification activity in soils with increasing concentrations of zinc, copper, and lead. Due to the essential nature of trace metals for human and ecological health and their natural occurrence in the environment, removing trace metals from all soils is not possible. Setting regulatory limits for the protection of ecological health is further complicated by the range of effects trace metal exposure generates in individual species in the environment. Trace metals are the primary contaminant of concern identified at 6,557 federally managed contaminated sites (Government of Canada, 2016).

Soils are a finite resource that provide vital services for the protection of human and ecological health. Contamination of soils places the services soils provide at risk. Soil functions such as nutrient transformations are a vital part of these services and provide support to ecosystems as a whole. Therefore, the contamination of soils with trace metals is not only a potential risk to human health directly, but also places ecological health and ecosystems supporting human health at risk. To protect human and ecological health from the detrimental effects of trace metals, regulatory limits for the presence of trace metals in soils are set. In Canada, soil quality guidelines for trace metal contamination are set by the Canadian Council of Ministers of the Environment (CCME) and based on dose-response studies. Further regulatory protection for trace metals is also provided by provincial contaminated site programs. Several standardized approaches to soil eco-toxicology have been outlined by Environment Canada (2012) to assess the health of soils. However, these methods focus primarily on higher trophic organisms and do not include soil microorganisms. They also focus on species relevant to agricultural soils and not those applicable to the range of soils, land-uses, and ecosystems present in Canada.

Regulatory soil quality guidelines (SQGs) and site specific assessments of soils should use species relevant to the ecosystems in which the contamination is present. However, only limited data are available for terrestrial ecosystems. The bulk of our present knowledge is derived from plant and invertebrate testing, and information on microorganisms is limited. This presents a knowledge gap in eco-toxicology assessment and with respect to the ability to set regulatory limits through dose-response studies. This knowledge gap means that regulatory limits to protect

ecosystems from trace metals could therefore be either overly protective or not protective of soil microbial health.

Base metal smelting is a part of Canada's industrial landscape. There are 50 non-ferrous metal smelting and refining facilities across Canada (National Resources Canada, 2013) that represent a primary source of trace metal contamination. The largest production in total tonnage is refined copper and slab zinc (National Resources Canada, 2013), and the highest revenues from non-precious or ferrous metal production are derived from zinc and copper production (National Resources Canada, 2013). Despite their economic importance, large-scale industrial mining and smelting activities have produced numerous contaminated sites and led to the degradation of soil health. One such example of this activity is the mining and smelting operation location in the Flin Flon-Creighton area spanning the Saskatchewan-Manitoba boarder. This mining site is just one example of numerous "industrial barrens" present across Canada (Kozlov and Zvereva, 2007). Zinc and copper are also the metals most often associated with the "industrial barrens" identified next to base-metal smelting facilities (Kozlov and Zvereva, 2007). It is for these reasons that zinc and copper are the metals of primary focus in the research described in this thesis.

The goal of this dissertation is to contribute to knowledge on the effect of trace metals, specifically zinc and copper, on long-term soil microbial function in Canadian soils. The focus of this dissertation is therefore on the results of long-term exposure (six months) of zinc and copper across a range of land-uses and soils found in Canada. The results are presented in three manuscript style chapters, presented in a format consistent with the research journal of the Soil Science Society of America, showing the effects zinc and copper have on a range of soil microbial process and examination of the effects of changes in functional activity on functional stability.

1.1 Research Objectives

The scope of this thesis is to examine soil enzyme activity across a range of different soil types and determine the long-term effects of zinc and copper contamination. This study focused on artificially managed and natural systems, such as agricultural, urban, and anthropic soils,

grassland, and forest soil. The primary goal of this research was to determine the varying effects of zinc and copper with respect to different land-uses and soil types across Canada. This study provides a comprehensive assessment of soil enzyme activity over time and eco-toxicological data for microorganisms relevant to Canadian land-uses. The main purpose of this research was to explore how different land-uses are influenced by toxicity of trace metals, zinc and copper by comparing several land-use types in Saskatchewan and Manitoba. The specific objectives of these studies were:

- To provide an eco-toxicological data for the soil microbial community to zinc and copper toxicity across a range of different soils.
- Demonstrate the potential application of soil microbial enzyme activity assays in the Canadian regulatory assessment of contaminated sites, specifically enzyme activities.
- Identify the role of land-use on the toxicity and adaptive capacity of soil microbial functions to zinc and copper.
- Determine the impact of adaptation to zinc and copper on the stability of microbial functions to additional stresses.

This study achieves these aims by testing several hypotheses.

1. The use of 0.1 vs. 1 g of soil will not significantly affect the results of measurements of enzyme activities of soil acid phosphatase, dehydrogenase, and sulphatase.
2. Soil heterotrophic nitrification activity and soil autotrophic nitrification activity will have significantly different responses to soil lime application in smelter contaminated soils.
3. Soil enzyme activity (acid phosphatase, dehydrogenase, and sulphatase) will significantly differ between soil remediation treatments in smelter contaminated soils and therefore be a viable method of measuring treatment effectiveness of large-scale eco-restoration projects.

4. Land-use is a factor that will produce significantly different toxicities to zinc and copper as measured by soil microbial functions

5. Nitrification is not the only microbial function to adapt to the presence of trace metals (zinc and copper).

6. Soils adapted to trace metal exposure (zinc and copper) will have significantly different responses (enzyme activity: acid phosphatase, dehydrogenase and sulphatase and soil nitrification) to secondary stresses (heat and moisture) than previously unexposed soil microbial populations.

7. Soil properties and land-use will significantly affect the response of soil microbial populations adapted to severe stresses (zinc and copper) to subsequent milder stresses (moisture and heat perturbation) With soil not exposed to stresses or land-use changes having a low resistance and resilience to stress, as prior exposure to stress if thought to be produce more resistant microbial ecosystems.

1.2 Organization of this Dissertation

This dissertation is written as a collection of articles that have been prepared for submission for peer-reviewed journals. Preceding the research chapters are the introduction to the dissertation and a review of the current state of literature related to this research topic. Each research chapter has an introduction; detailed material and methods section; summary of results and statistical analysis; discussion of the results in relation to other relevant literature; and finally a summary and conclusions of the implication of the research. The bulk of this research comes from laboratory analyses conducted during the summers of 2013 and 2014. The research experiments are described in Chapters 3, 4, and 5. The final chapter of this dissertation contains a summary of the research as a whole, as it relates to the research questions posed and to the wider literature, as well as a discussion of the implications of my research. Additionally, it examines areas of research not explored and the limitations of the studies conducted.

2 LITERATURE REVIEW

This literature review of peer-reviewed publications was carried out using the web-based search engine ISI Web of Knowledge (New York, USA). The search terms trace metals (zinc and copper), soil quality, nutrient cycling, contaminated sites, and metal tolerance were used. There was a specific focus on publications from the years 2000 to 2016. This literature review is divided into several sections outlining different concepts pertinent to the aims and objectives of this dissertation:

- Background,
- Soil quality,
- Soil nutrient cycling,
- Contaminated sites,
- Soil metal speciation,
- Metal tolerance development, and
- Adaptation of soil microbial functions

2.1 Background

Soils are a ubiquitous yet finite resource. Soil formation is an extremely slow process and soils can therefore be considered a non-renewable resource, on the time-scale of human civilizations. However, soils are integral to the provision of a range of services that support human

civilizations and society, including the production of food and biomass, carbon storage, contaminant storage, and basic raw materials. The services that soils provide are often referred to as soil derived ecosystem services. The protection of these functions is important due to a range of social-economic factors, as well as environmental considerations. The degree to which society can benefit from soils is dependent on their management and how we interact with them. As such, the protection of soil and its importance has been highlighted by the European Commission (EC), the United States Environmental Protection Agency (US-EPA) (United States, Environmental Protection Agency, 2007) and United Nations (UN) Rio 20 plus Summit, the Canadian Council of Ministers of the Environment (CCME) (Canadian Council of Ministers of the Environment, 2006), and Environment Canada (Environment Canada, 2013, 2012).

Soil is subject to a range of potential degradation processes or threats, including erosion, loss of organic matter, sealing, compaction, and contamination. Soil erosion is estimated at 2.2 million tonnes of soil lost per year in the United Kingdom (Defra, 2009) and 970 million tonnes of soil lost per year across Europe (European Commission, 2012). Soil degradation and loss presents a serious problem, often exacerbated by anthropogenic activity. This negative impact and degradation is associated with the loss of the capacity of soils to perform functions and services supporting humans and ecosystems. This loss of functional capacity in soil fertility, carbon, biodiversity, and water holding capacity (WHC) can cause disruption of natural cycling and the underlying capacity of a soil to perform vital ecosystem services. The focus in this thesis is on the threat to soils from contamination.

2.2 Soil Quality

The concept of soil quality was first introduced scientifically in the 1970s by Alexander (1971), but was not widely discussed until the 1990s (Gregorich and Carter, 1997). Due to the vast range of stakeholders that use soils and the interdisciplinary nature of soil science as an academic discipline, there is a vast range of definitions of this concept. Definitions of soil quality can be put

into two broad categories (1) soil functions, and (2) soil use (Garrigues et al., 2012). The primary definition of soil quality is set out by the Soil Science Society of America (SSSA), and is:

> *"the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation."* (Karlen et al., 1997)

An alternate definition and concept of soil health has arisen in recent years to move away from the concept of soils needing to perform their functions or use to be protected. This concept of soil health has led to a need to quantify and regulate to protect and support soil health. The health of soil, also known as soil quality, is the soil's ability to fulfill its functions, ensuring biological productivity and maintaining the quality of the environment. This is required, in part, to maintain the health of plants, animals, and microorganisms (Epelde et al., 2008). There are several different ways to define, consider, and determine soil health. With no direct measurement of soil quality, indicators of soil quality are used. A good indicator of the health of soil should be simple, easy to use, effective in any environment evaluated, reproducible, and insensitive to spatial and temporal variations. Additionally, a good indicator of soil health must be sensitive to changes in management and anthropogenic disturbances (Bêcaert et al., 2006).

2.3 Soil Nutrient Cycling

Microorganisms are critical to biogeochemical cycling of nutrients and therefore play a major role in supporting soil ecological functioning. Despite the importance of soil microorganisms and soil enzyme activities to soil fertility and nutrient cycling, they are often excluded from risk assessments for hazardous chemicals in both the remediation and assessment context. Soil enzyme activities have the potential to assess the impacts of hazardous chemicals. In addition, their use provides a broader picture of nutrient cycling and soil functioning. This assessment is done by determining the inhibitory effects of a chemical on the activity of soil enzymes. Specifically, this is done by creating dose-response models.

There are several factors to consider when selecting an end-point for the assessment of hazards from a chemical or chemical elements. Here, four different end-points were specifically selected for assessment: soil nitrifiers and phosphatase, sulfatase, and dehydrogenase enzyme activities. These end-points for assessment were selected for a range of different reasons. Soil nitrification and the enzyme activity of phosphatase and sulfatase play a central role in fundamental transformations in their respective biogeochemical cycles (N, P, and S). Dehydrogenase activity was selected due to its relationship with overall soil enzyme activity (Carter et al., 2007). These microbial enzyme activities are considered important for their role in not only maintaining but in assessing soil quality and soil health (Doran, 1994, 2002). The use of enzymes is widely advocated to assess soil health (Coppolecchia et al., 2011), for three main reasons: (1) activity rates are reflective of soil microbial activity, (2) their ease of assessment, and (3) their rapid response to environmental changes.

Soil enzymes are found in two locations within soils: as part of the viable cell (intracellular) and as extracellular enzymes found externally to the cell within the soil. The function of soil enzymes is to catalyze reactions. Often these reactions are vital for the survival of the soil microbial organisms involved in the production of these soil enzymes. Intracellular enzymes do this within the cells they have been produced. The specific benefits of extracellular enzymes may be less obvious than intracellular, but they are also vital for maintaining available nutrient forms for catabolism and eventual cellular uptake (Allison and Vitousek, 2005). The importance of soil nitrification and enzyme activity is the critical role each enzymatic process plays in the availability of nutrients for ecosystem functioning. Soil nitrification is the oxidation of reduced forms nitrogen to nitrate via nitrite, an important step in the regulation of available soil nitrogen. Soil phosphatase and sulfatase transform organic compounds in soil organic matter to inorganic forms available for plant uptake.

Dehydrogenases activity is one of most important soil enzymes to the soil microbial community (due to the role dehydrogenases plays in numerous functions, for example respiratory (C) metabolism and N metabolism (Trevors, 1984)). Soil dehydrogenases are representative of

the Oxidoreductase enzymes class (Trevors, 1984). Additionally, dehydrogenases are not active independently of the parent microbial cell meaning that dehydrogenase is an indicator of overall microbial activity (Obbard, 2001). Dehydrogenases activity is also thought of as the best indicator of microbiological redox systems and is considered an indicator of microbial oxidative activity (Carter et al., 2007). This is because dehydrogenase enzymes have a central role in the oxidation and breakdown of soil organic matter and catalyze the transfer of hydrogen from organic substrates to inorganic acceptors (Obbard, 2001). Due to their importance and their non-independent state in soils, their response to stresses is related to changes in the soil microbial community itself (Obbard, 2001). Meaning that environmental factors influencing the microbial community will intern effect the activity of dehydrogenase in soils. As a result dehydrogenases activity is often used to determine the impacts of stress on the soil microbial community (Rogers and Li, 1985). Specifically, it is often used to determine the extent of effects of anthropogenic factors on the soil microbial community (Doran, 1994, 2002).

Phosphorus is present in soils in numerous different forms. The distribution of these forms is governed by geochemical and biological processes (Cross and Schlesinger, 1995). Over the short term and during the life-cycle of terrestrial organisms biological processes govern the availability and distribution of the forms of phosphorus (Cross and Schlesinger, 1995). The majority of available phosphorus forms in soils are derived from the breakdown of soil organic matter (Balota et al., 2003). Soil phosphatase activity is critical in the liberation of P from soil organic matter. Phosphatase enzymes are critical to the reactions which transforms organic P present in soil organic matter into inorganic phosphate forms which can be taken up by plants or microorganisms. Specifically, they catalyze the hydrolysis of ester-phosphate and anhydrides bonds, leading to the release of phosphate (P) in more available forms (Tabatabai and Bremner, 1970; Eivazi and Tabatabi, 1977). The primary control on the availability of phosphorus is biological activity (Eivazi and Tabatabi, 1977; Cross and Schlesinger, 1995). The microbial community is the dominant synthesizer and supplier of soil phosphatase enzymes to soils (Balota et al., 2003). Therefore environmental stresses and impacts on the soil microbial community have also been linked to the

phosphatase enzyme activity in soils (Clarholm and Rosengren-Brinck, 1995; Balota et al., 2003), meaning that they are not only a vital component for maintaining health soils but also provide an indicator of environmental impacts.

Much like the nitrogen and phosphorus cycles in soils, sulfur is present in soils in a variety of forms. Sulfur is primarily present in soils in the organic form, with limited amount available for uptake by organisms (in-organic) (Fitzgerald, 1976). The breakdown of organic matter is therefore the main driver of available sulfur forms in soils. Soil sulfatase enzymes are a critical for is breakdown as they facilitate the transformation of organic compounds in the soil into inorganic and plant available forms (Margesin and Schinner, 1994). Catalyzing the hydrolysis of ester-sulfate (Fitzgerald, 1976). Specifically, sulfatase enzymes help the breakdown of C-O-S bonds in soil organic matter to inorganic sulfur forms (H_2S).

Soil nitrification is a critical step in the nitrogen cycle in relation to productivity of an ecosystem due to the role nitrification plays in regulating the availability of two inorganic pools of nitrogen (ammonium and nitrate) in soil. Nitrification influences these two pools as nitrification is the conversion of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-). The difference in charge of these forms of nitrogen is why nitrification is so important. Ammonium is positively charged and therefore bound to negative sites on soil organic matter and less available to plants; however, it is also less available for leaching by run-off. Nitrate is a negatively charged ion and the availability of nitrate for plants is more but it is also more available for leaching and run-off as well as being available for denitrification. This means nitrification is important for maintaining plant available forms of nitrogen as needed.

2.3.1 Effect of metals on soil nutrient cycling

The response of soil microbial communities to metal stress is not uniform. A review into the effect of metal stress on the soil microbial community by Giller et al. (1998) identified that there is significant variability. Similar to the conclusions and observations of earlier review by Bååth (1989) which also concluded that there were significant discrepancies between the observed

toxicity in soils to metals. Giller et al. (1998) identified several factors which contributed to the discrepancy, concluding that metal bioavailability, differences between laboratory and field analysis, and the sensitivity of soil microorganisms are all factors contributing to the apparent variability in the response of the soil microbial communities to metal stress (Giller et al., 2009).

Unlike other (animals, plants, and invertebrates) toxicity studies, soil microbes cannot be assessed at the individual level. Traditional toxicity assessment in other studies use responses identified at the individual level of a single species to generate dose-response data. The level of separation required for these types of studies cannot be achieved for soil microbes. So to assess soil microorganisms the effects observed to the sum of the activity of various species combined in a community is used and not done at the individual species level (Smolders et al., 2004; Speir et al., 2007; Kuperman et al., 2014). There is a range of microbial indicators which have been developed to assess the soil microbial community. The three main types of indicators relate to soil microbial biomass, microbial activity, and microbial diversity.

Microbial biomass is one method of assessing the effect of metals to soil microbial health. Several studies have found significant relationships between increases in soil metal concentrations and overall decrease in microbial biomass (Anderson et al., 2009; Li et al., 2009). However, the results of these studies have not been consistent, with several studies observing the opposite effect of increasing metal concentrations on microbial biomass (Anderson et al., 2009). This study used field (smelter) contaminated soils meaning variation in the results could be accounted for in part, by adaptation and metal bio-availability. To take account of adaptation of parts of the soil microbial community to metal stress it has been suggested that other methods of assessing the microbial community provide a more sensitive indicator than microbial biomass (Bååth, 1989; Giller et al., 1998, 2009). Microbial biomass can also show significant spatial and temporal variation which can limit the overall applicability of these types of studies to the assessment of metal stresses.

Microbial activity is another potential method for assessing the effects of metal stress on the soil microbial community. Enzymes form a significant part of the methods developed to

assess microbial activity, due to the central role they play in the metabolic functioning of the microbial community. Enzymes are therefore produced by the microbial community to help the overall metabolic functioning of the microbial community as a whole. Enzymes are highly specialized proteins produced by living cells (soil microbes) to catalyze reactions beneficial to the cells survival. Due to the range of metabolic functions living cells have there is a range of different analytical methods which have been developed to assess the various enzymes produced by the soil microbial community. These all relate to the various biogeochemical cycles which occur in soils to provide the energy and resource vital for the survival of the cell.

Enzymes activity is an additional function which can be used to assess the effects of metal stress. The measurement of enzyme activity is done by assessing the overall enzyme kinetics of a soil. The enzyme kinetics being an observation of the rate an enzyme can catalyze a reaction of a substrate into produce. There is a range of different enzymes which can be assessed in the soil, all of which perform various roles to aid the survival of the cell. This is, therefore, ecological significant. Several studies have applied soil enzymes to assess metal stress (Chaperon and Sauvé, 2007; Hinojosa et al., 2008; Coppolecchia et al., 2011; Trevisan et al., 2012). There is not a single enzyme assay that is effective to define the health status of all soils exposed to different metals (Epelde et al., 2008). It is, therefore, necessary to measure the activity several enzymes of soil covering the major biogeochemical cycles to ensure to have an overall picture of the health status of a soil. This inability to assess individual species does however, cause additional problems. Since there is only a small portion of the soil microbial community which is cultural independent of the soil, toxicity assessment has to be conducted done in-situ (Kuperman et al., 2014). Leading to a potential problem in assessing differences soil properties, specifically the differences causes by bio-availability and differences soil properties cause to the microbial community itself. With a need for research to disentangle the factors of soil contributing to the microbial sensitivity to metals and the factors contributing to the availability of the metal to cause a toxic effect.

2.4 Legislation & Regulation

Several different methods are applied by different regulatory regimes to protect soil ecological receptors (plant and soil organisms). Four main jurisdictions are responsible for outlining approaches to dealing with contaminated sites: the European Union (EU) Registration Evaluation Authorization and Restriction of Chemicals (REACH) Regulation; Australia's National Environment Protection Council (NEPC) program; the US-EPA (metals framework); and the CCME (soil quality guidelines).

The United States, at a national level, has outlined their approach to the assessment of metal contamination in the environment in the US-EPA's Framework for Metals Risk Assessment (United States, Environmental Protection Agency, 2007), which is specifically aimed at considering the risks posed from metals to human and ecological health. The framework puts forward five principles the US-EPA considers important to the assessment of metals (Greenberg et al., 2014); metals are naturally occurring, all sites contain metals in naturally occurring mixtures, metals are essential for maintaining health, metals are persistent in the environment, and metal bio-availability is transformed by naturally occurring reactions in soils (Greenberg et al., 2014; United States, Environmental Protection Agency, 2007).

The EC adapted a Soil Thematic Strategy in 2006, with the aim of protecting soils across the whole of the EU. This strategy identified a number of threats to soil quality and identified specific threats in soils in the EU: erosion, loss of soil organic matter, contamination, sealing, compaction, biodiversity losses, salination, flooding, and landslides. This strategy failed to be developed into the proposed Soil Framework Directive, and was withdrawn in May 2014. This is the only strategy to date to be adapted by the EC and not developed into a directive. The EU still recognized the threats of soil degradation in the 7th Environmental Action Programme and specified contaminated sites as a cause of soil degradation and therefore a challenge that needs to be address by individual member states to ensure E.U. member states have land that is managed sustainably by 2020. Therefore, the current legislative requirements for soils lie with individual member states. Most of the current regulation by individual member states related to soils is focused on contam-

inated land legislation (Bone et al., 2010). However, other legislation frameworks in place in the EU are related to contaminated land, specifically: EC Integrated Pollution Prevention and Control Directive (IPPC-2008/1/EC); EC Sewage Sludge Directive (86/278/EEC); EC Mine Waste Directive (2006/21/EC); and EC Registration, Evaluation, Authorization and Restriction of Chemical Substances (REACH-EC/1907/2006).

In Canada, soils are regulated at a national level by the CCME. The CCME has a tier-based framework for risk assessment (Canadian Council of Ministers of the Environment, 2006) that outlines how soil quality guidelines (SQGs) are developed for the protection of human and ecological health in Canada. This framework is based on three principles (Greenberg et al., 2014); a risk-based approach for the remediation of contaminated sites, a tiered framework for assessment incorporating generic and site specific methodologies, and, most importantly, equal protection for human and environment health (Canadian Council of Ministers of the Environment, 2006).

2.5 Contaminated Sites

The definition of what constitutes a contaminated site is not universally accepted. Contaminated sites are defined by the regulatory authority in which they are located. In Canada, a contaminated site is defined by two ways. The Government of Canada has set about investigating contaminated sites under the Federal Contaminated Sites action plan. First, a site can be considered contaminated and require additional assessment if concentrations of a chemical are present at sufficient levels to warrant further investigation. This could be concentrations of chemicals at a site in excess of natural background concentrations or at concentrations that appear to impair biological functioning. The focus is on the presence of chemicals in the environment and not on known toxicological implications of their presence at a site. Second, a site is considered contaminated if it exceeds explicitly defined concentrations set out in policies or regulations. A contaminated site is only however a problem requiring remediation or land-management if adverse long-term health effects are observed. It is this aspect of contaminated sites which has received the least attention.

Canada's federal contaminated site action plan (FCSAP) was set up by the Government of Canada in 2005 to identify federally owned contaminated sites. It followed from the NCSRP program with an aim to reduce the environmental and human health effects at sites managed by the Federal Government. The government set a budget of $3.5 billion and a time frame of 15 years to deal with the contaminated sites. This process identified 21,000 sites across Canada and, to date (2016), 9,000 sites are classified as closed, meaning the site are no longer considered a liability requiring interventions or management of the contamination present at the site.

Land-use is a factor often considered when dealing with contaminated sites. Land-use is included in the Canadian regulatory system under the CCME framework. Sites are split into four different classifications (agricultural, residential \ parkland, commercial, and industrial). The sensitivity of human and ecological receptors is considered with an increasing gradient from agricultural soils to industrial sites. However, there are limited data available with respect to why this is considered the case for ecological end-points. For human receptors, considering land-use is important due to the role it plays in exposure to a hazard, time and day cycle of human activity, work, and play.

Canada has no single regulatory measure for assessing contaminated sites, but has a collection of federal and provincial acts and programs. The key legislative tool used at the federal level is the Canadian Environmental Protection Act (Government of Canada, 1999), often refereed to simply as CEPA. This act is administered by both Health Canada and Environment Canada.

2.5.1 Soil contamination near non-ferrous metal smelters

Mining forms a fundamental part of the Canadian economy, contributing $44.7 billion to the GDP in 2014 (Mining Association of Canada, 2015). The industry is estimated to have paid $71 billion in taxes and royalties to Canadian governments (Mining Association of Canada, 2015). Several metal smelting facilities are located across Canada, with a total of 50 non-ferrous metal smelting facilities located in six provinces (British Columbia, Alberta, Ontario, Quebec, New Brunswick, and Saskatchewan) (Environment Canada, 2013). These are predominantly found in

association with areas rich in non-iron metals, such as copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) (Kozlov and Zvereva, 2007). The processing and smelting of various metal ores has led to the emission and deposition of sulphur dioxides (SO_2), hydrogen chloride (HCl), sulfuric acid (H_2SO_4), ammonia (NH_3), and trace metals into the surrounding environment (Environment Canada, 2013). The degradation of areas of land surrounding metal smelters as a result of emissions had led to the descriptive term "industrial barrens" (Kozlov and Zvereva, 2007). These areas are thought to arise as a result of a combination of both natural and anthropogenic stresses to the ecosystems (Kozlov and Zvereva, 2007). The contaminants that form the main focus of this dissertation are zinc and copper.

Zinc is a naturally occurring trace element present in soils. The mean zinc concentration in Canadian soils is 74 $mg\,kg^{-1}$as reported by McKeague and Wolynetz (1980). Zinc is present in most rock types and is concentrated through various natural geological processes into mineral ore bodies. Several minerals are associated with zinc ores bodies: zinc sulphides, zinc carbonates and zinc silicates. Specifically zinc is a chalophile metallic element and occurs most often in the following mineral forms: sphalerite (most common mineral form) (ZnS) and smithsonite ($ZnCO_3$). Zinc ore rarely forms in grades that can be smelted and often requires concentrating; specifically zinc ores are most often in the 5-10% concentration range and not the 55% or more required for smelting (Plant et al., 2012).

Copper is a naturally occurring trace element present in soils that is distributed through the compositional make-up of crustal materials. Copper is present in most rock types and is concentrated through various natural geological processes into mineral ore bodies. There are several minerals associated with copper ores bodies, in which copper occurs as: native copper (Cu); sulphides; carbonates and silicates. Specifically copper is a chalcophile metallic element and occurs in the following mineral forms: (1) sulphides: chalocopyrite ($CuFeS_2$), covellite (CuS), and bornite (Cu_5FeS_4) (2) carbonates: malachite ($Cu_2CO_3(OH)_2$) and azurite ($2\,CuCO_3\,.\,Cu(OH)_2$); and (3) sicilcates (least common): chrysocolla ($CuSiO_3 \cdot 2\,H_2O$). The most economically significant

deposits of copper are copper sulphide deposits. These deposits account for 90% of copper mined globally, with chalcopyrite forming the bulk of these deposits (British Geological Survey, 2007).

2.6 Hazard Assessment

The soil microbial community is under-represented in ecotoxicological databases because, unlike plants and soil invertebrates, there is no universal or standard method of assessment; yet, it is more sensitive to metals than the latter two categories (Li et al., 2009). So, it stands to reason that the development of a standard test for ecotoxicological metal contaminants using functions related to soil microorganisms is desired. In dealing with contaminated sites, exposure to a hazard needs to be considered. Microbial communities fall under the soil contact exposure pathway. This exposure pathway must be considered for all land-use types for CCME soil quality guideline derivation.

For each chemical, the CCME outlines six factors that will be considered here for zinc and copper: (1) Physical and chemical properties, (2) Sources and emissions, (3) Distribution in the environment, (4) Environmental fate and behavior, (5) Short- and long-term toxicity, and (6) Existing guidelines and standards.

2.6.1 Identifying toxicity

The fundamental principle around which any toxicological investigation is centered can be summarized as by a single conceptual phrase, credited to Paracelsus (1493-1541):

"All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy."

Toxicology and dose-response models relate to this concept. Dose-response relationships occur as a result of an observable biological effect documented in relation to increasing concentrations of a chemical. The simplest expression of a dose response model is summarized in Equation 2.1:

$$Y = f(x) + E \qquad \text{(Eq. 2.1)}$$

where *Y* is a measure of a biological response (toxicity), according to the function *f()* in relation to a concentration of a chemical mg kg^{-1}, *x*, with associated errors or uncertainties *E*. This equation can be altered to include a parameter of interest, to form Equation 2.2.

$$Y = EC_{50} \times f(x) + E \quad \text{(Eq. 2.2)}$$

The parameter of interest relates to the estimated effect that a chemical will have in response to a given concentration as calculated by the dose response equation. For most eco-toxicological investigation it is the effective concentration 50, which is the median effective concentration estimated to cause a 50 percent observed reduction in a specified toxic effect, as calculated by the dose response curve. However there can be any number of different estimates calculated from the equation. Different jurisdiction often used different estimates for regulation. The potential number of different estimates is often defined as EC_p is calculated the same as the expressed EC_{50}. The basic components of any dose-response analysis are (Roberts et al., 2015): test organism, measurable biological response, exposure to a chemical, observation of a response, and varying concentrations of exposure.

A "weight of evidence" approach and the use of Species Sensitivity Distributions to identify risk are preferred by the CCME (Zajdlik, 2006). This is done in order to reduce uncertainty in identifying risks. The weight of evidence approach aims to provide different lines of evidence to make an assessment. Firstly the guidelines for acceptable risks of a metal should be considered. These are produced for each individual metal by CCME. They indicate the acceptable levels of metals in soils at a site depending on where it is located. Acceptable standards are produced at various scales from eco-regions and provincial jurisdiction to the land-use at a specific site. This can mean that they are too conservative and don't take into account other site specific factors which could mitigate a hazard. This is why site monitoring of observed effects is also taken into account. The interrelationship of these components could hold the key to using soil enzymes as a means to assess contaminated sites, as there is a valid relevancy to the data obtained that is related to the soil

conditions of a hazard present at a site, as soil enzymes can be used in all lines of evidence which can be used.

The same factors considered for hazard assessment of a chemical also apply to contaminated sites. This means that demonstration of the applicability of a method for hazard assessment of a given chemical for the ecological nutrient cycling soil contact pathway will also serve as a demonstration of the applicability of the method to assess a chemical in the field at a contaminated site. The ecological nutrient cycling soil contact pathway is the exposure pathway identified by CCME relevant to soil microbial community. The CCME develops SQGs using a tiered hierarchical approach considering several factors: land-use, soil properties, and median effect concentration (toxicity) to calculate soil quality guidelines to protect nutrient cycling for a specific metal (Canadian Council of Ministers of the Environment, 2006). The tiered hierarchical approach consists of a first tier (Tier I) which is the direct application of SQGs a set by CCME, a second tier (Tier II) with a limited modification of CCME SQGs using some site specific factors known to influence toxicity (i.e. background concentrations and soil properties), and a third tier (Tier III) using a risk assessment approach to establish detailed site specific remediation guidelines (Canadian Council of Ministers of the Environment, 2006). Soil guideline levels are developed to sustaining the current and likely future uses of the site by ecological and humans receptors provide a healthy functioning ecosystems (Canadian Council of Ministers of the Environment, 2006).

There are few available data and methods of assessment of nutrient cycle end-points for Canadian contaminated sites. As a result, microbial data are only considered in the form of a "check mechanism" where professional judgment is used to assess whether nutrient cycles have been impacted (Checkai et al., 2014; Greenberg et al., 2014). Three main factors are most often cited as the reason for the limited inclusion of microbial end-points in SQG derivation: variability in dose-response analysis (Griffiths et al., 2000; Checkai et al., 2014), bio-availability, and uncertainty of appropriate controls (Bêcaert et al., 2006). Variability in the dose response analysis and the response of the microbial community to metal stress was reviewed by Kuperman et al. (2014). They reviewed the variation in the response of the microbial population to metal stresses in a num-

ber of studies and determined that there was no more significant than that of other trophic levels (plants and invertebrates) and that no single end-point was consistently the least or most sensitive to metal stress. This means that variation in end-point response is not sufficient to warrant their exclusion in the derivation of SQG. This is supported by earlier work by (Broos et al., 2005) which showed that the robustness (variation of control between soils) of the soil microbial community end-points was not significantly greater than plant end-points of assessment. However, they did demonstrate a negative correlation between the sensitivity to metal stress and robustness of the end-point assessed. Bio-availability is also a factor thought to introduce uncertainty in the use of soil microbial community response as a tool to assess toxicity. As the soil microbial community cannot be separated from the soil and assessed in artificial soils as invertebrates and plants are. This means confounding factors affecting the soil microbial community are harder to assess independently. The comparison of effect to the soil microbial community often uses reference sites which are area of undisturbed soil and vegetation located in areas close to that of the disturbed site. Often this is criticized as not providing an appropriate control for comparison (Bêcaert et al., 2006). Specifically, there may be differences in the soil microbial communities prior to the disturbance not accounted for in the reference soil. Despite the factors often cited for their limited inclusion numerous reviews (Checkai et al., 2014; Greenberg et al., 2014; Kuperman et al., 2014) have called for increased inclusion and use of soil microbial community end-points by the CCME and other regulatory bodies.

2.7 Metal Fate in Soils

Metals are typically persistent in the environment as they are not subjected to biodegradation. This puts soils at a particular vulnerability to metal contamination. As soils are a sink for a range of emissions into the environment. Soils have the potential for their metal concentrations to increase because unlike other potential contaminates soil metal concentration will not be reduced over time by biodegradation but will continue to increase until the sources of emission are removed. In order to understand this particular vulnerability of soils to metal persistence, several

studies have tried to identify the long term fate of metals introduced into soils (Lock and Janssen, 2003; Mertens et al., 2007; Lamb et al., 2009; Smolders et al., 2009). These studies have identified that although soil total metal concentrations do not change over time, the bio-available fraction of metals does. This introduces the concept of "aging" of metals within soils (Smolders et al., 2003). The aging of metals in soils is thought to occur as the result of the interaction between metals and the soils reactive surface (Smolders et al., 2003, 2009). This results in an alteration of metal speciation and aging is the cumulative effect of physiochemical interaction among: the soil, the soil solution, and the metal. As soils are dynamic in their nature and have a range of different properties influencing the reaction which occur within them, there is no single metal speciation characterization and bio-availability cannot always be viewed as decreasing overtime and is dependent on the site and contaminant conditions. The concept of aging and mimicking field assessment of trace metal hazards has been a goal of soil eco-toxicity assessment since the discrepancies between field and laboratory contaminated soils were first identified (Giller et al., 1998), because shifts in metal bio-availability and speciation may occur.

Metal speciation is a fundamental concept required to understand the long term fate and effect of metals in soils. There are several thoughts on the aging of metals after they are introduced to soils. Smolders et al. (2003) assumed that soil aging processes were reversible with pH changes in soils. Several possible explanations exist for how this reversible process would occur in soils. Aging may form phases such as double hydroxides and secondary phase metal formations. Factors such as soil pH may influence the solubility of these phases. Smolders et al. (2003) suggested that the diffusion rate of mineral phase formation is an important factor and that a slow decrease in the available metal concentration may occur as mineral phases are formed.

