SOIL MICROBIAL AND ECOSYSTEM SERVICE RESPONSES TO METAL MIXTURES IN CANADIAN SOILS

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By

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ABSTRACT

Metal contamination is a major environmental concern especially in metal mining countries like Canada. The assessment and cleanup of soils with elevated metal concentrations is an area that has been widely studied. A major challenge faced by environmental scientists when assessing metal toxicity in soils is the wide difference in toxic effects between laboratory spiked soils and field contaminated soils. Also, since contamination occur as mixtures, researchers are faced with understanding metal mixture interactions in soil to help quantify risks associated with metal mixture contamination. When assessing the toxic effects of metals in a laboratory setting, it is recommended to use fixed ratio rays, but maintaining desired metal ratios in soils is challenging because of metal loss from leaching metal salt-spiked soils. To eliminate leaching, which is a required step, two alternative metal types (metal oxides and spinel minerals) were evaluated. The main objectives of the thesis were to investigate the differences in toxicity of three metal types found in contaminated soils and to test the adherence of mixture toxicity to additivity models using the activity of soil enzymes as model toxicity endpoints. I also extended our understanding of the effects of metal mixtures on the quality of ecosystem services using soil properties as predictors.

First, the toxicity of metal salts, metal oxides and spinel minerals were assessed using acid phosphatases (ACP) and ammonia monooxygenases (AMO) as model processes in three Canadian soils. The activity of both enzymes in the soils were determined in leached and non-leached soils, as well as soils spiked with mixtures containing Pb, Cu, Ni, Co, and Zn in five fixed ratio rays. The results showed that the activity of AMO was inhibited when soils were leached with artificial rainwater. Generally, metal salts were the most toxic, while the spinel minerals were the least toxic. Two extractants, $CaCl_2$ and Diethylenetriamine Pentaacetic Acid (DTPA), were evaluated for their ability to link toxicity to metals across all three metal forms. Salt toxicity was closely linked to $CaCl_2$ extractable concentrations but DTPA was the most appropriate for oxides. I determined that combining fixed ratio rays with metal oxides for metal mixture studies was more appropriate for conducting mixture studies since soil ratios created using oxides were more precise and required less experimental effort compared to salts and spinel minerals.

Following the investigation into the differences in toxicity of metal mixture types, I evaluated the adherence of metal mixture toxicity to the concentration addition (CA) and response addition (RA) models. I assessed mixture toxicity using metal oxides (Cu, Co, Pb, Zn, and Ni) in two Canadian soils. The additivity models were used because current risk assessment is conducted assuming metals are non-interactive and have similar modes of action. I investigated the sensitivity of the carbon (C) and phosphorus (P) cycles to the mixtures using two soil enzymes, beta glucosidases (BGD) and ACP as model processes. In general, P cycling (ACP) was a more sensitive enzyme to both single and metal mixtures compared to C cycling (BGD). Upon exposure to quinary mixtures, both synergistic and antagonistic deviations from both reference models were observed. The antagonistic deviations were observed across all concentrations, thus from low to high, but synergism was only observed at lower concentrations for both additivity models. The results indicate that, the effects of metal mixtures are greater than singles at lower concentrations which is important in the risk assessment of metal mixtures. I also observed that Cu, an essential metal, may be protecting biogeochemical cycles from mixture toxicity.

In the third chapter, I developed adverse ecosystem service pathway (AESP) models to study the soil ecosystem’s response to a metal mixture containing Cu, Pb, Zn, Co, and Ni. I assessed the effects using the relationships between soil properties and ecosystem services (ES) in the presence and absence of the metal mixtures. Forty-seven (47) soils were sampled and 15 soil processes that represented five ES including food production and water purification were measured. Using a Pearson bivariate correlation matrix, I confirmed that ecosystem services were closely linked to soil properties, especially cation exchange capacity and organic carbon. Results from t-tests also showed that, except for the three soil enzyme activities measured ($p < 0.05$), the processes underlying ecosystem services are significantly reduced in metal-impacted soils. Using soil properties as the main predictors of ecosystem services, I built two AESP models: one for metal-impacted soils and another for control soils. These models showed adverse effects to ecosystem services in metal-impacted soils, depicted as changes in partial correlation coefficients. An AESP model, therefore, can be an important tool to better understand complex ecosystems and improve site specific risk assessment and natural resource management.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ACP	Acid phosphatases
AESP	Adverse ecosystem service pathways
AMO	Ammonia monooxygenases
AMPA	Aminomethylphosphonic acid
ANOVA	Analysis of variance
AOA/B	Ammonia oxidizing archaea or bacteria
AOP	Adverse outcome pathways
AT	Average time
BGD	Beta glucosidases
BLM	Biotic ligand model
BW	Body weight
CA	Concentration addition
CCME	Canadian Council of Ministers of the Environment
CEC	Cation exchange capacity
COPC	Chemicals of potential concern
CSF	Cancer slope factor
CSQG	Canadian soil quality guidelines
CSSC	Canadian system of soil classification
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DRA	Dose response assessment
DTPA	Diethylenetriaminepentaacetic acid

EC	Effective concentration
ED	Estimated duration
EDI	Estimated daily intake
EEC	Estimated environmental concentrations
EECR	Equal effect concentration ratios
ERA	Ecological risk assessment
ES	Ecosystem services
EU-REACH	European Union Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals
FA	Fulvic acid
FF	Flin Flon Ray
FIAM	Free ion activity model
GC	Gas chromatograph
GDP	Gross domestic product
GLP	Glyphosate
GOF	Goodness of fit
gSEM	Generalized structural equation models
HC	Hazard concentration
HSD	Honest significant difference
ICP-OES	Inductively coupled plasma optical emission spectroscopy
IPGR	Instantaneous population growth rate
IR	Ingestion rate
LT	Lifetime

MUB	Modified universal buffer
OC	Organic carbon
PEAT	Peaty ray
PM	Particulate matter
PNEC	Predicted no effect concentration
PNR	Potential nitrification rate
PTE	Potentially toxic elements
RA	Response addition
RAs	Risk assessment
RfD	Reference dose
RMSE	Root mean square error
ROS	Reactive oxygen species
SAVR	Surface area to volume ratio
SEM	Structural equation model
SES	Soil ecosystem service
SOM	Soil organic matter
SSD	Species sensitivity distribution
SUD	Sudbury ray
TBLM	Terrestrial biotic ligand model
TDI	Tolerable daily intake
TEA	Triethanolamine
TRV	Toxic reference value
TU	Toxic unit

UNEP	United Nations Environmental Program
USEPA	United States Environmental Protection Agency
WHAM	Windermere humic aqueous model
WHC	Water holding capacity
XRF	X-ray fluorescence

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Research guidelines for a manuscript-style thesis. Chapter 1 is a general introduction, including objectives and hypothesis, Chapter 2 is the literature review, and Chapter 6 reviews the principle findings, synthesis, general discussion and conclusions tying the chapters together. Chapters 3, 4 and 5 of this thesis are organized as manuscripts for publication in peer reviewed scientific journals. Chapter 3 has been published in Chemosphere, Chapter 4 has been formatted for submission to Science of Total environment, and Chapter 5 has been submitted to Environmental Science and Technology. Full citations for the published research manuscripts are provided in the preface section to each chapter. As a result of the manuscript-style format, there is repetition of material in the materials and methods sections of the thesis. Tables, figures, supporting information and references cited in these research chapters have been reformatted here to a consistent thesis style. References cites in each chapter are combined and listed in the References section of the thesis. Supporting information associated with research chapters are presented in the Appendix section at the end of this thesis.

1 INTRODUCTION

Metal concentrations in the environment have continuously been increasing above background since the industrial revolution. Subsequently, the surge in population and developments in technology has caused an increase in the demand for natural resources such as oil, gas, and metals. As a result, several mining explorations have been established to meet this high demand. Over the span of a century (between 1900 to 2000), global material extraction has increased from less than 10 billion tons, to over 90 billion tons (Carvalho, 2017). The explorations which include metal mining, smelting and refining have caused elevated metal concentrations in aquatic and terrestrial ecosystems around the world. For example, Hamilton et al. (2016) reported over 20,000 ppm of Zn in soils at a Canadian smelter in Flin Flon, Manitoba.

Elevated soil metal concentrations are deemed unsafe by environmental regulatory agencies like the Canadian Council of Ministers for the Environment (CCME) because of the potential toxicity of metals to humans and soil organisms. Metals such as Zn, Cu, Co, and Mg are required for normal metabolic activities and growth of biota at low concentrations. Others like Pb, Hg, and Cd, have no known biological role. In contaminated soils, metals usually occur as mixtures rather than singles. The effects of single metal exposure to soil biota have been investigated for several decades, and the knowledge of these effects is well established (Chaperon and Sauvé, 2007; Jegede et al., 2019; Versieren et al., 2017). However, the behavior and toxic effects of metal mixtures in soils is complex, and researchers are still preoccupied with attempts to comprehend metal interactions to improve the prediction of mixture toxicity (Farley and Meyer, 2015; Meyer et al., 2015). The complexity results from a combination of differences in the behavior of metals, and modification from soil properties. When environmental concentrations of each metal in a whole mixture are below regulatory limits, there is a question of additivity or possible interactions between the components (Cedergreen, 2014). Mixture effects are particularly important for metals since metals cannot be degraded or destroyed but can only be transformed from one species to another. The persistence of metals theoretically plays a role in estimating the toxicity, since the components can interact to produce lesser or stronger effects in the short or long term. Current risk assessment of metal mixtures in Canada assume concentration addition (CA) to be the best approach amidst the uncertainties. On the contrary, studies have often shown that the approach conservatively estimates toxic effects of metal mixtures. Another area of concern is how metal mixture tests and experiments are designed for soils, particularly with the selection of metal form and dosing method. Differences in dosing methods have been shown to affect toxicity estimates for single metal toxicity tests in soils (Schwertfeger, 2010). In this PhD thesis, I determined an appropriate experimental approach for dosing metal mixtures in soils while minimizing artefacts and disruptions to soil properties and microorganisms. I further investigated the toxicity of metal mixtures consisting of Pb, Cu, Ni, Zn, and Co to the Carbon and Phosphorus cycles, and developed an adverse ecosystem service pathway (AESP) that utilizes a site specific approach by using soil properties to predict the effects of mixtures on ecosystem services (ES). The AESP model can be used as a tool to improve site specific ecological risk assessment.

1.1 Objectives and hypotheses

The cardinal objective of this PhD research is to understand interactions between metal mixtures and soil biota to improve the accuracy of estimates derived from mixture models to improve the risk assessments of mixtures in Canada. Three main hypotheses were evaluated in this study: (1) The toxicity of metal mixtures to soil organisms is determined by the metal spiking method, (2) Concentration addition (CA) explains the toxicity of metal mixtures to carbon (C) and phosphorus (P) cycles, (3) Metal mixtures alter intimate relationships that exist between soil properties and the processes that generate ecosystem services.

The first hypothesis investigated the differences in toxicity between three metal mixture types; metal salts, metal oxides and spinel minerals in five mixture ratio rays in three soils. The toxicity of the metal types was assessed using the activity of two soil enzymes, acid phosphatases (ACP), and ammonia monooxygenases (AMO). The results for the first hypothesis are presented in Chapter 3 (Toxicity of metal mixtures to soil enzymes is determined by metal spiking method). Chapter 3 provided a levelled foundation for testing the second hypothesis where the toxicities of metal mixtures calculated for 10 fixed ratio rays in 10 doses were assessed in two soils using ACP and beta glucosidases (BGD) as model toxicity endpoints. The results are presented in chapter 4 (Response Addition (RA) is more protective of biogeochemical cycles compared to Concentration Addition (CA): a metal mixture modeling perspective). For the last hypothesis, 47 soils were used to investigate the intimate links between soil properties and 15 endpoints that underlie ecosystem services and is presented in Chapter 5 (Introducing adverse ecosystem service pathways (AESP) as a tool in predictive ecological risk assessment). In chapter 6, I discuss the implications and future directions of the study while opening avenues for further research.

2 LITERATURE REVIEW

2.1 History and overview of the Canadian metal mining industry

Metal mining and smelting in Canada began in the mid-1700s along the St. Lawrence River in Quebec, Canada (Cranstone, 2002). The industry initially started with the smelting of iron (Fe) ore till the first nonferrous metal (copper) production began in Ontario in the year 1848 (Cranstone, 2002). In the same century, gold (Au) was discovered in Quebec, Ontario, Nova Scotia, and British Columbia. Production of Au in Canada commenced in the late 1850s in British Columbia. Other metals that were subsequently produced included nickel (Ni), zinc (Zn), lead (Pb), silver (Ag), cadmium (Cd), and tin (Sn). Between 1900 and 2000, the industry annually produced up to 300,000 tons of Ni, 850,000 tons of Cu, 1.4 million tons of Zn, 60 million tons of iron ore, and 10 million tons of gypsum ($CaSO_4 \cdot 2H_2O$) (Natural Resources Canada, 2000). The nation's huge contribution to the world mineral market has made Canada a world leader in mining and one of the largest producers of minerals and metals. In 2001, Canada was a top five world producer of 16 minerals and metals (Marshall, 2016). Despite the displacement from the topmost position, the nation is still a leading producer of Au, Cd and diamond. The mining industry provides most of the materials that Canadians rely on to build infrastructure, instruments, electronics and automotive.

The industry contributes significantly to the growth of the nation's economy and infrastructural developments especially in the rural and northern regions. The industry currently employs almost 600,000 workers both direct and indirectly and contributed 56 billion dollars to the nation's gross domestic product (GDP) in 2015 (Marshall, 2016). It is obvious that the industry is a building block for the Canadian economy. Despite the huge positive contribution of mining operations to economic development, the process is accompanied with environmental degradation and contamination that can have huge impacts on humans and terrestrial organisms. These environmental problems occur even in the presence of environmental management techniques. Documented issues associated with metal mining in Canada include greenhouse gas emissions, acid rock drainage, metal leaching and groundwater contamination, soil contamination, erosion and sedimentation (Hatch, 2013). These concerns have both direct and indirect impacts on biodiversity and human health. Several contaminated sites have resulted from mining explorations in Canada. In 2012, a report from Canada's Environmental Commissioner identified four contaminated sites with the most expensive financial liabilities (Scott Vaughan, 2012). Interestingly, soils found at these sites were contaminated with metals and metalloids including radium (Ra), uranium (U) and arsenic (As).

2.2 The soil ecosystem

Soil is an indispensable part of human existence and is described as the foundation that nurtures life. It provides several functions such as the regulation and purification of water, serving as a medium that supports the growth of plants and waste recycling, provides a specie-rich habitat and support for human infrastructure. Serving as the most populated habitat, the soil contains about 360, 000 animal species (ITPS, 2015). An estimation of more than a thousand bacterial cells from more than 106 species exist in 10 g of soil (ITPS, 2015). Soils are therefore complex systems made up of a mixture of moisture, minerals, air, humus and organisms. Soils contain more C than both the atmosphere and terrestrial vegetation making it a major C reservoir. The economic value of agro-sylvo-pastoral products and services are supported by soils. Specifically, the production of food, meat and fiber will not be feasible without soil and its rich biodiversity.

The propensity of soils to provide these functions depend on the physical, chemical and biological components that are regularly in communication with each other. The ability of the soil to function within the scope for its given purpose is dependent on specific characteristics of the soil components collectively termed soil quality (Winder, 2003). The components are viewed as indicators that are measurable and influence the capacity of soils to function. These components inform and allow scientists to categorize soils on relative scales that differ in biological, chemical or physical characteristics. The Canadian System of Soil Classification (CSSC) defines soils as a naturally occurring unconsolidated mineral or organic material that form at the surface of the earth and is capable of supporting plant growth. Some authors have economically defined soil as a natural capital or stock that sustainably yields the flow of goods and services (Dominati et al., 2010).

2.2.1 Soil microbes

The soil's invisible majority has significantly shaped the environment since the beginning of evolution. The biosphere in its current state is even more dependent on the action of microorganisms. For example, microorganisms are involved in food production, degradation of xenobiotics, and remediation of contaminated sites through (co)metabolic pathways. Soil microbial abundance is higher in the rhizosphere compared to other parts of the soil. The increase in abundance is due to the secretions and root exudates from plants that contain chemicals that attract archaea, fungi, bacteria, viruses and oomycetes. Microbes are also able to adapt and to most extreme life conditions in terms of salinity, temperature, and pH due to their phenotypic plasticity. Soil microorganisms play multifaceted roles as consumers, producers and decomposers. They are significant organic matter decomposers and certain transformation processes solely depend on them. They play important roles in food webs and chains. The biogeochemical cycling of elements can continue in the absence of plants, animals and soil invertebrates. Soil microbes have proven to be good indicators of soil quality and soil health (Smolders et al., 2001a). This is because of their sensitivity to climate, contamination, changes in land management, their relationships with soil processes and ecosystem functions, and its affordability (Lukac et al., 2017).

2.2.2 Soil invertebrates

The diversity of soil invertebrates is wide. Recent studies have reported that soil microarthropods and other micro invertebrates represent about 23% of the entire diversity of biological organisms that have ever been described (Decaëns et al., 2006).These soil animals live in different parts of the soil but interact with each other. Microfauna like nematodes and protists consists of the smallest group in size (~200 µm) and live in soil pore water. Microarthropods (mesofauna) which include enchytraeids, mites and other groups of mesofauna live in the interstitial air-filled spaces of surface and some mineral soil. It’s been reported that collembolans are the most abundant soil mesofauna (George et al., 2017). The largest soil arthropods (macrofauna) which includes earthworms and pseudoscorpions live in burrows or surface litters of the soil. The activity of soil invertebrate communities plays a vital role in the maintenance of soil health and quality. These organisms are involved with several stages of soil formation, soil aggregation, and erosion control. They are active performers in the decomposition of soil organic matter, nutrient cycling, and climate regulation. Soil invertebrates have been successfully used as bioindicators in both disturbed and undisturbed soils. A decline in the abundance of mesofauna was observed on agricultural sites due to high disturbance (Rutgers et al., 2009). Another study reported that the abundance of collembola on agricultural lands is an indication of appropriate agricultural management and organic fertilizers (Cluzeau et al., 2011).

2.2.3 Ecosystem services

A wide range of goods and services utilized by humans are provided by the soil ecosystem. The United Nations Environmental Program (UNEP) defines Ecosystem Services (ES) as the benefits people obtain from ecosystems (Millenium Ecosystem Assessment, 2005). The various services encompass food and water provision, flood and disease control, nutrient cycling, spiritual, recreational, and cultural benefits that are necessary to maintain the right conditions for life on Earth. Ecosystem services are grouped into provisioning services, regulating services, cultural services and supporting services. Authors like Dominati et al. (2010) argue that the value of services and natural stocks of soils are poorly understood.

The processes and functions that occur in soils provide a multi-dimensional benefit that cover all ecosystem service categories outlined by UNEP. Apart from the service to humans, the lithosphere (pedosphere) plays a key role as an interface between components of the Earth which include the atmosphere, biosphere, and hydrosphere. The lithosphere serves as a major control of climate change and biodiversity. Several functional roles are provided by soils due to the wide diversity of organisms that inhabit the soil. Microbes are important because they provide functions that maintain all other lives. Hence, elucidating the relationships between ecosystem functions, microbial activity, processes and diversity is important to predict how ecosystems respond to environmental changes caused by stressors.

Depending on the functional roles, soil organisms can be viewed as chemical, biological, mechanical or ecosystem engineers (Fajana et al., 2019). Soil microbes and invertebrates degrade organic matter to release locked nutrients for plant uptake, growth and production of food, thereby regulating about 90% of the energy flux in soils (Lukac et al., 2017). When the simplest form of organic compounds like water and carbon dioxide are complexed into long organic structures, soil organisms are needed for their breakdown. For example, the conversion of urea, an organic compound, to nitrite for plant use is mediated by microbial enzymes including urease, ammonia monooxygenases and hydroxalamine oxidoreductase (Das and Varma, 2011). Furthermore, glucosidases and galactosidases in soils play a major role by degrading carbohydrates into simple sugars for use by other microorganisms. Plants and other soil organisms serve as a carbon sink that stores large quantities of carbon. Due to their role, a change in soil health can potentially reduce the ability of the soil to sequester organic carbon by up to 60%, nutrient cycling and habitat quality leading to losses of biodiversity (Ding et al., 2018). Interactions have been reported to exist between soil enzymes, plants and invertebrates that supports and enhances ecosystem functions. As plants grow and shed litter into soils, they serve as a source of carbon and energy that promotes the growth and diversity of fauna decomposers. The decomposers in turn enhance the supply of nutrients like phosphorus, nitrogen and potassium to the plants (Scheu et al., 2005). Due to natural resource explorations and the use of xenobiotics in agriculture, soils are exposed to several anthropogenic chemicals that, for the most part are degraded by soil organisms. Soil organisms also prevent diseases through the predation on other soil organisms that would otherwise cause harm to humans and other organisms.

2.3 Metals in soils

Soils serve as a major sink for heavy metals that are released into the environment from mining explorations. Unlike organic chemicals that can be degraded through oxidization or reduction, metals do not undergo biochemical degradation. Soil naturally contains a wide range metals at background concentrations. Increased metal concentrations above background in soils from metal mining can potentially adversely affect the environment and surrounding ecosystems. Elevated metal concentrations result from released dust that contain particulate heavy metals, and from tailings and waste rocks that contain heavy metals. Common heavy metals found in Canadian soils include Aluminum (Al), Manganese (Mn), Iron (Fe), Zinc (Zn), Lead (Pb), Nickel (Ni), Copper (Cu), and Cobalt (Co) (Frank et al., 1976; Gopalapillai et al., 2018; Ihnat et al., 1996). The rest of the review will emphasize on the last five metals (i.e. Zn, Pb, Ni, Cu, Co) because they were the metals of potential concern tested in this thesis. Concentrations of these metals (i.e. Zn, Pb, Ni, Cu, Co) in Canadian soils are usually found around background levels unless elevated from accumulated contamination from mining and smelting activities (Frank et al., 1976). Elevated concentrations of metals in soils can leach into groundwater or surface waters but can also be absorbed by soil invertebrates or plants and end up in the food chain. Reported cases of metal uptake in plants above regulatory limits include Cd and Pb uptake in lettuce, cauliflower, tomato, potato and spinach, and Cr uptake in rice, maize, beans and radish (Khan et al. 2015). The ability of plants like phragmites (reed) to accumulate metals has made it an option for phytoremediation (Etim, 2012). Generally, the remediation of heavy metals in soils are comparatively more expensive and time consuming due to the difficulty in extracting metals from the soil. The basic properties of the five heavy metals of interest in this study are discussed below.

2.3.1 Zinc

Zinc is a shiny bluish-white metal that is a brittle and crystalline at room temperatures with a 420^{o}C melting point and a 907^{o}C boiling point. When heated to temperatures above 100^{o}C and below the melting point, the metal becomes ductile and malleable. The metal has anti-rust properties that enables its use as a coating for steel and iron in a process called galvanization. It is also found in alloys like brass and bronze. Labelled as one of the most common metals on earth, Zn is found in all environmental media including food and water in low concentrations. It is an essential metal that is required for normal biological functions in living organisms. Elevated concentrations of Zn have resulted from mining and smelting activities across the globe. The solubility, mobility and bioaccessibility of Zn largely determines its toxicity to soil organisms. Zinc is released into the environment primarily as a mineral from smelting activities. Zinc is comparatively more soluble and mobile (McLean and Bledsoe, 1992). Common Zn minerals include franklinite, sphalerite and willemite. These minerals can undergo weathering releasing Zn into soil over long time periods (Hamilton et al., 2016). Zinc compounds can dissolve in acids to release Zn^{2+} or in alkaline solutions to form anions (e.g. $[Zn(OH)_3]^-$, $[Zn(OH)_4]^{2-}$, and $[Zn(OH)_4(H_2O)_2]^{2-}$) due to its amphoteric properties. When in solution, Zn can exist as a free ion or react with chloride and sulfate to form soluble compounds. Zinc can also be hydrolyzed to form hydroxides or hydrated zinc oxides or react with carbonates to form $ZnCO_3$. The toxicity of Zn to human and ecological health will be discussed in sections 2.5 and 2.6.

2.3.2 Lead

Lead is a soft silvery transition metal found in period 6 and group 14 (IVA) of the periodic table. Its solid at room temperature with melting and boiling points of 327.5°C and 1749°C respectively. Its anti-corrosive and low melting point have enabled its extensive use in pipes, batteries and weights. The metal can exist in 0, +2 and +4 oxidation states. Under normal environmental conditions, the metal exists in its divalent state switching into its tetravalent state in highly oxidizing environmental conditions. It is usually found in combination with elements such as Sulphur and Carbon and Phosphorus to form minerals. Lead concentrations in soils range from 10 parts per million to 67 parts per million and it is one of the top five metal mined in Canada (Ihnat et al., 1996). Lead is primarily released from smelting activities as stable sulfur compounds that include $PbSO_4$, $PbO.PbSO_4$ and PbS. Lead in soils can be found in the ionic, oxides or hydroxide forms. Lead (Pb^{2+}) is the more reactive and predominantly forms ionic bonds while Pb^{4+} is more stable and forms covalent bonds with ligands. Chaney et al. (1989) reported that $PbSO_4$ is unstable and is quickly transformed into other species of Pb when spiked in soils. According to Khan and Frankland (1983), only a small proportion of lead is water soluble in soils. The solubility of the metal is highly driven by soil pH. Its solubility at low pH correlates with the sorption and solubility of iron and manganese oxyhydroxides (Agency for Toxic Substances and Disease Registry, 2007). Lead can form insoluble compounds with phosphates and carbonates in soils (Chaney et al., 1989). The toxicity of Pb to human and ecological health will be discussed in Chapters 2.5 and 2.6.

2.3.3 Nickel

Described as a strong lustrous metal with high melting (1455°C) and boiling points (2913°C), Ni is considered the 24th most abundant metal on earth (Agency for Toxic Substances and Disease Registry, 2005). The average concentration of Ni within the Earth's crust is around 86 ppm (Duke, 1990). It is found in period 4 and group 10 of the periodic table with an atomic number of 28 and exists in the environment in three main oxidation states; 0, +2 and +3. It is found in combination with iron, zinc, chromium and copper, and is a popular metal in alloys and stainless steel. Nickel is extensively mined in Southwestern Ontario and is associated particularly with environmental contamination of soils. The average concentrations in soils in the vicinity of operations are around 25 mg per kg. Nickel is primarily produced from pentlandite, nickel silicates or oxides. The metal purifying process causes the release of different species of the metal into soils. Nickel can be found as chloride, nitrate or sulphate salt that are soluble in soils. Nickel can also exist in its subsulfide or sulfide form in anthropogenic nickel deposits (Duke, 1990). Other important species are nickel carbonates, hydroxides, sulfide and ferrite. Ni hydroxides species are predominant in alkaline soils while $NiSO_4$ and $NiHPO_4$ species dominate in acidic soils. Nickel ferrite has been reported to be the solid species of the metal that is likely to precipitate in soils. Free Ni^{2+} is found in both acidic and alkaline soils. The toxicity of Ni to human and ecological health will be discussed in sections 2.5 and 2.6.

2.3.4 Copper

Copper is a reddish non-ferrous metal that naturally occurs in rocks, air soil and water in relatively low levels. It is found in period 4 and group 11 of the periodic table. It's a solid at room temperature with melting and boiling points of 1084.6^{o}C and 2560^{o}C respectively. Common oxidation states found in the environment are +1 and +2. It's been reported that the average concentration of copper in soils is 50 part per million (Agency for Toxic Substances and Disease Registry, 2004a). Copper is very useful in the manufacturing of electronics and other products due to its corrosion resistance, and electrical and thermal conducting properties. Of all metals, Copper has the best electrical conductivity apart from silver. Copper is an essential metal that is naturally found at low levels in all known plants and animals. Copper exists in soils as both primary and secondary minerals. These minerals are primarily found bound to sulphide as found in tetrahedrite, chalcocite, chalcopyrite, and bornite. Secondary forms of copper in soils include cuprite, malachite, azurite, brochantite, and antlerite (Canadian Council of Ministers for Environment, 1999). Copper has a high affinity and forms complexes with organic matter, carbonates and clay minerals that reduces the metal's concentrations likely to be found in soil solution (Wuana and Okieimen, 2014). Davis-Colley et al. (1984) confirmed the latter in an experiment that tested the adsorption of Copper to organic matter, clay, iron and manganese oxides, and aluminosilicates. They found that Copper preferentially binds to manganese-iron oxides and organic compounds compared to aluminosilicates and clay. This suggests that Cu will have a high potential for leaching out of soils with low pH and low clay and organic matter content. The toxicity of Cu to human and ecological health will be discussed in Chapters 2.5 and 2.6.

2.3.5 Cobalt

A naturally occurring magnet with the ability to retain its magnetism at temperatures (~ 1100°C), cobalt is a transition metal that primarily exists in its stable isotope ^{59}Co (Agency for Toxic Substances and Disease Registry, 2004b). It is a hard-silvery colored metal. The metal is commonly found in its divalent oxidation state but can also be found in its tetravalent state (Collins and Kinsela, 2010). It's solid at room temperature with melting and boiling points of 1945°C and 2927°C respectively. Cobalt is naturally found in most rocks and living organisms in minute concentrations primarily as cyanocobalamin (Agency for Toxic Substances and Disease Registry, 2004b). Cyanocobalamin is biochemically important in animals and essential for good health. The average concentration of cobalt in the environment is 27 ppm and 110 ppm in ultramafic rocks. It is found in combination with elements such as nickel, iron, arsenic and copper. Examples of cobalt minerals are erythrite, skutterudite, spherocobaltite and cobaltite. The metal is used in high technology industries for the manufacturing of rechargeable batteries, generators, turbines and magnets. Cobalt speciation is controlled by the presence of both organic and inorganic ligands like humic acids, chlorides, hydroxides, carbonates and sulphates. Divalent cobalt dominates in soil solution because the tetravalent state is extremely insoluble. In highly oxidized soils, Co^{2+} may be oxidized to Co^{3+} which will then precipitate unless complexed with a strong chelating organic molecule (Collins and Kinsela, 2010). The mobility of Co in soils depends on its solubility which is primarily driven by soil pH. Cobalt (Co) can form an octahedral coordination complex with six molecules of water to form $Co(H_2O)_6^{2+}$, but can undergo hydrolysis to form $Co(H_2O)_5(OH)^+$ or $Co(H_2O)_4(OH)_2$ at alkaline pH. The toxicity of Co to human and ecological health will be discussed in Chapters 2.5 and 2.6.

2.4 Soil-metal interactions

Interactions between soil and metals control the fate and transport of metals in the environment. Like most xenobiotics, metals partition into the liquid, solid and gaseous phases in soils (McLean and Bledsoe, 1992). Specific kinetics control the partitioning of metals between the solid and aqueous phases and in turn determines metal availability in soils (Baker, 2008). The degree of metal mobility is influenced by desorption and sorption reactions. Apart from differences in metal properties, soil properties like pH, percent clay, percent organic carbon (OC), cation exchange capacity (CEC), reduction-oxidation (redox) potential and the concentration of base cations and anions control the rate of metal reactions in soil (Tatara et al., 1998). According to Shuman (1991), metals in soil can either exist as free metals in solution, can be exchangeably held or adsorbed onto inorganic soil constituents, complexed with insoluble organic matter, precipitate as oxides, or exist in the structure of both primary and secondary minerals. Furthermore, the existence of metals in primary or secondary minerals is unlikely to result from the anthropogenic contamination of soils. When considering fate and mobility of metals, the aqueous, labile and exchangeable fractions are of primary importance (Elikem et al., 2019; Laird et al., 2011). The concentration of metals in the aqueous phase is the most reactive, and the metals can be transferred into groundwater through leaching, taken up by plants or soils organisms, or can be chemically immobilized in the soil. The ability to predict metal behavior in soils requires an in-depth understanding of the major factors governing metal speciation in soils (i.e. soil properties) (McLean and Bledsoe, 1992).

2.4.1 Role of soil properties

Soil pH is a major driver of speciation, mobility and fate of heavy metals in the environment (Alamgir, 2016; McLean and Bledsoe, 1992; Rieuwerts et al., 1998b). The adsorption of cations to ligands and nucleophilic sites is generally pH-dependent because the quantity of electrical charges on the surface of soil colloids is controlled by the pH of the soil solution (Alamgir, 2016). The negativity of the colloidal surfaces increases with pH, which increases the adsorption of cations in soils (McLean and Bledsoe, 1992). The relationship between soil pH and adsorption is influenced by proton and Al^{3+} ions concentrations particularly at low pH (Figure 2-1). The increase in the concentration of protium/ Al^{3+} also affects the intensity of heavy metal mobilization in soils especially at high heavy metal concentrations. Protium competes with cations for sorption sites at low pH, resulting in a decrease in cation retention in soils. Acidic soils favor the mobilization of metals compared to neutral or alkaline soils. Harter's (1983) experiment on the adsorption of four metals (Pb, Cu, Ni, Zn) in two soils across an adjusted pH range showed an increase in cation adsorption with pH. The mobility of metals differs in acidic soils and reduces in the order of Cd>Ni>Zn>Mn>Cu>Pb (Fijalkowski et al., 2012). In alkaline or neutral soils complexation and immobilization of heavy metals become important drivers of metal transport. Hence, the adsorption of metal cations largely increases with pH as shown by Rieuwerts et al. (1998b) in Figure 2-1. Some authors have reported that the hydrolyzed concentration of metals increase with pH and are preferentially adsorbed compared to free metal ions (Elliott et al., 1986a, 1986b). The differences in pH-dependent hydrolysis explains the differences in adsorption between Cu at pH 6 and Cd at pH 8 (McLean and Bledsoe, 1992).

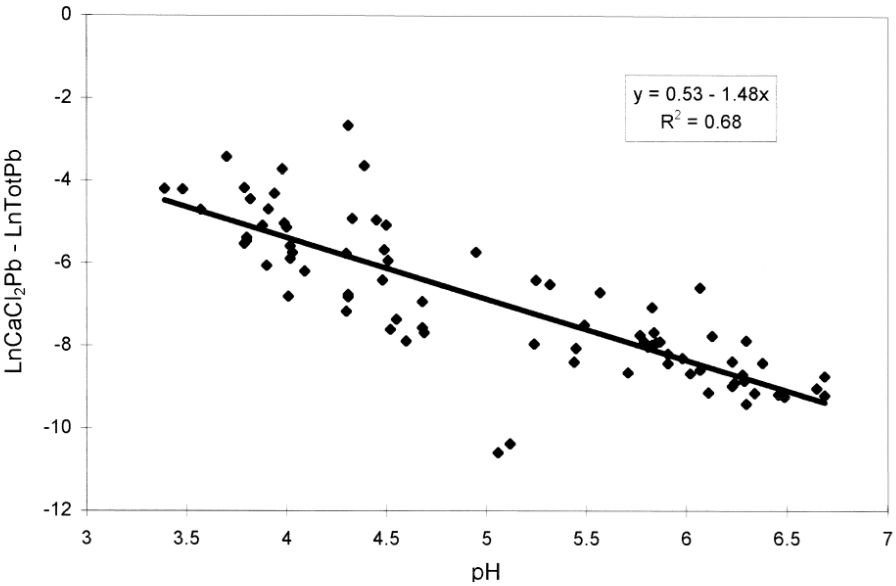

Figure 2-1. Graph showing decrease in metal (Pb) solubility with increasing soil pH. X-axis is soil pH and y-axis is the difference between the base log of total Pb concentration and the base log of 0.01 M $CaCl_2$ extracted Pb. Soils were collected from North Wales, UK (modified from Rieuwerts et al., 1998b)**.**

Soil organic matter (SOM) content is a combination of plant and animal residues undergoing decomposition in the soil. The decomposition of soil organic matter produces low molecular weight organic acids, stable and insoluble humic substances (Jackson et al., 2015). Humic substances include humic acids that are soluble at alkaline and fulvic acids that are soluble at all pH values. Alloway (1990) reported that the adsorption capacity of SOM is high at pH 5 and above. Metals have high affinity for SOM and tend to sorb or form strong complexes. An increase in soil organic matter content causes a decrease in the amount of available metals that might potentially cause toxicity to plants and soil organisms (Rieuwerts et al., 1998). The effect of SOM on metal availability depends on the quantity and quality of the SOM (Barančíková and Makovníková, 2003). It’s been reported that metal binding affinity to humic acids increased with increasing amounts of nonprotonated aromatic carbon (Preston, 1996). The high surface area to volume ratio increases the reactivity of SOM. SOM also contains sulfhydryl, carboxylic and amine functional groups with soft Lewis base characteristics (Jackson et al., 2015). An increase in the amount of SOM in soil solution will cause a decrease in the availability of the dissolved fractions of metal cations in soil solution. SOM is a major contribution to the water holding capacity (WHC) and cation exchange capacity (CEC) in soils.

Soil texture is an important factor that regulates heavy metal mobility in soils. Texture is defined as the relative proportion of sand, silt and clay. Clay consists of a large group of minerals that mainly contain silica, iron and alumina oxides (Uddin, 2017). Clay materials have particle sizes below 2 µm and belong to a family of minerals with similar chemical compositions and common crystal structural characteristics (Velde, 1995). The main types of clay minerals are iron and aluminum oxides, layer silicates, amorphous and allophones, and clay-humus particles (Uddin, 2017). The capacity of clays to adsorb heavy metals is highly dependent on the clay type (Singh et al., 2010). For example, kaolinite is a 1:1 clay mineral that consists of one octahedral aluminum sheet and one tetrahedral silica sheet (Barton and Karathanasis, 2002). The sheets are held together by van der Waals forces and hydroxyls of the octahedral sheet. Specific characteristics of kaolinite include high swelling, relatively large particle size (0.1-0.5 µm), and low cation exchange capacity (Barton and Karathanasis, 2002). On the other hand, smectite is a 2:1 clay mineral that consists of one aluminum octahedral sheet and two silica tetrahedral sheets also held together by van der Waals forces. Smectites are smaller in size (0.001-0.1 µm) and have a high cation exchange capacity (Uddin, 2017). The size and high CEC of smectite clays makes it a better heavy metal adsorbent compared kaolinite and other clay families (Singh et al., 2010). Furthermore, soil colloids that contain clay particles below 1 µm are considered the most reactive portion of clays. The net negative charge on clay particles explains their high sorption ability and affinity for metals. This explains the differences in sorption capacities and nutrient retention between sandy and clayey soils.

The cation exchange capacity (CEC) of soils is defined as the sum of the available negatively charged sites to which cations or positively charged ions can sorb. It is expressed as centimoles per kilogram soil. Soil CEC is dependent on major soil properties such as soil organic matter, percent clay, iron and manganese oxyhydroxides, and soil pH. Many studies have interestingly shown that percent clay, percent organic carbon and soil pH can predict CEC of soils (R^2=0.90) (Curtin and Rostad, 1997; Saidi, 2012). Organic matter alone can contribute greater than 200 meq per 100 g in surface (~30 cm) mineral soils. Mclean and Bledsoe (1992) reported that organic matter is the major contribution to CEC in surface mineral soils while the mineral constituents become more important as organic matter content decreases especially with depth. The higher the CEC, the more sorption sites available for cations. In Table 2-1, I show a list of properties for the major metals under study (Cu, Ni, Pb, Co, Zn) that control metal binding and availability in the environment.

Table 2-1. Selected metals and their properties

Metal	pKa	Radius (Å) r	Charge Z	Electronegativity X	Redox Potential Eh	Covalent Binding Index (Xr)	Ionic Binding Index (Z^2/r)
Cobalt	9.85	2.008	2	1.91	-0.28	3.83528	1.9920
Copper	8.0	1.865	2	1.65	0.337	3.07725	2.1447
Lead	7.8	2.36	2	2	-0.126	4.72	1.6949
Nickel	9.86	1.934	2	1.9	-0.25	3.6746	2.0683
Zinc	8.96	1.8	2	1.81	-0.7618	3.258	2.2222

(Ghosh, 2002; Haynes et al., 2016; Kortum et al., 1961; Perrin, 1965)

The oxidation-reduction (redox) potential of the soil measures the availability of electrons in the soil and is reported in millivolts as electrochemical energy. Redox reactions in soil are an important regulatory factor in the mobility and availability of heavy metals (McLean and Bledsoe, 1992). Some metals can exist in different oxidation states depending on environmental factors and the redox potential of the soil. For example, in highly oxidized conditions, Fe is oxidized to Fe (III) while Mn is oxidized to Mn (III/IV) causing them to form complexes with other metals and thereby reducing their availability (Roberts et al., 2005). The mobility of metals like Iron (Fe) and Manganese (Mn) is highly influenced by the redox state of soils. In the appropriate conditions, metals can gain electrons and be reduced or lose electrons and become oxidized. Measuring the redox potential of the soil can give an indication or help predict the valence state of metals in the soil. The redox potential of oxidized soils average around +500 mV while reduced soils average around -250 mV (Roberts et al., 2005). Factors that cause reduced conditions include flooding of oxidized soils (Alamgir, 2016). Flooding results in decreased transportation of oxygen which causes reduction and deoxygenation of hydrolyzed metals increasing their mobility.

