Effects of Bioavailability and Accumulation of Single Metal and Mixture Metal on Toxicity to the Mite, *Oppia nitens*

A Thesis Submitted to the College of Graduate and Postdoctoral Studies

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Toxicology Graduate Program

University of Saskatchewan

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Abstract

Canada has some of the largest metal deposits in the world and the Canadian mining industry is one the largest employers of labour in Canada. Consequently, mining and smelting operations in Canada are one of the sources of metal level increase in the environment. Metals pollute the terrestrial environment because of fall-out from the mining industry. Soils are major sinks for metals in the terrestrial environment. It is therefore important that metal risk assessment should clearly reflect the metal contamination in the soils.

The main objectives of this thesis were to generate more realistic metal toxicity data using a native Canadian invertebrate species that will help improve metal risk assessment in Canada. Firstly, toxicity of common metals (Cu, Pb, Zn, Co, Ni) found in contaminated sites in Canada was assessed on an oribatid mite, *Oppia nitens* which is abundant in Canadian soils. The metal toxicities were assessed as singles and as mixtures in five different soils. The metal mixture ratios were fixed such that it reflected ratios of metals found in contaminated sites. The patterns of sensitivity of the mite to metals by soils differed between single metals and metal mixtures. Nickel, which had not been tested with *Oppia nitens* before, was found to be the most toxic metal to the mite and zinc was less toxic. Concentration addition was protective of 53% of metal mixture toxicity due to antagonistic and concentration addition. Bioavailable metals existed as metals bound to fulvic acid.

After determining the toxicity of the metals in the five soils, the multigenerational effect of one of the metals on soil mites was investigated in the most sensitive soil to single metal contamination. Continuous and pulse zinc exposure effect on *O. nitens* populations was assessed in three generations of the mites. Using critical-effect levels (EC50s), pulse exposed mites seemed to be

tolerant and the continuous exposed mites were sensitive. However, the instantaneous population growth rate showed that both pulse and continuous exposures were more sensitive than their parents. The major finding from this study was that persistence of metals in soils can cause multigenerational adverse effects on continuously exposed mites in the soil.

The last chapter of this thesis investigated the direct effect of soil habitat quality as a site-specific feature on organisms and how it influenced their response to metal contamination. For this test, forty-seven (47) soils were ranked according to their habitat qualities from one to three (high to low), using standard soil invertebrate species (*Folsomia candida, Enchytraeus crypticus*) fitness and plant (*Elymus lanceolatus*) productivity as metrics to choose habitat qualities. From the ranked 47 soils, eighteen (18) soils comprising six soils making each habitat quality was chosen in a duplicated experiment. The soils were spiked with increasing concentrations of Zn and the Zn toxicokinetics, toxicodynamics, survival and reproduction of mites were assessed. The mites in the soils of high habitat quality were less stressed than mites in the low habitat quality soils despite being exposed to the same amount of bioavailable metals. The key findings from this study were that soil habitat quality has a direct influence on how its inhabitants cope with metal stress. Therefore, habitat qualities of soils can be considered as a site-specific feature in remediation of contaminated sites.

Acknowledgements

I would like to thank my supervisors Drs. Steven Siciliano and Beverley Hale who have been supportive to me as a person and their unwavering encouragement throughout my research work up to completion. I would like to extend my thanks to other members of my graduate committee: Dr. David Janz, Dr. Karsten Liber, Dr. Sina Adl, Dr. Som Niyogi and Dr. Elizabeth Haack who served as my external examiner. My supervisors and committee members have been helpful to me, serving as role models for me in developing into a scientist and researcher that I will like to be.

I will like to thank Dr. John Owojori who has been a role model to me in all aspects of life and for his advices and help throughout my graduate education life. I will like to thank my friends and colleagues on the same project; Kobby Awuah, Mathieu Renaud and Mark Cousins who have been supportive as we navigated this work together through the good and bad times. I will like to thank Hamzat Fajana who is a friend and brother for his support and help whenever I call for it. I am grateful to Amy Gainer who has my back always as a colleague and does not hesitate to offer help when needed. My thanks goes to Richard Nhan who was helpful to me during his time as a laboratory technician in the Siciliano lab. I am grateful to Alix Schebel and Curtis Senger for helping me with lab logistics. I am thankful to the entire Siciliano lab group, students and staff of the Soil Science Department and Toxicology Centre for the support all through my program.

I will like to appreciate the RCCG Grace Sanctuary Church that has been a family to me here in Saskatoon. My appreciation goes to my parents, Mr. Tope and Mrs. Elizabeth Jegede for giving me all I needed to succeed in life. I want to thank my wife, Grace and my children, Oreofeoluwa and Temidayo for their love and belief in me. You people make me happy and feel loved. Lastly, I want to thank God for being my overall support and grace to be alive. God bless!

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List of Abbreviations

CCME	Canadian Council of Ministers of Environment
EC25 OR EC50	Effective concentration (25 or 50% reduction compared to the control response)
LOEC	Lowest observed effective concentration
LC50	Lethal concentration causing 50% mortality compared to the control response
EU REACH	European Union Registration, Evaluation, Authorization and Restriction of Chemicals
CEC	Cation exchange capacity
WHAM	Windermere Humic Aqueous Model
DNA	Deoxyribonucleic acid
ISO	International Organization of Standardization
OECD	Organization for Economic Co-operation and Development
CSQG	Canadian Soil Quality Guideline

1. Introduction

Metals are ubiquitous because they occur naturally, and their use by humans is inevitable. Canada is one of the largest producers of metals, with a vast mining industry to show for it. Mining accounts for about 30% of the total GDP of Canada. Therefore, metal risk assessment in soils is a priority in Canada because of the associated release of metals into the environment during mining and smelting operations. Metals commonly occur in the environment as mixtures; however, current data for metal risk assessment does not reflect mixtures. In Canada, like in many other jurisdictions, the risk assessment of mixtures of metals in contaminated sites have been based on data for single components of the mixture as provided by CCME's soil quality guidelines, and not on the risk as a mixture. Moreover, when mixtures are assessed, they are by default assumed to be merely additive of single metal toxicity, thereby not factoring in interactions that could lead to synergism or antagonism.

Metals persist in the environment and do not succumb to degradation. Therefore, many soil organisms are exposed to metals for a period exceeding one life cycle. Whereas reproduction is often used as an endpoint, current soil remediation guidelines for metals reflect single generation laboratory studies not exceeding one life cycle.

Soil is heterogeneous and supports many life forms. The habitat function and quality of the soil are related to its heterogeneity, but habitat function has not been factored into ecotoxicity tests that are needed to develop guidelines. The habitat quality of soils as it influences metal toxicity needs to be incorporated into ecotoxicity studies because it can help to achieve an increased understanding of the response of ecological receptors to metal contaminants.

The research for this Ph.D. thesis investigates how to generate metal toxicity data for commonly occurring metals (copper, zinc, lead, cobalt and nickel) that are representative of the Canadian environment for metal risk assessment. To achieve this, I used soils representative of the Canadian agricultural and mining sites. The test species is a soil oribatid mite, *Oppia nitens,* which is vital to soil function processes and is abundant in Canadian soils.

Objectives and Hypotheses

The global objective of this Ph.D. research was to generate metal toxicity data that explicitly incorporate the effects of metal mixtures, metal persistence and habitat quality. In doing so, I aimed to improve the ecological relevance of test data used for site-specific metal risk assessment. To achieve this global objective, four hypotheses were tested, as follows: (1) Soil organism's sensitivity to a single metal will remain similar when that metal is present in a mixture, (2) At environmentally relevant ratios, metals will interact and not follow concentration addition, (3) Continuous and pulse-like exposures of *Oppia nitens* to a metal will increase the mite's sensitivity in successive generations, (4) Habitat quality will influence toxicity through toxicodynamics by increasing energy available to mites after accounting for soil's influence on metal speciation and bioavailability.

The hypotheses were addressed in the manuscripts making up this Ph.D. thesis. Each manuscript in this thesis was submitted for publication. The contents of Manuscript 1 was submitted to Environmental Science and Technology, Manuscript 2 was submitted to and published in Environmental Toxicology and Chemistry, and Manuscript 3 was submitted to and published in Chemosphere.

For the first hypothesis, *O. nitens* was exposed in five different soils to five single metals, and eleven unique mixtures of these metals using a fixed ratio ray to mimic environmentally relevant

ratios, which was presented in Manuscript 1 (Single Metal and Metal Mixture Toxicity of Five Metals to *O. nitens* in Five Different Canadian Soils). The second hypothesis was investigated by determining the frequency of toxicant-interactions in all the mixtures and in all the soils; this was also presented in Manuscript 1. To test the third hypothesis, the toxic effect of zinc on three generations of *O. nitens* was assessed after one-time exposure (pulse) and continuous exposure to zinc and it is presented in Manuscript 2 (Multigenerational Exposure of Populations of *Oppia nitens* to Zinc under Pulse and Continuous Exposure Scenarios). To investigate the fourth hypothesis, bioavailability and zinc toxicity to *Oppia nitens* was assessed in three levels of habitat quality from low to high and is presented in Manuscript 3 (The Forgotten Role of Toxicodynamics: How Habitat Quality Alters the mite, *Oppia nitens* Susceptibility to Zinc, Independent of Toxicokinetics). Chapter 6 discussed key findings from this study and suggested future directions.

2. Literature Review

2.1 Introduction

Canada is one of the countries in the world with the largest deposits of metals and large mining industry (MAC, 2018). Due to these large-scale mining and smelting operations, metals usually pollute the environment around these operations. There are more than 5,400 Federal sites (only soils) in Canada that are contaminated with metals and metalloids (Government of Canada, 2019). Therefore, metal risk assessment is important. It is also imperative to ensure that the risk assessment process and framework is widely applicable, relevant and provide onward remediation.

Ecological risk assessment (ERA) is the evaluation of potential hazards of pollutants on populations of organisms, and the determination of safe levels of these pollutants in the environment (USEPA, 1998). Environmental and exposure sets of data are therefore collected, organized and analyzed in order to estimate risks from contamination in ERA (Niemeyer et al., 2010). For contaminated site assessment (including metal-contaminated sites) and remediation, Canada adopts a three-tiered risk-based approach (CCME, 2006; Checkai et al., 2014). The first tier comprises the CSQGs, which is expected to protect most ecological receptors and preserve human health (Checkai et al., 2014). Organisms native to Canada are used in toxicity tests to generate data for deriving the CSQGs (CCME, 1999a). After factoring land use types for the soil, site-specific risk assessment of metals are triggered when metal levels exceed the lowest Tier 1 CSQG value for the metal. Site-specific risk assessment is the second tier in the contaminated site assessment process, and it allows for modification of the CSQG based on site-specific objectives (CCME, 1999a). An example of this are sites with high natural metal background levels, multiple exposure pathways, metal mixtures, and different soil textures (CCME, 2007).

In Canada, regulatory jurisdictions determine the processes for deriving the soil quality guidelines. For example, the Federal jurisdiction adopts the guidelines developed by the CCME for lands that fall under Federal jurisdiction. Provincial jurisdictions like Ontario, British Columbia and Alberta develop guidelines for their provinces, using the CCME CSGQ as a guide (CCME, 2007). In order to derive SQG, scientifically sound literature relevant to direct soil contact pathways at optimal bioavailability are extensively searched for and compiled (CCME, 1999a; Checkai et al., 2014). When there are many quality data, the weight of evidence approach (WOE) is adopted to create a threshold effect concentration (CCME, 2006). The threshold effect concentration (TEC) is established from at least three studies, using two invertebrate, two plant species, and ten data points; and using a species sensitivity distribution (SSD) within which effective inhibitory concentrations (ECx) e.g. EC25s are ranked (CCME, 2000; CCME, 2006). To create soil quality guideline that protects the agricultural/residential and parkland land uses, the 25^{th} percentile of the ranked ECx value is used, and 50^{th} percentile is used for industrial and commercial land uses (CCME, 2006). When data is limited, the lowest observed effective concentration (LOEC) from at least one soil invertebrate and one terrestrial plant species can be used and the TEC derived will be divided by an uncertainty factor of 1 to 5 at the discretion of the risk manager (Checkai et al., 2014). If this approach also fails, the lowest EC50 or LC50 value is divided by an uncertainty factor of 5 or 10 (Checkai et al., 2014).

Ecologically relevant endpoints are the environmental values made up of an ecological entities and their attributes, which need to be protected. The ecologically relevant endpoints are considered when deriving guidelines (CCME, 2006). Ideally, the best measure of the ecological impact comes from assessment endpoint data collected at the structure and function of ecosystem levels (CCME, 2006). However, this is an enormous challenge because of the variability in time and space

associated with biology and physicochemical conditions of terrestrial ecosystems (Pederson and Samsoe-Petersen, 1993; CCME 2006). Therefore, the assessment endpoints data are often restricted to the field population level. Most laboratory studies or data used in deriving soil quality guideline reflects population-level effects such as reproduction, even though single species are usually used. The endpoints traditionally assessed in ecotoxicology are survival, reproduction and growth (Van Gestel, 2012).

In recent years, an increasing number of short and long-term toxicity tests have been developed (CCME, 2006). For soil invertebrates, long-term tests should contain at least one reproductive stage (CCME, 2006). There are more short term tests data available than long term tests, but in order to derive soil quality guidelines, long term tests are preferred (CCME, 2006). However, no consensus has been reached by scholars on what constitute short or long-term tests from agency to agency (CCME, 2006). Nevertheless, it is important that a set of data truly representing reality is generated for metal risk assessment. One reason for this is the persistence of metal exposure to soil organisms and the generation of their offspring in the soil (Jegede et al., 2019a). Long term tests should reflect multigenerational exposures to metals for at least two generations.