2.7.1 Metal speciation in soils

Metal speciation is considered to be the distribution of a metal (i.e. zinc and copper) between its various chemical phases (Templeton et al., 2000). Changes in the distribution occur through the interaction of the metal within the soil environment. In the case of zinc, it can be found

distributed between soluble phases (such as chlorides, sulfates and, nitrates) in the soil solution and insoluble phases (carbonates, phosphates, and silicates). Depending on the soil condition and metal concentration present there are several physiochemical reactions which can influence the distribution of soil metal speciation between these different phases. One of the main factors influencing soil metal speciation is soil pH. As soil pH influences a soils ion balance, absorption and complexation and the concentration of dissolved organic matter (Sauvé et al., 2000). Soil consists of various reactive surfaces; zinc speciation is altered by interactions with these surfaces. Soil organic matter is the largest reserve of reactive surface in soils, followed by clay minerals and soil oxides. The largest reactive process that occurs in soils is adsorption. pH influences the reactions that occur in soils. Zinc is more sorbed and complexed in soils at pH >7. This affects the total amount of zinc in solution, with soil zinc concentrations shown to have an inverse relationship to soil pH (Janssen et al., 1997). This is thought to relate to the complexes and adsorption of zinc and the solubility of zinc minerals present in soils, as the solubility of zinc decreases with increasing pH (Janssen et al., 1997).

2.7.2 Methods of Assessing soil metal speciation

One method of identifying metal speciation in soils is X-ray absorption spectroscopy (XAS). Specifically, synchrotron-based X-ray absorption near-edge structures (XANES) and X-ray absorption fine structure (EXAFS) spectroscopy have been widely used to determine chemical speciation in soil systems. XAS use synchrotron radiation (X-rays) to probe an electron structure. This provides data on the oxidative state, coordination number and local bonding environment of an element and allows an assessment of the overall metal speciation in a sample (Lombi and Susini, 2009). Synchrotron-based XAS works by exposing (irradiating) a sample to X-rays, causing an excitement of an elements core electrons. As each element has a unique fluorescence produced upon the excitement of its core electrons this allows probing into the elemental properties and overall bonding environment in which the elements are present. The unique fluoresce is the result of differences in the core electron structure each element has, allowing targeting of x-rays to specific

elements (in this case zinc or copper) of interest. XAS can be divided into several stages. A sample is irradiated by x-rays produced by the synchrotron. The atom absorbs the incoming X-rays which excite the atoms core electrons. The electrons are then ejected from the atom to produce a photo-electron. This creates a vacancy in the electron orbit of the atom, which is filled by higher energy electrons surrounding the atom. A process of decay occurs as higher energy electrons (outer shell) lose their excess energy to become electrons in a lower energy state (core electrons). This excess energy is further released as photoelectrons, which in turn can be measured using an electron count detector and produces a spectrum which can be analyzed. The XAS spectra can be subdivided into different regions which are produced as this process occurs: pre-edge, the main peak, XANES and EXAFS. The XANES part of XAS spectra is the most sensitive to an elements oxidative states and the coordination of the electrons in the first shell surrounding the atom (Lombi and Susini, 2009). This means that the XANES region of the XAS spectrum can provide the fingerprinting information. This can be used to identify the oxidative state of an element in a sample. Comparison of EXAFS of known compositions the interactions of an atom and its neighbouring atoms to be determined (Lombi and Susini, 2009).

Bulk-XANES and EXAFS spectroscopy of a sample represent an average speciation of metals present in soils. This can therefore limit the amount of metal speciation information which can be obtained. As speciation can become obscured by other compounds present in the soil. Therefore numerous additional methods of determining metal speciation in more detail have also been developed, specifically methods such as spatially resolved microscopic X-ray fluorescence (μ-XRF), and scanning transmission X-ray microscopy (STXM) spectroscopy (Yang et al., 2014).

2.8 Metal Tolerance Development and Adaptation

2.8.1 Metal Tolerance Development

Trace elements occur in nature, are common contaminants of soils, and when present in sufficient concentrations, are toxic to living organisms (Adriano, 1986). Negative effects of trace elements on microbial growth and survival have been known since the beginning of the last century

(Lipman and Burgess, 1914). However, it was only once large effects of emissions from smelters on surrounding ecosystems were observed in the 1960s to 1970s that scientists started to realize how severely soil microorganisms and soil microbial processes can become disrupted by elevated concentrations of trace elements in soils (Giller et al., 1998).

More recently, numerous studies have examined the ability of soil functions to withstand stresses and the buffering capacity of soils to withstand the toxic effects of inorganic pollutants on soil biological functions (Gelsomino et al., 2006; Bamborough and Cummings, 2009); this has been the subject of a review by Puglisi et al. (2012). The key biological players in the buffering capacity of soil are microorganisms. Soil microbial populations, with their high degree of genetic malleability, can rapidly respond to changes in the soil environment and have evolved, and are still evolving, different ways to cope with the presence of toxic substances.

Microorganisms have a high surface to volume ratio in comparison to multicellular life forms (Puglisi et al., 2012). This is thought to have led to the development of metal resistant systems early in the development of prokaryotic life and their wide spread presence in bacteria cells. The toxic effect of trace metals occurs as a result of their entering the cell. This is thought to be the result of exposure of cells to trace metals and the subsequent uptake by systems present in cells for the assimilation of other essential elements. The toxic effect of trace metals occurs when their presence within a cell disrupts essential functions. This can occur by several mechanisms, such as substitution, oxidative stress, and alteration of nucleic acids (Nies, 1999; Puglisi et al., 2012). Specifically, subsituation occur when metals with similar properties to essential cations replace them in enzymes, inhibiting enzyme activity and disrupting the metabolic activity of the cell. Oxidative stress occur as a result of the binding of trace metal cations to glutathione in bacteria can form molecules (bisglutathionato complexes) reactive to oxygen forming oxidized molecules (bisglutathion) (Nies, 1999). These oxidized molecules are reduced again and release the metal ions forming more complexes and inducing oxidative stress. Metals also alter the conformational structure of the nucleic acids and proteins and alter with the balance of electroytes and non-electrolytes within the cell.

Two primary means by which microorganisms can mitigate the toxic effects of pollutant exposure have been identified. The first is microbially-based transformation of contaminants to more (microbially) benign species. This includes degradation, in the case of organic contaminants (Hussain et al., 2009), and chelation in the case of inorganic contaminants (Dimkpa et al., 2008); in some cases (e.g., arsenic and mercury), methylation (Loseto et al., 2004) can lead to more volatile species and thus reduce the exposure of soil microorganisms (Cattani et al., 2008; Rinklebe and Langer, 2010), or detoxification can occur due to a change of redox status (Borch et al., 2010). The second is a range of internal resistance processes that can apply to both organic and inorganic contaminants. This review focuses on the relevance of these latter resistance processes to soil microbial survival in the presence of trace element contamination.

The second process of mitigating the toxic effects of trace metals resistance can be further subdivided into several six different mechanisms (Bruins et al., 2000; Puglisi et al., 2012): exclusion, active transport, intra-cellular sequestration, extra-cellular sequestration, and enzymatic detoxification.

Exclusion occurs when trace metals cannot penetrate the cell membrane for uptake by the microorganism. This can occur as a result of a variety of different mechanisms. One example is microorganisms synthesizing extracellular ligands, which form complexes and prevent the cellar uptake of metals (Wood and Wang, 1983). This is relevant here as copper is a metal often reported as being excluded by permeable barriers of microorganisms (Puglisi et al., 2012). Specifically, copper has been reported to bind to the outer surface of microbial cells (outer membrane) of *Pseudomonas syringae* (Arnesano et al., 2003) and the *Frankia sp.* strain of *EuI1c* (Rehan et al., 2014).

Active transport out of a cell can occur as a result of a variety of different process. Active transport is an important factor to consider for zinc toxicity. Zinc is an essential element for the function and structure of many proteins required in cells (Eide, 2006). Zinc transport is mediated by plasmids by a mechanism similar to cadmium transport (Wood and Wang, 1983). Specifically, two separate plasmid genes have been identified as responsible for preventing zinc

toxicity (Wood and Wang, 1983). A specific example of this type of efflux system in place in a cell is in *Staphylococcus aureus*. Cadmium *cadA* gene encoding has been identified for an efflux pump that exports cadmium from the cell interior (Oger et al., 2003).

Intracellular sequestration is a mechanism by which microbial cells accumulate metals within their cytoplasm to prevent exposure of essential cellular components (Puglisi et al., 2012). Again, a specific example of this process is the genes *smetA* and *smetB*, which genes that act to regulate cellular zinc (Morby et al., 1993; Choi and Bird, 2014). Intracellular copper concentrations also need to be controlled to protect against toxicity. Extra-cellular sequestration can occur by a variety of mechanisms. One specific example is exclusion of cadmium in *Pseudomonas putida* (Ueshima et al., 2008; Puglisi et al., 2012).

Enzymatic detoxification occurs through the detoxification of a microbial cell by alteration of metal speciation to generate less toxic forms. For example, after copper in the form Cu^{2+} is in the periplasm, it is re-oxidized by *Cue0*, a multi-copper oxidize, to a less harmful form (Nies and Herzberg, 2013).

When studying the long-term effects of trace metals on microbial functions it is essential to consider the process of metal tolerance development. The development of tolerance is thought to occur not though a singular process but through a range of different mechanisms. Selective pressures after the exposure of microorganisms to trace metals lead to adaption though three main processes: increases in the activity and size of the resistant population, natural selection (random mutations), and horizontal gene transfer.

2.8.2 Adaptation of soil microbial communities to metal stress

The decreased in the toxicity of metals to soil microbial process have been attributed to the adaptation of the soil microbial community to metal stress over time (Giller et al., 1998, 2009). The process of adaptation is thought to occur as a result of structural and community-level changes to the microbial population to produce a soil microbial community which is more tolerant to metal stress. Specifically, more tolerant groups are thought to replace less tolerant groups over

time (Bååth, 1989). The adaptation process is thought to follow the conceptual framework outlined by Barkay et al. (1985) and Puglisi et al. (2012). First, a soil microbial community is exposed to a trace metal. This is followed by the loss of sensitive species, which leads to the development of novel ecological niches able to withstand metals. These niches then compete and proliferate with three main processes involved: an increase in the population resistant to metals, mutations of novel resistant strains in the community and the horizontal-gene-transfer of resistant genes located on the mobile genetic material.

The tolerance of soil microbial process to metal stress has been observed in numerous soils exposed to zinc and copper in the long term (Davis et al., 2004). Tolerance is thought to have been derived from the process outlined above and therefore the microbial diversity of these soils has often been of interest (Giller et al., 2009). The composition of these soils is thought to have been altered by their exposure to zinc or copper and subsequent adaptation. However, there has not been a microbial community structure identified which is distinctive of adapted metal stressed soils. As a result, several studies have tried to determine how soil microbial communities respond to metal stress and it has been the subject of a number of studies (Broos et al., 2004; Rusk et al., 2004; Fait et al., 2006; Mertens et al., 2006; De Brouwere et al., 2007; Mertens et al., 2007, 2009; Ruyters et al., 2010b,a; Trevisan et al., 2012). These studies have focused primarily on soil microbial functions related to the nitrogen cycle (Trevisan et al., 2012) for two reasons; firstly soil nitrification is the most sensitive microbial process to metal stress (Smolders et al., 2001) and secondly nitrogen is most often the limiting factor for plant growth (Bardgett et al., 1994). The responses observed for nitrogen cycling process to metal stress are thought to be caused by overall changes to the soil microbial population. From these studies, insights into the several different factors influencing adaptation have been gained. However, despite metal studies showing recovery (resilience) many ecosystems are still impacted and do not recover from the metal stress. Therefore the underlining factors influencing adaptation still require further investigation.

Soil properties are thought to be one of the factors affecting the recovery (resilience) of the soil microbial to metal stress. Soil properties not only influence the structure and composition

of the microbial community but also influence the bio-availability and therefore the severity of the metal stress. Mertens et al. (2009) showed that the recovery of nitrification activity is the result of shifts in the ammonia-oxidizing community structure over time. Specifically, that adaptation is the result of shifts in the microbial community structure by ammonia oxidizing bacteria (AOB) and not ammonia oxidizing archaea (AOA) (Mertens et al., 2009). Showing that soils adapted to zinc stress became dominated by AOB over time (1 year). As metal stress is not the only factor to cause structural changes of the microbial community other pressures can exist. Soil moisture can influence how adaptation occurs by causing changes in the activity of AOB and AOA in soils prior to zinc exposure (Vasileiadis et al., 2012). Microbial community structure is however not the only factor which soil properties influence. Changes to soil microbial activity as well as composition are also linked to soil properties Ruyters et al. (2010b). The availability of substrate for soil nitrification is a determining factor in the rate a which adaptation occurs Ruyters et al. (2010b). The rate of adaptation of different microbial end-points is not uniform. Denitrification activity has been shown to recover within days of exposure to Zn, Cu, and Cd (Holtan-Hartwig et al., 2002). However, Trevisan et al. (2012) noted no significant adaptation to zinc stress for denitrification after one year. The rate of recovery of nitrification has been observed after one year (Rusk et al., 2004). But adaptation has also been seen after up to two years (Mertens et al., 2009). Part of this discrepancy in adaptation rate is though to be the result of variations in activity as shown by (Ruyters et al., 2010b). However, this could also be caused by differences in incubation time of soils.

Giller et al. (2009) hypothesized that the response of soil microbial communities is dependent on the initial state of a soil and that the response varied due to the difference in the soil conditions. Soil properties are known to influence the microbial community. Factors such as, pH, fertility and texture have all been shown to influence the composition of the soil microbial community. Land-use influence on soil properties has also been shown to alter the structural and composition of the soil microbial community (Lauber et al., 2008). Different land-use and soil types have been shown to have differing relative importance of soil properties to microbial com-

position (Högberg et al., 2007). One specific example is land-use management practices, which influence microbial composition and resistance to stress (Chaer et al., 2009). Specifically, they examined how the effects of heat stress (40-70°C) compared between agricultural and forest soils. This type of experiment to compare how forest soils and agricultural soils respond to metal stress has not been carried out.

2.8.3 Consequences of Adaptation

As soil microbial process can adapt to soil toxicity, soil ecosystems are often thought of as resilient to metal toxicity. There is a lot of evidence showing that soil microbial functioning is affected by trace metal contamination in the short term, but that they are restored over time. Typically, exposure to metal stress decreases soil processes for a relatively short period followed by a recovery to pre-exposure functioning. This recovery is thought to be a result of biological adaptation to the metal exposure and not due to decreases in metal bio-availability. This restoration of functions could, therefore, mean that there is no detrimental effect on the wider ecosystem from metal addition to soils. However, as metal impacted ecosystems still occur, questions have been raised about the consequences of adaptation Giller et al. (2009). Specifically, the effect of metal stress has on the resistance and resilience of the soil microbial to additional stresses. Several studies have investigated whether exposure of soil microbial communities to persistent contamination (such as zinc and copper) decreases the resistance and resilience of the soil microbial community to subsequent stressors or disturbances (Brandt et al., 2010; Tobor-Kaplon et al., 2005, 2006; Philippot et al., 2008; Deng, 2012). There is however no consistent response of metal adapted microbial communities to additional stresses.

The tolerance of the soil microbial community which has adapted to one metal has been showed to influence the tolerance to other metals. Rusk et al. (2004) demonstrated that soil nitrifying bacteria which have developed a tolerance to zinc were more tolerant to Pb stress. This is thought to result from similar non-metal specific mechanisms for metal tolerance having been developed by the microbial community. Rusk et al. (2004) however hypothesized that increases in

microbial tolerance (adaptation) could lead to adverse effects in the microbial community. Specifically, that the increased metabolic burden of metal tolerance decreases the resilience of the microbial community to other stresses. This was however, contradictory to their observation that trace metal (Zn and Pb) adapted soil microbial communities were as resilient to soil pH changes as soils not exposed to trace metals.

Several theories have developed as to the effects of metal contamination on their response to subsequent stresses. The first concept is that a stressed system will have less functional redundancy than an unstressed system (Odum, 1985) and as a consequence will be less able to cope with additional stresses. The second concept is that a previously stressed system is more stable due to the effects of adaptation, with the physiological changes to cope with the previous stresses making the system more capable of withstanding additional stresses. Specifically, that soil microbial communities exposed to a persistent stress are more stable and undergo structural and physiological changes to produce a microbial population more resistant to subsequent stress (Brussaard et al., 2007). The first theory is supported by work of Tobor-Kaplon et al. (2005) who found that copper contaminated soils had a reduced stability when exposed to lead and salinity stresses compared to soil not previously exposed to copper stress. Additional studies by Dussault et al. (2008) also support this theory with normal enzyme activity (protease) observed in copper-contaminated soils exposed to subsequent heat stress compared to their unexposed counterparts. However, numerous studies support the alternative theory (Philippot et al., 2008). Rusk et al. (2004) found that Zn and Pb were significantly more tolerant to these when exposed to other metal stress. Specifically, that zinc tolerance nitrifiers were also tolerance to lead and vice versa. Fait et al. (2006) demonstrated that nitrifying bacteria tolerant to copper and nickel showed no adverse consequences to subsequent environmental stresses. Mertens et al. (2007) showed that the stability of nitrification to environmental stresses (biocide, freeze-thawing and wet-dry cycles) was not affected by zinc contamination and was more dependent on land-use.

The capacity of a soil ecosystem to withstand environmental disturbances is referred to as a soil ecosystems' functional stability (Seybold et al., 1999; Bêcaert et al., 2006). It can

be characterized by two factors: the ability to withstand a stress (resistance) and ability to return to normal functioning over time (recovery) (Bêcaert et al., 2006; Griffiths and Philippot, 2013). Bêcaert et al. (2006) have developed a method of quantifying differences in the stability of soil using a concept called the relative soil stability index (RSSI). The RSSI Relative soil stability index considers changes in enzyme activity after disturbance. This is measured in comparison to the changes in enzyme activity over time. This method is based on a time-integrated assessment of soil enzyme activity outlined by Bêcaert et al. (2006).

2.9 Conclusion

Zinc and copper exposure is a significant problem across Canada. Trace metal exposure though extraction and processing of ores has led to the existence of numerous contaminated sites in Canada. The effects in the short and long term from zinc and copper are unknown. Adaption to trace metals has been observed in European soils; however, there is little knowledge of the effect of zinc and copper addition in the short and long term as related to different land uses and soil types in Canada. This represents a significant obstacle with respect to the assignment of safe regulatory limits for zinc and copper in the environment. Significant amounts of eco-toxicological data are required to address this issue. This study therefore aims to address this knowledge gap.

3 VALIDATION OF LOW SOIL REQUIREMENT ENZYME ASSAY FOR USE IN NUTRIENT ASSESSMENT OF METAL CONTAMINATED SITES

3.1 Preface

This chapter is an initial research chapter to the further ensuing research experiments and forms the basis for the experimental design for studies discussed in the remaining research chapters of this thesis. Standard methods of assessing soil enzyme activity require significant quantities of soil to be collected to then assess contaminated sites and determine toxicity. This experiment aims to reduce the overall quantity of soil required by an order of magnitude.

3.2 Abstract

The assessment of soil nutrient cycling forms a part of all site investigations carried out by Canadian risk assessors. Despite their importance to nutrient cycling soil enzymes are underutilized in the assessment of risk at contaminated sites. One of the key factors influencing the cost and therefore the scope and size of any potential investigation is the transportation of soils from a site to laboratories for experimentation. Therefore, a low soil requirement assay would significantly expand the potential for site investigations into nutrient cycling using soil enzymes and allow their wider incorporation into contaminated site investigations. This work used soil enzyme assays (nitrification, phosphatase, sulfatase, and dehydrogenase) and artificially contaminated boreal forest soil to compare the effect of soil weight in determining EC_{50} values for zinc and copper. Soil enzymes were then used to assess in-situ remediation amendments of smelter (zinc and copper) contaminated soils from a boreal forest site located in Flin Flon, Manitoba. Soil liming amendments applied over a decade were assessed using soil nitrification identifying differences in the response of heterotrophic and autotrophic nitrification. Soils were also treated with trial plots 1 m^2 of hydro-seeding (fertilizer and bend grass) applied with amendments (biochar, smectite, and multch) and the response of soil enzyme activities was determined. This study demonstrated soil enzymes (phosphatase, sulfatase, and dehydrogenase) were not significantly effected by the weight of soil used in their assessment and that soil enzymes could be used to assess differences in the response of the soil microbial community to different in-situ amendment strategies.

3.3 Introduction

Canada's boreal forest and Arctic regions combined account for over a half of the nation's total land-mass (Princz et al., 2012). These regions are therefore environmentally and economically significant not just on a local scale but also globally (Princz et al., 2012), as Canada's boreal forest region accounts for one-third of all boreal forests. The extraction and processing of metals in these environments has often led to the inadvertent or intentional release of contaminants into the surrounding environment, leading to increases in soil metal concentrations. This has, in some cases, ultimately led to the degradation of soil quality and health, producing areas of extensive forest and vegetation die back. One such example is in Flin Flon, Manitoba (Owojori and Siciliano, 2015; Hamilton et al., 2016), where an area of northern boreal forest has undergone a transformation due to the impacts of decades of mining and smelting activities in the region. The area is home to a smelting complex that has evolved over time as demands have changed, as well as being a mining region with a legacy of extraction of numerous metals over the last century. The processing of ores and extraction of metals, including copper (Cu), zinc (Zn), and cadmium (Cd), has led to these and associated metals (lead (Pb), arsenic (As), and mercury (Hg)) being released into the environment (Zoltai, 1988). Zinc and copper are by far the most commonly distributed metals present in the environment and are thought to be the main contaminant present at the site (Owojori and Siciliano, 2015; Hamilton et al., 2016).

The Canadian Council of Environmental Ministers (CCME) requires the assessment of nutrient cycling as part of a contaminated site assessment. This is referred to as a "nutrient cycling check". With metals being persistent in the environment and extensively extracted across Canada, many sites need to be assessed. In addition, a single soil sample cannot be used as a valid representation of a contaminated site. To address the need for numerous samples and correspondingly large volumes of soil to be taken, this paper outlines an evaluation of methods to reduce the volume of soil required to perform soil enzyme assays. Soil enzyme activity is considered one potential method of reducing the soil volume requirement. Herein, the amount of soil required for a soil enzyme assay was reduced from 1 g down to 0.1 g in a validation experiment.

Metal contamination as a result of mining and smelting activities has negative effects on soil ecosystems as a whole (Kozlov and Zvereva, 2007). The effects of trace metals on soil microbial community structure and functions have been well documented (Azarbad et al., 2013). The role of soil microbes and their functions and structure in soil rhizospheres for phytoremediation of trace metal contaminated soils has been the subject of a review (Khan, 2005). Additionally, considerable research has been aimed at finding not only how trace metals affect the soil microbial community but how to assess these effects. Research has also been conducted to identify suitable end-points for assessment (Ramsey et al., 2006). Northern boreal forests often experience forest dieback as a result of metal ore mining and smelting (Kozlov and Zvereva, 2007). The most commonly applied remediation technique is to lime metal-contaminated soils, which aims to increase the pH and thereby reduce metal toxicity, and encourage ecosystem recovery (United States, Environmental Protection Agency, 2007). In Flin Flon, however, this method has yielded only moderate benefits and soils have been completely unresponsive to lime addition in some areas. To prompt eco-restoration in Flin Flon-Creighton, the local community started treating soil surfaces with dolomite limestone in 1999. However, this method resulted in mixed responses from the site (10,000 hectares of contaminated soil), with no consistent greening of the area. To identify the unique reasons for this, the University of Saskatchewan conducted numerous detailed investigations of soil chemistry and amendment evaluations.

To improve eco-restoration effectiveness and aid contaminated site investigations, the present study aimed to use soil enzyme activity measurements demonstrating there applicability. A series of experiments were carried out using a range of different boreal forest soils. A single soil was artificially spiked with trace metals (zinc or copper). This soil was a boreal forest soil collected from an area of uncontaminated boreal forest close to the Flin Flon in Manitoba. This site was selected as a reference site to an area of extensive trace metal contamination. The site was select as the soils were similar to the disturbed site but undisturbed. Theses soils were used for the initial validation of 0.1 g and 1 g. In this validation experiment, the amount of soil required for the soil enzyme assays was dropped from 1 g down to 0.1 g. The reduced soil mass assays were

still effective and worked across a range of different soils as an adequate method to assess soil enzyme activities. Soil enzymes were selected because they are easily measurable in rapid assays, both advantages in an assessment method, and they are sensitive to trace metal toxicity. A range of soil biochemical properties was measured, including phosphatase, sulphatase, and dehydrogenase activities. The utility of the enzyme activity assays was also compared to other biological measures of soil function, in this case soil nitrification. Soil nitrification was determined by two methods: heterotrophic and autotrophic assessment.

Here, additional amendment treatments were evaluated for their effect on soil nutrient cycling and enzyme activity using the low soil requirement tests developed. The amendments investigated were biochar, lime, smectite, and mulch in differing treatment application rates on areas of zinc- and copper-impacted soils for four different soil types identified at the site (Owojori and Siciliano, 2015; Hamilton et al., 2016). To identify treatments suitable to be scaled-up to hydro-seeding treatments suitable for large-scale eco-restoration (10,000 hectares). Amendments were selected based on local availability in an effort to reduce one of the largest costs to remediation operations: transportation of raw materials. For example, biochar could be locally produced on site using waste materials (United States, Environmental Protection Agency, 2007). Additionally, the treatment should only involve a single application. All treatments tested were therefore applied as a single mixed slurry, similar to the process used in a large-scale hydro-seeding application. This application method was also suitable for biochar (Lehmann and Joseph, 2009).

Biochars are biological combusted (charred) materials, formed by combustion in low -oxygen conditions (pyrolysis) (Sohi et al., 2010; Beesley et al., 2011; Kloss et al., 2012). Combustion via this manner produces a new porous, low density, and carbon-rich material that is an effective treatment for organic and inorganic soil contamination (Beesley et al., 2011). The product formed as a result varies depending on the material used to form the biochar; starting materials can include wood (e.g., spruce (*Picea*) and willow (*Salix*)), straw, fish, and bone meal. In addition to varying the material used in production, the pyrolysis temperature affects the final biochar product (Kloss et al., 2012). Biochar application as a soil treatment is increasingly being investigated

for reasons such as improving soil fertility and carbon sequestration (Lehmann and Joseph, 2009; Laird et al., 2010; Sohi et al., 2010).

Smectite clays are important in the chemical speciation and fate of heavy metals in soils. Zinc is thought to bind to the mineral surface and diffuse into the mineral inter-layers where it is transformed into less available forms (Ma and Uren, 1998). This reaction is pH dependent, with alkaline soils generally resulting in greater specific adsorption of Zn (Ma and Uren, 1998). Smectite addition was therefore paired with lime application to increase the pH and provide an adsorption surface for metals in the contaminated soils.

The ecotoxicological assessment of metal-contaminated field-collected soils based on the estimated activity of several soil enzymes appears to be a suitable approach to develop routine terrestrial eco-toxicological tests (Epelde et al., 2008). Soil enzyme activities are the rate-limiting step of biogeochemical cycles and are critical to the decomposition of soil organic matter; this means they offer a method of assessment of soil microbial process and soil quality that is fast, reliable, and reproducible (Nannipieri et al., 2012). Biochemical analyses of metal-contaminated field-collected soils constitute a powerful approach to identify trace element interactions in soils, thus assessing soil speciation and bio-availability of metal toxicity in ecosystem studies (Sauvé et al., 2000). Labile (free metal) concentrations are the most widely accepted means for estimating metal bio-availability. The labile metals in soil solution include free metal and metal ion-pairs (mainly inorganic but also some organic ligands) showing rapid dissociation association kinetics (Nannipieri et al., 2012).

3.4 Materials & Methods

3.4.1 Soils

Flin Flon, Manitoba and Creighton, Saskatchewan, are located on the Saskatchewan-Manitoba border (54.7658°N, 101.8762°W) in the Churchill River Upland Boreal Ecoregion of Western Canada. A zinc and copper mining and smelting operation owned and operated by Hud-Bay Minerals Inc. (formerly Hudson Bay Mining and Smelting Company) has been in operation in this area since the 1930s. This followed from the earlier discovery and exploration for ore deposits in this area in the late 19th and early 20th century, with mining beginning in the area in some form in 1915 after the discovery of a copper deposit (Stauffer, 1974). This area was then identified as rich in metal ore deposits, featuring a series of sulphidic ore deposits formed in the Flin Flon greenstone belt (Syme et al., 1999) that is part of the extensive surface outcropping of the Canadian Shield in the region. Development of this area occurred long before environmental regulation or an understanding of the wider effects a smelting operation can have on the landscape had been developed. The first smokestacks (six) were 53-76 m high leading to localized deposition of metal emissions from the smelting process. More recently (1974), a single 251-m stack was installed (Franzine et al., 1979), which increased the range of deposition up to 100 km from the smelter location (Zoltai, 1988; Henderson et al., 1998; McMartin et al., 1999). The smelting operation produced cadmium (Cd), copper (Cu) and zinc (Zn) until July 2010 when it was decommissioned. Locally extracted ores are now shipped globally for processing, significantly reducing the local environmental impact.

The Churchill River Upland Boreal Ecoregion is characterized and dominated by a mixture of jack pine (*Pinus banksiana*), black spruce (*Picea mariana*), white spruce (*Picea glauca*), subalpine fir (*Abies lasiocarpa*), and trembling aspen (*Populus tremuloides*) (Henderson and McMartin, 1995; Henderson et al., 1998). Mining and smelting in this area coincided with the exploitation of the surrounding forest, with logging fueling the smelting operation. In addition to the exploitation of this forest at the start of mining operations (1939), a significant (1 in 150 years)

forest fire greatly impacted the area (Peng and Apps, 1999). This led to limited regrowth of the forest and the development of tree and vegetation species that are distinct from the rest of the region and dominated by metal-tolerant grasses such as colonial bent grass (*Agrostis capillaris*) and stunted relic boreal tree species disconnected from their natural growth cycle (Winterhalder, 2003). Poor vegetation cover and soil quality in the region have led to erosion of soils, exposure of the bedrock, and formation of a landscape dominated by unique soil types, specifically Orthic Regosol or Brunisols on fluvial plains and Organic soils dominating depression with-in the exposed bed-rock (Mycock, 2011).

Smelter emissions into the atmosphere have led to the deposition and build-up of a range of different trace metals in soils, specifically arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni) and zinc (Zn) (Henderson and McMartin, 1995). Additional sulfur dioxide (SO_2) deposition has led to a reduction in soil pH by soil acidification. In an effort to re-vegetate the affected areas and stabilize soil quality decline, a local initiative called the Flin Flon-Creighton Green Project was established in 1999 based on work carried out in Sudbury, Ontario that was successful in revegetating Ni-Cu smelter-contaminated soils (Gunn et al., 1995). Both projects aimed to increase soil pH by applying coarse grain-sized crushed dolomitic limestone ($CaMg(CO_3)_2$), with the lime neutralizing soil acidity through its reaction with water (H_2O) to forming hydroxyl (OH^-) ions available to combine with H^+ ions in the soil. A reduction in soil pH should reduce pH-dependent metal mobility in the soils as well as metal toxicity. Treatments as part of this project could not be applied to areas closest to the mine complex due to it being a community-led project using local volunteers.

Three different sets of soil were used in a series of experiments: (1) uncontaminated boreal forest soils, (2) smelter-contaminated boreal forest soil subject to *in situ* lime treatment with varying times since application, and (3) smelter-contaminated boreal forest soil subject to *in situ* hydro-seeding remediation treatments.

First, three uncontaminated boreal forest soils were collected (fall of 2012) from an area of boreal forest with similar soil properties and climatic conditions to the area where the contaminated boreal forest soils were collected. These soils were then artificially contaminated with zinc and copper. Field-contaminated soils were collected from several areas in the Flin Flon and Creighton region. An initial investigation of the area was done using samples collected from two 3-km transects, running north and south of the smelter. Soil samples were collected 100 m apart, starting 1.1 km from the smelter location. This initial investigation showed differences in nitrification activity depending on organic matter content. A series of locations in and around the town of Flin Flon and located close to the smelter complex was then selected: two locations were selected based on soil metal content (high) and organic matter content (low vs. high). Two different field assessments were carried out in this investigation. The first examined the long-term (decadal) effects of liming on soil heterotrophic versus autotrophic nitrification rates. This was conducted on samples of soil collected from areas of Flin Flon limed by the green project. The second examined the effects of a series of alternative amendment strategies applied at four different soil locations in Flin Flon. The potential for use of the low requirement soil assays to assist with large-scale eco-restoration efforts was examined. Differences in enzymatic activity across several (N, S, and P) nutrient cycles were determined in response to liming and a range of amendment strategies under differing soil conditions.

3.4.1.1 Soil Properties

Data on the initial characterization of the soils is summarized in Table 3.1. Soil total metal concentrations at these sites are higher than typical for uncontaminated boreal forest in the region. Available metal concentrations are higher in the lime non-responsive (NR) vs. responsive (R) soils, but highest in the un-limed soils. At the NR site, limed soils had noticeably increased soil pH. However, this increase was small with an average 0.2 pH increase observed at the site. This translated into a decrease in available metal content in limed soils compared to un-limed soils. At

the R site, soil pH increased on average from 3.46 to 5.12 in the 10 years since lime application. Liming the R site also resulted in significant decreases in available metal concentrations.

Trace element concentrations in soils were determined using the ETHOS One microwave digestion system (Milestone Srl, Sorisole, Bergamo, Italy) for metal extraction. For each soil a sample (0.5 g) of air-dried soil was ground and placed into a Teflon coated vessels with 12 ml of aqua regia. The vessesls were sealed and digested at a temperature of 200°C. The digests were then filtered using Whatman No. 41 filter paper and diluted to 50 mL with deionized water. These solutions were analyzed for zinc and copper using a microwave plasma-atomic emissions spectrometer, (MP-AES; Agilent 4100, Agilent Technologies, Melbourne, Vic, Australia) and MP Expert software (Agilent Technologies, Melbourne, Vic, Australia). Analyses of zinc and copper were carried out at a set wavelength of 324.75 nm for copper and 213.86 nm for zinc. Analyses followed quality control procedures outlined by Wightwick et al. (2010).

Soil CEC in this study was determined by ALS laboratories (Saskatoon, Saskatchewan) using the ammonium acetate method of McKeague (1978). Subsamples were separated from the bulk collected soils and weighed (10 g). These soils were then used for each analysis, along with blanks and quality control samples. Ammonium acetate (1M) (NH_4OAc) was added to samples to saturate them with ammonium at pH 7. This was then displaced with sodium in the form of 1 M NaCl. Analysis of the NH_4 content was then determined colorimetrically.

Table 3.1 Mean (n=5,± 1 standard deviation) of soil pH, Cation exchange capacity, extractable Zn & Cu, NO_3^- and NH_4^+ for soils used in this study.

Soil	Experiment	Treatment	pH	CEC $cmol\,kg^{-1}$	Zinc $mg\,kg^{-1}$	Copper $mg\,kg^{-1}$	NO_3^- $mg\,kg^{-1}$	NH_4^+ $mg\,kg^{-1}$
Reference Site	Enzyme Assay Validation	Zn and Cu	4.5	3.24	1.24 ± 0.21	0.12 ± 0.01	0.02	0.52
Non-Responsive	Liming	Control	4.01	4.25	417.4 ± 23.21	51.68 ± 5.31	0.07	7.235
Non-Responsive	Liming	10 years	4.25	4.35	154.8 ± 6.54	9.16 ± 1.49	0	14.34
Responsive	Liming	Control	3.36	7.39	56.89 ± 5.52	102.5 ± 21.34	8.85	8.58
Responsive	Liming	10 years	5.1	9.27	6.05 ± 1.78	1.65 ± 0.12	1.69	8.45
HOMHM	Enzyme Activity	Hydro-seeding	5.05	3.21	6800.33 ± 234.87	5483.28 ± 55.84	0.01	12.31
LOMHM	Enzyme Activity	Hydro-seeding	3.78	9.58	5700.29 ± 321.23	6801.25 ± 327.83	0.08	8.54

3.4.2 Enzyme Assays

A series of soil enzyme activities often used as soil quality indicators were used in this study. Soil phosphatase (EC 3.3.2), sulfatase (EC 3.16.1), and dehydrogenase (EC 1.1) were analyzed using modified versions of published methods. Phosphatase (Eivazi and Tabatabi, 1977) and sulfatase (Whalen and Warman, 1996) activities were determined by the *p*-nitrophenol method, using *p*-nitrophenylphosphate for phosphatase activity and *p*-nitrophenylsulphate for sulfatase activity. Dehydrogenase activities were measured using *p*-iodonitrotetrazolium chloride and iodonitrotetrazolium formazan using methods outlined by Trevors (1984). All methods were modified to use only 0.1 g instead of 1 g of soil with the reagent to soil ratio maintained. In an effort to eliminate the confounding effects caused by changes in metal availability that occur when using pH buffered solutions, these were not used in the assays, as per Lessard et al. (2013); activities can still be measured using water and not pH buffered solutions. All measurements were carried out on 96-well plates in a 96-well plate reader, with the wavelength used for detection varied depending on the substance being analyzed.