2.4.2 Metal speciation

Metal speciation is a broad term used to denote the chemical form of metals in soils and consists of the solid, liquid and gaseous phases. Metals in the liquid and gaseous phases are supplied by the solid phase to establish chemical equilibrium (Fijalkowski et al., 2012; McLean and Bledsoe, 1992; Roberts et al., 2005). The concentration of metals in all three phases are determined by processes that include, adsorption-desorption, dissolution-precipitation, reduction-oxidation, occlusion-sequestration, migration-diffusion, immobilization-mobilization by soil organisms, and metal competition (Alamgir, 2016; McLean and Bledsoe, 1992). These processes result in metals undergoing several transformations due to interactions among chemical, biological and physical components of the soil. Metals tend to exist as free ions in solution, form complexes with either organic or inorganic ligands, sorb to soil surfaces, and precipitate as oxides, hydroxides or carbonates. Sorption kinetics and dissolution-precipitation reactions determining metal partitioning between the different phases while redox and metal complexation control the solubility and bioaccessibility of metals (Chaney et al., 1989; Rieuwerts et al., 1998).

2.4.3 Bioaccessibility and bioavailability

Understanding the factors that drive the bioavailability of heavy metals in soil is important in toxicity estimation and risk assessment. Bioavailability is even more important when heavy metals exist as mixtures in soils and individual concentrations are below regulatory limits. The incorporation of bioavailability in assessing the risk of metal mixtures has proven difficult because of the protective role of metals like Zn in mixtures, hence, their bioavailability might not necessarily mean toxicity (Versieren et al., 2017). The bioavailable fraction as defined by toxicologists is the proportion of a chemical that reaches the systemic circulation in an unchanged form (Semple et al., 2004). Bioaccessibility in soils is defined as the concentration of free metals that can be mobilized from soil and potentially taken up by soil organisms (Semple et al., 2004). When modeling the toxicity of heavy metals to soil organisms, both schools of thought need to be considered in order to more accurately predict the actual concentrations that interact with biological targets to cause the measured apical effects. As previously discussed, bioavailability of metal ions in soils is controlled by pH, cation exchange capacity (CEC), organic matter, and clay, although pH is the main determinant of speciation (Roberts et al., 2005).

Due to the high influence of soil properties on metal bioavailability and the wide variation in soil properties, appropriate models have been developed to predict the bioavailability and toxicity of single metals and metal mixtures to soil organisms (Gopalapillai et al., 2018; Tipping, 1994; Tipping et al., 2011). These equilibrium-based models predict toxicity by determining the speciation and the concentration of free metal ions that can potentially bind and interact with biotic ligands (Tipping, 1994; Tipping et al., 2011). The free ion activity and biotic ligand models (BLM) will be briefly discussed while highlighting their pros and cons.

2.4.3.1 Free ion activity and biotic ligand models

There has been massive development and improvements in our understanding of metal speciation and the predictive power of toxicity models in both aquatic and terrestrial ecosystems (Cedergreen, 2014; Farley et al., 2015; Nys et al., 2018; Tipping et al., 2011). Earlier studies conducted to determine the toxicity of metals identified that the effect might be related to a fraction of the total metal concentrations, speciation and chemical complexation (Black, 1973; Vuceta, 1979). Further research introduced the importance of speciation of metals, and suggested that metal speciation and not total concentrations needed to be considered in order to fully understand toxicity to organisms (Martyn, 1988; Vuceta, 1979). A study by Sunda and Guillard (1975) confirmed the importance of speciation when they related the toxicity of Cu to algae by a measure of free ion activity using a Cu selective electrode and observed better correlations with toxicity. In defining the bioavailable portion which was assumed to be the dissolved concentration of metals in solution, the pore water hypothesis was proposed and suggested that the uptake of metals is mediated by pore water concentrations (Crommentuijn et al., 1997; Van Gestel, 1997). Robert et al. (2005) also reported that soil aqueous phase determined the soils reactivity, nevertheless, the solid phase contains majority of the metals in soils and supplies them accordingly.

The free ion activity model (FIAM) has been widely used in elucidating the uptake of nutrients and metals by cells (Hare and Tesier, 1996; Parker and Pedler, 1997). The FIAM describes metal-organism interactions that determine the toxicity of metals and assumes that the primary form of metal uptake is the free fraction in pore water. Furthermore, FIAM assumes that the plasma membrane is the primary metal interactive site in cells and the biological response is strictly dependent on the concentration of the metal bound to the cell surface. This model further explains that the concentration of metals bound to a surface is proportional to the free ions in solution and not the ligand concentration, which is only true when the speciation of the metal is not altered by the introduction of a ligand (Hare and Tesier, 1996). The FIAM has been successfully used in predicting toxicity of Cd to gram negative soil bacteria and also the inhibition of nitrification by Cd and Ni in a lab bioreactor (Hu et al., 2002; Slaveykova et al., 2009). A major weakness of the model is its failure to fully account for metal-metal competitions and metal ligand complexes. This was confirmed in a study by Ytreberg et al. (2011) where FIAM failed to predict copper accumulation to *Ceramium tenuicorne*. Another study has reported wide deviations from FIAM predictions because of the addition of chelators (Zitko et al., 1973). Metal-metal competitions and absorption of complexed metal forms can possibly explain the modification of toxicity and the weak prediction from FIAM. For example, it was discovered by Moberley et al. (2010) that metal complexes may enter cells through diffusion and transportation by endogenous anion transporters.

The biotic ligand model (BLM) was initially developed to predict acute and chronic toxicity to aquatic organisms and was coined from both the gill interaction model and the FIAM (Stefaniak, 2007). The main difference between BLM and FIAM is that, the former includes competition and interaction among metals, protons and essential cations such as Ca and Mg (Qiu et al., 2013; Steenbergen et al., 2005). Furthermore, the bioavailability of a metal is dependent on the concentrations of Ca, Mg and other protons due to competition for ligands (Srivastava et al., 2010). Also, high organic matter content in soils has been linked to lower toxicity of the metals to the mite *Oppia nitens* (Jegede et al., 2019). The toxicity is dependent, however, on the affinity of the metal ion to the organic ligand. The characteristicx of metal ions have been used to predict metal toxicity with success using the properties like the log of the first hydrolysis constant, charge and radius (Tatara et al., 1998). The BLM also accounts for metal complexes with inorganic ligands and humic substances and has been widely accepted following extensive validation (Thakali et al., 2006).

The BLM has been successfully applied to soils in predicting toxicity of metals to *Folsomia candida*, *Aporrectodea caliginosa* and microbes (Slaveykova and Wilkinson, 2005; Steenbergen et al., 2005; Van Gestel and Koolhaas, 2004). However, a study by Ponizosky et al. (2006) pointed out that when the moisture content of soils is below their maximum water holding capacity the pore water concentration is insignificant. To account for the differences in soil properties, Thakali et al. (2006) applied some modifications to the BLM, which he named the terrestrial BLM (TBLM). They applied the TBLM to a study using non-calcareous soils amended with Cu and showed that Cu ions significantly adsorb to soil organic matter. Their results agreed with the modeling results from Windermere Humic Aqueous Model (WHAM). It has hence been accepted as a model that sufficiently predicts bioavailability of single metals across different soils and suitable for risk assessments (Stefaniak, 2007).

2.5 Ecological Health

Heavy metals found in contaminated soils in Canada that are of potential concern include lead, copper, nickel, zinc and cobalt. Metal concentrations in soil pose a wide range of threats to plants and soil organisms. Concentrations of Pb as low as 1 ppm affect 16S and nirK genes (Sobolev and Begonia, 2008). Metals at toxic concentrations generally result in the deactivation of enzymes and cause damage to microbial cells by acting as antimetabolites, forming precipitates or chelating essential metabolites (Sobolev and Begonia, 2008). Soil enzymes such as ammonia monooxygenases and beta glucosidases that are involved in biogeochemical cycles are sensitive and inhibited by metals in soils (Ruyters et al., 2013; Wang et al., 2018). Despite the differences in toxicokinetics and sensitivity of enzymes to metals, substantial effects on enzymes can occur even at sub-lethal concentrations, that can indirectly affect ecological health (Hayes et al., 2018). The impacts of metals on soil enzymes can potentially affect nutrient uptake by plants, because, some plants secrete enzymes (e.g. acid phosphatases) to enhance the solubilization and mobilization of nutrients (e.g. phosphorus) during stressed conditions(Das and Varma, 2011). Apart from the direct effects of metals on plant exoenzymes, metals such as lead are easily absorbed and accumulated in different parts of plants. The mode of action of these metals in plants is through the inhibition a number of key processes in plants including photosynthesis, osmotic balance, hormonal balance, and cell integrity (Sharma and Dubey 2005). Metals like lead and zinc induce the generation of reactive oxygen species that overwhelm the detoxifying enzymes in plants and cause toxicity. Manifestation of lead toxicity in plants include reduction in growth, darkening of the root system and chlorosis (Sharma and Dubey 2005).

Metals accumulated in plants are found in cell walls, vacuoles, and vascular bundles (Etim, 2012). These metals are localized in their salts form or complexed with proteins and carbohydrates (Göhre and Paszkowski, 2006). Continuous accumulation of metals in plant parts cause increases in concentrations that may be toxic to plants (Gamalero et al., 2009). Hyperaccumulators have developed specific metal resistant and detoxifying mechanisms (Göhre and Paszkowski, 2006). Some metals are detoxified in the cytosol by amino or organic acids produced by the plants. Phytochelins and metallothioneins are two cysteine-rich detoxifying proteins that have high affinities for metals. Salt et. al (1995) reiterated that the detoxicification of Cd is achieved by complexation with phytochelatins, while Cu forms complexes with metallothionein and other similar compounds (Salt et al., 1995). In an experiment where

Brassica juncea was exposed to high Pb concentrations, phytochelatins were produced in roots, which suggests that as a detoxifying mechanism for Pb (Salt et. al, 1995). Furthermore, the Zn accumulator *Thlaspi caerulescens* precipitates the metal into Zn-phytate. In the detoxicification of Ni, *Alyssum lesbiacum* releases dose-dependent amounts of histidine that forms stable complexes with Ni (Verbruggen, 2009).

2.6 Human health

Elevated concentration of metals can cause a wide range of toxicological effects to humans. Effects range from subtle symptoms like skin irritation or nausea, to tumor and cancer and sometimes death (US Environmental Protection Agency, 2007). Some metals are known to target specific organs while others exhibit a wide range of targets. Humans are exposed to metals in contaminated soils through direct skin contact with contaminated soil and by the ingestion and inhalation of dust. Indirect routes of exposure include accumulation in ingested plants and ground or surface waters that have elevated metal concentrations. The route of exposure is important because it can be used to predict the toxicodynamics and toxicokinetics of the metal.

The route of exposure for lead is less important because its effect on the target organ; the nervous system is not altered by differences in exposure routes (Agency for Toxic Substances and Disease Registry, 2007). Lead concentrations in the body after exposure has been shown to accumulate in bones; 95% for adults and 70% for children (Agency for Toxic Substances and Disease Registry, 2007). The absorption of lead in the gastrointestinal tract is controlled by a person’s diet and the role of phosphate, calcium and iron as lead reducers (Agency for Toxic Substances and Disease Registry, 2007). Studies showed that the cognitive performance of adults that were exposed to lead were decreased (Dietrich et al., 1993, 1987). Exposure to lead also caused high blood pressure and weakness in joints. Lead causes hypertension, anemia and miscarriage in pregnant women (Borja-Aburto et al., 1999; Rabinowitz et al., 1987). In high concentrations, the metal causes death in children. Even though lead is not classified as a carcinogen, it has been reported to cause tumor in the kidneys of rats and mice (IARC, 2006).

Zinc is a micronutrient that is required for metalloenzymes and for a variety of functions that include cell division and growth, metabolism of proteins and maintenance and function of membranes in the animals and humans (Agency for Toxic Substances and Disease Registry, 2013). Metalloenzymes are enzymes with metal cofactors and include deoxyribonucleic acid polymerase, ribonucleic acid polymerase, superoxide dismutase, alcohol dehydrogenase, carbonic anhydrase, and leucine aminopeptidase (McCall et al., 2000; Vallee and Williams, 1968). Hence, the toxicity of Zinc does not only occur at high doses but also at concentrations below that required for normal body functions. There are no sensitive indicators for marginal zinc deficiency in humans but some research has shown signs of impairment that include both physical and neuropsychological abnormal developments in children (Hambidge, 2000). Zinc deficiency has also been linked with attention deficits and disorders in motor neurons in infants that can persist into adulthood (Hambidge, 2000). Prasad (2012) reported stunted growth and development in humans with severe Zinc deficiency, a finding that was derived from patients with acrodermatitis enteropathica, a genetic condition that results in the impaired uptake and transport of zinc. Exposure to high concentrations of Zinc can cause toxicity to a wide range of organs. Zinc can affect the reproductive and nervous systems. The metal exhibits immunological, systematic, genotoxic and carcinogenic effects (Agency for Toxic Substances and Disease Registry, 2013).

The essentiality of Nickel to humans is debatable, but studies have reported that the metal is needed in concentrations below 35 µg per day by humans (Anke et al., 1995). It is believed that Nickel deficiency affects the metabolism of calcium, iron and vitamin B12 (Dwivedi et al., 2015). Symptoms of the deficiency includes dysfunction of the liver, hormonal imbalance and anomalies in bone growth. High concentrations of Nickel affect the skin, respiratory tracts, immune and reproductive systems (Agency for Toxic Substances and Disease Registry, 2005). Dermatitis is frequently encountered from Nickel exposure through skin contact from nickel-containing jewelry and prosthetics or contaminated soil (Nielsen et al., 1999).

Copper is an essential component of several metalloenzymes that are required for normal body functions in humans (Vallee and Williams, 1968). Copper dependent metalloenzymes include super oxide dismutase, monoamine oxidase, cytochrome c oxidase, and ferroxidases (Cobine et al., 2006). These metalloenzymes are involved in metabolic reactions that control the metabolism of xenobiotics and carbohydrates, hemoglobin synthesis and defense against antioxidants. In Canada, the estimated daily intake of the metal in children and adults is up to 66 µg kg $^{-1}$ per body weight and 74 µg kg $^{-1}$ per body weight respectively (CCME, 2004). The absorption of copper occurs in the stomach and intestines. The synthesis of the metal binding protein metallothionein is activated when absorbed Copper concentrations are above that required by the body (Cobine et al., 2006). Metallothionein binds, stores, transports and aids in the excretion of copper and other metals (Hamer, 1986). Overwhelming concentrations of copper can cause damage to the liver and kidney, and cause toxicity to development and the immune system (Agency for Toxic Substances and Disease Registry, 2004a). Copper’s mode of action is by binding to cysteine rich macromolecules, creating reactive oxygen species and interfering with cellular protection (Wuana and Okieimen, 2014).

Cobalt is needed in minute concentrations by humans for nutrition and growth, making it an essential metal (Agency for Toxic Substances and Disease Registry, 2004b). The estimated daily consumption of cobalt from diet in the general human population is between 5-40 µg (Agency for Toxic Substances and Disease Registry, 2004b). Cobalt is a component of cobalamin (vitamin B12) and is involved in the synthesis of DNA, and the metabolism of amino and fatty acids (Andrès et al., 2004). The highest concentrations of cobalt in the body are found in the liver. Exposure to high concentration of Cobalt can affect the nervous and respiratory systems through ingestion and inhalation respectively (Agency for Toxic Substances and Disease Registry, 2004b; Leyssens et al., 2017). Humans that were occupationally exposed to cobalt metal (0.015–0.13 mg Co/m^3) developed health effects that included asthma, lung diseases, dyspnea, and decreased pulmonary function (Leyssens et al., 2017).

2.7 Metal toxicity and mixture modeling

Metals and metallic compounds are potentially toxic to all biological organisms including humans (Dietrich et al., 1993; Jegede et al., 2019; Qiu et al., 2016). Even though the toxicity of metals depends on the concentration, speciation is of critical importance when estimating their toxicity (Nys et al., 2017; Tipping, 1994). Metals in their solid state (zero valence) are insoluble compared to metals in their free or ionic state. This plays a significant role in the bioavailability and reactivity of the metal, and subsequent toxicity. Metals typically exist in environmental media as a mixture of metals (Farley and Meyer, 2015; Meyer et al., 2015). The toxicity of metal mixtures is very complex due to a variety of reasons. First, the metals comprising the mixture might or might not interact (Ross and Warne, 1997; Warne and Hawker, 1995). If they do not interact, the toxicity of the mixture could be estimated from their individual concentrations or responses. However, if interactions occur between metals in a mixture, it could result in lesser or stronger effects compared to their individual effects as usually observed from the traditional mixture models (i.e. concentration addition and response addition models).

2.7.1 Modeling metal mixture toxicity

2.7.1.1 Concentration addition

The German pharmacologist Loewe originally articulated this in 1926 for a mixture with non-interactive components that simply act similarly in their effects. For a mixture of n chemicals, it can be arithmetically illustrated as:

$$\sum TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1 \qquad \textit{Equation 2-1}$$

Where c_i is the concentration of chemical i in the mixture containing n components exhibiting an effect x% and ECx_i is the concentration of i that exhibits same x% effect when singly applied. The quotient of c_i and ECx_i is referred to as the toxic unit (TU) which represents the fractional potency contributed by each component in the mixture. If the TU's of all the components in the mixture add up to 1 at a mixture concentration provoking x%, then the CA model holds. On the other hand, if it's less or greater than 1, then it is either antagonistic or synergistic respectively. Following the assumptions of CA, one component of the mixture can be replaced with an equitoxic (same TU) chemical with same mode of action without altering the overall effect.

2.7.1.2 Response addition

The response addition concept is based on dissimilarly acting components in a mixture, hence it is expressed mathematically as a product of the probability of nonresponse, thus:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \qquad \textit{Equation 2-2}$$

Where $E(c_{mix})$ is the proportional effect of the total mixture at a particular concentration

$$c_{Mix} = \sum_{i=1}^{n} c_i \qquad \textit{Equation 2-3}$$

and $E(c_i)$ is the proportional effect of the individual components when applied singly at the same concentrations present in the mixture and c_{Mix} is the sum of concentration of the components in the mixture (Van Gestel et al., 2016). Equation 2.4 can be rewritten as:

$$E_{activity} = Max_{activity} \prod_{i=1}^{n} q_i(c_i) \qquad \textit{Equation 2-4}$$

Where $E_{activity}$ represents the observed enzyme activity while $Max_{activity}$ represents the enzyme activity in control soils and $q_i(c_i)$ replaces $E(c_i)$ as in Equation 2.4 as the the proportional effect of the individual components when applied singly at the same concentrations present in the mixture.

2.8 Human health and ecological risk assessment

Risk is defined as the likelihood of an event or substance to cause harm, coupled with its ability to be severe (Canadian Standards Association, 1997). Mathematically, it is a function of exposure and hazard. Hazard is when a substance has the potential to cause harm, injury or damage (Schierow, 2002). The assessment of risks associated with contaminants is conducted to protect either the lives of human beings (i.e. human health risk assessment) or population of organisms and ecological integrity (Golder Associates, 2013). Both forms of assessments follow similar structures (Figure 2-2) and use similar models to inform the characterization of risks associated with environmental concentrations of xenobiotics. The framework for estimating risks to humans and ecology following exposure to chemicals is described in detail below.

2.8.1 The risk assessment process

As defined by the United States Environmental Protection Agency (USEPA), risk assessment is a process that is used to evaluate the nature and probability of adverse effects on humans health and ecology upon exposure to chemicals through contaminated environmental media now or in the future (Department for environment food and rural affairs, 2011; United States

Environmental Protection Agency, 1986). The approaches applied in RA have developed progressively over the last few decades. The framework is divided into four (4) main steps namely: the problem formulation step, the hazard identification step, the exposure and dose-response assessment steps, and the risk characterization step.

The various steps are structured to answer simple questions like;

- Problem Formulation Step: This is the planning stage where basic questions like what the problem is, who are being exposed, how are people or organisms being exposed are answered.
- Hazard Identification Step: At this stage, the most important question is defining what level of exposure is safe.
- Exposure Assessment & Dose-Response Assessment Step: This answer questions such as the exposure level, the duration of exposure, frequency of exposure. The dose-response assessment step quantifies the relationship between effects and degree of exposure.
- Risk Characterization Step: This step answers how exposure compares to what is deemed safe.

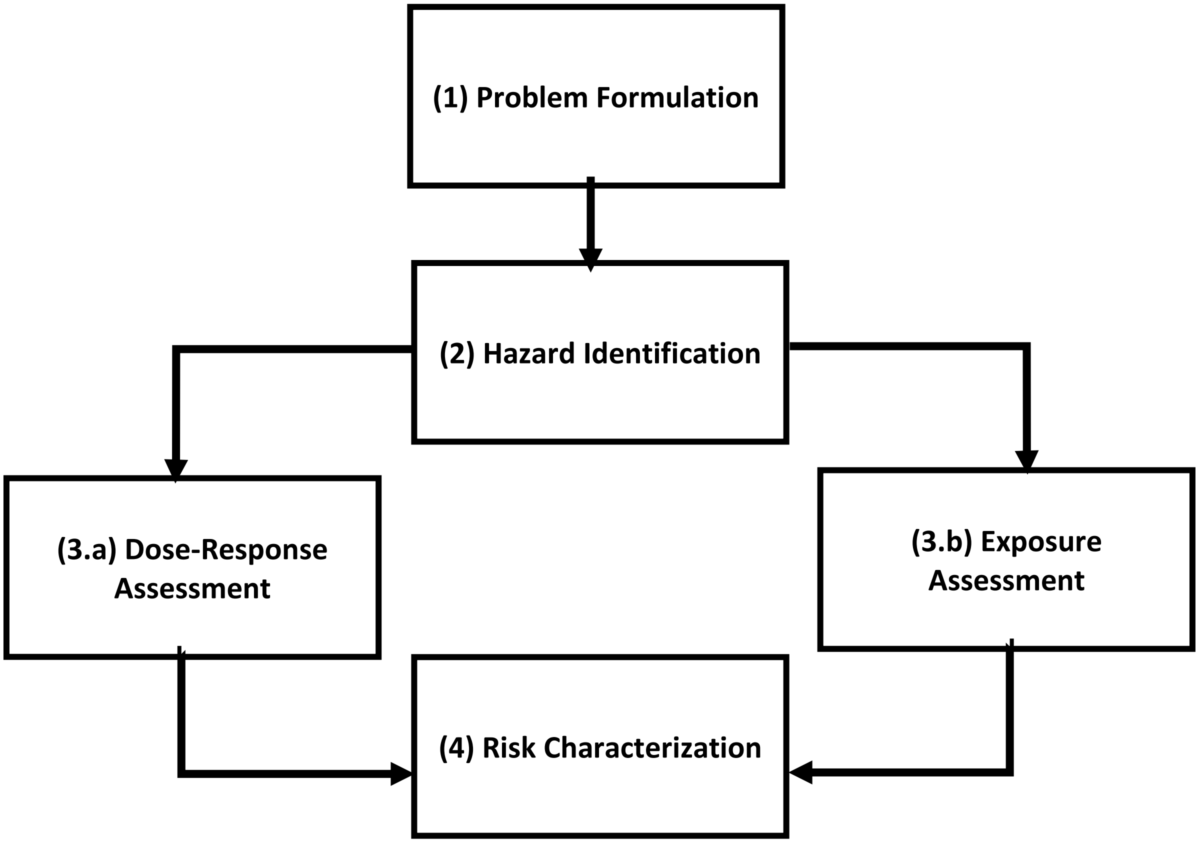

Figure 2-2. A flow diagram of the risk assessment framework. Modified from Canadian Council of Ministers for the Environment (Canadian Council of Ministers for Environment, 1997), United States Environmental Protection Agency (United States Environmental Protection Agency, 1998) and Department for Environment, Food and Rural Affairs (Department for environment food and rural affairs, 2011).

2.8.1.1 Conducting a Risk Assessment

Once concerns are raised about a potential chemical hazard, the source of the hazard is determined, potential receptors are identified, and concentrations are measured or estimated. Risk assessment exists in a tiered approach (Tier 1, Tier 2, Tier 3), and all four steps are part of each tier. Overall, the complexity, costs, amount of data, and characterization of sites, receptors, and contaminants of the assessment increases from Tier 1 to Tier 3. The following provides an overview of the steps involved in risk assessment.

2.8.1.2 Problem Formulation

The problem formulation stage involves identifying chemicals of potential concern (COPC). The maximum estimated or measured concentrations of chemicals are compared to the allowable concentrations from guidelines. Regulatory limits/values of most chemicals in environmental media (soil, air, water, food) are available in guidelines that can be found on government websites (Canadian Water Quality Guidelines, Canadian Soil Quality Guidelines, Canadian Food and Drugs Act, USEPA, WHO, IPCS). The contaminant of potential concern undergoes different levels of screening to determine if the risk assessment process should proceed (Canadian Council of Ministers for Environment, 1997). Three main steps are used in the contaminant screening stage;

1. If the concentration of the chemical exceeds the maximum regulatory allowable concentration in the media, then the chemical is of potential concern and vice versa.
2. If the concentration of the chemical exceeds normal or natural background levels especially for inorganics, the chemical is considered a COPC and vice versa.
3. If a chemical has no guideline and considered nocuous but exceeds background or has no background information, then it is considered a COPC.

2.8.1.2.1 Receptor Identification

After identification of COPC’s, potential receptors such as employees, community member and general members of the public are identified. There is a variation in sensitivity of receptors to the COPCs. Health Canada defines five age classes that can be used in identifying the most sensitive receptors based on the nature of the COPC. These classes are;

a. Infants 0-6 months
b. Toddlers 7 months-4 years
c. Child 5 years-11 years

d. Teen 12years-19 years
e. Adult 20 years and over

For situations of soil contamination, identification of sensitive population is further divided based on land use (Canadian Council of Ministers for Environment, 1997). The guidelines enumerate four different land uses and their sensitive receptors with respect to age. Generally, toddlers are considered the most sensitive population due to their large surface area to volume ratio (SAVR) compared to adults.

- Agricultural Land-use: - Toddlers are designated as the most sensitive population due to their pica behavior. It is estimated that toddlers ingest 80 mg of soil in a day while adults ingest 20 mg. If COPCs end up in agricultural produce toddlers will be the ones most at risk. Time of exposure is calculated as 24 hours per day.
- Residential Land-use: - Toddlers are designated as the most sensitive in for similar reasons as outlined above. Time of exposure is calculated as 24 hours per day.
- Commercial Land-use: - Toddlers are designated as the most sensitive for similar reasons as outlined above. Time of exposure is calculated as less 24 hours per day.
- Industrial Land-use: - Adults are designated as the most sensitive population for industrial land-use due to their exposure during work shifts calculated as 8-hour per day and 40-hour per week of exposure.

Receptors can also be identified based on the mode of action, toxicokinetic or toxicodynamic of the COPC (Canadian Council of Ministers for Environment, 1997). Adults are the most sensitive population if the COPC is a known carcinogen, infant and women of child-bearing ages are the most sensitive population if the COPC is a known neurotoxin. Other sensitive populations are also identified on the merit of specific lifestyles. For example, if the COPC is a persistent pollutant that bioaccumulates and biomagnifies, fishing communities and eaters are considered the most sensitive population. If the COPC is in a consumer product, users of the products are designated as the most sensitive population.

2.8.1.2.2 Exposure Pathways

After identification of the sensitive populations, exposure pathways and routes of exposure to receptors need to be defined. Three main routes of exposure have been identified;

- Inhalation: - Of ambient air contaminated with vapors or PM
- Ingestion: - Contaminated soil or dust
- Dermal absorption: - Contaminated soil or water

2.8.1.2.3 Conceptual Model

A conceptual model is developed after identifying COPCs, receptors, and pathways of exposure. The model gives a visual representation of the COPC's origin, release and medium of transfer to the receptor that could result in potential adverse effects. Below is an example of a conceptual model developed for lead (Pb) as a COPC in contaminated soil. The model incorporates the media of exposure for Pb and the exposure pathways that define possible doses associated with each pathway.

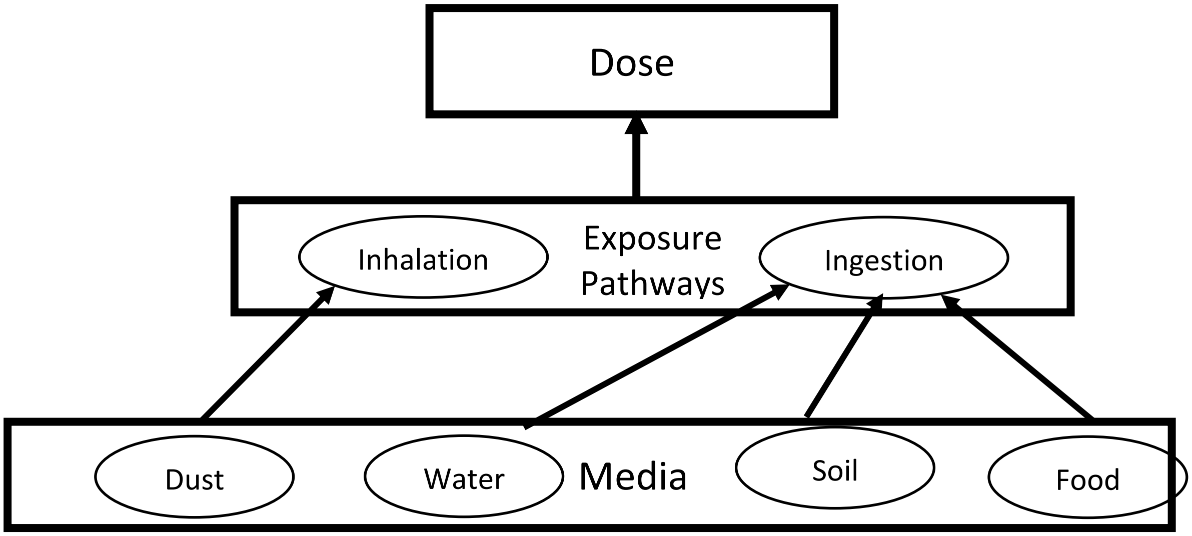

Figure 2-3. A conceptual model of Pb exposure from soil contamination, and how the fate and transport of Pb contaminates groundwater and food for human health risk assessment.

2.8.1.3 Hazard Identification

This is the process of describing the likely increase in the incidence of a particular adverse effect (or important endpoints) upon exposure to the COPC (United States Environmental Protection Agency, 1998). Adverse effects could include cancer, birth defects, other diseases. Mode of action and the absorption, distribution, metabolism, and excretion of the COPC are employed because the cardinal objective is to find a relationship between the COPC and the biological response or adverse effect. Information collected from the Problem formulation step is combined with weight of evidence identified in publications and other sources of data on the COPC to establish this link.

2.8.1.4 Exposure Assessment

This step is done to evaluate how receptors are being exposed, how much they are being exposed to, and the duration of exposure (Means, 1989). Exposure can either be speculated (predicted) or measured (empirical). The estimated environmental concentrations (EEC) and reasonable maximum exposure are determined for each chemical of potential concern (COPC). Concentrations of COPC's measured within 20 cm of the soil surface are used in determining EEC's (United States Environmental Protection Agency, 1998). Toxic or potency equivalency factor is an approach that compares the toxicity of COPCs with the same mode of action and expresses the toxicity relative to the most potent compound (United States Environmental Protection Agency, 1986). It can be used when dealing with multiple contaminants only if they have the same modes of action and are additive (e.g. dioxin-like compounds) (United States Environmental Protection Agency, 2010).

Exposure is calculated as a function of ingestion or inhalation rate (IR), estimated duration (ED), exposed concentration (C) or EEC, body weight (BW) and average time (AT). The reference dose (RfD) or the tolerable daily intake (TDI) from guidelines are compared to the estimated daily intake (EDI) to estimate risks (Means, 1989). The duration used in calculating exposure is different for threshold and non-threshold contaminants. For threshold contaminants, exposure is averaged across the length of exposure, but for non-threshold contaminants, exposure is averaged over a lifetime (LT). As a result, adults are the most sensitive population for a possible carcinogen while children are the most sensitive population for non-carcinogens.

$$Exposure\ for\ non-carcinogens\ (EDI) = \frac{[C]*IR*ED}{BW*AT} \qquad Equation\ 2\text{-}5$$

$$Exposure\ for\ carcinogens\ (EDI) = \frac{[C]*IR*ED}{BW*LT} \qquad Equation\ 2\text{-}6$$

2.8.1.5 Risk Characterization

This is the final risk assessment step. It combines all the previous steps, particularly hazard identification and exposure assessment to estimate potential risks associated with the exposure of the COPC to receptors (Means, 1989). Risk is calculated for different routes of exposure for threshold and non-threshold toxicants. This process involves the comparison of exposure estimates to toxic reference values (TRV) also known as TDI or RfD. Hazard quotients are used for estimating risks of non-carcinogens while the cancer slope factor (CSF) is used for carcinogens (Means, 1989; United States Environmental Protection Agency, 1998).

$$Risk\ for\ a\ non-carcinogen\ (HQ) = \frac{EDI}{TDI} \qquad \text{Equation 2-7}$$

$$Risk\ for\ carcinogen\ = EDI * Cancer\ Slope\ Factor\ (CSF) \qquad \text{Equation 2-8}$$

2.8.2 Human health risk assessment

Even the indispensables of life (e.g. water and air) can be harmful at high concentrations. People have died from water intoxication, and babies exposed to high oxygen levels in incubators have experienced defects in blood vessels of the eye, increasing the risk of losing their sight (Terry, 1942; Williams, 2007). Paracelsus established that "everything is poisonous, and nothing is not poisonous, it is factually the dose that defines the poison". Several activities from both natural and anthropogenic sources release chemicals into the environment. These chemicals classified as hazards potentially cause adverse effects to humans and the environment (receptors) when they exceed levels that are deemed "safe".

Once an identified hazard overlaps with a receptor and an exposure pathway, risk is defined (Cooper et al., 2000). Risk assessment (RAs) is therefore undertaken to acquire an in-depth knowledge of the contaminant or hazard, its effects on the environment or humans and its associated risks (Berglund and Järup, 2001; Gentile et al., 1991). RAs is also done to determine a solution that combines an optimum balance of risk control and societal benefit usually through a cost-benefit analysis. The precautionary principle is applied to identify appropriate balances when managing risks and making decisions (Cooper et al., 2000; Gentile et al., 1991). A systematic approach and framework were developed several decades ago to be used in assessing the risks of chemicals to humans and ecology. It is a multi-step process that employs the organization and analyses of knowledge from both scientific and social contexts.

2.8.3 Ecological risk assessment

The ecological risk assessment (ERA) process is used to evaluate the possibility of adverse ecological effects that might occur after exposure to stressors (United States Environmental Protection Agency, 1998). The definition of adverse effects in ecological risk assessment is complex because of the differences in sensitivity of ecological components to metals and mixtures (United States Environmental Protection Agency, 1998). Hence, selecting an assessment endpoint in ERAs are based on susceptibility of the endpoint, ecological relevance of the endpoint, and management goals (United States Environmental Protection Agency, 1998). An endpoint is ecologically relevant, when it represents significant physiognomies of the system, and is functionally associated to other endpoints (United States Environmental Protection Agency, 2003). The identification of relevant endpoints by risk assessors can be decided at the individual, population, community or ecosystem levels. The relationship between the level of the selected endpoint and stressor level can be used in the analysis of ecological responses to determine plausible impacts from the stressor. A more holistic approach however, will be to incorporate all relevant endpoints into a statistical distribution that can describe the variation of exerted toxic effects among the endpoints (Posthuma et al., 2002). From the distribution, the potentially affected fraction (PAF) defined as the fraction of endpoints that will be adversely affected by the stressor, can be estimated (Klepper et al., 1998). This approach to ERAs is termed the species sensitivity distribution (SSD) and aims to protect a wide range of organisms (endpoints). A major advantage of SSD is that, it utilizes entire knowledge on the toxic effects of xenobiotics (Posthuma et al., 2002). Despite the advantages of an SSD, it fails to incorporate the effects of the toxicant on interactions that occur between components of the ecosystem (i.e. between microbes and plants, or invertebrates). Hence, an approach that incorporates direct effects of toxicants including metal and mixtures on component interactions will be a better representation of risks.

There are certain aspects of our present risk assessment practice that can be improved. For example, the current RA practice does not entirely consider the unique properties of metals in risk assessment. Furthermore, the persistent nature of metals is typically underestimated when characterizing associated risks. The valence state of metals is largely not considered when characterizing risks. Regulation largely focuses on total metal concentrations, when the soluble fractions are the actual culprits (United States Environmental Protection Agency, 1986). This implies that, there should be a tiered level approach that associates different risk levels to valence states of metals in the environment, to improve site specific risk assessment.

3 TOXICITY ASSESSMENT OF METAL MIXTURES TO SOIL ENZYMES IS INFLUENCED BY METAL DOSING METHOD

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3.1 Preface

The following chapter has been published as a peer-reviewed article in the Chemosphere journal with the following co-authors:

Mark cousins (University of Saskatchewan) – involved with experimental design, data analysis, and editorial;

Mathieu Renaud (University of Saskatchewan) – involved with experimental design, statistical analysis, and editorial;

Olukayode Jegede (University of Saskatchewan) – involved with experimental design and editorial;

Beverley Hale (University of Guelph) – co-supervisor involved with all aspects of project oversight.

Steven D Siciliano (University of Saskatchewan) – supervisor involved with all aspects of project oversight.

As the lead author, Kobby Awuah, was involved in every aspect of the article. More specially, Kobby performed approximately 30% of soil preparation and dosing, 80% of the lab work (enzyme assays, metal extractions and analysis), 90% of the data analysis and 95% of the manuscript writing.

This chapter focuses on how the differences in toxicity of metals dosed as salts, oxides and spinel minerals. The objective of the chapter was to determine an appropriate experimental method for carrying out metal mixture toxicity tests in soils.

3.2 Abstract

Metals are present as mixtures in the environment, yet testing such complex mixture poses design and technical challenges. One possible solution is the use of fixed ratios, i.e. rays of increasing metal concentrations. But fixed ratios rays are compromised when soils dosed with metal salts are leached due to metal-soil selectivity rules. Two alternative metal forms, metal oxides and spinel minerals of quinary metal mixtures (Pb, Cu, Co, Ni, Zn), were evaluated for their toxicity to soil microorganisms measured by the activity of ammonia monooxygenases and acid-phosphatases in three soils. Leaching, a required step for salts, had a larger effect on ammonia monooxygenases than metals. Generally, metal salts were the most toxic form, while the spinel minerals were the least toxic form. Two extractants, $CaCl_2$ and DTPA, were evaluated for their ability to link toxicity to metals across all three metal forms. Salt toxicity was closely linked to $CaCl_2$ extractable concentrations but DTPA was the most appropriate for oxides. I strongly recommend combining fixed ratio rays with metal oxides for metal mixture studies, since soil ratios created using oxides were more precise and required less experimental effort compared to salts and spinel minerals. Furthermore, because DTPA and $CaCl_2$ closely tracked the toxicity of more realistic metal forms (i.e. oxides), I recommend that field studies investigating metal mixtures use both DTPA or $CaCl_2$.

Keywords: Fixed ratio ray, metal oxides, spinel minerals, leaching, metal salts, enzymes

3.3 Introduction

The study of heavy metal exposure to soil biota beyond binary mixtures has received increased attention in the last two decades (Khalil et al., 1996; Nys et al., 2017). The interest is growing because we have yet to decipher how to address metal mixtures in risk assessment. Despite the improved understanding of chemical exposures, legislation still largely focuses on assessing individual chemicals while excluding possible interactions. Much of the advancements in metal mixture studies apply and improve existing predictive models to help advance the risk assessment of metal mixtures (Cedergreen et al., 2008; Jonker et al., 2005; Thakali et al., 2006). The classic empirical models widely used to predict mixture toxicity are concentration addition (CA) and response addition (RA), but there are concerns that these models have significant drawbacks. For example, Zwart and Posthuma (de Zwart and Posthuma, 2005) stated that the models were ineffective beyond binary mixtures. Modeling the toxicity of a chemical mixture on an organism while assuming a one-compartment distribution kinetics and not accounting for the toxicokinetics of the chemicals in other compartments of the organism is a major flaw of both models (Cedergreen et al., 2008; de Zwart and Posthuma, 2005). Furthermore, inferences from the models are typically based on statistically significant evidence and ignore the biology of the endpoint of interest. To bridge this knowledge gap, extensive modifications of the mixture models and their deviations (interactions) by including bioaccessibility and free ion activity has improved the prediction of metal mixture toxicity in soils (Cipullo et al., 2018; Jonker et al., 2004; Nys et al., 2017).

However, an area that has received little attention despite its pertinence in elucidating mixture toxicity is the metal type or form used when conducting metal mixture tests in soils. Soil organisms are usually exposed to metals dosed as salts in laboratory experiments (Mertens et al., 2009; Ruyters et al., 2013). This method, including ageing and leaching of the soils before the toxicity testing, is widely accepted by the European Union's Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (EU-REACH) (Assessing risks of chemicals, 2017). The major issue with metal salts is the fact that the counterions increase metal accessibility and may be toxic to soil dwelling organisms especially ammonia oxidizing bacteria (Cui et al., 2016; Dinçer and Kargi, 1999; Owojori et al., 2008). To reduce the salt effect, metal salt-spiked soils are usually leached with artificial rain or deionized water to return the electrical conductivity of the spiked soils to control levels (Bongers et al., 2004; Schwertfeger and Hendershot, 2012). Metal salt-spiked soils are also aged and leached in some studies to mimic natural attenuation of the metals thereby reducing their accessibility (Lock et al., 2006; Oorts et al., 2007; Van Gestel et al., 2012).

Leaching removes essential ions and clays from soil, alters the soil's physicochemical properties (Gordon et al., 2008; Haynes and Swift, 1986), and reduces the nominal metal concentrations in spiked soils (Schwertfeger and Hendershot, 2013; Stevens et al., 2003). Apart from the effects of salts and leaching on the physicochemical properties of the soil, the microbial community can also be compromised (Chen et al., 2017; Gordon et al., 2008). Metal loss via leaching is also uneven due to differences in the physicochemical properties of metals and soils, which determines metal speciation and availability (Langdon et al., 2015; Li et al., 2011). Soil properties like pH and cation exchange capacity (CEC), and metal properties like lability and covalent binding indices affect metal loss (Laird et al., 2011; Tatara et al., 1998). Differential metal loss is a problem in mixture toxicity testing because it causes significant changes to metal ratios in dosed soils and ultimately confounds links between nominal and actual concentrations.