Up until now, and like in many other jurisdictions, the Canadian metal ERA is commonly determined on metal-by-metal basis even if metals are present as a mixture (CCME, 2006). The omission to consider metal interactions as a possible outcome with metal mixtures could result in much uncertainty in the actual risk of metals. In addition, the Canadian ERA does not explicitly incorporate metal bioavailability, unlike the European Union (EU) REACH, which incorporates bioavailability for metal ERA. The EU incorporates bioavailability by considering metal speciation, ageing and soil characteristics. Despite that the Canadian ERA does not incorporate bioavailability like the EU does, technical reports reflect the rationale and data for each metal in

deriving the SQG, which makes the Canadian ERA, process more transparent (Checkai et al., 2014).

2.2 Common Metals at Contaminated Sites

Metals constitute more than 70% of known elements (Sparks, 2005). Metals are also important in daily life. Therefore, to meet the demands of a growing global population, there is a tendency to increase the production of metals. This production increase results in increased dumping of metals in the environment. The deposited metals have accumulated in high concentrations in many cases in aquatic and terrestrial ecosystems, thus posing threats to the existence of plants, animals and humans (Adriano et al., 2004). The elevated concentrations of metals in the terrestrial environment is largely due to anthropogenic activities (Qui et al., 2014). Examples of anthropogenic activities that may lead to high concentrations of metals in soils include the application of organic and inorganic fertilizers, pesticides, and improper disposal of industrial solid wastes and effluents (Zhang et al., 2011; Alloway, 2012; Su et al., 2014). Others include fall-outs from mining and smelting operations such as ore tailing dumps (Alamgir, 2016). In Canada, like in many other jurisdictions, metal contamination is a concern because of its significant mining and smelting operations (MIHR, 2011).

2.2.1 Copper (Cu)

Copper is a metal with an atomic weight of 63.546, which exists in four oxidation states Cu, Cu^{1+}, Cu^{2+}, Cu^{3+} but commonly exists as the oxidation state of Cu^{2+}. Cu usually occurs in the form of $CuFeS_2$, Cu_2S, Cu_5FeS_4, $(CuFe)_{12}Sb_4S_{13}$ (CCME, 1997). Copper is used in industries to manufacture textiles, paints, pipes in plumbing, electrical conducting wires, fungicides, and monetary coins (CCME 1999b). Copper makes up a broad range of primary and secondary

minerals in mineral deposit types (CCME, 1999b). An average of 60 mg/kg of copper is found in the earth's crust (Oorts, 2012).

Canada is one of the countries of the world with large deposits of copper ore (NRCAN, 2018). In Canadian soils, copper concentrations range between 2 and 100 mg/kg with an average concentration of 20 mg/kg (CCME, 1999b). The highest average concentration of copper (46 mg/kg) in Canadian soils are found in mountainous regions of Canada like British Columbia, the Yukon, Southwest Alberta and some part of Northwest territories (McKeague and Wolynetz 1980).

Copper strongly absorbs soil particles, accumulates in soils, and has low mobility when compared to other trace metals (Alloway, 1990; Slooff et al., 1989). The concentration of copper in soil varies according to soil type, distance from anthropogenic disturbance, natural ore bodies, and the composition of the parent material (CCME, 1999b). Copper has higher affinity for soil organic matter (OM) than other metals, and copper is retained in the soil by binding strongly with OM (Adriano, 1986). Another way by which copper is retained in the soil is through precipitation or adsorption on soil surfaces (McLean and Bledsoe, 1992). However, copper precipitates are unstable at the concentrations commonly found in native soils (McLean and Bledsoe, 1992). For non-calcareous soils, the clay mineral exchange phase is most important in retaining copper through adsorption (Mcbride, 1977). In calcareous soils, copper adsorbs to the calcium carbonate surfaces as a retention mechanism (Cavallaro and Mcbride, 1978; Dudley et al., 1991). Although organic matter makes copper to be immobile in soils, it can also contribute to the mobility of copper in soils. This mobility is because copper can form complex with soluble organic ligands due to copper's strong affinity with OM (CCME, 1999b).

Copper is one of the essential metals needed for normal functioning of plants, animals and humans. Some proteins and enzymes like cytochrome C oxidase, superoxide dismutases contain copper

(Oorts, 2012). Copper could have an adverse effect on the health of plants and animals if it is either too much or deficient. Elevated copper levels in soil can be toxic to soil organisms, thereby disrupting soil ecosystem function (Oorts, 2012). Toxicity of copper to soil organisms is highly dependent on copper bioavailability and sensitivity of the organisms. Some studies have shown that the toxic concentration of copper ranges from 28 to 122 mg/kg on soil earthworm species (CCME, 1999b).

2.2.2 Zinc (Zn)

Zinc is a divalent transition metal with an atomic weight of 65.38. It is the 24th most abundant element there is and is naturally found in the soil as part of rocks or zinc-rich ores in the earth crust (Mertens and Smolders, 2012; CCME, 2018). For the economically important zinc ores, 5-15% of zinc occurs as sphalerite or wurzites (Zinc sulphides) (Mertens and Smolders, 2012). Zinc is useful for galvanizing in the automobile and construction industries (CCME, 1999c).

Canada produces about 600,000 metric tons of zinc and is one of the largest producers of zinc in the world (NRCAN, 2018). McKeague and Wolynetz (1980) reported an average concentration of 74 mg/kg of zinc in Canadian soil. The highest concentration of zinc (81 mg/kg) is found in the regions of the Appalachian Mountains such as Newfoundland, Quebec, Nova Scotia, and New Brunswick.

Zinc is very reactive in soils. It can be absorbed by non-specific ion exchange to metallic oxides or clay minerals in the soil (Sachdev et al., 1992). It can also sorb to ionized groups of soil organic matter (Mertens and Smolders, 2012). In soil solution, zinc can form a complex with inorganic matter such as sulphates or organic ligands such as humic acids, which reduces its charge and makes it more soluble (CCME, 1999c). Soil pH is one of the factors that influence zinc mobility and sorption in soils (Davis-Carter and Shuman, 1993). The solubility of zinc

increases as soil pH decreases, as it does for many other metals (CCME, 1999c). Zinc compounds have varying degrees of solubility (Environment Canada, 1996a). For example, zinc sulphate is more soluble in soil solution than zinc oxide (CCME, 1999c). Although, in most zinc contaminated sites, zinc primarily exists as oxide minerals such as franklinite, sphalerite or willemite (Hamilton et al., 2016).

Zinc is one of the metals essential for the proper functioning of plants, animals and humans, as it is a major constituent of more than 200 metalloenzymes (Vallee, 1959). As a characteristic of all essential metals, the deficiency or excess of zinc in the body of an organism can produce adverse effects. When concentrations of zinc are high, it could elicit toxic effects on microorganisms, soil-dwelling organisms and plants. Studies have shown some of their toxic values on some organisms, such as LC50 of 80 mg/kg concentration on earthworm (CCME, 1999b). Zinc is very reactive in soils and is found in the primary minerals of the soil parent material (Sachdev et al., 1992).

2.2.3 Lead (Pb)

Lead has an atomic weight of 207.2 and exists primarily as a stable plumbous ion (Pb^{2+}) oxidation state (CCME, 1999d). Lead is used to make alloys, pipes, bend and blocks for caulking ammunition, and batteries (Environment Canada, 1996b). Lead and zinc often occur together in the ore, and they are usually produced together (CCME, 1999d).

Environment Canada (1996b) reported that as of 1991, 5% of world-refined lead was from Canada. The production of lead in Canada decreased in the 1990s (CCME, 1999d). However, Canada was the seventh largest producers of lead in the world in 2017 (NRCAN, 2018). Average background level of lead in Canada is estimated as 20 mg/kg (McKeague and Wolynetz, 1980).

The highest concentrations of lead (25 mg/kg) were found in the St. Lawrence lowlands (McKeague and Wolynetz, 1980).

Lead has a high affinity for organic matter in the soil and binds strongly to organic matter from pH 4 and above (Kerndorff and Schnitzer 1980). Mercury and copper are the only metals that have a stronger affinity for organic matter than lead (Steinnes, 2012). When organic matter is low in soils, lead sorbs strongly to iron oxides and clay minerals (Steinnes, 2012). The adsorption of lead to clay minerals is stronger than for metals such as copper, zinc, nickel and cadmium (Usman, 2008). Lead sorbs to clay surface, or forms lead carbonate at a pH of 6 and above (McLean and Bledsoe, 1992). However, in the presence of competing cations and complexing ligands, sorption of lead decreases (McLean and Bledsoe, 1992). Under alkaline conditions, the formation of soluble lead is possible when lead binds to soluble organic and hydroxy complexes (McBride, 1994).

Lead is not an essential metal and can be toxic at elevated levels in the soil (CCME, 1999d). Moreover, lead with its compound tends to accumulate and remain available in soil for a long time (CCME, 1999d). Therefore, there is a high likelihood for soil organisms to be exposed to lead in soil. Several studies have reported the toxicity of lead to soil invertebrates. For example, LC25 and LC50 of lead to earthworms was 2067 mg/kg and 2500 mg/kg (Environment Canada, 1995) while the 50% inhibitory effect of lead on reproduction of another invertebrate, *Folsomia candida,* was 2970 mg/kg (Sandifer and Hopkin, 1996). On exposure, lead inhibits the reproduction of *Oppia nitens* by 50% at 1678 mg/kg soil concentration (Owojori and Siciliano, 2012). The clean-up criteria for lead in Canadian soils was set at 70 mg/kg for the agriculture/residential and parkland land use (CCME, 1999d).

2.2.4 Nickel (Ni)

Nickel has an atomic number of 28 and an atomic mass of 58.71. Nickel exists in oxidation states of -1, 0, +1, +2, +3 and +4 but predominantly exists in the soil as Ni^{2+}(CCME, 2015) Nickel is used in more than 250,000 application as a constituent of alloys (MAC, 1991). Nickel is also used in making nickel-powered batteries that are used in electric cars. Therefore, nickel use will continue to increase in the coming years, consequently heightening environmental risk.

Canada is one of the major countries of the world where nickel is mined in large quantities (NRCAN, 2018). Most of the nickels produced in Canada are got from Sudbury in Ontario and Thompson in Manitoba (CEPA, 1994). The average nickel level in Canadian soils is 26.8 mg/kg (Rencz et al., 2006; Grunsky, 2010). Nickel has a relatively strong affinity for soil organic matter, as 5% of total nickel in soil is usually related to the organic matter. Nickel is generally retained in the soil via its affinity for charged surfaces e.g. clay mineral surfaces, organic compounds, and hydroxides (Gonnelli and Renella, 2012). The affinity of nickel for these charged surfaces makes nickel to be removed from soil solution quite easily. However, in soil solution, the affinity of nickel also plays its role. Nickel forms complexes with dissolved inorganic and organic ligands in soil water (Gonnelli and Renella, 2012).

Nickel is known to be essential to some bacteria, plants and animals (CCME, 2015). However, there has been no report of nickel's essentiality to normal body functioning in soil invertebrates. Anthropogenic sources such as oil and coal combustion, nickel mining and smelting have been responsible for elevated nickel levels in soils (McGrath, 1995). Elevated levels of nickel cause toxicity to soil invertebrates. The toxicity of nickel to some soil invertebrates is well documented. Based on EC50 of nickel to earthworms, springtails and enchytraeids, nickel is one of the most toxic metals for soil invertebrates. The EC50 of nickel to *Eisenia fetida* is 362 mg/kg (Lock and

Janssen, 2002a). The 50% reproduction inhibitory nickel concentration to *Folsomia candida* is 476 mg/kg and 275 mg/kg to *Enchytraeus albidus* (Lock and Janssen, 2002a). The CSQG value of nickel is 45 mg/kg for agricultural/residential and parkland land use (CCME, 2015).

2.2.5 Cobalt (Co)

Cobalt has a molar mass of 58.93. It has one stable isotope and 26 known radioisotopes (WHO, 2006). Cobalt has three valence states (0, +2 and +3) and Co^{2+} is the most stable. Cobalt, like other metals, occurs naturally (Environment Canada, 2017). It is used in making alloys, manufacturing pigment, and making rechargeable batteries (Environment Canada 2017; CDI, 2006). Cobalt as cobalt sulphate is used as a nutritional supplement in cattle feed (Environment Canada, 2009). The global average concentration of cobalt in the soil is 20-25 mg/kg (WHO, 2006; IPCS, 2006).

Cobalt is retained in the soil through its binding with clay minerals, oxides and organic matter. Generally, cobalt is adsorbed very rapidly by the soil, doing so within 1 to 2 hours (WHO, 2006). Cobalt binds more to oxides than other soil constituents do, and its desorption from oxides is very low. Clay minerals adsorb a small amount of cobalt, and the adsorption is basically due to cation exchanges (McLaren et al., 1986). Increase in pH leads to the formation of more insoluble cobalt hydroxides and carbonates (WHO, 2006).

Cobalt is an essential micronutrient, which is involved in many enzymatic processes such as the formation of vitamin B12 (Gal et al., 2008). Environmental elevated levels of cobalt mainly as cobalt oxide come from anthropogenic sources, which include burning of fossil fuels, mining and smelting of cobalt ores, and industrial wastes from nickel processing (WHO, 2006). Elevated levels of cobalt can be toxic to soil invertebrates. Hartenstein et al. (1981) reported that *E. fetida'*s growth was inhibited at about 300 mg/kg of cobalt. In a mixture of marshland soil and horse manure spiked with different cobalt concentrations, 100% reproduction of *E. fetida* was inhibited,

and 77% mortality occurred at 4720 mg/kg of cobalt (Fischer and Molnar, 1997). In a 28-day test, cobalt inhibited 50% reproduction of *Folsomia candida* at about 1480 mg/kg and 409 mg/kg in OECD artificial soils and LUFA soils respectively (Lock et al., 2004). The toxic effect of cobalt was also reported for *Caenorhabditis elegans* at LC50 of 1274 mg/L (Tatara et al., 1998).