3.4.2.1 Nitrification

Nitrification in each test soil was analyzed using two different protocols of assessment: ISO 14238 (International Standards Organization, 2012a) and ISO 15685 (International Standards Organization, 2012b). The ISO 15685 method was conducted following the published protocol. The ISO 15685 method was modified with respect to quantities of soil required and to allow for assessment of soil heterotrophic activity. All assays were carried out following 7 d of incubation in darkness at approximately 20°C. The ISO 14238 method was carried out using $(NH_4)_2SO_4$ as a nitrogeneous substrate to facilitate the assessment of nitrification only. For each test, 99 g of soil were incubated with 100 mg of NH_4-N kg, applied as $(NH_4)_2SO_4$ from a 100 mg $(NH_4)_2SO_4$ mL stock solution.

Nitrification activity was measured by two different methods and varied not just in the quantity of reagents used in the method but also via methods of measurement. The two methods

were the ISO standard method for measurement of soil nitrification via rapid ammonia oxidation test for the determination of potential nitrification rates and the ISO Standard method for determination of biological nitrification and measurement of chemical inhibition. To the author's knowledge, this is the first comparison of these two methods for assessing soil nitrification. If the methods show a similar response, this will allow the incorporation of nitropyrine and give risk assessors the ability to determine the heterotrophic and autotrophic nitrification potential of soils. Additionally, it will allow for the use of a more rapid method of nitrification assessment, similar to that of other enzyme assays considered.

Soil heterotrophic nitrification activity can be estimated using a chemolithoautotrophic ammonia oxidation inhibitor. Nitrapyrin is a known chemolithoautotrophic ammonia oxidation inhibitor at a concentration of 80 $\mu g\, g^{-1}$ soil (Islam et al., 2007; Banerjee and Siciliano, 2012). The inhibited nitrification activity was used to calculate heterotrophic nitrification activity (HNA) (equation (3.1). Where HNA is heterotrophic nitrification activity calculated by subtracting a nitrification activity from nitrification activity with nitropyrin inhibiting autotrophic nitrification activity in the soil.

$$HNA = (TotalN) - (NitrapyrininhibitedN) \quad \text{(Eq. 3.1)}$$

3.4.2.2 Arylsulfatase activity

Arylsulfatase activity was measured in soils using a modified version of protocols set out by Whalen and Warman (1996). This protocol was developed from work carried out by Tabatabai and Bremner (1970). In the current study, the protocol was modified to use only 0.1 g instead of 1 g of soil with the reagent to soil ratio maintained. A comparison of the scaled-down method was validated for use in determining dose-response relationships in both artificially and field contaminated soils.

Soils were incubated in glass vials, in the dark at 20°C, for one week prior to analysis. Soils (0.1 g) were weighed into 2 mL polypropylene Eppendorf® tubes. Toluene (20 µL) was added to inhibit further enzyme proliferation and enzyme synthesis (Whalen and Warman, 1996).

Soils were then incubated for a further 1 hour. Substrate was then added to each tube, in the form of 0.1 mL of a 10 nM solution of *p*-nitrophenylsulphate. Deionized water (0.4 µL) was added to each tube followed by vigorous mixing. Soils were incubated at 37°C for 1 h, the temperature was maintained using a water bath. After 1 h, with activity was stopped by transferring tubes to an ice water bath for 5 min. Tubes were then centrifuged at 14,000 rpm for 2 min and the supernatant (150 µL) extracted and combined with 100 µL of a 0.5 M NaOH solution. The supernatant-NaOH solution was transferred to a clean Pierce™ 96-well polystyrene plate, for measurement of *p*-nitrophenol content. Control samples and reagent blanks were included, to account for natural variation due to soil color.

Soils were also tested following the methods outlined by Whalen and Warman (1996) for comparison to the 0.1 g method of assessment. Soil samples (1 g) were weighed into Falcon™15 mL conical centrifuge tubes with 4 µL of toluene added. Soils were then incubated for a further 1 h. Substrate was then added to each tube in the form of 1 mL of a 10 mM solution of *p*-nitrophenylsulphate. Deionized water (4 µL) was added to each tube and the tubes mixed vigorously. Soils were incubated at 37°C for 1 h, with the temperature maintained using a water bath. After 1 h, activity was slowed down by transferring tubes to an ice water bath for 5 min. Tubes were then centrifuged at 14000 rpm for 2 min. The supernatant (150 µL) was then extracted and combined with 100 µL of 0.5 M NaOH solution and transferred to clear Pierce™ 96-well polystyrene plates for measurement of *p*-nitrophenol content.

P-nitrophenol content in the supernatant was determined using a SpectraMax 384 microplate reader (Molecular Devices, San Francisco, CA) measuring absorptivity. Absorbance of each sample was measured at 405 nm and the concentration of *p*-nitrophenol compared to a standard calibration curve. Standards (10 to 50 nm *p*-nitrophenol) were produced from a stock solution of 4.5 µg *p*-nitrophenol (Acros Organics, Fisher Scientific, NJ) added to 100 mL of 0.2 M NaOH.

3.4.2.3 Phosphatase activity

Phosphatase activity was measured using a modified version of protocols set out by Eivazi and Tabatabi (1977). This method was modified to use both 0.1 g and 1 g samples, with the reagent to soil ratio maintained, and with respect to the use of deionized water instead of a buffer solution. The protocol also differed with respect to the use of *p*-nitrophenylphosphate as the substrate.

3.4.2.4 Dehydrogenase activity

Dehydrogenase activity was measured using a modification of the protocol set out by Trevors (1984). Soils were incubated in glass vials in the dark at 20°C and 60% water holding capacity (WHC) for 7 d prior to analysis. Soils (0.1 g) were weighed into 2 mL polypropylene Eppendorf® tubes and *p*-iodonitrotetrazolium chloride (INT) solution (200 µL of 0.4%) was then added. The soil solution mixtures were then incubated in the dark at 20°C for 48 h. Methanol (1 mL) was added to allow extraction of iodonitrotetrazolium formazan (INTF) formed during the incubation. The mixtures were then vigorously vortexed and centrifuged at 14,000 rpm for 2 min. The supernatant (250 µL) was then extracted and transferred to clear Pierce™ 96-well polystyrene plates, for measurement of INTF concentrations. Control samples and reagent blanks were included, to account for natural variation due to soil color.

Concentrations of INTF were determined spectrophotometrically, by measuring absorptivity at 480 nm and comparison with a standard calibration curve. A standard curve was created using a stock solution of INTF (Sigma-Aldrich, Oakville, ON) created using 2.4 mg INTF added to 100 mL methanol. A standard curve between 0 and 24 $\mu g\,mL^{-1}$ of INTF was produced, with individual standard concentrations of 0, 1, 5, 7, 10, 15, 20, and 24 $\mu g\,mL^{-1}$ of INTF.

3.4.3 Statistical Analysis

3.4.3.1 Effect of assay soil weight

This experiment was carried out with artificially-contaminated soils. Boreal forest soils were dosed with a treatment of zinc or copper. Soils had been air dried after collection (fall 2012) and stored in bulk for one year prior to analysis. Soil samples (10 g) were placed in 500-mL glass jars and re-hydrated to 60% WHC. Soils were then incubated in the dark at 20°C for 7 d. Soils were then dosed with a treatment, in the form of zinc or copper sulphate. Increasing doses of 0, 250, 500, 1000, 2000, 3000, 4000, and 5000 mg kg^{-1} of zinc ($ZnSO_4$) or copper ($CuSO_4$) were applied to each 10 g test unit. Soils were then divided into sub samples (n=5) with 2 g of soil placed in glass test tubes. Each test unit was then incubated for a further 7 days in the dark with WHC maintained at 60%. Following incubation each test unit was analyzed using both the 0.1 g and 1 g methods of analysis for enzyme activity.

Effective concentrations (EC_{50}) were determined using dose-response models. The dose response of each treatment was modelled using the drc package (Ritz and Streibig, 2005) available for the R statistical program (R Development Core Team, 2013). Models were evaluated and fitted to each curve using comparisons of Akaike's information criteria (AIC) and an analysis of variance (ANOVA) to identify significant differences between each model used to fit the data. The effective concentration values were a 50% inhibition of the population (EC_{50}) and were calculated using the model with the best fit to the data.

An ANOVA comparing the two methods was then carried out. Statistical analyses were performed using the R statistical program (R Development Core Team, 2013). All data were checked for normality using the Shapiro-Wilk test ($P \leq 0.05$) and for homogeneity of variance using the Levene's test ($P \leq 0.05$). Uni-variate ANOVA testing and a least significant difference (LSD) test were used to assess treatment differences.

3.4.3.2 Soil liming

This experiment was carried out with field-contaminated (zinc and copper smelter emissions) soils treated with lime applications. These boreal forest soils were collected in the fall of 2012, chilled (4°C) after collection at each site prior to freezing and storage at -80°C. The soils selected were from two areas of soils characterized as either non-responsive (NR) vs. responsive (R), with two sites selected which had received lime application in the year 2000 or 2011.

Statistical analyses were performed using the R statistical program (R Development Core Team, 2013). All data were checked for normality using the Shapiro-Wilk test ($P \leq 0.05$) and for homogeneity of variance using the Levene's test ($P \leq 0.05$). An ANOVA of the results was carried out using responsiveness of the soils to liming, time since liming, nitrification type, and their interactions.

3.4.3.3 Alternative amendments

This experiment was carried out with field-contaminated (zinc and copper smelter emissions). This experiment examined the use of soil enzymes as a method of assessment for alternative amendment strategies in smelter contaminated soils. The alternative amendment strategies in this case were four (biochar, smectite, multch, lime, and fertilizer) different hydro-seeding (water and bent grass) amendments applied in six (biochar + lime + fertilizer, smectite + lime + fertilizer, biochar + smectite + lime + fertilizer, multch + lime + fertilizer, biochar + smectite + multch) alternative combinations. An area of soil was divided into 1 m^2 plots with the amendments applied. These amendments were applied to sites one characterized as having low organic matter (LOHM) and one characterized as having high organic matter (HOMHM) soils with high metal contents. For each soil The low requirement soil assays measuring enzymatic activity across several (N, S, and P) nutrient cycles were determined. The soils were treated with amendments in the spring of 2012 and samples were then collected in the fall. Soil samples were chilled (4°C) after collection and for transport to the University of Saskatchewan for freezing (-80°C) and storage.

Enzyme activity was measured using the scaled down 0.1 g methods outlined earlier. An aggregated mean result of all enzyme activities was then calculated to produce a single result for comparison between treatments. This follows similar methods of enzyme assessment using aggregated means set out by (Bêcaert et al., 2006; Zhang et al., 2010).

3.5 Results

3.5.1 Effect of assay soil weight

Figures 3.1-3.4 shows a comparison of all enzyme assay methods using either 1 g of soil or using 0.1 g of soil. For copper, dehydrogenase activity (Fig. 3.1A) measured using the 0.1 g and 1 g methods resulted in EC_{50} values of 434 and 433 mg Cu kg^{-1}, respectively. Phosphatase activity (Fig. 3.3A) measured using the 0.1 g and 1 g methods resulted in EC_{50} values of 4231 and 4232 mg Cu kg^{-1}, respectively. Sulphatase (Fig. 3.2A) activity measured using the 0.1 g and 1 g methods resulted in EC_{50} values of 1618 and 1620 mg Cu kg^{-1}, respectively.

For zinc, dehydrogenase activity (Fig. 3.1B) measured using the 0.1 g and 1.0 g methods resulted in EC_{50} values of 340 and 344 mg Zn kg^{-1}, respectively. Phosphatase activity (Fig. 3.3B) measured using the 0.1 g and 1.0 g methods resulted in EC_{50} values of 2495 and 2512 mg Zn kg^{-1}, respectively. Sulphatase (Fig. 3.2B) activity measured using the 0.1 g and 1.0 g methods resulted in EC_{50} values of 1132 and 1143 mg Zn kg^{-1}, respectively.

Nitrification activity was consistent using the low soil requirement method of a solid state assay. Nitrification activity (Fig.3.4) measured using the 0.1 g and 1.0 g methods resulted in EC_{50} values of 334 and 398 mg $kg^{-1}$for zinc, respectively. Although similar results and variation were observed at low metal concentrations (0-500 mg kg^{-1}). The EC_{50} values (Table 3.2) showed the most difference between the two tests. With concentrations above 3000 showing significantly different enzyme activity.

Decreasing the soil requirement from 1 g to 0.1 g did not increase the variable of the response. The standard deviation of phosphotase activity varied between 29 and 2 pmol g^{-1} h^{-1} for 0.1 g test and 59 and 11 pmol g^{-1} h^{-1} for the 1 g. The standard deviation of arylsulphatase activity varied between 1.64 and 0.49 pmol g^{-1} h^{-1} for 0.1 g test and 1.71 and 0.3 pmol g^{-1} h^{-1} for the 1 g. The standard deviation of dehydrogenase activity varied between 68 and 1.3 pmol g^{-1} h^{-1} for 0.1 g test and 45 and 2.5 pmol g^{-1} h^{-1} for the 1 g.

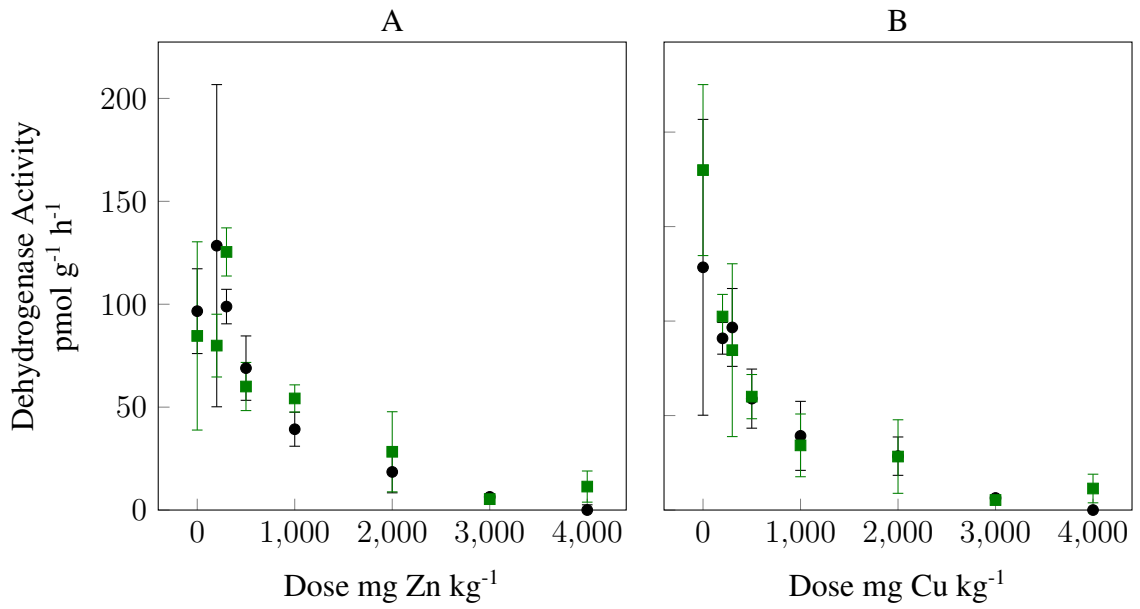

Fig. 3.1 Enzyme validation test for dehydrogenase activity in artificially contaminated boreal forest soils. A comparison of the effects of zinc (A) or copper (B) on the effectiveness of different enzyme activity measurement methods. Dose response model of artificially contaminated boreal forest soil. This graph shows dehydrogenase activity of 0.1g (circle) and 1g (square) assay results for each dose. Error bars represent ± 1 standard deviation.

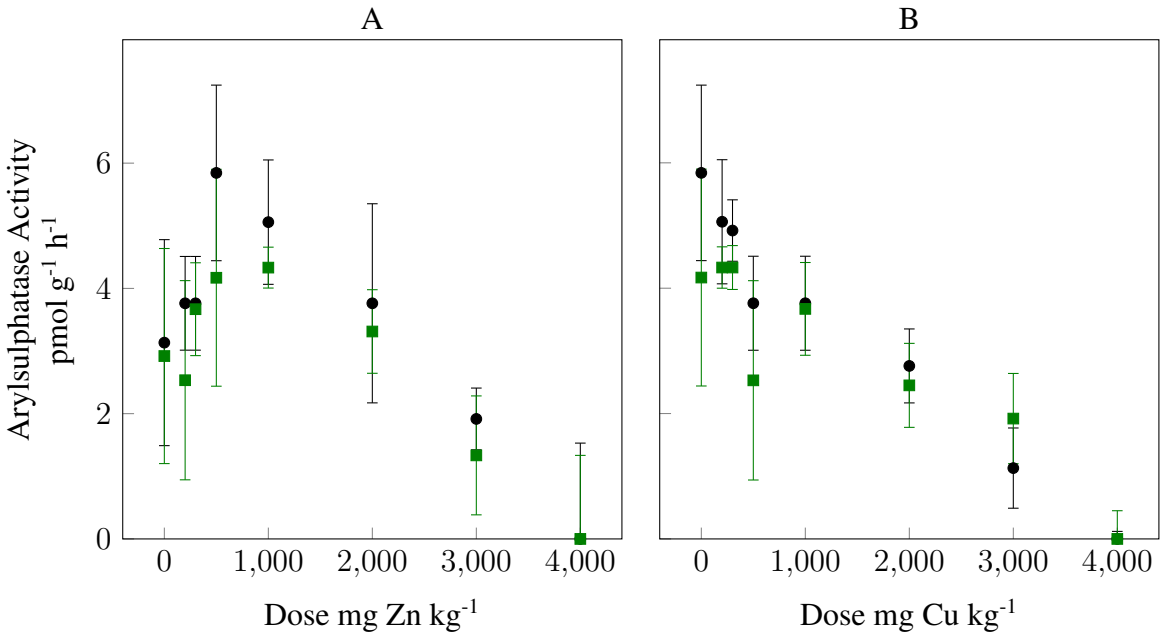

Fig. 3.2 Enzyme validation test for arylsulphatase activity in artificially contaminated boreal forest soils. A comparison of the effects of zinc (A) or copper (B) on the effectiveness of different enzyme activity measurement methods. Dose response model of artificially contaminated boreal forest soil. This graph shows arylsulphatase activity of 0.1g (circle) and 1g (square) assay results for each dose. Error bars represent ± 1 standard deviation.

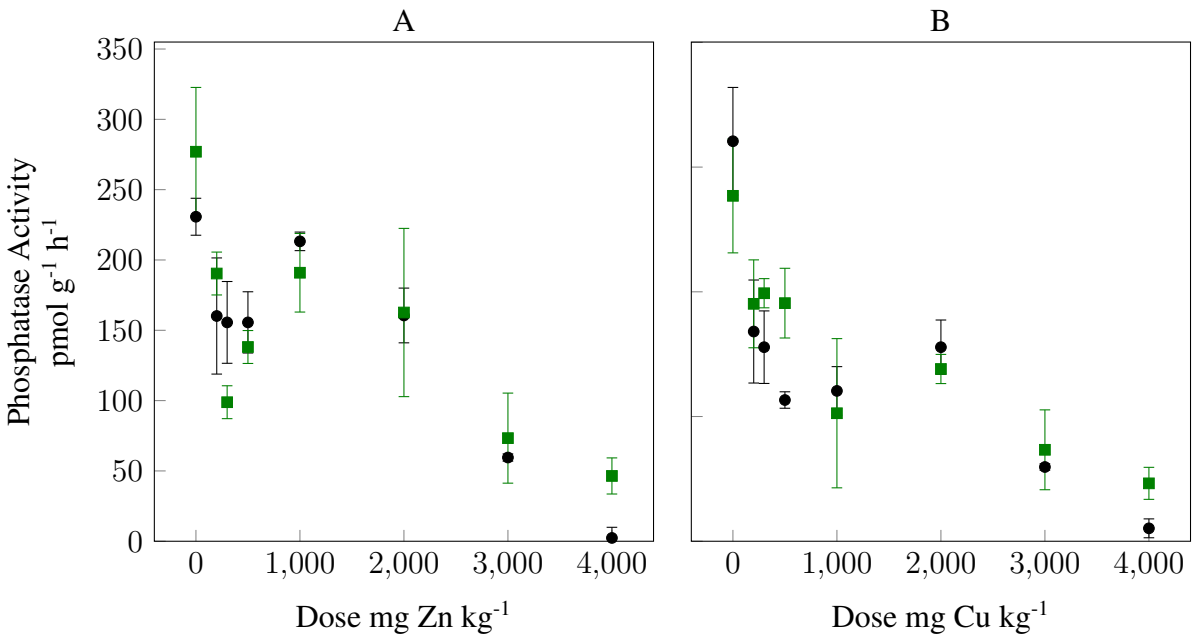

Fig. 3.3 Enzyme validation test for phosphatase activity in artificially contaminated boreal forest soil. A comparison of the effects of zinc (A) or copper (B) on the effectiveness of different enzyme activity measurement methods. Dose response model of artificially contaminated boreal forest soil. This graph shows phosphatase activity of 0.1g (circles) and 1g (squares) assay results for each dose.Error bars represent ± 1 standard deviation.

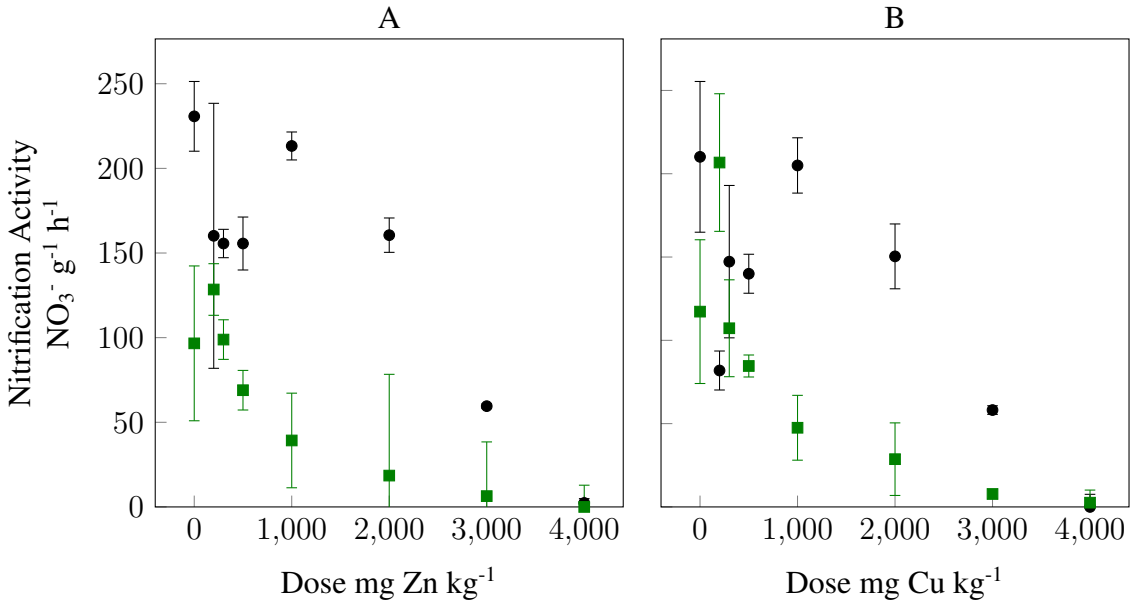

Fig. 3.4 Enzyme validation test for nitrification activity in artificially contaminated boreal forest soil. A comparison of the effects of copper (A) or zinc (B) on the effectiveness of different enzyme activity measurement methods. Dose response model of artificially contaminated boreal forest soil. This graph shows nitrification activity of 0.1g (circle) and 1g (square) assay results for each dose. Error bars represent ± 1 standard deviation.

Table 3.2 ANOVA table of the effect of weight of soil (0.1 vs. 1 g) and microbial response on the EC_{50} values.

Source of Variations	DF	SS	MS	F	Model *p*
Weight of soil	1	24.46	24.46	4.41	0.7
Weight of soil*dehydrogenase	1	20.29	19.79	4.38	0.4
Weight of soil*arylsulphatase	1	12.92	12.43	0.31	0.6
Weight of soil*nitrification	1	7.37	7.36	4.07	>0.05
Error	21	36.86	25.93		
Lack of fit	1	10.17	13.27	8.63	<0.01
Pure error	22	42.16			
Total	21	28.91			

3.5.2 Soil liming

Differences in nitrification activity in soils previously limed compared with un-limed soils in similar location were determined. Two sites show differences in soil autotrophic and heterotrophic activity: soils from sites that were unresponsive to liming had a lower nitrification rate dominated by heterotrophic nitrification activity while soils from sites that were responsive to liming had increased soil pH (as seen in Table 3.1 and Table 3.3) and nitrification activity (Figure 3.5). Soil nitrification was predominately increased by changes in autotrophic nitrification activity, with the greatest changes in nitrification activity occurring after a decade of increased soil pH (Figure 3.5).

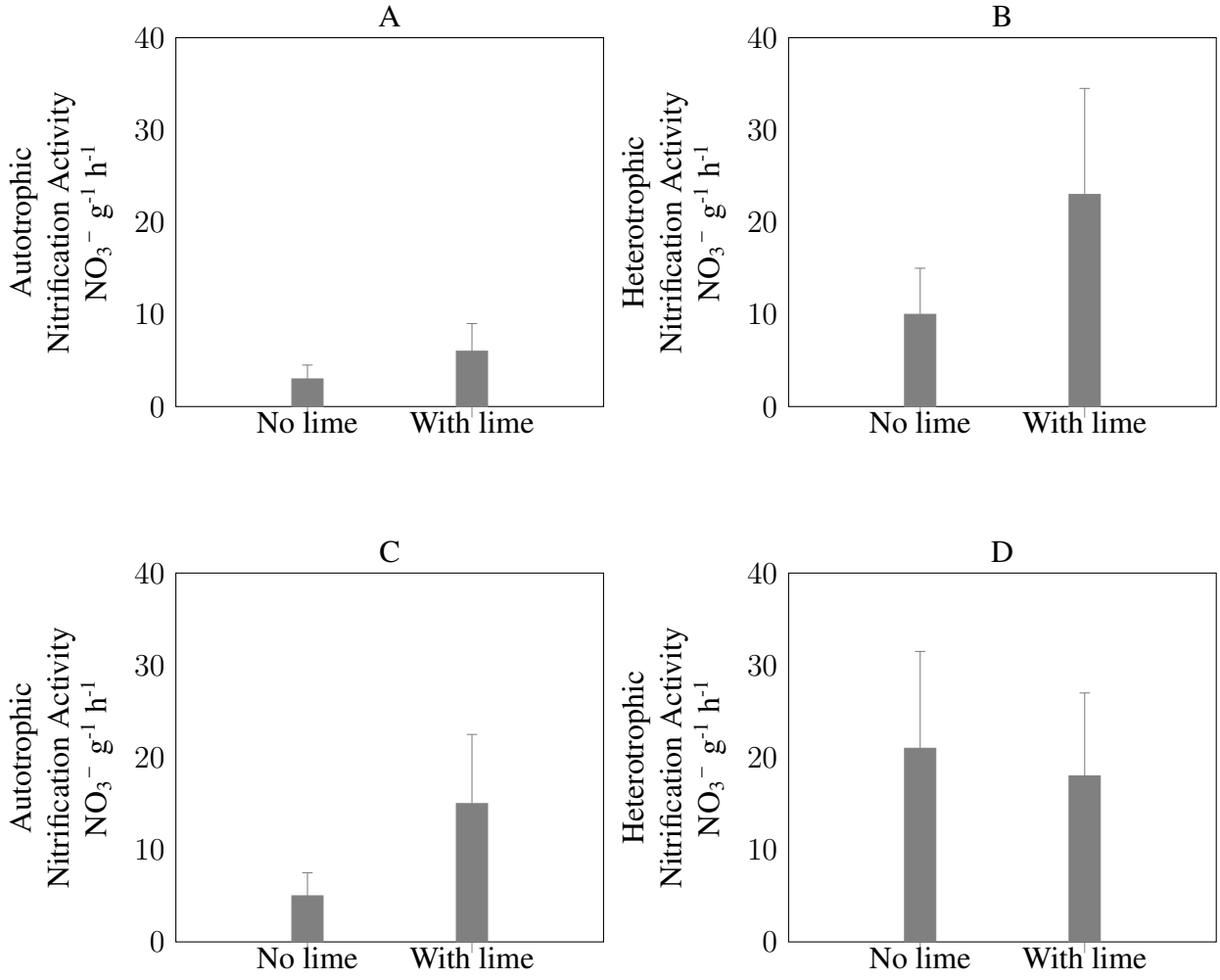

Fig. 3.5 Soil nitrification activity in field contaminated boreal forest soils from Flin Flon, Manitoba, Canada. Plots A and B show autotrophic and heterotrophic nitrification activity for site NR, the non-responsive site. Plots C and D show autotrophic and heterotrophic nitrification activity for site R, the responsive site. This is shown for different soils, one series of contaminated control soils with no lime (control) added and a series of lime amended soils (with lime) after a decade since lime application. Error bars represent +1 standard deviation from the mean.

Table 3.3 ANOVA table of the effects of lime responsiveness, time since liming and nitrification type (heterotrophic vs. autotrophic nitrification) on nitrification activity in soils from four sites

Source of Variations	DF	SS	MS	F	Model *p*
Responsivness	1	246.44	246.44	9.51	0.04
Responsivness*Nitrification type	1	2.07	2.07	0.08	>0.05
Responsivenss*Lime application timing	1	15.12	15.12	0.58	>0.05
Nitrification type	1	147.88	147.88	56.81	<0.01
Nitrification type*Lime application timing	1	63.34	63.34	2.44	>0.05
Lime application timing	1	252.75	252.75	9.75	<0.01
Error	33	856.56	25.93		
Lack of fit	1	213.27	213.27	10.63	<0.01
Pure error	32	642.29			
Total	39	2908.15			

3.5.3 Alternative amendments

Soil enzyme activity varied across both locations as shown in Fig.3.6. The lowest soil enzyme activities were seen in low organic matter high metal soils (LOMHM). All treatments showed an increase in activity relative to the control for enzyme activities, which was the fertilizer treatment (N-P-K).

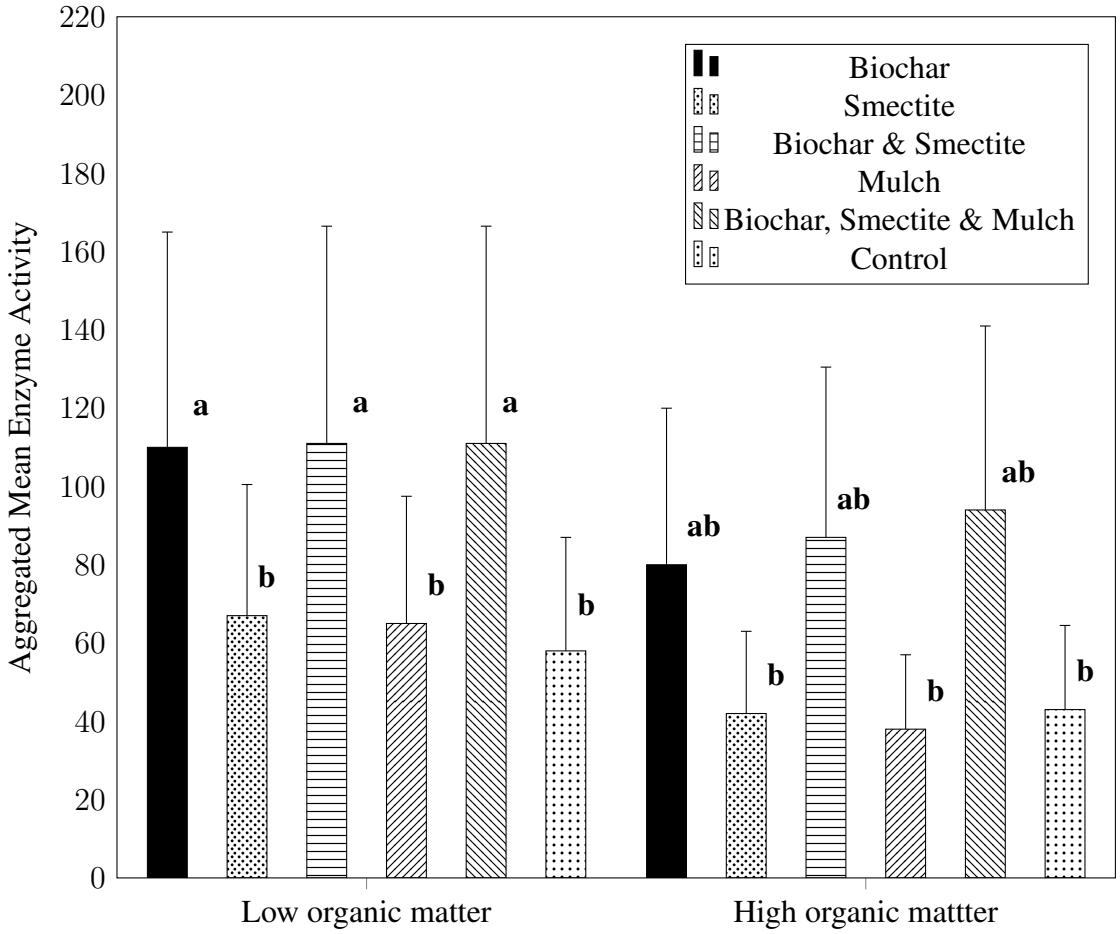

Fig. 3.6 Aggregated mean of enzyme activity in two smetler contaminated boreal forest types: low and high organic matter content. Six treatments were applied: biochar; smectite; biochar & smectite; mulch; biochar, smectite & mulch and a control plot. All plots had lime and fertilizer (19-19-19). Error bars indicate +1 standard deviation, letters indicate significant differences ($p<0.01$) between treatments

3.6 Discussion

3.6.1 Effect of assay soil weight

A reduction in the total soil requirement for the assessment of toxicity adds to the advantages of using a soil enzymes assay over other existing biological test methods for assessing soil ecological health. Soil enzyme-based methods are favorable due to their lower costs and short incubation periods, and thereby offer a cheap and rapid assessment tool for contaminated sites. Soil enzyme sensitivity and response to metal toxicity also add to their effectiveness. A comparison between the full and smaller scale test showed very favorable results, with the EC_{50} values for all assessment end-points being comparable regardless of sample size.

Overall, the results suggest that soil enzyme activities provide a useful end-point for assessing trace metal toxicity to soil ecological functions, regardless of the soil sample size used. This method provided EC_{50} values within the range of acceptable metal concentrations in Canadian soils (CCME SQG: 300 mg $kg^{-1}$for Zn and 200 mg $kg^{-1}$for Cu). The data also provide a valuable indication of the number of control samples required for the study of contaminated sites to account for natural factors influencing the biogeochemical properties of soils (e.g., soil pH, cation exchange capacity (CEC), and nutrient content).