When conducting metal mixture studies, it is better to use an optimized fixed ratio ray design. This design ensures that metals are tested as rays, with each ray consisting of a specific number of metals tested in specific ratios at different concentrations. It is an economical design that allows estimation of both additivity and interactions with reduced laboratory efforts (Coffey et al., 2005). The optimized fixed ratio design also has an increased power of determining departures from additivity compared to standard non-optimized designs (Yeatts et al., 2010). A fixed ratio can be calculated based on environmentally relevant concentrations of one or more metals, or regulatory concentrations to represent a ray. Thus, one can select rays of interest to all stakeholders in a risk assessment. However, the uneven metal loss associated with leaching compromises the fixed ratio ray design by altering ratios in a concentration dependent and ratio dependent manner. Therefore, an exposure method that maintains ratios without compromising soil properties and biota would increase the reliability and precision of effective concentrations estimated from concentration-response curves when coupled with the optimal fixed ratio ray design.

Maintaining the innate property of soil and its biota is even more essential when investigating microbial endpoints. Soil microbes play significant roles in the biogeochemical cycling of nutrients on a global scale (Madsen, 2011). Microbes mineralize nutrients like phosphorus and nitrogen allowing plants to acquire these nutrients from the soil. Furthermore, fungal filaments (hyphae) and other microbial secretions influence soil properties and as a result, the activity and composition of the microbial community, structure and diversity are intimately linked to the soil's physicochemical properties (Goyal et al., 2003; Klimmek et al., 2001; Mapolelo et al., 2005). Microbes respond quickly to stressors such as metals because of their high surface to volume ratio and rapid metabolic responses (Matheron and Caumette, 2015; Polonenko et al., 1981; Ruyters et al., 2013).

Here I evaluate spiking alternatives that are environmentally relevant and avoid leaching and aging. According to research conducted by Hamilton et. al (Hamilton et al., 2016) and Thorn (Thorn, 2015), metal species found in either aged metal salt-spiked soils or field contaminated soils include aqueous metals (Me^{2+}), iron spinel minerals ($MeFe_2O_4$), metal hydroxides ($Me (OH)_2$), and metal oxides (MeO). Spinel minerals are naturally occurring minerals that also form at hot temperatures during smelting and can persist in the environment due to low rates of weathering (Alloway, 1990; Scheinost et al., 2002). The spinel minerals and metal oxides were chosen as possible alternatives to spiking with metal salts. Metal oxides were chosen based on their commercial availability compared to hydroxides, and spinel minerals were selected because of their crystalline structure and persistence (Alloway, 1990). This study had two objectives; the first was to determine the relative toxicities of the three-metal species (metal salts, metal oxides and spinel minerals) to soil microbes in three Canadian soils. The second was to determine a spiking method that could improve estimations of exposure concentrations from mixture models through the effective usage of the optimized fixed ratio ray design in soils. The five metals of interest cobalt, copper, lead, nickel and zinc were chosen because there were the metals of potential concern in sampled soils from Canadian smelting sites. The activities of ammonia monooxygenases and acid phosphatases were investigated because previous studies have established that they are good indicators of soil health (Eivazi and Tabatabai, 1977; Sharma et al., 2014; Smolders et al., 2001b). Therefore, I evaluated effects on the potential nitrification rates (PNR) and the activity of acid phosphatases in leached and metal exposed soils. I hypothesized that, metal salts would be more toxic to soil microorganisms compared to spinel minerals and metal oxides.

3.4 Materials and methods

3.4.1 Soil sampling and treatments

Three Canadian soils were used for the study (Table 3-1). Topsoil (~30cm depth) was collected to cover a wide range of soil properties. The soils were air-dried and sifted using a 2mm sieve. Background metal concentrations were determined by reverse Aqua Regia (rAR) method and metals were measured with an Agilent 5110 SVDV inductively coupled plasma optical emission spectrometer (ICP-OES) (Topper and Kotuby-Amacher, 1990). The CEC was determined by the methylene blue method (Yukselen and Kaya, 2008), soil pH was determined by 0.01 M $CaCl_2$ in a ratio of 1:5 (solid: liquid) and measured with a Mettler Toledo pH meter (Conyers and Davey, 1988), soil texture was determined by the pipette method (Bouyoucos, 1962), soil organic carbon was determined by the LECO-C632 carbon analyzer (Wang and Anderson, 1998), and water holding capacity (WHC) was measured with open ended test tubes with soil retained by filter paper placed at the bottom (Jaabiri Kamoun et al., 2018). The soils were stored in plastic containers at room temperature until they were used for the experiment (rewetting 2 weeks prior to toxicity testing). The soils were named S1, S2, and S3 with pH values of 3.4, 4.6 and 6.8 respectively. The soils were selected to cover a wide range of pH and CEC because they are modulators of metal bioaccessibility and toxicity (Brallier et al., 1996).

Table 3-1. Physicochemical properties of experimental soils.

Soil	pH	CEC (meq/100 g)	Clay Content (g/kg)	Organic Carbon (g/kg)	Water Holding Capacity (ml/g)	Texture	Background Zn	Cu	Ni	Co	Pb
							(mg/kg)				
S1	3.4	8	45	17	0.29	Sandy Loam	967	303	7	7	397
S2	4.6	16	110	25	0.35	Sandy Loam	635	107	11	9	23
S3	6.8	20	58	27	0.30	Loamy Sand	195	11	49	4	15

CEC=Cation Exchange Capacity.

3.4.2 Rays, metal types and dosing

Soils were spiked with metal mixtures at one concentration level in five equal effect concentration ratio (EECR) rays (Table 3-2). Each EECR ray consisted of five heavy metals at different ratios calculated to exhibit equitoxic effects.

3.4.2.1 Calculation of equal effect concentration ratios (EECR)

3.4.2.1.1 Ratio rays

The five mixture ratio rays were calculated from a combination of Pb, Cu, Ni, Co, and Zn. The ratio of the metals was determined from either regulatory concentration (for the CSQG and Peaty rays) and environmentally relevant concentrations (for Flin Flon, Sudbury and Port Colborne rays). The maximum allowable concentrations of the five metals from the Canadian Soil Quality Guidelines (CSQG) for agricultural sites was used for the CSQG ray. The predicted no effect concentrations (PNEC) for each of the five metals from the EU-REACH PNEC calculator for clayey and peaty soils was averaged and computed to acquire the Peaty ray (Assessing risks of chemicals, 2017). For the environmental rays (Flin Flon, Sudbury and Port Colborne), soil samples from field contaminated soils were collected and analyzed for total concentrations of the metals of interest and computed into rays. Hence, each ratio ray consisted of 5 metals in different ratios (Table 3-2).

3.4.2.1.2 Equal effect concentrations

The concentration of the metals in each ray was determined from EC_{50} values of the five metals (Pb, Cu, Ni, Co, and Zn) derived from literature and the calculated ratio as shown in Table 3-2 (Lock and Janssen, 2002a, 2002b, 2003; Sandifer and Hopkin, 1997a). Assuming concentration addition (CA) from Eq. 3-1, the EC_{50} values of the metals were computed to acquire a toxic unit (TU). The quotient of c_i and ECx_i which is the TU, represents the fractional potency contributed by each metal in the mixture. The mixture of *n (n=5)* chemicals was arithmetically illustrated as:

$$\sum TU_{EC_{50}} = \sum_{i=1}^{n} \frac{c_i}{EC_{50i}} = 1 \qquad \textit{Equation 3-1}$$

Where c_i was the concentration of each metal i in the mixture containing n components exhibiting an effect 50%, and EC_{50i} is the concentration of metal i that exhibits same effect when singly applied. The concentration of each metal in the mixture derived from Eq. 3-1 was quadrupled for each mixture ray, such that the sum of their individual TUs equaled 4. A high TU was chosen to guarantee an observation of toxic effects despite the possible metal losses from leaching metal salt spiked soils, and low solubility of metal oxides and spinel minerals. Also, since this study was part of a larger mixture experiment, 4TU was chosen as the midpoint dose. The EC_{50} of a ray at a TU can be derived by combining the quotient of the ratio of the metals in the ray by their EC_{50}s (Table 3-2), then dividing 1 by the results since I assumed concentration addition (CA).

3.4.2.2 Preparation of metals for spiking soils

Metal nitrate salts (>99% purity) and metal oxides (>99% purity) were purchased from Sigma Aldrich. Spinel minerals were synthesized in the laboratory from metal nitrates salts (Li-Zhai et al., 2010). Prior to spiking, metal oxides were exposed to gaseous HNO_3 in a desiccator to remove inorganic carbon and weighed into soils. Stock solutions were prepared from metal nitrate salts for metal salt spiking. Briefly, metal concentrations in stock solutions were added to soils without exceeding 60% of the WHC of the soils. Soils were leached to remove excess salts per EU-REACH guidelines with artificial rainwater (Li et al., 2011). Soils were leached until electrical conductivity levels in control soils were reached. For spinel minerals, the stock solutions used for spiking metal salts were mixed in their respective ratios. Iron was added as Fe

$(NO_3)_3$ at 70% wt/wt of the total metals in the mixture. The pH of the mixture was adjusted to about 7.0 with NH_4OH and shaken for 24 hours. The mixture was centrifuged at 1128 g forces for 30 minutes. The supernatant was separated, and the residue was air dried for about 72 hours. The residue was heated to 600^o C in a muffle furnace for an hour and ground into a fine powder after cooling. For all spiking methods, soils were mixed with plastic spoons for five minutes to ensure homogenization. Enzyme activity was determined 48 hours after spiking.

Table 3-2. Metal mixtures rays and dose at 4 times the Toxic Unit (TU). Metal mixture rays in the first column with their corresponding nominal metal doses and ratios in brackets. The TU was calculated from EC_{50} values of individual metals (Cu, Co, Pb, Ni, Zn) derived from literature in mg/kg of soil (Lock and Janssen, 2002a, 2002b, 2003; Sandifer and Hopkin, 1997a, 1997b).

Metal/ Ray EC_{50}	Copper 700 (mg/kg)	Cobalt 1480 (mg/kg)	Lead 1600 (mg/kg)	Nickel 475 (mg/kg)	Zinc 750 (mg/kg)	**Total (Ratio) (mg/kg)**
CSQG	483 (0.151)	306 (0.096)	536 (0.167)	345 (0.108)	1532 (0.478)	3202 (~1)
Flin Flon	619 (0.202)	9 (0.003)	202 (0.066)	9 (0.003)	2223 (0.726)	3062 (~1)
Peaty	663 (0.206)	354 (0.110)	612 (0.190)	396 (0.123)	1199 (0.372)	3224 (~1)
Port Colborne	381 (0.178)	28 (0.013)	56 (0.026)	1513 (0.707)	163 (0.076)	2141 (~1)
Sudbury	161 (0.039)	153 (0.037)	2314 (0.561)	297 (0.072)	1196(0.29)	4121 (~1)

CSQG=Canadian Soil Quality Guidelines.

3.4.3 Metal Concentrations

3.4.3.1 Total metals concentration

Total metals concentration in spiked soils was determined using an X-Ray Fluorescence (XRF) method as described by Margui et al. (MarguÃ- et al., 2009). Soil (4 g dry) was ground and homogenized with 0.8 g of Chemplex spectroblend 44µm powder to act as an adhesive.

Samples were transferred into Chemplex pellet cups, covered with polypropylene thin-films and placed into a pellet die set. The pellet set was mounted on a hydraulic press and a force of about 10,000 psi was applied to the samples for 5 minutes to form discs. The samples were analyzed on the Thermofisher ARL Optim-X X-ray analyzer for total metal concentrations. Six soil samples analyzed with an Agilent 5110 SVDV inductively coupled plasma optical emission spectrometer (ICP-OES) for metal concentrations (reverse Aqua Regia) were re-analyzed with the XRF for confirmation. Montana II obtained from Sigma-Aldrich was used as a standard reference material (SRF) on the XRF. Metal recoveries from the Montana II SRF was 90%.

3.4.3.2 Extractable metals

3.4.3.2.1 Calcium chloride extractable

The mobilized metal fractions were extracted using a 0.01 M calcium chloride ($CaCl_2$) solution (Quevauviller, 1998). Briefly, 2.5 g of dry soil was weighed into 50 ml test tubes, 25 mL of $CaCl_2$ solution was added and shaken on an end-over-end shaker for 180 minutes. Samples were centrifuged at 4704 g forces for 10 minutes, filtered with a Whatman 0.45μm syringe filter and analyzed with the Agilent 5110 SVDV ICP-OES for dissolved metal concentrations. Standard metal mixture solutions (VWR Zn, Cu, Ni, Pb and Co standards) prepared from a serial dilution (1, 5, 15, 30, and 50 mg/ L) with 0.01 M calcium chloride were used as standards. The quality control included blanks, duplicates and calibration standards were run every 21 samples.

3.4.3.2.2 DTPA-TEA extractable metals

The mobilized and mobilizable metal fractions in spiked soils were extracted using a solution comprising 0.005 M diethylenetriaminepentaacetic acid (DTPA), 0.01 M calcium chloride ($CaCl_2$), and 0.1 M triethanolamine (TEA). Following Kulikov (Kulikov, n.d.), 3 g of dry soil was weighed into 15ml test tubes, 6 ml of DTPA-TEA solution was added and shaken for 120 minutes on an end-over-end shaker. Samples were filtered with a Whatman 0.45µm syringe filter and the metal concentrations determined with the Agilent 5110 SVDV ICP-OES. Standard metal mixture solutions (VWR Zn, Cu, Ni, Pb and Co standards) prepared from a serial dilution (1, 5, 15, 30, and 50 mg/ L) with DTPA were used as standards. The quality control included blanks, duplicates and calibration standards were run every 21 samples.

3.4.4 Soil enzymes

3.4.4.1 Potential nitrification rate

The potential nitrification rate as measured by ammonia monooxygenase activity in the soils was measured (Berg and Rosswall, 1985). Briefly, 2g of each soil was weighed into 15ml test tubes, to which 10ml of 1mM NH_2SO_4 and 0.5ml of 1.5 M $NaClO_3$ were added. This was shaken end-to-end for 6 hours at room temperature, and 2ml of 2M KCl was added and shaken again for about 2 minutes. The samples were then centrifuged with an Eppendorf miniSpin Plus at 3011 g forces for 4 minutes and read colorimetrically at 545nm with the Biorad iMark 96-well-plate reader (Smolders et al., 2001b).

3.4.4.2 Acid Phosphatases

Soil (0.1g) was weighed into 1.5ml test tubes, 20µl of toluene was added, mixed and left in the fume hood for an hour (Eivazi and Tabatabai, 1977). After an hour, 400µl of 0.5 M acetate buffer (pH~5.8) and 100µl of 10mM p-nitrophenyl phosphate were added to each sample. The samples were incubated in a water bath at 37^o C for one hour (Eivazi and Tabatabai, 1977). The samples were then removed and placed on ice to stop the reaction. Samples were centrifuged with an Eppendorf miniSpin Plus at 13148 g forces for 2 minutes and read colorimetrically at 410nm with the Biorad iMark 96-well-plate reader.

3.4.5 Data Analysis

The activity of the soil enzymes was determined from the slope of the standard curve derived from known concentrations of p-nitrophenyl (ACP) and nitrate (AMO). The percentage activity was determined relative to control soils using the Equation 3-2 below.

$$Activity = \frac{S_{a,j,} - S_{b,j,}}{C_a - C_b} * 100\% \qquad Equation\ 3\text{-}2$$

Where $S_{a,j}$ and $S_{b,j}$ represent analyte (p-nitrophenol or nitrate) concentrations extracted from incubated and non-incubated (negative control) samples respectively for a ray j, while C_a and C_b represent analyte concentrations in controls. The relative activity of dosed samples was determined separately for leached and non-leached soils. All data were statistically analyzed with either a 1-way or 2-way ANOVA using the R (studio) program using a significance level of $\alpha=0.05$ (R Core Team, 2018). Data was tested for normality and homogeneity of variance to meet ANOVA assumptions. The Tukey HSD test was used for testing multiple comparisons between soils, metal types, and rays. Pearson product-moment bivariate correlations were performed between soil pH or activity and $CaCl_2$ or DTPA extractions.

3.5 Results

3.5.1 Activity of AMO and ACP in non-metal spiked soils after leaching.

In all three soils, leaching non-metal-spiked soils with artificial rainwater reduced ammonia monooxygenases activity (AMO), but not acid phosphatases (ACP) activity (Figure 3-1). The potential nitrification rates (PNR) of leached non-metal-spiked soils showed decreases (27%-58%) compared to non-leached non-metal-spiked soils ($p<0.01$). The PNR in S1 was reduced by 57.5±2.2%, PNR in S2 was reduced by 56.6±7.5%, and PNR in S3 by 26.8±3.9% (Figure 3-1). The extent of PNR reduction from adding artificial rainwater to soils S1 and S2, were higher in some instances, compared to the activity in non-leached metal spiked soils.

Leaching however didn’t significantly inhibit acid phosphatases (ACP) activity in any of the experimental soils (Figure A-1).

3.5.2 Sensitivity of AMO and ACP to metals

3.5.2.1 Sensitivity of AMO and ACP to metal mixtures

AMO was generally more sensitive to the metal mixtures in comparison to ACP. The differences in sensitivity of both enzymes to metal mixtures was soil dependent (Figure 3-2), (grids a and c). In the low pH soil (S1), metal mixtures stimulated the activity of AMO (i.e. AMO > Control), while the highest toxicity to ACP was observed in this same soil. There was a 10-fold reduction in PNR from S1 to the medium pH soil (S2). In this same soil (S2), the average AMO inhibition was about 90%. while ACP activity was not altered significantly compared to control. At high pH (S3), a 35% increase in PNR was observed, while ACP activity remained unchanged.

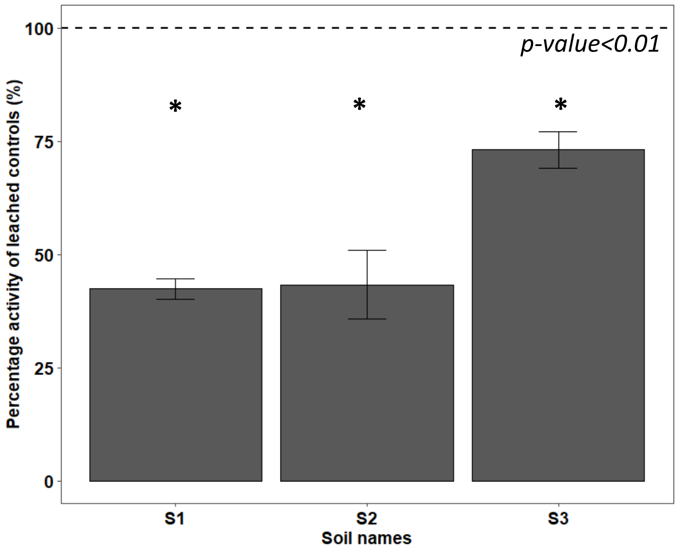

Figure 3-1. Leaching by itself reduced AMO activity in all three Canadian soils. Leaching was designed to remove excess salts from the soils. Control soils were leached once to determine an electrical conductivity baseline for metal salt spike soils. The potential nitrification rate was measured 7 days after leaching 210 mg of soil with 200ml of artificial rainwater. S1, S2 and S3 represent the names of the three Canadian soils used. Horizontal broken line indicates percentage activity in non-leached soils. Gray vertical bars represent average AMO activity of 9 replicates (3 per soil) in the three soils. The standard error (SE) of the mean are represented by error bars. Asterisks indicate significant differences from non-leached soils (controls).

3.5.2.2 Differences in toxicity of metal mixture types.

Metal mixture salts were generally the most toxic (reduction in activity) metal type to AMO but not ACP. In the low pH soil (S1), none of the metal mixture types significantly inhibited AMO activity, in fact, a stimulatory effect was observed for salts and spinels minerals (Figure 3-2. Grid b). In the medium pH soil (S2), where AMO sensitivity to the metal mixtures was highest, metal salts exerted a near complete inhibition on AMO, whereas spinel minerals and metal oxides exhibited an equitoxic effect of 80% inhibition. Conversely, in the high pH soil (S3), metal oxides and salts exerted a 50% and 75% inhibition respectively to AMO, while no significant inhibition was observed for spinel minerals.

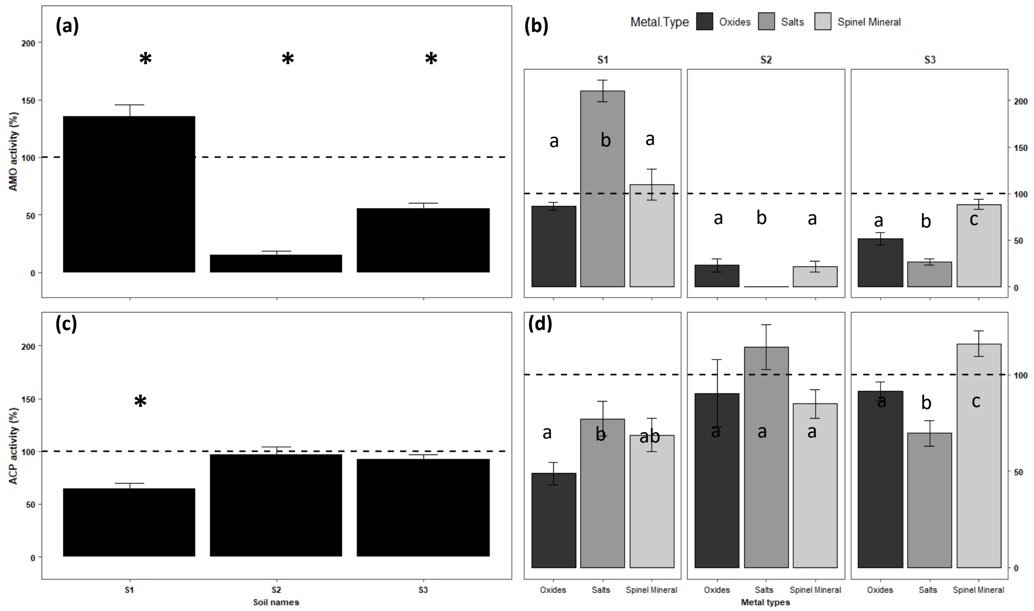

Figure 3-2. Sensitivity of the soil enzymes ammonia monooxygenases (AMO) and acid phosphatases (ACP) to metal mixtures is affected by soil and metal mixture type. Grid (a): potential nitrification rate (PNR) in three metal mixture spiked Canadian soils. Grid (b): PNR of three metal mixture types in three Canadian soils. Grid (c): Activity of ACP in three metal mixture spiked Canadian soils. Grid (d): Activity of ACP of three metal mixture types in three Canadian soils. Horizontal broken line indicates percentage activity in non-metal spiked soils. Gray vertical bars (Grids (a) and (c)) represent average AMO (top) and ACP (bottom) activity of 54 replicates (9 per soil per enzyme) in the three soils. Black-Gray vertical bars (Grids (b) and (d)) represent average AMO (top) and ACP (bottom) activities in 3 soils spiked at one dose with three metal mixture types in 9 replicates (3 per soil) in three soils. The standard error (SE) of the mean are represented by error bars. Asterisks in grids (a) and (c) represent significant differences between control metal spiked soils in the three soils. Alphabets a, b and c in grids (b) and (d) represent significant differences between metal types. Bars with same alphabetic insets within the same grid are not significantly different ($p > 0.05$).

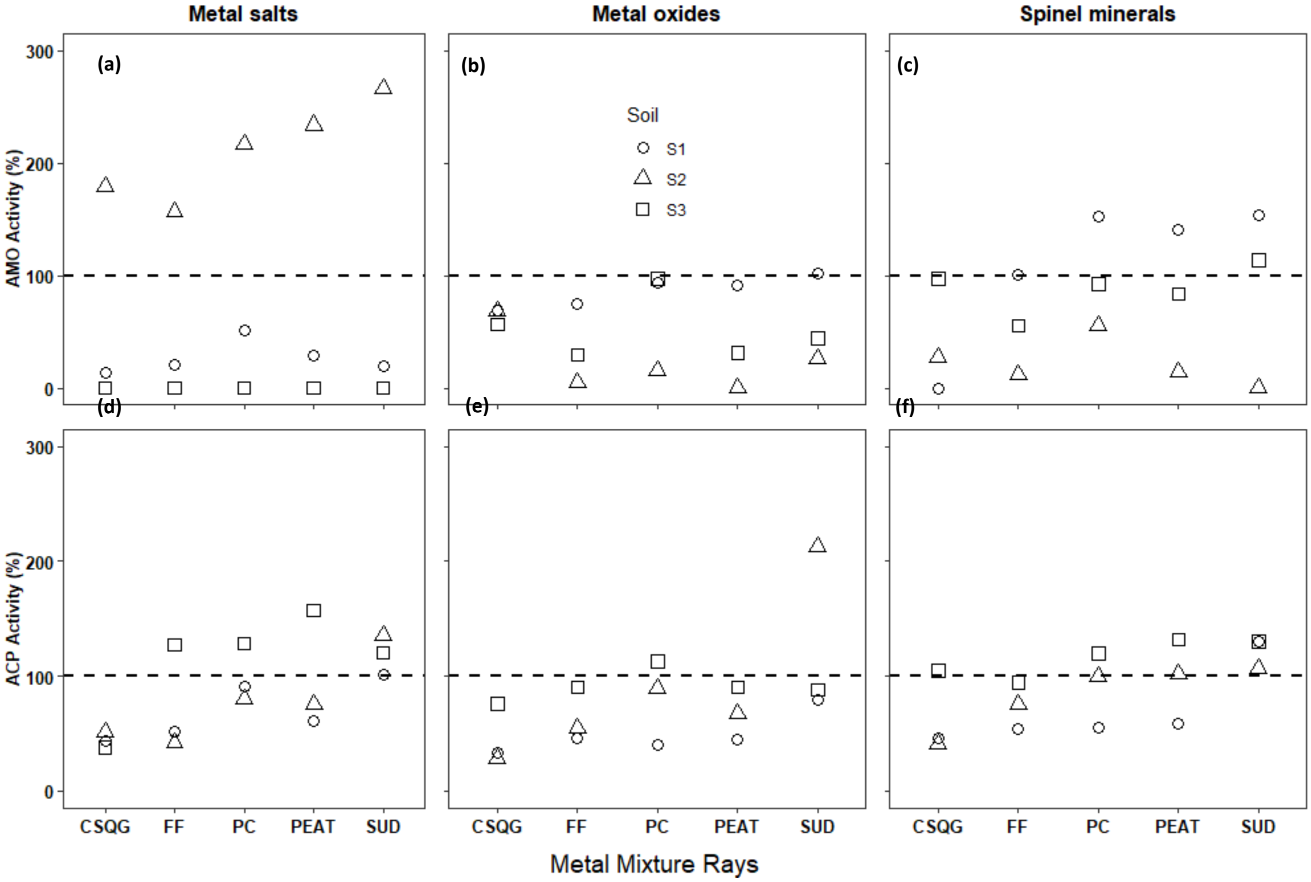

Figure 3-3. Differences in toxicity of equal effect concentration ratios (EECR) of metal mixture types as driven by metal type to soil enzymes ammonia monooxygenases (AMO, top) and acid phosphatases (ACP, bottom). Left grids represent salt-spiked soils, middle grids represent oxide-spiked soils, and right grids represent spinel mineral-spiked soils. Triangular symbols represent activity in soil S1, square symbols represent activity in soil S2, and round symbols represent activity in soil S3. Y-axes represent AMO (top) and ACP (down) activity while x-axes represent metal mixture rays (CSQG: SUD). Activity in each soil is connected by either a solid or broken line. The activity is normalized to non-metal spiked soils (leached or non-leached). The standard error (SE) of the mean are represented by error bars. Grid (a): Toxicity of metal salts to AMO in all three soils. Grid (b): Toxicity of metal oxides to AMO in all three soils. Grid (c): Toxicity of spinel minerals to AMO in all three soils. Grid (d): Toxicity of metal salts to ACP in all three soils. Grid (e): Toxicity of metal oxides to ACP in all three soils. Grid (f): Toxicity of metal salts to ACP in all three soils. Horizontal broken line indicates percentage activity in non-metal spiked soils. Rays have been splined within soil to ease visualization.

In contrast, ACP was moderately sensitive to all three metal types in S1, particularly the oxides (Figure 3-2. Grid d). There was no significant difference in ACP responses to spinel minerals and metal salts. In S2, ACP activity was not altered significantly between metal mixture types and control. Nevertheless, metal oxides exerted a 5% toxic effect on ACP in S3, while metal salts exerted a 35% toxic effect (Figure 3-2d). As expected, spinel minerals were not toxic to ACP in the same soil due to the high soil pH and low metal solubility. Overall, the toxicity of the metal types was both soil and enzyme dependent.

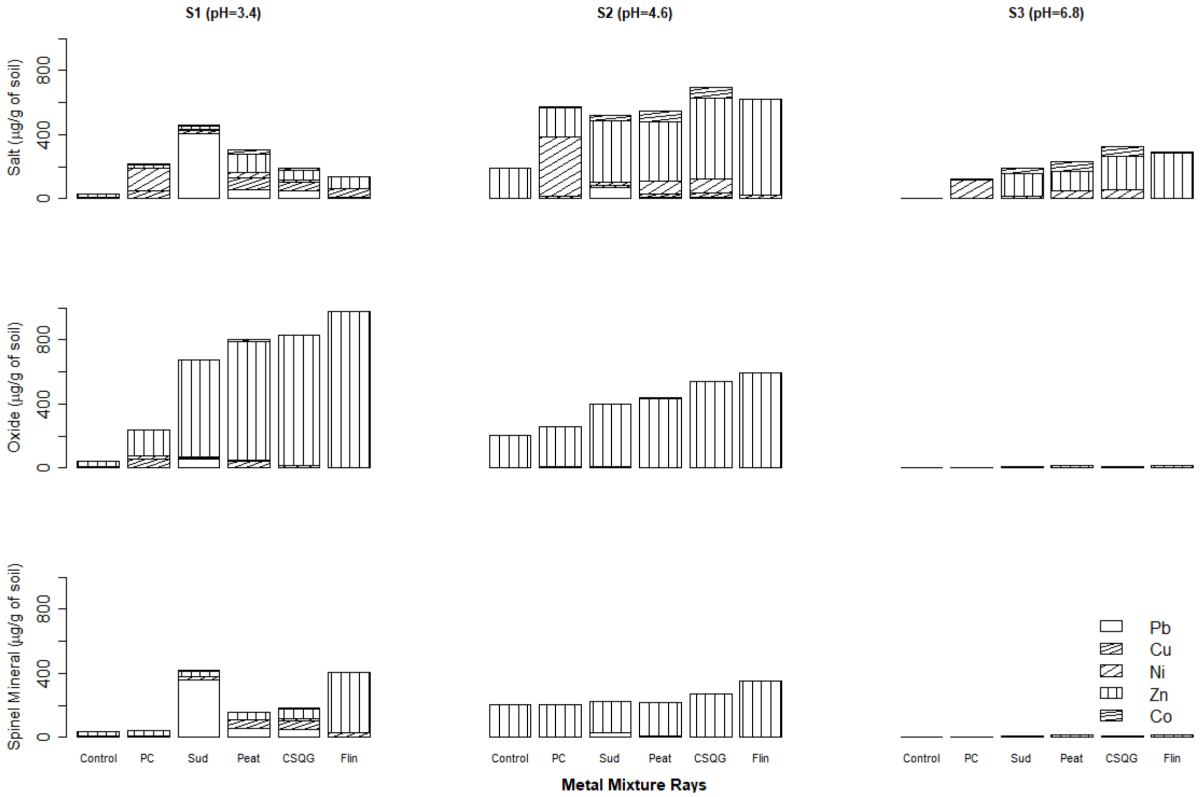

Figure 3-4. Stacked barplot showing concentration of $CaCl_2$ extractable metals from metal-salt spiked (top), metal-oxide-spiked (middle) and spinel mineral spiked (bottom) soils (3) from Canada. To the left S1 with a pH value of 3.4, centered is S2 with a pH value of 4.6, and to the right is S3 with a pH value of 6.8. The vertical bars show the total concentration of extracted metals (5) from two mixture types from three Canadian soils. Each texture pattern represents a metal. For example, vertical patterns represent Zn, horizontal pattern represent Cu, no pattern represent Pb etc. as shown by the legend.

3.5.3 AMO and ACP responses to equal effect concentration ratio rays for metal types in spiked soils

Soil and rays modulated absolute toxicity of metal mixtures to the enzymes, but the relative toxicity of the rays remained similar between soils and metal types. For example, the rays from all three metal types were most toxic only in acidic soils S1 and S2 (Figure 3-3). Toxicity of the rays in S3 was none or slight depending on the enzyme. At least two out of the five rays from all metal types were equitoxic to both enzymes in all the soils (Table 3-3). The toxicity of a specific ray differed in some cases between metal types, but similar trends were observed when all rays within a metal type were compared to the other. For example, the toxicity of the PC ray for S1 was different for metal types, but the ray was consistently either more or equally toxic compared to the PEAT and SUD rays, and less toxic compared to the CSQG and FF rays. The PEAT and SUD rays had the highest ratios of Cu and Pb respectively, and both metals have high affinity for SOM. The similarity in trend was more pronounced for metal oxides and spinel minerals.

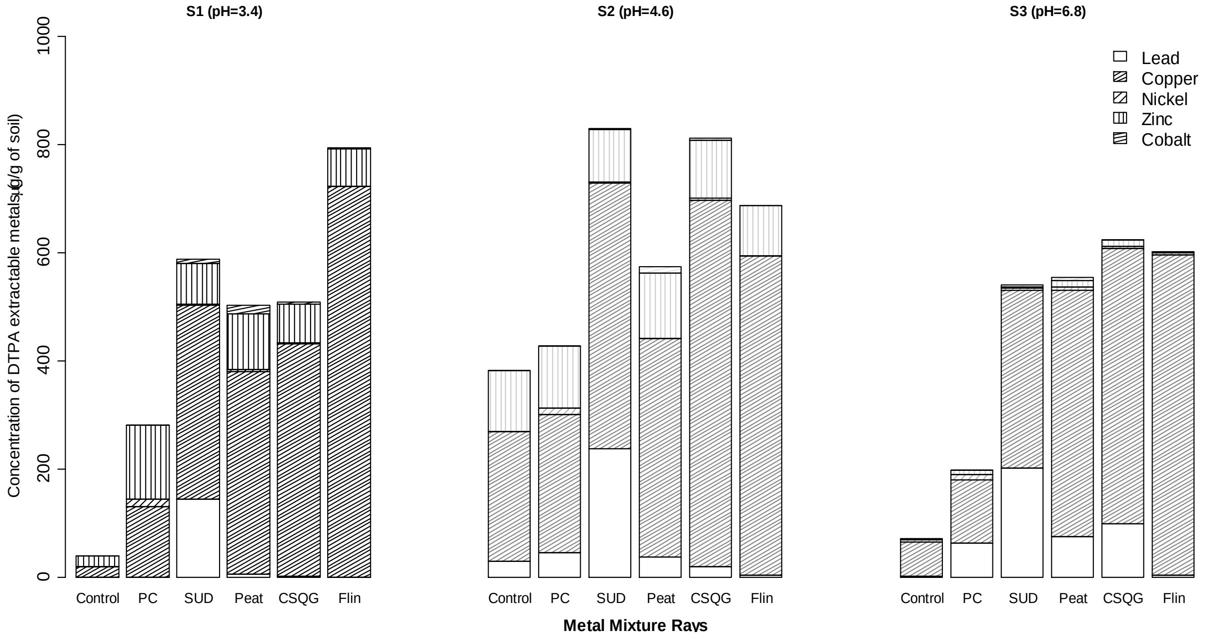

Figure 3-5. Stacked bar plots showing concentration of DTPA extractable metals from metal-oxide-spiked soils (3) from Canada. To the left is S1 with a pH value of 3.4, centered is S2 with a pH value of 4.6, and to the right is S3 with a pH value of 6.8. The vertical bars show the total concentration of extracted metals from one mixture type from three Canadian soils. Each texture pattern within a bar represents a metal. For example, vertical patterns represent Zn, horizontal pattern represent Cu, no pattern represent Pb etc. as shown by the legend.

3.5.4 $CaCl_2$ extractable metals

Zn was the most mobilized metal in metal-salt, metal-oxide and spinel mineral spiked-soils (Figure 3-4). Only the SUD ray in S1 showed substantial concentrations of Pb. The concentration of metals extracted from oxides and spinel metal mixtures followed known paradigms of pH dependence. As the pH and CEC of the experimental soils increased, the concentration of metals in the extracts decreased rapidly. Extracted metal concentrations were lowest in the high pH soil with concentrations below 100 µg/g of soil. The concentration of metals in metal salt-spiked soils weakly adhered to pH dependence compared to oxides and spinel minerals.

3.5.5 DTPA extractable metals in oxide-spiked soils

Cu was the most mobile and mobilizable metal extracted with DTPA from metal oxide spiked soils, followed by Pb and Zn (Figure 3-5). The concentration of extracted Ni and Co were below 50 μg/g in all soils for all rays. The concentration of DTPA extracted metals didn't show any strong relationship with soil pH.

3.5.6 Total metal concentrations

Measured metal oxides concentrations were most similar to nominal concentrations in all three soils (Figure 3-6a). The concentrations of metals measured in salt spiked soils were below 50% of the nominal concentrations after leaching in S1 (pH 3.4) when pre-leached metal spiked soils were compared to leached metal spiked soils (Figure 3-6). The metal retention (i.e. metals retained in soil after leaching) in the salt spiked soils however increased as the pH of the soils increased i.e. S1<S2<S3. The concentrations of metals measured in oxides and spinel mineral spiked soils remained constant across all three soils as expected.

For individual metals, only Cu and Pb retained their actual concentrations in salt spiked soils (Figure 3-6b). At least 50% of Co, Ni, and Zn were lost in metal salt spiked soils. The concentrations of all metals but Co in spinel mineral spiked soils were below concentrations measured in metal salt spiked soils. The metal concentrations for Ni, Zn, and Co was greatly improved in metal oxides compared to leached salts.

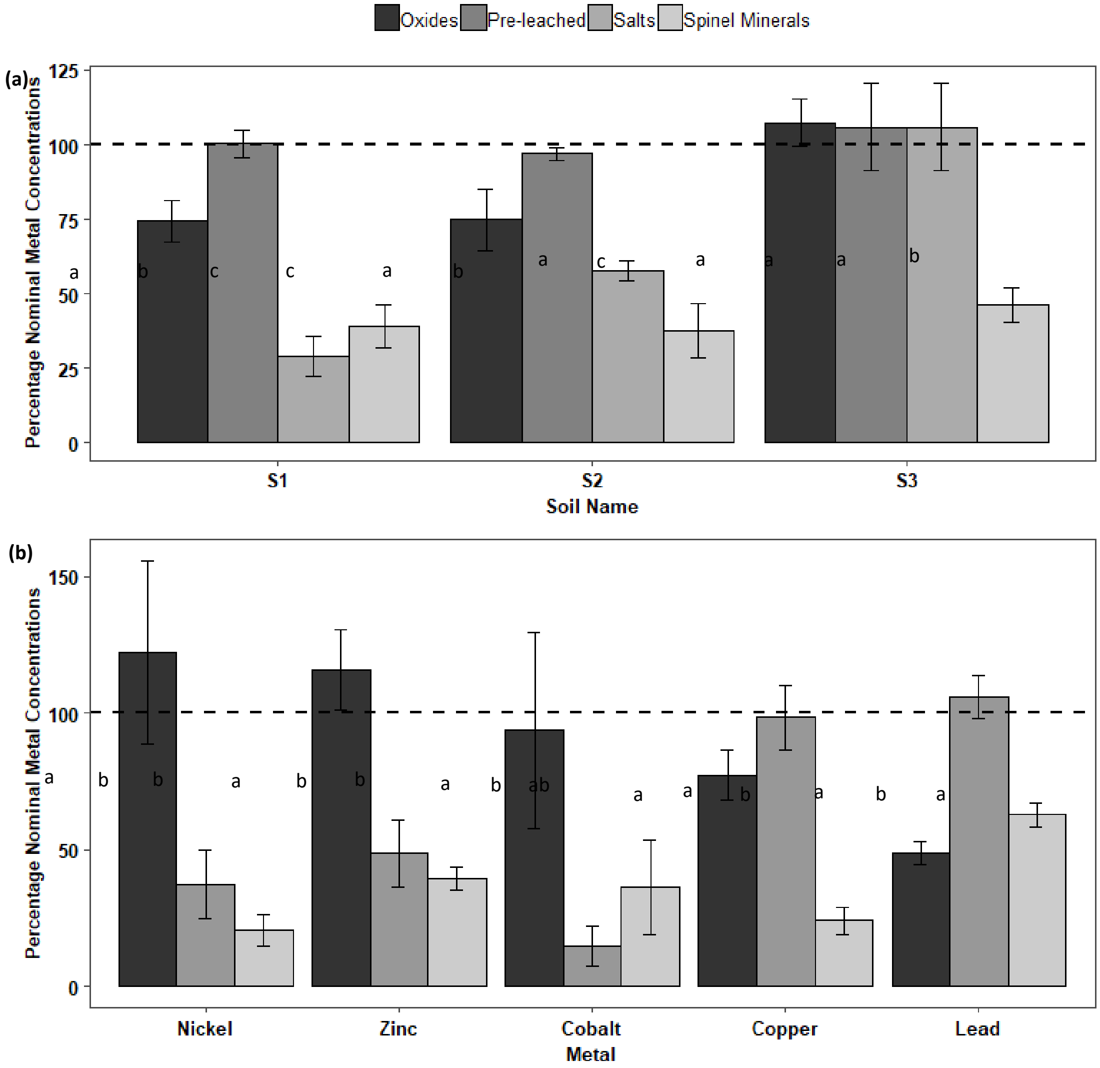

Figure 3-6. Metal retention relative to nominal concentrations for all metals and metal types in all three soils. Grid (a): Percentage metal mixture retention relative to nominal concentrations across mixture types in three spiked Canadian soils. Grid (b): Percentage metal retention relative to nominal concentrations across mixture types in three spiked Canadian soils. Black-Gray vertical bars represent average measured concentrations (corrected for background) of oxides, pre-leached salts, leached salts and spinel minerals in all soils. Horizontal broken line represents nominal concentrations. The standard error (SE) of the mean are represented by error bars. Alphabets a, b and c represent significant differences between metal types within soil for grid (a), and metal for grid (b). Bars with same alphabetic insets within the same grid indicate no significant difference (p-value >0.05).