The clean-up criteria of cobalt in Canada have not been updated since developing the interim soil criteria in 1991. The criteria values are not risk-based but rather based on professional judgment, because cobalt toxicity data is limited. Since trace element like cobalt can be found in contaminated sites, there is a need for updated guidelines.

2.3 Metal Bioavailability

For a metal to be toxic, it has to be bioavailable. In order to improve metal ERA, there must be a sound understanding of how to account for or incorporate metal bioavailability. Moreover, soil quality guidelines do not explicitly consider metal bioavailability. Metals can be bioavailable depending on the species of metals present in the soil. The free metal ion is commonly related to metal toxicity (McLean and Bledsoe, 1992). However, other forms of metal other than free metal ion might be bioavailable and could correlate with toxicity. For example, Zhao et al. (2016) reported that there are cases where intact complex metals are internalized by organisms, cases where metal complexes react with biotic ligands, and cases where the complex metals dissociate close to the biotic ligands to increase the level of free ions that binds to the biotic ligands. Because of the importance of metal speciation in determining bioavailability, models have been developed to calculate metal speciation. One of such models is the WHAM.

The WHAM simulates how metals react in soil or water systems (CEH, 2019). The model calculates the equilibrium speciation of chemicals in water, sediment and soils (Tipping, 1994). The WHAM combines humic ion-binding model, inorganic solution chemistry models, cation

exchange on clay, precipitation of aluminium, manganese, silicon and iron oxyhydroxides, and adsorption-desorption reactions of fulvic acids (Tipping, 1994; CEH, 2019). The WHAM is currently made up of 248 data sets and 17116 data points and is used by more than a hundred laboratories around the world (CEH, 2019). Updates are available from time to time as more data are inputted, and the current version is the WHAM 7. The model considers soil parameters that influence metal speciation from which bioavailable metal species can be determined. Some of the parameters that are fed into the WHAM in order to calculate metal speciation relating to toxicity are competing ions like Mg^{2+}, K^{+}, Na^{+}, Ca^{2+} and anions of carbonates, sulphates, nitrates, phosphates, and organic matter in form of humic acid or fulvic acid (Gopalapillai and Hale, 2017; Jegede et al., 2019b). Some studies have reported the use of WHAM in calculating metal speciation and determining metal bioavailability to soil invertebrates. Using WHAM (WHAM 7), Jegede et al. (2019b) reported the calculation of zinc speciation in soils of different habitat qualities as potential metal bioavailability estimates. In the study, predicted free zinc ion concentrations did not correlate with toxicity, whereas the total concentrations of zinc in the soil did correlate with toxicity. Using WHAM 6, free Ni^{2+} ion was calculated and found to be the toxic nickel species to *E. crypticus* (He et al., 2014). Competing ions like H^{+}, Mg^{2+}, Ca^{2+}, K^{+}, and Na^{+} at sites of uptake influence the amount of metals that are taken up by organisms (He et al., 2014).

The biotic ligand model was developed to account for competing ions at the site of toxic action (Niyogi and Wood, 2004). The fish gill has been modelled as the biotic ligand for fishes, and the idea is applied to other organisms. For soil invertebrates, only earthworms have been explored for biotic ligand modelling. It is challenging to identify biotic ligands in other soil invertebrates because of their small body sizes (He et al., 2014). Therefore, whole metal body concentration is

used for approximating the amount of metals bound to toxic target sites in many soil invertebrates. Metal uptake by organisms demonstrates the potential of the metal to be bioavailable.

Soil invertebrates can accumulate metals, and this phenomenon has been demonstrated in several studies (Heikens et al., 2001). Oribatid mites are efficient bioaccumulators for metals (Skubala and Kafel, 2004). For example, *O. nitens* accumulated zinc to about 2118 µg/ g bodyweight of the mite when exposed to zinc soil concentration of 2000 mg/kg (Owojori and Siciliano, 2012). Owojori and Siciliano (2012) also reported a substantial accumulation of cadmium, lead but a reduced accumulation of copper based on their biota soil accumulation factor (BSAF). However, in a study with nine different oribatid mites, the mites accumulated copper the most with bioaccumulation factor (BAF) ranging from 1.3 to as high as 22.7 in *Oppiella nova* (Skubala and Kafel, 2004). The BAF of nickel in the earthworm *Lumbricus terrestris* was between 0.6 and 0.91 (Ardestani et al., 2014).

2.4 Mechanisms of Metal Toxicity

Metals cause toxicity, and the toxicity is often measured at the organismal level using reproduction and growth as sub-lethal effects and mortality. Metals do this through many mechanisms that may be detectable at the molecular level. One way to detect the effect of metals on soil invertebrates is by the production of protective enzymes to counteract the effect of metals. For example, the increase in the level of metallothionein and metallothionein-like proteins is well documented in soil invertebrates that are exposed to metals (Hodson, 2012). Zinc stimulated the induction of metallothionein-like proteins in *Porcellio scaber* that was exposed to zinc through feeding (Znidarsic et al., 2005). Zinc at 654 ug/g caused a 50% increase in the induction of the gene encoding metal-binding protein (mt-2) in *Lumbricus rubellus* (Spurgeon et al., 2005). On exposure

to copper, metallothionein (MT) levels in *F. candida* and *E. albidus* increased significantly in order to scavenge copper and mitigate against the toxicity (Maria et al., 2014).

When *Lumbricus rubellus* was exposed to increasing concentrations of copper, lysosomal membrane was damaged, which correlated to increased body burden and decreased reproduction (Svendsen and Weeks, 1997). Another related study using *Eisenia fetida* also reported the reduction in the lysosomal membrane stability of coelomocytes on exposure to copper at about 8 mg/kg (Scott-fordsmand et al., 2000). The increase in lysosomal membrane damage corresponding to significant reproduction inhibition was also observed when *Eisenia venetta* was exposed to Nickel (Scott-fordsmand et al., 1998).

One traditional way by which metals cause toxicity is by causing oxidative stress characterized by the generation of reactive oxygen species (ROS) (Novais et al., 2011). Maria et al. (2014) reported that copper caused increased generation of hydrogen peroxide in *Folsomia candida* cells, which was evident from the induction of catalase (CAT) and glutathione reductase (GR) enzymes. In the same study, a consistent observation was made for *Enchytraeus albidus*. Glutathione S-transferase (GST) activity reduced in *Enchytraeid albidus* that was exposed to 100 mg/kg of zinc after 8 days indicating direct interaction of ROS with the enzyme (Novais et al., 2011). The generation of superoxide dismutase (SOD) after copper exposure shows that superoxide anion radical was produced and the effect had to be counteracted (Gomes et al., 2012). In addition, copper as a salt and as a nanoparticle both caused an increase in lipid peroxidation in *E. albidus* (Gomes et al., 2012).

Lead caused an increase in the expression of heat shock proteins (hsp) in the mite, *Archegozetes longisetosus,* which coincided with severe leg malformation in its larvae (Kohler et al., 2005). Elevated levels of hsp 70 were also observed in the isopod, *Oniscus asellus,* when exposed to lead,

zinc and cadmium singly, and as mixtures (Eckwert et al., 1997). Copper at 400 mg/kg was also found to cause significant elevation of hsp 70 in *E. fetida* after about 15 days exposure (Wenguang et al., 2014).

The genotoxicity of metals has also been reported in some studies. In a toxicity study with nickel on *Eisenia fetida*, there was an increase in DNA strand breaks, indicative of DNA damage (Reinecke and Reinecke, 2004). This DNA damage is not surprising, as the carcinogenicity of nickel to mammalian cells is well documented (Reinecke and Reinecke, 2004). Although the effect of copper on DNA methylation in *F. candida* was assessed, the results showed that copper did not cause DNA methylation (Noordhoek et al., 2018).

Some studies reported the effect of metals on energy metabolism. For example, Jegede et al. (2019b) reported an increase in induction of lactate dehydrogenase and glucose 6 phosphate dehydrogenase activities in *Oppia nitens* after exposure to toxic levels of zinc. Another study showed the increase in the energy consumption and protein budget of *E. crypticus* when exposed to EC20 levels of copper (Gomes et al., 2015a).

Metals also tend to bioaccumulate in particular regions of soil invertebrate bodies. Earthworms preferentially accumulate metals in their posterior alimentary canal, collembolans preferentially accumulate metals in their midgut epithelium, and isopods are known to accumulate metals in their hepatopancreas (Hodson, 2012). These regions of metal storage or accumulation are rich in metal-rich granules (Hodson, 2012). Cotter-Howells et al. (2005) demonstrated that the earthworm, *Dendrodrilus rubidus*, has calcium metal-rich granules that have high affinity for lead and zinc. The earthworm *D rubidus* also has sulphur-rich granules that have a high affinity for cadmium and copper. The sulphur-rich granules in hepatopancreas of *Porcelio scaber* that have a high affinity for copper have also been reported (Kohler, 2002). The collembola, *Orchesella cincta* possess the

calcium metal-rich granules in its midgut, which are usually excreted with the gut epithelium during moulting (Joosse and Verhoef, 1983; Van Straalen et al., 1987). There are suggestions that the excretion of the gut epithelium during moulting is responsible for tolerance to high concentrations of lead and cadmium in some populations of *O. cincta* (Kohler, 2002).

2.5 Metal Mixture Toxicity

Metal mixture toxicity has recently gained more attention because the reality of how metals occur in nature or from anthropogenic sources has dawned on many scholars. Due to the varying ways by which metals could exist and perhaps interact in mixtures in the environment, hazard assessment of all mixtures is not possible to achieve (Heys et al., 2016). The alternative is to rely on the knowledge of the toxicity of individual components of metal mixtures. Expectedly, metals often exist in the environment in low concentrations except in high metal-contaminated sites (Kortenkamp et al., 2009).

Many studies suggest that mixture effect occurs when chemicals are combined at low concentrations (below single contaminant threshold). The mixture effect may be due to the additivity of the individual toxicities of the chemicals in the mixture (Kortenkamp et al., 2009). This type of mixture effect is called “concentration addition”. The individual toxicities are expressed as toxic units. The toxic unit is the concentration of a metal in the mixture divided by its effective concentration. When the toxic units are added together and equal to 1 (one), it means that concentration addition is valid for the particular mixture.

$$\sum_{i=1}^{n} \frac{c_i}{EC_{Xi}} = 1 = \text{Concentration Addition}$$

Where c_i = concentration of a metal, i in the mixture, EC_{Xi} = X% reproduction inhibition concentration of metal, i derived from the single metal dose response.

Concentration addition (CA) theory postulates that contaminants in the mixtures have a similar mode of action. It is a concept that was first introduced by Loewe, a German pharmacologist in 1926 in a publication by Loewe and Muischnek (Cleuvers, 2003). Another concept of mixture toxicity is the Independent action or response addition. The independent action (IA) theory suggests that individual components of a mixture have a different mode of action, but that their responses can be added up (Cleuvers, 2003). The CA and IA assume that there are no interactions between the mixture components (Backhaus and Faust, 2012). Due to the complex nature of assessing metal toxicity, regulators adopt CA as the default conservative estimate of metal mixture toxicity for risk assessment especially if none of the metals in the mixture exceeds its single metal threshold.

A number of studies have been done on metal mixture toxicity to soil invertebrates. However, like many mixture studies, there is always no clear-cut way of predicting whether a particular mixture will be additive, synergistic or antagonistic on a particular species. Some studies have reported that CA is protective of mixtures. For example, the equitoxic binary mixture of zinc and cadmium have been found to result in antagonistic effects on *E. albidus* (Lock and Janssen, 2002b). The mixture effect of copper and zinc on *E. crypticus* was also antagonistic (Posthuma et al., 1997). When the earthworm, *Aporrectodea calignosa*, was exposed to a mixture of cadmium, copper and zinc, the effect was antagonistic (Khalil et al., 1996; Qui et al., 2011). Van Gestel and Hensbergen (1997) reported an additive effect of cadmium and zinc on the reproduction of *F. candida*.

In contrast to these, some studies have shown that mixture effects are more than additive. A study looking at the mixture effects of cadmium and copper on *C. elegans* found out that the effect was synergistic (Jonker et al., 2004). The binary combinations of Cu, Ni and Mn were synergistically toxic to *Paronychiurus kimi* (Son et al., 2016). Some of the differences in type of mixture effects

are related to the effect levels considered in a mixture study. For example, there could be differences in the effect depending on if individual metals are expressed at low effect level (e.g EC10) or high effect level (EC50). Amorim et al (2012) reported antagonism of a mixture of Cd and Zn to *F. candida* at lower doses and synergism at higher doses.

In some cases, metal toxicity depends on if metals were expressed as total metal or extractable metals. Weltje (1998) reported that metal mixture toxicity shifted from antagonism with total metal concentration to CA when metals were expressed as extractable metal concentrations. Using water-soluble concentrations, the toxicity of Cd and Zn on springtail was higher than when the metals were expressed as total concentration (Van Gestel and Hensbergen, 1997). The joint effect of chemicals may also be specific to species tested. For example, using the same set of metals Cd Zn, dose-level dependent (antagonism at low concentrations and synergism at high concentrations) effects were observed with *F. candida* and *Porcellionides pruinosus* but was only antagonistic when tested on *Enchytraeus albidus* (Amorim et al., 2012).