Decreasing the soil requirement from 1 g to 0.1 g did not increase the variable of the response. This is consistent with the standard deviation reported for enzyme assays previously reported (Saiya-Cork et al., 2002; Weintraub et al., 2007). In determining soil enzyme activity it is often considered that more soil will provide more heterogeneity in the results. This is because a larger sample size this thought to capture more within-sample variation in soil enzyme availability. This study demonstrated a protocol that soil slurries generated from lower sample size (0.1g) did not differ from those of a larger sample size (1g). This allows a reduction in the overall amount of soil used in each test. It is however important to note that a larger amount of individual samples (n=5) was used compared with that of others (Saiya-Cork et al., 2002; Weintraub et al., 2007). Typically soil enzyme activity is reported using a single value using between 5 and 1 g of soil,

I used a higher amount of 5 g. It is thought that this increase in the number of measurement replicates for the individual sub-sample accounts for the heterogeneity in results observed using single larger soil slurries to generate a result.

For nitrification activity however, although the variation of each individual result was comparable between the two different methods (0.1 g vs. 1 g). There was a difference in the dose-response curve with nitrification showing an apparent increased sensitivity to metal stress in the 1 g sample method. Nitrification is different from the other enzyme assays in a number of ways. Firstly tests of nitrification activity often use much higher sample sizes than the other enzymes assessed. Several studies (Smolders et al., 2001; Rusk et al., 2004; Mertens et al., 2006, 2007, 2009; Ruyters et al., 2010a) all report nitrification activity using sample of 50 to 1 g. Also, the analysis of nitrification uses a different method of assessment than soil enzymes. With the soil enzyme test targeting a specific breakdown product not normally present in soils. This could mean that there is increased variability between soils found in the nitrification assay than in other soil enzyme activities. It is therefore not recommended to use a smaller scaled down variation of assay for the assessment of soil nitrification. A study by (Trevisan et al., 2012) provides a comparison of different methods of assessing soil nitrification activity in metal contaminated soils. That study also showed that different methods of assessing nitrification activity and different sample sizes showed differing results.

3.6.2 Soil liming

Differences between heterotrophic and autotrophic nitrification responsiveness to lime application were identified. The mechanisms by which soil nitrifiers derive energy from the nitrification process differs for heterotrophic vs. autotrophic nitrification (De Boer and Kowalchuk, 2001). This means that the optimum conditions for nitrification also differ for these two mechanisms. Soil heterotrophic nitrification is best achieved in lower pH soils, and soil autotrophic nitrification perform optimally in soils with a pH of 6 to 7 (Ward et al., 2011). Liming may have produced more favorable conditions in terms of soil pH (3 to 4) for heterotrophic vs. autotrophic (4

to 5) nitrification in NR soils with lower pH changes. R soils showed the greatest overall recovery as a result of changes autotrophic nitrification being greater than heterotrophic.

3.6.3 Alternative amendments

Biochar has a clear potential for the reduction of contaminants in mobile forms present in soils (Lehmann et al., 2011). However, this is only one aspect; biochar has also been investigated for its potential to improve soil health in terms of biodiversity, soil structure, nutrient cycling, aeration, disease resistance, and carbon storage capacity (Lehmann et al., 2011). The effects of biochar on soil biota are well documented at numerous trophic levels (Warnock et al., 2010; Lehmann et al., 2011).

The abundance of microorganisms has been investigated in biochar amended soils in various studies by various methods. Numerous reasons for alterations to soil microbial abundance have been suggested. Mycorrhiza respond positively to biochar addition (Warnock et al., 2007), and this is thought to occur through direct interaction with biochar particles within the soil. The porous nature of biochar is thought to protect microbial communities from grazers (collembola, nematodes, and protozoa), which promotes soil total biomass and thus influences the biodiversity of the soil microbial community. As the biodiversity of contaminated soils are poorly understood, biochar has the potential to offer a significant biological benefit to contaminated soils and therefore promote overall soil health. However, the response of soils to biochar was not consistent with several studies showing decreases in microorganism abundance in response to biochar addition (Warnock et al., 2010). Alterations to soil conditions can produce unwanted soil property alterations (pH, water retention, organic matter availability) that can reduce microbial activity (Lehmann et al., 2011). However, sorption of compounds that would otherwise inhibit microbial community growth rate and abundance are still regarded as beneficial to the community overall. Biochar addition influences several microbial functions in soil (Lehmann et al., 2011) that, in turn, affect soil biogeochemical cycles. Biochar is thought to affect soil microbial function directly (via changes to the abundance and structure of the soil microbial community) as well as indirectly (by

altering the metabolic environment in which soil function process take place) (Lehmann et al., 2011). A challenge often encountered in soil microbial assessment is separating biotic and abiotic factors causing alterations to soil processes (Lehmann et al., 2011).

3.6.4 Conclusion

Given the critical role enzymes play in soil ecosystem functioning, their limited application in deriving site specific soil quality guidelines undermines the reliability of these assessments. Here, the validation of a new method for assessing soil enzyme activities (phosphatase, sulfatase, and dehydrogenase) that only requires low (0.1 g) amounts of soil demonstrated that enzyme assays can be incorporated into site specific assessment with a negligible amount of soil required with little to no impact on the overall cost of transportation or environmental impact (loss of soil from a site).

For soil nitrification this type low weight assay could not be validated. This could indicate that not all enzyme assays can be scaled down to low weight requirements and their could be a minimum weight of soil required for some enzyme assays. Soil nitrification assays with the use of nitrapyrin was however successfully applied to examine the effect of lime on the soil microbial community of smelter contaminated soils with varying physiochemical properties. This was able to identify the differences in response of the soil heteotrophic and autotrophic nitrification activity to liming.

Soil enzymes (phosphatase, sulfatase, and dehydrogenase) proved reliable at identifying the treatment effectiveness of biochar and smectite amendments applied on a range of soils with varying zinc and organic matter content. This research also demonstrated the applicability of a low soil requirement assessment method for use in large scale eco-restoration projects, confirming the potential for soil enzyme assays as a tool for identifying soil microbial responsiveness to amendment strategies. This allows for the evaluation of soil amendment strategies for large-scale eco-restoration projects in an affordable and effective manner.

4 EFFECT OF LAND-USE ON THE TOXICITY OF ZINC AND COPPER TO SOIL MICROBIAL FUNCTIONS

4.1 Preface

This chapter is the central chapter of this thesis. This paper incorporates the methods of soil enzyme activity developed and outlined in the previous research chapter (3) and calculates EC_{50} values for zinc and copper of nitrification, dehydrogenase, arylsulphatase, and acid phosphatase activity in 18 soils from across Western Canada. This provides a clear demonstration of the capability of soil enzyme assays for terrestrial eco-toxicity assessment and how this method meets the requirements of the CCME for the assessment of contaminated sites and determining soil quality criteria. This experiment also demonstrates the importance of soil properties and land-use. The experiment provides a range of eco-toxicity data and the first steps towards making soil enzyme assessment a part of the Canadian regulatory regime.

4.2 Abstract

Extraction, processing, and use of trace metals has led to increases in surface soil concentrations. The present study investigates the effect of land-use on the toxicity of both zinc (Zn) and copper (Cu) to a range of different microbial enzyme activity EC_{50} values. Toxicity across soils varied significantly and by orders of magnitude. This study used North American soils from a range of land-uses to identify differences in adaptation rate and sensitivity, and aimed to evaluate methods used to assess the hazards from trace metals using spiked soils. First, soils (n=18) were spiked with zinc and copper at eight different concentrations. Dose-response curves were then determined for soil nitrification and enzyme activity (dehydrogenase, arylsulphatase, and acid phosphatase). Land-use was shown to be a factor ($P < 0.01$) affecting the toxicity of zinc and copper. Boreal forest soil nitrification rates had the most sensitive end-point to zinc (EC_{50} = 202 $\pm$ 44 mg kg^{-1}) and copper (EC_{50} = 197 $\pm$ 23 mg kg^{-1}) compared to other enzyme activities and land-uses. Across all land-uses and soil types, a similar pattern of sensitivity was noted: potential nitrification > dehydrogenase > arylsulfatase > acid phosphatase. Land-use was a significant factor influencing the short- and long-term toxicity of zinc and copper across a range of land-uses. Land-use influences soil properties such as pH and CEC, which in turn affect the toxicity of zinc and copper. Land-use should therefore be considered for not only the protection of human health but also ecological health. The demonstration of adaptation across a range of land-uses and the variation in toxicity observed will serve to assist the future assessment of trace metal risk in soils and the interpretation of site specific toxicity compared to benchmark dose-responses.

4.3 Introduction

Trace metals impact the activity of soil microorganisms and microbial functions (Giller et al., 2009). Soil microorganisms are sufficiently sensitive and important to warrant their inclusion in risk assessment processes (Kuperman et al., 2014). However, determining the extent of the impact of trace metals on soil microorganisms presents a challenge, because soil properties and adaptation alter the sensitivity of microorganisms to trace metals (Puglisi et al., 2012). The toxicity of metals varies across soil types (Smolders et al., 2004; Oorts et al., 2006). Several studies have tried to identify the primary cause for differences in toxicity observed across soil types (Smolders et al., 2004; Oorts et al., 2006; Broos et al., 2005, 2007). Cation exchange capacity (CEC), pH, soil organic matter (SOM) content, and background metal load are often cited as factors influencing soil toxicity to trace metals. CEC and pH are cited as the primary drivers of soil toxicity (Smolders et al., 2004; Broos et al., 2007). CEC influences the absorptivity of trace metals important for soil health as well as the bioavailability of metals. Soil pH can be thought of as the prime driver because of its overriding influence on metal speciation and reactivity.

Microorganisms are critical to soil biogeochemical cycling and play a fundamental part in ecosystem services. Ecosystem services provided by soils should be thought of as the functions that soils can provide. This can be split into numerous different types of interrelated services: provisional, environmental regulation, cultural, and supporting services to ecosystems at large (Haygarth and Ritz, 2009). Microbial communities contribute significantly to the overall enzyme activity in soils, which is central to regulating the availability of inorganic nutrient forms supporting primary productivity. This has often led to soil enzyme activities being utilized as indicators of soil quality and soil health (Karlen et al., 2003). The health of a soil is often described as a soil's capacity to support ecosystem functioning and the capacity of a soil to regulate environmental processes (Schoenholtz et al., 2000).

The ability of soils to mitigate the toxic effects of pollutants on biological functions has been repeatedly observed (Fait et al., 2006; Mertens et al., 2009; Puglisi et al., 2012). Microorganisms are the key trophic level providing the buffering and adaptive capacity attributed to soils.

Microbial populations have the genetic variability to be able to rapidly respond to environmental changes and thereby mitigate the toxic effects of metals. There are two primary mechanisms by which microorganisms mitigate these toxic effects (Puglisi et al., 2012). First, microorganisms can immobilize or mobilize chemical forms present in the soil. For example, chelation (Dimkpa et al., 2009), methylation (Loseto et al., 2004), and microbially mediated reduction of certain metal species (White et al., 1997) all lead to a reduction in the exposure of the soil microbial community to metals. Second, internal resistance mechanisms are used to withstand the pressures of metal toxicity. For example, this can involve the physical exclusion of metals by electronegative components in cell membranes and exopolymers, metal efflux systems to remove toxic metals from cells, and intercellular sequestrations with lower molecular weight proteins (Ledin, 2000).

In Canada, soil processes are often termed a "nutrient cycling check" and, in the regulatory context, land-use is a key determinant of the receptors and pathways considered during a chemical risk assessment (Canadian Council of Ministers of the Environment, 2006). Adaptation to trace metals in soils has been demonstrated (Rusk et al., 2004; Mertens et al., 2006; Trevisan et al., 2012). Evidence of adaptation has, however, been limited to European agricultural soils and to date has not been demonstrated across a range of land-use types. Here I assessed the effects of land-use and soil properties on the sensitivity and adaptation of soil processes to zinc and copper. I hypothesized that land-use would be a determinant of soil process sensitivity and adaptive capacity compared to simply assessing soil properties, all the while recognizing that land use would interact with soil properties.

This experiment was designed to meet several objectives simultaneously, including determining the effect of zinc and copper concentration on soil enzyme activities in Canadian soils and the effect of adaptation on the relative sensitivity of differing enzyme activities to zinc and copper toxicity. The aim of this experiment was to provide a set of eco-toxicity data for enzyme activities and demonstrate their utility for risk assessment in the Canadian regulatory regime. To achieve this, several soils were artificially contaminated with of zinc and copper at varying concentrations. These soils were then aged for a period of six months (180 d).

4.4 Materials & Methods

4.4.1 Soil samples

Eighteen sites representative of five different land-use types were selected from across Western Canada, representative of boreal forest (n=6), cultivated agricultural (n=3), native grassland (n=3), urban parkland (n=3), and urban industrial (n=3) (Table 4.1 and Figure 4.1). Whenever possible, sampling locations were selected to avoid metal contamination, with soils collected from sites more than 100 m away from all obvious man-made structures and anthropogenic disturbances. This was possible for boreal forest and native grassland locations but was unavoidable at sites representative of agricultural and urban land-uses. Over the summer of 2012, soil samples were collected from each location. Soils were sampled in bulk from the surface layer (top 0-10 cm) using a metal spade. All sampling equipment was cleaned with deionized water and a 5% methanol solution. All equipment was cleaned between each site and pre-contaminated by exposure to soil at the location by digging a small hole and discarding this material, prior to sampling.

Boreal forest soils were collected from six sites, three sites in Manitoba (one location) and three sites (two locations) in Saskatchewan. In Manitoba soils were collected from an area of undisturbed boreal forest close to Sheriddon, Manitoba. The area is vegetated with trembling aspen (*Populus tremuloides*) and birch (*Betula papyrifera*) trees. This is typical of vegetation observed in the surrounding area (Eilers and Swidinsky, 1989). The area is dominated by two soil groups, Brunisols and Grey Luvisols (Eilers and Swidinsky, 1989). Saskatchewan boreal forest soils were collected from two locations. All close to Meadow Lake Provincial Park, collected from an area of established mixed boreal forest vegetated with a mixture of white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*). The first location was Alcott Creek, approximately 40 km southeast of Meadow Lake. The second location was near Keeley Lake, approximately 20 km northeast of Meadow Lake.

Agricultural soils were collected from three locations. The first location (one site) was approximately 15 km north of the town of Outlook, SK, in the central prairie eco-region of Sas-

katchewan. Soils in the region are broadly classified as a mix of Orthic Dark Brown Chernozems. The area is cultivated with a range of different crops, predominantly wheat and canola, in rotation cropping systems. Soils were collected post-harvest after wheat. The second location (two sites) was in the prairie-forest eco-region in west Saskatchewan, in an area of mixed native grasslands, agricultural fields, and jack pine forest, with soils collected from the cultivated agricultural fields. Soils were collected post-harvest after wheat.

Native grassland soils were collected from three sites in two areas with similar site histories. Soil in these locations were formed on glacial till deposits, following glacial retreat around 10,000 years before present (Williams et al., 2009). The sites were located in an area of open grassland surrounded by aspen and areas of cultivated agricultural land.

Urban parkland soils and urban industrial soils were collected from the City of Saskatoon. The City of Saskatoon features a mix of land-use divisions set by its City Council. Soils representative of urban parkland were collected from areas of predominantly residential land-use, specifically from two small parks and a residential garden. Soils representative of urban industrial land-use were collected from areas of predominately industrial land-use, in areas of open unmanaged and uncultivated grassland.

Table 4.1 Location of the 18 sites across Saskatchewan and Manitoba where soils were sampled in bulk. Sites came from five land-use types with three replicates for each land-use and for boreal forest soils, three from the province of Saskatchewan and Manitoba.

Land-Use	Sites	Lat N°	Long W°
Agricultural	1	53.255	103.552
	2	53.347	108.782
	3	51.393	106.563
Native Grassland	4	53.293	108.76
	5	53.321	108.777
	6	53.341	108.764
Boreal Forest (SK)	7	53.46	108.215
	8	54.513	108.162
	9	54.141	108.445
Boreal Forest (MB)	10	55.032	101.276
	11	54.949	101.267
	12	54.942	101.263
Urban Residential	13	52.114	106.639
	14	52.116	106.636
	15	52.148	106.562
Urban Industrial	16	52.17	106.657
	17	52.165	106.674
	18	52.165	106.674

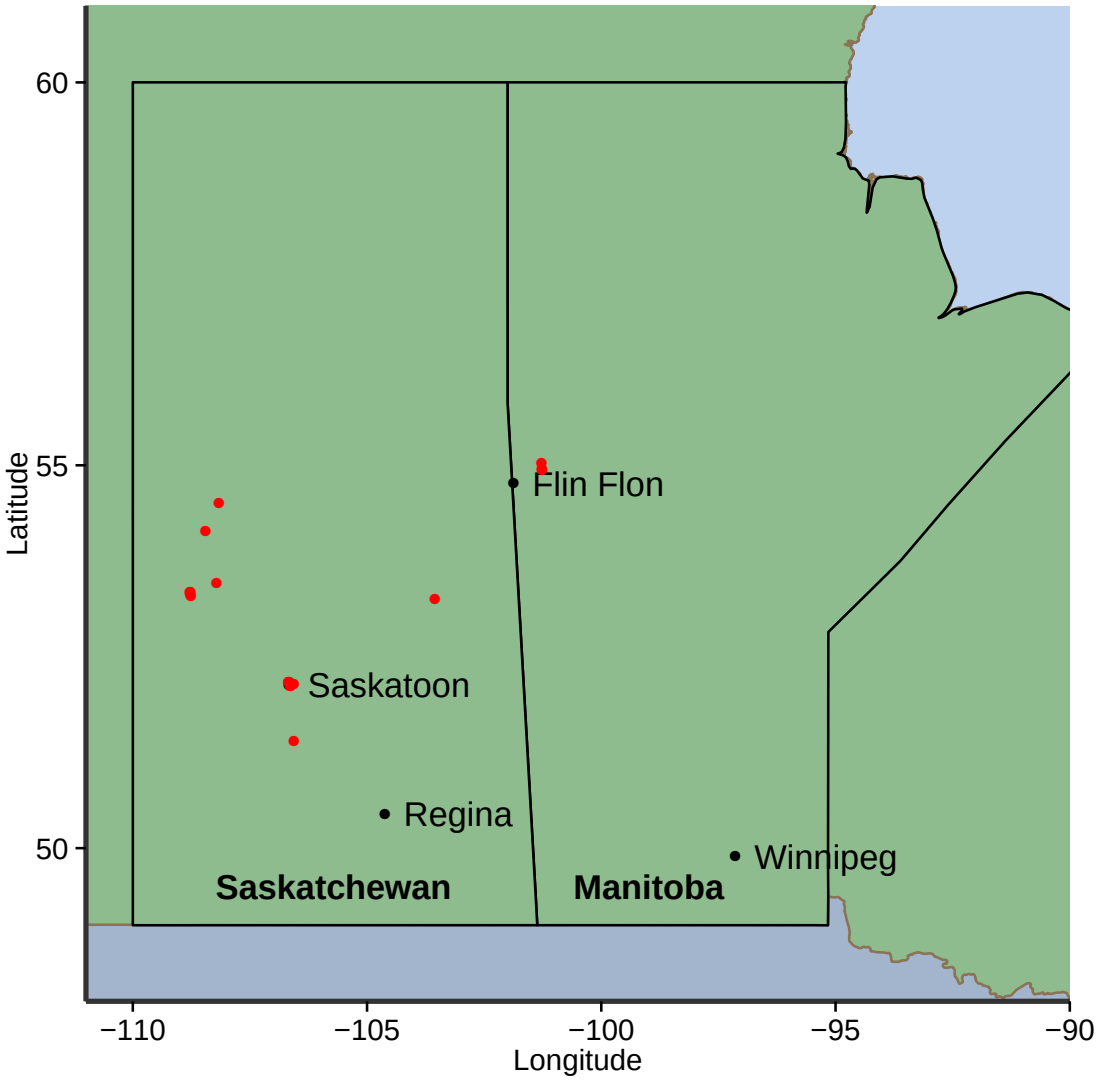

Fig. 4.1 Map of an area of Canada with the 18 soil sample sites highlighted (in red). Samples were taken from Saskatchewan and Manitoba (provinces outlined). This map was created using the R statistical program (R Development Core Team, 2013), plotting vector and raster map data obtained from Natural Earth open source data available in the public domain.

4.4.2 Soil characterization

Soils were collected in the fall of 2012 and stored at 4°C prior to the experimentation. Selected soil properties were analyzed from subsamples of each bulk sample. Subsamples were air-dried and sieved to 2-mm prior to analysis.

Soil pH was determined using a Oakton PC700 bench-top meter and a single probe (Thermo Fisher Scientific, Burlington, ON) and 0.01 M $CaCl_2$ (1:5 (w/v)), following methods modified by Hendershot et al. (2008). Hydrated soil (1 g) was placed into Falcon™ 50 mL conical centrifuge tubes. Samples were shaken horizontally for 1 h after 0.01 M $CaCl_2$ (5 mL) was added. Soils were then left to settle for 1 h prior to taking a pH reading. A $CaCl_2$ solution was used to account for the potential effect of metal sulphide concentration compared to alternative methods (Hendershot et al., 2008). This method of pH analysis is recommended for assessing contaminated soils (Environment Canada, 2012). The pH of each bulk sample was measured five times (n=5), using five sub-samples taken from each soil type (n=18). Further analysis of pH was carried out using a single test measurement with three analytical replicates. Soil conductivity was measured using a Oakton PC700 bench-top meter and probe (Thermo Fisher Scientific, Burlington, ON), following the methods outlined by Environment Canada (2012).

Soil moisture content of all soils were determined by weighing 1 g of field moist soil into pre-weighed aluminum weigh boats. Each soil was then oven dried for 24 h at 105°C. Samples were then cooled in a desiccator for a minimum of 1 h. After cooling, the dry soil was then re-weighed and soil moisture content determined using Equation 4.1.

$$SMC = \frac{wetsoilweight(\mathrm{g}) - drysoilweight(\mathrm{g})}{drysoilweight(\mathrm{g})} \times 100 \qquad \text{(Eq. 4.1)}$$

The WHC of each bulk soil was measured in triplicate (n=3), following protocols similar to those outlined by Environment Canada (2012). Soil (50 g) was weighed into aluminum boats and oven dried at 105°C for 24 h. Samples were then cooled in a desiccator for a minimum of 1 h. Then 25 g was placed into a 500 mL Bernardin™ mason jar with 25 mL of deionized H_2O and then

mixed thoroughly. Soil slurries were then placed into Fisherbrand™ P8 filter papers and plastic funnels. After filter papers were hydrated with 9 mL of deionized H_2O, completely covering the filter paper surface, soils were drained into 250 mL plastic vials. After 3 h the filter paper and funnels were then weighed. WHC was calculated using Equation 4.2.

$$WHC = \frac{F - I}{D} \times 100\% \quad \text{(Eq. 4.2)}$$

where F (g) is the mass of the funnel, filter paper and soil, I (g) is the mass of the funnel and filter paper and D is the dry mass of the soil (g).

Soil CEC in this study was determined by ALS laboratories (Saskatoon, Saskatchewan) using the ammonium acetate method of McKeague (1978). Subsamples were separated from the bulk collected soils and weighed (10 g). These soils were then used for each analysis, along with blanks and quality control samples. Ammonium acetate (1M) (NH_4OAc) was added to samples to saturate them with ammonium at pH 7. This was then displaced with sodium in the form of 1 M NaCl. Analysis of the NH_4 content was then determined colorimetrically.

Soil pore water concentrations of zinc and copper were measured using a desorption method with a 0.01M $CaCl_2$ extracting solution following methods outlined by Houba et al. (2000). The principle of the method is to use $CaCl_2$ to provide a constant electrolyte concentration with a similar binding concentration to $Zn^{2+}$and Cu^{2+}, so Ca^{2+} will replace other cations in soil adsorption complexes. Air-dried soil (10 g) sieved to <2-mm was extracted with 100 mL of 0.01M $CaCl_2$ at a 1:10 (w/v) soil solution ratio in a 200 mL polypropylene container. Samples were then shaken for 12 h at 160 rpm, centrifuged at 900 rpm for 15 min and, then the clear supernatant extracted and filtered through a 0.45 µm Millipore filter. The extract was then acidified with HNO_3 and stored for analysis. The solutions were analyzed using a microwave plasma-atomic emissions spectrometer, (MP-AES; Agilent 4100, Agilent Technologies, Melbourne, Vic, Australia) and MP Expert software (Agilent Technologies, Melbourne, Vic, Australia). Analyses of zinc and copper

were carried out at a set wavelength of 324.75 nm for copper and 213.86 nm for zinc. Analyses followed quality control procedures outlined by Wightwick et al. (2010).

Soil organic carbon content was determined by ALS laboratories (Saskatoon, Saskatchewan). Organic carbon content was calculated as the difference between total carbon content and inorganic carbon content for each soil. Organic carbon content was determine using a combustion method for CO_2 production, analyzed using a carbon analyzer, at 842 and 1142°C to determined organic and total carbon, respectively.

Exchangeable inorganic nitrogen content was determined using a 1 M KCl extraction (soil: solution ratio 1:5), following the protocol set out in international standard ISO 14238 (International Standards Organization, 2012a). Field moist soil (5 g) were added to 250 mL Erlenmeyer flasks to which 50 mL of 1M KCl was added. Samples were shaken at 160 rpm for 30 min and filtered using Whatman No. 42 filter paper. The NH_4^+ and NO_3^- concentrations of the solutions were then determined using a SmartChem 200 auto-analyzer (Westco Scientific Instruments Inc, Brookfield, CT).

4.4.3 Soil microbial analysis

Three enzyme assays were conducted, associated with three key bio-geochemical cycles: the soil phosphorus, sulfur, and carbon cycles. The enzyme assays followed modified versions of protocols available in the published literature. Activities associated with soil phosphatase (EC 3.3.2), sulfatase (EC 3.16.1), and dehydrogenase (EC 1.1) were determined. In addition to measurement of enzyme activities a functional assay associated with the nitrogen cycle was also conducted. This was a soil nitrification activity assay.

Nitrification in each test soil was analyzed using two different assessment protocols: ISO 14238 (International Standards Organization, 2012a) and ISO 15685 (International Standards Organization, 2012b). The ISO 15685 method followed the published protocol (International Standards Organization, 2012b) but the ISO 14238 method was modified for both quantities of soil re-

quired and to allow assessment of soil heterotrophic activity. All assays were carried out following 7 d of incubation in darkness at approximately 20°C.

The ISO 14238 method was carried out using $(NH_4)_2SO_4$ as a nitrogeneous substrate to allow the assessment of nitrification only. For each test, 99 g of soil were incubated with 100 mg NH_4^+-N kg, applied in the form of $(NH_4)_2SO_4$, added from a 100 mg $(NH_4)_2SO_4$ mL stock solution. Soils were incubated for 28 d with 5 g subsamples collected after 0, 4, 7, 14, 21, and 28 d. Bulk soil samples were then analyzed using only a 7 d incubation. Then, 2 M KCl was used to extract NH_4^+ and NO_3^- + NO_2^-, which are collectively referred to as NO_x. The concentrations of NH_4^+ and NO_3^- + NO_2^- were determined using a SmartChem 200 auto-analyzer (Westco Scientific Instruments Inc., Brookfield, CT, USA).Soils were maintained at a WHC of 60% for the duration of their incubation.

For the dose-response experiments, toxicity testing method ISO 14238 was applied to calculate the inhibited value as a percentage of the control (ID_x) for each treatment level, according to Equation 4.3:

$$ID_x = 100 - \left(\frac{\mathrm{NO_3}^- (1)}{\mathrm{NO_3}^- (2)} \right) \times 100 \qquad \text{(Eq. 4.3)}$$

where $\mathrm{NO_3}^- (1)$ is the rate of formation of NO_3^-in a treated soil, and $\mathrm{NO_3}^- (2)$ is the rate of formation of milligrams of NO_3^-per kilogram of soil per day in the untreated control soil. This was calculated for each of several individual time sets 0-4 days; 4-7 days; 7-14 days; 14-21; and 21-28 days. The slope (rate of formation of NO_3^-) was determined using a linear regression of NO_3^- formation over time. The linear regression was calculated using the R statistical program (version 3.02.) (R Development Core Team, 2013)

Arylsulfatase activity was measured in soils using a modified version of protocols set out by Whalen and Warman (1996). This protocol was developed from work carried out by Tabatabai and Bremner (1970) and modified further for this study to use only 0.1 g instead of 1 g of soil while reagent to soil ratios were maintained. Solutions buffering pH were not used in an effort to eliminate the confounding effects caused by changes in metal availability (Lessard et al.,

2013), with de-ionized water used instead. A detailed validation and comparison of this scaled down method is provided in Chapter 3.

Soils were incubated in glass vials, in the dark at 20°C, for one week prior to analysis. Soils (0.1 g) were then weighed into 2 mL polypropylene Eppendorf® tubes. Toluene (20 µL) was added, to inhibit further enzyme synthesis (Whalen and Warman, 1996). Soils were then incubated for a further one h. Substrate was then added to each tube, in the form of 0.1 mL of a 10 mM solution of *p*-nitro-phenol-sulfate. Deionized water (0.4 mL) was added to each tube followed by vigorous mixing. Soils were incubated at 37°C for 1 h, with the temperature maintained using a water bath. After 1 h, was activity stopped by transferring tubes to an ice water bath for 5 min and then centrifuging at 14000 rpm for 2 min. The supernatant (150 µL) was then extracted and combined with 100 µL of 0.5 M NaOH solution and transferred to a clear Pierce™ 96-well polystyrene plate, for measurement of *p*-nitro-phenol content. Control samples and reagent blanks were included, to account for natural variation due to soil color.

Phosphatase activities were measured using a modified version of protocols set out by Eivazi and Tabatabi (1977). This method was modified as noted above use of only 0.1 g of soil and the use of deionized water instead of a buffering solution. The same protocol as described above was followed except for the use of *p*-nitro-phenylphosphate as the substrate.

Dehydrogenase activities were measured using a modified version of the protocol set out by Trevors (1984). Soils were incubated in glass vials in the dark at 20°C and 60% WHC for one week prior to analysis. Soils (0.1 g) were weighted into 2 mL polypropylene Eppendorf® tubes to which 20 µL of 0.4% *p*-iodonitrotetrazolium chloride (INT) solution was added. Soils were then incubated in the dark at 20°C for 48 h. Methanol (1 mL) was then added and the soils were then vigorously vortexed and centrifuged at 14000 rpm for 2 min. The supernatant (250 µL) was then removed and transferred to clear Pierce™ 96-well polystyrene plates for measurement of iodonitrotetrazolium formazan (INTF) concentrations. Control samples and reagent blanks were included, to account for natural variation due to soil colour.

Concentrations of INTF were determined spectrophotometrically, with measurement of absorptivity at 480 nm and comparison to a standard calibration curve. The standard curve was created using a stock solution of INTF created using 2.4 mg of INTF added to 100 mL of methanol.

4.4.4 Experimental design and set up

There is no standardized method for the artificial contamination of soils, no method advocated in the published literature is widely accepted, and no single method favored by regulating authorities. For this study, soils were spiked with six different concentrations of zinc or copper. A control was also included. Soils were subdivided from bulk into microcosms of 1 kg of soil. Each 1 kg microcosm had varying amounts and concentrations of stock solution applied so the same dose was received by all soils. Soils were incubated in a complete block design with treatments randomly assigned incubation locations. The treatments were incubated in darkness at 22 20°C. Nominal rates of treatment application were determined by a range finding experiment using nitrification activity in soils to determine dose concentrations. The range was selected to achieve an equal number of response and 100% lethal concentrations. Treatments were applied by allowing them to slowly percolate into the base of the microcosms though a muslin cloth and the perforated base of each container. The treatments were applied as sulphate salts of each metal. Each treatment was applied at eight nominal concentrations (0, 300, 1,000, 2,000, 3,000, 5,000, 10,000, and 20,000 mg kg^{-1} of total salt applied). Number of doses applied was valued over number of replicates for this design, as analysis of dose-response was done using regression analysis and it is of more advantage to increases the number of doses over replications (Environment Canada, 2007). Doses were added to soils from a stock solution of varying concentrations to allow saturation (at WHC) of each soil with the required treatment dose. The concentration of stock solution required for this experiment was calculated using Equation 4.4.

$$SSC\,\mathrm{g\,l^{-1}} = \frac{Dose_{max}\,\mathrm{mg\,kg^{-1}}}{500 \times WHC_{min} \times f_{ms}\frac{\mathrm{g\,mol^{-1}}}{\mathrm{g\,mol^{-1}}}} \qquad \text{(Eq. 4.4)}$$

where SSC is the stock solution concentration, $Dose_{max}$ is the maximum dose applied to the soil, WHC_{min} is the minimum water holding capacity of any soil and f_{ms} is the molar mass of each metal to the molar mass of each salt applied.

Spiking soils with soluble metal salts leads to increases in salt concentration. Soils were therefore leached with an artificial rain water solution (Lock et al., 2006; Oorts et al., 2007; Langdon et al., 2014), consisting of 5×10^{-4} M $CaCl_2$, 5×10^{-4} M $CaNO_3{}^-$, 5×10^{-4} M $MgCl_2$, 10^{-4} M Na_2SO_4, and 10^{-4} M KCl using a method outlined by Li et al. (2010). The microcosms were submerged into 22 L plastic containers of artificial rainwater and equilibrated for 24 h. Soils were then allowed to free drain for a further 24 h and were partially air dried, allowing soil moisture to be restored to 60% WHC prior to incubation.

4.4.5 Incubation and monitoring

Soils were incubated for a total of six months (180 d). Soils were stored in a test unit consisting of a 1-kg plastic pot with a perforated base to allow free drainage. A plastic container was placed around each plot to collect any drainage and avoid cross contamination between units. The soils were incubated in darkness at approximately 22°C. Several parameters were monitored during this incubation.

Soil moisture content was maintained gravimetrically throughout. Soils were tested for changes in moisture content every two weeks. Soils were weighed (soil and container) and the mass of each unit identified and recorded. Soils were then re-weighed and artificial rainwater added to restore the mass of each unit. After samples of soil were collected from each incubation units for analysis, each unit's weight was re-recorded.

To examine toxicity of zinc and copper of each soil, nitrification activity was used as a indicator. The toxicity of soil nitrification and the potential for adaptation has been most widely characterized. This test was used to monitor changes in soil microbial activity over time, prior to further testing of other enzyme activities and experimentation. Soil samples were collected at four time points (0, 30, 90, 180 d) during the incubation. Nitrification activity was determined using

the methods outlined in Section 4.4.3. Soil sub samples were placed in 500 mL glass Mason jars with soil moisture set at 60% WHC and incubated for one week prior to analysis. The soils were incubated in darkness at approximately 22°C.

Soil pH and electrical conductivity were monitored for changes over time, with samples collected at 0, 30, 90, 180 d. Analysis was carried out using method described in Section 4.4.2. Samples of soil for further enzyme testing and experimentation were collected at 0, 30, 90, 180 d. Soils were placed into 500 mL glass vials and stored at -20°C for up to one year. Soils were re-hydrated and incubated in darkness at approximately 22°C for one week prior to any further microbial analysis.

4.4.6 Statistics

4.4.6.1 Effective concentrations and land-use

Statistical analysis for the determination of effective concentrations was done according to the guidelines outlined by Environment Canada (2007). The data for each microbial end point (nitrification activity; phosphatase activity; dehydrogenase activity and arylsulphatase activity) were fitted to a dose-response curve to determine the concentration of zinc or copper that produced a 50% reduction relative to the control (EC_{50}). Equation 4.5 was applied using the R statistical program (R Development Core Team (2013)) and the drc package (Ritz and Streibig, 2005).

$$y = c + \left(\frac{d - c}{1 + \frac{x}{e}^{b}} \right) \qquad \text{(Eq. 4.5)}$$

where, y is the response at concentration x, d is the response of the untreated control, c is the minimum effect, e is the dose at which the value d-c is reduced by 50 percent (EC_{50}) and is the point of inflection of the curve, and b is the slope around the EC_{50} value.