3.6 Discussion

3.6.1 Leaching affected both AMO activity and concentration of metal ratios

To return electrical conductivity of metal salt-spiked soils back to their original conductivity, soils must be leached (Table A-1) (Oorts et al., 2007).Yet, AMO activity decreased in all leached control soils that contained no metals. The extent of AMO inhibition from the leaching process was greater than the metal effect in some cases (>50% for both acidic soils). A probable reason for this observation was that leaching reduced microbial biomass in the soils (Gordon et al., 2008; Griffiths et al., 2003; Thion and Prosser, 2014) and contributed to the loss of NH_4^+ which resulted in decreased nitrification rates (Gordon et al., 2008; Haynes and Swift, 1986; Schwertfeger and Hendershot, 2013). In S1 and S2, the effect of leaching was amplified because at soil pH <6, there is a higher concentration NH_4^+ compared to NH_3, resulting in a higher loss of the nitrification substrate (NH_4^+) from leaching. This could explain why I observed greater than 50% reduction of AMO activity in S1 and S2 compared to S3 (~25%).

Leaching altered relative concentrations of metal mixture ratios in metal salt-spiked soils. Differences in metal loss was ray (metal) and soil (pH and CEC) dependent (Haynes and Swift, 1986; Huang et al., 2015; Smolders et al., 2015). There was a higher loss of metals in S1 and S2 due to possible competition by H^+, Ca^{2+}, and Mg^{2+} for sorption sites, resulting in a greater deviation of ratios in metal salt-spiked soils of S1 (pH=3.4) and S2 (pH=5.5). This is analogous to what has been reported in several studies (Haynes and Swift, 1986; Huang et al., 2015; Smolders et al., 2015). More metals were lost in acidic soils when leached (Smit and Van Gestel, 1998), but relative retention of metals is determined by the binding affinities of the metals (Tatara et al., 1998). Higher concentrations of metals with low binding affinities i.e. (Zn, Co and Ni) were lost compared to Pb and Cu (Figure 3-6b). Lead (Pb) and Cu have high affinities for organic matter, clay and other ligands, which explains their comparative retention (Huang et al., 2015; Lock et al., 2006; Schwertfeger and Hendershot, 2013). The loss of metals from leaching metal salt spiked soils ultimately altered the concentration of total metals in soils as reported by Stevens et al. (Stevens et al., 2003), Smolders et al. (Smolders et al., 2009) and Bongers et al. (Bongers et al., 2004).

Table 3-3. Equitoxicity of equal effect concentration ratios from metal types to soils enzymes in all three soils. Effects of mixture rays on enzyme activity (X) was either classified as Toxic, Slightly Toxic or Non-Toxic. If the activity was not significantly different from activity in control or higher (X≥100%), the ray was classified as Non-Toxic. If the activity was not significantly different or lower than 75% of the activity measured in control (X≤75%), the ray was classified as Toxic. If the activity was significantly higher than 75% of the activity in control, but significantly lower than activity in control (100%>X<75%), the ray was classified as Slightly Toxic.

Soil Name	Soil Enzyme	Metal Type Type	Equitoxic rays	Effect	Most Toxic Ray
S1	AMO	Salts	PC=PEAT=SUD	Non-Toxic	None
S2	AMO	Salts	All Rays	Toxic	All
S3	AMO	Salts	CSQG=FF=SUD	Toxic	CSQG
S1	AMO	Oxides	CSQG=FF=PEAT=PC	Slightly Toxic	CSQG
S2	AMO	Oxides	FF=PC=PEAT	Toxic	FF=PEAT
S3	AMO	Oxides	FF=PEAT=SUD	Toxic	FF=PEAT
S1	AMO	Spinel Mineral	FF=PEAT=PC=SUD	Non-Toxic	CSQG
S2	AMO	Spinel Mineral	CSQG=FF=PEAT	Toxic	SUD
S3	AMO	Spinel Mineral	CSQG=PC=PEAT	Slightly Toxic	FF
S1	ACP	Salts	CSQG=FF & PEAT=PC	Toxic & Slightly Toxic	CSQG=FF
S2	ACP	Salts	FF=PEAT=PC=SUD	Non-Toxic	CSQG
S3	ACP	Salts	CSQG=FF=PEAT	Toxic	CSQG=FF
S1	ACP	Oxides	All Rays	Toxic	CSQG
S2	ACP	Oxides	CSQG=FF=PEAT	Toxic	CSQG
S3	ACP	Oxides	CSQG=FF=PEAT=SUD	Slightly Toxic	CSQG
S1	ACP	Spinel Mineral	CSQG=FF=PEAT=PC	Toxic	CSQG=FF=PEAT=PC
S2	ACP	Spinel Mineral	FF=PC=PEAT=SUD	Non-Toxic	CSQG
S3	ACP	Spinel Mineral	All Rays	Non-Toxic	None

The "=" sign was used to denote rays of equal effects. Rays were classified as equitoxic if the responses were statistically similar (p-value>0.05). AMO=ammonia monooxygenases activity, ACP=acid phosphatases activity, PC=Port Colborne ray, PEAT=peaty ray, SUD=Sudbury ray, FF=Flin Flon ray and CSQG=Canadian soil quality guideline ray.

3.6.2 Toxicity of metal types

Apart from S1, metal salts were the most toxic metal type to both AMO and ACP in soils where toxicity was observed. The high dissolution rate of metals in salt-spiked soils is thought to be the reason for the high toxicity compared to other metal types (Lock and Janssen, 2003). Smolders et. al reported that leaching and ageing lead (Pb) salt spiked soils for five years reduced or eliminated their toxicity to plants, soil invertebrates and microorganisms (Smolders et al., 2015). More metals were leached as the major modifying factors of toxicity (i.e. pH and CEC) of the soils decreased, S3<S2<S1. As a result, the toxicity of metal salts to both soil enzymes in S1 was lower compared to other metal types in the same soil. These observations agree with previous studies where the toxicity of leached Ag, Ni, Pb and Zn spiked soils was reduced compared to unleached soils (Langdon et al., 2015; Lock and Janssen, 2003; Oorts et al., 2007; Smit and Van Gestel, 1998). It is important to note here that the low total metal concentration (background + fraction of nominal) in salt spiked soils did not explain differences in toxicity but the $CaCl_2$ extractable concentrations did. This confirms that speciation i.e. metal type is more important than total metal concentration in elucidating mixture toxicity.

AMO was more sensitive to metal mixtures in S1 and S2, and less sensitive in S3 compared to ACP. This finding agrees with Smolders et al. (Smolders et al., 2001b) who found AMO to be sensitive to metal toxicity and Borowik et al. (Borowik et al., 2012) who found ACP to be less sensitive compared to other soil enzymes. Ammonia oxidation is driven by autotrophic ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) that differ in niche preferences (Prosser and Nicol, 2012). Soil pH and ammonia/ammonium availability are factors that enhance niche specification and differentiation between the two microbes (Prosser and Nicol, 2012). Hence, the tolerance of AMO to metal mixtures at low pH could be explained by the presence of extremophilic AOA (Lu et al., 2015). This is because, high expression of autotrophic amoA genes (subunit A) by thermophilic Crenarchaea has been recorded particularly in low pH soils, resulting in an increase in the ratio of AOA to AOB gene abundance from less than 1 to greater than 100 with decreasing pH (Nicol et al., 2008).

3.6.3 Factors modifying extractable metals and toxicity of mixture rays

Mixture rays were not equally toxic between soils because of differences in leaching for salts and because of pH and CEC effects on oxide and spinel dissolution. As noted by others (Smolders et al., 2015; Stevens et al., 2003), leaching has very different effects across soils, and as a result, nominally equitoxic rays were not actual equitoxic rays (based on total) and thus, microbial responses differed. Even for oxides and spinel minerals, where leaching is not a concern, toxicity of metal oxides and spinel minerals have been linked to pH and CEC (Brallier et al., 1996; Smolders et al., 2004). Rays from spinel minerals were generally the least toxic because metals were strongly bound to iron oxides making them largely unavailable. The toxicity of rays from metal oxides was neither the most nor least toxic because metals were less soluble compared to metal salts and more soluble compared to spinel minerals.

Stronger correlations between pH and $CaCl_2$ extracted metals for metal oxides and spinel minerals were observed compared to metal salts (Figure 3-4). This was because metals are readily available when spiked as metal salts making surface complexation reactions with ligands a major determinant of availability (McBride et al., 2009). This is the probable reason why metal salts were comparatively more toxic. Hence, the availability of metal salts was controlled not only slightly by pH, but mainly by the availability of sorption sites (organic matter and clay) and other ligands in the soil. Unlike salts, the dissolution and desorption of metals in oxides and spinel minerals is highly dependent on soil pH, hence the strong correlation.

Extractions with DTPA and $CaCl_2$ extractions showed significant correlations with ACP activity in S1 and S2 but not S3 (Figure 3-4 and 3-5). The concentrations of $CaCl_2$ extractable metals for oxides was linked by soil pH (r = -0.59), but this was not the case for DTPA extractable metal concentrations (r = 0.14). As a result, higher concentrations of Cu and Pb with high affinities for ligands were extracted in DTPA in all soils (Figure 3-5). The strong correlations between enzyme activities in oxide spiked soils and $CaCl_2$ and DTPA extractable metals suggests that both DTPA and $CaCl_2$ are reliable methods for assessing metal availability of field contaminated soils, but toxicity of the metals may be dependent on the biological endpoint as observed in our study (McBride et al., 2009).

3.7 Conclusions

This is the first time spinel minerals have been synthesized and used in microbial toxicity testing. The role of metal mixture type and experimental design in modifying toxicity responses of soil enzymes has been established in this study. The study confirms that spiking soils with metal salts and leaching does not only inhibit microbial activities beyond metal effects but also compromises metal ratios in soils. The metal alternatives (i.e. oxides and spinel minerals) do not have to be leached or aged, and their toxicities are more dependent on soil properties compared to salts. It should be noted that the synthesis of spinel minerals required a significant laboratory effort to attain the desired nominal metal concentrations. Oxides are therefore recommended for metal and metal mixture toxicity tests when using a ratio ray design.

4 RESPONSE ADDITION (RA) IS MORE PROTECTIVE OF BIOGEOCHEMICAL CYCLES COMPARED TO CONCENTRATION ADDITION (CA): A METAL MIXTURE MODELING PERSPECTIVE

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4.1 Preface

The following chapter has been formatted for submission to Science of Total Environment journal with the following co-authors:

Mark cousins (University of Saskatchewan) – involved with experimental design, data analysis, and editorial;

Mathieu Renaud (University of Saskatchewan) – involved with experimental design, statistical analysis, and editorial;

Olukayode Jegede (University of Saskatchewan) – involved with experimental design and editorial;

Beverley Hale (University of Guelph) – co-supervisor involved with all aspects of project oversight.

Steven D Siciliano (University of Saskatchewan) – supervisor involved with all aspects of project oversight.

As the lead author, Kobby Awuah, was involved in every aspect of the article. More specially, Kobby performed approximately 30% of soil preparation and dosing, 80% of the lab work (enzyme assays, metal extractions and analysis), 90% of the data analysis and 95% of the manuscript writing.

This chapter focuses on evaluating the toxicity and sensitivity of quinary metal mixtures to the C and P cycles. The objective of the chapter was to assess CA as an appropriate mixture model for assessing the risks of metal mixtures to biogeochemical cycles.

4.2 Abstract

In soils, enzymes are crucial to catalyzing reactions and cycling elements such as carbon (C), nitrogen (N), and phosphorus (P). Although these soil enzymes are sensitive to metals, they are often disregarded in risk assessments, and regulatory laws governing their existence are unclear. Nevertheless, there is a need to develop regulatory standards for metal mixtures that protect biogeochemical cycles because soil serves as a sink for metals and receptor exposures occur as mixtures. Using a fixed ratio ray design, we investigated the effects of 5 single metals and 10 quinary mixtures of Zn, Cu, Ni, Pb, and Co metal oxides on two soil enzymes (i.e., acid phosphatases [ACP] and beta glucosidases [BGD]) in two acidic Canadian soils (S1: acid sandy forest soil, and S2: acid sandy arable soil), closely matched to EU REACH standard soils. Compared to BGD, ACP was generally the more sensitive enzyme to both the single metals and the metal mixtures. The EC_{50} estimates for single Cu (2.1 to 160.7 mmol kg^{-1}) and Ni (12 to 272 mmol kg^{-1}) showed that both metals were the most toxic to the enzymes in both soils. For metal mixtures, response addition (RA) was more conservative (overestimated mixture effects) in predicting metal effects compared to concentration addition (CA). For both additivity models, synergism was predicted at low effect levels, such as 10%, but antagonism occurred irrespective of effect levels. At higher concentrations, free and $CaCl_2$ extractable Cu protected both enzymes against the toxicity of other metals in the mixture. The results suggest that (1) assuming CA at concentrations less than EC_{50} does not protect biogeochemical cycling of C and P, and (2) Cu in soil may protect soil enzymes from other toxic metals and thus may have an overall positive role in soils with elevated metal concentrations.

Keywords: Acid phosphatases, beta glucosidases, metal mixtures, risk assessment, biogeochemical cycling.

4.3 Introduction

Soils play a major role in the biogeochemical cycling of carbon (C) and nutrients such as nitrogen (N) and phosphorus (P), but this role depends on soil health. Soil health is evaluated by considering a combination of soil physicochemical properties and biological parameters, including survival, reproduction, and activity of soil invertebrates and microbes. Soil microbes drive biogeochemical cycling of C, P and N because they synthesize and excrete several enzymes that are crucial to the decomposition of soil organic matter (SOM) (Burns, 1977; Madsen, 2011; Schimel and Schaeffer, 2012). During SOM decomposition, promoted by microbes, nutrients are released, which in turn fill the microbes' energy demands for growth (Luo et al., 2017). Similar to soil biogeochemical cycles, microbial efficiency requires optimal combinations of pH, organic carbon (OC), and cation exchange capacity (CEC) (Bartram et al., 2014; Insam and Domsch, 2013; Sarapatka et al., 2004; Yan et al., 2015). Since changes in soil health affect microbial activity, soil enzymes are a quick and robust indicator of soil health (Borowik et al., 2012; Chaperon and Sauvé, 2008; Smolders et al., 2001a).

Elevated metal concentrations can affect soil physicochemical qualities and be toxic to microorganisms and enzymes crucial to biogeochemical cycles (Chaperon and Sauvé, 2007; Hagmann et al., 2015; Wiatrowska et al., 2015). Metals alter these cycles by generating reactive oxygen species (ROS) which precipitate essential metabolites or deactivate enzymes (Khalid and Jin, 2016; Sobolev and Begonia, 2008). Microbes can detoxify metals through redox, methylation, and dealkylation reactions (Gadd, 1993; White et al., 1995). However, at higher concentrations, these detoxifying mechanisms are overwhelmed. Although the effects of single metals such as Zn, Cu, and Pb on soil enzymes are known, metal mixtures are responsible for most soil contamination and soil organism exposures to metal (Chaperon and Sauvé, 2008; Mertens et al., 2009; Oorts et al., 2007; Smolders et al., 2015). As a result, risk assessment of metals has shifted to accommodate typical exposures to metals as mixtures (Cedergreen, 2014; Nys et al., 2018).

The two reference models most widely used for estimating mixture toxicity are the concentration addition (CA) model and the response addition (RA) model. A common assumption for both models is that components of a mixture are non-interactive. However, the assumption for toxic modes of action (MoA) of mixture components differs for both models. For the CA model, there is an assumption that arithmetically, the overall effect of a mixture can be predicted from the addition of the potencies of the individual components of the mixture relative to their individual potencies when applied alone (Equation 4-1). Implicit in the CA model is the assumption that each component can be replaced by an equally effective component without altering the overall effect. The toxic unit (TU) is the ratio of the concentration (*c*) of a component (*i*) in a mixture eliciting a combined effect (EC_x) to the concentration of the same component eliciting EC_x when applied alone (Equation 4-2).

$$\sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = TU \qquad Equation\ 4\text{-}1$$

$$\sum TU_{EC_x} = \sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = 1 \qquad Equation\ 4\text{-}2$$

In Equations 4-1 and 4-2, c_i/EC_{xi} is the TU of a metal (i), which represents the fractional potency contributed by the metal (i) in the mixture. When used for assessing the risk of mixtures, antagonism at an effect level is inferred when the summation of the TUs (∑TU) is greater than 1 (less than additive), synergism (more than additive) when ∑TU is more than 1, and additive ∑TU is equal to 1.

In contrast to CA, RA assumes that the toxicity of each component of the mixture is independent of the others. Thus, the effects of the mixture will be less than the effects of the individual metals acting alone due to probability. RA is mathematically expressed in Equation 4-3, where $E(c_{mix})$ is the effect of the total mixture at a total concentration (c_{mix}), and $E(c_i)$ is the effect of the component (i) when applied singly at the same concentration as is present in the mixture. The effect of each metal is subtracted from 1 (i.e., control or no inhibition) to determine noneffects. The products of all noneffects is subtracted from 1 (probability of effect) to determine the marginal effect of the mixture.

$$E(c_{mix})=1-\prod_{i=1}^{n}[1-E(c_i)] \qquad \text{Equation 4-3}$$

It has been reported that 70% of mixtures have additive toxicity, 10-15% have antagonistic toxicity, and 10-15% have synergistic toxicity (Warne and Hawker, 1995). According to Ross (1996) and Ross and Warne (1997), additivity deviates by a factor greater than 2.5 for only 6% of the mixtures. Other studies have reported that CA is overly conservative and overestimates mixture toxicity, especially for metals (Nys et al., 2016; Versieren et al., 2016). Metal interactions produce either synergistic or antagonistic effects, rendering the current practice of applying CA in the risk assessment of metals quite problematic (Chaperon and Sauvé, 2007, 2008; Nys et al., 2016, 2017; Versieren et al., 2016). For example, Chaperon and Sauvé (2007) reported that the activity of urease and dehydrogenase enzymes were affected by exposure to a quaternary mixture containing Ag, Cu, Hg, and Zn. Furthermore, they found the effects of the metals on enzymes deviated from additivity and exerted a higher toxicity in agricultural soils. In aquatic systems, Nys et al. (2017) concluded that the combined toxicity of metals that individually exert less than 10% effect was greater than simply adding their individual effects.

The aim of this study was to improve our understanding of metal mixture toxicity to soil enzymes and to determine the sensitivity of two biogeochemical cycles (i.e., C and P) to metal mixtures containing Pb, Cu, Ni, Zn, and Co. These metals match what are typically found at Canadian base metal mining and smelting sites. We measured the effects of the mixtures on two soil enzymes, beta-glucosidase (BGD) and acid phosphatase (ACP), which are involved in the C and P cycles, respectively. BGD is a predominant soil enzyme and is responsible for catalyzing the hydrolysis of glucosidic bonds and biodegradation of plant debris to release glucose, an important C energy source for microbial metabolism (Eivazi and Tabatabai, 1988). ACP is a ubiquitous soil enzyme with strong correlations to P deficiency in soils and thus is a good indicator of soil fertility (Eivazi and Tabatabai, 1977). We determined the effects of single metals and 10 quinary mixtures on both soil enzymes and compared the responses to predictions from the two mixture toxicity reference models (i.e., CA and RA). We hypothesized that 1) CA, which is the current method used in mixture assessment, will hold true for all mixtures, and 2) the differences in metal mixture compositions will not alter toxicities to both enzymes.

4.4 Materials and methods

4.4.1 Soil sampling and treatments

Two Canadian soils were sampled for the study. Topsoil (~30cm depth) was collected, air-dried, and sifted using a 2-mm sieve. Background metal concentrations were determined by reverse aqua Regia (rAR) method, and metals were measured with an Agilent 5110 SVDV inductively coupled plasma optical emission spectrometer (ICP-OES) (Topper and Kotuby-Amacher, 1990). The CEC was determined using the methylene blue method (Yukselen and Kaya, 2008); soil pH was determined using 0.01 M $CaCl_2$ in a ratio of 1:5 (solid: liquid); soil texture was determined by the pipette method (Bouyoucos, 1962); soil organic carbon was determined by the LECO-C632 carbon analyzer (Wang and Anderson 1998); and, water holding capacity (WHC) was measured with open-ended test tubes with filter paper placed at the bottom to retain soil (Jaabiri Kamoun et al., 2018). The soils were stored in plastic containers until they were used for the experiment (rewetting to 65% of the WHC 2 weeks prior to toxicity testing). The soils were named S1 (Acid Sandy Forest) and S2 (Acid Sandy Arable) and had pH values of 3.4 and 4.6, respectively.

Table 4-1. Physicochemical properties of experimental soils

Soil	pH	CEC (meq/100 g)	Clay Content (g/kg)	Organic Carbon (g/kg)	Water Holding Capacity (ml/g)	EU PNEC Closest Reference	Background (mg/kg of soil)				
							Zn	Cu	Ni	Co	Pb
S1	3.4	8	45	17	0.29	Acid Sandy Forest	967	303	7	7	397
S2	4.6	16	110	25	0.35	Acid Sandy Arable	635	107	11	9	23

CEC = Cation exchange capacity

4.4.2 Mixture rays and metal dosing

Commercial metal oxides for Pb, Cu, Zn, Co, and Ni (≥97% purity) were purchased from Sigma Aldrich and used to spike the soils. Prior to soil amendment, the metal oxides were exposed to gaseous HNO_3 in a desiccator to remove inorganic carbon. The oxides were ground and weighed into the soils in their respective ratios (Table 4-2). Soils were spiked with single metals (12 doses) and 10 metal mixture rays (10 doses) (Table 4-2). Each mixture ray consisted of 5 heavy metals (Pb, Cu, Ni, Co, and Zn) at different ratios. The ratio of the metals was determined from regulatory concentrations (rays 1-4, 8), environmentally relevant concentrations (rays 7, 9, 10), and experimentally derived or arbitrary concentrations (rays 5, 6). For example, ray 4 was determined using the maximum allowable concentrations of the 5 metals according to the Canadian Soil Quality Guidelines (CSQG) for agricultural sites. Also, ray 3 was determined by averaging the predicted-no-effect-concentrations (PNEC) for each of the 5 metals from the EU-REACH PNEC calculator for clayey and peaty soils (Assessing risks of chemicals, 2017).

For the environmental rays (i.e., 7, 9, 10), samples from field-contaminated soils were collected and analyzed for total concentrations of the metals of interest and computed into rays. Ray 5 was computed following the CA assumption that the concentration of each metal in the mixture, relative to its individual effect when dosed alone, contributes equally to the overall effect. In contrast, ray 6 was computed so that the ratio of each metal in the mixture remained the same irrespective of effect. Hence, each ray consisted of 5 metals in fixed ratios (see Table 4-2 and Appendix B Table [B-2]).

Table 4-2. Fixed rays used for the full metal mixture toxicity tests by weight-by-weight (w/w) and molar (mol) ratios of the metals in the mixture.

		Dose composition									
		Co		**Ni**		**Cu**		**Zn**		**Pb**	
Ray	**Source**	**w/w**	**mol**	**w/w**	**mol**	**w/w**	**mol**	**w/w**	**mol**	**w/w**	**mol**
1	Reg.	0.097	0.123	0.109	0.138	0.155	0.182	0.421	0.479	0.218	0.078
2	Reg.	0.100	0.122	0.118	0.144	0.184	0.208	0.423	0.465	0.175	0.061
3	Reg.	0.110	0.135	0.123	0.152	0.206	0.234	0.372	0.412	0.190	0.066
4	Reg.	0.013	0.013	0.707	0.736	0.178	0.172	0.076	0.071	0.026	0.008
5	Exp.	0.090	0.110	0.110	0.134	0.160	0.181	0.470	0.516	0.170	0.059
6	Arb.	0.037	0.065	0.072	0.128	0.039	0.064	0.290	0.461	0.561	0.282
7	Env.	0.200	0.271	0.200	0.235	0.200	0.217	0.200	0.211	0.200	0.066
8	Reg.	0.294	0.396	0.088	0.119	0.147	0.183	0.147	0.178	0.324	0.124
9	Env.	0.050	0.080	0.064	0.102	0.157	0.232	0.260	0.374	0.469	0.212
10	Env.	0.003	0.003	0.003	0.003	0.202	0.216	0.726	0.755	0.066	0.021

Reg. = Rays derived from regulatory concentrations (e.g., CCME or EU-REACH)
Env. = Rays derived from environmentally relevant concentrations (e.g., metals in field-contaminated soils)
Exp. = Rays derived from experimentally derived concentrations (e.g., EC_{50})
Arb. = Randomly derived ray based on equal metal ratios

To calculate the 10 equitoxic dosing concentrations for each mixture ray, EC_{50} values of the 5 metals (Pb, Cu, Ni, Co, and Zn) derived from literature were used to calculate toxic units (TUs) ranging from 0 to 16 (Lock and Janssen, 2002a, 2002b, 2003; Sandifer and Hopkin, 1997a, 1997b).

4.4.3 Metal concentrations

4.4.3.1 Total metal concentration

Total metal concentrations in spiked soils were determined using x-ray fluorescence (XRF) (Margui et al., 2009). Soil (4 g air dried) was ground and homogenized with 0.8 g of Chemplex SpectroBlen 44µm powder to act as an adhesive. Samples were transferred into Chemplex pellet cups, covered with polypropylene thin-films, and placed into a pellet die set. The pellet set was mounted on a hydraulic press and a force of about 10,000 psi was applied to the samples for 5 minutes to form discs. The samples were analyzed on the Thermofisher ARL OptimX XRF analyzer (1 ppm detection limit) for total metal concentrations. For confirmation, we used the XRF to re-analyze 6 soil samples that had been previously analyzed with an Agilent 5110 SVDV inductively coupled plasma optical emission spectrometry (ICP-OES) for metal concentrations (reverse aqua Regia). Montana II was used as a standard reference material for the XRF with metal recoveries were between 90% to 95%.

4.4.3.2 Metal speciation

4.4.3.2.1 Calcium chloride extractable

The mobilizable metals were extracted using a 0.01 M calcium chloride ($CaCl_2$) solution (Quevauviller, 1998). Briefly, 2.5 g of dry soil was weighed into 50 mL test tubes, 25 mL of $CaCl_2$ solution was added, and the tubes were shaken on an end-over-end shaker for 180 minutes. Samples were centrifuged at 4704 g for 10 minutes, filtered (Whatman 0.45µm syringe), and analyzed with the Agilent 5110 SVDV ICP-OES for metal concentrations. Single element atomic absorption standards obtained from VWR and diluted with 0.01 M $CaCl_2$ to obtain standard concentrations of 0, 1, 5, 15, 30 and 50 mg/L of element. Duplicates, blanks and calibration standards run every 21 samples were used as quality control. The pH of the supernatant was measured prior to metal analysis.

4.4.3.2.2 Base anions and cations

Soil anions (i.e., Cl^-, NO^{3-}, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}) and major cations (i.e., Ca^{2+}, Mg^{2+}, K^+) were determined following Quevauviller's water method (Quevauviller, 1998). The concentration of anions was measured using a Dionex ICS-2000, an ion chromatograph equipped with a Chromeleon 7 software. The base cations were analyzed with the Agilent 5110 SVDV ICP-OES. Duplicates, blanks and calibration standards run every 21 samples were used as quality control.

4.4.3.2.3 Dissolved organic carbon (DOC)

The DOC was determined following Zsolnay, 2003. Briefly, 15 g of dry soil was weighed into 50 mL centrifuge tubes, and 30 mL of 0.005 M $CaCl_2$ solution was added. The sample was gently stirred with a glass rod for about a minute. The sample was centrifuged at 12,000 g for 10 minutes and filtered into 30-mL dram vials with a 0.45 µm polycarbonate filter and using a vacuum suction. Samples in the dram vials were analyzed for DOC using a Mandel total carbon analyzer. Percent coefficient of variation for injected replicates was less than 2%.

4.4.3.2.4 Free ion speciation modeling

The free ion speciation in the soil solutions was determined using Windermere Humic Aqueous Model (WHAM VII) for both soils (Nys et al., 2016; Tipping et al., 2011). When calculating speciation, the following assumptions were applied: room temperature was 298 K, partial pressure was 0.00038 atm; 65% of DOC was active as fulvic acid (FA); the dissolved organic matter contained 50% carbon by weight (Nys et al., 2016); and colloidal precipitates controlled the activities of ferric ($Fe [OH]_3$) cations (Tipping et al., 2011). WHAM VII to calculate metal speciation in soil solution. We derived free metal ions (Me^{2+}), metal hydroxides ($MeOH$, $MeOH_2$), metal complexes with anions, and FA-bound metal fraction (FA-Me) because free ion activity is more representative for metal mixture toxicity than total metal concentrations.

4.4.4 Soil toxicity tests

4.4.4.1 Beta glucosidases

Soil (0.1g) in 4 replicates that included a negative control, was weighed into 1.5 mL test tubes, 20 µL of toluene was added, and the samples were mixed and left in the fume hood for 15 minutes (Eivazi and Tabatabai, 1988). After 15 minutes, 400 µL of modified universal buffer (MUB, pH~6) and 100 µL of 50mM P-nitrophenyl-B-D-glucopyranoside were added to each sample. The samples were then incubated in a water bath at 37^{o} C for one hour (Eivazi and Tabatabai, 1977). Next, 100 µL 0.5 M $CaCl_2$ and 400 µL of 0.1 M Tris (hydroxymethyl) aminomethane (THAM) buffer (pH 12) were added to the samples. Samples were centrifuged at 13,148 g for 2 minutes and were read colorimetrically at a wavelength of 410 nm with the Biorad iMark 96-well plate reader.

4.4.4.2 Acid phosphatases

Soil (0.1g) in 4 replicates that included a negative control, was weighed into 1.5-mL test tubes, 20 µL of toluene was added, and the samples were mixed and left in the fume hood for an hour (Eivazi and Tabatabai, 1977). Next, 400 µL of 0.5 M acetate buffer (pH~5.8) and 100 µL of 10mM p-nitrophenyl phosphate were added to each sample, and the samples were incubated in a water bath at 37 ^{o}C for one hour (Eivazi and Tabatabai, 1977). The samples were then removed and placed on ice to stop the reaction. Samples were centrifuged at 13,100 *g* for 2 minutes and read colorimetrically at a wavelength of 410 nm with the Biorad iMark 96-well plate reader.

4.4.5 Data analysis

4.4.5.1 Soil enzyme activity

Soil enzyme activity was determined from the slope of the standard curve derived from concentrations of p-nitrophenyl (ACP and BGD read colorimetrically at a wavelength of 410 nm). The percentage activity was determined relative to control soils using Equation 4-4 below.

$$Activity = \frac{S_{a,j,} - S_{b,j,}}{C_a - C_b} * 100\% \qquad Equation\ 4\text{-}4$$

In Equation 4-4, $S_{a,j}$ and $S_{b,j}$ represent analyte (p-nitrophenol) concentrations for ray j extracted in incubated and non-incubated samples (negative control), respectively, and C_a and C_b represent analyte concentrations in controls.

All data were statistically analyzed with a 2-way ANOVA with the R (studio) program using significance levels of $\alpha = 0.05$ and 0.10 (R Core Team, 2018). Data were tested for normality and homogeneity of variance to determine if ANOVA assumptions were meet. To meet the assumptions of ANOVA data transformation was applied where necessary. The Tukey HSD test was used for testing multiple comparisons between rays. Pearson product moment bivariate correlations were performed between free and $CaCl_2$ metal concentrations and enzyme activity.

4.4.5.2 Dose response models

The dependent variable for the dose-response relationship was percent activity in dosed soils relative to control (Equation 4-4) and independent variable was XRF estimates of total metal concentration (Ritz and Strebig, 2016).Using a 3-parameter log-logistic function (Equation 4-5) we determined effective concentrations (EC_x) at 10%, 25%, and 50% for each metal from the dose-response models using the R package drc (Ritz and Strebig, 2016).

$$f(x) = 0 + \frac{d - 0}{1 + exp^{(b(log(x) - log\,(e))}} \qquad Equation\ 4\text{-}5$$

In the above quation (4-5), *b* represents the steepness of the dose-response curve, *d* is the upper limit of the response, *c* is the concentration variable, and *e* is the median concentration (EC_{50}).

4.4.5.3 Mixture analysis

Mixture effects and interactions between metals were evaluated using CA and RA. For CA, TUs for all rays were calculated using Equation 4-1 for effective concentrations EC_x (EC_{10}, EC_{25}, EC_{50}). A four-parameter log-logistic function was used in fitting relative activity in mixture-spiked soils and the sum of TUs at each EC. Deviations from 1 (i.e., $\sum$TU with confidence intervals) were classified as antagonistic (overestimation by mixture model) or synergistic (underestimation by mixture model) using a t-test. For RA, the predicted activity from the RA model (Equation 4-3) was subtracted from the observed activity. The computed difference was defined as synergistic when negative values were obtained, while positive values were defined as antagonistic. Computed values within ±10 were defined as precise predicted by the RA model. The performance of both models was evaluated by comparing the observed versus the predicted mixture effects using the root mean square error (RMSE).

4.5 Results

4.5.1 Single metal experiments.

Well-defined dose response relationships were observed for the single metals in both soils, except for Ni (ACP in S1) and Co (BGD in S1 and S2), where no defined sigmoidal responses were observed (Figure 4-1). As a result, EC_{10} and EC_{25} estimates were not available for both enzymes in S2, and the reported estimates for Ni (ACP in S1) and Co (BGD in S1 and S2) will not be discussed in our results. Table 4-3 summarizes the effective concentrations EC_x

(EC$_{10}$, EC$_{25}$, EC$_{50}$) of enzyme activities (ACP and BGD) expressed as mmol kg^{-1} of total metal (Pb, Cu, Ni, Zn, and Co) in both soils.

Table 4-3. Single metal effective concentrations (EC$_{10}$, EC$_{25}$, EC$_{50}$) in soils S1 and S2 for acid phosphatases (ACP) and beta glucosidases (BGD)

	S1	**Lead (mmol kg^{-1})**	**Copper (mmol kg^{-1})**	**Nickel (mmol kg^{-1})**	**Zinc (mmol kg^{-1})**	**Cobalt (mmol kg^{-1})**
	EC10	1.43	0.03	-----	7.8	127.5
ACP	EC25	6.9	0.25	0.01	26.7	230
	EC50	33	2.1	13.6	91.7	416
	EC10	3.4	0.03	4.6	37	-----
BGD	EC25	13	0.4	112	77	-----
	EC50	52	5.3	272.3	160.2	2.7
	S2					
	EC10	46.7	88.1	12.3	30.8	3.4
ACP	EC25	112.8	91.8	15.7	33.4	11.9
	EC50	250	95.3	19.5	40	36.2
	EC10	91.8	23.6	0.003	16.5	-----
BGD	EC25	97	61.6	0.18	39.3	-----
	EC50	102.7	160.7	12	93.4	47.7

----- = Not estimated from model

The mean activity of both soil enzymes decreased as the concentrations of single metals increased (Figure 4-1). Complete inhibition of either enzyme, however, was not observed for any of the single metals. The toxicity thresholds for the single metals varied widely between soils and enzymes, with the EC$_{50}$ for ACP ranging from 2.1 to 416 mmol kg^{-1} and 19.5 to 250 mmol kg^{-1} for S1 and S2 respectively, representing a variation of 198 and 13-fold respectively (Table 4-3). Effective concentrations (EC$_{x}$) expressed in a molar basis showed that in S1, ACP was generally more sensitive to all metals than to BGD. In S2, enzyme sensitivity varied with metal and EC$_{x}$. For example, at EC$_{10}$, BGD was more sensitive to Cu, Ni, and Zn, while at EC$_{50}$, it was less sensitive to Cu and Zn (Table 4-3). Comparing only EC$_{50}$ values, the results revealed that Cu was the most toxic single metal in S1, while Ni was the most toxic in S2.

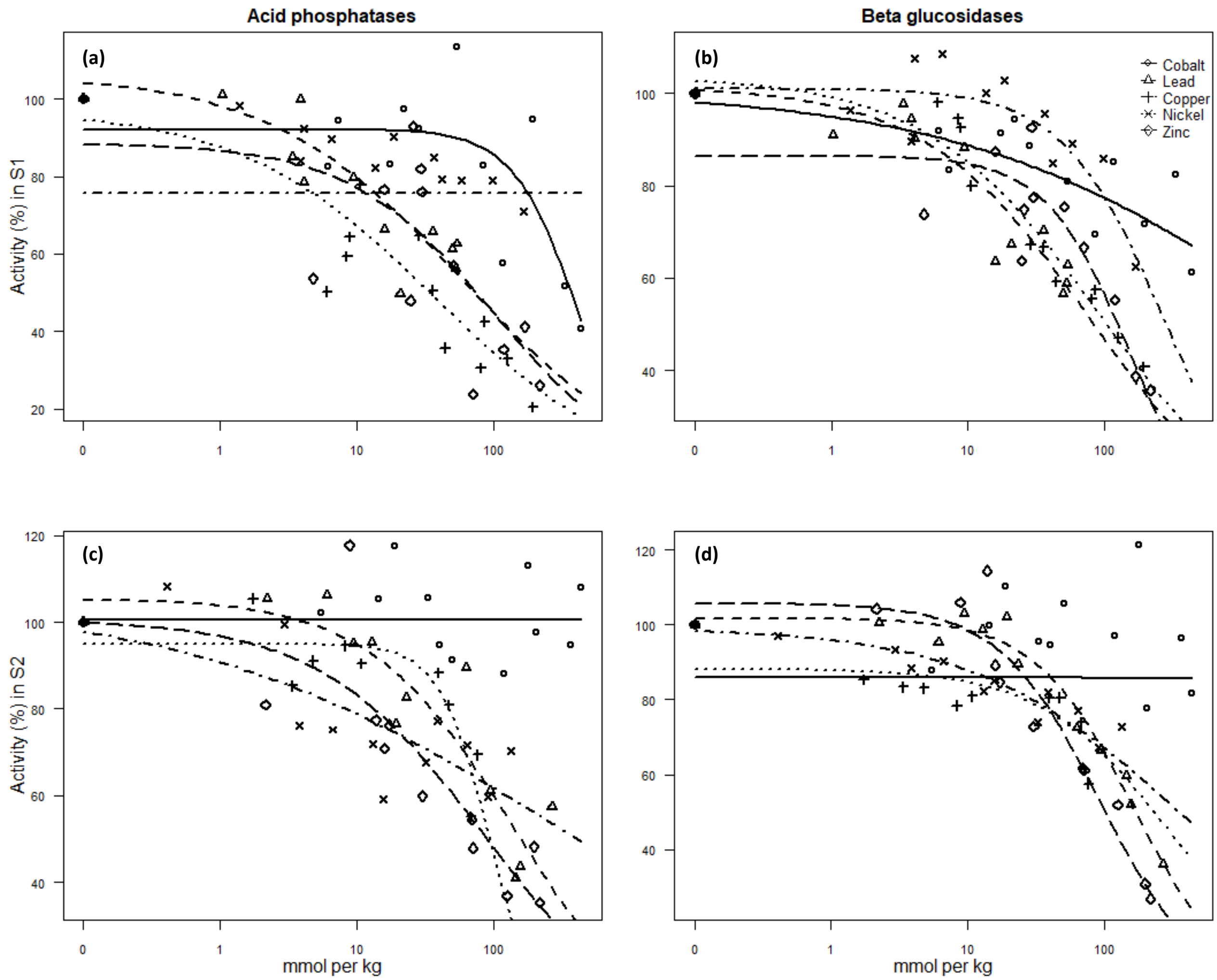

Figure 4-1. Acid phosphatases (ACP) shows a higher sensitivity to single metals than beta glucosidases (BGD). The figure shows the concentration response relationship for the two soil enzymes (acid phosphatases [ACP] and beta glucosidases [BGD]) and five metals (Co, Pb, Cu, Ni, Zn) in two soils (S1 and S2), fitted by a log-logistic model. Y-axis represents the percentage activity of enzymes in soils, and x-axis represents the concentration of metals in soils. Grid (a) ACP response to five metals in S1, Grid (b) BGD response to five metals in S1, Grid (c) ACP response to five metals in S2, Grid (d) BGD response to five metals in S2. Well defined DRC were not derived for the solid and dashed horizontal lines i.e. Co and Ni respectively.