There are current efforts at developing statistical models to explain metal mixture toxicity. Jonker et al (2005) reported four patterns of metal mixture toxicity in respect to the additivity, which is the conservative estimate. The study reported that metal mixtures might not deviate from additivity, as it may be synergistic or antagonistic, and dose-level dependent or dose-ratio dependent. Although models are very important for interpreting or predicting metal mixture toxicity, there have been reports of inconsistency when applying complex statistical models to metal mixtures (Liu et al., 2017). For example, using parameters derived from one experiment that adopted the isobole-based statistical models, to detect deviations from additivity were not reproducible in three consecutive experiments (Sorensen et al., 2007; Liu et al., 2017). This phenomenon might be due to the sensitivity of the models in predicting metal mixture toxicity in

terms of significant statistical levels (between alpha = 0.05 and alpha = 0.01) (Liu et al., 2017). Liu et al. (2017) suggested that a more stringent alpha level (e.g. 0.01) might be adopted to reduce variability between models and raise the predictive power of models for metal mixture interactions. There are other factors such as discussed earlier; differences in species, and differences in the measure of metal bioavailability, which can constitute sources of variability in model predictions.

2.6 Soil Habitat Quality

The soil is a biologically active, permeable medium found in the uppermost layer of the earth's crust (Birkeland, 1999), which serves as habitat for many organisms. The maintenance of the soil system as a living and biologically active entity could be attributed to the range of organisms that inhabit the soils (Kamin, 2010; Ruiz et al., 2008). Apart from the biological make-up of soils, the physicochemical properties of soils mainly influence soil as habitat for a wide range of species. The heterogeneity of soil horizontally and vertically, which also influences biota diversity, is attributed to the various ways in which the soil properties are distributed (Voroney, 2007).

The habitat quality of soils is an important characteristic of soil that should be taken into account when interpreting differences between toxic responses in soils. The International Organization for Standardization (ISO) 15799 suggests that habitat function of soils should be preserved when doing tests. Soils must be able to conserve biodiversity, be suitable to grow crops in Agriculture, and be useful for assessing the potential ecotoxicity of chemicals which chemical analysis cannot detect (ISO, 2019a). In order to preserve biodiversity or populations, the fitness (survival and reproduction) of organisms need to be sustained. The fitness of organisms is linked to the resources available within their habitat (Johnson, 2007). The resources could be in the form of food, access to food, space or shelter, which protects them from predators or adverse environmental conditions (Hope, 2001). For a soil organism, fitness is related to the soil habitat quality, which in turn is

related to the soil properties such as soil pH, texture and organic matter (Jegede et al., 2019b). For example, at a pH of 3.5, *Folsomia candida'*s reproduction reduced by half of its optimum reproduction at pH range 5.4 to 6.6 (Jansch et al., 2005). The earthworm, *Eisenia Andrei,* reproduced significantly higher in clay loam and forest organic soils than in clayey agricultural and sandy soils (Jansch et al., 2005). Princz et al. (2010) reported that *Oppia nitens* laid more eggs in soils with higher organic matter. The effect of soil properties in mitigating toxicity is well established in soil ecotoxicology (Kuperman et al., 2009; Van Gestel, 2012; Son et al., 2007; Madani et al., 2015; Owojori et al., 2010). Therefore, many soil ecotoxicology studies account for soil properties or differences, thereby incorporating habitat quality partly or indirectly through toxicokinetics (toxicokinetics is how an organism influences uptake, metabolism and excretion).

However, studies that examined the direct effect of habitat quality on toxic potentials of metals on soil invertebrates are scarce. Only one study (Jegede et al., 2019b) assessed the direct effect of habitat quality on metal toxicity to mites. In the study, the response of mites in high habitat quality soils, and those in low habitat quality soils were different. For example, mites in high habitat quality were able to withstand higher metal body burden than mites in low habitat quality. Energy-based biochemical responses also showed that mites in high habitat quality were less stressed, hence, expended less energy. Although the mechanism by which habitat quality influenced metal toxicity in the mites is not clear at the moment, studies have shown that food quality can influence metal toxicity in soil invertebrates. When *F. candida* was fed with food amended with 2% or higher glucose, the gene coding for metallothionein expression levels was lower than when fed with lower glucose that exposed it to same cadmium concentrations (Nakamori and Kaneko, 2013). This phenomenon may be related to the energy budget allocated for detoxification (Kooijman, 2010) where high-quality foods supply more energy, thus making more energy available to detoxify

metals at toxic concentrations. How habitat quality influences toxicity needs to be further studied in order to reduce the uncertainties in interpreting some soil ecotoxicology results, especially when comparing toxicity between soils.

2.7 Species Selection

In an ideal situation, the toxic effects of chemicals should be tested on all species inhabiting the soil. However, the diversity of organisms inhabiting the soil is very high, and some have not been identified nor characterized; therefore, it is not practical to use all the species for tests. Relative to soil biodiversity, only a few species are tested for the effect of chemicals for risk assessment (Princz, 2014). The species used in toxicity testing are selected based on some criteria. The criteria for test species selection are summarized from Rombke et al. (2009) as follows:

- Species should be ecologically relevant. The species should play critical roles in food webs within the ecosystem.
- The species should be easy to breed in the laboratory and should have rapid generation succession.
- Species must be such that they are in close contact with soil, plants or plant residues
- Sensitivity to stress: Species must be moderately sensitive to a broad spectrum of stress.
- Ecological tolerance: The species must have low sensitivity to fluctuations in soil properties, and abiotic factors like temperature and moisture.
- Distribution: The species must be well distributed in the environment.

A number of toxicity tests with invertebrates are standardized for soils (e.g. with springtails, *F. candida* (ISO, 1999; OECD, 2009), earthworms *Eisenia fetida/andrei* (ISO, 1998; OECD, 2004a), enchytraeids *Enchytraeus albidus/crypticus* (ISO, 2004; OECD, 2004b), predatory mite *Hypoaspis aculeifer* (OECD, 2008), and Mollusca: *Helix aspersa* (ISO, 2006). Environment Canada and

International Organization for Standardization (ISO) have finalized the addition of the oribatid mite *O. nitens* to the retinue of standard test species, and the protocol was released as ISO/DIS 23266 (ISO, 2019b).

2.7.1 Oppia nitens

Oppia nitens (C.L. Koch, 1836) is an oribatid mite belonging to the Oppioidea superfamily and Oppiidae family (Norton and Behan-Pelletier, 2009). The Opiiddae family is the largest family of the oribatid order (Princz et al., 2010). *O. nitens* was first described in 1836 by C.L Koch (Baran and Ayyildiz, 2004). The species *O. nitens* can be found in Palearctic and Holarctic soils (Baran and Ayyildiz, 2004). Adult *O. nitens* are dark brown, and the length of the adult species varies between 467 to 533 µm and 260 to 320 µm in width (Baran and Ayyildiz, 2004; Michael 1888). The juvenile stages of *O. nitens* from larvae to tritonymphs are characterized by white/translucent colour (Hernandez, 2014). Apart from its smaller body size, the larvae of *O. nitens* is usually distinguished by its three pairs of legs, while at its advanced stages, it has four pairs of legs typical of acari (Krantz, 2009). The length of the larvae is approximately 200 µm, and width, 105 µm and the length of the tritonymphs is approximately 372 µm and width, approximately 195 µm (Seniczak 1975, Princz, 2014). Eggs laid by *O. nitens* eggs are about 90 to 150 µm in size, oval in shape, smooth and whitish (Seniczak, 1975; Princz, 2014). After hatching and at about 22-23^{o}C and 60% humidity, it takes about 28 days for *O. nitens* to fully develop- this is after it might have gone through five instars (Sengbusch and Sengbusch, 1970). Until the present time, it is not yet clear if *O. nitens* exhibits sexual dimorphism (Princz et al., 2010). This lack of clarity is probably because males or females cannot be distinguished both as juveniles and as adults. *Oppia nitens* mainly feeds on fungi; however, lichens, humus, and carrion can be a part of its diet (Luxton, 1972; Princz et al., 2014). Nonetheless, *O nitens* are reared in the lab on yeast (Jegede et al., 2019a).

As a K-strategist, *O. nitens* produces few offspring and most live up to the nearly maximum life span, that is survival is preferred to reproduction. An individual *O. nitens* adult reproduces more than once in its lifetime. The adults prefer to lay eggs in their food or in crevices (Sengbush and Sengbish, 1970). *Oppia nitens* are heavily sclerotized, and their sclerotized cuticle might help to protect them from toxic chemicals. The mites can avoid metals (especially essential metals) at elevated or toxic concentrations in the environment (Owojori and Siciliano, 2012). A recent analysis of the lipid content of *O. nitens* showed that they have higher lipid contents than other soil invertebrates like *F. candida*, *E. crypticus*, *E. fetida* by about one to ten orders of magnitude (Gainer et al., 2018). The higher lipid content is a measure of more energy available for *O. nitens* to cope with the environment.

Oppia nitens are widely distributed. They are abundant in soils and play vital roles in food webs. *Oppia nitens* is easy to breed and can be cultivated in the laboratory, thereby meeting the criteria for use in ecotoxicological tests (Rombke et al., 2009; Huguier et al., 2015). It has been used in a number of toxicity tests with chemicals such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), pesticides, perfluorinated compounds, biopesticides, rare earth elements and metals (Princz et al., 2012; Gainer et al., 2018; Owojori and Siciliano, 2012; Princz et al., 2018; Yu et al., 1997; eSilva et al., 2017; Keshavarz Jamshidian et al., 2017; Owojori and Siciliano, 2015; Jegede et al., 2019a, b).

2.7.1.1 Standardized Ecotoxicological Tests with O. nitens

Although some oribatid mites such as *Archegozetes longisetosus*, *Platynothrus scaber* and *Oppia nitens* have been used in toxicity tests, there are no standardized protocols available for their use. Recently, efforts have been made by Environment Canada to include *O. nitens* as a standardized

species in ecotoxicological studies. The efforts have been successful, and the protocols for *O. nitens* is now available (ISO, 2019b).

Some of the adaptations for *O. nitens* that were adopted in the protocol were about the validity criteria of tests. For example, the reproductive capacity of mites was taken into account. Soil invertebrates like *F. candida* or *E. crypticus* have more fecundity than *O. nitens*, as their validity criteria for reproduction is set as ≥ 50 juveniles per 10 adults in control soils. In the case of *O. nitens*, the validity criteria is set at ≥30 juveniles per 15 adult mites in control soils. The survival was set ≥ 70% just like other soil invertebrates. The conditions for the tests are same as tests with *F. candida* (OECD, 2009) such as 16-h light: 8-h dark, light intensity of 400-800 lux equivalent to a quantal flux of 5.6−11.2 μmol/($m^2 \cong s$) for cool-white fluorescent, the temperature of 20±2°C for 28 days.

It is noteworthy that metal risk assessment relies on data got from toxicity tests, with the assumption that they are protective of the long-term effect of metals. However, most toxicity tests only account for single generational exposure of organisms to contaminants, which are often short term. Therefore, the effect on multigeneration is often ignored. For contaminants such as metals, which are known to be non-destructible but persist in soil, current toxicity tests, might not reflect the full potential of metal toxicity to soil organisms. Jegede et al. (2019a) demonstrated that the sensitivity of *O. nitens* to metals increased in successive generations that were continuously exposed to metal thereby showing that tests need to be extended beyond single-generation when assessing metal toxicity in soils.

2.7.1.2 Oppia nitens in Metal Toxicity Tests

Like many soil invertebrates, *O. nitens* reproduction is more sensitive to metal toxicity than survival. The effects of Zn, Pb, Cu and Cd to *O. nitens* in standard artificial soil are well

documented. Cadmium is the most toxic of the metals to *O. nitens* with an EC50 of 137 mg/kg, which is at least ten places more toxic than Zn, Cu and Pb in the order of magnitude (Owojori and Siciliano, 2012). Next to Cd in terms of toxicity to *O. nitens* is Zn (1562 mg/kg). Although Zn is an essential metal, it exhibits toxicity to *O. nitens* at elevated concentrations (which are below Zn concentrations typical of some contaminated sites in Canada). In terms of inhibitory effects on *O. nitens* reproduction, copper is the least toxic of the metals (EC50 = 2896 mg/kg) (Owojori and Siciliano, 2012). However, *O. nitens* is the least sensitive to lead in terms of survival (LC50 = 6761 mg/kg) (Owojori and Siciliano, 2012). Toxicity data of metals with *O. nitens* is relatively scarce when compared to standardized invertebrate species like *F. candida*. For example, there is no toxicity tests data available for Ni nor Co with *O. nitens* in standard artificial soils. Although *O. nitens* is adjudged to be somewhat less sensitive compared to *F. candida* or earthworms, the sensitivity of *O. nitens* to metals is comparable to established standard soil invertebrate species (Table 1).

Table 2- 1. Summary of the sensitivity of some soil invertebrates (*Oppia nitens, Folsomia candida, Eisenia fetida, Enchytraeus albidus/crypticus*) to six metals (Cu, Zn, Pb, Cd, Co, Ni) in Organization for Economic Cooperation and Development (OECD) artificial soil and some described natural soils.