Statistical analyses were performed using the R statistical program (R Development Core Team, 2013). All data were checked for normality using the Shapiro-Wilk test ($P \leq 0.05$) and for homogeneity of variance using the Levene's test ($P \leq 0.05$). The conditions of normality (P

≤ 0.05) and homogeneity ($P \leq 0.05$) of variance were met for each metal (zinc and copper) EC_{50} values. Analyses of variance (ANOVA) and multiple comparison Fisher's LSD test were done to determine significant differences among mean values for EC_{50} and land-use.

4.4.6.2 Effective concentrations and soil properties

Statistical analyses were performed using the R statistical program (R Development Core Team, 2013). All data were checked for normality using the Shapiro-Wilk test ($P \leq 0.05$) and for homogeneity of variance using the Levene's test ($P \leq 0.05$). The conditions of normality ($P \leq 0.05$) and homogeneity ($P \leq 0.05$) of variance were not met for soil pH, CEC and initial enzyme activity of the soil. Linear regression analysis were used to determine significant relationships between pH, CEC and initial enzyme activity with EC_{50} values. Where significant relationships could not be found the data were log transformed.

4.5 Results

4.5.1 Effective concentration and land-use

An ANOVA (Table 4.2) of pH and CEC with land-use showed no significant differences between land-use for CEC; however, pH was significantly different ($P<0.01$) between land-uses. A Fisher's LSD test shows that boreal forests and native grassland soils (with a mean pH of $\sim$5) were significantly more acidic than agricultural, urban residential, and urban industrial soils (with a pH of $\sim$7). All soils showed a decrease in potential nitrification and soil enzyme activities at the first sampling time point (Fig. 6.3 in the appendix (Chapter 6) is an example dose response after 7 d). Soil properties varied both within and across land-uses (Table 4.3).

Nitrification, phosphatase, dehydrogenase, and sulphatase activity were sensitive to zinc or copper across land-uses (Figs. 4.2-4.5). For nitrification activity, boreal forest soils were the most sensitive to zinc (EC_{50} 202 $\pm$ 44 mg kg^{-1}) and copper (EC_{50} 197 $\pm$ 23 mg kg^{-1}). Urban residential soils were the least sensitive to zinc (EC_{50} 339 $\pm$ 60 mg kg^{-1}) and copper (EC_{50} 333 $\pm$ 31 mg kg^{-1}). The average EC_{50} across all land-uses was 273 $\pm$ 60 mg kg^{-1} for zinc and 266 $\pm$ 56 mg kg^{-1} for copper. Copper or zinc effects did not differ across land-uses ($P < 0.05$), with copper and zinc causing similar effects to each land-use.

The sensitivity of phosphatase and sulphatase activity to zinc and copper also differed ($P < 0.01$) across land-uses. For phosphatase activity, boreal forest soils were the most sensitive to zinc (EC_{50} 3358 $\pm$ 185 mg kg^{-1}) and copper (EC_{50} 3362 $\pm$ 283 mg kg^{-1}) and urban residential was the least sensitive to zinc (EC_{50} 5524 $\pm$ 125 mg kg^{-1}) and (EC_{50} 5217 $\pm$ 97 mg kg^{-1}) copper. Sulphatase activity in boreal forest soils was the most sensitive to zinc (EC_{50} 1075 $\pm$ 181 mg kg^{-1}) and (EC_{50} 1079 $\pm$ 70 mg kg^{-1}) copper and native grassland being the least sensitive to zinc (EC_{50} 1903 $\pm$ 107 mg kg^{-1}) or (1920 $\pm$ 18 mg kg^{-1}) copper. Dehydrogenase activity did not differ across land-uses and the mean sensitivity was 446 $\pm$ 86 mg kg^{-1}for zinc and 429 $\pm$ 83 mg kg^{-1}for copper.

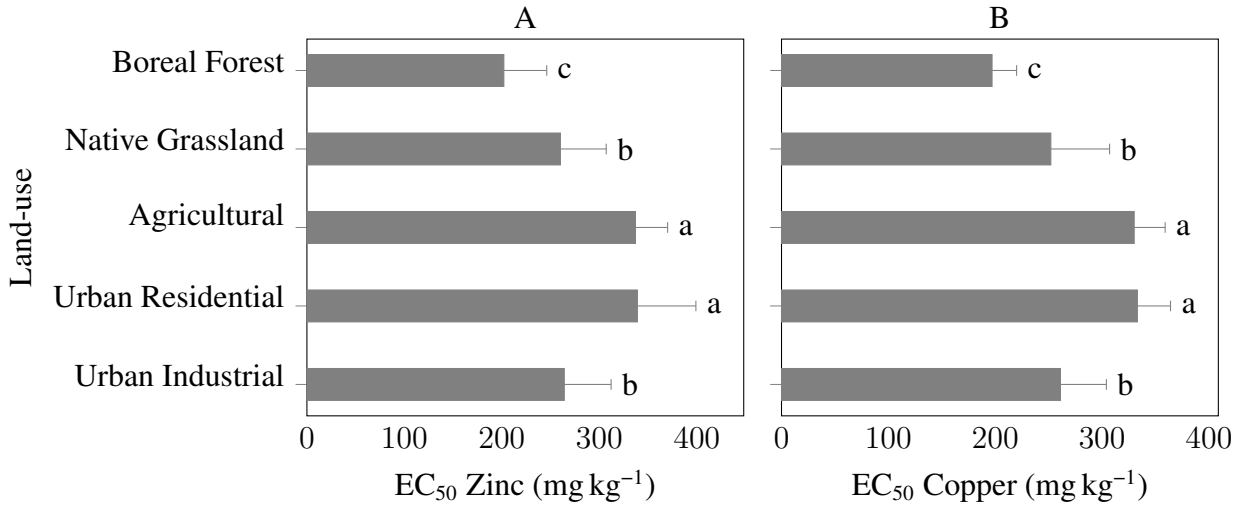

Fig. 4.2 Effective concentration of zinc (A) or copper (B) to soil nitrification potential across five land-uses. Error bars indicate plus one standard deviation from the mean of n=3 soils, except for boreal forest n=6. Letters indicate the results of a Fisher's LSD test, a, b, and c denoting groups with significantly different means.

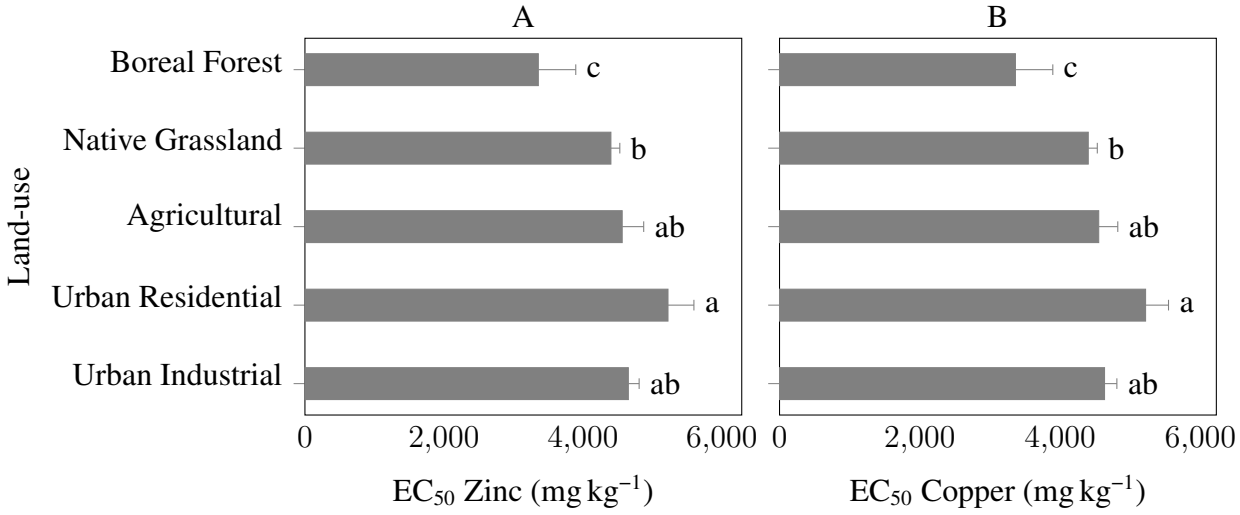

Fig. 4.3 Effective concentration of zinc (A) or copper (B) to soil phosphatase activity across five land-uses. Error bars indicate plus one standard deviation from the mean of n=3 soils, except for boreal forest n=6. Letters indicate the results of a Fisher's LSD test, with a, b, and c denoting groups with significantly different means.

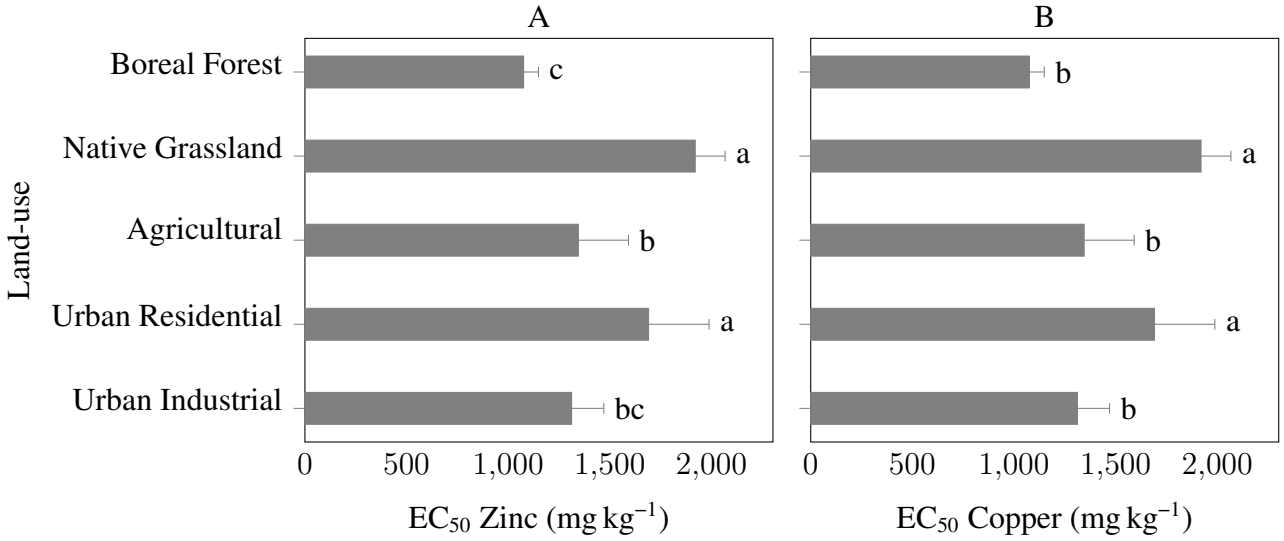

Fig. 4.4 Effective concentration of zinc (A) or copper (B) to soil arylsulfatase activity across five land-uses. Error bars indicate plus one standard deviation from the mean of n=3 soils, except for boreal forest n=6. Letters indicate the results of a Fisher's LSD test, with a, b, and c denoting groups with significantly different means.

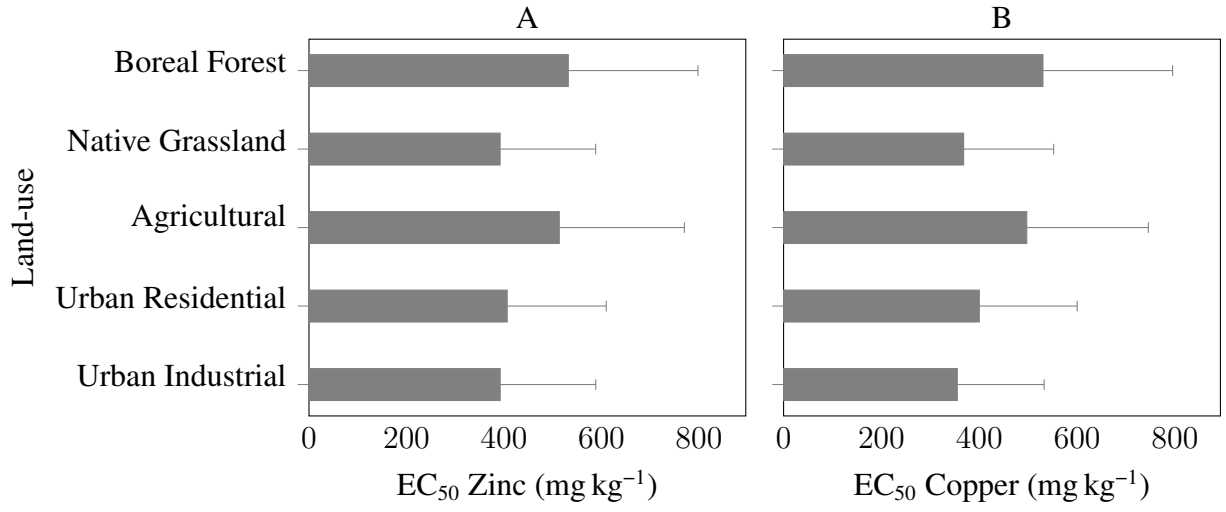

Fig. 4.5 Effective concentration of zinc (A) or copper (B) to soil dehydrogenase activity across five land-uses. Error bars indicate plus one standard deviation from the mean of n=3 soils, except for boreal forest n=6. Mean groupings not shown because all treatments were not significantly different (P>0.05).

Table 4.2 ANOVA table for zinc and copper mean median effective concentrations (EC_{50}) and land-use (Boreal Forest, Urban Industrial, Urban Residential, Agricultural, and Native Grassland)

Metal	Microbial Response	Source of Variations	DF	SS	MS	F	Model *p*
Zinc	Nitrification	Land-use	4	110718	27679.4	87.24	<0.001
		Error	31	9835	317.3		
		Total	35	120553			
	Sulphatase	Land-use	4	1614448	403612	8.64	0.009
		Error	31	1448663	46731		
		Total	35	3063111			
	Phosphatase	Land-use	4	16783984	4195996	33.51	0.004
		Error	31	3881383	125206		
		Total	35	20665366			
Copper	Nitrification	Land-use	4	110618	27579.4	4.27	<0.001
		Error	31	9735	217.3		
		Total	35	120453			
	Sulphatase	Land-use	4	1614348	403512	4.86	<0.001
		Error	31	1448563	46631		
		Total	35	3063011			
	Phosphatase	Land-use	4	16783884	4195896	51.32	<0.001
		Error	31	3881283	125106		
		Total	35	20665266			

4.5.2 Effective concentration and soil properties

Soil properties varied both within and across land-uses (Table 4.3) with the majority of these not strongly linked to EC_{50} values. However, CEC and pH were predictors of EC_{50} values (Table 4.4). Soil properties such as CEC varied across land-uses with organic carbon content and pH. Soil pH also regressed and was a predictor of EC_{50} values (Table 4.4), however with a distinct deviation from this trend for the six boreal forest soils. Boreal forest soil variation also strongly predicted initial nitrification activity ($logEC_{50}$ = 6.576 (0.14) + 207.9 (35.4) log initial nitrification activity, r^2=0.63, n=18, p<0.001). This was also observed for copper (EC_{50} = 87.9 (19.6) + 94.7 (10.0) log initial nitrification activity, r^2=0.83, n=18, p<0.001), for potential nitrification rate (PNR) this did not occur for some land-uses with initial nitrification activity not a significant driver for all 18 soils. The boreal forest nitrification activity and sulphatase activities were lower (mean nitrification activity of boreal forests = 3.30 $\pm$ 0.32 $NO_3{}^-$ mg $kg^{-1}day^{-1}$; mean sulphatase activity = 9.51 $\pm$ 2.91 *p*-mol product g h^{-1}) than for the remaining soils (mean nitrification activity of all remaining land-uses = 9.56 $\pm$ 2.91 $NO_3{}^-$ mg $kg^{-1}day^{-1}$; mean sulphatase activity = 11.21 $\pm$ 4.50 *p*-mol product g h^{-1}). Initial enzyme activity was regressed with sulphatase activity for zinc EC_{50} values (EC_{50} = 1437.4 (78.9) - 20.36 (7.7) initial sulphatase activity, r^2=0.30, n=18, p=0.018).

Table 4.3 Mean values of soil properties (n=5) for five land-use types (n=18). This includes soil fertility measurements and background metal concentrations. Values in parentheses represent one standard deviation from the mean.

Land-use	pH	EC	WHC	CEC	Zinc	Copper	NO_3^-	NH_4^+	Total N	Total C	Organic C	Available P	Available S
		$dS\,m^{-1}$	$gH_2O\,g^{-1}Soil$	$cmol\,kg^{-1}$	$mg\,kg^{-1}$	$mg\,kg^{-1}$	$mg\,kg^{-1}$	$mg\,kg^{-1}$	$mgN\,g^{-1}$	$mgC\,g^{-1}$	$mgC\,g^{-1}$	$mg\,kg^{-1}$	$mg\,kg^{-1}$
Agricultural	7.90 (0.33)	0.33 (0.03)	0.36 (0.1)	2.47 (0.98)	0.40 (0.48)	0.48 (0.34)	0.10 (0.05)	0.99 (0.47)	0 (0.21)	13 (1.25)	16 (2.05)	13 (1.25)	705 (20)
Native Grassland	5.27 (0.17)	0.34 (0.05)	0.30 (0.14)	8.16 (0.27)	0.56 (0.34)	0.79 (0.39)	0.03 (0.01)	0.35 (0.11)	1 (0.22)	7 (1.25)	18 (2.05)	7 (1.25)	767 (13)
Boreal Forest	5.00 (0.55)	0.16 (0.02)	0.33 (0.1)	5.95 (1.88)	0.76 (0.51)	0.20 (0.16)	0.02 (0.01)	0.36 (0.1)	1 (0.17)	14 (2.38)	15 (2.36)	14 (2.38)	641 (263)
Urban Residential	7.27 (0.48)	0.35 (0.08)	0.47 (0.03)	5.43 (2.41)	0.14 (0.12)	0.41 (0.23)	0.02 (0.0)	0.24 (0.07)	0 (0.08)	2 (1.25)	15 (1.25)	2 (0.82)	676 (30)
Urban Industrial	7.10 (0.33)	0.40 (0.08)	0.26 (0.06)	5.40 (1.2)	1.45 (0.17)	1.74 (0.31)	0.02 (0.01)	0.18 (0.06)	0 (0.08)	2 (0.82)	17 (0.82)	2 (0.82)	770 (15)

Table 4.4 Statistically significant linear regressions for potential nitrification activity and enzyme activities for zinc and copper mean median effective concentrations (EC_{50}). Values in parenthesis are the standard error of parameter estimates.

Metal	Microbial End-point	Regression Equation	n	Adjusted r^2	Model *p*
Zn	Nitrification	Log EC_{50} = 4.7 (0.2) + 0.13 (0.2) pH	18	0.53	<0.001
		Log EC_{50} = 5.81 (0.16) - 0.507 log CEC	18	0.21	0.032
		EC_{50} =103.7 (30.1) + 207.9 (35.4) log (activity)[1]	18	0.63	<0.001
		Log EC_{50} = 6.576 (0.14) + 0.08 (0.02) pH	18	0.45	0.001
	Sulphatase	Log EC_{50} = 7.378 (0.118) - 0.338 (0.154) log CEC	18	0.23	0.044
		EC_{50} = 1437.4 (78.9) - 20.36 (7.7) activity [2]	18	0.3	0.018
		Log EC_{50} = 7.6 (0.2) + 0.11 (0.02) pH	18	0.51	<0.001
	Phosphatase	Log EC_{50} = 2.01 (0.06) + 0.06 (6.78) pH	18	0.72	<0.001
Cu	Nitrification	EC_{50} = 378.4 (37.6) -67.1 (21.4) Log CEC	18	0.37	0.006
		EC_{50} = 87.9 (19.6) + 94.7 (10.0) Log (activity)	18	0.83	<0.001
	Phosphatase	Log EC_{50} = 3.31 (0.07) + 0.05 (0.04) pH	18	0.54	0.001

[1]initial activity of bulked soil
[2]initial activity of bulked soil

4.5.3 Adaptation

The sensitivity of soil nitrifiers to zinc or copper decreased ($P < 0.05$) over the course of the 180 days incubation. The change in sensitivity was biphasic with no significant changes of EC_{50} ($P < 0.05$) in the first 30 days of incubation followed by a steady increase from day 30 to 180 (Fig. 4.7). An ANOVA and Fisher's LSD test were carried out on the change in EC_{50} values over time. The rate of decreasing sensitivity was dependent upon land-use ($P < 0.01$) specifically. Urban industrial soils had the lowest mean change in EC_{50} zinc values (833 ± 151.8 mg kg^{-1}). The greatest sensitivity change was seen in boreal forest soils with an increase in EC_{50} zinc values (1328 ± 193.7 mg kg^{-1}) that was not significantly different from the changes observed in urban residential (1319 ± 178 mg kg^{-1}) and agricultural (1133 ± 33.90 mg kg^{-1}) soils. The average adaptation rate of nitrification was 11.1 ± 0.77 mg kg^{-1}day^{-1} for zinc and 10.98 ± 0.49 mg kg^{-1}day^{-1} copper over the 150 d. These changes were not linked to changes in labile metal concentration (Fig. 4.6; Fig. 6.4), labile metals remained constant at doses of 1,000 mg kg^{-1}and below. EC_{50} values for zinc and copper were not significantly different when using labile metal concentrations from 1 day and 180 days of exposure.

Despite these changes in absolute sensitivity, the relative sensitivity between land-uses did not change (Table 4.5), with one exception, boreal forest nitrification activity which was the most sensitive to zinc at 30 d became the least sensitive at 180 d. The remaining metals and enzyme activities did not demonstrate any changes in differences in relative sensitivity. The adaptation rates of soil phosphatase did not vary significantly with land-uses with a mean change of EC_{50} value for zinc of 949 ± 448 mg kg^{-1}and for copper of 393 ± 282 mg kg^{-1}.

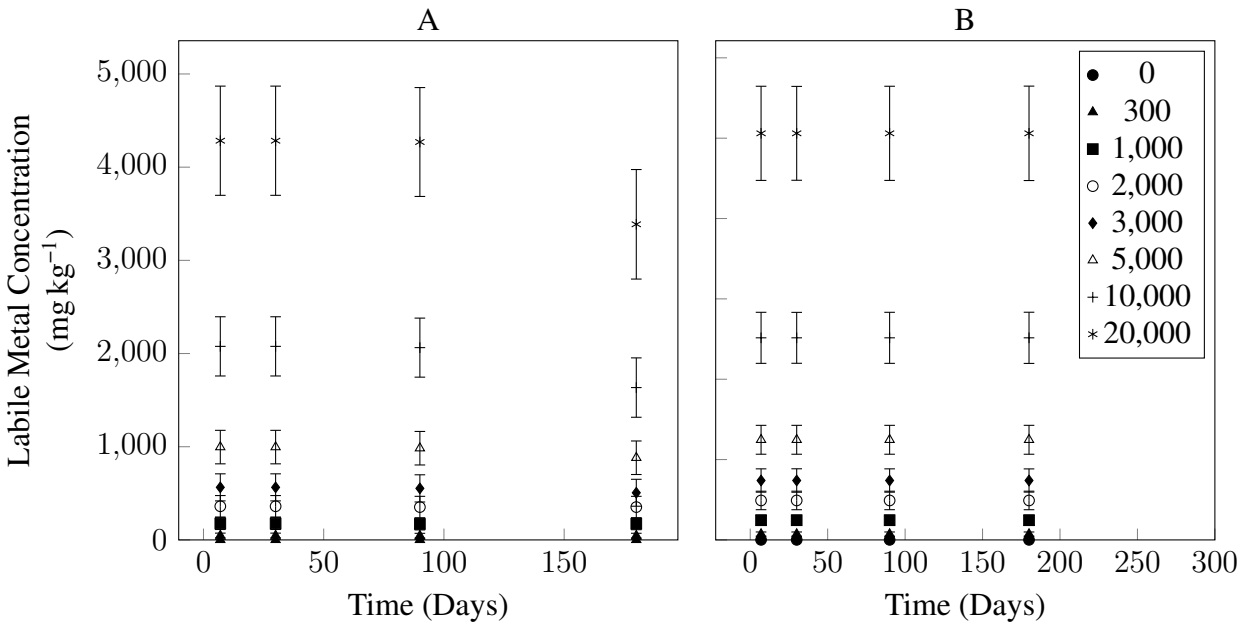

Fig. 4.6 Mean microcosm labile metal concentration of zinc (A) and copper (B) against time for eight doses (mg kg^{-1}of metal salt applied). Error bars indicate $\pm$ one standard deviation from the mean.

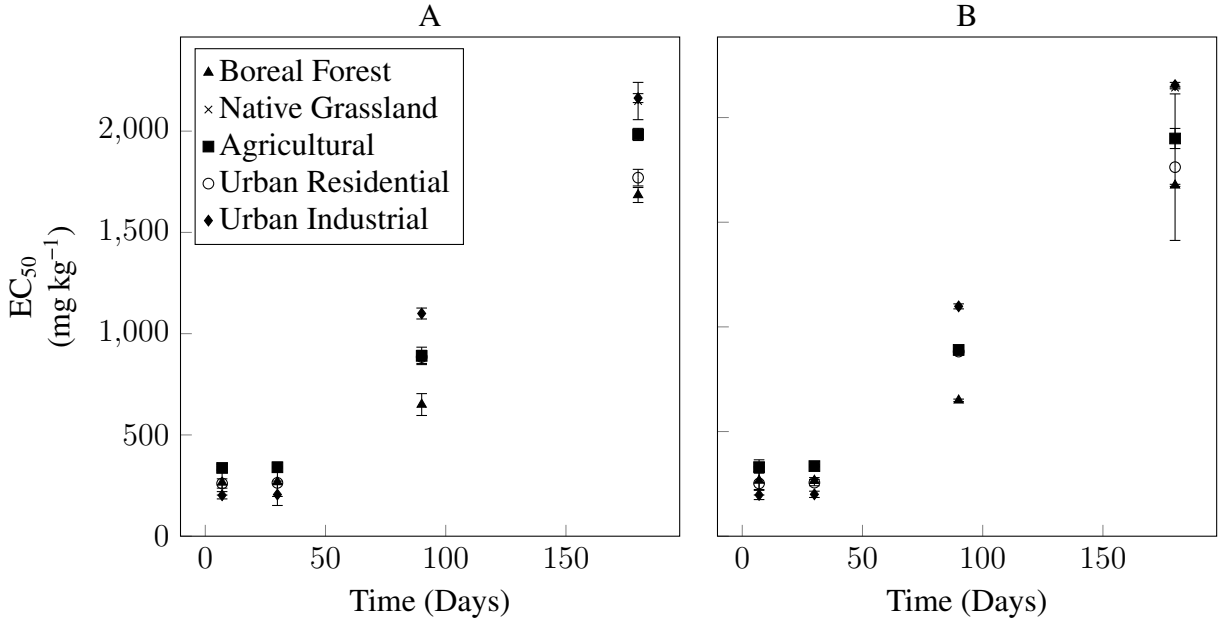

Fig. 4.7 Effect concentration (EC_{50}) of zinc (A) and copper (B) against time for five land-uses to nitrification activity. Error bars indicate $\pm$ 1 standard deviation from the mean.

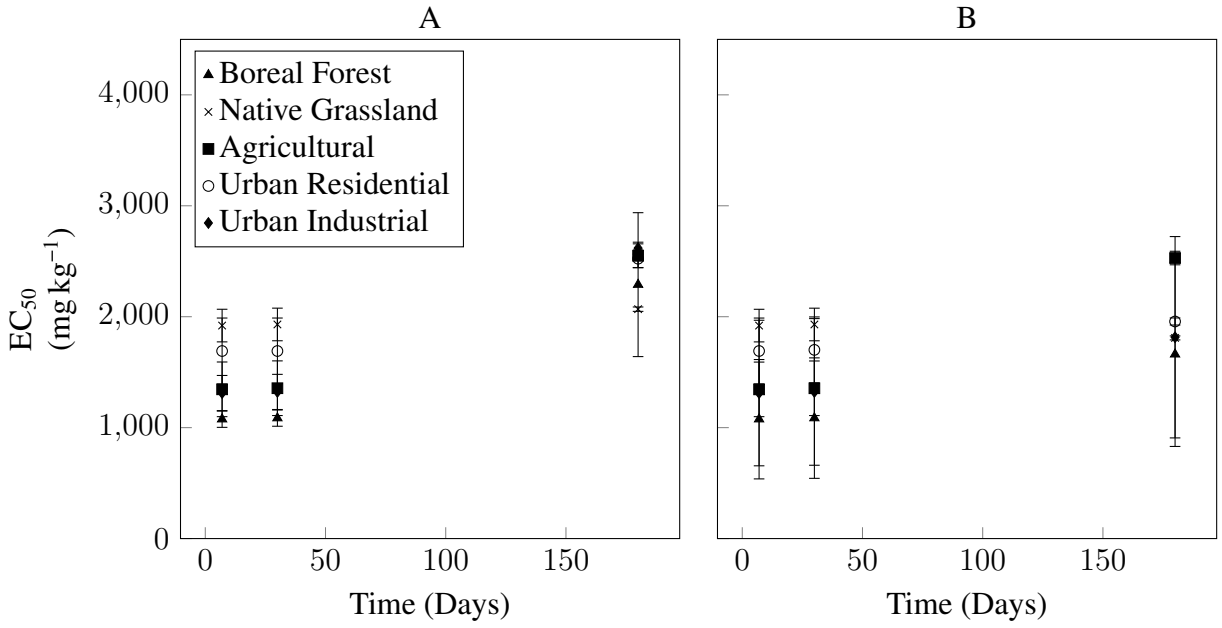

Fig. 4.8 Effect concentration of zinc (A) and copper (B) against time for five land-uses to sulphatase activity. Error bars indicate ± 1 standard deviation from the mean.

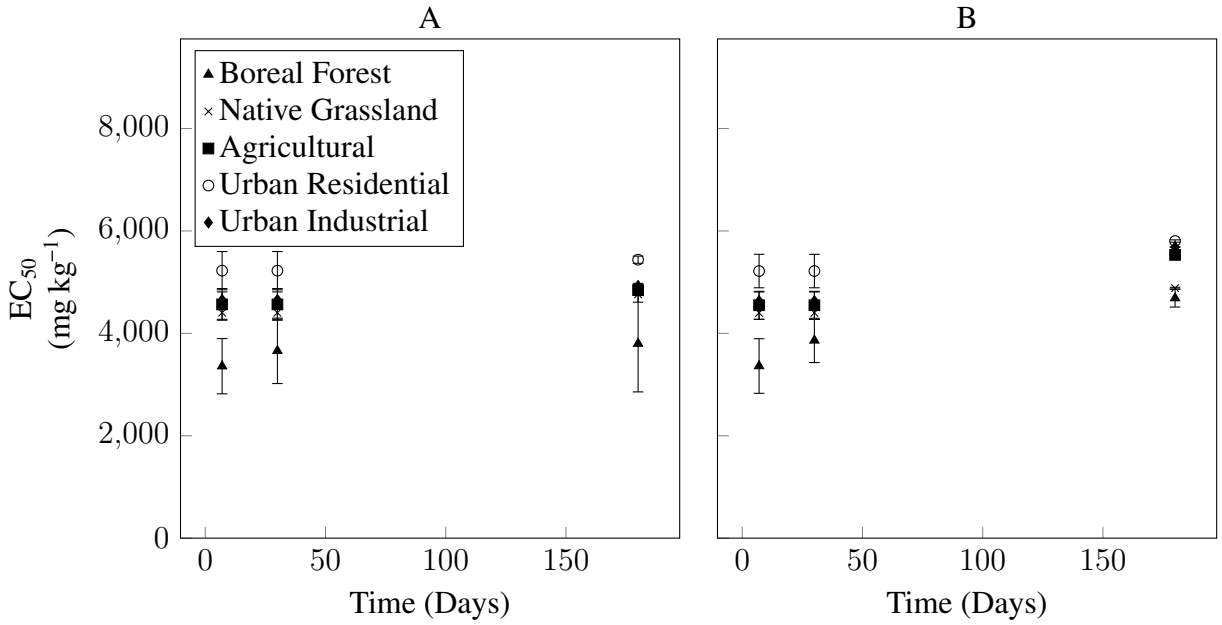

Fig. 4.9 Effect concentration (EC_{50}) of zinc (A) and copper (B) against time for five land-uses to phosphatase activity. Error bars indicate ± one standard deviation from the mean.

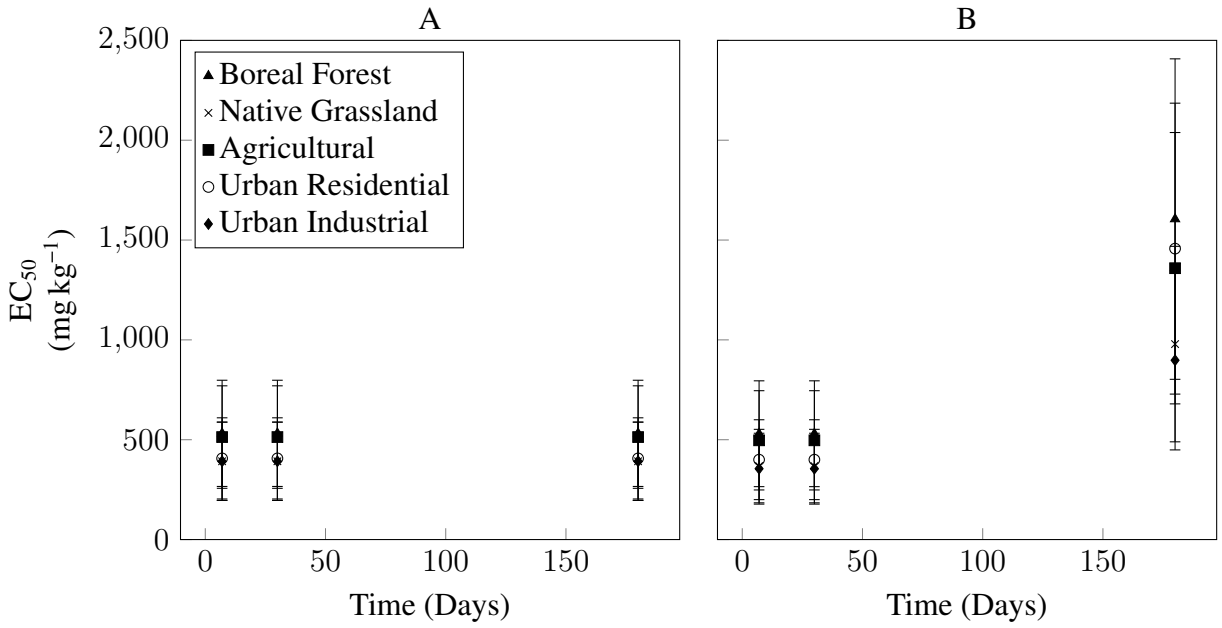

Fig. 4.10 Effect concentration (EC_{50}) of zinc (A) and copper (B) against time for five land-uses to dehydrogenase activity. Error bars indicate $\pm$ one standard deviation from the mean.

Table 4.5 Relative ranking of land-use sensitivity to zinc and copper. This is based on the EC_{50} calculated after 7, 30, and 180 days of exposure to either zinc or copper. The most sensitive is ranked as 1 and the least sensitive with the highest EC_{50} value ranked as 5. Dehydrogenase did not show a significant land-use effect thus values were not ranked.