4.5.2 Combined toxicity of metal mixtures

4.5.2.1 Concentration addition

For each ray, the observed percentage activity of both enzymes in single-metal and metal mixture-spiked soils was expressed as TU following Equation 4-1 (calculated from total metal concentrations). Figure 4-3 shows the toxicity response for the 10 metal mixture rays consisting of Pb, Cu, Ni, Zn, and Co and the response of the enzyme ACP (Appendix B Figures [B-1] and [B-3] for BGD response). TUs were calculated for EC_{10}, EC_{25}, and EC_{50} for all 10 mixture rays and compared the results to the CA model (Table 4-4). TUs calculated for BGD (TU_{50} for S1 and $TU_{10,25}$ for S2) and ACP (TU_{10} for S2) were not presented because we felt that the large magnitude of the results was unreliable. Deviations from 1 were classified as interactions between metals (i.e., synergism or antagonism).

Table 4-4. Toxic units (∑TUs) calculated for 10 mixtures rays for acid phosphatases (ACP) and beta glucosidases (BGD) in both soils.

S1		**Ray 1**	**Ray 2**	**Ray 3**	**Ray 4**	**Ray 5**	**Ray 6**	**Ray 7**	**Ray 8**	**Ray 9**	**Ray 10**	**Concentration Addition 95%CI**
ACP	$\sum TU_{10}$	6.6e-03^{S}	1.5e-03^{S}	1.5^{CA}	1.6e-03^{S}	3.5e-03^{S}	1.3^{CA}	6.1e-05^{S}	5.9e-04^{S}	2.8e-03^{S}	15^{A}	0.9 - 3.8
	$\sum TU_{25}$	0.007^{S}	0.003^{S}	0.92^{CA}	0.04^{S}	0.02^{S}	1.8^{CA}	0.001^{S}	0.002^{S}	0.02^{S}	3.8^{A}	0.9 - 2.2
	$\sum TU_{50}$	0.03^{S}	0.01^{S}	0.66^{CA}	0.03^{S}	0.08^{S}	1^{CA}	0.02^{S}	0.01^{S}	0.04^{S}	1^{CA}	0.6- 2.7
BGD	$\sum TU_{10}$	1.3^{CA}	0.75^{CA}	5.9^{A}	8.4^{A}	13.4^{A}	0.4^{CA}	2.2^{A}	1.4^{CA}	4.8^{A}	16.1^{A}	-23.5-1.6
	$\sum TU_{25}$	0.53^{CA}	2.0e-04^{CA}	1.4^{CA}	2.98^{A}	3.5^{A}	7.6^{A}	2.2^{A}	2.9^{A}	4.98^{A}	1.7^{CA}	-1.3-2.0
S2												
ACP	$\sum TU_{25}$	0.01^{S}	5.23^{CA}	0.003^{S}	0.62^{S}	4.3^{A}	0.00^{S}	0.04^{S}	1.3^{CA}	0.23^{S}	2.13^{CA}	1.0- 3.5
	$\sum TU_{50}$	0.36^{CA}	10.2^{CA}	0.1^{CA}	2.6^{CA}	10^{CA}	0.01^{CA}	0.3^{CA}	6.6^{CA}	0.9^{CA}	6^{CA}	-12.1-13.8
BGD	$\sum TU_{50}$	>20^{CA}	>20^{CA}	11^{A}	9^{CA}	6^{CA}	7^{CA}	12^{CA}	6^{CA}	5^{CA}	>20^{CA}	-1.3 – 10.5

Values with superscript “CA” follow the CA model. Values with superscripts “S” synergistically deviated from CA, and values with superscript “A” antagonistically deviated from CA.

$\sum TU_{10}$ = TUs calculated from EC_{10}
$\sum TU_{25}$ = TUs calculated from EC_{25}
$\sum TU_{50}$ = TUs calculated from EC_{50}

The CA model consistently underestimated the mixture effects on ACP activity in S1; thus, the CA model predicted lower effects. For rays 3 and 6, CA precisely predicted mixture effects irrespective of the EC, while the predictions for ray 10 were only accurate at the median concentration (EC_{50}) but overestimated at lower ECs. In general, the predictions from the CA model for ACP were only 20-30% accurate at all three ECs, while the actual effects were 70% synergistic and ≤10% antagonistic (Table 4-4). For ACP in S2, the deviations at EC_{25} were 60% synergistic and 10% antagonistic, while 30% followed CA. At the median concentration (EC_{50}), 100% of the mixture effects followed CA.

For BGD in S1 where only two EC_x (EC_{10} and EC_{25}) were obtained from the model, only antagonistic deviations from CA were observed (Table 4-4). For both ECs, CA predicted 40% of the mixture effects to BGD, while the deviations constituted 60% of the predictions. For S2 (BGD) CA predicted 90% of the mixture effects at EC_{50} with only 10% antagonistic deviations. In general, we observed that CA underestimated the effects of the mixtures at lower EC_x (EC_{10}, EC_{25}) and either predicted or overestimated the effects at higher EC_x. When we compared the combined mixture effects on both soil enzymes at all ECs, we observed that 25% of the effects followed CA, 25% of the effects were overestimated, and 50% of the effects were underestimated (Figure 4-2a).

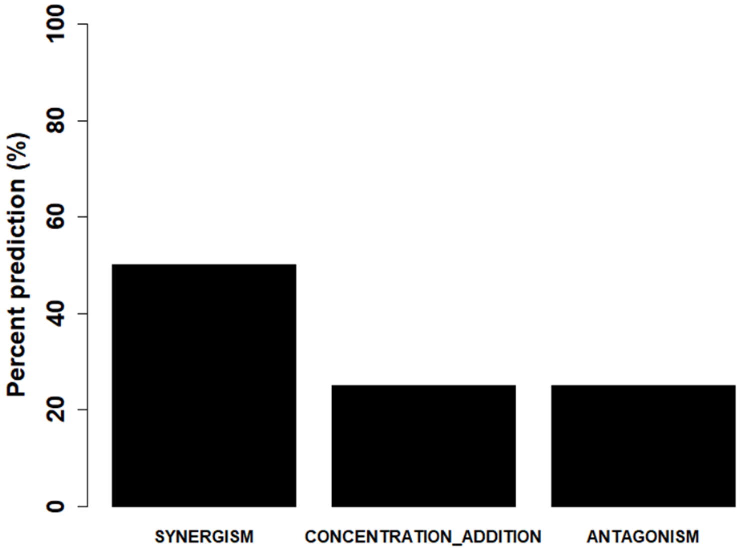

Figure 4-2a. Predictive performance of the concetration addition model shows a 50% underestimation of metal mixture effects on the biogeochemical cycling of C and P.

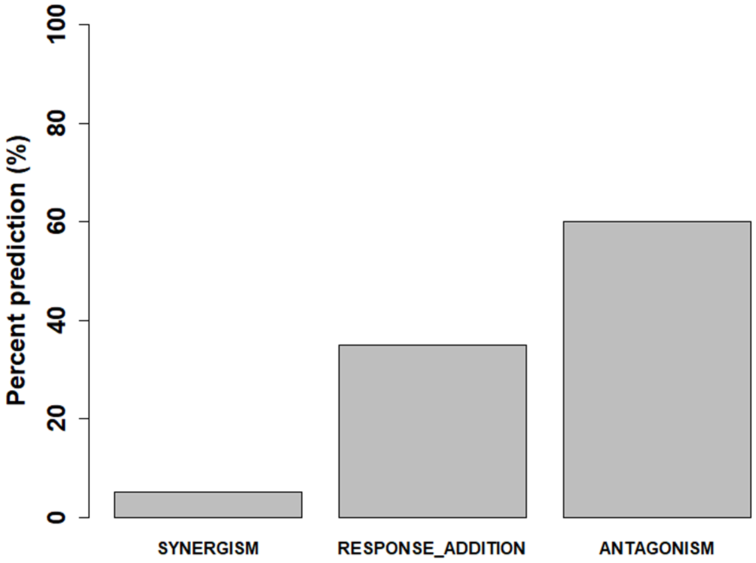

Figure 4-3b. Predictive performance of the response addition model shows a 60% overestimation of metal mixture effects on the biogeochemical cycling of C and P.

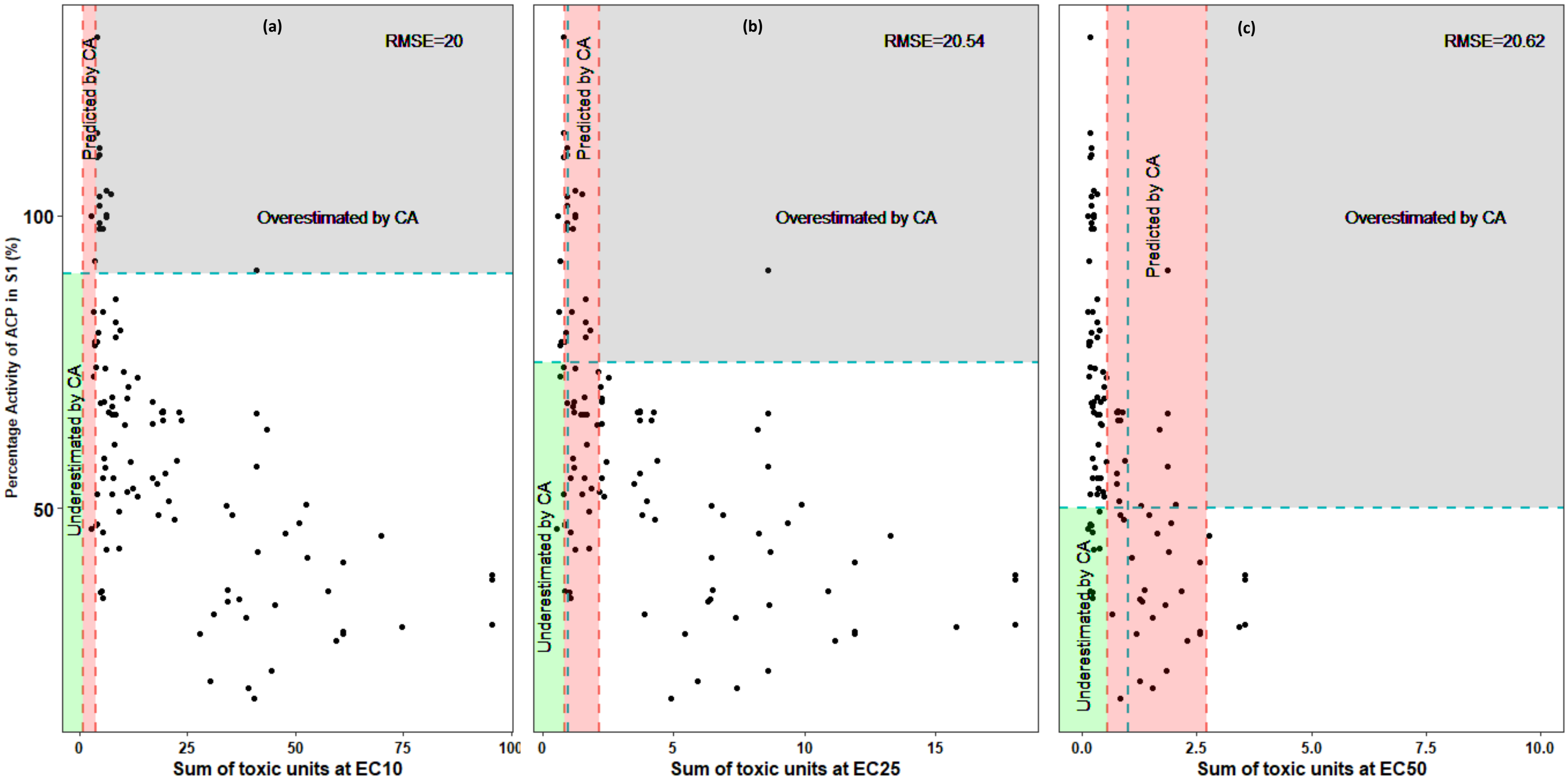

Figure 4-4. Concentration addition (CA) does not predict mixture toxicity to ACP below EC_{50}. The figure shows the concentration response relationships between acid phosphatases (ACP) and 10 mixture rays in S1 fitted by a log-logistic model. Y-axis represents the percentage activity of the enzyme, and X-axis represents the toxic units (TUs) of the metals calculated from effective concentrations. Grid (a) TUs calculated from EC_{10}, Grid (b) TUs calculated from EC_{25}, Grid (c) TUs calculated from EC_{50}. The intersection between the two blue dashed lines represent the point where CA occurs for the specified effective concentration, and the red shaded area represents the 95% confidence interval. The green-shaded quadrant represents underestimation by the CA model, and the grey-shaded quadrant represents overestimation. The root mean squared error (RMSE) for each TU is reported.

4.5.2.2 Response addition

Overall, RA overestimated the effect of the mixtures on the enzymes in both soils, compared to CA. Based on the root mean square errors (RMSE), better predictions from the RA models were obtained for S2 compared to S1 (Figure 4-4). Also, lower RMSE values were obtained for the enzyme BGD compared to ACP, signifying better predictions for the latter. Observed and predicted data show both antagonistic and synergistic deviations of the mixtures from RA to ACP and BGD. The deviations were more antagonistic than synergistic for both enzymes. Specifically, RA overestimated the effects of the mixtures 60% of the time, underestimated the effects 5% of the time, and predicted the effects 35% of the time. The underestimated effects were observed mostly at total metal mixture concentrations below 10,000 mg/kg soil, but antagonistic deviations were observed across all concentrations. When we compared the combined mixture effects on both soil enzymes, we observed that 50% of the effects followed RA, 60% of the effects were overestimated, and 5% of the effects were underestimated (Figure 4-2b).

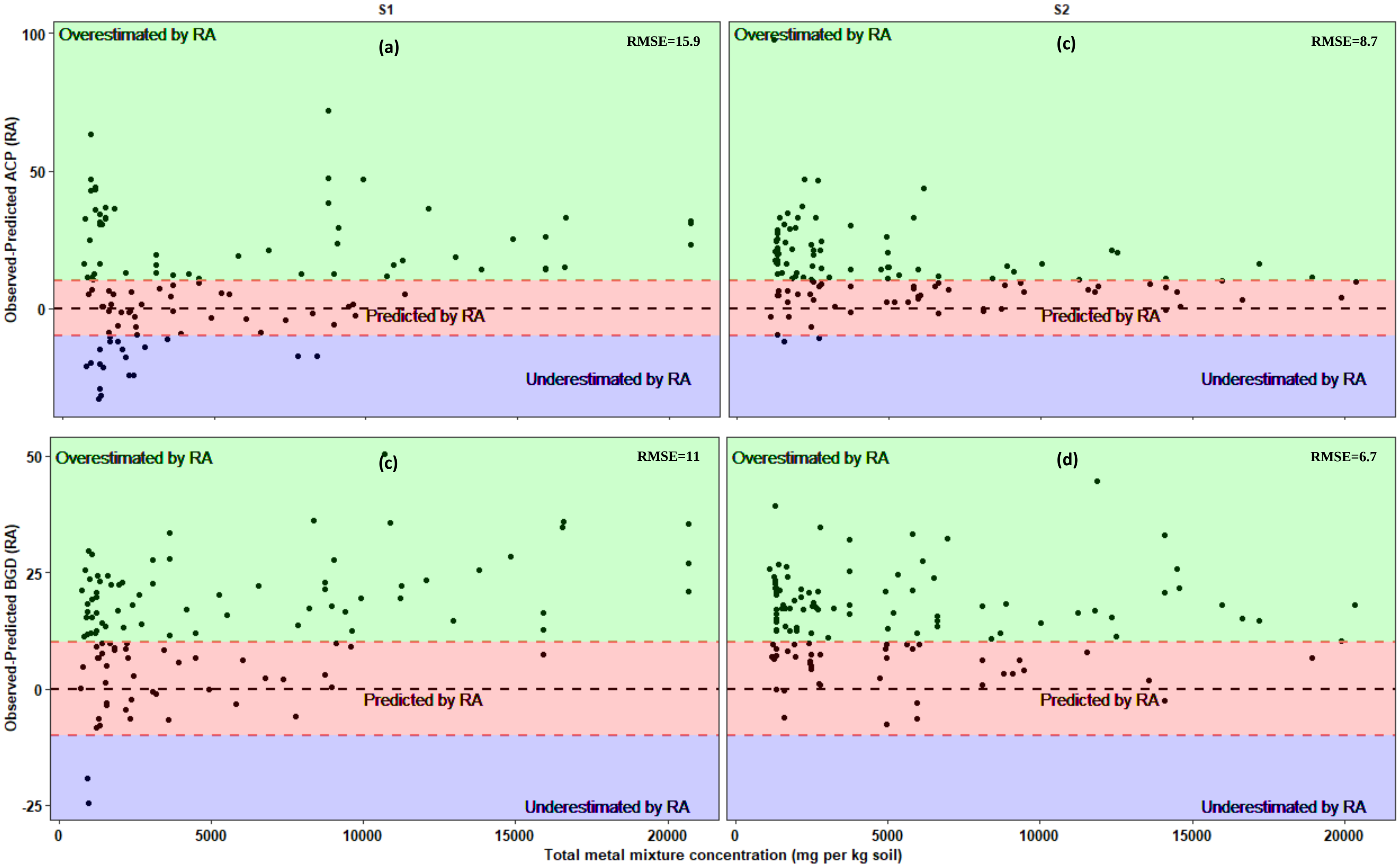

Figure 4-5. RA predicts antagonism at all dose levels and synergism at lower concentrations (<10000 mg kg^{-1}). The x-axis shows the total concentration of mixtures in mg kg $^{-1}$, and the y-axis shows the difference between observed and predicted activity for ACP (Grids a and b) and BGD (Grid c and d), S1 on the left, and S2 on the right. Broken black line indicates the RA model, and the red shaded portion represents 95% confidence interval. Points in the green shaded portion are overestimated by RA, while points in the blue shaded area are underestimated by RA.

4.5.3 Effects of soils and rays on enzyme activity

Soil was a significant factor in determining the effect of metals on both ACP and BGD in this study (Table 4-5). The activity of both enzymes in metal-spiked soils was comparatively lower in S2 relative to S1. No differences in mixture rays were observed for the ACP enzyme in both soils. Differences in rays, however, were observed for the BGD enzyme. Specifically, in S1, ray 9 was significantly less toxic to BGD in S1 compared to rays 1 and 4, while in S2, ray 9 was significantly less toxic to BGD compared to rays 3 and 10 (Appendix B Table [B-1]).

Table 4-5. Two-way ANOVA table for enzymes, soils, and rays

Activity	Predictors	SS	Df	MS	F	*p*-value
ACP	Soil	7515	1	7515	12.047	0.00***
	Ray	9180	9	1020	1.635	0.11
	Soil: Ray	5081	9	565	0.905	0.52
	Residuals	136002	218	624		
BGD	Soil	12139	1	12139	55.875	0.00***
	Ray	6622	9	736	3.387	0.00***
	Soil: Ray	3647	9	405	1.865	0.06
	Residuals	47361	218	217		

Significance codes: * < 0.05, ** < 0.01, *** < 0.001
*SS = sum of squares
*Df = degrees of freedom
*MS = mean square
*ACP = acid phosphatases
*BGD = beta glucosidases

4.5.4 Metal speciation as related to toxicity.

4.5.4.1 Relationship between enzyme activity and total, free, $CaCl_2$ extractable and FA-bound metal concentrations

Both enzymes showed significant decreased activity with increases in total mixture concentrations of Pb and Zn. In S1, increases in Ni concentrations exerted significant toxic effects on ACP activity, while increases in Co concentrations exerted toxic effects on BGD. The free and $CaCl_2$ extractable metal concentrations also showed that increases in the concentrations of Pb, Zn, and Ni caused decreases in the activity of both enzymes in both soils (Table 4-6). In contrast, the enzymes increased with concentrations of Cu in both soils, signifying a possible interaction and alleviation of toxicity by Cu. Free and CaCl$_2$-extractable Ni exerted toxic effects on ACP in both soils, but no significant toxic effects were observed for BGD (Table 4-6). FA-bound Zn and Co increased with increasing enzyme activity in both soils (Table 4-6). Conversely, FA-bound Pb in both soils and FA-bound Ni in S2 decreased with increasing enzyme activity.

Table 4-6. Influence of metal mixtures in total, CaCl$_2$ extractable, free ion, and fraction-bound to fulvic acid on soil enzyme activities in both soils; no interactions specified in the models

S1	**Total mg kg^{-1}**	**R^2**	**CaCl$_2$ µg g^{-1}**	**R^2**	**Free ion (M)**	**R^2**	**FA bound**	**R^2**
ACP	-Pb** -Zn** -Ni**	0.44	+Cu* -Zn* -Ni** -Pb*	0.48	-Ni** +Cu* -Zn* -Pb*	0.45	+Co** +Zn*	0.57
BGD	-Pb** -Co** -Zn**	0.62	-Zn**	0.47	-Zn**	0.45	+Co** -Pb**	0.46
S2								
ACP	-Pb** -Zn**	0.56	+Cu* -Zn** -Ni*	0.53	-Ni* +Cu** -Zn**	0.42	-Ni** +Zn** -Pb*	0.57
BGD	-Pb* -Zn**	0.31	-Zn**	0.43	-Zn*	0.33	-Pb*	0.31

Positive sign (+) = increase in enzyme activity with increase in metal concentration
Negative sign (-) = decrease in enzyme activity with increase in metal concentration
ACP = acid phosphatases
BBG = beta glucosidases
Significance codes: * *< 0.05, * < 0.1

4.6 Discussion

4.6.1 Single metal toxicity to soil enzymes

In order to understand the effects of single metal and metal mixtures on the global biogeochemical cycling of C and P, we used ACP and BGD enzymes as model endpoints. Results showed less sensitivity of the soil enzymes to metal oxides compared to what has been reported in other studies where metals were spiked as salts (Chaperon and Sauvé, 2008, 2007). We found that the EC$_{50}$ for Cu (2.1 to 160.7 mmol kg^{-1}) and Zn (12 to 272 mmol kg^{-1}) were up to 6-fold higher compared to what has been reported for metal-salt-spiked soils (Chaperon and

Sauvé, 2008, 2007). The differences in ECs were explained by others to be a result of increased solubility of metals spiked as salts and the indirect effects of salts on microbial toxicity and soil ionic strength (Awuah et al., 2019; Schwertfeger and Hendershot, 2012; Stevens et al., 2003). For metal oxides, solubility and availability is primarily driven by soil solution pH and CEC, and higher toxicity is expected in soils with low pH and CEC (Schwertfeger and Hendershot, 2012; Stevens et al., 2003). This explains the higher sensitivity observed for both enzymes in S1, which has a lower pH and CEC compared to S2. EC_{50} estimates of the metals in both soils (Table 4-3) showed that ACP were more sensitive to metal oxides compared to the BGD estimates, which is consistent with results from several studies (Borowik et al., 2012; Moreno et al., 2003). Others reported that Ni and Cu were the most toxic metals to soil enzymes including phosphatases, beta glucosidases, and dehydrogenases, while Zn was the least toxic (Chaperon and Sauvé, 2008, 2007; Khalil et al., 1996; Moreno et al., 2003). As reported by Feng et al.(2016) we also found Cu and Ni as the most toxic single metals to both enzymes (Figure 4-1 and Table 4-1). For Co, the model unsatisfactorily fitted the data in both soils, hence the estimates were unreliable despite our efforts to test other models.

4.6.2 Predictability of metal mixture effects by CA and RA

The predictions from both additivity models (i.e., CA and RA) showed significant synergistic and antagonistic deviations, suggesting possible interactions between metals (Khalil et al., 1996; A. Liu et al., 2017; Nys et al., 2017; Versieren et al., 2016). This finding supports Cedergreen et al. (2008), who observed similarities in predictions derived from both additivity models for metal mixtures. Even though the models were largely inadequate in predicting mixture effects to both enzymes, the results derived may have profound implications on risk assessment. For example, the toxicity of the mixtures to the less sensitive endpoint (BGD) in both soils followed CA 60-70% of the time with mostly antagonistic deviations. In contrast, the more sensitive endpoint (ACP) followed CA only 30% of the time in both soils with mostly synergistic deviations. Furthermore, RA mostly overestimated (60% antagonistic) the toxic effects of the mixtures to both endpoints. This suggests that RA might more conservative and protective of both endpoints, while CA will only be accurate for the less sensitive endpoint. It should be acknowledged that the assumptions of the models are completely reliant on an understanding of the MoAs of the mixture components, which are currently not fully understood. However, the differences in slopes obtained from our single dose response data suggests that the metals have dissimilar modes of action (Table 4-7) (Chaperon and Sauvé, 2008, 2007).

Table 4-7 Single metal dose response slopes for two soil enzymes in soils

	Lead	Copper	Nickel	Zinc	Cobalt
ACP/S1	0.62	0.61	0.00	0.82	1.84
BGD/S1	0.72	0.69	1.17	1.41	0.35
ACP/S2	2.28	0.83	0.32	0.72	5.22
BGD/S2	0.92	1.21	0.57	1.17	0.00

ACP/S1 = dose response for acid phosphatases in S1
BGD/S1 = dose response for beta glucosidases in S1
ACP/S2 = dose response for acid phosphatases in S2
BGD/S2 = dose response for beta glucosidases in S2

Additionally, the observed deviations from the models conformed with the funnel hypothesis suggested by Warne (2003) and Warne and Hawker (1995), where simple mixtures have highly variable toxicities (i.e., antagonism or synergism), while complex mixtures with greater than 10 components follow additive toxicity This hypothesis implies that assuming additivity at the regulatory level for fewer than 10 metals, particularly at low effective concentrations, might not be protective. Furthermore, Nys et al. (2017) showed that combining Zn, Pb, and Cd in individual effect concentrations <10% can yield a combined effect of up to 66%. Our study accordingly showed that, despite the occurrence of antagonism across all tested concentrations, mixture effects more than additive were only observed at lower concentrations

(Figures 4-3 and 4-4). The results of this study imply that CA might only be a conservative tool for 1) assessing mixtures at high concentrations, and 2) assessing mixtures using less sensitive endpoints (Cobbina et al., 2015).

4.7 Effect of soil and ray on mixture toxicity

Soil was the dominant factor determining enzyme activity and sensitivity to the metals. In contrast, mixture composition had no detectable effect on dose response. Studies have shown that ACP activity decreases with an increase in soil pH, which possibly increases its sensitivity to stressors (Liu et al., 2017; Sarapatka et al., 2004; Wang et al., 2018). Thus, soil properties like pH and CEC can modulate metal mixture toxicity to enzymes (Awuah et al., 2019; Wang et al., 2018; Ying et al., 2017). In the study by Awuah et al. (2019), the equitoxicity of the mixture rays was questionable due to differences in metal types and leaching effects. In the current study, however, we found no differences in the overall effects of the mixture rays to ACP and found very few differences for BGD (3 differences in rays per soil) (Table 4-5). Hence, the ratios of metals in the mixture did not alter the overall effect of the mixture, particularly for ACP. This finding suggests that, the presence of the metal in the mixture might be more significant than the relative concentration of the metal for soil enzymes (Luo and Rimmer, 1995; McKenna et al., 1993; Nys et al., 2018). Furthermore, its been reported that the presence Zn in metal mixture combinations determines the adherence of the effects to either RA or CA (Nys et al., 2017). The results further confirm the role of metals in modifying mixture toxicity (Awuah et al., 2019).

When we compared TUs, we observed differences between enzymes, soils, and concentration levels (Table 4-4), suggesting that interactions and deviation from additivity may be dependent on dose, endpoint sensitivity, and soil characteristics (Karaca et al., 2010). According to the funnel hypothesis, less biologically sensitive endpoints that require high concentrations of mixture components to exert a toxic effect would deviate from additivity (Warne, 2003; Warne and Hawker, 1995). We can also infer that more sensitive endpoints requiring low concentrations to exert toxic effects will deviate from additivity. In our case, synergistic deviations were observed for the more sensitive enzyme (ACP), while antagonistic deviations were observed for the less sensitive enzyme (BGD). Chaperon and Sauvé (2007, 2008) observed that synergistic effects are likely to occur in soils with high metal availability. Again, for the more sensitive enzyme, more synergistic deviations were observed at lower concentrations for CA (EC_{10}, EC_{25}) and RA (<10,000 mg kg^{-1} of total mixture) in both soils, with greater synergism observed in S1 (lower pH and CEC) compared to S2.

4.8 Ability of copper (Cu) to protect against mixture toxicity

Despite Cu being one of the most toxic single metals in the study, observed trends suggested that Cu in a mixture with Pb, Co, Zn, and Ni may be interacting to indirectly protect biogeochemical cycles against other metals. It has been reported that some biomarkers can be more sensitive to single metals compared to mixtures, hence the most toxic single metal could be protective in a mixture (Versieren et al., 2017). In the regression analysis, we observed that the concentration of Cu in both $CaCl_2$ and free metal extracts positively correlated with the activities of ACP and BGD in both soils, while Zn, Pb, and Ni exerted toxic effects (negative correlation) in the mixture (Table 4-6). We hypothesize that the presence of other metals (i.e. Zn, Co, Pb, and Ni) reduces the availability and uptake of Cu, causing lower toxic effects compared to Cu alone. Also, Cu strongly binds to organic matter, and can cause the displacement and increased availability of less toxic metals like Zn (Cedergreen, 2014).

Furthermore, it has been reported that Cd and Cu can increase Zn availability (Luo and Rimmer, 1995; McKenna et al., 1993). When the bioavailability of less potent metals like Zn are increased by other metals in a mixture due to competitive binding, the overall effect of the mixture would be expected to be lower than the most toxic metal alone. Because the average concentration of Cu in all mixture rays was about 16%, with Zn and Pb at 34% and 24% respectively, it is possible that Cu could be acting as an antioxidant, potentially protecting the enzymes from other metals in the mixture. Versieren et al. (2017) reported that Zn alleviates the toxicity of other metals in mixtures, which is contrary to what was observed in this study. However, both Cu and Zn are essential metals necessary for the function of several metalloenzymes that act as antioxidants (Agency for Toxic Substances and Disease Registry, 2013, 2004a). Furthermore, Zn concentrations in $CaCl_2$-extracted and free metals were consistently higher than other metals (Table 4-2), supporting the finding that metals like Cu increase the ability to extract Zn due the strong binding affinity of Cu. Excessive Zn has been reported to stimulate generation of reactive oxygen species and subsequent cellular damage (Lee, 2018). This is a possible reason why Zn was not observed to be a protective metal in this study.

4.9 Conclusions

In this study, we showed that the biogeochemical cycling of C and P in soils are both sensitive to metal mixtures, with P cycling being more sensitive than C cycling. Furthermore, the findings show that both RA and CA are inadequate in predicting metal mixture effects on both biogeochemical cycles, especially at lower concentrations of environmental concern. However, compared to the CA model, the RA model might be more conservative for both cycles and will be more appropriate for risk assessment schemes if the goal is to be protective of these cycles. Despite the accuracy of CA in predicting the effects of metal mixtures, the concentrations were high and will be unprotective of sensitive endpoints. The conservativeness of the RA model despite its comparative inaccuracy and overprotectiveness might be a better approach to characterize risks associated with biogeochemical cycles.

5 INTRODUCING ADVERSE ECOSYSTEM SERVICE PATHWAYS (AESP) AS A TOOL IN ECOLOGICAL RISK ASSESSMENT.

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5.1 Preface

The following chapter has been submitted to Environmental Science and Technology journal with the following co-authors:

Olukayode Jegede (University of Saskatchewan) – involved with experimental design and editorial;

Beverley Hale (University of Guelph) – co-supervisor involved with all aspects of project oversight.

Steven D Siciliano (University of Saskatchewan) – supervisor involved with all aspects of project oversight.

As the lead author, Kobby Awuah, was involved in every aspect of the article. More specially, Kobby performed approximately 100% of soil preparation and dosing, 80% of the lab work (enzyme assays, metal extractions and analysis), 90% of the data analysis and 95% of the manuscript writing.

This chapter focuses on identifying relationships between soil properties and measured soil processes and functions in the presence and absence of metal mixture contamination. The objective of the chapter was to determine relationships between soils properties and processes and use the relationships as a tool to improve site specific risk assessment of metal mixtures.

5.2 Abstract

When properly functioning, soils provide people with numerous ecosystem services (i.e., benefits), such as food production and water purification. These ecosystem services result from soil organism interactions and activities, which are supported by the soil physicochemical properties. Risk assessment for this complex system requires understanding the relationships among its components, both in the presence and absence of stressors including potential toxic elements (PTE). To better understand the soil ecosystem and how exposure to PTE impacts ecosystem services, I developed a novel quantitative technique, the adverse ecosystem service pathway (AESP) model that uses soil properties to evaluate effects of contaminants on ecosystem services. I sampled 47 soils across the Canadian Prairies and analyzed them for soil properties that included pH, cation exchange capacity, organic carbon, and percent clay. I spiked the samples with a mixture containing Pb, Cu, Ni, Zn, and Co and then measured 15 soil processes representing five ecosystem services. Using a Pearson bivariate correlation matrix, I confirmed that ecosystem services are closely linked to soil properties, especially cation exchange capacity and organic carbon. Results from t-tests also showed that, except for the three soil enzyme activities measured ($p < 0.05$), the processes underlying ecosystem services are significantly reduced in metal-impacted soils. Using soil properties as the main predictors of ecosystem services, I built two AESP models: one for metal-impacted soils and another for control soils. These models showed adverse effects to ecosystem services in metal-impacted soils, depicted as changes in partial correlation coefficients. An AESP model, therefore, can be an important tool to better understand complex ecosystems and improve risk assessment and natural resource management.

Keywords: Ecosystem services, adverse ecosystem service pathways, structural equation models, metal mixtures, risk assessment.

5.3 Introduction

Human industrial and agricultural activities release chemical environmental stressors, including petroleum hydrocarbons, fluorinated chemicals, bisphenols and phthalates, and potentially toxic metals (PTE), that significantly impact regional and global ecosystems. These chemical stressors potentially affect organisms that influence key ecosystem processes and functions, particularly microbes, invertebrates, and plants (Defarge et al., 2018; Gainer et al., 2019; Motta et al., 2018; Nys et al., 2017). Of all chemical stressors, PTE are of particular interest because they are not biodegradable, and although they can be toxic (Awuah et al., 2019; Jegede et al., 2019), some are essential for daily metabolic activities (Agency for Toxic Substances and Disease Registry, 2013, 2005, 2004a, 2004b). Although organisms are usually able to tolerate relatively high concentrations of essential potentially toxic metals (PTE) (e.g., Cu, Zn), they can only tolerate low concentrations (Haferburg and Kothe, 2007; Lenart-Boro and Boro, 2014) of non-essential PTE with no known biological roles (e.g., Cd, Pb). Toxic effects are inevitable with any metal when concentrations exceed toxicity thresholds and the detoxification and repair mechanisms of an organism are overwhelmed (Awuah et al., 2019; Jegede et al., 2019).

Many researchers have studied the toxicodynamics of PTEs at the lowest level of biological organization (i.e. molecular level) that adversely impact soil organisms (Buekers et al., 2007; Van Gestel, 1997). The adverse outcome pathway (AOP) depicts the cascade of events after an organism is exposed to a stressor (e.g., metal contaminant), that leads to adverse changes at higher levels of biological organizations (i.e., population, community, and ecosystem) (Ankley et al., 2010). These adverse changes to organisms impact their activities, processes, and roles in the ecosystem, which potentially reduce the benefits that people derive from the ecosystem.

Humans derive many and diverse benefits from a well-functioning ecosystem. These benefits are collectively known as ecosystem services (ES) (Hanson et al., 2012), and they maintain conditions for life on earth. These benefits include, for example, the provision of food and water, nutrient recycling, purification of air and water, and waste decomposition and detoxification (Hanson et al., 2012). Furthermore, ES are produced from ecosystem functions, including biogeochemical processes resulting from microbial, invertebrate, and plant activities in ecosystems (Buggenhoudt, 2017). For instance, soil invertebrates feeding on detritus (an activity), release locked nutrients (a process) for plant and microbial use (an activity) and for livestock, wildlife, and human consumption (a benefit). Several studies have demonstrated that soil properties influence biological processes and functions that underlie ES, while others have confirmed that PTEs are toxic to soil biota and that soil properties alter its bioavailability and thus impact metal toxicity(Awuah et al., 2019; Bergamin et al., 2015; Insam and Domsch, 2013; Murphy et al., 2011; Princz et al., 2010; Saidi, 2012; Smolders et al., 2015; Tueche, 2014; Van Eekeren et al., 2010). Because of the strong links between ES and soil organism activities, several ES can be compromised when soil organisms are exposed to metal stressors (Ding et al., 2018; Hayes et al., 2018).

I wanted to explore the cascade of events that affect soil processes, functions, and provision of ES. Thus, I developed a conceptual model—called an adverse ecosystem service pathway (AESP)— representing the physical, chemical, and biological events that adversely impact ES in soil (Figure 5-1). Our AESP systematically assesses the impacts of chemical stressors. While both AOPs and AESPs investigate outcomes after an organism is exposed to a stressor, an AOP identifies the mechanisms leading to impaired functions, whereas an AESP identifies how impaired functions impact ES (Figure 5-1). With an AESP, therefore, I examined how exposing soil organisms to PTE impacts soil ES. The AESP approach is beneficial because it assesses the function of the entire system rather than its individual components, and it can be used to evaluate and predict the effects of multiple stressors on a regional or global scale. This approach would significantly improve terrestrial risk assessment.

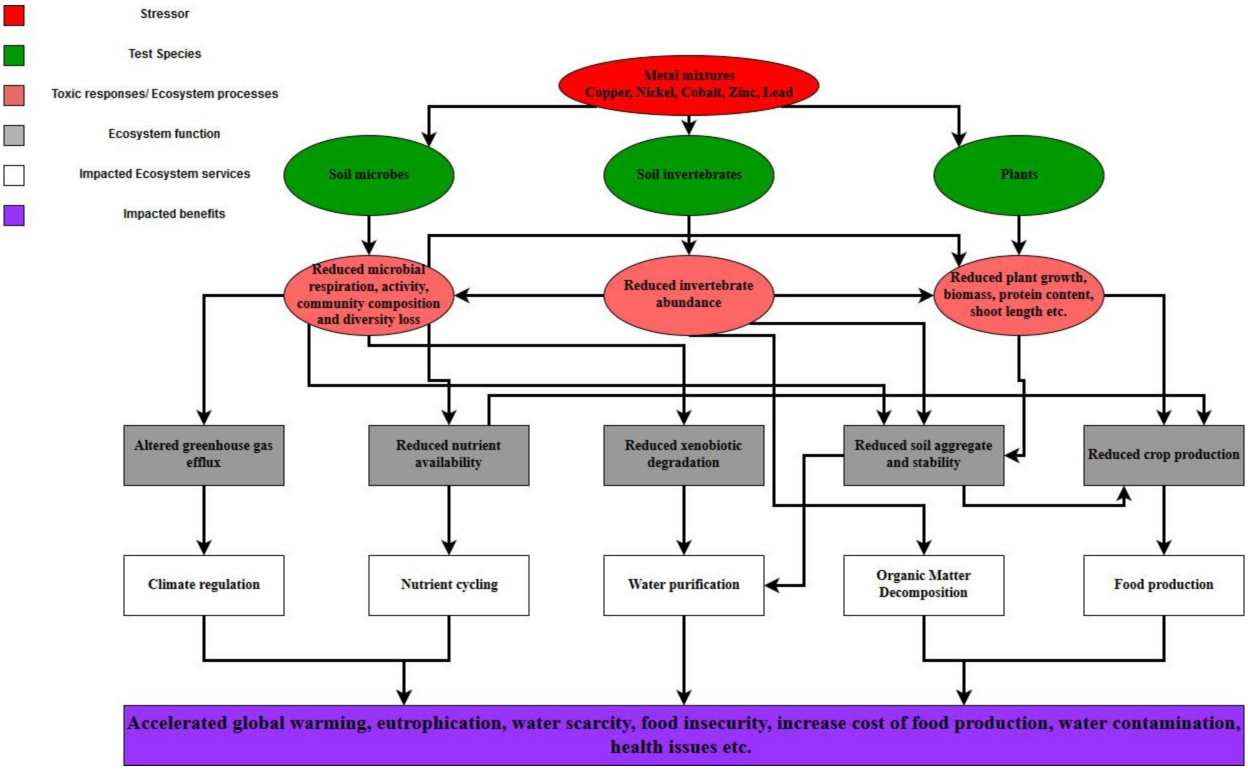

Figure 5-1. Theoretical illustration of direct and indirect pathways leading to potential impacts on soil ecosystem services from metal mixtures; an Adverse Ecosystem Service Pathway.

Complex biological, chemical, and physical interactions among soil components create various soil functions (Delgado and Gomez, 2016). For example, soil enzyme activity that support biogeochemical processes (e.g., acid phosphatases) positively correlates with percent soil organic carbon but negatively correlates with soil pH (Kumari et al., 2017; Wang et al., 2018). Also, organic matter decomposers *E. fetida* and *E. cripticus* show strong survival and reproduction relationships with soil pH between 4 and 9 (Jänsch et al., 2005) but up to 100% mortality outside of this pH range. The mortality relationship between decomposers and pH, however, is complicated in soils with elevated concentrations of PTEs, as the bioavailability (and thus toxicity) of the PTEs to the organic decomposers can be pH-dependent, depending on which PTEs are present. Thus, an effective statistical approach to modeling adverse effects of soil contaminants is to partition how soil properties (such as pH) directly affect organism survival and reproduction and how the effects are modified both directly and indirectly in the presence of contaminants. Jegede et. al (2019) reported that, survival and reproduction of mites exposed to Zn is determined by the habitat quality of the soil, and not concentration of Zn. Due to the organizational complexity of the soil ecosystem, a structural multivariate analysis that can model more than one dependent variable is useful to risk assessment. Furthermore, an analysis that can handle more than one fixed or random independent variable will be advantageous for evaluating how stressors alter the links among soil properties and soil functions.