Species	Guideline reference	Metal	EC50 (mg/kg)	Duration (d)	Soil	Reference
Oppia nitens	Not available	Cu	2896	35	OECD Artificial soil	Owojori and Siciliano, 2012
Folsomia candida	OECD, 2009		700	28	OECD Artificial soil	Sandifer and Hopkin, 1996
Eisenia fetida	OECD, 1998		316	28	OECD Artificial soil	Owojori et al., 2009
Enchytraeus albidus	OECD, 2004		305	28	OECD Artificial soil	Lock and Janssen, 2002b
Oppia nitens	Not available	Zn	1562	35	OECD Artificial soil	Owojori and Siciliano, 2012
Folsomia candida	OECD, 2009		750	28	OECD Artificial soil	Sandifer and Hopkin, 1996
Eisenia fetida	OECD, 1998		705	28	OECD Artificial soil	Lock and Janssen, 2001d
Enchytraeus albidus	OECD, 2004		267	28	OECD Artificial soil	Lock and Janssen, 2001d
Oppia nitens	Not available	Pb	1678	35	OECD Artificial soil	Owojori and Siciliano, 2012
Folsomia candida	OECD, 2009		1600	28	OECD Artificial soil	Sandifer and Hopkin, 1996
Eisenia fetida	OECD, 1998		1940	28	OECD Artificial soil	Spurgeon and Hopkin, 1995
Enchytraeus albidus	OECD, 2004		320	28	OECD Artificial soil	Lock and Janssen, 2002b
Oppia nitens	Not available	Cd	137	35	OECD Artificial soil	Owojori and Siciliano, 2012

Folsomia candida	OECD, 2009		315	28	OECD Artificial soil	Sandifer and Hopkin, 1996
Eisenia fetida	OECD, 1998		108	28	OECD Artificial soil	Lock and Janssen, 2001c
Enchytraeus albidus	OECD, 2004		158	42	OECD Artificial soil	Lock and Janssen, 2001b
Oppia nitens	Not available	Co	1213 - 14000	28	Natural soil (pH = 3.4 - 6.8, %OC = 17 – 29, CEC = 8 – 20 mmol/100g)	Present study
Folsomia candida	OECD, 2009		409	28	OECD Artificial soil	Lock et al., 2004
Eisenia fetida	OECD, 1998		300	28	Sludge (pH = 6.5 – 7.0 + Teel silt loam soil.	Hartenstein et al., 1981
Enchytraeus crypticus	OECD, 2004		200	28	LUFA 2.2	Ribeiro et al., 2018
Oppia nitens	Not available	Ni	133 - 3600	28	Natural soil (pH = 3.4 - 6.8, %OC = 17 – 29, CEC = 8 – 20 mmol/100g)	Present study
Folsomia candida	OECD, 2009		476	28	OECD Artificial soil	Lock and Janssen, 2002a
Eisenia fetida	OECD, 1998		362	21	OECD Artificial soil	Lock and Janssen, 2002a
Enchytraeus albidus	OECD, 2004		275	42	OECD Artificial soil	Lock and Janssen, 2002a

3. Manuscript 1: Single Metal and Metal Mixture Toxicity of Five Metals to *Oppia nitens* in Five Different Canadian Soils.

3.1 Preface

The single and metal mixture toxicity of five metals to the survival and reproduction of *Oppia nitens* in five soils were assessed after 28 days. Metal concentration in mixtures followed fixed-ratio rays. Metal speciation in soils was determined to explain the variation among soils in mite response. Multivariate statistics were used to explain interactions of soil properties on metal mixture toxicity to the mites.

Jegede OO, Awuah KF, Renaud M, Cousins M, Hale BA, Siciliano SD. Single metal and metal mixture toxicity of five metals to *Oppia nitens* in five different Canadian soils *(Submitted to Environmental Science and Technology)*.

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3.2 Abstract

Metal mixture toxicity across soil types is a daunting challenge to risk assessment. Here, we tested some simple assumptions regarding metal mixture toxicity, namely: (i) concentration addition would predict metal response, (ii) soils where the mites are sensitive to single metals would also be the soils where the mites are susceptible to mixtures, (iii) the specific metal composition in mixtures would influence toxicity, and (iv) speciation of metals in mixtures could explain toxicity better than total metals. We evaluated metal mixture toxicity in *Oppia nitens*, using ten fixed metal mixture ratios in five Canadian soils that closely matched some of the EU PNEC reference soils. Soils were dosed with five metals (Cu, Zn, Pb, Co, Ni) as single metals (ten concentrations) and as mixtures (eight concentrations). Synchronized adult mites were exposed to metals, with survival and reproduction assessed after 28 days. Total metal concentrations were determined, and speciation was calculated using WHAM 7. We found out that: (i) response to about 90% of the metal mixtures deviated from concentration addition, (ii) the differences among soils in mite sensitivity and single metals were not consistent when mites were exposed to metal mixtures, (iii) the specific metal composition in mixtures had little effect compared to differences among soils with regard to metal toxicity, but Zn emerged as a protective metal in most mixtures, and (iv) in combination with soil properties, metal speciation explained 57% of the variation in toxicity among soils but metal speciation alone only explained 14% of the variation in toxicity response. Instead, soil properties such as CEC, independent of effects on metal speciation, explained 23% of the variation. Both CEC and Zn alter *O. nitens* toxicodynamics, and thus suggest that the toxicity of metal mixtures in soils is likely driven more by toxicodynamics than toxicokinetics. Further work is needed to insure that by protecting soil-dwelling organisms from single metals, the risk from metal mixtures is appropriately protected for.

3.3 Introduction

Metals co-occur in the environment and the myriad potential combinations preclude a hazard assessment of each and every mixtures (Heys et al., 2016). The alternative is to rely on the toxicity of individual components of the mixtures and incorporate the typical conservativism of those single-metal toxicity thresholds into a reference mixture-toxicity concept. Concentration addition (CA) is one reference mixture-toxicity concept adopted by regulators (Jonker et al., 2005; Nys et al., 2017). The CA concept assumes that individual components of the mixture do not interact and that they have the same mode of toxic action (Qui et al., 2015).

In many cases, the mode of action is unknown; nonetheless, the CA is applied to all mixtures because of the ease of interpretation (Qui et al., 2015). Mathematically, the CA is determined by simply adding the toxicities of each metal in the mixture after expressing the metals as toxic units. The toxic unit is the ratio of the concentration of a metal in the mixture to the metal's effective concentration (ECx). When the sum of the toxic units of each metal in the mixture equals one, then such mixture toxicity follows concentration addition. However, when the sum is significantly higher or less than one, it means that the metals in the mixtures interact.

Concentration addition predicts an increase in toxicity at low concentrations (concentrations below individual toxicity thresholds) due to additivity (Kortenkamp et al., 2009). However, metal interactions leading to synergism (CA < 1) enhance toxicity of mixtures more than additivity (Jonker et al., 2005). It is also possible that metal interactions can reduce mixture toxicity through antagonism (CA > 1). For example, an equitoxic binary mixture of zinc and cadmium resulted in antagonistic effects on *E. albidus* (Lock and Janssen, 2002b). In contrast, Jonker et al. (2004) reported that cadmium and copper's effect on *C. elegans* was synergistic. Synergism is a source

of concern to regulators because it implies higher risk with a particular mixture. For the proponents of contaminated sites, antagonism is also a concern because it can lead to greater risk management than needed, at greater cost. The interplay between metals in soils and soil properties is one of the reasons why interactions occur more often than not (Qui et al., 2015).

Soil properties influence the performance of organisms in soils (Jansch et al., 2005; Kuperman et al., 2009). Soil properties determine the soil habitat quality, which relates to the fitness of soil organisms (Jegede et al., 2019b). For example, at a pH of 3.5, *Folsomia candida'*s reproduction was reduced to half of its optimum reproduction at pH range 5.4 to 6.6 (Jansch et al., 2005). High habitat quality as a function of soil properties protects organisms by providing more energy for its inhabitants to cope with metal exposure. The high cation exchange capacity (CEC) in some soils was given as the reason for the increased ability of *O. nitens* to withstand metal through toxicodynamics (Jegede et al., 2019b). Studies have demonstrated that soil organism response is related to fundamental soil properties like soil texture, pH, organic matter content and clay (Van Gestel, 2012). For example, soil properties influenced the toxicity of metals like copper and lead on survival and reproduction of the enchytraeid, *Enchytraeus albidus* (Lock and Janssen, 2001a), and the toxicity of copper to the springtail, *Folsomia candida* (Criel et al., 2008). Generally, soil organism responses in metal-contaminated soils is more closely related to the bioavailable, rather than the total concentration of metals.

Soil properties drive metal bioavailability. For example, keeping other soil properties constant, a soil with a lower pH and CEC tends to have a higher cationic metal bioavailability and vice versa (Alloway, 2012; Hasegawa et al., 2016). Soil properties influence metal bioavailability by altering the ratio of free to bound metals in soil solution. For example, soils with lower pH, organic carbon and CEC have a greater proportion of total metal as free ion or very labile species and therefore

possess increased toxicity when compared with soils with high pH, organic carbon and CEC (McLean and Bledsoe, 1992). He et al. (2014) reported that toxicity of nickel to *Enchytraeus crypticus* was due to free nickel [Ni^{2+}] species because the [Ni^{2+}] explained much of the accumulation and toxicity of nickel to the enchytraeid. Other metal complexes such as organic-matter bound metals relate to metal toxicity because organisms can internalize intact metal-complexes. For example, Stockdale et al. (2010) reported that metals bound to humic acid correlated to metal-mixture body burdens causing toxicity in stream macroinvertebrate species. It is thought that these metal-complexes react with biotic ligands, releasing free metal species, which are then absorbed by the organism (Zhao et al., 2016).

Though mixture toxicity data are increasingly becoming available (Baas et al., 2010), mixture data are still relatively uncommon. Many of the metal mixture data in literature e.g. (Norwood et al., 2003; Vijver et al., 2011) were based on nominal concentrations and acute effects rather than measured metal concentrations and chronic effects, which are more relevant for risk assessment of metals (Nys et al., 2018). Moreover to incorporate measures of bioavailability, it is important that properties of receiving environment, in our case soil properties, should be accounted for (Nys et al., 2018). To test the conservativeness of CA for risk assessment or how deviated mixtures are from CA, a mixture interaction factor (MIF) is used (Nys et al., 2018). In this study, we sought to ascertain if: (i) metal mixtures followed CA and if not, how the mixtures deviated from CA, (ii) if soil properties could be used to predict mixture effects, (iii) if the composition of the metal mixture significantly altered toxicity outcomes, and (iv) if metal speciation could explain mixture toxicity better than total metals. Here for the first time, we assessed metal mixture toxicity effect of five metals on reproduction of *Oppia nitens* in five Canadian soils. We used *Oppia nitens* as our model organism due to its importance in the boreal forest and Arctic regions where the majority of

Canadian mines are located, as well as the growing body of literature describing this organism's response to toxicants. The five metals (Zn, Pb, Cu, Ni, Co) used in this study are often found together in metal contaminated sites (CEM, 2004; Pourret et al., 2016). To increase the relevance of this study for metal risk assessment, we mimicked metal ratios found in soils near mining or metal smelting operations. We also tested how deviated from CA are the mixtures in all the five soils.

3.4 Materials and Methods

3.4.1 Soil collection

Eighteen (n=18) soils were collected from sites across Canada. The soils spanned residential, agricultural and mining sites. The soils were air-dried until constant weight and stored as dry soils for about two weeks. The physicochemical properties of the soils were determined (Jegede et al., 2019b). Five of the 18 soils were then selected as reference soils for their similarity in properties to five of the soils used in the EU REACH for the PNEC calculator (Table 3-1).

Table 3- 1. The physicochemical properties of soils selected to mirror the European Union predicted no-effect concentration (EU PNEC) reference of the five reference soils.

	Soil	**pH ($CaCl_2$)**	**Water Holding Capacity (%)**	**Organic Carbon (g kg^{-1})**	**Clay Content (g kg^{-1})**	**CEC (mmol/ 100g)**	**Closest EU PNEC Reference**
1	3.22	3.4	29	17	45	8	Acid Sandy Forest
2	Elora	6.7	30	21	200	21	Loamy Alluvial
3	KUBC	5.6	20	12	24	28	Loamy
4	WTRS	4.6	23	25	110	16	Acid Sandy Arable
5	PC7	6.8	30	29	58	20	Loam Sandy

Two of the soils (3.22 and WTRS) were collected from mining sites in Flin Flon, Manitoba. Two other soils (Elora and PC7) were collected from Elora and Port Colborne, both in Ontario; while the fifth soil (KUBC) was a mixed soil from Kernen, which is an agricultural research field in Saskatchewan, and UBC soil from Iqaluit, Nunavut, mixed in a 1:1 ratio to create the desired soil properties. Henceforth, for easy identification, the soils are named according to their closest EU PNEC reference: 3.22 = Acid Sandy Forest, Elora = Loamy Alluvial, KUBC = Loamy, WTRS = Acid Sandy Arable, PC7 = Loamy Sand.

3.4.2 Test Species

3.4.2.1 Oppia nitens

Oppia nitens is an oribatid mite, which is fungivorous. They are important nutrient recyclers in the soil and are the most abundant microarthropod in the boreal forest soils (Princz et al., 2010). They have been used in toxicity tests with metals (Owojori and Siciliano, 2012; Keshavarz Jamshidian et al., 2017; Jegede et al., 2019a). The *O. nitens* used for this study was taken from the already established laboratory cultures in the soil toxicology laboratory at the Soil Science department in the University of Saskatchewan, Canada. Adult mites were cultured on a medium made of Plaster of Paris (POP) and activated charcoal in an 8:1 ratio. The POP was moistened twice in a week, and the mites were fed with bread yeast ad-libitum. The mites were age-synchronized; after about 5-6 weeks, newly emerged amber-colored adult mites were selected and placed in a new medium of POP and allowed to mature fully. The fully matured mites had dark brown sclerotized pigments. The fully matured mites were then used for the tests.

3.4.3 Metals

Five metals were used for this test as singles and were also combined as mixtures. The metals were purchased as metal oxides from Sigma Aldrich: zinc oxide (puriss p.a American Chemical Society

(ACS) reagent ≥ 99%), lead (II) oxide (ACS reagent, ≥ 99%), cobalt (II, III) oxide (powder, < 10 µm, 98%), copper (II) oxide (powder , < 10 µm, 98%), and nickel oxide. The metal oxides were further ground to finer particles with mortar and pestle. The ground oxides were placed in contact with concentrated nitric acid in a desiccator for 48 hours to remove trace carbonates. Further, the oxides were air dried in a fume hood for 24 hours.

3.4.4 Fixed Ratio Ray Determination and Rationale

A fixed ratio ray is the combination of chemicals in a particular ratio with increasing doses maintaining this ratio. A fixed ratio ray design increases the ease of interpreting and visualizing experimental results (Kortenkamp et al., 2009). For example, predictions and observations of effect level deviations are better observed with a fixed ratio ray design (Crofton et al., 2005; Kortenkamp et al., 2009). Moreover, a fixed ratio ray design helps to reduce the amount of experimental effort associated with mixtures, and to easily evaluate environmentally relevant mixtures (Casey et al., 2005).