	Zinc									Copper								
	Nitrification			Sulphatase			Phosphatase			Nitrification			Sulphatase			Phosphatase		
Land-use	7	30	180	7	30	180	7	30	180	7	30	180	7	30	180	7	30	180
Agricultural	1	1	4	1	1	2	1	1	1	1	1	1	5	5	5	1	1	1
Native Grassland	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2
Boreal Forest	4	4	3	4	4	4	4	4	3	3	3	3	4	4	3	3	3	3
Urban Residential	5	5	5	5	5	3	5	5	5	5	5	5	3	3	4	5	5	5
Urban Industrial	3	3	1	3	3	5	3	3	4	4	4	4	1	1	1	4	4	4

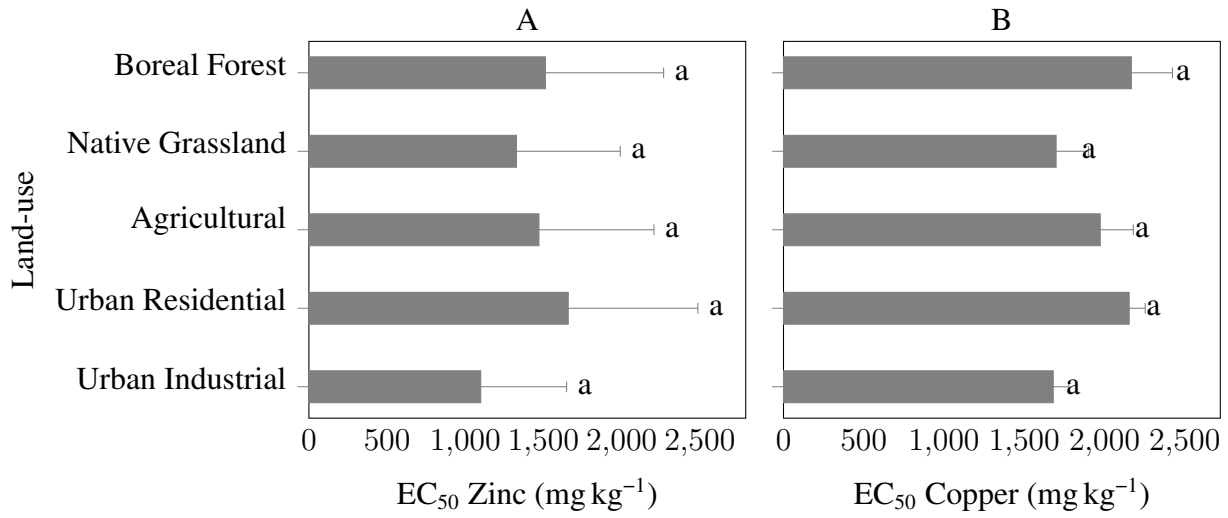

Fig. 4.11 Effective concentration (EC_{50}) of zinc (A) or copper (B) to soil nitrification potential across five land-uses, after 180 days of incubation. Error bars indicate one standard deviation from the mean of n=3 soils, except for boreal forest n=6.

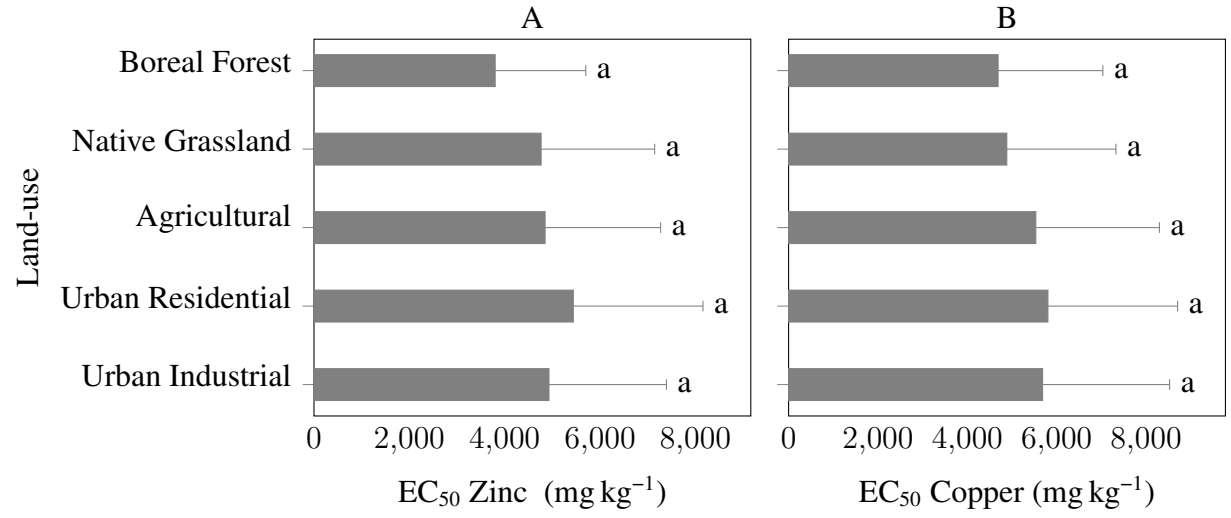

Fig. 4.12 Effective concentration (EC_{50}) of zinc (A) or copper (B) to soil phosphatase activity across five land-uses, after 180 days of incubation. Error bars indicate one standard deviation from the mean of n=3 soils, except for boreal forest n=6.

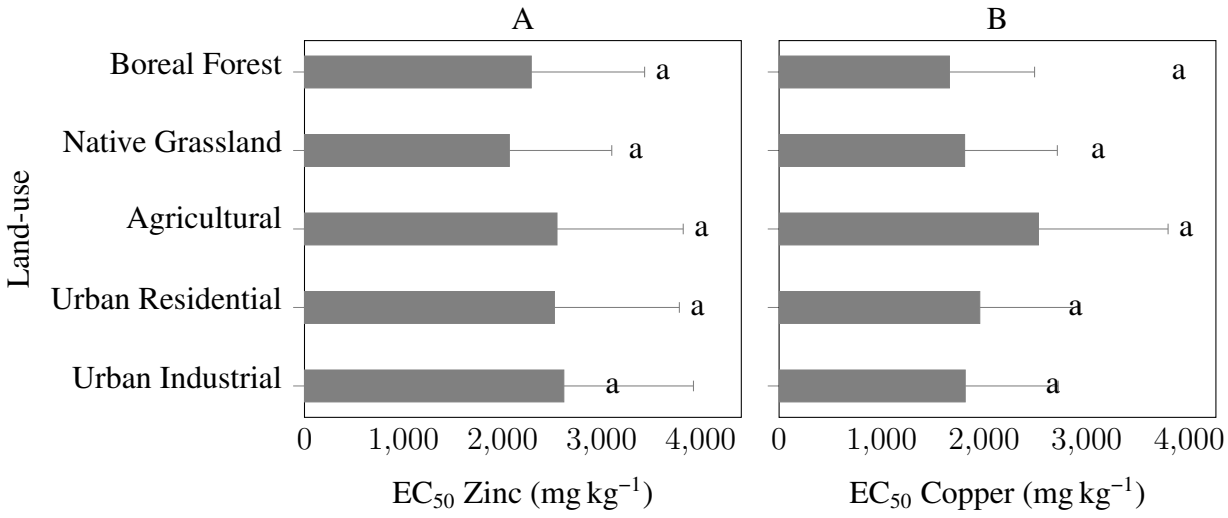

Fig. 4.13 Effective concentration (EC_{50}) of zinc (A) or copper (B) to soil arylsulfatase activity across five land-uses, after 180 days of incubation. Error bars indicate one standard deviation from the mean of n=3 soils, except for boreal forest n=6.

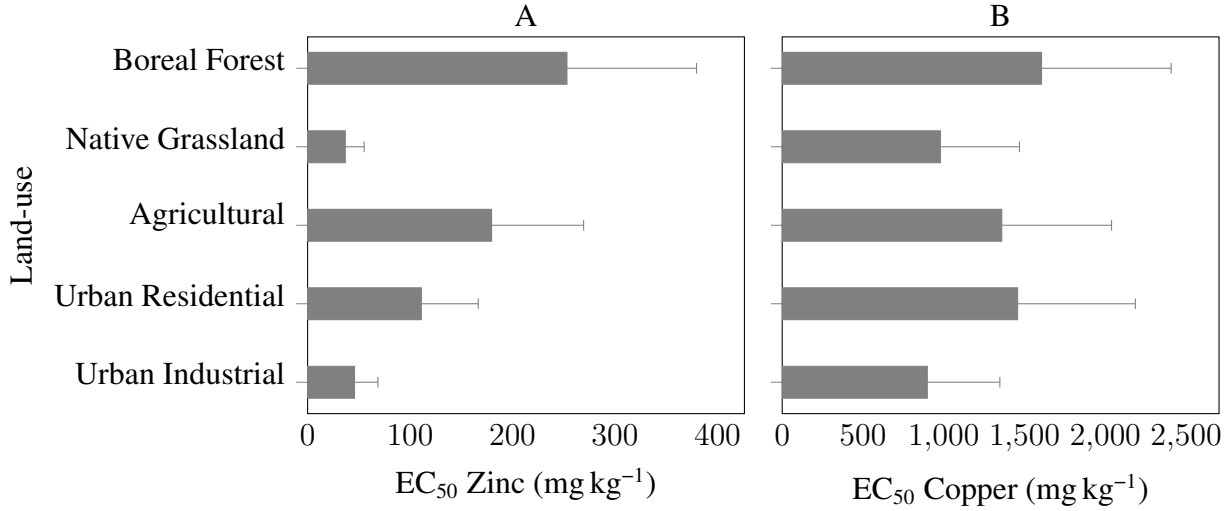

Fig. 4.14 Effective concentration (EC_{50}) of zinc (A) or copper (B) to soil dehydrogenase activity across five land-uses, after 180 days of incubation. Error bars indicate one standard deviation from the mean of n=3 soils, except for boreal forest n=6.

4.6 Discussion

This is the first study to examine a range of Canadian soils and show that land-use influences the toxicity of trace metals. Similar to other soils across Australia and Europe, pH and CEC were important drivers of toxicity. The results of this study show that soil metal concentrations alone cannot be used to predict toxicity of zinc and copper. Instead soil properties and land-use influence the toxicity of both zinc and copper to soil microbial functions. Soil pH is often thought of as the master variable controlling the mobility and fate of zinc and copper in soils (Smolders et al., 2009; Oorts et al., 2006; Broos et al., 2007). Despite this, CEC has been observed as the greatest factor controlling the toxicity of trace metals in European soils (Smolders et al., 2009). These data however, show that (Canadian) soils may be more affected in response by pH than CEC. This is similar to results seen by Broos et al. (2007) (in Australian soils), were pH was the most significant factor controlling the toxicity of zinc and copper. In this study CEC was only the third most significant factor after land-use and pH. These data show that European and Australian soil models for the protection of soils from trace metals could be adapted and used to protect Canadian soils.

The toxic effect of trace metals in soils is through the soil solution and is reflected by $CaCl_2$ extractable concentrations (Pueyo et al., 2004). There can be greater variation in dose-response modelling when using $CaCl_2$ extractable concentrations (Broos et al., 2007; Smolders et al., 2009) but (Coppolecchia et al., 2011) found that dose-response models calculated using $CaCl_2$ concentrations more accurately reflected observed inhibitions when they validated different methods of dose-response modelling. No significant changes in $CaCl_2$ extractable zinc or copper content were seen for six of the applied dose levels (control; 300; 500; 1000; 2000 and 5000 $mg\,kg^{-1}$). Two very high dose levels did show significant changes in metal content over time; however, this will have had limited impact on the results as these dose levels showed no observed enzyme activity and therefore will not have altered the modelled EC_{50} concentrations. It should also be noted that there was a linear increase in $CaCl_2$ extractable metal content between 1000 and 5000 $mg\,kg^{-1}$. This is consistent with Coppolecchia et al. (2011) who, observed a non-linear increase in $CaCl_2$ extractable metal content with increases in dose up to 1000 $mg\,kg^{-1}$, followed

by a linear increase in metal content. This study indicates that there may be an upper limit to this relationship below 10,000 mg $kg^{-1}$for zinc and copper. This linear relationship has been attributed to saturation in soil binding sites (Coppolecchia et al., 2011). In this study, however, there was no significant variation in $CaCl_2$ metal content relative to the dose applied across soils (n=18). There was variation observed for two doses (300 and 500) but not other dose levels.

In addition, this study provides evidence of adaptation of a range of soil microbial function to elevated trace metal concentrations. While evidence of adaptation has been widely reported for soil nitrifiers (Rusk et al., 2004; Mertens et al., 2006, 2007, 2009; Ruyters et al., 2010a; Trevisan et al., 2012) here I show that a wider range of soil microbial functions (sulphatase, dehydrogenase, and phosphatase activity) also adapt to metal addition. Trevisan et al. (2012) demonstrate that adaptation not only occurs in soil nitrifier populations but also in β-galactosidase activity, and suggest this was in response to the entire microbial community developing a tolerance to zinc.

The advantages of using soil enzymes to assess soil health and as a toxicity end-point for the soil microbial community are numerous, particularly when compared to other methods of assessment. The methods demonstrated here have low soil requirements, short incubation times, and simple requirements for chemical analysis. Enzymes were easily assessed across a wide range of soil types and land-uses present in Canada. Importantly, enzymes are sensitive to trace metals and provide a measure of trace metal toxicity. The enzyme data gained from this study show a similar response to the more time- consuming assessment of nitrification activity. This is true for both the short- and long-term consequences of zinc and copper exposure to the soil microbial community.

The results from this study show that Canadian soils have a range of toxicity to zinc and copper similar to that observed in Australia and European countries. The toxicity of zinc and copper to soil microbial processes varies significantly (>1000 fold). This has been suggested as a reason to limit their use in regulatory risk assessment (Broos et al., 2007). However, many soil toxicity assessments use soil total metal concentrations and not labile soil metal concentrations as

used here. The variation in toxicity values is much less when considering only those studies using labile metal concentrations (Trevisan et al., 2012). Alternatively, this variation in toxicity could be due to adaptation and is more reflective of individual toxicity to the microbial community and the length of time used in each assessment. Changes in enzyme activity over time could be a result of two changes within soils: adaptation of the soil microbial community and changes in soil metal bioavailability over time. The $CaCl_2$ extractable metal concentrations did not change and, thus, the changes observed here are likely due to changes within the community.

Few studies have focused on functional adaptation of soil microbial communities and those conducted primarily consider functions related to the soil nitrogen cycle. These studies (Broos et al., 2004; Rusk et al., 2004; Fait et al., 2006; Mertens et al., 2006; De Brouwere et al., 2007; Mertens et al., 2007, 2009; Puglisi et al., 2012) report similar trends with respect to the effects of trace metals. First, a short-term loss in soil microbial functions is observed, followed by a long- term recovery. Adaptation has thus been viewed as a positive effect for soil microbial functions and overall soil health. The maintenance of soil microbial functions is thought to be the result of the adaptation of soil metabolic process and is therefore essential for the overall survival of individual organisms and the community as a whole (Puglisi et al., 2012). If this assertion is true, other soil microbial functions such as soil enzyme activity should follow a similar long-term response to trace metals. This study showed that soil microbial activities important to soil microbial community metabolic processes have a response to trace metals similar to that previously observed for soil nitrification.

Soil nitrification has often been the preferred measurement end-point at trace metal concentrations due to the relative sensitivity of soil nitrifiers to trace metals. This study showed that soil nitrification was the most sensitive soil enzymatic process across 18 soils tested. A trend in the sensitivity of soil enzymatic processes (PNR > dehydrogenase > sulphatase > phosphatase) was observed across all soils (n=18). The trends remained consistent even when changes in the sensitivity across land-uses were observed. The relative sensitivity remained after adaptation oc-

curred despite differences in the rate of adaptation also being observed for the different microbial enzyme activities.

In conclusion, soil properties such as CEC, soil pH, and organic C content affected zinc and copper toxicity to soil microbial processes, and land-use was recognized as an additional factor. Therefore, land-uses already considered for the protection of human health should be considered for the protection of soil ecological health. Land-use affected the initial sensitivity of soil microbial processes to Zn and Cu and their long-term sensitivity after adaptation. However, alteration of land-use to reduce ecological risk as carried out when managing human health risk will not result in a reduction in toxicity and therefore risk; this can only be achieved by alteration of soil properties. This means that land-use and soil properties are both important factors influencing zinc and copper toxicity and both need to be taken into account. Land-use was the only consistent method of explaining differences observed in the sensitivity of different soils to trace metals. This is most likely due to the influence of land-use on soil microbial structure.

5 THE LONG TERM EFFECT OF ZINC AND COPPER ON SOIL MICROBIAL FUNCTIONS: BIOLOGICAL AND CHEMICAL IMPLICATIONS FOR HAZARD ASSESSMENT

5.1 Preface

This work follows on from the long-term exposure assessment of zinc and copper across a range of different land uses outlined in Chapter 4. To further expand on this work, this study assessed the effects of a secondary stress on soils from the previous experiment that have adapted to zinc and copper exposure. This study also relates to the first research experiment, using the soil enzyme assays validated in Chapter 3.

5.2 Abstract

The effects of trace metal contamination on the factors regulating soil microbial stability (resistance and resilience) to additional stresses are poorly understood, despite the significance of soil microbial functions to overall ecosystem functioning. This study tested whether adaptation to a metal stress (zinc and copper contamination) affects resistance or resilience to subsequent environmental stresses. The activities of three enzymes (dehydrogenase, arylsulphatase, and acid phosphatase) were selected as microbial test responses for measurement to determine the toxicity to the microbial community of zinc and copper. Heat and moisture stress were used as the secondary stresses applied to soil microbial communities already affected by a primary stress of zinc and copper and shown to adapt to this exposure after 180 d. Data were compared to the same soils after 30 days of exposure to zinc and copper but which had not adapted. Results show that soils that have adapted to zinc and copper are more resilient to environmental stresses after adaption to metal stress as opposed to before. This experiment suggests that microbial activity is a good indicator of soil microbial health while metal concentrations are not. Metal speciation was determined using synchrotron-based X-ray Absorption Spectroscopy (XAS) at the Canadian Light Source Inc. Specifically Zn k-edge near-edge structures (XANES) data were collected at the HXMA beamline (06-ID1). Zinc metal speciation was determined in soils with differing soil properties (Boreal forest and urban residential) were determined for 7 and 180 d of incubation with $ZnSO_4$. This showed that soils did not undergo the changes in metal speciation to become less available which may have been expected. Soil zinc speciation remained consistent overtime and across soils either over time or soil in the most available form of zinc (aqueous zinc). Overall this study provides new insights into the long-term consequences to the soil microbial community of of Zn and Cu in soils and the changes which occur overtime.

5.3 Introduction

The pollution induced community tolerance concept (PICT) was introduced by Blanck (1988), as an eco-toxicology tool for aquatic environments (Blanck, 2002). This concept was then expanded upon for use in the terrestrial environment and soils by Siciliano and Roy (1999); Davis et al. (2004); Díaz-Raviña et al. (2007). The concept of PICT is that communities develop a tolerance to a contaminant as a response to a selective pressure. The approach is to compare communities with a contaminant present to communities that have not been exposed, so there is not a need to find similar reference soil. Shifts in microbial community structure have often been regarded as the indicator of negative effects of a contamination event. However, this shift in community structure could be regarded as an indicator of restoration of natural functioning in response to pollution (Díaz-Raviña et al., 2007). There has been a significant debate as to the utility of the PICT concept, as covered in a review of adaptation (Puglisi et al., 2009) that highlighted this in a single question: is adaptation an ecological risk? If adaptation alters the resilience of functions, then it is clearly an ecological risk.

Ecosystems are reliant on soil microbial communities to perform soil functions responsible for maintaining long-term soil fertility. Specifically, microbial communities are responsible for regulating soil nutrient cycling and litter decomposition (Van Der Heijden et al., 2008). Soil ecosystems are often exposed to multiple stresses through natural cycles (e.g., wild fires, wet and dry cycles) (Van Der Heijden et al., 2008). Healthy soils are able to withstand and recover from such short-term stresses by being functionally stable (Griffiths and Philippot, 2013). Soils are also exposed to long-term stresses such as trace metal contamination. These stresses could have an effect on the functional stability of microbial communities. The fitness of soil microbial communities could be affected, with a reduced ability to withstand (resistance) and recover from (resilience) short-term stresses that subsequently occur (Philippot et al., 2008; Bissett et al., 2013). There is no consistent theory or concept defining how stresses on ecosystems affect their ability to withstand further stresses, but data linking diversity to functional stability suggest that reductions in diversity may adversely affect functional stability (Wittebolle et al., 2009).

Several studies have investigated whether exposure of soil microbial communities to persistent contamination (such as zinc and copper) decreases the resistance and resilience of the soil microbial community to subsequent stressors or disturbances (Brandt et al., 2010; Tobor-Kaplon et al., 2005, 2006; Philippot et al., 2008; Deng, 2012). Two theories have developed as to the effects of metal contamination on subsequent stress to metals. The first theory is that a stressed system will have more functional redundancy than an unstressed system. The view is that a previously stressed system is more stable due to the effects of adaptation, with the physiological changes to cope with the previous stresses making the system more capable of withstanding additional new stresses (Odum, 1985). The second theory is that soil microbial communities exposed to a persistent stress are more stable and undergo structural and physiological changes to produce a microbial population more resistant to subsequent stress (Brussaard et al., 2007). The first theory is supported by work of Tobor-Kaplon et al. (2006) who found that copper contaminated soils had a reduced stability when exposed to lead and salinity stresses compared to soil not previously exposed to copper stress. Additional studies by Dussault et al. (2008) also support this theory with normal enzyme activity (protease) observed in copper contaminated soils exposed to subsequent heat stress compared to their unexposed counterparts. However, numerous studies support the alternative theory (Philippot et al., 2008).

Adaptation to trace metals has most widely been studied on a narrow group of species, such as those involved in nitrification (Philippot et al., 2008; Puglisi et al., 2012). Nitrification is defined as the biological oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-) (Ward et al., 2011). Nitrification is considered a biologically mediated process, which covers several individual biochemically mediated reactions. These biochemical reactions can be driven into several separate sub-processes, depending on the microorganisms involved. The first step of nitrification is production of hydroxylamine (NH_2OH) by the oxidation of ammonia (NH_3); the reaction is catalyzed by ammonia monooxygenase (AMO) (Ward et al., 2011). First ammonia oxidizers mediate the reaction of ammonia (NH_3) to nitrite (NO_2^-), which is the rate limiting step in nitrification. These organisms are referred to as ammonia oxidizing bacteria (AOB) and archaea

(AOA) or primary nitrifiers (Ward et al., 2011). The second step in nitrification is the conversion of nitrite (NO_2^-) to nitrate (NO_3^-). This secondary process is carried out by nitrite oxidizing bacteria or secondary nitrifiers (Ward et al., 2011; Philippot et al., 2012).

Soil enzymes form a major part of biogeochemical cycles because they often form the rate limiting steps in soil biogeochemical reaction and control the rates of nutrient cycling within soils (Bååth, 1989). Here, soil enzymes were selected as they have not widely been used to characterize the effects of stress on soil ecological processes despite their importance in regulating ecological functions. Soil enzymes also have the advantages of being representative of the whole soil microbial community (Bååth, 1989). Soil enzymes are often thought of as critical due to the role they play in the transformation of soil nutrients into more available forms for uptake by the soil microbial community and the soil ecosystem as a whole. The enzymes selected for this study are often reported as good indicators of soil quality (Bêcaert et al., 2006; Nannipieri et al., 2012; Burns et al., 2013; Paz-Ferreiro and Fu, 2016). Soil enzymes have a rapid response in activity to biological changes within the soil (Bandick and Dick, 1999). This is one of the reasons for their selection as an assessment end-point for this study. However, there are differences in the opinion with respect to whether or not soil enzymes provide a good indicator of soil health. Soils with higher enzyme activity potential are thought by some to not represent healthier soils (Trasar-Cepeda et al., 2000; Alkorta et al., 2011).

Soil phosphatase and soil sulfatase enzymes are also responsible for critical steps in their respective cycles (P and S cycles) as they facilitate the transformation of organic compounds from soil into inorganic and plant available forms (Margesin and Schinner, 1994). Specifically phosphatase performs the function of transforming organic P form soil organic matter into the inorganic phosphate forms (HPO_4^{-1} and H_2PO_4).

The first objective of this study was to apply a quantitative method to evaluate the relative stability of key soil functions (biogeochemical cycles: N, P, C, and S) performed by specific enzymes, based on the soil response to heat and moisture perturbation. Soil microbial communities are often exposed to these stresses though natural processes (Bissett et al., 2013). Gleeson

et al. (2010) show that nitrification and ammonia oxidizers are particularly sensitive to moisture changes. Specifically they demonstrated that changes in effect of water filled pore space (WFPS) significantly alter nitrification in terms of rate and population levels, resulting in changes in an overall change in nitrification activity. This supported earlier work by Stark and Firestone (1995) that showed water stress affected nitrification activity through dehydration and substrate limitation. Heat stress to soil microbial communities can occur through a variety of means: climatic and seasonal changes in temperature, forest fires, burning of surface vegetation, and artificial heat treatments for land management (Griffiths and Philippot, 2013). Heat stress then alters the microbial community, via cell death and protein denaturing (Dell et al., 2012). Soil microbial communities can be resistant to heat stress, through protein folding and thickening of cell walls (Ramos et al., 2001; Tobor-Kaplon et al., 2006). The capacity of a soil ecosystem to withstand environmental disturbances is referred to as a soil ecosystems' functional stability (Seybold et al., 1999; Bêcaert et al., 2006). It can be characterized by two factors: ability to withstand a stress (resistance) and ability to return to normal functioning over time (recovery) (Bêcaert et al., 2006; Griffiths and Philippot, 2013). Here I used an index developed by Bêcaert et al. (2006), called the relative soil stability index (RSSI). The RSSI considers changes in enzyme activity after disturbance. This is measured in comparison to the changes in enzyme activity over time. This method is based on a time-integrated assessment of soil enzyme activity outlined by Bêcaert et al. (2006). This method works by assessing the enzyme activity of two sub-samples collected from a single sample, one sub-sample has a stress applied and the other remains undisturbed. The stress applied most often is heat stress (Griffiths and Philippot, 2013). Enzyme activity between these two samples is then compared over at several intervals over a period of time. This is done over a period of time when enzyme activity is thought to be affected by the stress applied, meaning that resistance and recovery of the soil microbial community to the stress applied is observed. Most often this time period for recovery to heat stress is between 11 and 14 days Bêcaert et al. (2006). This present study investigated the effects of 180 days of exposure and adaptation to severe stress (copper and zinc) on the functional stability of microbial communities to subsequent milder stresses and distur-

bances. Two potential environmental stresses that metal adapted soil ecosystems could be exposed to were investigated: soil moisture changes, which may occur seasonally and with increased frequency due to climate change (Davis et al., 2003; Lessard et al., 2014), and heat exposure (60°C for 48 h) similar to a forest fire, to which soils are exposed as part of natural successional pressures and which are predicted occur with increased frequency in the future (Aven, 2016), in part due to anthropogenic climatic change.

Additionally, this experiment aimed to assess long-term changes in zinc speciation that occur during the spiking and leaching procedures used in the assessment of the hazards zinc poses to soil ecosystems. Specifically, this study compared freshly spiked soils and leached soils to leached and aged soils to identify changes in metal speciation that could account for differences in observed toxicity. The aim was to separate the different factors that could account for alterations in soil toxicity observed over time, e.g., salinity changes, acidification, and equilibration of zinc with soil over time.

5.4 Materials & Methods

5.4.1 Experimental design: soils

Two different treatments were applied to soils: (1) addition of copper (2,000 mg kg^{-1}) salts ($CuSO_4$), and (2) addition of zinc (2,000 mg kg^{-1}) salts ($ZnSO_4$), leaching, and aging for 180 days. In addition to laboratory spiked soils, soils from a smelter-contaminated site were assessed. Toxicity was measured with four different enzyme assays with thresholds reported as labile metal concentrations measured after each soil treatment. Soil resistance and resilience (stability) to additional stresses was the determined for these soils.

Soils were collected from two primary sources: uncontaminated soils from a range of different land-uses from Western Canada (n=18) and field-contaminated soils (n=6), specifically smelter-contaminated boreal forest soils, were collected from the Flin Flon-Creighton area at the Saskatchewan-Manitoba border. The uncontaminated soils from sites representative of five different land-use types from across Western Canada were selected from sites representing boreal forest (n=6), cultivated agricultural (n=3), native grassland (n=3), urban parkland (n=3), and urban industrial (n=3). Soils were collected in bulk from the surface layer of each site (top 10-cm) using a metal spade. A total of ten locations was selected with a total of 18 individual sites. Sites were selected with the aim of avoiding contamination, but this was not possible for urban sites surrounded by potential anthropogenic sources of a variety of soil pollutants. These soils are the same as the soils outlined in more detail in the previous chapter (Chapter 4).

As stated previously after collection, uncontaminated soils were separated and treated with zinc or copper and then incubated for a total of six months. As there is no standardized method for the artificial contamination of soils, metal contamination followed methods outlined in section 4.4.4. Control soils were also maintained at laboratory conditions for (22 °C) for the 180 days. Soils were maintained at a WHC of 60% for the duration of their incubation. The soil moisture content and water holding capacity were determined by the methods outlined in detail previously in Chapter 4.4.2, using equations 4.2 & 4.1. Soils for this experiment were taken after

180 days of incubation and frozen at -20°C prior to use. Frozen soils from 180 days were thawed and incubated in darkness for one week prior to use analyses.

5.4.1.1 Soil properties

Table 5.1 shows the soil properties (n=5) of each soil. Table 5.2 shows the enzyme activity of each soil. Soils treated with zinc and copper after 180 days showed significantly ($P<0.01$) lower enzyme activity than unexposed soil (Fig. 5.1). This was observed across all 18 soils, where a control with no exposure to zinc or copper was available. Soil properties were broadly similar between untreated soils and soils treated with trace metals (zinc or copper). This comparison was not possible for smelter-contaminated soils. This exposure to zinc and copper was historic and sampling of soils prior to contamination was not possible (n=6). Soil properties are also shown for smelter-contaminated soils collected from Flin Flon, Manitoba.

Table 5.1 Soil property data, including soil fertility measurements and background metal concentrations, for all soils

Land-Use	Soil	NO_3 mg kg^{-1}	NH_4 mg kg^{-1}	Total N mg-Ng^{-1}	Total C mg-Cg^{-1}	Organic C mg-Cg^{-1}	Available P mg kg^{-1}	Available S mg kg^{-1}
Agricultural	1	10	9.7	0.3	13	13	13	682
Agricultural	2	16	15.8	0.6	14	16	14	701
Agricultural	3	4	4.3	0.1	11	18	11	732
Native Grassland	4	5	4.9	0.8	6	16	6	782
Native Grassland	5	3	3.3	1.2	9	21	9	768
Native Grassland	6	2	2.3	0.7	7	18	7	751
Boreal Forest	7	3	2.9	0.4	16	12	16	471
Boreal Forest	8	3	2.7	0.8	13	17	13	392
Boreal Forest	9	3	2.5	0.5	12	18	12	284
Boreal Forest	10	0	4.8	0.6	11	13	11	892
Boreal Forest	11	3	3.7	0.3	14	15	14	887
Boreal Forest	12	2	5.2	0.7	18	12	18	921
Urban Residential	13	2	2.2	0.2	2	14	2	641
Urban Residential	14	3	3.4	0.1	1	15	1	670
Urban Residential	15	2	1.6	0.3	3	17	3	716
Urban Industrial	16	1	1.4	0.2	2	16	2	788
Urban Industrial	17	1	1.3	0.3	3	17	3	770
Urban Industrial	18	3	2.6	0.1	1	18	1	751
Flin Flon (Smelter Contaminated)	19	3	5.1	12	11	13	11	902
Flin Flon (Smelter Contaminated)	20	2	4.9	11	10	14	12	885
Flin Flon (Smelter Contaminated)	21	1	2.1	13	12	17	14	889
Flin Flon (Smelter Contaminated)	22	3	4.5	15	14	11	15	893
Flin Flon (Smelter Contaminated)	23	2	4.9	16	11	10	16	892
Flin Flon (Smelter Contaminated)	24	1	5.3	12	10	9	12	882

Table 5.2 Mean soil enzyme activity each soil (n=3) for 24 soils, 18 soils were treated with artificial zinc and copper treatment and 6 were from a smelter contaminated (zinc and copper) boreal forest soils. Values in parentheses represent one standard deviation from the mean

Land-Use	**Soil**	**Nitrification** NO_3–N kg^{-1} day^{-1}	**Dehydrogenase** pmol product g^{-1} h^{-1}	**Arylsulphatase** pmol product g^{-1} h^{-1}	**Acid Phosphatase** pmol product g^{-1} h^{-1}
Agricultural	1	10.1 (1.3)	0.3 (0.02)	13 (1.3)	13 (1.1)
Agricultural	2	11.2 (1.7)	0.6 (0.01)	14 (1.6)	16 (1.3)
Agricultural	3	10.5 (1.2)	0.1 (0.02)	11 (1.3)	18 (0.7)
Native Grassland	4	5.3 (1.3)	0.8 (0.03)	6 (1.2)	16 (0.3)
Native Grassland	5	4.7 (1.6)	1.2 (0.03)	9 (1.2)	21 (1.8)
Native Grassland	6	5.8 (1.2)	0.7 (0.01)	7 (0.9)	18 (1.4)
Boreal Forest	7	4.2 (0.8)	0.4 (0.02)	16 (0.7)	12 (1.3)
Boreal Forest	8	3.7 (1.1)	0.8 (0.03)	13 (0.1)	17 (1.1)
Boreal Forest	9	3.4 (0.5)	0.5 (0.1)	12 (1.1)	18 (0.7)
Boreal Forest	10	3.1 (0.9)	0.6 (0.02	11 (1.2)	13 (0.9)
Boreal Forest	11	3.5 (0.6)	0.3 (0.03)	14 (1.3)	15 (2.1)
Boreal Forest	12	2.8 (0.7)	0.7 (0.01)	18 (1.3)	12 (1.9)
Urban Residential	13	10.1 (1.2)	0.2 (0.01)	2 (0.8)	14 (1.7)
Urban Residential	14	12.9 (1.3)	0.1 (0.03)	1 (0.8)	15 (2.5)
Urban Residential	15	14.1 (1.7)	0.3 (0.02)	3 (0.7)	17 (2.8)
Urban Industrial	16	7.7 (0.97)	0.2 (0.03)	2 (0.6)	16 (1.3)
Urban Industrial	17	12.1 (1.5)	0.3 (0.01)	3 (0.8)	17 (1.4)
Urban Industrial	18	10.2 (1.1)	0.1 (0.03)	1 (0.7)	18 (0.9)
Flin Flon (Smelter Contaminated)	19	2.9 (0.8)	0.6 (0.01)	19 (1.2)	12 (0.7)
Flin Flon (Smelter Contaminated)	20	2.91 (0.8)	0.58 (0.02)	21 (0.3)	12 (0.2)
Flin Flon (Smelter Contaminated)	21	3.06 (0.7)	0.53 (0.01)	17 (0.2)	13 (1.3)
Flin Flon (Smelter Contaminated)	22	2.8 (0.9)	0.63 (0.02)	21 (1.1)	12 (1.2)
Flin Flon (Smelter Contaminated)	23	2.7 (0.9)	0.68 (0.03)	25 (1.8)	11 (1.4)
Flin Flon (Smelter Contaminated)	24	2.53 (0.8)	0.73 (0.08)	28 (1.7)	10 (1.7)

5.4.2 Experimental design: secondary stress

The experimental design consisted of 27 microcosms for each of the 24 soils with three replicates per treatment. Heat, moisture perturbation and a control (no stress or no secondary stress) was applied to three microcosms containing zinc contaminated, copper contaminated, and uncontaminated soil, respectively.

Microcosms were prepared and incubated in the dark at room temperature for 7 d prior to the start of the experiment. Sub-samples were collected three times over the course of the experiment. Enzyme activities were measured immediately following the application of stresses, then 1, 4, and 11 days later. The duration of the experiment was based on previous experiments (Lessard et al., 2014) and work carried out by Griffiths et al. (2001); Degryse et al. (2003); Orwin and Wardle (2004) that show functional stabilization occurs within a 15-day period.