Structural equation modeling (SEM) is a powerful multivariate tool that can analyze relationships among variables in complex systems (Stenegren et al., 2017), hence the interactions and underlying mechanisms that drive ES derived from soils were investigated with SEM. I selected SEM because unlike most multivariate methods that are generally descriptive and suitable for exploratory analyses, SEM is capable of quantifying networks of causal hypotheses, making it more suitable for studying systems (Grace et al., 2018; Mamet et al., 2017). It allows the testing of simultaneous interactions and effects, both directly and indirectly, instead of single causal effects (Lefcheck, 2016; Mamet et al., 2017). The building block of SEMs is determined from the researcher’s assumptions that are derived from reported studies, which are then tested with derived data (Grace et al., 2012). The hypothesized network is represented as series of (non)linear equations that produce an expected covariance matrix which is compared with the observed covariance matrix derived from experimental data (Mamet et al., 2017). The chi-square statistic (or any other statistical measure of congruence) is then used to compare both covariance matrices for validation and acceptance.

SEM has been successfully used to elucidate relationships between N_2 fixation and greenhouse gas emissions in the Arctic; study links between N_2 fixation and plant community structure; and examine the effects of seed size and seeding density on oat yields (Lamb et al., 2011, 2014; Siciliano et al., 2014; Stewart et al., 2013). Furthermore, a SEM study conducted in the Arctic and Antarctic regions linked soil microbial community richness to soil fertility (defined as organic matter, nitrogen, and chloride content) and community composition to soil pH (Siciliano et al., 2014). Traditional SEM assumes that links between components are linear and that the data display multivariate normality (Lefcheck, 2016). Generalized SEM (gSEM) extends traditional SEM to include non-normal, non-linear links between structural components; therefore, probability density functions other than normal can be modelled (Lefcheck, 2016; Lombardi et al., 2017; Stenegren et al., 2017). Other multivariate methods have been proposed for investigating soil functions, ecosystem services and effects of contaminants in soils (Parelho et al., 2016; Piva et al., 2011). Those models are based on biological indicators, whereas AESP is built using soil properties as predictors of ES which allows AESP to leverage existing soil mapping resources in a predictive fashion.

I sampled 47 soils from most of the agricultural regions across the Canadian Prairies and exposed these samples to metal mixtures. Using SEM, I evaluated direct and indirect theoretical causal relationships between 4 soil properties (i.e., pH, percent clay, organic carbon [OC], and cation exchange capacity [CEC]) and 5 ES (organic matter decomposition, nutrient cycling, water protection, food production, and climate regulation). I also evaluated the response of the five ES to quinary metal mixtures. These four soil properties were chosen for their reported relationships with activity and survival of soil organisms and their role in modulating metal bioavailability and toxicity (Awuah et al., 2019; Jänsch et al., 2005; Kumari et al., 2017; Wang et al., 2018). The link between individual soil sample ecosystem services and aggregate ecosystem services was established in Bru et al. (2010) in which individual soil properties were linked to N cycling across a 31,500 km^2 area in France. Like our samples, the samples in Bru covered a largely agricultural region with varying properties. Thus, I feel that linking soil properties to ecosystem service indicators on an individual soil sample basis, and then extrapolating those links to larger geographical areas, is a reasonable approach.

I consecutively determined (1) the survival and reproduction of the following three soil invertebrates: *Oppia nitens*, *Folsmia candida*, and *Enchytraeus crypticus* (proxy for organic matter decomposition); (2) the activity of three soil enzymes: acid phosphatases (ACP), ammonia monooxygenases (AMO), and beta glucosidases (BGD) (proxy for nutrient cycling); (3) the degradation of glyphosate (proxy for water protection); (4) the biomass and percent protein of *Elymus lanceolatus* (proxy for food production); and (5) the fluxes of three greenhouse gases: CH_4, CO_2, and N_2O (proxy for climate regulation). The endpoints were selected because 1) they represent provisional, regulation and supportive services that are essential to human survival; and 2) there are existing standardized methods for their determination. Our objectives were to test hypothetical causal relationships between soil properties and the endpoints measured in control soils. The effects of sub-lethal metal mixture concentrations on these relationships (i.e., using the AESP model) were to be explored and the effects of metal mixtures on soil-ecosystem service relationships (soil-ES) were to be established. Metal oxide mixtures were used to reflect scenarios in which large areal extents are impacted, such as those typically found around metal extraction and processing facilities. The mixture was selected to represent one such facility and a dose, known to cause effects, chosen. I hypothesized that metal mixtures would alter soil-ES relationships in the 47 test soils.

5.4 Materials and methods

The study was conducted in the major agricultural zones of three Canadian prairie provinces: Alberta, Saskatchewan, and Manitoba, which cover approximately 1,780,650 km^2. A stratified random sampling method was used to sample a total of 47 soils from different soil zones (brown, dark-brown, black, dark gray, and gray) to include a diverse range of soil properties. Most of the soils were within the Prairie and Boreal Plain ecozones, with a few from the Boreal Shield ecozone (Figure 5-2).

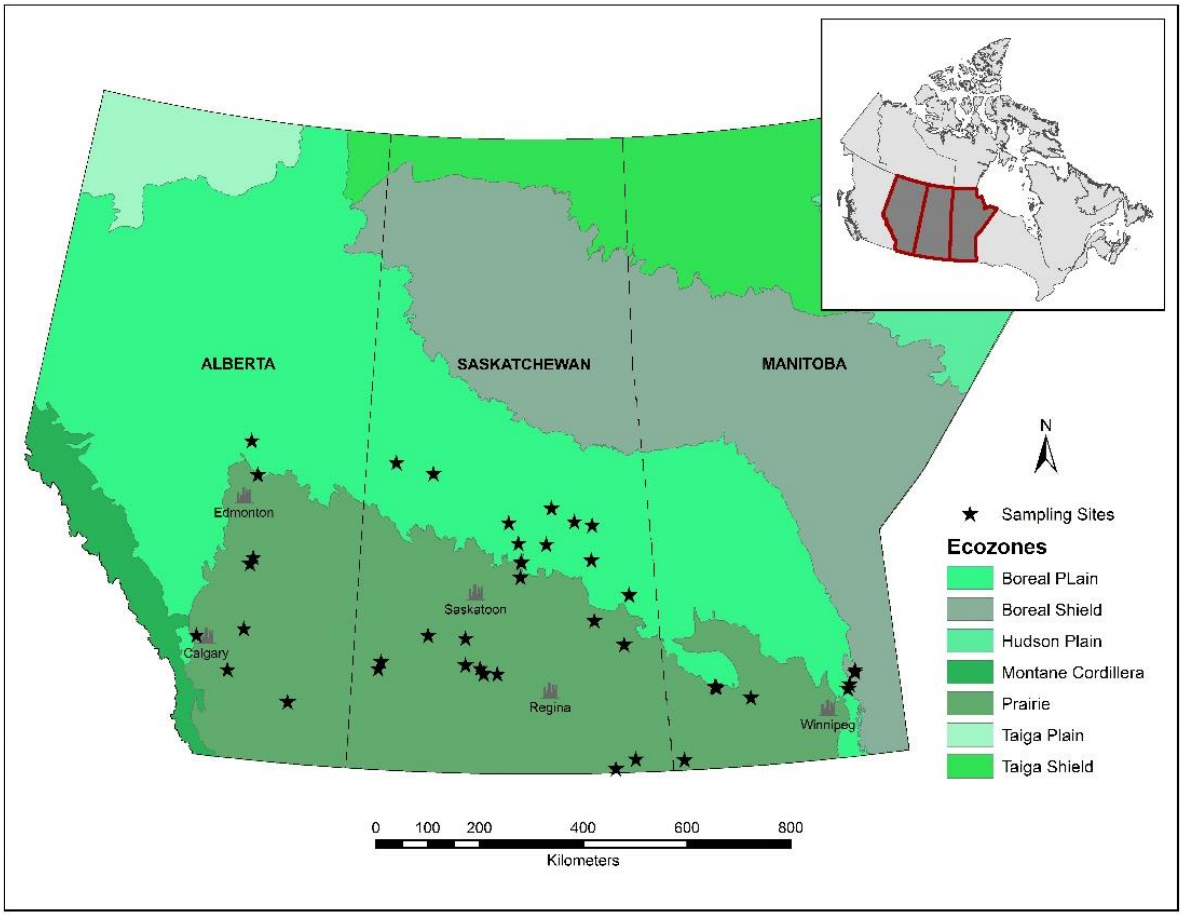

Figure 5-2. Map of the study area in Canada showing major cities in the three provinces where soils were sampled. Black stars represent soil sampling sites. Map inset at the top right shows the map of Canada with the Prairie Provinces highlighted in gray with red boundaries to ease geographical visualization.

5.4.1 Soil sampling and treatments

Topsoil (~30cm depth) from all 47 sites was collected, air-dried, and sieved to 2 mm. About 250 kg of topsoil was collected from each sampling location and homogenized. Background concentrations of N, P, K, S, Cu, Mn, Zn, B, and Fe were determined at ALS Laboratory Group in Saskatoon. Available Ca, Mg, K, P, Fe, Mn, Cu, Zn, B, S, Pb, Al, Cd, NO_3-N, and NH_4-N were determined using plant root simulator probes (Card, 2012). CEC was determined using the methylene blue method (Yukselen and Kaya, 2008); soil pH, by placing a pH meter in a 1:5 soil:0.01 M $CaCl_2$ mixture (Environment Canada, 2005); the percentage of clay in the soil (texture), using the pipette method (Bouyoucos, 1962); soil OC, using the LECO-C632 carbon analyzer (Wang and Anderson, 1998); and water holding capacity (WHC), using open-ended test tubes (Jaabiri Kamoun et al., 2018). Soil pH ranged from 4.55 to 8.34; percent clay, 2.35 to 62.2; percent OC, 0.27 to 36; CEC, 9.9 to 34.8 meq $100g^{-1}$; and WHC, 0.15 to 0.73 ml g^{-1}. The soils were stored at room temperature in closed plastic containers in the dark until they were used for the experiment. The soils were re-wetted (to 65% of the WHC) two weeks prior to dosing with metal mixtures and for toxicity testing.

5.4.2 Soil dosing

All 47 soils were dosed (w/w) with a dry mixture of Pb, Cu, Ni, Zn, and Co that reflected the dominant ratios of heavy PTE in contaminated soils at Port Colborne, Ontario (Awuah et al., 2019; Dan et al., 2008). PTE were added as commercial metal oxides (>99% purity) purchased from Sigma-Aldrich. Prior to dosing, the metal oxides were exposed to gaseous HNO_3 in a desiccator for 48 hours to remove inorganic carbon. The PTE were then dried, ground, and weighed into the soils in their respective ratios. Using a toxic unit (TU) approach and assuming these PTEs were behaving under conditions of concentration addition, soils were dosed at 12 TU (Table C-2) (Awuah et al., 2019). They were dosed at a single total concentration of 6,250 mg of PTE per kg of soil, with Pb, Cu, Ni, Zn and Co in ratios of 0.026, 0.178, 0.707, 0.076, and 0.013 respectively (Awuah et al., 2019). Moisture content of the soils was kept at 60% of their WHCs after dosing for all tests. Total metal concentration in spiked soils was determined using an X-ray fluorescence method (MarguÃ- et al., 2009). Calcium chloride extractable PTE were measured with the Agilent 5110 ICP-OES instrument as described earlier in this thesis (Awuah et al., 2019; Quevauviller, 1998). Briefly, 2.5 g of dry soil was weighed into 50 ml test tubes, 25 mL of $CaCl_2$ solution was added and shaken on an end-over-end shaker for 180 minutes (Awuah et al., 2019).

Samples were centrifuged at 4704 g forces for 10 minutes, filtered with a Whatman 0.45μm syringe filter and analyzed with the Agilent 5110 SVDV ICP-OES for dissolved metal concentrations. Standard metal mixture solutions (VWR Zn, Cu, Ni, Pb and Co standards) prepared from a serial dilution (1, 5, 15, 30, and 50 mg/ L) with 0.01 M calcium chloride were used as standards. The quality control included blanks, duplicates and calibration standards that were run every 21 samples.

5.4.3 Measurement of ecosystem services (ES)

5.4.3.1 Climate regulation

Greenhouse gases were determined by measuring soil fluxes of CO_2, CH_4, and N_2O. Briefly, 5 g of soil (i.e., control and spiked) was incubated in triplicates at room temperature in serum vials sealed with a rubber septum lid and a metal crimped-on seal. Gas samples were taken at 0, 6, and 72 hours with a syringe, and injected into vacuumed vacutainers. To maintain vial pressure, the air was replaced by pure N_2 after sampling. Samples in the vacutainers were analyzed with a Scion 456-GC (a gas chromatographer) for concentration of greenhouse gases. See appendix C section 10.1 for information on how actual greenhouse gas effluxes were back calculated to account for N_2 dilutions.

5.4.3.2 Food production

To analyze food production, I used *Elymus lanceolatus*, an approved test species commonly known as thickspike or northern wheatgrass. Prior to testing, a seed viability test was conducted with tetrazolium chloride(Bennett and Loomis, 1948; Porter et al., 1946). Seeds were then planted in pots (400g dry soil) with metal-spiked and control soils (no replication), following planting procedures described in Environmental Canada protocols (Environment

Canada, 2005). The test was performed in controlled chambers at 24 ^{o}C (± 2 ^{o}C), with full spectrum fluorescent lighting (18,750 ± 6250 lx) and a 16 h of light/8 h of darkness cycle. After 35 days, the above-soil biomass was harvested, oven dried at 65^{o}C for 5 hours, ground and weighed in grams for total N analysis (Thomas et al., 1967). The total N was then converted to crude protein using a factor of 5.7 (Mariotti et al., 2008).

5.4.3.3 Nutrient cycling

Colorimetric methods described in Awuah et. al (Awuah et al., 2019; Berg and Rosswall, 1985; Eivazi and Tabatabai, 1988, 1977) were used for both spiked and control soils to determine the activity of three soil enzymes: ammonia monooxygenases (AMO), acid phosphatases (ACP), and beta glucosidases (BGD). These enzymes play significant roles in the biogeochemical cycling of nitrogen (AMO), phosphorus (ACP), and carbon (BGD). For AMO, 2g of each soil was weighed into 15 mL test tubes (4 replicates), to which 10 mL of 1mM NH_2SO_4 and 0.5 mL of 1.5 M $NaClO_3$ were added. This was shaken end-to-end for 6 hours at room temperature, and 2 mL of 2 M KCl was added and shaken again for about 2 minutes. The samples were then centrifuged with an Eppendorf miniSpin Plus at 3,011 g for 4 minutes and read colorimetrically at 545nm with the Biorad iMark 96-well-plate reader (Smolders et al., 2001b). For ACP and BGD 0.1g of soil (4 replicates each including a negative control) was weighed into 1.5 mL test tubes, 20 µL of toluene was added, mixed and the tubes were left in the fume hood for an hour for ACP and 15 minutes for BGD (Eivazi and Tabatabai, 1988, 1977). To ACP, 400 µL of 0.5 M acetate buffer (pH~5.8) and 100 µL of 10mM p-nitrophenyl phosphate were added to each sample, while 400 µL of modified universal buffer (MUB) (pH~6.0) and 100 µL of 10mM p-nitrophenyl-B-D-glucopyranoside were added to the BGD test tubes. The samples were incubated in a water bath at 37^{o} C for one hour (Eivazi and Tabatabai, 1977). The

ACP samples were then removed and placed on ice to stop the reaction. For BGD samples, 100 µL of 0.5 M $CaCl_2$ and 400 µL of 0.1 M Tris(hydroxymethyl)aminomethane (THAM) buffer were added to the samples and mixed thoroughly. Samples were centrifuged with an Eppendorf miniSpin Plus at 13148 g for 2 minutes and read colorimetrically at 410nm with the Biorad iMark 96-well-plate reader.

5.4.3.4 Water protection

The xenobiotic degradation potentials of spiked and control soils were determined by amending 5 g of soil with 0.01 µM of ^{13}C-labelled glyphosate in 160 mL serum bottles. At 0, 1, 3, and 5 days, a syringe was used to sample gas in the headspace and inject it into two sets of vacutainers. One set was analyzed with a Scion 456-GC for total CO_2, while the second set was analyzed for the ratio of $^{13}CO_2$ to $^{12}CO_2$ with a Picarro G2201-I analyzer. The concentration of $^{13}CO_2$ from the degradation of glyphosate was calculated in nmol per g soil per day.

5.4.3.5 Organic matter decomposition

Soil organic matter decomposition was determined by exposing three soil invertebrates—*Oppia nitens* (mites), *Folsomia candida* (collembola), and *Enchytraeus crypticus* (enchytraeids)—to control and metal-spiked soils (ISO, 2014; ISO Guideline 11267, 2001; Princz et al., 2010). These invertebrates were chosen because they decompose organic matter and are important nutrient recyclers. Specimens used for the study were taken from established cultures grown in the soil toxicology laboratory at the University of Saskatchewan. The instantaneous population growth rate (IPGR, or r_i), which integrates both survival and prolificacy in measuring population rate, was calculated using survival and reproduction results (Equation 5-1) (Herbert et al., 2004); n_f is the number of animals at the end of the test, n_o is the number of animals exposed, and ΔT is the number of days of the exposure. Positive values represent growing populations, negative values represent declines in populations and possible extinction, while neutral values represent a stable population.

$$r_i = ln\left(\frac{n_f}{n_o}\right)/\Delta T \qquad \textit{Equation 5-1}$$

5.4.4 Statistical analysis

A total of 15 endpoints representing 5 ecosystem services were obtained from the experiment. The endpoints included three greenhouse gases, three enzyme activities, plant biomass, plant crude protein, concentration of degraded glyphosate, and survival and reproduction of three soil invertebrates. Bivariate scatter plots were examined to determine linear relationships between endpoints and soil properties. Comparisons between endpoints in control and metal-spiked soils were accomplished with a Welch two-sample t-test. I examined how the soil properties (pH, OC, percent clay, CEC) influenced ES by using an exploratory SEM that translates each path diagram into a linear equation. Specifically, an open source R package (i.e., piecewiseSEM) was used to build the structural models (Jon et al., 2018). A major advantage of piecewiseSEM is that it can use smaller data sets with different sampling and experimental designs (Lefcheck, 2016).

I first used piecewiseSEM to establish the causal network and relationships between soil properties and the endpoints measured in control soils. The structure of the model was specified based on prior knowledge and theory about the soil ecosystem and its processes. For example, relationships have been reported to exist between OC and soil respiration, and CEC has been reported to be influenced by soil pH, clay and OC (Curtin and Rostad, 1997; Riches et al., 2013). Pearson correlations derived from preliminary analysis (Table 5-1 & Figure 5-3) were also utilized in the model development process. Please see Lamb (2011) and Grace (2018) for detailed steps involved in the development of SEM models. The validity of the model was confirmed by testing the goodness of fit (GOF) between the covariance structures and the data set. The entire process was iterative. Prior to building the SEM models, identified outliers were removed ($n \leq 5$). Mite, collembola, and enchytraeid survival were rescaled to percentage, AMO was multiplied by a factor of 100, BGD was square rooted, and CO_2 was rescaled to remove negative values and then divided by a factor of 100 in order to achieve a common scale (Stewart et al., 2013). For control soils, equation 5-2 was used to build the model used to examine the causal relationship between soil properties and ES (the control model was slightly modified for metal-spiked soils, see Appendix C Equation [C-1]):

$$
\begin{aligned}
\textit{Control model (ES)}= \quad & [\mathbf{CEC}_i = \beta_0 + \beta_1\text{pH}_{,i} + \beta_2\text{Clay}_{,i} + \beta_3\text{Organic}_{,i} + \varepsilon_{\text{CEC}},\\
& \mathbf{Phosphatase}_i = \beta_4 + \beta_5\text{Organic}_{,i} + \varepsilon_{\text{Phosphatase}},\\
& \mathbf{AMO}_i = \beta_6 + \beta_7\text{CEC}_{,i} + \beta_8\text{pH}_{,i} + \varepsilon_{\text{AMO}},\\
& \mathbf{Glucosidase}_i = \beta_9 + \beta_1\text{CEC}_{,i} + \beta_{10}\text{Organic}_{,i} + \beta_{11}\text{AMO}_{,i} + \beta_{12}\text{Phosphatase}_{,i} + \varepsilon_{\text{Glucosidase}},\\
& \mathbf{Carbon\ dioxide}_i = \beta_{13} + \beta_{14}\text{Glucosidase}_{,i} + \varepsilon_{\text{Carbon dioxide}},\\
& \mathbf{Methane}_i = \beta_{15} + \beta_{16}\text{CEC}_{,i} + \varepsilon_{\text{Methane}},\\
& \mathbf{Nitrous}_i = \beta_{17} + \beta_{18}\text{pH}_{,i} + \beta_{19}\text{Phosphatase}_{,i} + \beta_{20}\text{Carbon dioxide}_{,i} + \varepsilon_{\text{Nitrous oxide}},\\
& \mathbf{Biomass}_i = \beta_{21} + \beta_{22}\text{CEC}_{,i} + \beta_{23}\text{Clay}_{,i} + \beta_{24}\text{pH}_{,i} + \beta_{25}\text{Glucosidase}_{,i} + \varepsilon_{\text{Biomass}},\\
& \mathbf{Mite\ survival}_i = \beta_{26} + \beta_{27}\text{pH}_{,i} + \varepsilon_{\text{Mite survival}},\\
& \mathbf{Mite\ reproduction}_i = \beta_{28} + \beta_{29}\text{CEC}_{,i} + \varepsilon_{\text{Mite reproduction}},\\
& \mathbf{Collembola\ reproduction}_i = \beta_{30} + \beta_{31}\text{Collembola survival}_{,i} + \varepsilon_{\text{Collembola reproduction}},\\
& \mathbf{Collembola\ survival}_i = \beta_{32} + \beta_{33}\text{Organic}_{,i} + \varepsilon_{\text{Collembola survival}},\\
\mathbf{Glyphosate\ degradation}_i = {} & \beta_{34} + \beta_{35}\text{Phosphatase}_{,i} + \beta_{36}\text{CO}_{2,i} + \varepsilon_{\text{Glyphosate degradation}}]
\end{aligned}
\tag*{\textit{Equation 5-2}}
$$

On the left side of each equation is the response variable, and on the right are the predictor variables. The β(s) are the standardized regression coefficients, and the ε(s) are the error terms. Response variables in one equation could be a predictor in another equation, and endpoints that significantly reduced the model fit were removed from the entire model. In building the second model for metal-spiked soils (Appendix C Equation [C-1]), I substituted data from the spiked soils into the same model and tested the model fit. An additional equation with enchytraeid reproduction as a response variable was added to the model specification. All other response variables remained the same, with very few changes in predictor variables.

5.5 Results

5.5.1 Relationships between soil properties and ecosystem services (ES)

Using a Pearson product-moment bivariate correlation matrix, all ES showed strong relationships with soil properties. The quality of some ES increased as the values of the soil properties increased. For example, as CEC increased, mite survival, CO_2 production, glyphosate degradation, wheat biomass, and potential nitrification rates also increased (Table 5-1).

Table 5-1. Pearson correlation coefficients between soil properties and ecosystem services in control soils

	Ecosystem Services				
	Climate Regulation	**Water Protection**	**Food Production**	**Organic Matter Decomposition**	**Nutrient Cycling**
	Indicator Measurement for Ecosystem Service				
Soil properties	CO_2 production	Glyphosate degradation	Wheat biomass	Mite survival	AMO activity
pH	0.30*	0.33*	0.28	0.68***	0.66***
Percent clay	0.57***	0.54***	0.24	0.33*	0.40**
CEC	0.58***	0.58***	0.57***	0.41**	0.72***
Percent OC	0.96***	0.90***	0.39**	0.06	0.39**

Significance codes: * < 0.05, ** < 0.01, *** < 0.001. AMO= ammonia monooxygenase

As CEC increased, all five ES also showed significant increases, as shown in Figure 5-3 ($p<0.01$). On average, CEC showed the highest correlation with these services ($r = 0.57$), followed by OC ($r = 0.54$). I observed increases in all five services with increases in soil pH, percent clay, and OC, but the correlation was significant for only four services (Table 5-1). Furthermore, CH_4 consumption, ACP activity, and IPGR of enchytraeids and collembola, showed negative relationships with some soil properties, particularly with soil pH (Appendix C Table [C-1]). These correlations, however, were not significant.

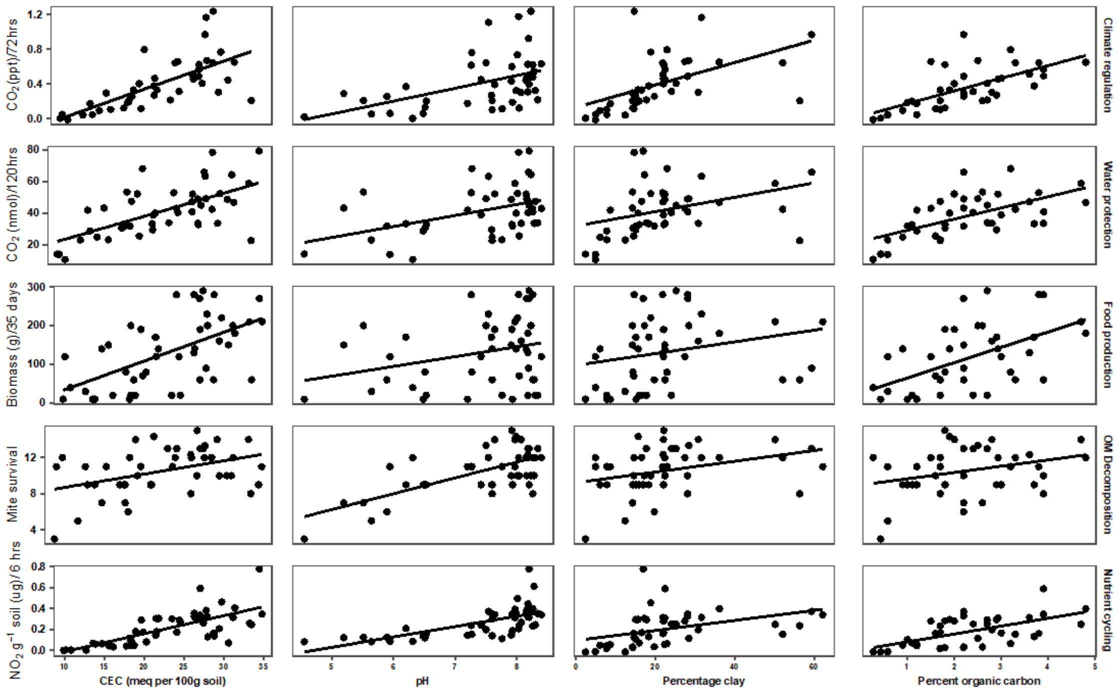

Figure 5-3. A multi-panel scatterplot showing the relationship between ecosystem services and soil cation exchange capacity (CEC), soil pH, percentage clay, and percentage organic carbon for 47 soils. A trend line was added to ease graphical interpretation. The first row shows links between CO_2 production (parts per thousand per gram soil per 72 h) and soil properties, second row shows links with glyphosate degradation by a measure of $^{13}CO_2$ (nmol per gram soil per 120 h), third row shows links with wheat biomass (grams) after 35 days of seeding, fourth row shows links with the survival of an oribatid mite *Oppia nitens* (count), and the fifth row shows links with ammonia nitrification (NO_2 per gram soil/ 6 h in ug). The right side of the y-axis shows the ecosystem service that each measured endpoint represented.

5.5.2 Impacts of metal mixtures on ES quality

The quality of ES was significantly reduced in metal-spiked soils across the most critical endpoints ($p < 0.05$) (Figure 5-4), which were food production, water protection, and climate regulation. For instance, the mean CO_2 production and CH_4 consumption in control and metal-spiked soils was 0.44± 0.4 ppt (parts per thousand) and 0.28± 0.3 ppt, and -145.8± 47 ppb and -128.3± 33 ppb respectively. All three nutrient cycling endpoints (AMO, ACP, and BGD activities) showed no sensitivity to the metal-spiked soils (6250 mg kg^{-1}) when compared to control soils ($p > 0.05$).

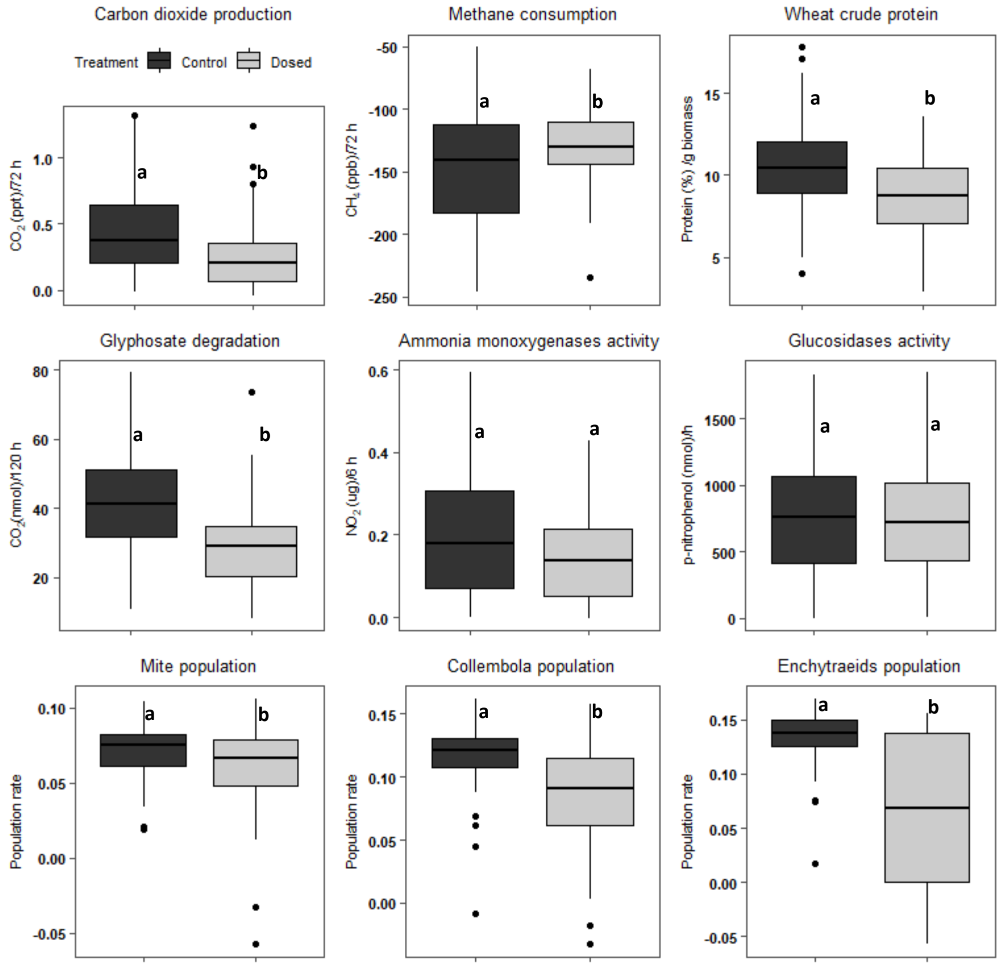

Figure 5-4. Boxplots showing the sensitivity of nine endpoints that represent five ecosystem services to metal mixtures in 47 Canadian soils. Black and gray plots represent services in control and metal spiked soils respectively. Middle line in the box represents median, while the lower and upper ends of the box represent the first and third quartiles respectively. Points beyond the lines are considered as statistical outliers. Alphabets a and b within plots represent significant differences ($P<0.05$) between treatments. Plots with same alphabetic insets indicate similarities while those with different letters represent differences. The first row shows CO_2 production (left) in parts per thousand (ppt), CH_4 consumption (middle) in parts per billion (ppb), and the crude protein content as percentage biomass in *E. lanceolatus* (right). Second row shows the concentration of glyphosate degraded by a measure of $^{13}CO_2$ produced (left) (nmol), the activity of ammonia monooxygenases (middle) (µg), and the activity of beta glucosidases (right) (nmol). The last row shows the instantaneous population growth rates (r_i) of mites (left), collembolans (middle), and enchytraeids (right).

The instantaneous population growth rates (r_i) for collembola, enchytraeids, and mites showed extinction values ($r_i < -0.05$) in metal-spiked soils. The mean r_i in metal-spiked soils was statistically different when mites were compared to collembola (Tukey HSD, $p < 0.01$). The mean r_i for mites and collembolan in metal-spiked soils were 0.06±0.03 and 0.08±0.05 respectively.

5.5.3 Relationships between soil properties and ES using a piecewise SEM

An exploratory SEM model was used to develop a network of relationships between soil properties and the measured variables representing ES in control soils. It was established that the specified SEM model was a strong fit for the data (χ^2 =143, df= 184, p=0.99) (Figure 5-5). The three exogenous variables, soil pH (0.32, p<0.001), OC (0.40, p<0.001), and clay (0.59, p<0.001), directly influenced soil CEC, accounting for 87% of the variability in CEC in the 47 soils. Furthermore, CEC had a strong positive influence on the production of plant biomass (0.70, p<0.05) and a moderate influence on the activities of AMO (0.38, p<0.01) and BGD (0.27, p<0.05). However, soil pH was found to have a positive influence on mite survival (0.56, p<0.001) and AMO activity (0.39, p<0.01), and a negative influence on nitrous oxide effluxes (-0.41, p<0.01). Percent clay had a strong negative influence on wheat biomass production (-0.55, p<0.05). Percent OC had weak to moderately strong positive relationships with BGD activity (0.28, p<0.01) and ACP activity (0.33, p<0.05), and a negative relationship with collembola survival (-0.30, p<0.05). No significant links were observed for mites and enchytraeids reproduction.

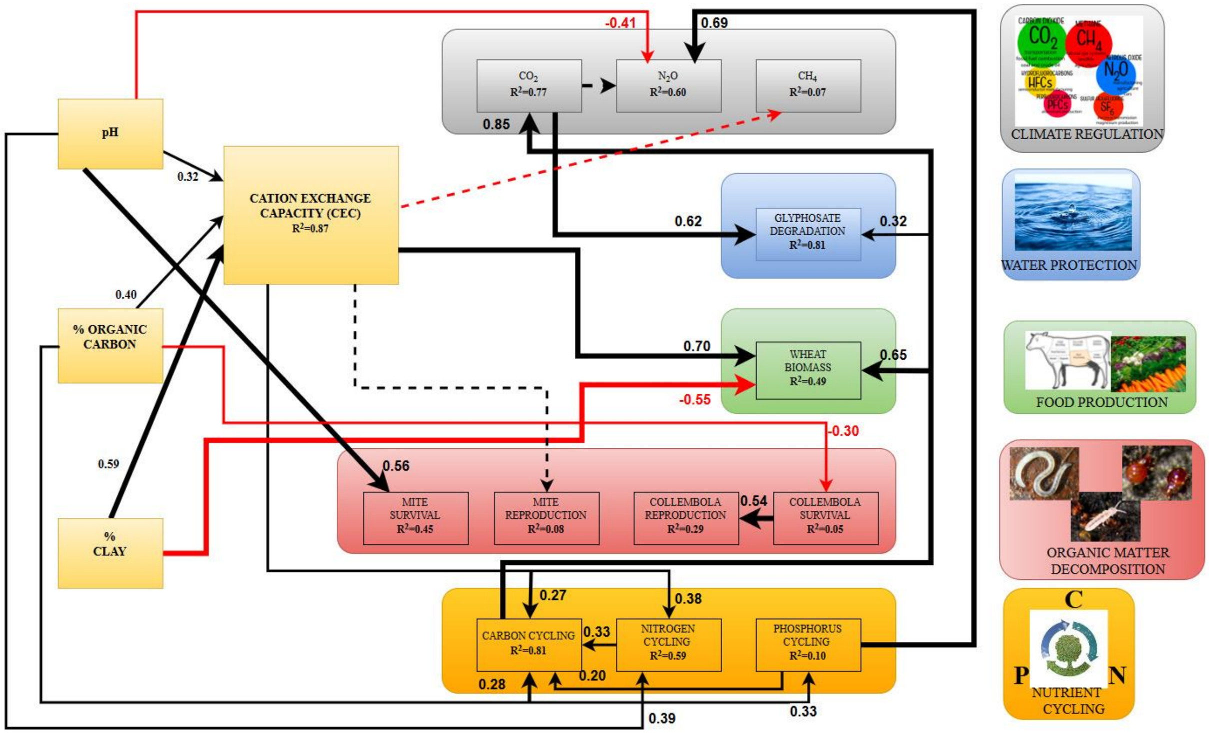

Figure 5-5. Fitted piecewise SEM using general linear models to examine the relationships between soil properties (pH, %Clay, %OC, CEC) and ecosystem services in 47 Canadian non-metal impacted soils. All standardized path coefficients are significant at $p < 0.05$. Black and red lines represent positive and negative path coefficients respectively. Weight of the lines are proportional to the strengths of the path coefficients. Light yellow boxes represent soil properties, gray boxes are a measure of greenhouse gases that represent climate regulation, blue box is a measure of glyphosate degradation that represents water protection, green box is a biomass measurement of *E. lanceolatus* that represents food production, pink boxes are a measure of survival and reproduction of soil invertebrates that represent organic matter decomposition, and yellow boxes are a measure of soil enzymes that represent nutrient cycling. Inserted within the box of each response variable is the r^2. The chi-square test of SEM model fit for the data was $\chi^2 = 143$, df= 184, p=0.99.

Apart from the survival and reproduction of invertebrates, the quality of all ES was dependent on soil enzyme activity (Table 5-2) in control soils. For example, greenhouse gas fluxes CO_2 (0.85, $p<0.001$) and N_2O (0.69, $p<0.001$) were positively influenced by the activity of BGD ($r^2 = 0.81$) and ACP ($r^2 = 0.10$), respectively. BGD activity also influenced the wheat biomass production (0.65, $p<0.01$), while degradation of the herbicide glyphosate was positively dependent on ACP activity (0.31, $p<0.001$). The low r^2 values for mite reproduction ($r^2 = 0.08$) and collembola survival ($r^2 = 0.09$) were expected, since no significant links were found between them and other ES variables in the control model. The variability around the means for glyphosate degradation ($r^2 = 0.81$), CO_2 production ($r^2 = 0.77$), N_2O fluxes ($r^2 = 0.60$), BGD activity ($r^2 = 0.81$), AMO activity ($r^2 = 0.59$), and wheat biomass ($r^2 = 0.49$) were at least 50% explained by the SEM model for control soils.

Table 5-2. SES relationships and partial correlation coefficients as modified by metal mixtures

Predictor variable	Response variable	Non-Impacted soils	Impacted soils	Ecosystem service affected
CEC	CH_4 consumption	--------	-0.37**	Climate regulation
CEC	Biomass production	0.70*	---------	Food production
CEC	CO_2 production	--------	-0.48*	Climate regulation
CEC	AMO activity	0.38**	0.73***	Nutrient cycling
CEC	BGD activity	0.27*	0.26*	Nutrient cycling
pH	AMO activity	0.39**	------	Nutrient cycling
pH	Mite survival	0.56***	0.47***	OM decomposition
pH	Nitrous flux	-0.41**	-0.33*	Climate regulation
% clay	CO_2 production	--------	0.56**	Climate regulation
% clay	Biomass production	-0.55*	--------	Food production
% OC	ACP activity	0.33*	0.38*	Nutrient cycling
% OC	BGD activity	0.28**	0.30*	Nutrient cycling
% OC	Collembola survival	-0.30*	-0.38*	OM decomposition
% OC	CO_2 production	-------	0.54***	Climate regulation
% OC	Biomass production	-------	0.52***	Food production
% OC	Enchytraeids reproduction	-------	0.32*	OM decomposition
AMO activity	BGD activity	0.33**	0.25**	Nutrient cycling
AMO activity	CO_2 production	--------	0.33**	Climate regulation
ACP activity	BGD activity	0.20*	0.17*	Nutrient cycling
BGD activity	CO_2 production	0.85***	--------	Climate regulation
ACP activity	Nitrous flux	0.69***	--------	Climate regulation
BGD activity	Nitrous flux	--------	0.36*	Climate regulation
BGD activity	Biomass production	0.65**	-------	Food production
AMO activity	Biomass production	--------	0.26*	Food production
Mite survival	Mite reproduction	--------	0.44***	OM decomposition
Enchytraeids survival	Enchytraeids reproduction	--------	0.65***	OM decomposition
ACP activity	Enchytraeids reproduction	--------	-0.38*	OM decomposition
Collembola survival	Collembola reproduction	0.54***	0.45*	OM decomposition
ACP activity	Glyphosate degradation	0.32***	0.16*	Water protection
CO_2 production	Glyphosate degradation	0.62**	0.54***	Water protection
BGD activity	Glyphosate degradation	-------	0.16*	Water protection

Significance codes: * < 0.05, ** < 0.01, *** < 0.001
OM= Organic Matter

5.5.4 Impacts of metal mixtures on soil ecosystem service (SES) relationships

Metal mixtures significantly altered soil-ES relationships. Novel relationships were established, which were otherwise not captured in the SEM model for control soils, while the strength of correlation coefficients for previously observed relationships were either weakened, negated, or eliminated (Table 5-2). The specified model for the data set was a moderate fit ($\chi^2 = 231$, df= 236, p=0.57) (Figure 5-6). From the model, the influence of the trio (pH, OC, clay) on

CEC was not altered, but their effects on ES were significantly transformed when PTEs were present. For example, the effects of CEC on biomass production, pH on AMO activity, and clay on biomass production were eliminated in metal-spiked soils (Figure 5-6).