Ratios relevant to existing regulations (regulatory ratios) were developed. One of the five ratios was created from the Canadian soil quality guidelines (CSQG) for each metal, for agricultural land use. The other four ratios were selected based on the PNEC estimates for individual metals in eight of the EU REACH soil types and the CSQG land use classes. The ten potential regulatory ratios were combined into four, based on the similarity in the ratios between the soils/land uses (Table 3-2). Three environmental ratios were based on the average metal concentrations in Flin Flon, Sudbury, and Port Colborne soils (Johnson and Hale, 2004; Hamilton et al., 2016; Gopalapillai et al., 2018). Ratios based on organism sensitivity (toxicity ratios) were created from the *Folsomia candida* EC50 values of each metal in artificial soil (there was not enough *O. nitens* data to build such a ratio) (Sandifer and Hopkin, 1996; Sandifer and Hopkin, 1997; Lock and Janssen, 2002a;

Lock et al, 2004). The *Folsomia candida* EC50 values were used because it is a standard test species, as it is a microarthropod like mites, and has literature EC50 values for all the metals of interest. Two toxicity ratios were determined. One was determined based on the EC50 values of the metals, and the other ratio was based on equal concentrations of the metals. For easy visualization, metal concentrations in each fixed ratio ray are expressed as a percentage (w/w) and by molar ratios (Table 3-2).

Table 3- 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**.** Regulatory ray **=** Ratio relevant to existing regulations, Environmental ray = Ratio based on average metal concentrations found in sites at or near mining and smelting operations in Canada, Toxicity ray = Ratio based on a standard species' (*Folsomia candida*) sensitivity to the individual metals in the mixture

	Fixed Ratio Ray	**Type of Ray**	**Dose composition**									
			Co		**Ni**		**Cu**		**Zn**		**Pb**	
Mixture			**w/w**	**mol**	**w/w**	**Mol**	**w/w**	**Mol**	**w/w**	**mol**	**w/w**	**mol**
1	CSQG	Regulatory	0.090	0.110	0.110	0.134	0.160	0.181	0.470	0.516	0.170	0.059
2	Clayey/Peaty		0.110	0.135	0.123	0.152	0.206	0.234	0.372	0.412	0.190	0.066
3	Ag/Res/Loamy		0.100	0.122	0.118	0.144	0.184	0.208	0.423	0.465	0.175	0.061
4	Loamy/Sand/Industry		0.097	0.123	0.109	0.138	0.155	0.182	0.421	0.479	0.218	0.078
5	Acid Sandy Arable		0.050	0.080	0.064	0.102	0.157	0.232	0.260	0.374	0.469	0.212
6	Flin Flon	Environmental	0.003	0.003	0.003	0.003	0.202	0.216	0.726	0.755	0.066	0.021
7	Sudbury		0.037	0.065	0.072	0.128	0.039	0.064	0.290	0.461	0.561	0.282
8	Port Colborne		0.013	0.013	0.707	0.736	0.178	0.172	0.076	0.071	0.026	0.008
9	EC50	Toxicity	0.294	0.396	0.088	0.119	0.147	0.183	0.147	0.178	0.324	0.124
10	Equal Ratio		0.200	0.271	0.200	0.235	0.200	0.217	0.200	0.211	0.200	0.066

3.4.5 Test Design

The experiment was designed such that at least 50 test units were randomly assigned into each of 15 blocks. Each test unit contained each of the five soils, each of the single metals, and each of the ten mixture rays. Therefore, the single and metal mixtures were performed concurrently in this randomized fashion to minimize variation within treatment conditions in the laboratory, and to improve the reliability of the tests. Dose replications were also randomized such that the controls and some concentrations had replications. In all, there were 870 test units including the replicates. Each of the single metals tests had 11 doses including control and each mixture test had 9 treatments, including control.

3.4.6 Test Performance

All the soils were moistened with 50% water holding capacity, while the test soils were spiked with single metals and metal mixtures. Some of the soils were not spiked with any metal, for the control. Twenty-five (25) g of the spiked and control soils were weighed into a 2 cm diameter glass vials and labelled accordingly. Fifteen synchronized adult mites were then introduced into the vials containing the soils. The mites were fed with yeast once every week starting with week 0. The experiment was run under constant conditions for 28 days; 21^{o}C, 50 - 60% humidity, >800 Lux, day to light; 16: 8h regime (Princz et al., 2010). The loss in moisture was also adjusted each feeding day. After 28 days, the mites were extracted using a modified Berlese tullgren extractor for 48 hours (Jegede et al., 2019a). The number of surviving adults and the juveniles produced were counted and recorded against each concentration.

3.4.7 Chemical Analysis

3.4.7.1 Total Metal Concentrations

The total metal concentrations in the test soils were determined by X-Ray Fluorescence (XRF) (Margui et al., 2016). Four (4) g of dry soils were weighed and ground. The ground soils were homogenized with 0.8 g of 44 µm powdered Chemplex spectroblend, acting as adhesive to hold the soils together. The homogenized samples were transferred into Chemplex pellet cups, covered with polypropylene thin-films and vacuum-sucked into a pellet die set. The pellet set was mounted on a hydraulic press and the samples were pressed (10,000 pounds per square inch) for 5 minutes to form soil discs. The soil discs were analyzed on the Thermofisher ARL Optim-X X-ray analyzer for total metal concentrations. For quality control, the recoveries of each metal from a certified reference material (Montana 2710a) were determined. The recoveries of the metals ranged between 90-95%.

3.4.7.2 Bio-accessible and Free Metal ion Concentration

Free metal ion concentrations were predicted using bioaccessible metal concentrations, anions, cations and the dissolved organic carbon. These were inputted into the WHAM 7 to calculate the free metal ions (Gopalapillai and Hale, 2017). The bio-accessible metal concentrations were determined by the calcium chloride extraction method (Quevauviller, 1998). Soil (2.5g) was weighed into a 50 ml centrifuge tube and 25 ml of 0.01M $CaCl_2$ added. The $CaCl_2$ and soil mixture were shaken for 3h at 15 rpm using a rotary shaker. A subsample of the solution was used for pH and the remaining sample centrifuged for 10 minutes at 5000g, filtered through a 0.45 µm filter, and was refrigerated prior to analysis. The filtered samples were then analyzed using an Agilent Microwave Plasma Atomic Emission Spectrometer (MP-AES) at the Department of Soil Science, University of Saskatchewan, Canada. Standard solutions of the metals (VWR atomic absorption

standards) were diluted with 0.01M calcium chloride serially from 0, 1, 5, 15, 30 and 50 mg/L as standards. The quality control included blanks, duplicates and calibration standards for every set of 21 samples.

3.4.7.3 Anions and Cations

The anions and cations in the soil were determined using water instead of calcium chloride (Quevauviller, 1998). The filtered extracted-samples were divided into two; while one part was analyzed for anions, the other part was analyzed for base cation concentrations. The anions were analyzed by ion chromatography (IC) with a Dionex ICS-2000 using the Chromeleon 7 software at the Department of Soil Science, University of Saskatchewan, Canada. The base cations (Ca^{2+}, K^{+}, Mg^{2+}) were analyzed with an Agilent MP-AES at the Department of Soil Science, University of Saskatchewan, Canada. Standards for the cations were run randomly in the MP-AES, and the calibration curve of the absorbance (Ca^{2+} = 616.21 nm, K^{+}, = 769.89 nm, Mg^{2+} = 383.80 nm) at different concentrations was determined. The quality control included blanks, duplicates and calibration standards in every set of 21 samples.

3.4.7.4 Dissolved Organic Carbon

Dissolved organic carbon (DOC) was determined by a method described by Chantigny et al. (2008). Soil (15 g) was mixed with 30 ml of 0.005M $CaCl_2$ in a 50 mL centrifuge tube. The soil and the $CaCl_2$ were mixed gently for a minute with a glass rod. After this, the soil-water mixture was centrifuged at 12000*g* for 10 minutes. The supernatants from the centrifuged samples were filtered with 0.4 µm polycarbonate through vacuum suction into 30 mL dram vials. The filtered samples were immediately analyzed for DOC using a Mandel Total Organic Carbon analyzer at the Department of Soil Science, University of Saskatchewan, Canada. Percent coefficient of variation for replicate injections was less than 2%.

3.4.7.5 Speciation Calculations.

The Windermere Humic Aqueous Model version 7 (WHAM 7) (Tipping et al., 2011) was used to determine how metals were speciated in the soil solutions. The input parameters were: dissolved organic carbon (DOC), cations (Ca^{2+}, Mg^{2+}, K^{+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}) and the anions (Cl^{-}, NO^{3-}, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}). The reaction conditions were: temperature at 298K, the partial pressure of CO_2 at 0.00038 atm, and pH, which was as, measured (Peng et al., 2018). Fulvic acid (FA) was estimated from the DOC by assuming 65% of the DOC is the active FA and that DOC is 50% of dissolved organic matter in the soil (Tipping et al., 2003; Rooney et al., 2007). The output parameters were the free metal ion species (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}), metal bound to fulvic acid (FA-Me) and other metal (Me) species (Me-SO_4, Me-$(OH)^{+}$, Me-$(OH)_2$, Me-CO_3, Me-Cl_2, Me-$(HCO_3)_2$)

3.4.8 Statistics

The total metal concentrations were expressed as mmol/kg. The effective concentration inhibiting 10% and 50% mite reproduction (EC10, and EC50) of the individual metals and the metal mixtures for reproduction were estimated with a non-linear regression model (three or four-parameterized log-logistic model or Weibull 1 and 2 models using R.) The models were chosen based on which better fits the dose-response data (lower Akaike Information Criteria (AIC) values), using the DRM package in R (Ritz, 2016). The PNEC calculator (Version 3) was used to calculate the bioavailable PNEC value of each metal based on the soil properties (Arche, 2015). Analysis of variance (ANOVA) was used to check the difference ($p < 0.05$) in mean EC10 and EC50 values for mixtures among soils, while a Tukey post hoc test was used to identify the pair(s) of soils with difference. The number of toxic units of a metal in the mixture was calculated as (Equation 1):

$$\frac{c_i}{EC_{Xi}} = \text{Toxic unit (TU)} \quad (1)$$

Where c_i = concentration of a metal, i in the mixture, and EC_{Xi} = X% reproduction inhibition concentration of metal, i derived from the single metal dose response.

The normalized mixture exposure concentrations were calculated as the sum of the toxic units for each metal (TU mixture) (Equation 2).

$$\sum_{i=1}^{n} \frac{c_i}{EC_{Xi}} = \text{TU mixture} \quad (2)$$

The TU Mixture was plotted as a dose against mite reproduction to fit a dose-response curve, using the DRM package to determine the TU10 and TU50. The TU10 and TU50 are the TU mixtures at which 10% and 50% reproduction inhibition were observed. The TU10 and TU50 values were compared to additivity, namely where TU mixture = one (1). The type of response was determined by if the TU10 or TU50 is equal to, greater or less than one. A one-sided T-test was used to determine if the observed TU10 or TU50 with its estimated standard error (error derived from the dose response analysis) differed ($p < 0.05$) from one. When TU10 or TU50 is equal to one, the mixture response type is additive. When TU10 or TU50 is > 1, it is antagonism, when TU10 or TU50 is < 1, it is synergism. The non-interactions or interactions of metals in the mixture were expressed as the percentage frequencies of each type of response in the soils. A mixture interaction factor (MIF) for each soil was derived at EC10 and EC50 level as the median of the TU10s and TU50s of all the ten mixtures in each soil.

Multivariate analysis was used to determine which soil properties or measured metals better explained mixture toxicity or mixture interactions. Before performing the multivariate analysis, the sets of data were checked for normality with a QQplot, and transformed accordingly. The multivariate analysis was performed using variation partitioning of matrices of soil properties and

measured metals (total, free and % metals bound to Fulvic acid) with EC10 and EC50/TU10 and TU50 of metal mixtures as response variables (Borcard et al., 2018). A forward selection of a redundancy analysis (RDA) with the VEGAN package in R (Oksanen et al., 2013) was used to determine which soil property or metal significantly ($P < 0.05$) explained variation.

3.5 Results

3.5.1 Metal Toxicity

Nickel was the most toxic metal (Average EC10 = 14.6 ± 8 mmol/kg) in four of the five soils used for the test, (Figure 3-1 & Table 3-3) while Zinc was the least toxic (68 ± 34 mmol/kg). The Acid-Sandy-Forest soil had the highest toxicity (EC10 = 3.7 ± 0.9 mmol/kg and EC50 = 11.2 ± 3.3 mmol/kg), while the Loamy soil was the least toxic (EC10 = 55 ± 40 mmol/kg, EC50 = 209 ± 81 mmol/kg) (Figure 3-1). However, Loamy-Alluvial soil had the highest metal mixture EC10 level (7.6 ± 3.5 mmol/kg) while Acid-Sandy-Forest soil had the highest metal mixture EC50 level (17.8 ± 2.4 mmol/kg) (Table 3-3). The Acid-Sandy-Arable soil had the lowest metal mixture toxicity (EC10 = 52.9 ± 14.3 and EC50 = 97.8 ± 19.0 mmol/kg) (Table 3-3). Toxicity irrespective of the mixture ratio was similar (within an order of magnitude) in the Acid-Sandy-Forest soil except in one mixture where it was within two orders of magnitude (Table 3-3). The single metal EC10, EC25 and EC50 values from this study were also expressed as mg/kg (Appendix A, Table A-1).