The heat perturbation was an increase in temperature to 60°C. This was selected due to previous observations that this is the most effective treatment for impacting the microbial community and enzyme activity (Lessard et al., 2014). This treatment is also similar to potential natural disturbances and stress to which a soil may be subjected, being a temperature consistent with a fire or heat wave (Davis et al., 2003; Zeleznik and Dickmann, 2004; Lessard et al., 2014). To apply the heat stress, soils were weighted out into aluminum weigh boats and heated in an oven at 60°C for 24 h. Soils were cooled and returned to glass test tubes and restored to a WHC of 60%.

Moisture stress was applied by placing soils in a desiccation chamber for 48 h. Moisture levels in the desiccation chamber were set to reduce the moisture content of each individual soil to a set WHC at 35% for each individual soil. This was done by varying the salt (NaCl) concentration in each desiccation chamber for each individual soil.

Three enzyme assays were associated with three key bio-geochemical cycles: the soil phosphorus; sulfur and carbon P, S, and C cycles. The enzyme assays followed modified versions of protocols avail-able in the published literature. Soil enzymes associated with soil phosphatase (EC 3.3.2), sulfatase (EC 3.16.1), and dehydrogenase (EC 1.1) were analyzed as previously described. Modifications made for methods of assessment of arylsulfatase activity (Whalen and War-

man, 1996) based on a protocol developed by Tabatabai and Bremner (1970), phosphatase activity by a protocol outlined by Eivazi and Tabatabi (1977), and dehydrogenase activity by a protocol set out by Trevors (1984) are outlined in section 4.4.3.

5.4.3 Soil relative stability index (RSSI)

Enzyme stability was assessed using a time integrated method of enzyme stability developed by Bêcaert et al. (2006). First, resistance and recovery calculations were applied to each set of samples (undisturbed and disturbed). Equation 5.1 was used to calculate the immediate effect of perturbation on each individual soil. Then, the activity of soils after 11 days in disturbed and undisturbed soils was compared. This was done to determine the recovery of enzyme activities after perturbation. Equation 5.2 was then used to calculate recovery rates. The relative soil stability index (RSSI) compares enzymatic activity in disturbed and undisturbed soils and is calculated using Equation 5.3.

$$Resistance = 100\% \times \frac{EA_{\ day2}}{EA_{\ day2control}} \qquad \text{(Eq. 5.1)}$$

$$Recovery = 100\% \times \frac{EA_{\ day11}}{EA_{\ day11control}} \qquad \text{(Eq. 5.2)}$$

$$RSSI = \frac{\int_{day\ 2}^{day\ 11} EA_{\ perturbed}(t)dt}{\int_{day\ 2}^{day\ 11} EA_{\ control}(t)dt} \times 100\% \qquad \text{(Eq. 5.3)}$$

5.4.4 Chemical analysis

Soil pore water concentrations of zinc and copper were measured with a 0.01 M $CaCl_2$ extracting solution following methods outlined by Houba et al. (2000). The principle of the method is to use $CaCl_2$ to provide a constant electrolyte concentration with a similar binding concentration to $Zn^{2+}$and Cu^{2+}, so Ca^{2+}will replace other cations on soil adsorption complexes. Air-dried soil (10 g) sieved to <2-mm was extracted with of 100 mL of 0.01 M $CaCl_2$ at a 1:10 (w/v) soil solution ratio in a 200 mL polypropylene container. Samples were then soil were shaken horizontally for 12 h at 160 rpm, centrifuged at 900 rpm for 15 min, and the clear supernatant extracted and filtered through

a 0.45 µm, Millipore filter. The extract was then acidified with HNO_3 and stored for analysis. The solutions were then analyzed using a microwave plasma-atomic emissions spectrometer (MP-AES; Agilent 4100, Agilent Technologies, Australia) and MP Expert software. Analyses were carried out at a set wavelength of 324.75 nm for copper and 213.86 nm for zinc. Analysis followed quality control procedures outlined by Wightwick et al. (2010).

5.4.5 Metal speciation

Zinc metal speciation was determined using synchrotron-based XAS targeted at the zinc K-edge. All XAS measurements were carried out at the Canadian Light Source (CLS) synchrotron in Saskatoon, Saskatchewan. This synchrotron operates with a storage ring between 2.9 GeV and 150 mA. Measurements were collected at the Hard X-ray Micro-Analysis beamline (HXMA) beamline (06-ID1) of the CLS. Spectra were collected using a Si (220) monochromator with detuning of 50% at 12k to remove higher order harmonics. Calibration of the beamline was carried out using the Zn content of metal foil located behind the sampler to the Zn K-edge (9659 eV). Calibration was carried out continuously during the sample run. Spectra were collected at the same time as an extensive library of Zn reference compounds were measured using X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS). The details of this library and it's collections have been extensively outlined by Hamilton (2013) and Hamilton et al. (2016).

Bulk XANES data of each sample was processed and analyzed using the ATHENA software package Ravel and Newville (2005). Data was processed to remove of background and calibration each spectra scan. A visual comparison using semi-qualitative approach was then applied. Comparison to an XANES library of 16 zinc reference standards of zinc species which are commonly present in soils, soil contaminated zinc, and zinc minerals produced during the mining and smelting of zinc. The reference standards were Zn^{2+}, Zn-sorbed Smectite, Zn-sorbed Goethite, Zn Oxalate, Zn citrate, Zn-sorbed Binessite, Zn-sorbed SiO_2, $ZnSO_4$, $ZnPO_4$, $Zn(OH)_2$,

ZnO, $ZnCO_3$, Zn coppt HIM,Zinc aluminum layered double hydroxide (Zn-AL-LDH), Franklinite ($ZnFe_2O_4$), Willemite (Zn_2SiO_4), and Sphalerite (ZnS).

5.4.6 Statistics

Statistical analyses were performed using the R statistical program (R Development Core Team, 2013). All data were checked for normality using the Shapiro-Wilk test ($P \leq 0.05$) and for homogeneity of variance using the Levene's test ($P \leq 0.05$). The conditions of normality ($P \leq 0.05$) and homogeneity ($P \leq 0.05$).

Linear regression analysis were used to determine significant relationships between microbil activity (dehydrogenase, arylsulphatase, and acid phosphatase) after exposure to soil moisture and heat stress with EC_{50} values.

To evaluate the soil enzyme activity stability of each soil to heat stress (°C), RSSI for dehydrogenase, phosphatase and arylsulphatase activity was created and was done for soils that underwent a range of treatments; uncontaminated control soils, zinc and copper addition (two treatment levels), and smelter-contaminated soils.

RSSI value comparison (n=18) for heat stress was done for: A control group of soils with no metals added, two treatments of zinc and copper at two dose levels, and a smelter contaminated soils with both zinc and copper. Two treatments of zinc (Zn-1: 2,000 mg kg^{-1}& Zn-2: 5,000 mg kg^{-1}) and two treatments of copper were applied (Cu-1: 2,000 mg kg^{-1}: Cu-2 5,000 mg kg^{-1}). All artificially contaminated soils were incubated for 180 days prior to analysis and field contaminated soils were treated the same after there collections in the field as artificially contaminated soils. In addition to soils (n=18) were collected from a zinc and copper smelter contaminated boreal forest landscape (Flin Flon soils). Analyses of variance (ANOVA) and multiple comparison Fisher's LSD test were carried out to determine significant differences among mean values of RSSI for each soil (n=24).

5.5 Results

5.5.1 Enzyme Activity Inhibition

The inhibition of enzyme activity by the presence of zinc and copper in soils is shown in Figs. 5.1 & 5.2. The control activity of each enzyme is shown in Table 5.2 from which percent activity is calculated. The control sample in this experiment is enzyme activity from the same soil (n=18) incubated for the same time (180 d), under the same experimental conditions. Here, the concentrations of zinc and copper showed no significant relationship with enzyme activity.

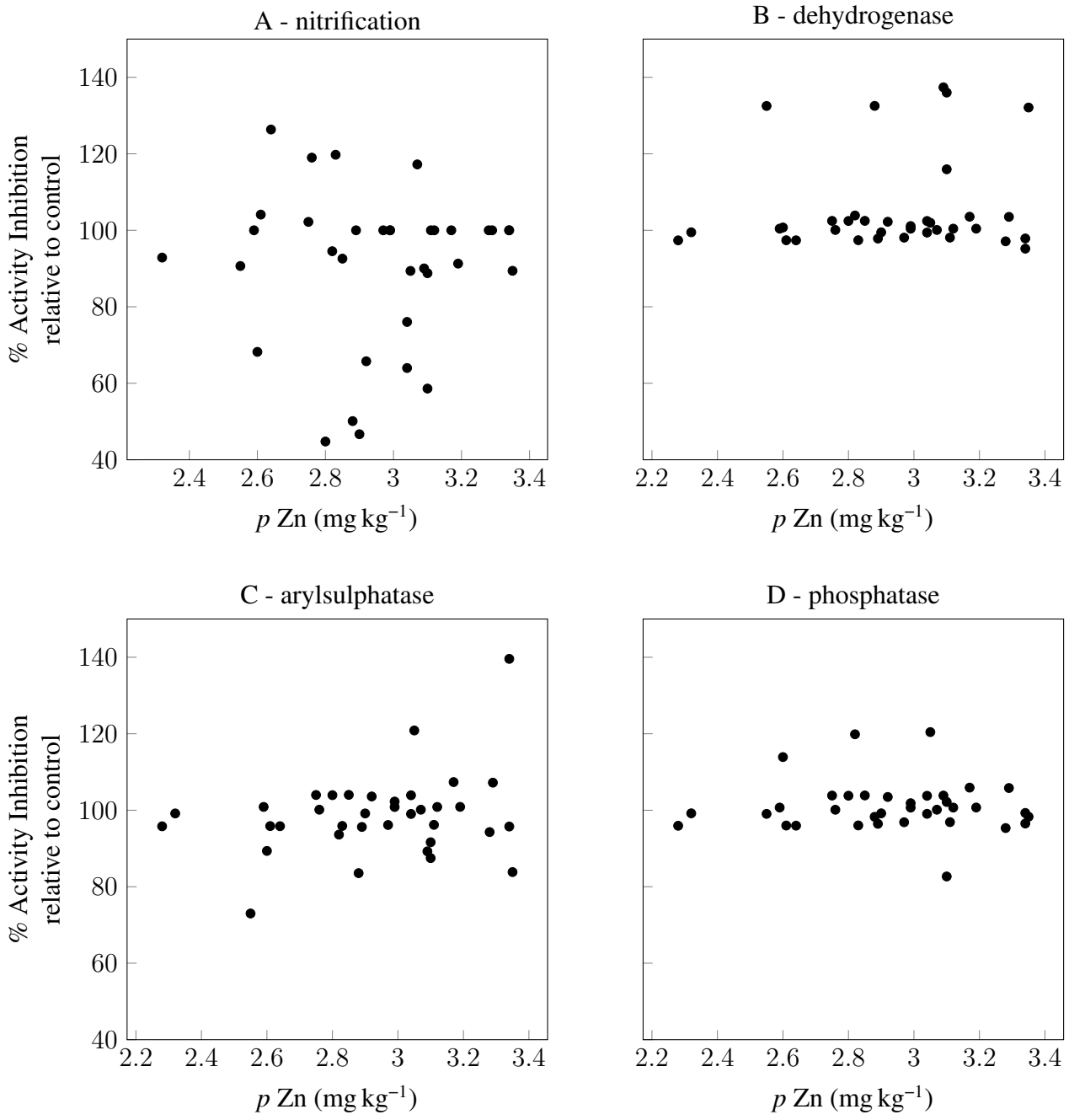

Fig. 5.1 Enzyme activity inhibition as a percentage of control against log zinc concentration in the soils (n=18). All soils enzyme activity were measured after 180 days of exposure to zinc. The control soils were stored in the same manner as the soils exposed to zinc. This plot shows activity for four different enzymes from the same soils: (A) nitrification; (B) dehydrogenase; (C) arylsulphatase and (D) phosphatase activity.

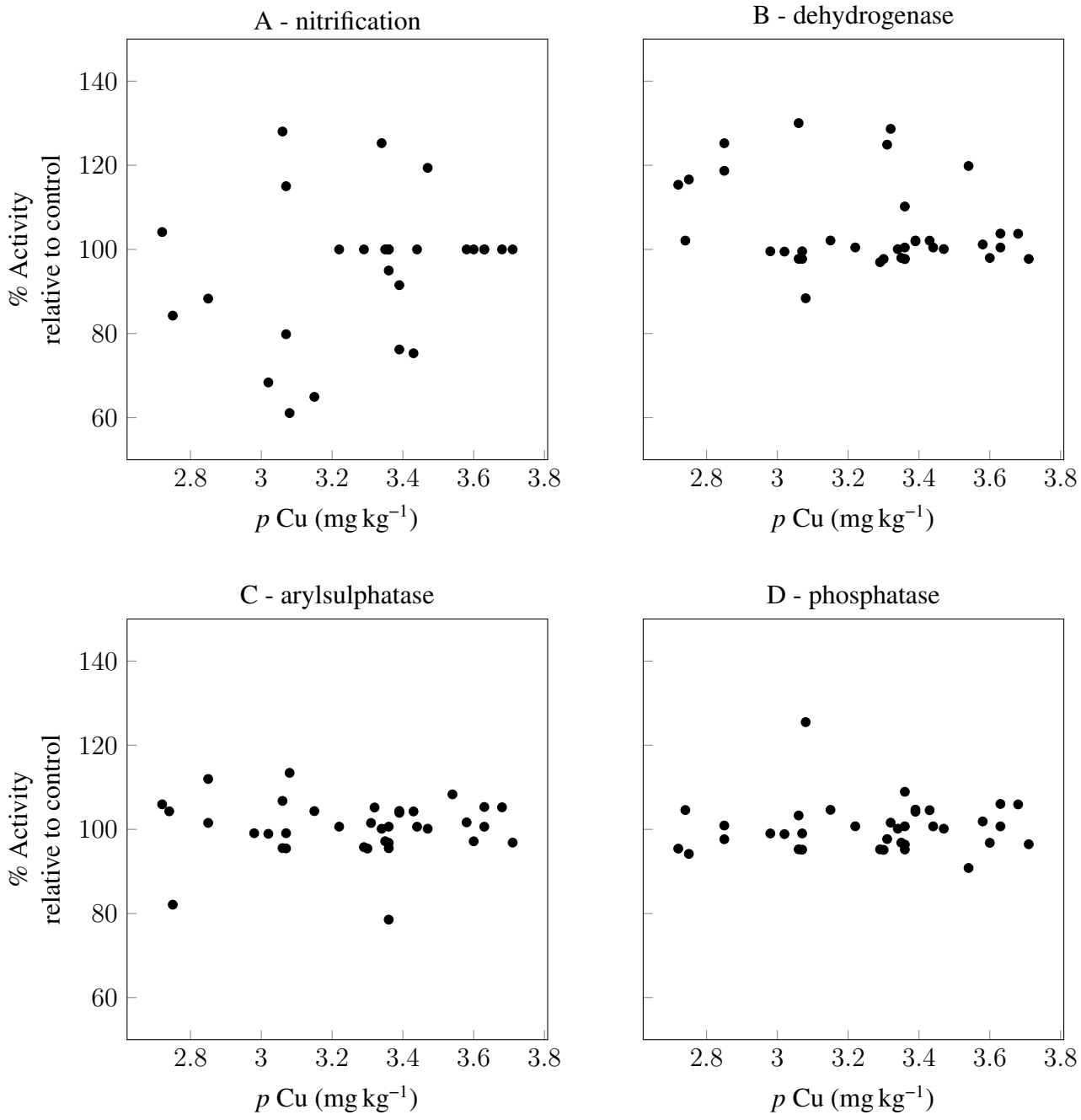

Fig. 5.2 Enzyme activity inhibition as a percentage of control against log of copper concentration in the soils (n=18). All soils enzyme activity were measured after 180 days of exposure to copper. The control soils were stored in the same manner as the soils exposed to copper. This plot shows activity for four different enzymes from the same soils: (A) nitrification; (B) dehydrogenase; (C) arylsulphatase and (D) phosphatase activity.

5.5.2 Effect of soil properties: Metal Speciation

A semi-quantitative visual comparison of Zn XANES data for two soils showed similarities between all the Zn speciations present in the soils. Each XANES data plot (Figs. 5.3 and 5.4) shows the zinc speciation for zinc present in each soil after 0 d of incubation and six months (180 d). This was done for two soils to allow comparison of the effects of soil properties on the process of "ageing" of zinc in different soils over time. Firstly a boreal forest soil (soil 10, Fig. 5.3) and secondly an urban land-use soil (soil 16, Fig 5.4) are provided. Zinc speciation showed relatively small changes over time in both soils. Also, no speciation differences were identified between the two soil types. A comparison of this XANES data to a range of standard zinc forms on CEC sites in soils showed that aqueous zinc (Zn^{2+}) provides the best fit to the spectra. This is evident by the similar absorption features present in the whole spectra. Aqueous zinc is, therefore, the most likely metal speciation of zinc present in the soils, at all times. No other absorption features or evident scattering of the spectra are apparent across the spectra. Aqueous zinc provides a good visual match for zinc metal speciation and therefore further EXAFS analysis was not carried out.

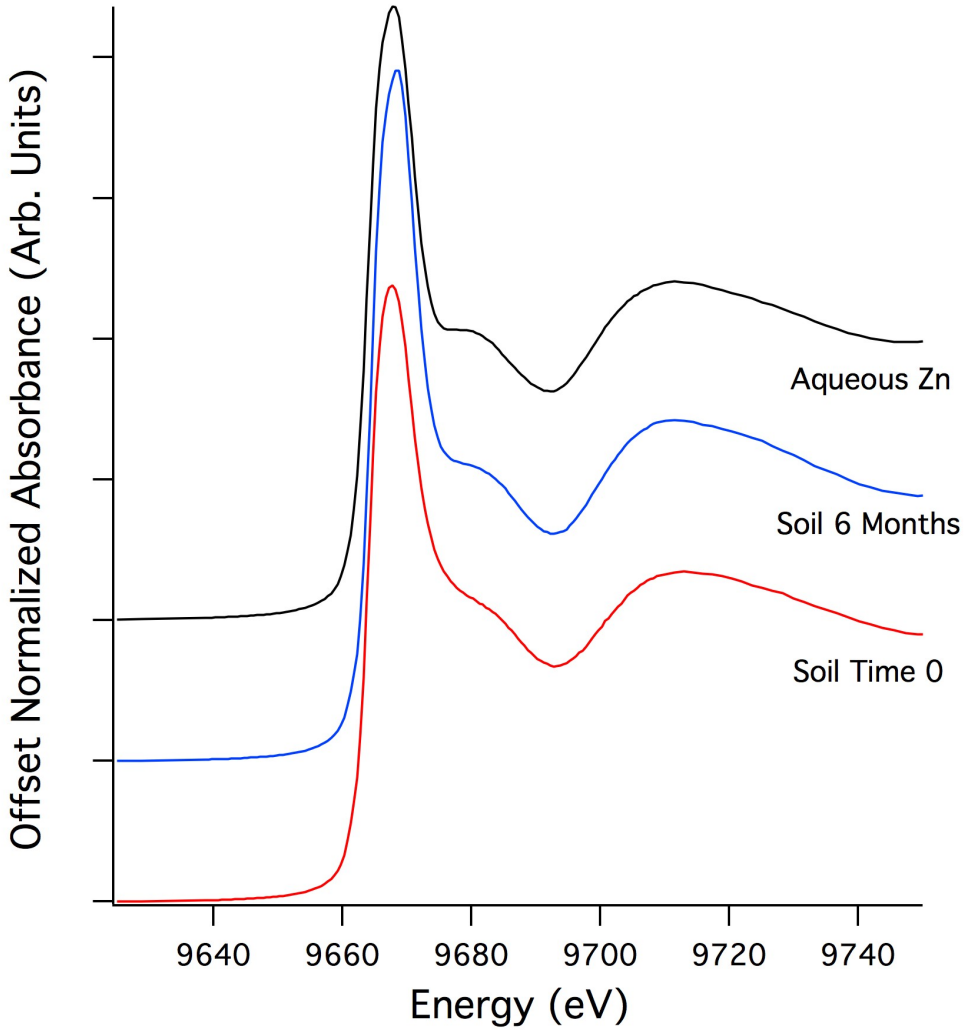

Fig. 5.3 Soil K-edge x-ray absorption spectroscopy (XAS). The x-ray absorption near-edge structure (XANES) is displayed for boreal forest soil land-use (soil 10) and aqueous zinc.

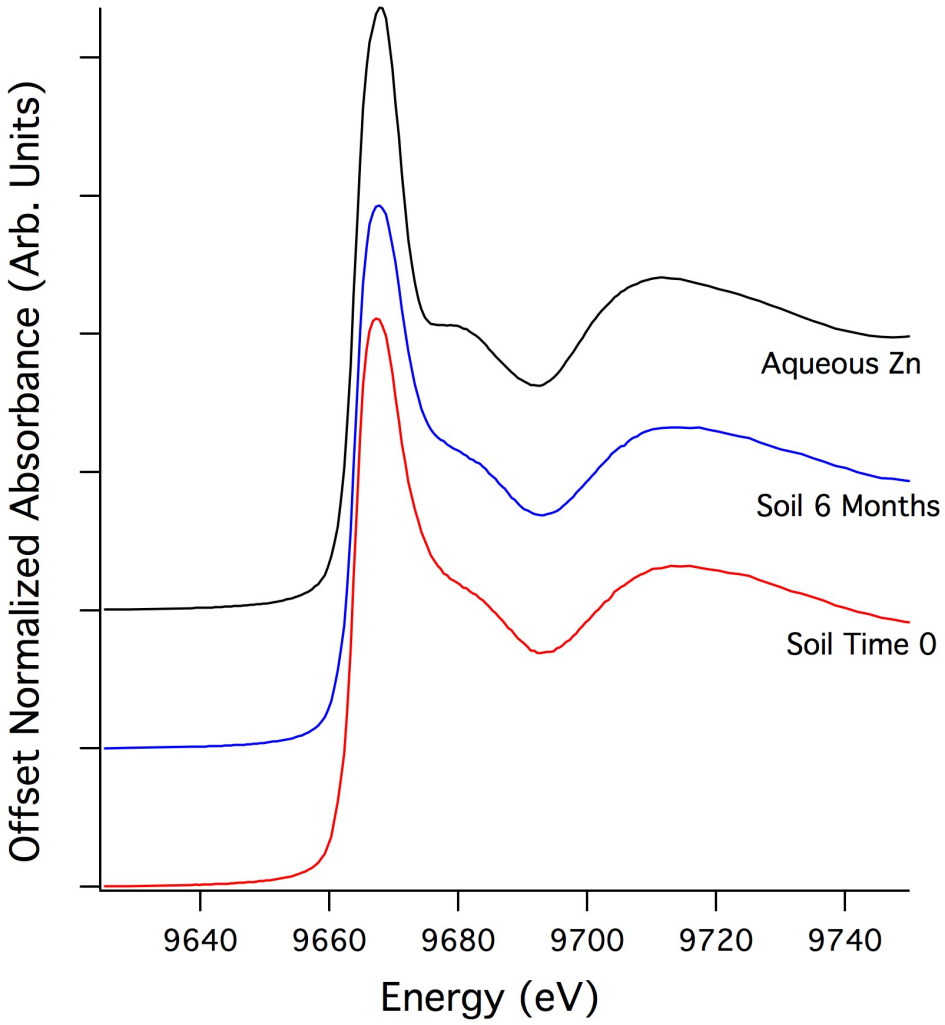

Fig. 5.4 Soil K-edge x-ray absorption spectroscopy (XAS). The x-ray absorption near-edge structure (XANES) is displayed for urban soil land-use (soil 16) and aqueous zinc.

5.5.3 Effect of Soil Properties: Stress, Disturbance, and Stability

The resistance of individual soils regressed with the sensitivity to zinc and copper as determined in toxicity tests (EC_{50} values). Figures 5.5 & 5.6 show that zinc and copper toxicity for nitrification significantly regressed (r^2=0.89 P<0.05) with the percent resistance relative to the control for moisture stressed soils.

The resistance of individual soils to heat stress was compared to the sensitivity to zinc and copper as determined in toxicity tests (EC_{50} values). Figures 5.7 & 5.8 show that zinc and copper toxicity for nitrification significantly correlates with the percent resistance relative to the control for heat stressed soils. No significant effects of senstivity to metal stress (EC_{50} values) to resistance to heat stress was observed.

To evaluate the soil enzyme activity stability of each soil to heat stress (°C), RSSI for dehydrogenase, phosphatase and arylsulphatase activity was created. This is shown in Fig. 5.9; and was done for soils that underwent a range of treatments; uncontaminated control soils, zinc and copper addition (two treatment levels), and smelter-contaminated soils. The results of a Fisher's LSD test showed that there were significant differences for enzyme activity RSSI in artificially copper contaminated soils (Cu1 and Cu2), as well as field contaminated soils (Flin Flon) to uncontaminated control soils when subjected to heat stress. Significant differences between artificially contaminated zinc soils (Zn1 and Zn2) to uncontaminated soils and copper contaminated soils was also observed. An ANOVA for enzyme activity RSSI and moisture shows no significant differences between contaminated and uncontaminated soils.

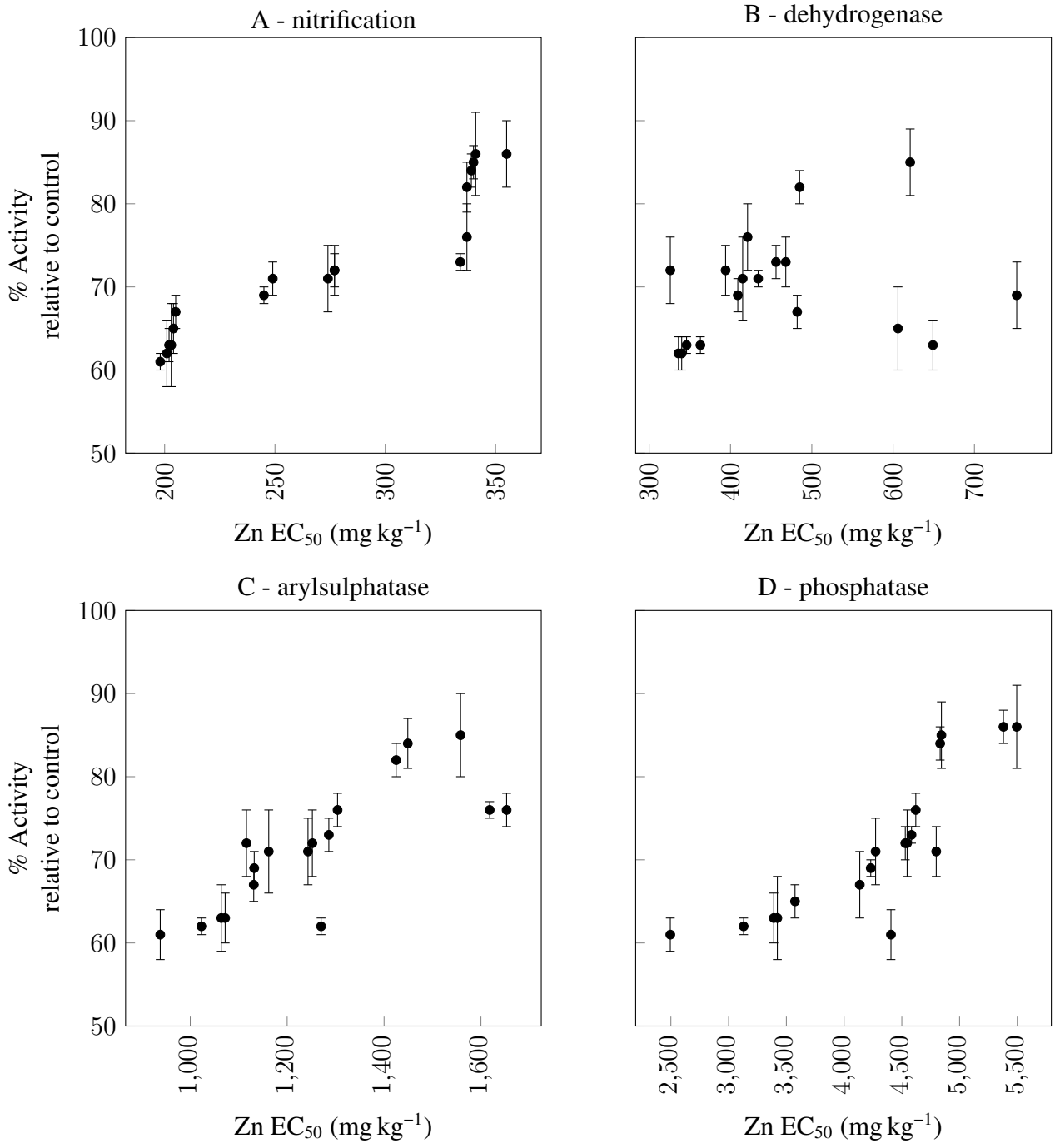

Fig. 5.5 Enzyme activity inhibition as a percentage of the control (no moisture stress) to soils after moisture stress compare against zinc EC_{50} mg kg^{-1} values determined for each individual soils for (A) nitrification; (B) dehydrogenase; (C) arylsulphatase and (D) phosphatase activity. Error bars indicate one standard deviation from the mean.

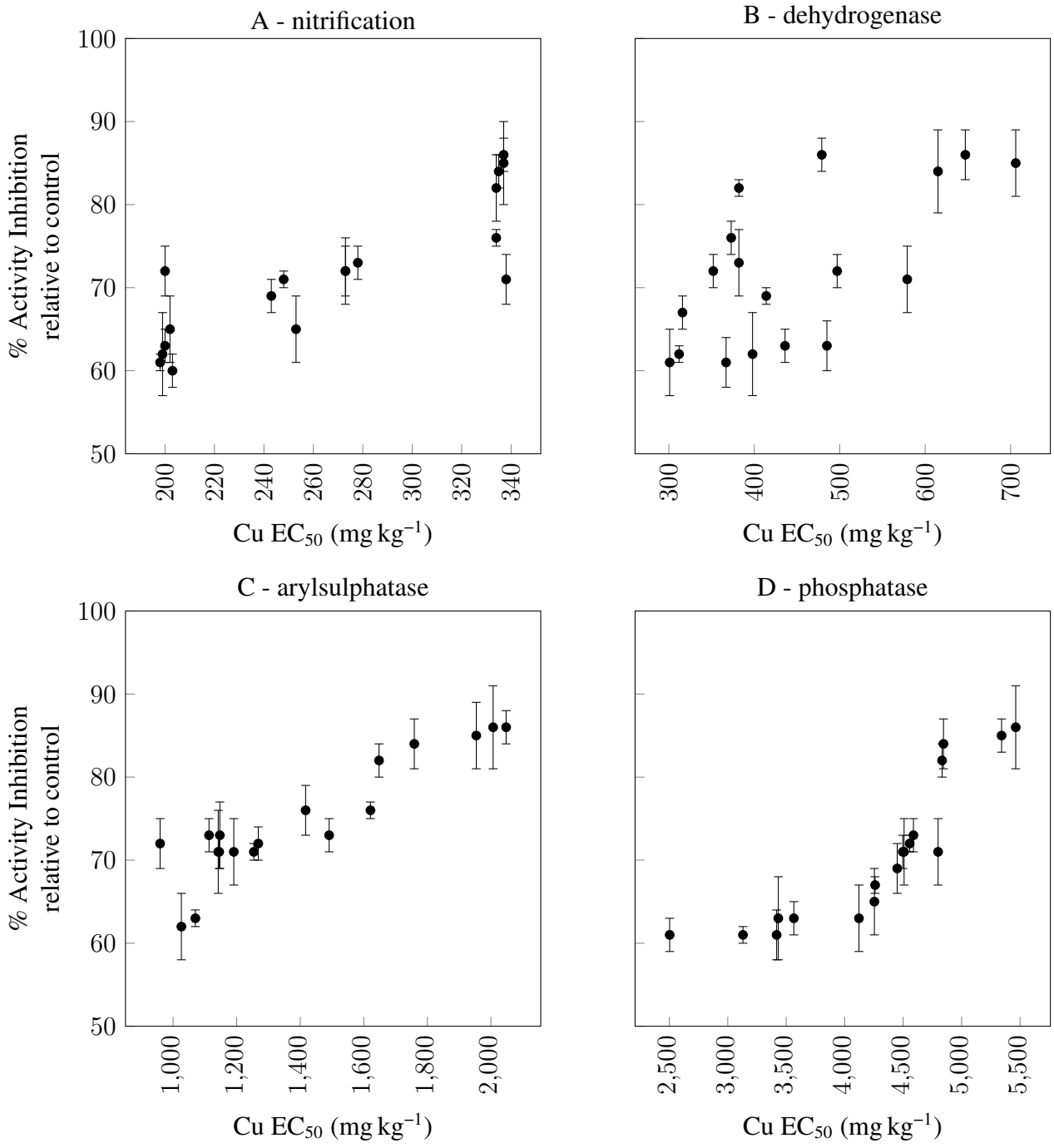

Fig. 5.6 Enzyme activity inhibition as a percentage of the control (no moisture stress) to soils after moisture stress compare against copper EC_{50} values determined for each soils for (A) nitrification; (B) dehydrogenase; (C) arylsulphatase and (D) phosphatase activity. Error bars indicate one standard deviation from the mean.

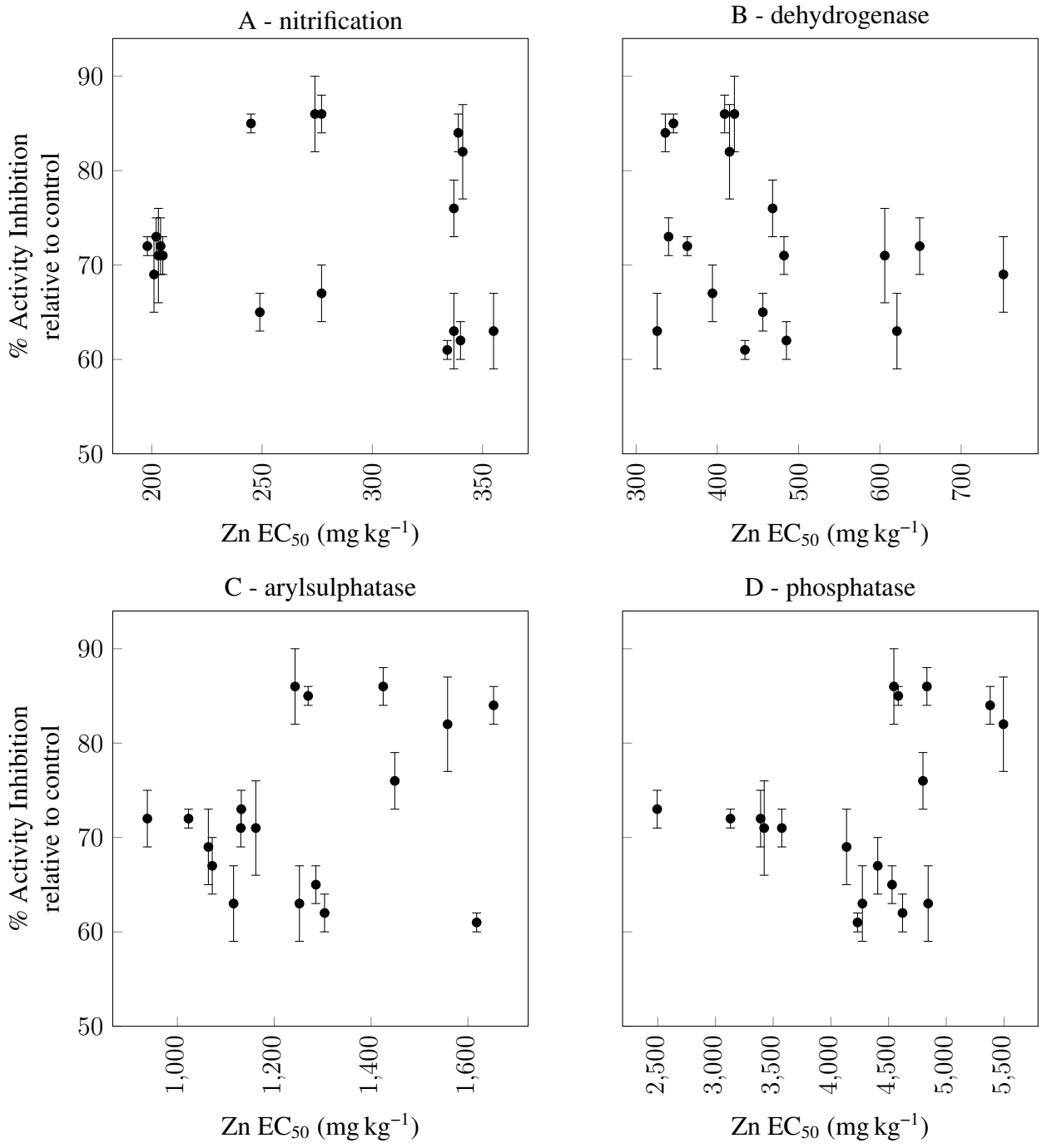

Fig. 5.7 Enzyme activity inhibition percentage of control after exposure to heat stress compare against zinc EC_{50} mg kg^{-1} values from the same soils for (A) nitrification; (B) dehydrogenase; (C) arylsulphatase and (D) phosphatase activity. Error bars indicate one standard deviation from the mean.