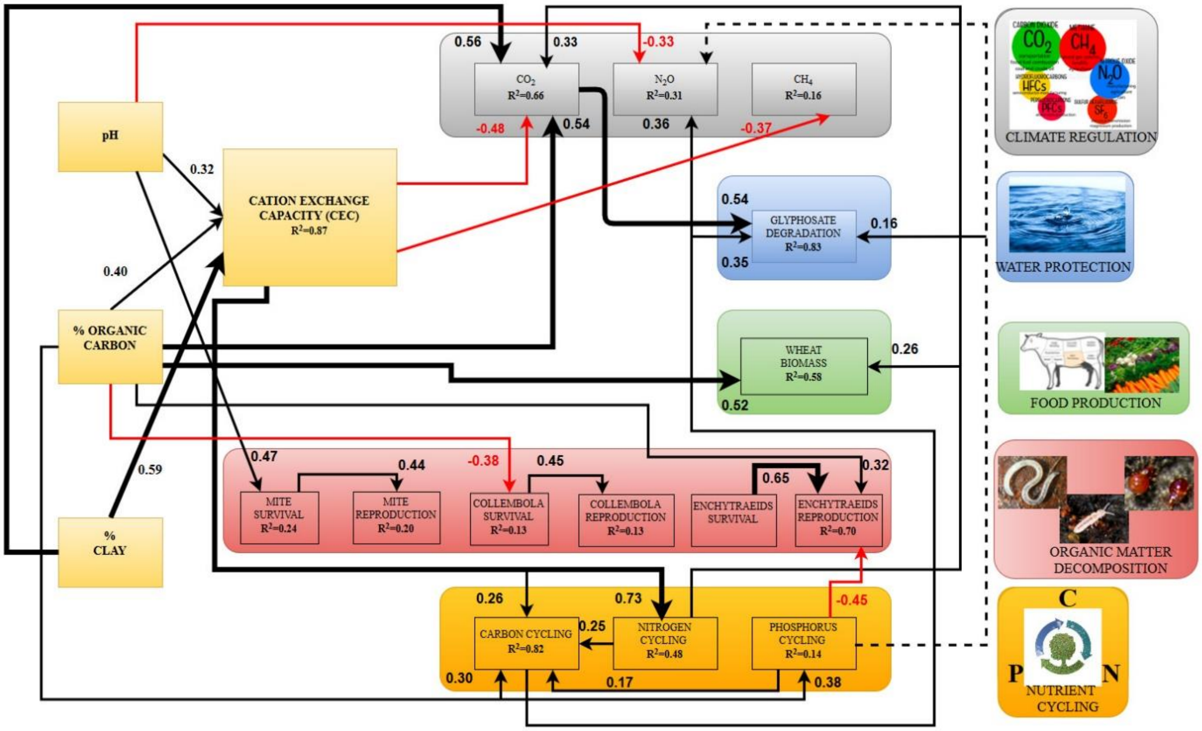

Figure 5-6. Fitted piecewise SEM using general linear models to examine the relationships between ecosystem services (15 endpoints) and soil properties (pH, clay, OC, CEC) in metal mixture impacted soils. All standardized path coefficients are significant at $p < 0.05$. Black and red lines represent positive and negative path coefficients respectively. Weight of the lines are proportional to the strengths of the path coefficients. Blacklines that connect redlines and vice versa is a change in relationship for a variable and should be traced back to the predictors. Light yellow boxes represent soil properties, gray boxes are a measure of greenhouse gases that represent climate regulation, blue box is a measure of glyphosate degradation that represents water protection, green boxes are a biomass measurement of *E. lanceolatus* and its crude protein content that represents food production, pink boxes are a measure of survival and reproduction of three soil invertebrates that represent organic matter decomposition, and yellow boxes are a measure of soil enzymes that represent nutrient cycling. Inserted within the box of each response variable is the r^2. The chi-square test of SEM model fit for the data was $\chi^2 = 231$, df= 236, p=0.57.

However, the same soils showed moderate to strong positive effects of OC on CO_2 production (0.54, $p < 0.001$), biomass production (0.52, $p < 0.001$), and enchytraeid reproduction (0.32, $p < 0.05$) (Table 5-2). Furthermore, a negative relationship was established between CEC and CO_2 production (-0.48, $p < 0.05$) and CH_4 consumption (-0.37, $p < 0.01$). For clay, only a strong positive relationship on CO_2 production (0.56, $p < 0.01$) was observed.

I also observed an increase in the interdependence of ecosystem functions in metal-spiked soils. For example, as expected, I observed mite survival (0.44, $p < 0.01$) positively affected mite reproduction, and enchytraeid survival (0.65, $p < 0.001$) positively affected enchytraeid reproduction. However, in control soils I observed a reduction in the magnitude of influence that collembola survival (0.54 to 0.45) had on collembola reproduction.

Moreover, I observed similar changes in relationships between ES in the SEM model for metal-spiked soils. For instance, I observed new relationships between BGD activity and glyphosate degradation (0.35, $p < 0.001$), AMO activity and CO_2 production (0.33, $p < 0.01$), and ACP activity and enchytraeids reproduction (-0.45, $p < 0.01$). The magnitude of beta coefficients was reduced for ACP activity on BGD activity (0.20 to 0.17), collembola survival on collembola reproduction (0.54 to 0.45), and CO_2 production on glyphosate degradation (0.62 to 0.55) (Table 5-2). Additionally, the influence of ACP activity on glyphosate degradation changed from (0.32, $p < 0.001$) to (0.16, $p < 0.05$). The variability around the means for glyphosate degradation ($r^2 = 0.83$), BGD activity ($r^2 = 0.82$), enchytraeids reproduction ($r^2 = 0.70$), CO_2 production ($r^2 = 0.66$), and wheat biomass ($r^2 = 0.58$) were more than 50% explained by the SEM model for metal-spiked soils.

5.6 Discussion

5.6.1 Soil ES intimacy confirmed.

The role of soil properties in driving soil functions is explicitly known, and the impact of metal contamination on soil functions has been established in several studies (Hayes et al., 2018; Princz et al., 2010; Smolders et al., 2001b). However, no study has quantitatively established the changes in soil functions and ES caused by PTEs as a function of soil properties. I proposed a novel quantitative technique, Adverse Ecosystem Pathway (AESP), to address and predict the direct and indirect effects of PTEs on ES by using soil properties and functions.

In this study, I confirmed defined relationships reported in literature and revealed some undefined associations between soil properties and ecosystem functions in both metal-spiked and control soils (Murphy et al., 2011; Van Eekeren et al., 2010). The results indicate that the three soil properties tested (i.e., pH, OC, and clay) directly influence CEC and can account for about 90% of the variability as has been reported (Curtin and Rostad, 1997; Saidi, 2012). I observed strong correlations between CEC and nutrient cycling, crop productivity, C-mineralization, and xenobiotic degradation in control soils (Bergamin et al., 2015; Insam and Domsch, 2013; Murphy et al., 2011; Saidi, 2012; Tueche, 2014; Van Eekeren et al., 2010). Clay, OC, and pH positively influence CEC and define the WHC, nutrient supply and bioavailability of xenobiotics in soils (Curtin and Rostad, 1997; Saidi, 2012). As a result, high soil microbial activity and crop productivity are associated with increases in these soil properties through the provision of optimum conditions, i.e. soil structure, nutrients, carbon, and moisture availability (Curtin and Rostad, 1997; Saidi, 2012). Low pH soils usually have elevated bioavailable concentrations of PTEs that might reduce bacterial abundance, thus decreasing pesticide degradation and nutrient cycling (Bååth and Arnebrant, 1994; Bartram et al., 2014).

Riches et al. (2013) reported a direct relationship between OC and potential mineralizable nitrogen, soil respiration, and levels of biomass carbon. I similarly observed that increases in soil microbial energy (i.e., OC) resulted in a cascade of events that confirmed the supportive role of microbes in soils. Table 5-2 shows that the five ES included in this study all depend on the activity of soil microbes (i.e., enzymes). As a result, the strongest correlations between ecosystem functions were observed for OC. However, not all OC is created equal, and future work is needed to explore if OC quality modulates AESP results (Grigal and Vance, 2000).

5.6.2 Direct and indirect impacts on ecosystems unraveled.

Metal mixtures decreased soil functions and altered its supportive and regulatory services (Figure 5-4). The impact of PTEs caused significant decreases in the population of soil invertebrates, degradation of glyphosate, consumption of CH_4 and the production of CO_2. Similar observations have been identified for several metal studies including Ding et. al (2018), Moreno et. al (2003) and others (Hayes et al., 2018; Li-Zhai et al., 2010; Versieren et al., 2017). The direct impact on soil enzymatic activity, however, was not captured by the traditional t-tests when functions in control soils were compared to metal-spiked soils (Figure 5-4 and Appendix C Figure [C-3]). This could either result from the low solubility and availability of oxides or from functional redundancy, where changes in microbial community composition prevents large changes in community functions and hence less sensitive (Awuah et al., 2019; Kuperman et al., 2014). Instead, the impacts were captured in the AESP, portrayed as changes in the effects (i.e., beta coefficients) of soil properties and supportive functions on ES (Figure 5-6 and Table 5-2). I observed no significant relationships between the reproduction/survival of soil invertebrates and enzyme activity in control soils. I postulate that this occurred because soil invertebrates are not reliant on exoenzymatic processes for nutrient mineralization (Coleman et al., 2018). Soil pH had a positive effect on the AMO activity (β=0.39) in control soils, supporting the finding that AMO activity increases with soil pH. The increase in AMO activity with soil pH is due to the increase in the abundance of ammonia oxidizing bacteria (AOB), which is a more effective ammonia oxidizer compared to ammonia oxidizing archaea (AOA) (Nicol et al., 2008; Prosser and Nicol, 2012). Interestingly, the relationship between AMO activity with soil pH was not observed in the metal-spiked soils. This was because, despite the increase in AOB abundance, AOB is more sensitive compared to AOA, resulting in higher toxicity at higher soil pH despite the decrease in metal bioavailability (Nicol et al., 2008; Prosser and Nicol, 2012; Rieuwerts et al., 1998b). As expected, CEC, a proxy for soil fertility, strongly influenced wheat biomass (Saidi, 2012). Studies have shown that metal contamination reduces CEC in soils (Eurola et al., 2015). A combination of the direct effect of PTEs on wheat growth and reduction in CEC could be a plausible explanation for the zero relationship between CEC and plant biomass in impacted soils (Gopalapillai et al., 2018).

PTEs reduced soil habitat quality and extinction values ($r_i < 0$) with all three organic matter decomposers (*O. nitens, F. candida, E. crypticus*) in impacted soils. Filzek et al. (2004) directly linked decreases in organic matter decomposition with reductions in invertebrate abundance and diversity (r_i) caused by metal stressors. In this study, the reproduction of *O. nitens*, *F. candida*, and *E. crypticus* was dependent on adult survival, but the survival-reproduction dependences for *O. nitens* and *E. crypticus* were not observed in control soils, which could be attributed to either differences in their sensitivity to heavy PTEs or growth reproduction strategies (Blakely et al., 2002). The less sensitive *O. nitens* is a *k*-strategist, while the more sensitive *E. crypticus* is an *r*-strategist, with reported increases in reproduction in the presence of a stressor (Blakely et al., 2002). Increased reproduction could also reduce the body burden of PTEs in adults through maternal transfer, which might increase adult survival chances. The influence of OC on the reproduction of *E. crypticus* was only observed in impacted soils, while the role of pH on the survival of *O. nitens* was observed in both soils. This suggests that survival and fecundity of *E. crypticus* in contaminated soils is directly controlled by organic matter, while *O. nitens* is controlled by soil pH (Jänsch et al., 2005). The positive relationships suggest that pH and organic matter could be reducing metal availability to these decomposers and contributing to their coping mechanisms, either as a specified niche or as a food source (Blakely et al., 2002).

Metal mixtures also reduced the ability of microbes to degrade glyphosate (GLP), increasing the half-life of the xenobiotic in soils (la Cecilia and Maggi, 2018). GLP and its formulations have endocrine-disrupting properties toxic to bacteria, plants, and human cell lines(Defarge et al., 2018). Motta et al. also reported that the chemical inhibits weight gain and increases pathogen susceptibility and mortality in honey bees by reducing gut bacteria in worker bees(Motta et al., 2018). Since symbiotic bacteria are predominantly found in the gut microbiome of soil arthropods, the same could be true for soil-dwelling arthropods and invertebrates, especially those with reduced degradation potential caused by metal exposure. In fact, Niemeyer et al. (2018) reported reduced feedings by soil invertebrates after exposure to four GLP formulations.

There are two major pathways of GLP degradation: the C-P lyase pathway, which releases sarcosine and PO_4; and the oxidation pathway, which breaks the C-N bond to release aminomethylphosphonic acid (AMPA) and CO_2 (la Cecilia and Maggi, 2018). Interestingly, our results showed that ACP (a lyase enzyme) and CO_2 were good predictors of GLP degradation, even though I observed a reduced influence in impacted soils. Furthermore, GLP degradation in impacted soils relied on the activity of BGD, which catalyzes the hydrolysis of glycosidic bonds in glucosides and oligosaccharides to release glucose (Eivazi and Tabatabai, 1988). Since BGD is comparatively less sensitive to PTEs, the glucose released from its activity was possibly being used by other microbes as an energy (C) source to degrade GLP, particularly in metal-contaminated soils (Moreno et al., 2003).

Some methanotrophs can potentially use NH_4 as an energy source, making NH_4 a competitive inhibitor of CH_4 oxidation in soils (Bedard and Knowles, 1989). In the absence of metal contamination, soils with high CEC have increased cation fixation (NH_4^+), which might reduce the competitive inhibition of CH_4 oxidation by NH_4 (Lehmann J, 2009). In this study, CEC negatively influenced both CH_4 consumption and CO_2 production in contaminated soils. Apart from the direct effect of PTEs on soil enzymes, competitive desorption of NH_4 by heavy PTEs in contaminated soils possibly increased the inhibition CH_4 oxidation. Hence, increases in CEC resulted in increases in NH_4 displacement in metal-spiked soils, decreases in the consumption of CH_4 and the production CO_2, and increases in NH_4 oxidation, as observed in the study (Table 5-2). Based on other reported studies (Fomsgaard and Kristensen, 1999; Mitra et al., 2002), I expected to see a significant correlation between OC and clay percentages and CO_2 production and CH_4 in the control soils. Instead, I found a positive pathway for OC and clay on CO_2 production only in metal-spiked soils. Because OC is a source of energy and CEC determines nutrient availability, this positive pathway confirms that CEC and OC help to modulate metal bioavailability and toxicity, especially to soil microbes (Grigal and Vance, 2000).

5.7 Role of AESP models in ecotoxicological studies and risk assessment

AESP models could play a major role in predictive risk assessment and management. The method outlines relevant endpoints and parameters needed to predict the ecological functions of a system in the presence and absence of stressors. AOPs focus on cellular and molecular interactions that lead to ecosystem changes (Ankley et al., 2010), and AESPs can predict regional effects on ES. A fundamental strength of the AESP model is its ability to use the inherent characteristics and functions of the interactive parts of a system in predicting the overall status of an ecosystem. When a chemical disrupts an ecosystem, the AESP combines the effects of all chemically initiated molecular events (portrayed as reductions in activity, growth, growth rate, and mortality) with the indirect effects of the stressor (characterized by the changes in the chemical and physical attributes of the system); both are intrinsically encapsulated in the AESP. The complexity of AESP model could be expanded to include concentration-dependent variables as well as explicit spatially dependences (Lamb et al., 2014).

The AESP model is one of the first models that explicitly integrates habitat quality indicators with ecosystem services in a holistic fashion. Thus, site managers that wish to maintain ecosystem services can identify key habitat quality indicators that will support ecosystem services at an impacted site during restoration and remediation activities. The AESP model further establishes that, the actual risks associated with soil contaminants are not necessarily the direct effects on functions, but potentially the indirect effects that go unidentified. Thus, AESP can serve as a powerful tool to inform risk assessment and decision making through its comprehensive approach.

6 SYNTHESIS

Since exposures to metal contamination of soils occur as mixtures, there is a necessity for a shift in the process of evaluating metal mixture effects to inform the risk assessment process. Currently, the risk assessment of metal mixtures in Canada assumes similar modes of action and concentration addition (CA). Concentration addition is preferred because of two main reasons. Firstly, majority of the toxicity data exists for both single metals and simple mixtures (binary), and secondly, the addition of concentrations to derive TUs is much simpler and easier to extrapolate and evaluate compared to other mixture models. The cardinal issue with the CA approach is that interactions are likely to occur, causing deviations from the reference model that can lead to an over/underestimation of the toxic effects and the characterization of risks. An overestimation of metal mixture effects might result in the establishment of lower guideline concentrations and subsequently the setting up of unnecessarily high remediation goals. In contrast, an underestimation of mixture effects will result in the lack of protection and subsequently, high risks to humans and the environment. The primary goal of the thesis was to improve our current knowledge of metal-metal and metal-organism interactions and the prediction of the effects to organisms and ecosystem services of mixtures that contain common metals found in contaminated soils around smelting operated sites in Canada.

I investigated experimental parameters that have been reported to possibly confound results derived from mixture toxicity tests for Cu, Pb, Ni, Zn and Co. I further characterized the mixture effects along several rays and doses and determined the in/direct effects on important benefits that humans derive from soils. The specific objectives of the thesis included:

- Assessing the differences in toxicity of metal mixtures spiked in the form of metal salts, metal oxides and spinel mineral oxides to soil enzymes, and the modifying role of soil properties in three Canadian soils;
- Quantifying the effects of metal mixtures on the carbon and phosphorus cycles using soils enzymes as biomarkers and to determine the differences in sensitivity to the metals;
- Investigating the effects of metal mixtures on the relationships among soil properties and processes that underlie ecosystem services (ES) in Canadian soils;

Several important questions arose in addressing the above objectives, these questions included:

- Are metal salts more toxic to ecological receptors and services compared to other metal types? Does leaching metal salt spiked soils affect metal ratios and soil microbial activity? Does the dosing method used in mixture toxicity testing affect toxicity estimates?
- Does the toxicity of metal mixtures to soil enzymes (ACP, BGD) that contribute to biogeochemical cycles follow CA? Are there any differences in the sensitivity of C and P cycles as represented by the BGD and ACP respectively?
- Do metal mixtures affect soil ecosystem service relationships?

6.1 Principal findings

The findings from Chapter 3 were very critical to the formation of the thesis because the entire experimental design of the subsequent chapters was dependent on the outcome of Chapter 3. Pertinent information was obtained from comparing the metal mixture types and the experimental feasibility of using the three metal mixture types in Chapter 3. I found out that, the metal mixture types behaved differently across the different soils. I also confirmed that leaching soils with artificial rainwater affected soil enzyme activity and mixture ratios in soils spiked with metal salts. Metal concentrations after leaching in salt spiked soils were below 50% of their pre-leaching concentrations in acidic soils. The extent of leaching for metal components of the mixture were dependent on the hydrolysis constant and binding affinities to soil organic matter and hydroxides. Another factor that controlled the extent of metal loss from leaching was the differences in soil properties, particularly pH and CEC. More metals were lost from soils with low pH and CEC and vice versa. Interestingly, the sensitivity of the activity of both soil enzymes to leaching followed known paradigms, thus the less metal tolerant enzyme was more sensitive to leaching with artificial rainwater.

An important determinant of metal toxicity to soil organisms including enzymes is how bioaccessible metals are in soils. I found out that metal salts were most bioaccessible, but the bioaccessibility of the metal salts were not as pH dependent as the oxides and spinel minerals. This was probably because metal salts were spiked in their free form but unlike the metal salts, the dissolution and solubility of the metal oxides depended on the pH of the soil. Hence increasing soil pH resulted in a decreased dissolution and bioaccessibility and lesser toxicity of the oxides. The spinel mineral oxides required huge laboratory efforts to anneal and were least toxic compared to metal oxides and salts. I concluded that metal oxides were more suitable for conducting metal mixture toxicity tests in soils if concentrations and ratios of metals are to be maintained. Overall, the results from Chapter 3 demonstrated that:

- Metal salts were more mobile in soils and more toxic to soil enzymes compared to metal oxides and spinel minerals;
- Leaching compromises soil enzyme activity and distorts desired metal concentrations and ratios in soils;

- The solubility and toxicity of metal oxides and spinel minerals were dependent on soil pH.

Once the discrepancies associated with experimental design had been resolved, I evaluated the accuracy of the currently used reference model (i.e., CA or the TU approach) in assessing risks associated with metal mixture contaminated sites in Canada, and the less used RA model. In Chapter 4, I tested the single and combined effects of five metals namely; Cu, Pb, Zn, Ni and Co in 10 fixed ratio rays and different dose levels to represent a more complex mixture treatment than what is usually reported in the literature using metal oxides. An important modification from the status quo in literature was the use of metal oxides as our preferred spiking method. By using metal oxides, I avoided compromising the status of soil enzymes, leaching of metals that alter metal concentrations and ratios, and more importantly reduced artefacts from salts that could modify toxicity in soils. Hence, I was confident that the observed effects of the mixtures on soil enzyme activity was only driven by metal concentrations in the soil. In fact, this was the first time metal toxicity had been evaluated as quinary mixtures and coupled with a fixed ratio ray design across a wide range of doses with soil enzymes as an endpoint or model process.

I discovered that, enzymes in the low pH soil were more sensitive to single metals than enzymes in the higher pH soil. I also found that, P cycling as measured by enzyme activity was generally more sensitive to single metals compared to C cycling. I compared the predictions from both reference models to the observed enzymatic responses and found that there were both differences and similarities between the models. I observed antagonistic deviations at all levels of spiked metal mixture concentrations, but the synergistic deviations were only observed at lower concentrations from both models. An interesting finding was that more synergism was found for the sensitive P cycle compared to the C cycle. I also found that, the ability of CA to accurately predict mixture effects depends on the resisitance of the endpoint being measured, hence CA accurately predicted mixture effects to C cycling especially at the median concentration. Predictions from the RA model were more conservative compared to the CA model, suggesting that the former will be protective of biogeochemical cycles. Overall, the results from Chapter 4 demonstrated that:

- Phosphorus cycling is more sensitive to single and metal mixture compared to Carbon cycling;
- The interaction of metal mixtures can produce synergistic effects to both cycles especially at lower concentrations;
- Copper in the mixture provides a protective effect to both enzymes.
- Response addition is conservative and protective of C and P cycling.

Chapters 3 and 4 demonstrated that metal mixtures inhibit soil enzyme activities in soils, but the effects of metal mixtures have far reaching effects. In Chapter 5 I expanded the scope of biological endpoints and linked the endpoints to benefits that humans derive from soil. The chapter demonstrated that, metal mixtures affect all soil biological processes that form the basis of the benefits that are ultimately enjoyed by humans. A major finding from Chapter 5 was the identification of relationships that existed between soil properties, particularly CEC and OC, and the processes that underlie ecosystem services in soils. Using an SEM, I also found out that soil ecosystem service relationships were altered in the presence of metal mixture contamination. Results from the AESP model serve as a major addition of knowledge that was formerly derived from SSDs. Even though both approaches report effects of toxicants from a community or ecosystem prespective, AESP reports the effects on interactions that directly impact the status of individual organisms. This makes AESP a more powerful tool for ecological risk assessment.

A major finding was that, the indirect effects of metals that were not captured by the traditional t-tests were revealed using the SEM approach. I also found that certain SES relationships are only established during contamination, while others are eliminated as a result of stressors. These results interestingly showed that, soil properties can be used as predictors of metal impacts on ecosystem services and can be used as a tool in site specific risk assessment. Overall, the results from Chapter 5 demonstrated that:

- Intimate links exist between soil properties and soil processes that underlie ecosystem services;
- These soil processes are directly and indirectly affected by metal contamination;
- The indirect effects can only be identified with the use of SEM's;
- We can use soil properties to predict the local or regional impacts of metal contamination on ecosystem services.

6.2 Future directions

The results generated from Chapter 3 indicate that the toxicity of metals is influenced by the dosing form or method. Currently, concentrations used as guidelines are based on laboratory research that was conducted using metal salts, which indicates that guideline concentrations may probably be overestimating risks associated with single metals, thus being overly conservative. This is because the more dominant and environmentally relevant metal forms (i.e., oxides and spinels) proved to be less toxic to soil enzymes compared to salts. For example, the guideline for residential Ni, Cu, Pb and Zn are 45, 63, 70, and 200 mg kg^{-1} respectively, but the EC25's of the same metals dosed as oxides were 6210, 456, 2430 and 1485 mg kg^{-1} accordingly. Further research must be conducted to derive specific metal factors that can correct for the differences in spiking method to improve the environmental relevance of guideline concentrations.

An aspect that can be improved in Chapter 1 will be ageing and modeling the free ion activity of the three metal types to explore the effects of speciation and weathering on their toxicities. The influence of ageing on speciation will ultimately modify metal exposure in soils and might result in more interesting findings. Also, with respect to differences in enzyme sensitivity to leaching and metals, additional molecular research is recommended as our study was only limited to the measurement of activity. As an example, it may be interesting to explore the differences in expression of antioxidant genes and osmotic stress induced genes between the microorganisms that express genes that code for acid phosphatases (ACP) and ammonia monooxygenases (AMO). The same may be done for Chapter 2 for ACP and beta glucosidases (BGD). Results from chapter 2 also raised several concerns about the current method used in assessing risks associated with metal mixture contaminated sites and the need for more research. The fact that synergism was only observed at lower concentrations requires probing to better quantify risks at such concentrations.

We need to improve our understanding on soil-metal, metal-metal, and metal-organism interactions to identify specific interactions between metals that cause toxicity and metals that alleviate toxicity. In addition, since the mixtures contained the same number of metals but behaved differently in different ratios, there is a possibility that the interactions are dependent on the ratios of specific metals in the mixture. Does toxicity increase with increasing concentrations of highly bioavailable metals (i.e., Zn) or metals that have high affinity (i.e. Pb and Cu) for clays and SOM? The number of ratios I had did not allow in-depth analysis to identify conclusive trends. Also, it will be interesting to know how the metal cations tested in the mixture will interact with oxyanions like selenium, arsenic, chromium in a mixture since they are metals of potential concern commonly found in contaminated sites. Will the differences in solubility with respect to soil pH and mode of toxic action result in a reduction or potentiation of toxic effects, especially if spiked as metal oxides? Will the combination result in stronger deviations from both additivity models compared to what was observed in this study? All these questions raise important ideas about mixture toxicity that need to be addressed through further research.

The discovery from chapter 5 used four soil properties to predict 15 biological endpoints necessary for human survival on earth. The research or AESP model could benefit from several improvements to increase the precision of the method. First, the AESP model could benefit from an increase in the number of soils from 47 to about 200 soils to cover a wider range of soils properties than what was observed in this study. Secondly, soil properties in the current study were only limited to pH, CEC, OC and clay. Soil properties in the model could be expanded to include concentration of base cations, anions and electrical conductivity that can possibly improve on the robustness and precision of the model. Thirdly, only one mixture concentration was tested in the study, but the concentrations of metals found in the environment may vary. To increase the applicability and environmental relevance with respect to concentration, a modification parameter that can control for the effect at different concentration can be added to the model. This could be done by conducting a full dose response experiment for the least, medium and most sensitive endpoints in the AESP model, scoring the endpoints and using the information to adjust predictions for different concentrations or effect levels. Furthermore, a confirmation of the sensitivity predictions from the model by using real life data could help in improving the model.

Overall, the future directions for improvements in the area of mixture toxicity will include a deeper understanding of interactions among the physical, chemical and biological components in soils. Despite our deeper understanding of the role and influence of soil properties, we need more research to improve AESP models because they represent the indirect and actual effects of metals on benefits that humans derive from ecosystems.

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8 APPENDIX A: CHAPTER 3 SUPPLEMENTARY MATERIAL

Table A-1. Electrical conductivity of soils after metal spiking before leaching (µS/cm). Dosed soils were leached with artificial rainwater till electrical conductivity in control soils were reached.

Rays Soil	Control	CSQG	Flin-Flon	Sudbury	`Peaty	PC
S1	601	5674	5380	4456	4784	5170
S2	483	3264	2547	3440	4031	4084
S3	544	2396	2801	2356	3532	3304

CSQG=Canadian soil quality guideline.
PC=Port Colborne.

Table A-2. Measured and nominal metal concentrations (excluding background concentrations) in dosed soils and $CaCl_2$ extracted metal concentrations (mg/kg of soil).

Soil Name	Metal Type	Ray	Nominal	Actual	$CaCl_2$	Percentage nominal	DTPA
S1	Salt	CSQG	3141.6	654.0	187.4	20.8	0.0
S1	Salt	FF	2914.2	514.0	135.7	17.6	0.0
S1	Salt	SUD	3977.1	2185.6	457.2	55.0	0.0
S1	Salt	PC	2066.9	450.7	214.8	21.8	0.0
S1	Salt	PEAT	3034.8	921.9	307.5	30.4	0.0
S3	Salt	CSQG	3141.6	3173.2	325.8	101.0	0.0
S3	Salt	FF	2914.2	2274.6	288.1	78.1	0.0
S3	Salt	SUD	3977.1	4302.3	188.8	108.2	0.0
S3	Salt	PC	2066.9	3309.5	123.7	160.1	0.0
S3	Salt	PEAT	3034.8	2500.6	228.7	82.4	0.0
S2	Salt	CSQG	3141.6	1920.8	693.5	61.1	0.0
S2	Salt	FF	2914.2	1785.4	622.9	61.3	0.0
S2	Salt	SUD	3977.1	2664.8	520.4	67.0	0.0
S2	Salt	PC	2066.9	1071.9	574.0	51.9	0.0
S2	Salt	PEAT	3034.8	1468.4	545.8	48.4	0.0
S1	Spinel	CSQG	3141.6	1219.6	316.6	38.8	0.0
S1	Spinel	FF	2914.2	1242.3	406.6	42.6	0.0
S1	Spinel	SUD	3977.1	2401.1	416.8	60.4	0.0
S1	Spinel	PC	2066.9	344.8	46.5	16.7	0.0
S1	Spinel	PEAT	3034.8	1113.7	157.5	36.7	0.0
S3	Spinel	CSQG	3141.6	2028.9	0.6	64.6	0.0
S3	Spinel	FF	2914.2	1182.9	4.0	40.6	0.0
S3	Spinel	SUD	3977.1	2114.8	0.7	53.2	0.0

S3	Spinel	PC	2066.9	626.8	0.0	30.3	0.0
S3	Spinel	PEAT	3034.8	1309.1	1.1	43.1	0.0
S2	Spinel	CSQG	3141.6	608.6	274.4	19.4	0.0
S2	Spinel	FF	2914.2	1365.4	350.9	46.9	0.0
S2	Spinel	SUD	3977.1	2330.6	228.0	58.6	0.0
S2	Spinel	PC	2066.9	268.9	208.2	13.0	0.0
S2	Spinel	PEAT	3034.8	1542.8	218.7	50.8	0.0
S1	Oxides	CSQG	3141.6	2468.0	831.1	78.6	508.6
S1	Oxides	FF	2914.2	2508.3	977.2	86.1	793.1
S1	Oxides	SUD	3977.1	1916.3	677.7	48.2	587.3
S1	Oxides	PC	2066.9	1558.5	236.7	75.4	282.0
S1	Oxides	PEAT	3034.8	2535.7	801.2	83.6	502.3
S3	Oxides	CSQG	3141.6	2812.3	12.3	89.5	623.8
S3	Oxides	FF	2914.2	3401.8	16.9	116.7	601.9
S3	Oxides	SUD	3977.1	3985.8	11.3	100.2	539.6
S3	Oxides	PC	2066.9	2757.9	1.8	133.4	198.0
S3	Oxides	PEAT	3034.8	2925.5	14.4	96.4	554.3
S2	Oxides	CSQG	3141.6	2073.4	540.8	66.0	812.1
S2	Oxides	FF	2914.2	2666.9	596.8	91.5	686.6
S2	Oxides	SUD	3977.1	1895.0	398.9	47.6	830.1
S2	Oxides	PC	2066.9	2178.7	259.7	105.4	427.8
S2	Oxides	PEAT	3034.8	1947.8	439.9	64.2	573.6

PC=Port Colborne ray.
PEAT=peaty ray.
SUD=Sudbury ray.
FF=Flin Flon ray.
CSQG=Canadian soil quality guideline ray.
DTPA=diethylenetriaminepentaacetic acid.

Table A-3. Two-way ANOVA table for AMO and ACP enzyme activity

Activity	Predictors	SS	Df	MS	F	*p*-value
AMO	Metal type	0.00025	2	0.00012	41.5	0
	Soil Type	0.00112	2	0.00056	188	0
	Metal Type: Soil Type	0.00024	4	0.00006	20.0	0
	Residuals	0.00046	153	0.000003		
ACP	Metal type	964	2	482.1	1.408	0.25
	Soil Type	29650	2	14825	43.28	0
	Metal Type: Soil Type	2644	4	661.1	1.930	0.108
	Residuals	52403	153	342.5		

SS=sum of squares.
Df=degrees of freedom.
MS=mean square.
AMO=ammonia monooxygenases.
ACP=acid phosphatases.

Table A-4. Pearson correlations between metal type, enzyme activity, soil pH, DTPA extracts, $CaCl_2$ extracts, and soils.

Metal Type	$CaCl_2$ metal concentrations vs Soil pH	
Salts	-0.05	
Oxides	-0.76***	
Spinel minerals	-0.73***	
	DTPA Extracts	$CaCl_2$ Extracts
Soil pH	0.14	-0.59***
Enzyme	DTPA Extracts	$CaCl_2$ Extracts
	S1	
AMO	-0.06	-0.13
ACP	-0.53***	-0.63***
	S2	
AMO	-0.27	-0.28
ACP	0.44**	0.48**
	S3	
AMO	-0.38*	-0.34*
ACP	0.03	0.17

Significance codes: * < 0.05, ** < 0.01, *** < 0.001.
DTPA=diethylenetriaminepentaacetic acid.
AMO=ammonia monooxygenases.
ACP=acid phosphatases.

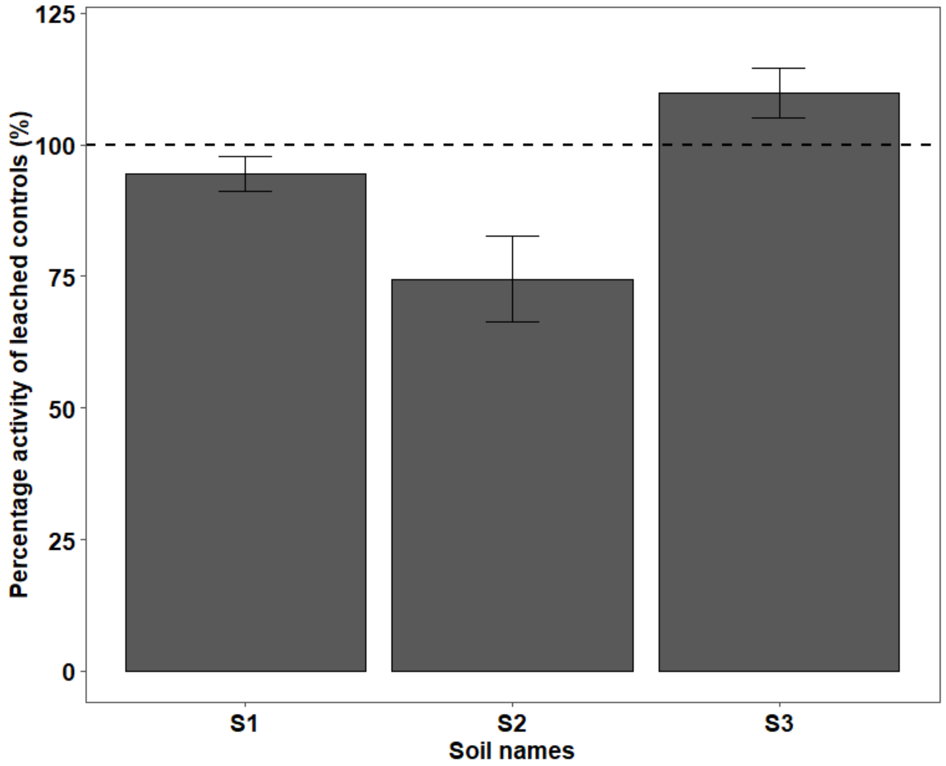

Figure A-1. Leaching did not reduce acid phosphatases (ACP) activity in all three Canadian soils. Control soils were leached once to determine an electrical conductivity baseline for metal salt spike soils. The activity of ACP was measured 7 days after leaching 210 mg of soil with 200ml of artificial rainwater. SI, S2 and S3 represent the names of the three Canadian soils used. Horizontal broken line indicates percentage activity in non-leached soils. Gray vertical bars represent average ACP activity of 9 replicates (3 per soil) in the three soils. The standard error (SE) of the mean are represented by error bars. Asterisks indicate significant differences from non-leached soils (controls).

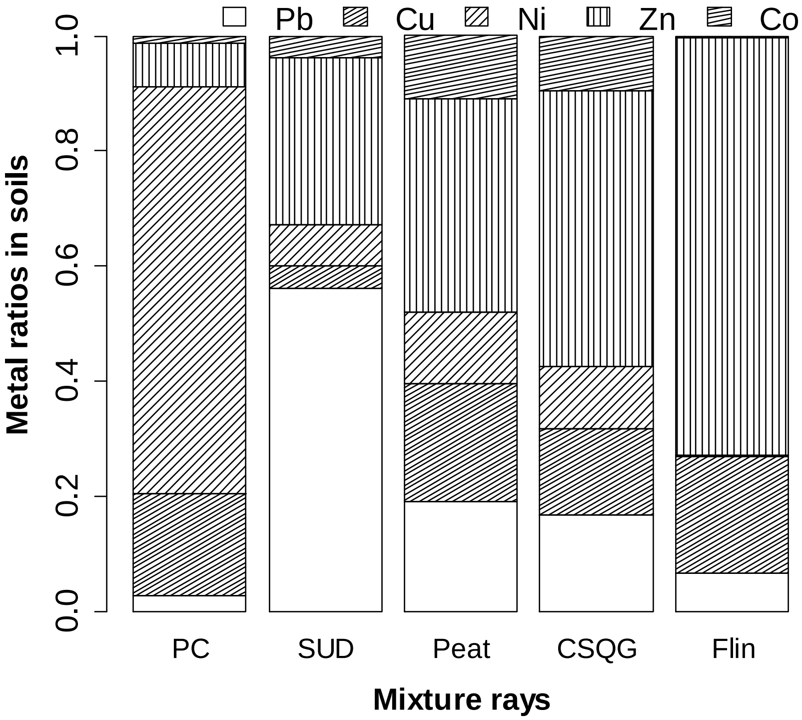

Figure A-2. Stacked bar plots showing ratios of metals in five fixed ratio rays. The vertical bars represent the ratios of each metal, each texture pattern within a bar represents a metal in the mixture. For example, vertical patterns represent Zn, horizontal pattern represent Cu, no pattern represent Pb etc. as shown by the legend above.

9 APPENDIX B: CHAPTER 4 SUPPLEMENTARY MATERIAL

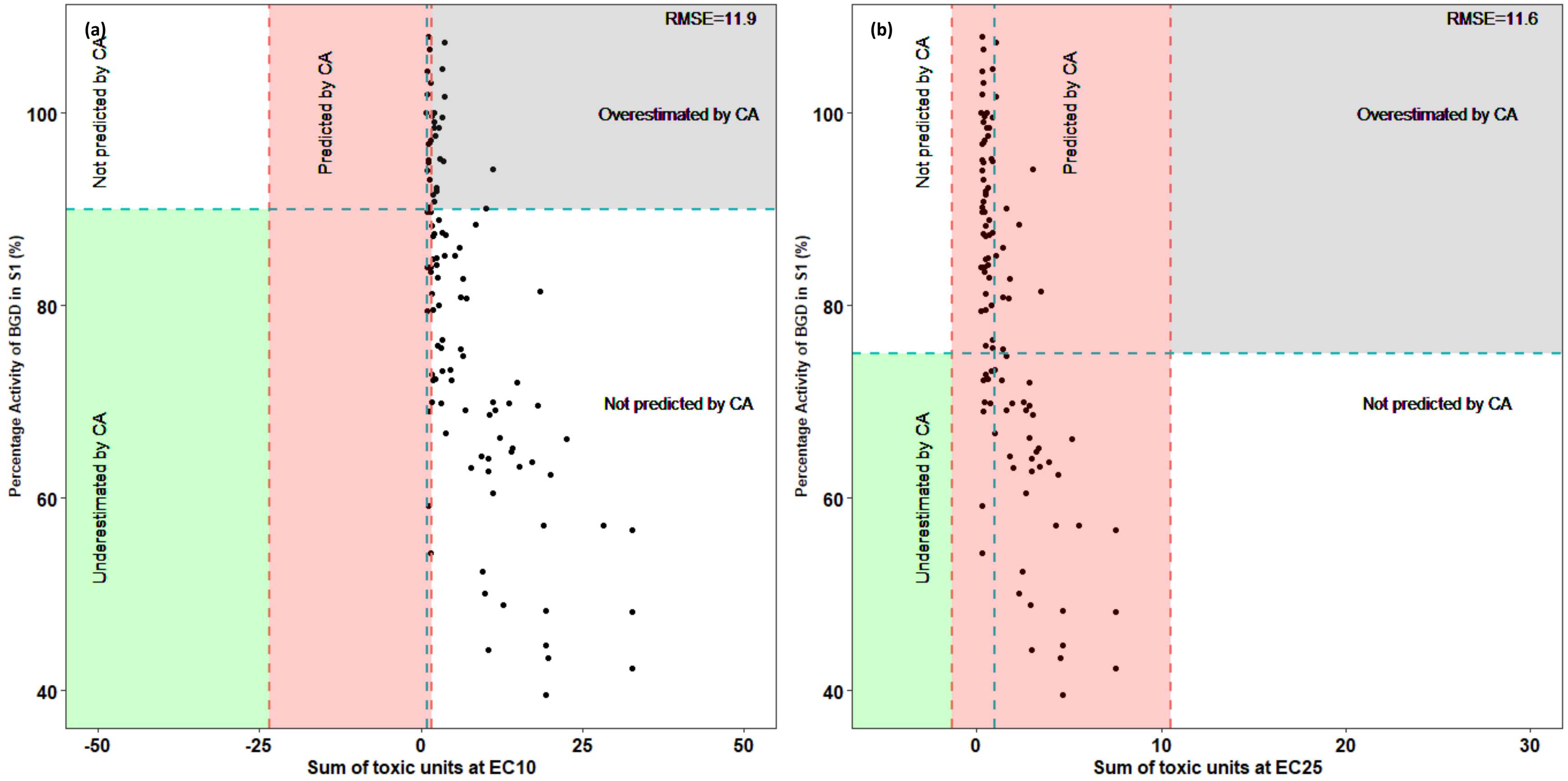

Figure B-1. Concentration addition (CA) predicts mixture toxicity to BGD at sum toxic units at EC_{25}but not EC_{10}. The figure shows the concentration response relationships between beta glucosidases (BGD) and 10 mixture rays in S1 fitted by a log-logistic model. Y-axis represents the percentage activity of enzyme in soil, and the X-axis represents the toxic units (TU) of the metals calculated from effective concentrations. Grid (a) TUs were calculated from EC_{10}; Grid (b) TUs were calculated from EC_{25}. The intersection between the two blue dashed lines represent the point where CA occurs for the specified effective concentration, while the red shaded area represents the 95% confidence interval. The green-shaded quadrant represents underestimation by the CA model, and the grey-shaded quadrant represents overestimation by the CA model. The non-shaded areas cannot be predicted by CA. The root mean squared error (RMSE) for each TU is reported.