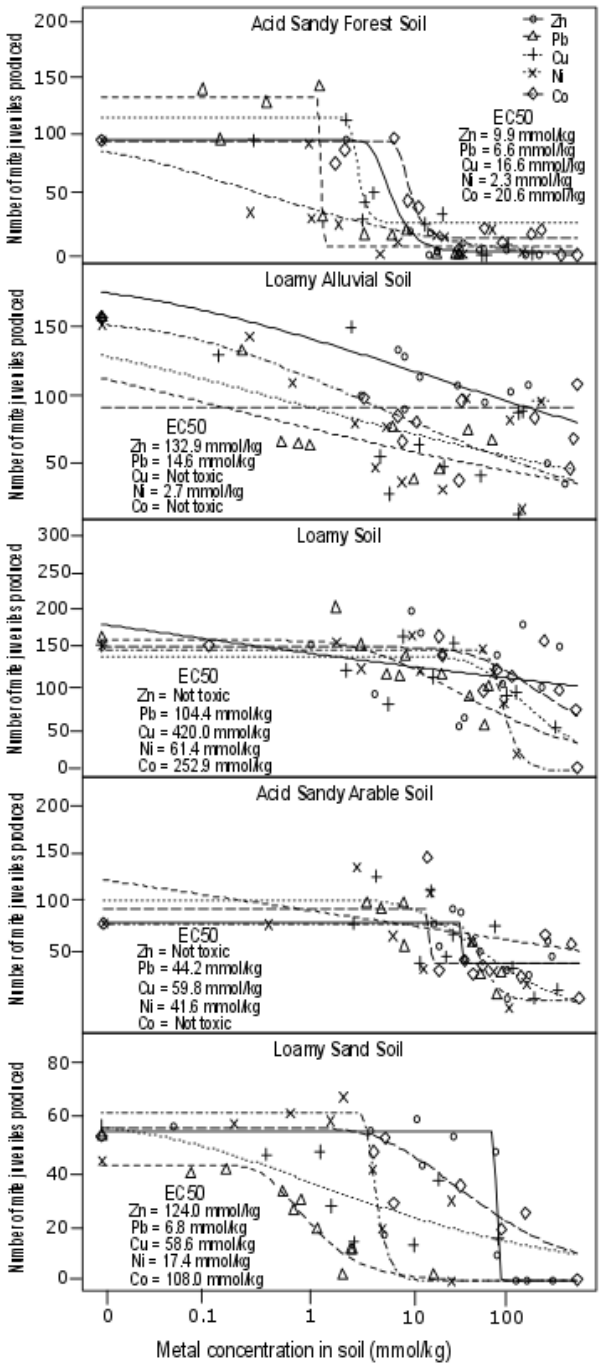


Figure 3- 1. Dose response curves for response of *Oppia nitens* to five metals (Zn, Pb, Cu, Ni, Co) in each of five soils (Acid Sandy Forest, Loamy Alluvial, Loamy, Acid Sandy Arable, Loamy Sand) and the effective concentrations inhibiting 50% reproduction (EC50) expressed in mmol/kg of metal.

Table 3- 3. The effective concentrations ± SE of five single metals and ten metal mixtures inhibiting 10% (EC10) and 50% (EC50) mite reproduction expressed as mmol/kg of soil in five soils (Acid Sandy Forest, Loamy Alluvial, Loamy, Acid Sandy Arable, Loam Sandy). Not calculated = data that could not be fitted with the model due to errors. Not toxic = data that could not be fitted because there was no toxicity, even at the highest concentrations.

		Acid Sandy Forest		**Loamy Alluvial**		**Loamy**		**Acid Sandy Arable**		**Loam Sandy**	
		EC10 (mmol/kg)	EC50 (mmol/kg)	EC10 (mmol/kg)	EC50 (mmol/kg)	EC10 (mmol/kg)	EC50 (mmol/kg)	EC10 (mmol/kg)	EC50 (mmol/kg)	EC10 (mmol/kg)	EC50 (mmol/kg)
Single metals	Zn	3.80 ± 1.20	9.90 ± 3.30	78.62 ± 27.24	133 ± 18.50	Not toxic	Not toxic	Not toxic	Not toxic	121.36 ± 6.90	124.36 ± 3.78
	Pb	4.20 ± 1.60	6.60 ± 3.18	0.83 ± 0.46	14.64 ± 6.08	3.08 ± 3.61	104.36 ± 100.00	22.44 ± 18.80	44.23 ± 18.65	1.31 ±0.46	6.77 ± 1.83
	Cu	5.10 ± 3.71	16.60 ± 8.35	Not toxic	Not toxic	2.27 ± 0.91	420 ± 51.20	8.44 ± 23.40	59.78 ± 20.33	0.88 ± 0.33	54.58 ± 17.80
	Ni	0.09 ± 0.03	2.26 ± 3.10	1.36 ± 0.48	2.71 ± 1.31	43.90 ± 5.86	61.43 ± 4.41	16.37 ± 14.22	41.55 ± 24.44	11.42 ± 2.52	17.41 ± 1.33
	Co	5.51 ± 5.20	20.60 ± 11.30	Not toxic	Not toxic	172.00 ± 57.47	253.32 ± 28.56	Not toxic	Not toxic	14.35 ± 29.46	108.20 ± 102.00
Metal mixtures	Mixture 1	7.88 ± 21.06	12.99 ± 23.90	0.14 ± 0.4	15.30 ± 14.00	34.90 ± 92.75	38.70 ± 77.00	68.00 ± 99.87	169.98 ± 102.00	1.25 ± 4.01	25.88 ± 26.00
	Mixture 2	13.06 ± 8.80	19.00 ± 4.30	13.00 ± 13.00	59.30 ± 27.00	5.50 ± 4.99	50.01 ± 36.87	33.36 ± 43.70	136.00 ± 62.90	Not calculated	Not calculated
	Mixture 3	8.55 ± 4.75	13.67 ± 7.64	2.00 ± 4.00	97.00 ± 89.00	5.09 ± 17.22	74.45 ± 61.59	40.88 ± 9.91	43.10 ± 3.18	51.80 ± 9.60	54.00 ± 9.98
	Mixture 4	6.64 ± 3.84	9.69 ± 5.56	29.80 ± 42.01	33.10 ± 37.90	20.20 ± 12.00	21.33 ± 27.67	35.01 ± 22.00	59.10 ± 36.77	11.70 ± 9.00	36.88 ± 18.01
	Mixture 5	8.00 ± 18.00	17.18 ± 12.00	Not toxic	Not toxic	22.11 ± 9.00	37.15 ± 14.86	155.22 ± 24.79	161.98 ± 8.01	Not calculated	Not calculated
	Mixture 6	17.87 ± 9.00	18.60 ± 3.80	Not toxic	Not toxic	0.78 ± 0.06	97.49 ± 33.93	35.78 ± 1.80	35.89 ± 2.20	1.80 ± 1.40	10.00 ± 5.13

	Mixture 7	11.30 ± 6.02	14.48 ± 3.41	Not toxic	Not toxic	9.98 ± 44.00	13.31 ± 3.01	26.90 ± 36.87	27.99 ± 33.01	9.30 ± 7.01	10.11 ± 2.00
	Mixture 8	10.30 ± 4.40	38.00 ± 16.50	4.50 ± 13.00	129.00 ± 118.00	Not toxic	Not toxic	Not toxic	Not toxic	Not toxic	Not toxic
	Mixture 9	17.80 ±2.87	18.44 ± 17.00	0.20	44.87 ± 61.80	81.01 ± 39.90	125.22 ± 17.90	71.89 ± 14.00	94.33 ± 27.21	Not toxic	Not toxic
	Mixture 10	9.43 ± 14.20	16.26 ± 8.80	11.98 ± 20.90	71.00 ± 43.97	27.98 ± 24.34	123.00 ± 44.01	9.70	142.91 ± 70.01	1.76 ± 2.61	3.30 ± 1.81

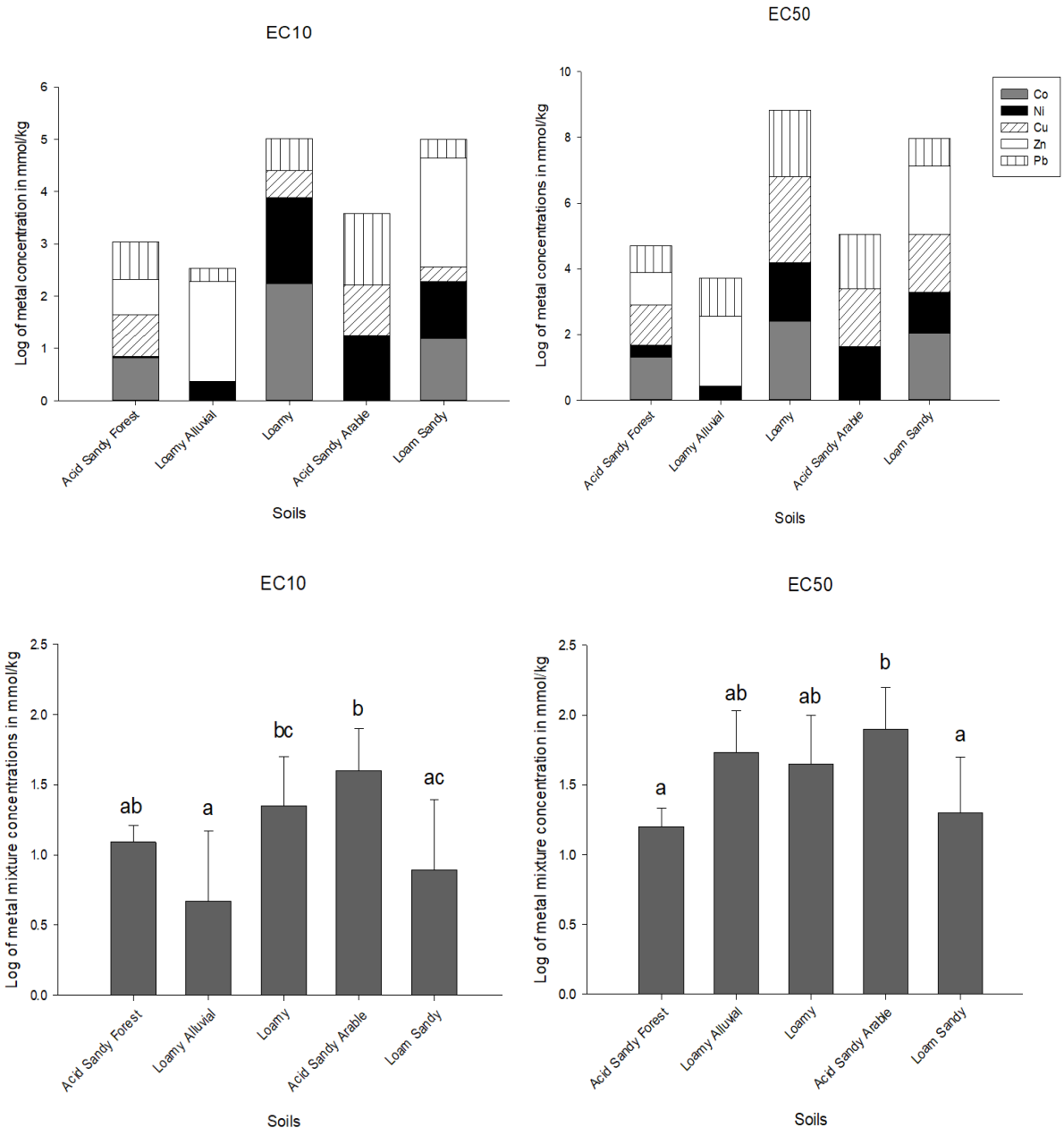


Figure 3- 2. Toxic responses of mite to single metals (Co, Ni, Cu, Zn and Pb) [upper panels] and metal mixtures [lower panels] at effect levels 10% and 50% (EC10 and EC50 in mmol of metal per kg of soil) in five soils (Acid Sandy Forest, Loamy Alluvial, Acid Sandy Arable, Loamy, and Loamy Sand). Metal concentrations that caused effect levels were expressed as the sum of all metals in the mixtures, with the bars in lower panel indicating the average concentration in 10 different mixtures and the associated standard error. The letters "a, b, c" represents significant difference ($p < 0.05$) when there is no overlap but no significant difference ($p > 0.05$) when there is an overlap.

3.5.2 Metal Interactions in Mixtures

Synergistic responses were the most common (57% - 67%) at the EC10 level in all the soils except Acid-Sandy-Arable soil, which was the only soil that had more antagonistic responses (67%). Concentration addition (CA) was only observed in the Loamy-Alluvial and Loamy soils at EC10 level. The Loamy soil had the highest frequency of synergistic responses (89%) at EC50 level, while the Loamy-Alluvial soil had 100% antagonistic responses at the EC50 level. CA was observed in all soils except the Loamy-Alluvial soil at EC50 level, while the Acid-Sandy-Forest soil had the highest frequency (30%) of CA at EC50 level compared to other soils. Adding EC10 and EC50 levels together, the Loamy soil had the highest frequency of synergistic responses (73%) and the Loamy-Alluvial soil had the highest incidence of antagonistic responses (64%) (Figure 3-3). CA was observed for all the soils with the highest frequencies occurring in the Acid-Sandy-Forest (15%) and Loamy (13.3%) soils. In general, synergism was the most frequent (47%) type of mixture toxicity response, followed by antagonism (43%), and concentration addition trailing behind with 10%. CA underestimated mixture toxicity the most in Loamy-Alluvial soil at 10% effect levels and overestimated mixture toxicity the most in the same soil at 50% effect levels (Figure 4). The dose-response of the metal mixture toxicity is shown (Appendix A, Figure A-1) and types of mixture responses (Appendix A, Table A-2).