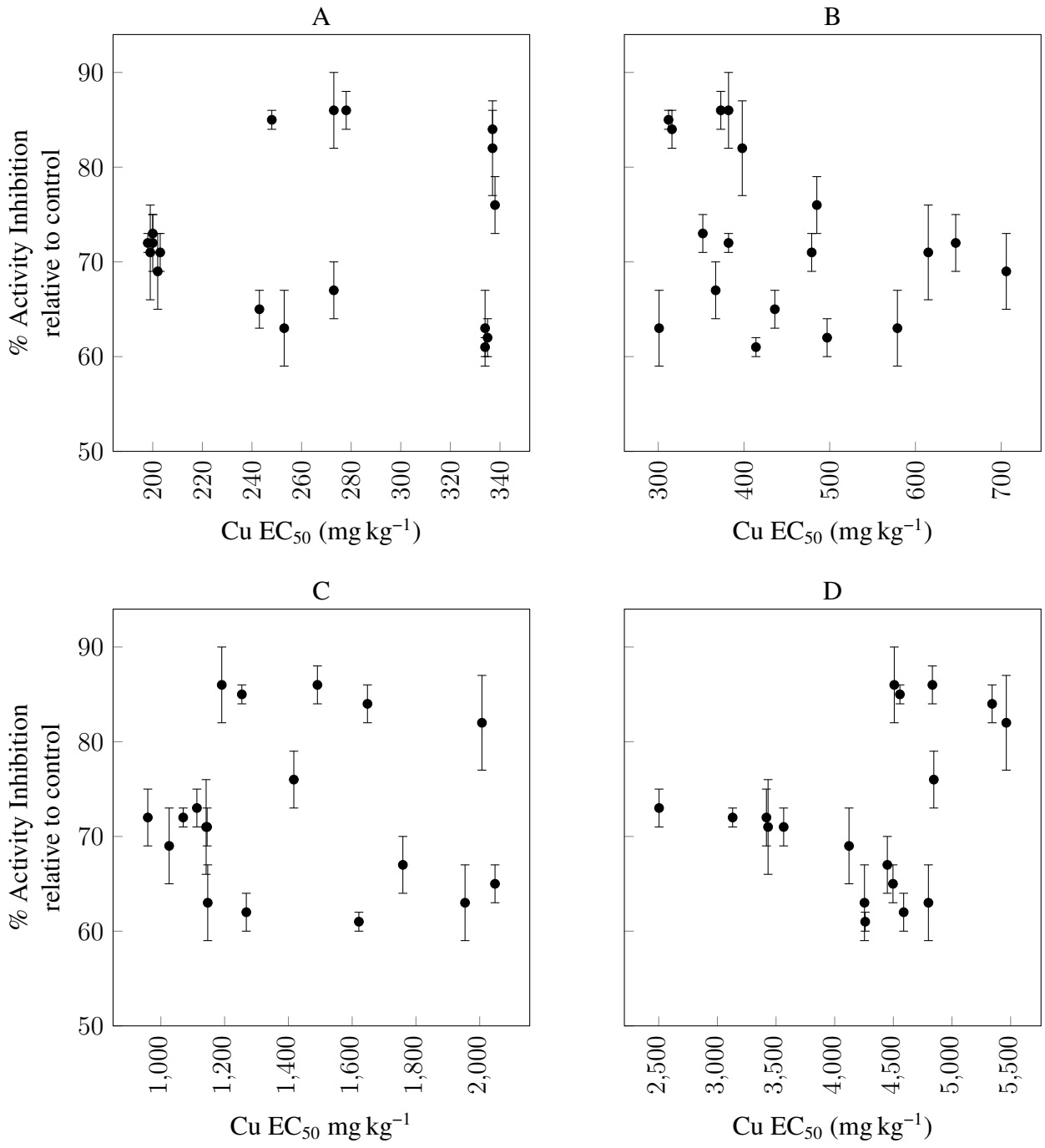

Fig. 5.8 Enzyme activity inhibition percentage of control after exposure to heat stress compare against copper EC_{50} mg kg^{-1}values from the same soils for (A) nitrification; (B) dehydrogenase; (C) arylsulphatase and (D) phosphatase activity. Error bars indicate one standard deviation from the mean.

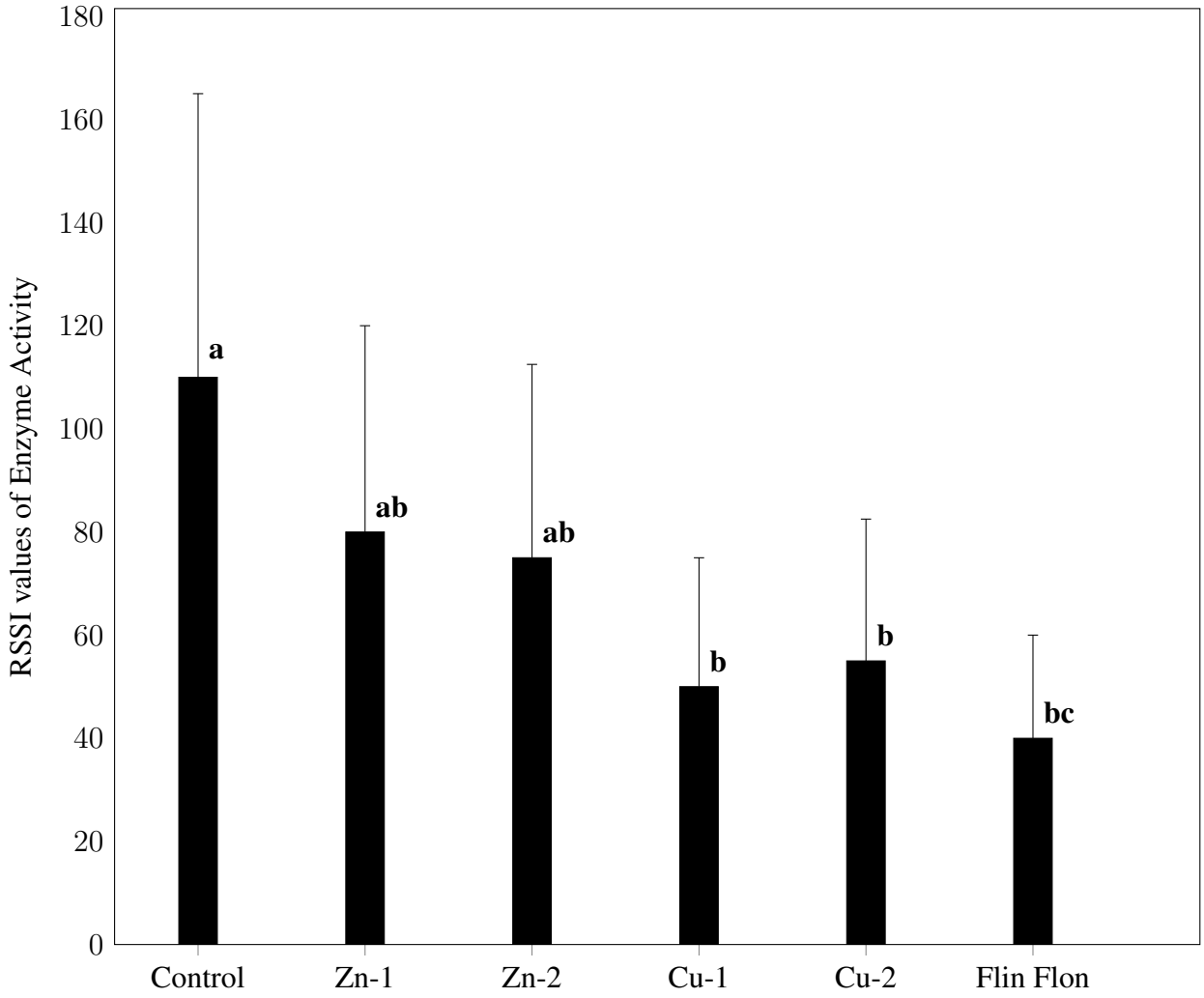

Fig. 5.9 RSSI value comparison (n=18) for heat (60°C) stressed soils. A control group of soils with no metals added, two treatments of zinc and copper at two dose levels, and a smelter contaminated soils with both zinc and copper. Two treatments of zinc (Zn-1: 2,000 mg kg^{-1}& Zn-2: 5,000 mg kg^{-1}) and two treatments of copper were applied (Cu-1: 2,000 mg kg^{-1}& Cu-2: 5,000 mg kg^{-1}). All artificially contaminated soils were incubated for 180 days prior to analysis and field contaminated soils were treated the same after there collections in the field as artificially contaminated soils. In addition to soils (n=18) were collected from a zinc and copper smelter contaminated boreal forest landscape (Flin Flon soils). Letters indicator significant differences between groups from a Fisher's LSD test

5.6 Discussion

5.6.1 Moisture and heat: stress, disturbance, and stability

The aim of this study was to determine the effect adaptation to metal stress (zinc and copper) has on the resistance and resilience of soil enzymes (enzyme activity: phosphatase, dehydrogenase and sulphatase, and nitrification activity) to secondary stresses (heat and moisture). Specifically, to compare soils incubated for 180 days with five treatments of metal stress: control uncontaminated soil, two treatments of zinc and copper and a smelter-contaminated soil. First the resistance to moisture stress. The two central theories to the long-term consequences of adaptation are that the alterations caused by adaptation and the increased metabolic stress lead to the development of soil microbial community being less resistant to secondary stresses (Tobor-Kaplon et al., 2005). The second theory is that stressed systems will have developed more resistance mechanisms and thus be more able to resist additional stresses (Odum, 1985). This is in line with several other studies which have shown that there are consequences to the adaptation to metal stress by the microbial community (Griffiths and Philippot, 2013)

The resistance of the soil microbial community to moisture stress was compared to the sensitivity of soils to metal stress. This was done using EC_{50} values for the soil. The resistance was correlated to the initial toxicity to zinc and copper for nitrification; dehydrogenase; arylsulphatase, and phosphatase activity (shown in Fig. 5.5). Nitrification, arylsulphatase, and phosphatase activity showed the most significant correlation. This could be reflective of a soil microbial community's resistance and resilience to any stress applied being related. This supports the first theory that changes to the soil microbial community to cope with one stress can effect with the resistance to additional stresses (Odum, 1985; Bååth, 1989; Tobor-Kaplon et al., 2005, 2006). This is related to changes in the energy budget of the organisms which have developed during the exposure to the first stress and therefore relate to how adaptation occurred (Odum, 1985). Soils that have developed resistance to a stress that requires energy for detoxification and stress resistance will be more stable.

The difference in response patterns between enzyme activity was relatively small. All microbial enzyme activities were negatively affected by secondary moisture stress. This is consistent with studies carried out by Tobor-Kaplon et al. (2006) and Griffiths and Philippot (2013) with all microbial enzyme activities showing a similar response in stability to the stresses. This suggests that changes in enzyme activity are indeed reflective of changes to the microbial community and not alterations of the individual enzymatic pathways themselves.

The resistance of the soil microbial community was compared to the sensitivity of soils to metal stress. This was done using EC_{50} values for the soil. The resistance was correlated to the initial toxicity to zinc and copper for nitrification, dehydrogenase, arylsulphatase, and phosphatase activity (shown in Fig. 5.7). The response of the microbial community could not be demonstrated using this method. The relationship between the sensitivity of enzymes to metal stress was not related to the sensitivity to heat stress. This could be that the mechanisms for resistance to the two stresses are not related. The response of metal stress microbial communities has been studied in numerous other studies (Griffiths et al., 2000, 2004, 2005; Kuan et al., 2007; Griffiths et al., 2008; Gregory et al., 2009; Zhang et al., 2010). Kuan et al. (2007) examined soil biological resilience measured by CO_2 respiration in soils treated with heat and copper stress. Specifically, they showed that resistance to heat stress was not correlated to soil properties. They hypothesised, that microbial heat resistance was the result of changes to the soil microbial population (Kuan et al., 2007). They also theorised, that the microbial populations resistance to heat and the microbial population resistance to copper are different. Meaning that the response of the microbial populations to these two stresses would also differ. This theory is supported by the results here, showing that the resistance to metal stress is not correlated to the resistance to metal stress. However, over time repeated exposure to heat stress could cause adaptation to this stress. The resistance of heat stress in this study was similar to the effects observed by Kuan et al. (2007); Griffiths et al. (2008) who both observed between a 100-50% effect of heat stress compared to non heat-stressed soils. Kuan et al. (2007); Griffiths et al. (2008) however, used denitrification and nitrite oxidation functions to determine the effect on the microbial population. This study has shown that similar responses

to heat stress can be observed in a wider range of microbial responses. Supporting the idea that soil enzymes and soil microbial process are indicative of changes in the over-all soil microbial community. The effects of heat stress on resilience as well as resistance were further compared with the use of RRSI values.

5.6.2 RRSI of soil enzymes to heat stress

Soil properties are often cited as a factor influencing the response of the soil microbial community to stresses (Kuan et al., 2007; Griffiths et al., 2008). In order to remove this factor and allow comparison between soils Bêcaert et al. (2006) developed the relative soil stability index (RRSI) method of analysis. The RSSI Relative soil stability index considers changes in enzyme activity after disturbance. This method is based on a time-integrated assessment of soil enzyme activity. Heat exposure (60°C for 48 h) similar to a forest fire, to which soils are exposed as part of natural successional pressures and which are predicted occur with increased frequency in the future (Aven, 2016), in part due to anthropogenic climatic change was the method used to assess the RRSI of each soil. The use of RSSI allows assessment of the effects of stresses irrespective of soil type. The effects of adaptation to metals (zinc and copper) at two concentrations (2,000 & 5,000 mg kg^{-1}) showed different responses to heat stress (60°C). Zinc (2,000 & 5,000 mg kg^{-1}) soils showed no significant differences to uncontaminated soils to heat stress, whereas copper adapted soils were significantly different to soils not exposed to metal stress. This shows that adaptation of soils and severity of a primary stress does not significantly influence the stability of soils. However, the type of stress (Zn or Cu) did influence the stability. This was not supported by our assessment of field (smelter-contaminated soils Flin Flon, Manitoba), as these soils showed a significantly lower RSSI to subsequent stress. This could indicate that metal concentrations in these soils have a significant effect on their stability.

This study does not support the concept that exposure to one stress can make a disturbance more resistant to a secondary stress, as proposed by Odum (1985). Therefore, supporting the observation and alternative theory that adaptation causes increased metabolic stress led to the

development of soil microbial community being less resistant to secondary stresses (Tobor-Kaplon et al., 2005). Soils previously exposed to zinc and copper showed more resistance and resilience to moisture stress than unexposed soils. This could be because changes that occurred in the microbial community due to exposure to the primary stress of zinc and copper led to a more resilient microbial community. This is also supported by a correlation between resistance to one stress and the sensitivity of soils to another stress, as expressed by EC_{50} values. However, assessment of heat stress did not support this concept. Soils showed no difference in resistance to heat stress after exposure to the primary metal stress regardless of the severity of the stress applied. This supports the alternative theory that says that unstressed systems are more stable. All field-contaminated soils showed less resistance and resilience to all subsequent stresses. This could be related to how the stress has occurred. This may suggest that soils that may not have adapted to the original stress are less stable to subsequent stresses. Laboratory-contaminated soils were stressed under optimum conditions, allowing adaptation to occur. This idea is supported by the lowest observed enzyme activity observed in these soils compared to others. However, they were still in the range observed for other soils and no correlation between metal content and enzyme activity could be seen. This relationship could, however, be obscured due to the presence of multiple metals in these soils. Our study indicates that there is probably no general response to stresses exhibited by soils and that each soil has a unique response.

5.6.3 Soil metal speciation

Metal speciation of zinc in soils has been characterized in a range of different soils. Metal speciation is influenced by the source of zinc (Voegelin et al., 2011) and soil properties (Jacquat et al., 2009). Metal speciation is an important factor in determining the toxicity of a metal. Specifically, metal speciation determines the availability of zinc to be taken up by microorganisms and thus is an important factor in the bio-availability of a metal (Jacquat et al., 2009). As metal speciation plays an important part of toxicity of a metal, the speciation of metals in numerous contaminated sites has been determined using synchrotron-based X-ray absorption spectroscopy

(XAS) techniques (Jacquat et al., 2009; Hamilton, 2013; Hamilton et al., 2016). Here the metal speciation changes of artificially zinc contaminated soils was assessed to determine the changes which occur over time and the affect that leaching has on the availability of metals in soils.

Soils spiked with metals are often leached to mimic natural field conditions and the changes that occur naturally to soils over time (Smolders et al., 2003, 2009). The protocol used in this study followed a similar approach to that used by others (Smolders et al., 2003; Lock et al., 2006; Oorts et al., 2007; Langdon et al., 2014). Smolders et al. (2003) studied the reactions that occur after spiking (lixiviation) of zinc (added as $ZnCl_2$) and showed that spiked soils have increased ionic strength ($\sim$0.05M) that is considerably higher than that formed by natural processes. This showed leaching provides a lower sensitivity in soil microbial processes and was more reflective of the natural environment than only spiked soils. This lowering of sensitivity was thought to be not just related to a change in ionic strength and the removal of salts but also alterations to metal toxicity and changes in metal speciation (Smolders et al., 2003). Although hypothesized, metal speciation in soils artificially spiked with metals was not assessed. Soil metal speciation in leached soils did not show the expected change to a less available forms of zinc. The dominant metal speciation observed in soils was aqueous zinc (Zn^{2+}). This is the most available form of zinc. This suggests that although leaching reduces the observed toxicity of zinc in spiked soils this is not the result of the formation of less available zinc forms as suggested. However, as leaching reduces other confounding factors such as alteration to ionic strength and pH changes in the soils (Smolders et al., 2003; Oorts et al., 2007; Li et al., 2009), which affect the activity of microbial process it should still be a method used in the assessment of metal toxicity.

The six months incubation was also expected to cause alterations in zinc metal speciation. This would be consistent with other artificially contaminated soils spiked with zinc (Voegelin et al., 2011). This was not however observed zinc metal speciation remain dominated by Zn^{2+} even after 6 months of exposure. The effect of soil properties was also not observed, changes in metal speciation between the two soils would also have been expected. Soil properties did not appear to have influenced metal speciation over time. This is an important factor to consider as although

metal speciation in these soils did not change over time the response of the microbial population did. This means that adaptation of the soil microbial population can occur in soils at a rate faster than metal speciation changes. This also has important implication for are understanding of adaptation. If in short term assays soil metals are more readily available for uptake by microorganisms, this could help explain the discrepancies between field contaminated soils and field contaminated soils (Oorts et al., 2007).

There are several thoughts on the aging of metals after they are introduced to soils. (Smolders et al., 2003) assumed that soil ageing processes were reversible with pH changes in soils. Several possible explanations exist for how the process of aging would occur in soils. This study showed that soils dosed with zinc in the form of $ZnSO_4$ did not undergo the changes which have been observed in other hazard assessments of zinc speciation in soils. Artificially dosing of soils use soluble metal salt is often done for the hazard assessment of metals (Smolders et al., 2003; Broos et al., 2004; Rusk et al., 2004; Fait et al., 2006; Lock et al., 2006; Oorts et al., 2007; Smolders et al., 2009; Trevisan et al., 2012; Langdon et al., 2014). It should be noted that the soluble metal salts used in these studies may not be reflective of the metal species found in field contaminated soils. Aging is though to form phases such as double hydroxides and secondary phase metal formations. These forms were not present in the soils after 6 months of exposure. This could indicate that the long term effects of soil properties is best studied after this time. (Smolders et al., 2003) suggested that the diffusion rate of mineral phase formation is an important factor and that a slow decrease in the availability of metal concentration may occur as mineral phases are formed. Our study did not show this process and the available concentration of zinc in soils did not decrease over 180 days. This data are similar to that published by others (Lock et al., 2006; Oorts et al., 2007; Li et al., 2009; Voegelin et al., 2011) that suggests aging is a poorly defined amalgamation of processes, and soil treated in the laboratory may not be fully reflective of field contaminated soils.

This study tested whether adaptation to a severe stress (trace metals) affects resistance or resilience to a subsequent mild stress. The resistance, resilience, and relative soil stability index (RSSI) of soils were determined for heat (60°Cfor 24h) and moisture stress, applied as short-term secondary stresses. Soils that had adapted to zinc and copper were not less resilient to additional stresses after adaption, than before recovery. The results of this experiment suggest that activity is a good indicator of soil microbial health but metal concentration is not. Metal speciation was determined using synchrotron-based X-ray Absorption Spectroscopy (XAS) at the Canadian Light Source Inc. Specifically Zn k-edge near-edge structures (XANES) data were collected at the HXMA beamline (06-ID1).

In Conclusion, detailed analysis of soils artificially contaminated with zinc and copper in this study allowed the long term consequences for soil microbial functioning to be studied in greater detailed than they have been before. This gives an improved understanding of the effects of zinc and copper to a range of soils. It also improved the understanding of how soils artificially contaminated with metals for hazard testing differ from field contaminated soils. This study provides an improvement in the understanding of how microbial data can be incorporated into soil quality guidelines for Canadian soils.

6 SYNTHESIS AND OVERALL CONCLUSIONS

The overall goal of this dissertation was to contribute to the understanding of the effect trace metals (zinc and copper) have on soil microbial functions in the short and long term, in Canadian soils. More specifically, it aimed to determine how soil properties and land-use influence the short and long-term activity of soil enzymes. This was achieved by first validating a new method for assessing soil enzyme activities (phosphatase, sulfatase, and dehydrogenase) that requires low (0.1 g) amounts of soil. This method was then applied to smelter-contaminated soils to assess remediation treatment effectiveness. Having developed new microbial enzyme activity assays for toxicity assessment, a range of soils were artificially contaminated with zinc and copper to allow the assessment of short- and long-term toxicity and further understand adaption of a range of soil functional processes to trace metals. To further assess the process of adaption and its effect on the stability of soil microbial functions, soils were assessed for their response to secondary environmental stresses (moisture and heat).

The validation study aimed to develop low soil requirement enzyme assays, as well as other assessment tools that could be applied to the assessment of large-scale contaminated sites, specifically in remote areas. This is a particular problem for Canada due to its size, population distribution, and number of contaminated sites. The investigation also considered the effectiveness of remediation treatments on soils affected by trace metal contamination in the vicinity of a smelter in the Flin Flon-Creighton area on the Saskatchewan-Manitoba border. This study demonstrated that autotrophic and heterotrophic nitrification respond differently to liming and significant differences between activities can be identified in the short (one year) and long term (10 years). Soil

enzymes (phosphatase, sulfatase, and dehydrogenase) proved reliable at identifying the treatment effectiveness of biochar and smectite amendments applied on a range of soils with varying zinc and organic matter content. This research also demonstrated the applicability of a low soil requirement assessment method for use in large scale eco-restoration projects, confirming the potential for soil enzyme assays as a tool for identifying soil microbial responsiveness to amendment strategies. This allows for the evaluation of soil amendment strategies for large-scale eco-restoration projects in an affordable and effective manner.

Eighteen soils were investigated to determine the toxicity of zinc and copper to soil microbial processes. The study provided data for both the short- and long-term responses of different soil microbial enzyme activities to these trace metals, the first to do so for Canadian soils. Predictive models and evidence for adaptation in response to zinc and copper have been outlined. Land-use and soil pH were the main factors influencing the toxicity of zinc and copper across soils. The results show that similar methods for regulating and screening for trace metal contamination at sites could be adapted for use in the Canadian regulatory system, following similar models for the protection of soils used in European and Australian regulatory processes. The capacity of adaptation of Canadian soils was shown to be similar to that observed in European agricultural soils. Soil properties such as CEC, soil pH, and organic C content affected zinc and copper toxicity to soil microbial processes, and land-use was recognized as an additional factor. Therefore, land-uses already considered for the protection of human health should be considered for the protection of soil ecological health. Land-use affected the initial sensitivity of soil microbial processes to Zn and Cu and their long-term sensitivity after adaptation. However, alteration of land-use to reduce ecological risk as carried out when managing human health risk will not result in a reduction in toxicity and therefore risk; this can only be achieved by alteration of soil properties. This means that land-use and soil properties are both important factors influencing zinc and copper toxicity and both need to be taken into account. Land-use was the only consistent method of explaining differences observed in the sensitivity of different soils to trace metals. This is most likely due to

the influence of land-use on soil microbial structure. However, more detailed methods to determine soil microbial community structure will be required to further explore this hypothesis.

Each research chapter has built up a picture that provides clear evidence for the adaptation of a range of soil microbial functions and enzymatic process to zinc and copper contamination. This is demonstrated as occurring across a range of Canadian soils and is not restricted by land-use; therefore, it can be considered a ubiquitous process and a new function ascribable to soils. This further clarifies the position that use of soil enzyme activities as a measure of metal bio-availability in soils is not possible. This means that hazard assessment based on short- term toxicity exposure to metals currently used to set bench mark levels of exposure are still based on sound assessment of the impact trace metals can have on soil microbial process. However, the fact that adaptation does occur may mean that these limits are overly conservative in nature and do not reflect the microbial communities found in nature. Their application in assessing site specific risk is, however, validated and may be essential, as predicting enzyme activity based on short-term exposure may not provide an adequate picture of field microbial sensitivity to trace metal stress.

Finally, these studies were able to assess soil microbial activity and determine adaption effects on the stability of soil enzyme activities to secondary applied environmental stresses. This supports both concepts with respect to stability. Soils stressed by zinc and copper have gained genetic abilities through adaptation but soils that have not been exposed have a more diverse microbial community to rely on in times of stress. This means that there is no general reason for soils developing stability and, rather, it is dependent on factors contributing to the development of a particular soil ecosystem. Additionally, this study provided zinc metal speciation data that showed these soils did not form additional mineral species. These results have particular significance because they were observed across a range of soil types. This study also suggests that hazard assessment metal spiking may not be reflective of metal speciation observed in field-contaminated sites. This provides a clear area for future research. Identifying metal speciation between across hazard assessment methods for assessing metal toxicity and field-contaminated soils should be a priority in further research.

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APPENDIX

Appendix A

Supplementary material for Chapter 3: Validation of low soil requirement soil enzyme assay for use in nutrient assessment of metal contaminated soils

Fig. 6.1 Photograph showing biochar amendment in high organic matter smelter contaminated soils, image was taken south of the smelter facility. Plots were established and sampled six months later, plots were 1 m^2

Fig. 6.2 Photograph B shows mulch and biochar soil amendments in low organic matter soils, this image was taken 700 m east of the smelter facility. Plots were established and sampled six months later, plots were 1 m^2

Appendix B

Supplementary material for Chapter 4: Effect of Land-use on the toxicity of Zinc and Copper to soil microbial functions

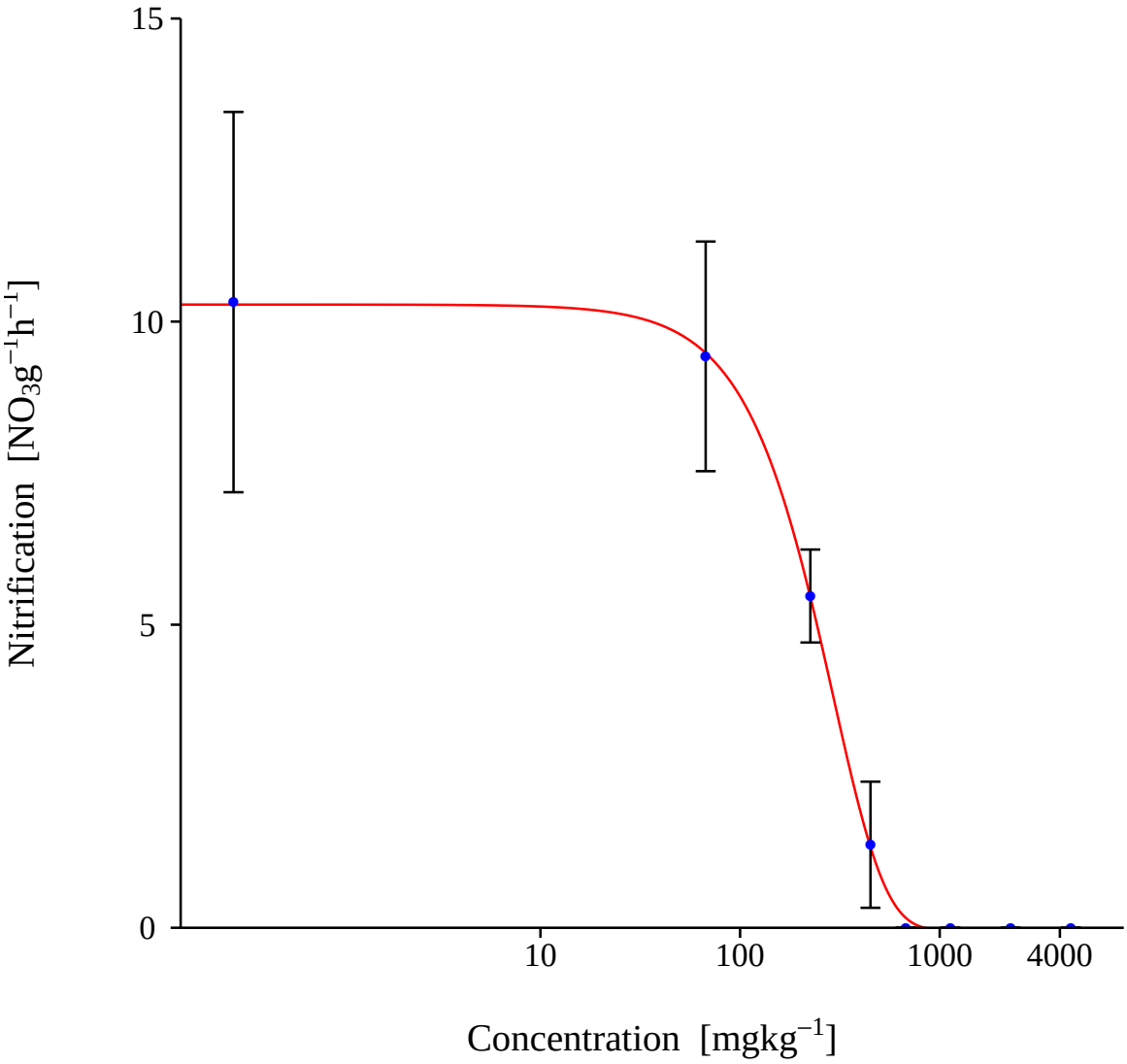

Fig. 6.3 Example of a dose response curve for nitrification (soil 13) to labile zinc concentration used to determine median effect concentration (EC_{50}) for each soil (1-18)

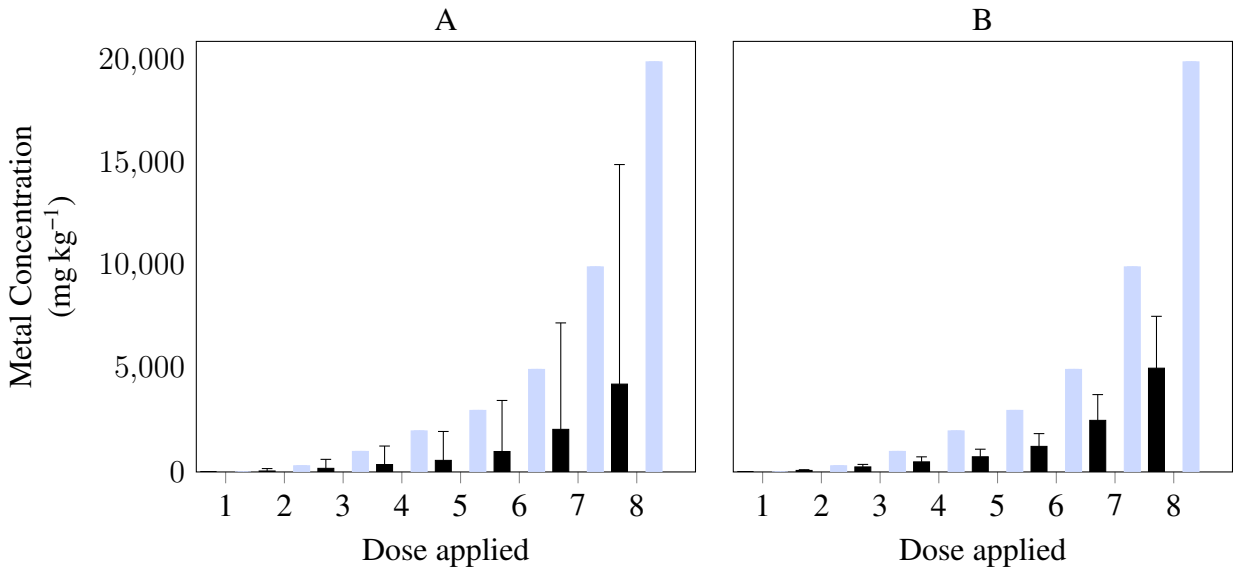

Fig. 6.4 Mean microcosm labile metal concentration zinc (A) and copper (B) against doses applied (8) Dose are, 1:0; 2:300; 3:1000; 4:2000; 5:3000; 6:5000; 7:10,000; 8:20,000 mg kg^{-1}of total salt applied. Grey bars indicate applied dose and black+ bars indicate measured labile metal concentration. Error bars show one standard deviation from the mean.

Table 6.1 Soil Classification of the 18 soils separated by land-use. All soils were classified according to the Canadian System of Soil Classification

Land-use	Soil	Classification
Agricultural	1	Orthic light Brown Chernozem
	2	Orthic Black Chernozem
	3	Orhtic Dark Brown Chernozem
Native Grassland	4	Orthic Black Chernozem
	5	Orthic Black Chernozem
	6	Orthic Black Chernozem
Boreal Forest	7	Orthic Gray Luvisol
	8	Brunisolic Gray Luvisol
	9	Brunisolic Gray Luvisol
	10	Eluviated Dystric Brunisol
	11	Eluviated Dystric Brunisol
	12	Eluviated Dystric Brunisol
Urban Residental	13	(Anthroposolic) Orthic Dark Brown Chernozem
	14	(Anthroposolic) Orthic Dark Brown Chernozem
	15	(Anthroposolic) Orthic Dark Brown Chernozem
Urban Industrial	16	(Anthroposolic) Orthic Dark Gray Chernozem
	17	(Anthroposolic) Orthic Dark Gray Chernozem
	18	(Anthroposolic) Orthic Dark Black Chernozem