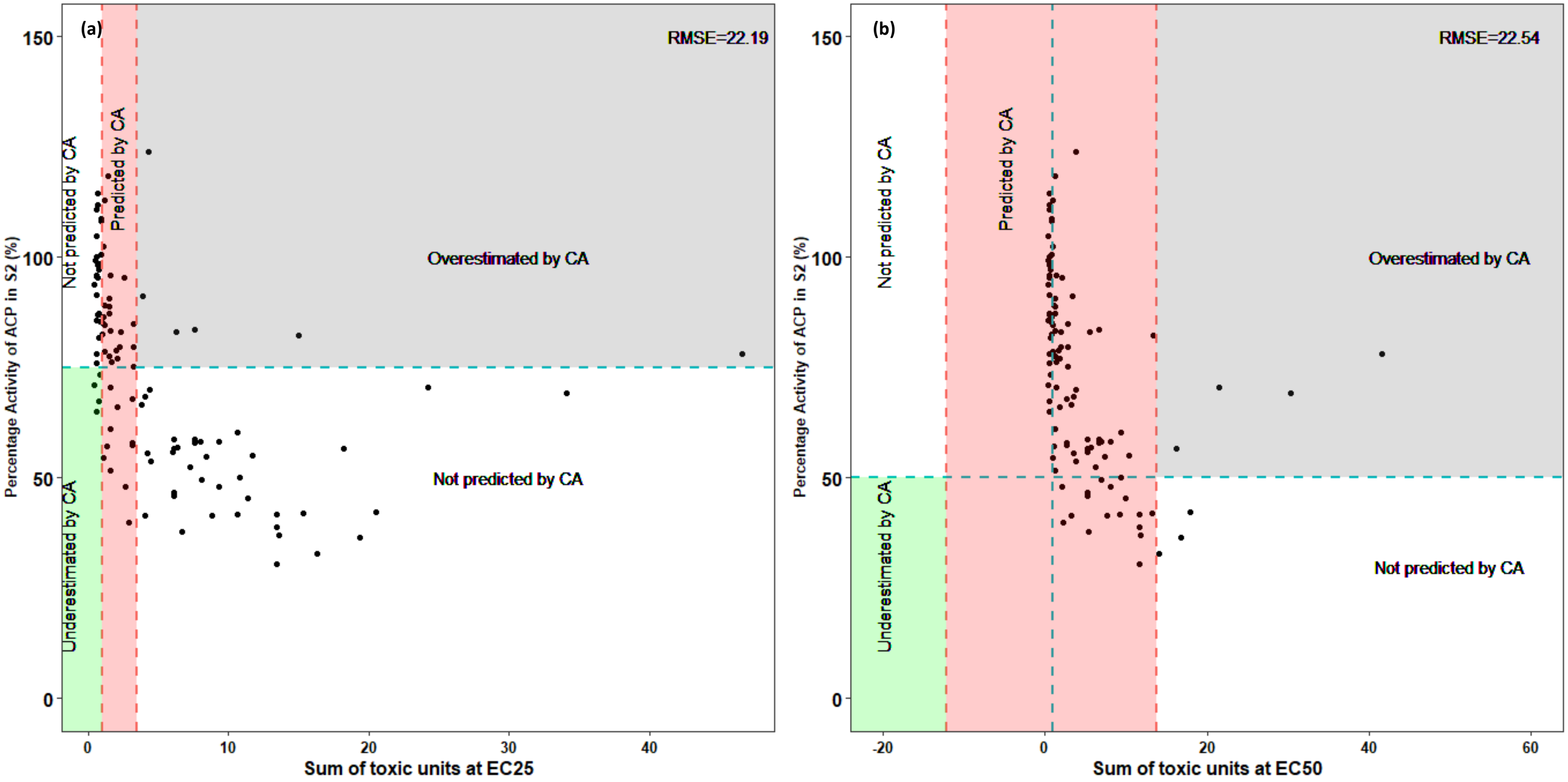

Figure B-2. Concentration addition (CA) predicts mixture toxicity to ACP at sum of toxic units at EC_{50}, sparingly at EC_{25}. The figure shows the concentration response relationships between acid phosphatases (ACP) and 10 mixture rays in S2 fitted by a log-logistic model. Y-axis represents the percentage activity of enzyme in soil, and X-axis represents the toxic units (TU) of the metals calculated from effective concentrations. Grid (a) TUs calculated from EC_{25}. Grid (b) TUs calculated from EC_{50}. The intersection between the two blue dashed lines represent the point where CA occurs for the specified effective concentration, while the red shaded area represents the 95% confidence interval. The green-shaded quadrant represents underestimation by the CA model, and the grey-shaded quadrant represents overestimation by the CA model. The non-shaded areas cannot be predicted by CA. The root mean squared error (RMSE) for each TU is reported.

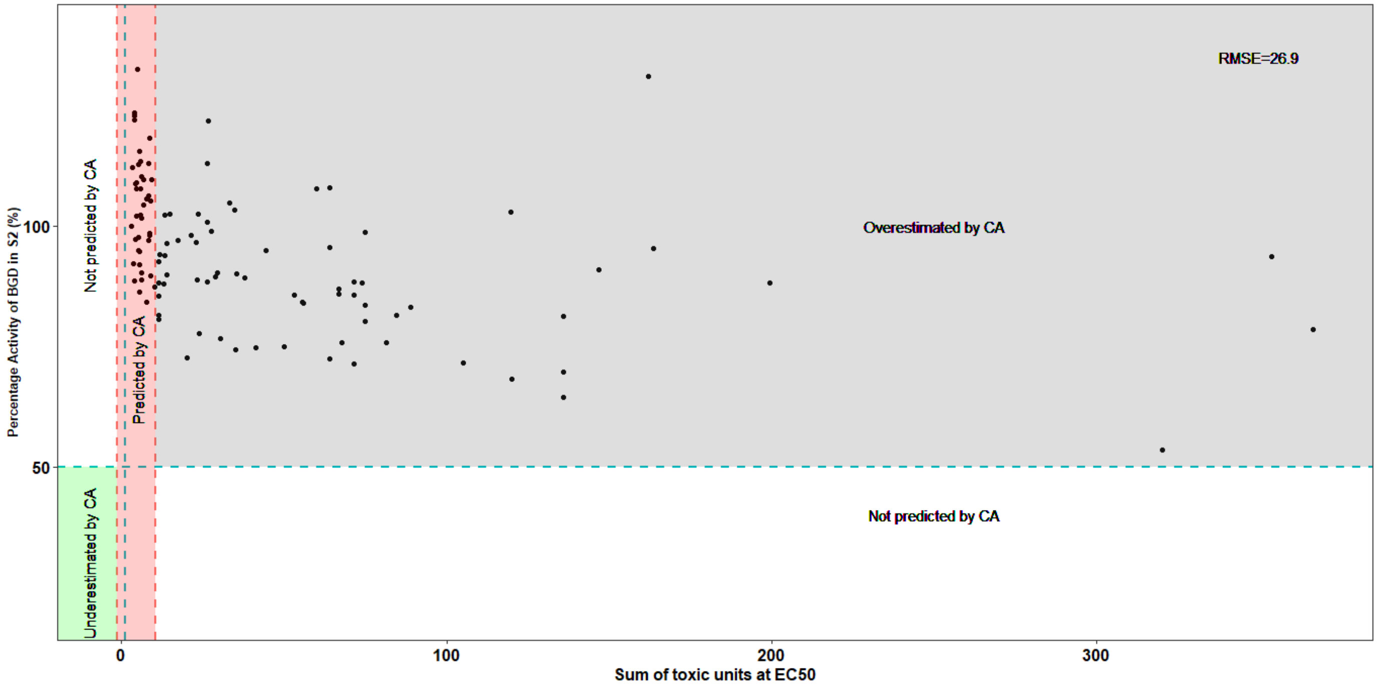

Figure B-3. Concentration addition (CA) either predicts or overestimates mixture toxicity to BGD at EC_{50}. The figure shows the concentration response relationships between beta glucosidases (BGD) and 10 mixture rays in S2 fitted by a log-logistic model. Y-axis represents the percentage activity of enzyme in soil, and X-axis represents the toxic units (TU) of the metals calculated from effective concentrations. The intersection between the two blue dashed lines represent the point where CA occurs for the specified effective concentration, and the red shaded area represents the 95% confidence interval. The green-shaded quadrant represents underestimation by the CA model, and the grey-shaded quadrant represents overestimation by the CA model. The non-shaded areas cannot be predicted by CA. The root mean squared error (RMSE) for each TU is reported.

Table B-1. Tukey HSD comparison between rays for beta glucosidases in S1 and S2

S1				
Rays	**Difference**	**Lower**	**Upper**	***p*-value**
Ray 10-Ray 1	8.2	-13.8	30.3	1.0
Ray 2-Ray 1	12.5	-9.0	34.0	0.7
Ray 3-Ray 1	11.2	-10.4	32.7	0.8
Ray 4-Ray 1	-1.2	-22.8	20.3	1.0
Ray 5-Ray 1	8.0	-13.5	29.6	1.0
Ray 6-Ray 1	14.3	-7.2	35.8	0.5
Ray 7-Ray 1	4.1	-17.4	25.7	1.0
Ray 8-Ray 1	4.2	-17.3	25.8	1.0
Ray 9-Ray 1	22.5	0.9	44.0	0.03*
Ray 2-Ray 10	4.3	-17.8	26.3	1.0
Ray 3-Ray 10	2.9	-19.1	25.0	1.0
Ray 4-Ray 10	-9.5	-31.5	12.6	0.9
Ray 5-Ray 10	-0.2	-22.2	21.8	1.0
Ray 6-Ray 10	6.1	-15.9	28.1	1.0
Ray 7-Ray 10	-4.1	-26.1	17.9	1.0
Ray 8-Ray 10	-4.0	-26.0	18.0	1.0
Ray 9-Ray 10	14.2	-7.8	36.3	0.5
Ray 3-Ray 2	-1.3	-22.9	20.2	1.0
Ray 4-Ray 2	-13.7	-35.3	7.8	0.6
Ray 5-Ray 2	-4.5	-26.0	17.1	1.0
Ray 6-Ray 2	1.8	-19.7	23.3	1.0
Ray 7-Ray 2	-8.4	-29.9	13.2	1.0
Ray 8-Ray 2	-8.3	-29.8	13.3	1.0
Ray 9-Ray 2	10.0	-11.6	31.5	0.9
Ray 4-Ray 3	-12.4	-34.0	9.1	0.7
Ray 5-Ray 3	-3.2	-24.7	18.4	1.0
Ray 6-Ray 3	3.1	-18.4	24.7	1.0
Ray 7-Ray 3	-7.0	-28.6	14.5	1.0
Ray 8-Ray 3	-7.0	-28.5	14.6	1.0
Ray 9-Ray 3	11.3	-10.3	32.8	0.8
Ray 5-Ray 4	9.3	-12.3	30.8	0.9
Ray 6-Ray 4	15.5	-6.0	37.1	0.4
Ray 7-Ray 4	5.4	-16.2	26.9	1.0
Ray 8-Ray 4	5.5	-16.1	27.0	1.0
Ray 9-Ray 4	23.7	2.2	45.2	0.02*
Ray 6-Ray 5	6.3	-15.3	27.8	1.0
Ray 7-Ray 5	-3.9	-25.4	17.7	1.0
Ray 8-Ray 5	-3.8	-25.3	17.7	1.0
Ray 9-Ray 5	14.4	-7.1	36.0	0.5

Ray 7-Ray 6	-10.2	-31.7	11.4	0.9
Ray 8-Ray 6	-10.1	-31.6	11.5	0.9
Ray 9-Ray 6	8.2	-13.4	29.7	1.0
Ray 8-Ray 7	0.1	-21.5	21.6	1.0
Ray 9-Ray 7	18.3	-3.2	39.9	0.2
Ray 9-Ray 8	18.2	-3.3	39.8	0.2

S2

Rays	**Difference**	**Lower**	**Upper**	***p*-value**
Ray 10-Ray 1	-16.5	-34.0	0.9	0.1
Ray 2-Ray 1	-6.3	-23.8	11.1	1.0
Ray 3-Ray 1	-14.2	-31.6	3.3	0.2
Ray 4-Ray 1	-4.1	-21.5	13.4	1.0
Ray 5-Ray 1	-8.0	-25.4	9.5	0.9
Ray 6-Ray 1	-5.4	-22.9	12.1	1.0
Ray 7-Ray 1	-8.7	-26.2	8.8	0.8
Ray 8-Ray 1	-8.8	-26.2	8.7	0.8
Ray 9-Ray 1	7.0	-10.5	24.5	1.0
Ray 2-Ray 10	10.2	-6.9	27.3	0.6
Ray 3-Ray 10	2.4	-14.7	19.4	1.0
Ray 4-Ray 10	12.5	-4.6	29.5	0.4
Ray 5-Ray 10	8.6	-8.5	25.7	0.8
Ray 6-Ray 10	11.1	-6.0	28.2	0.5
Ray 7-Ray 10	7.8	-9.2	24.9	0.9
Ray 8-Ray 10	7.8	-9.3	24.8	0.9
Ray 9-Ray 10	23.5	6.5	40.6	0.00*
Ray 3-Ray 2	-7.8	-24.9	9.2	0.9
Ray 4-Ray 2	2.3	-14.8	19.3	1.0
Ray 5-Ray 2	-1.6	-18.7	15.5	1.0
Ray 6-Ray 2	0.9	-16.2	18.0	1.0
Ray 7-Ray 2	-2.4	-19.5	14.7	1.0
Ray 8-Ray 2	-2.4	-19.5	14.6	1.0
Ray 9-Ray 2	13.3	-3.7	30.4	0.3
Ray 4-Ray 3	10.1	-7.0	27.2	0.7
Ray 5-Ray 3	6.2	-10.9	23.3	1.0
Ray 6-Ray 3	8.8	-8.3	25.8	0.8
Ray 7-Ray 3	5.5	-11.6	22.6	1.0
Ray 8-Ray 3	5.4	-11.7	22.5	1.0
Ray 9-Ray 3	21.2	4.1	38.3	0.00*
Ray 5-Ray 4	-3.9	-21.0	13.2	1.0

Ray 6-Ray 4	-1.3	-18.4	15.8	1.0
Ray 7-Ray 4	-4.6	-21.7	12.5	1.0
Ray 8-Ray 4	-4.7	-21.8	12.4	1.0
Ray 9-Ray 4	11.1	-6.0	28.2	0.5
Ray 6-Ray 5	2.6	-14.5	19.6	1.0
Ray 7-Ray 5	-0.7	-17.8	16.3	1.0
Ray 8-Ray 5	-0.8	-17.9	16.3	1.0
Ray 9-Ray 5	15.0	-2.1	32.0	0.1
Ray 7-Ray 6	-3.3	-20.4	13.8	1.0
Ray 8-Ray 6	-3.4	-20.4	13.7	1.0
Ray 9-Ray 6	12.4	-4.7	29.5	0.4
Ray 8-Ray 7	-0.1	-17.1	17.0	1.0
Ray 9-Ray 7	15.7	-1.4	32.8	0.1
Ray 9-Ray 8	15.8	-1.3	32.8	0.1

Significance codes: *< 0.05

Table B-2. Ratio of metal mixture rays and nominal concentrations calculated at 1 Toxic Unit (TU) in mg per kg soil. Metal mixture rays are in the first column with their corresponding nominal metal doses and ratios in brackets. The TU was calculated from EC_{50} values of individual metals (Cu, Co, Pb, Ni, Zn) derived from literature in mg/kg of soil. Lower and higher TU's were determined from dividing or multiplying concentrations in a ray by a desired factor or dose level (0 and 16)

Metal/ Ray EC_{50}	**Lead 1600 (mg/kg)**	**Copper 700 (mg/kg)**	**Nickel 475 (mg/kg)**	**Zinc 750 (mg/kg)**	**Cobalt 1480 (mg/kg)**	**Total (Ratio) (mg/kg)**
1	139.9 (0.469)	147.3 (0.157)	94.2 (0.109)	337.5 (0.260)	79.7 (0.050)	968 (1)
2	453.8 (0.175)	152.2 (0.184)	62.1 (0.118)	251.7 (0.423)	48.3 (0.1)	799 (1)
3	153.0 (0.190)	165.6 (0.206)	98.9 (0.123)	299.8 (0.372)	88.4 (0.110)	806 (1)
4	134.1 (0.167)	120.6 (0.151)	86.2 (0.108)	383.0 (0.478)	76.6 (0.096)	801 (1)
5	326.5 (0.324)	148.1 (0.147)	88.7 (0.088)	148.1 (0.147)	296.2 (0.294)	1008 (1)
6	162.1 (0.2)	162.1 (0.2)	162.1 (0.2)	162.1 (0.2)	162.1 (0.2)	811 (1)
7	50.5 (0.066)	154.7 (0.202)	2.3 (0.003)	555.8 (0.726)	2.3 (0.003)	766 (1)
8	181.2 (0.218)	128.6 (0.155)	90.1 (0.109)	349.6 (0.421)	80.4 (0.097)	830 (1)
9	13.9 (0.026)	95.2 (0.178)	378.3 (0.707)	40.7 (0.076)	7.0 (0.013)	535 (1)
10	578.6 (0.561)	40.2 (0.039)	74.3 (0.072)	299.1 (0.29)	38.2 (0.037)	1030 (1)

(Lock and Janssen, 2003, 2002a, 2002b; Sandifer and Hopkin, 1997a, 1997b).

10 APPENDIX C: CHAPTER 5 SUPPLEMENTARY MATERIAL

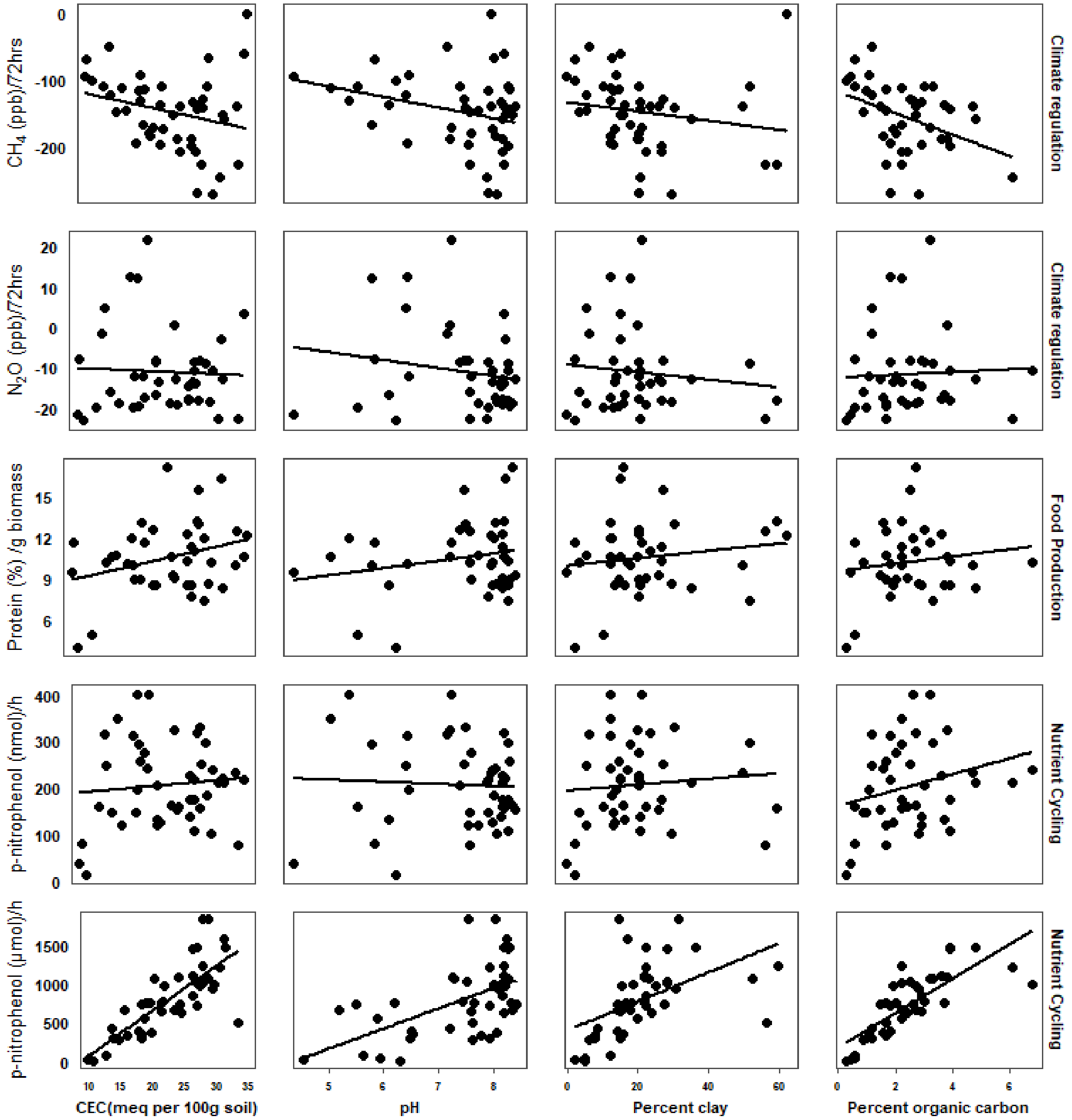

Figure C-1. A multi-panel scatterplot showing the relationship between ecosystem services and soil cation exchange capacity (CEC), soil pH, percentage clay, and percentage organic carbon for 47 soils. A trend line was added to ease graphical interpretation. The first row shows links between CH_4 production (parts per billion per gram soil per 72 h) and soil properties, second row shows links with N_2O fluxes (parts per billion per gram soil per 72 h), third row shows links with wheat crude protein (percent per gram) after 35 days of seeding, fourth row shows links with the activity of Acid phosphatases (nmol p-nitrophenol/hr/g soil), and the fifth row shows links with activity of beta glucosidases (µmol p-nitrophenol/hr/ g soil). The right side of the y-axis shows the ecosystem service that each measured endpoint represented.

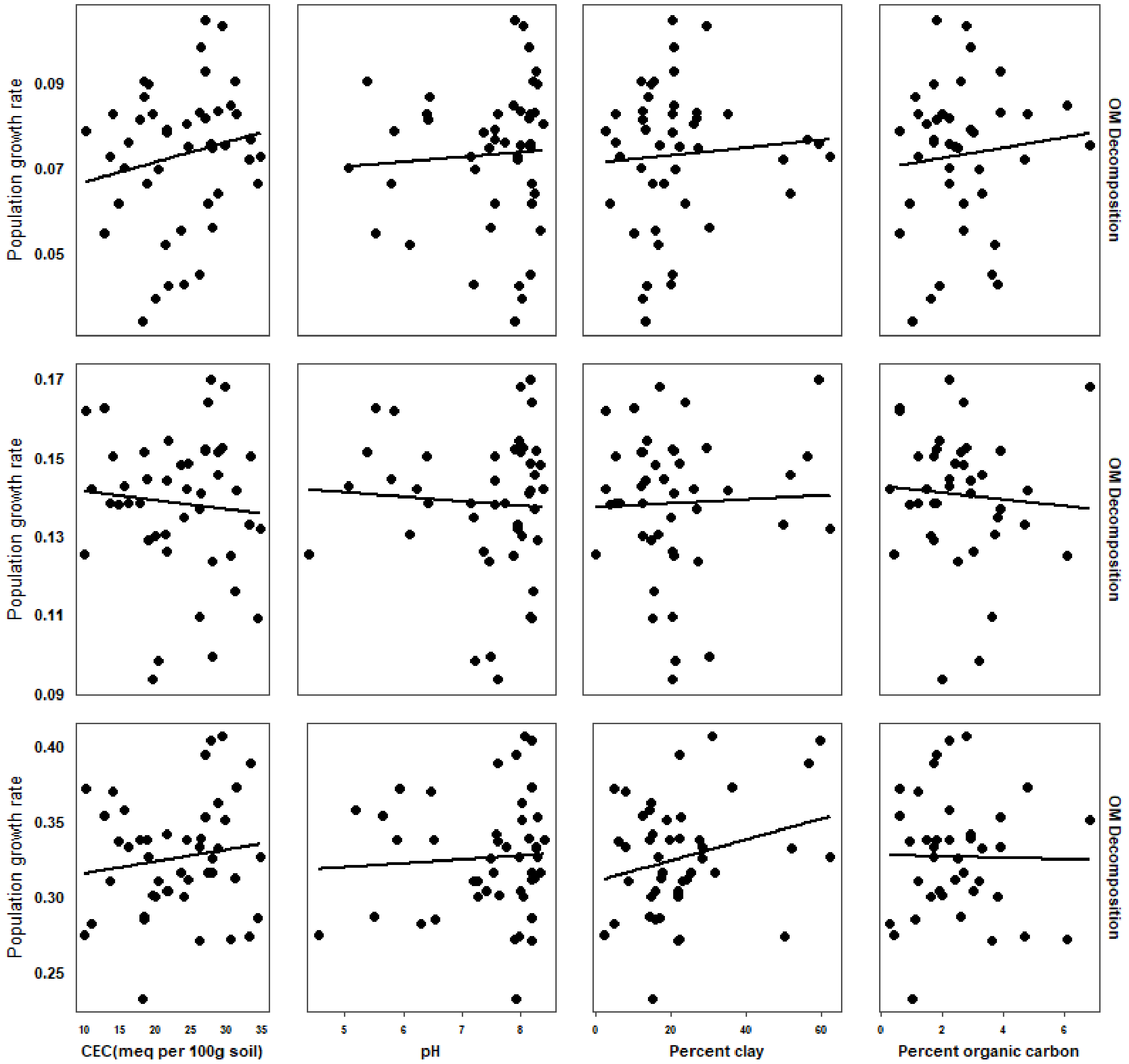

Figure C-2. A multi-panel scatterplot showing the relationship between ecosystem services and soil cation exchange capacity (CEC), soil pH, percentage clay, and percentage organic carbon for 47 soils. A trend line was added to ease graphical interpretation. The first row shows links between the instantaneous population growth rates (IPGR) of *Oppia nitens* and soil properties, second row shows links with IPGR of *Enchytraeus cripticus*, third row shows links with IPGR of *Folsomia candida*. The right side of the y-axis shows the ecosystem service that each measured endpoint represented.

Table C-1. Pearson correlation coefficients between soil properties and ecosystem services in control soils

Ecosystem Services	**Climate Regulation**	**Nutrient Cycling**	**Food Production**	**Organic Matter Decomposition**	**Organic Matter Decomposition**
Soil properties	CH_4 consumption	ACP activity	Percent protein	IPGR of collembola	IPGR of enchytraeids
pH	-0.27	-0.01	-0.14	0.11	-0.10
Percent clay	-0.11	0.26	-0.01	0.21	0.04
CEC	-0.28*	0.19	-0.02	0.11	-0.04
Percent OC	0.22	0.46***	0.02	-0.02	-0.06

Significance codes: * < 0.05, ** < 0.01, *** < 0.001.
AMO= ammonia monooxygenase

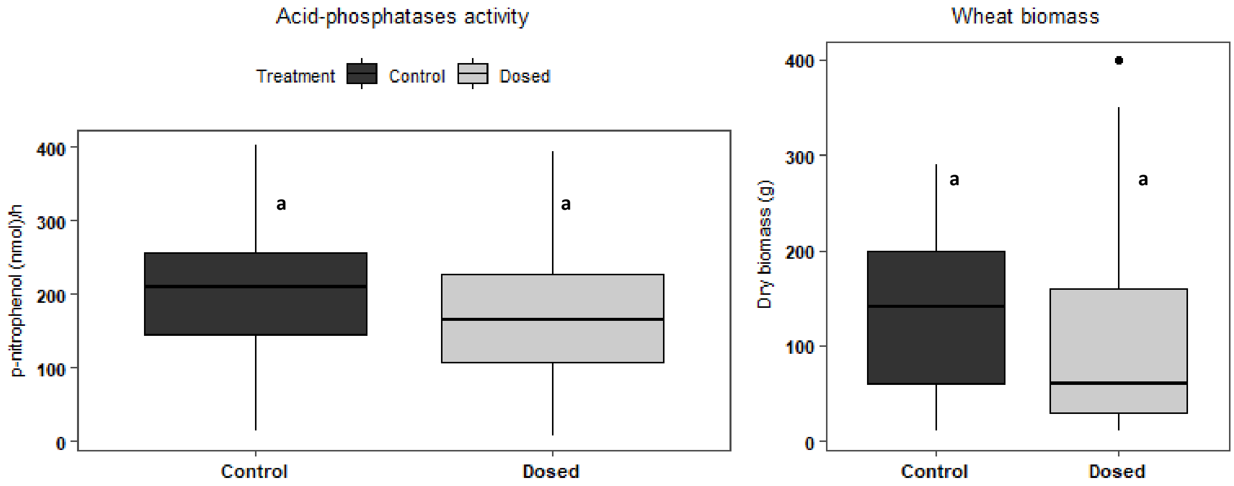

Figure C-3. Boxplots showing the sensitivity of two endpoints that represent two ecosystem services to metal mixtures in 47 Canadian soils. Black and gray plots represent services in control and metal spiked soils respectively. Middle line in the box represents median, while the lower and upper ends of the box represent the first and third quartiles respectively. Points beyond the lines are may be considered outliers. Alphabets a and b within plots represent significant differences (P<0.05) between treatments. Plots with same alphabetic insets indicate similarities while those with different letters represent differences. On the left is the activity of ACP in nmol per hour and to the right is dry wheat biomass in grams.

10.1 Derivation of greenhouse gas concentrations

The actual concentrations of greenhouse gas emission or consumption had to be back calculated to account for N_2 dilutions. At time 0, the concentrations of greenhouse gases (CO_2, CH_4 and N_2O) was assumed to be 0 ppm. Calculations for hours (time) 6 and 72 were determined as follows;

For Time 6;
Volume of serum vial = 125 mL
Volume of N_2 added = 15 mL
Percentage volume of N_2 in serum vial = $\frac{15\ mL}{125\ mL} \times 100\% = 12\%$
Volume of GHG taken with syringe= 15 mL
Volume of N_2 in syringe = $12\% \times 15\ mL = 1.8$ mL
Hence, for Time 6, 1.8 mL of each sample taken was nitrogen (N_2).
Actual [x] for Time 6= $\frac{15x}{13.2}$

For Time 72;
Volume of serum vial = 125 mL
Volume of N_2 from Time 6 = 13.2 mL
Volume of N_2 added = 15 mL
Total volume of N_2 = 15 mL+13.2 mL = 28.2 mL
Percentage volume of N_2 in serum vial = $\frac{28.2\ mL}{125\ mL} \times 100\% = 22.6\%$
Volume of N_2 in syringe = $22.6\% \times 15\ mL = 3.39$ mL
Hence, for Time 72, 3.39 mL of each sample taken was nitrogen (N_2).
Actual [x] for Time 72= $\frac{15x}{11.61}$

$$
\begin{aligned}
\text{Dosed model (ES)} = \quad [&\mathbf{CEC}_i = \beta_0 + \beta_1 \text{pH}_{,i} + \beta_2 \text{Clay}_{,i} + \beta_3 \text{Organic}_{,i} + \varepsilon_{\text{CEC}},\\
&\mathbf{Phosphatase}_i = \beta_4 + \beta_5 \text{Organic}_{,i} + \varepsilon_{\text{Phosphatase}},\\
&\mathbf{AMO}_i = \beta_6 + \beta_7 \text{CEC}_{,i} + \beta_8 \text{pH}_{,i} + \varepsilon_{\text{AMO}},\\
&\mathbf{Glucosidase}_i = \beta_9 + \beta_1 \text{CEC}_{,i} + \beta_{10} \text{Organic}_{,i} + \beta_{11} \text{AMO}_{,i} + \beta_{12} \text{Phosphatase}_{,i} + \varepsilon_{\text{Glucosidase}},\\
&\mathbf{Carbon\ dioxide}_i = \beta_{13} + \beta_{14} \text{Glucosidase}_{,i} + \varepsilon_{\text{Carbon dioxide}},\\
&\mathbf{Methane}_i = \beta_{15} + \beta_{16} \text{CEC}_{,i} + \varepsilon_{\text{Methane}},\\
&\mathbf{Nitrous}_i = \beta_{17} + \beta_{18} \text{pH}_{,i} + \beta_{19} \text{Phosphatase}_{,i} + \beta_{20} \text{Carbon dioxide}_{,i} + \varepsilon_{\text{Nitrous oxide}},\\
&\mathbf{Biomass}_i = \beta_{21} + \beta_{22} \text{CEC}_{,i} + \beta_{23} \text{Clay}_{,i} + \beta_{24} \text{pH}_{,i} + \beta_{25} \text{Glucosidase}_{,i} + \varepsilon_{\text{Biomass}},\\
&\mathbf{Mite\ survival}_i = \beta_{26} + \beta_{27} \text{pH}_{,i} + \varepsilon_{\text{Mite survival}},\\
&\mathbf{Mite\ reproduction}_i = \beta_{28} + \beta_{29} \text{CEC}_{,i} + \varepsilon_{\text{Mite reproduction}},\\
&\mathbf{Collembola\ reproduction}_i = \beta_{30} + \beta_{31} \text{Collembola survival}_{,i} + \varepsilon_{\text{Collembola reproduction}},\\
&\mathbf{Collembola\ survival}_i = \beta_{32} + \beta_{33} \text{Organic}_{,i} + \varepsilon_{\text{Collembola survival}},\\
&\mathbf{Glyphosate\ degradation}_i = \beta_{34} + \beta_{35} \text{Phosphatase}_{,i} + \beta_{36} \text{CO}_{2,i} + \varepsilon_{\text{Glyphosate degradation}}] \quad \text{Eq. C-1}
\end{aligned}
$$

On the left side of each equation is the response variable and on the right are the predictor variables, the β's are the standardized regression coefficients, and the Ɛ's are the error terms. Response variables in one equation could be a predictor in another equation.

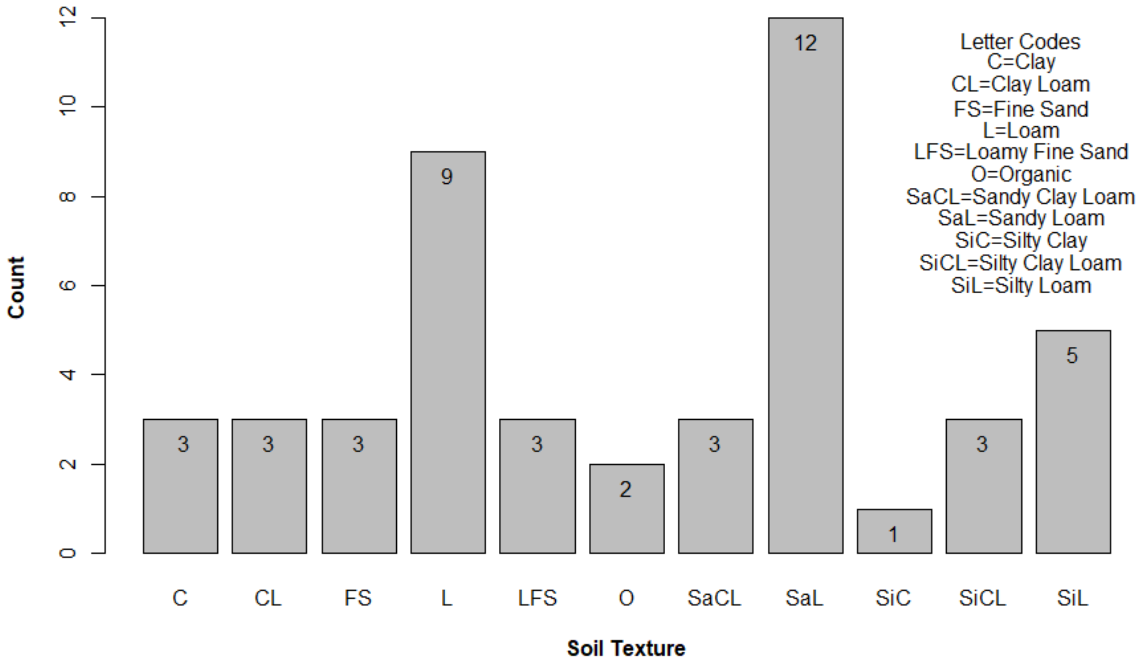

Figure C-4. Barplots textural classes of 47 Canadian soils.

Table C-2. Nominal and actual (average) doses of metals in 47 soils (mg/kg of soil)

Metal	**Lead**	**Copper**	**Nickel**	**Zinc**	**Cobalt**
Nominal dose	167	1143	4539	488	84
Actual dose	17	866	4718	581	10

Table C-3. $CaCl_2$ extractable metals in 47 metal spiked soils (mg/kg of soil)

Soil	Cobalt	Copper	Nickel	Lead	Zinc	Total
S1	0	0	0	0	19.183673	19.183673
S2	0	0	0	0	7.5510204	7.5510204
S3	0	0	0	0	30.510204	30.510204
S4	0	0	0	0	1.4285714	1.4285714
S5	0	0	0	0	1.2244898	1.2244898
S6	0	0	0	0	1.8367346	1.8367346
S7	0	0	0	0	0	0
S8	0	0	0	0	10.714285	10.714285
S9	0	0	0	0	0	0
S10	0	0	0	0	2.0408163	2.0408163
S11	0	1.7346938	4.2857142	0	115	121.02040
S12	0	0	0	0	9.8979591	9.8979591
S13	0	0	0	0	8.7755102	8.7755102
S14	0	0.9183673	2.7551020	0	94.897959	98.571428
S15	0	0	0	0	0	0
S16	0	0	0.7142857	0	11.428571	12.142857
S17	0	0	0	0	0	0
S18	0	1.9387755	2.7551020	0	101.02040	105.71428
S19	0	0.8163265	0	0	9.2857142	10.102040
S20	0	0	0	0	4.8979591	4.8979591
S21	0	0	0	0	7.5510204	7.5510204
S22	0	4.1836734	4.1836734	0	111.22449	119.59183
S23	0	2.3469387	4.0816326	0	110	116.42857
S24	0	0	0	0	1.8367346	1.8367346
S25	0	0	0	0	12.959183	12.959183
S26	0	17.959183	6.7346938	0	167.55102	192.24489
S27	0	0	0	0	1.8367346	1.8367346
S28	0	0	0	0	2.6530612	2.6530612
S29	0	0	0.9183673	0	29.081632	30
S30	0	0	0	0	4.7959183	4.7959183
S31	0	0	0	0	0	0

S32	0	0.9183673	2.4489795	0	62.653061	66.020408
S33	0	0.7142857	1.9387755	0	100.20408	102.85714
S34	0	0	1.3265306	0	25.918367	27.244898
S35	0	0	2.4489795	0	52.959183	55.408163
S36	0	0	0	0	0	0
S37	0	0	0	0	1.0204081	1.0204081
S38	0	0	0	0	1.2244898	1.2244898
S39	0	0	0	0	0	0
S40	0	0	0	0	3.4693877	3.4693877
S41	1.1224489	7.8571428	6.1224489	0	135.10204	150.20408
S42	0	0	0	0	14.591836	14.591836
S43	0	2.0408163	2.2448979	0	79.387755	83.673469
S44	0	1.0204081	3.2653061	0	106.53061	110.81632
S45	0	0	0	0	0	0
S46	1.4285714	8.2653061	10.204081	0	291.53061	311.42857
S47	0	0	0	0	2.5510204	2.5510204