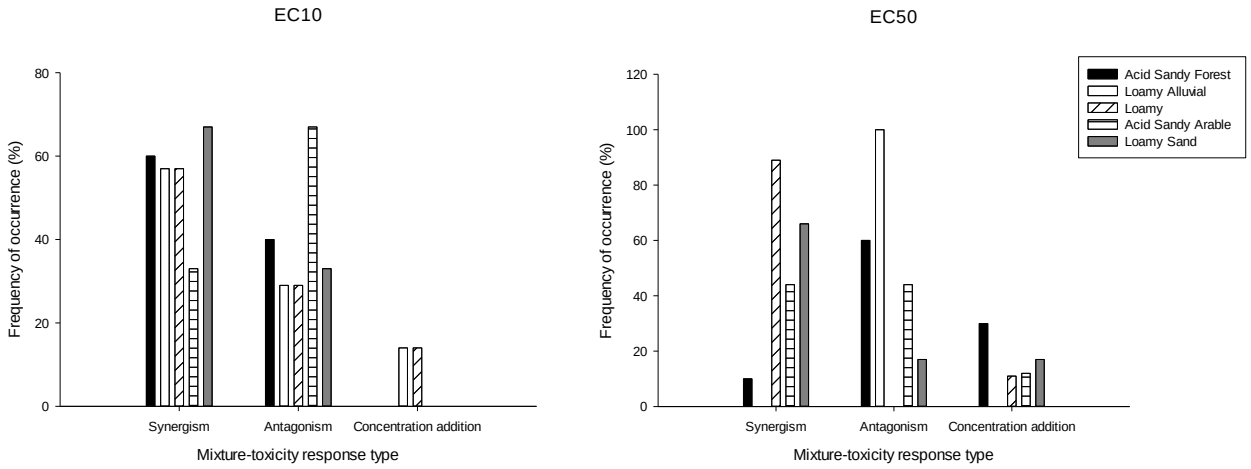


Figure 3- 3. Frequency (%) of occurrence from eighty (80) trials of mixture toxicity response types (synergism, antagonism and concentration addition) of metal mixtures in five soils (Acid-Sandy-Forest, Loamy-Alluvial, Loamy, Acid-Sandy-Arable, Loam-Sandy) at EC10 and EC50 levels. Synergism represents (TU < 1), antagonism represents (TU > 1) and concentration addition represents (TU =1). TU = Toxic unit.

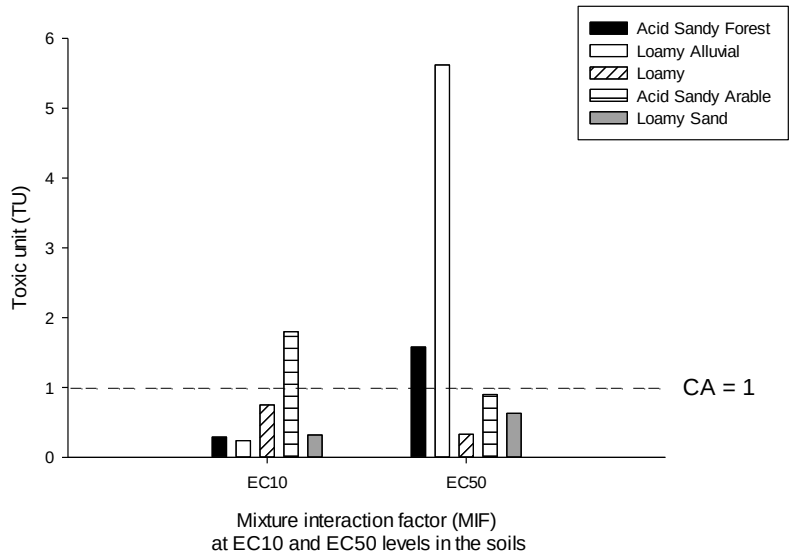


Figure 3- 4. The mixture interaction factor (MIF) of metal mixtures to *Oppia nitens* in five soils (Acid-Sandy-Forest, Loamy-Alluvial, Loamy, Acid-Sandy-Arable, Loam-Sandy) at 10% and 50% reproduction inhibition effect levels (EC10 and EC50). The broken line showed where the mixture is not interactive (CA = 1). When MIF is lower than 1, CA underestimates mixture toxicity (Synergism) and when MIF is higher than 1, CA overestimates mixture toxicity (Antagonism). The MIF is expressed as toxic units.

3.5.3 Metal Speciation Differed Among Soils

Metal speciation did not have particular patterns both for metal bound to Fulvic acid and free metal ion concentration. In some cases, there were differences between soils; in some cases, there were differences between the single and metal mixtures; and in other cases, there were similarities. The Loamy soil had the highest proportion of lead bound to Fulvic acid (FA-lead), and together with Loam-Sandy had the highest FA-copper. However, Loamy-Alluvial had the highest FA-cobalt, nickel and zinc (Figure 3-5a). For mixtures, Loamy-Alluvial had the highest FA-nickel and FA-zinc, in consistence with single metal speciation. Loamy-Sand had the highest FA-copper and FA-lead, but Acid-Sandy-Arable had the highest FA-cobalt (Figure 3-5b) as against what was observed with single metals where it had the second lowest FA-cobalt after Acid-Sandy-Forest (Figure 3-5a). Speciation of the single metals was similar for the Loamy and Acid-Sandy-Arable soils, which also were similar in pHs (5.6 and 4.6 respectively) (Figure 3-5a). However, this pattern was not consistent for mixtures; rather, Acid-Sandy-Forest and Acid-Sandy-Arable soils had similar patterns and had the lowest pHs (3.4 and 4.6 respectively) (Table 3-1) of all the soils. Only zinc maintained the same speciation pattern from single to mixtures across all the five soils, while nickel also maintained the same speciation pattern from single to mixtures in four soils (Figure 3-5). The other metals did not maintain the same speciation from single to mixtures across the soils. The behaviour of free metals were expectedly the exact opposite of what was observed with FA-Metal species (Figure 3-5c and 3-5d). In general, the Acid-Sandy-Forest soils had the highest free metal species as single and mixtures. The Loamy-Alluvial soil had the lowest free metal species both as single metal and metal mixtures. For metal mixtures, the pattern of percentage metal bound to Fulvic acid was Co < Ni < Zn < Pb < Cu (Figure 3-5b) which was the opposite pattern for the free metals (Figure 3-5d).

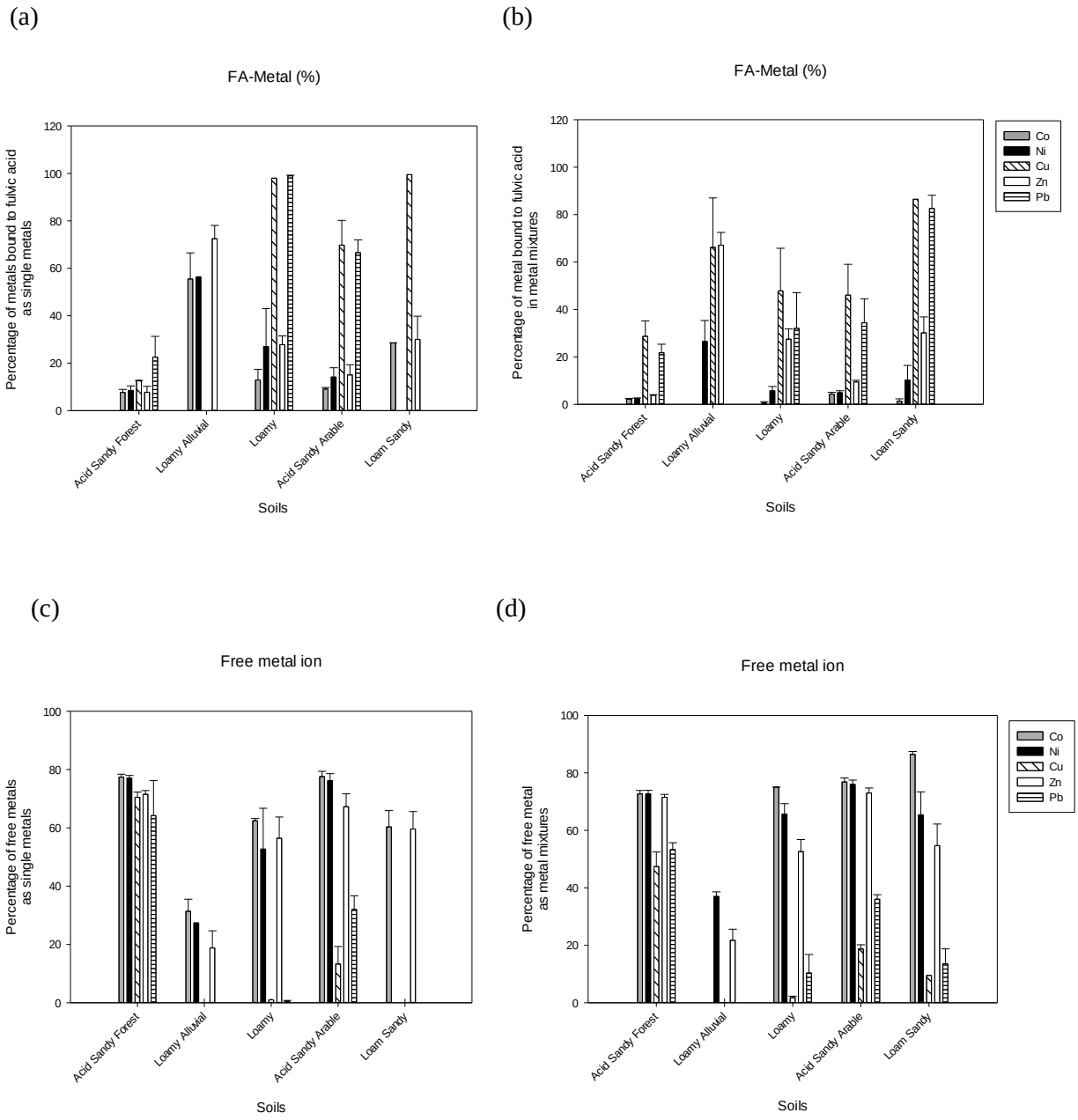
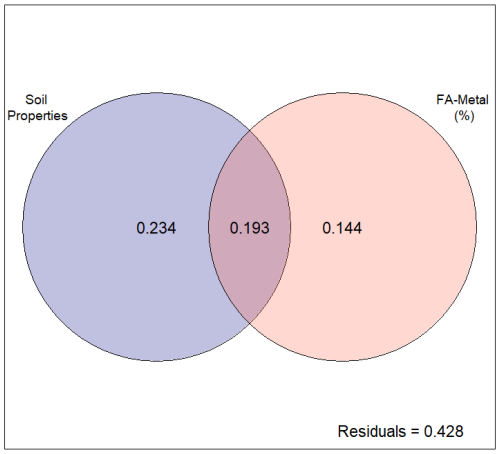


Figure 3- 5. The bioaccessibility of Co, Ni, Cu, Zn, and Pb as percentage ± SE of (a) single metals bound to fulvic acid (b) metal mixtures bound to fulvic acid (c) free single metals in solution (d) free metal mixtures in solution that were assessed in five soils. The soils are Acid-Sandy-Forest, Loamy-Alluvial, Loamy, Acid-Sandy-Arable, and Loamy-Sand.

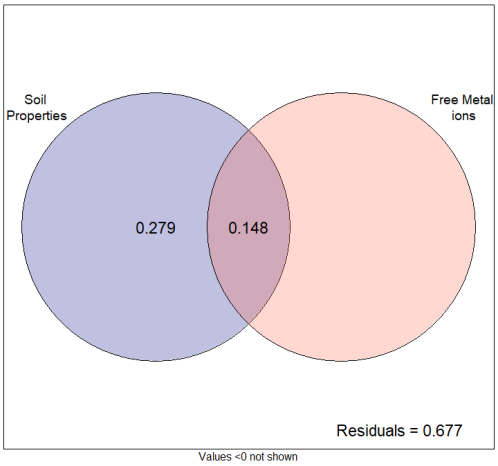
3.5.4 Soil Properties Influenced the Toxic Response Better than Measured Metals

Soil properties explain either 2 to 180 times more variation in toxicity than measured or speciated metals (Figure 3-6). Total metals were the least successful in predicting toxicity (0.2%), while fulvic-acid (FA) bound metals (33.7%) were the best metal speciation based predictor of toxicity followed by free metals (14.8%) (Figure 3-6, Table 3-4). Soil properties, independent of their effect on speciation, explained an additional 20%. When combined with soil properties, FA bound metal explained 57% of the variation in toxicity. At EC10, soil pH, CEC and OC ($p < 0.01$) in combination with FA bound metals predicted EC10 mixture response (Appendix A, Table A-2); only FA bound cobalt and lead drove toxicity response at EC10 mixture level (Appendix A, Table A-3). Free metal concentrations were not significantly predictive of EC10 mixture toxicity (Appendix A, Table A-3). FA bound zinc and nickel influenced ($p < 0.01$) interactions of metals in the mixtures only at TU50 and not TU10, but free metal concentrations did not influence interactions both at TU10 and TU50 (Appendix A, Table A-4).

(a)



(b)



(c)

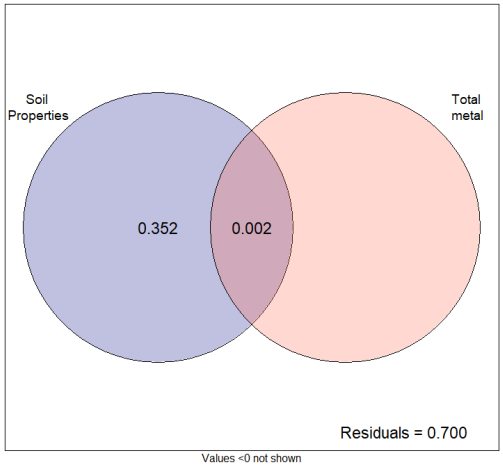


Figure 3- 6. Venn diagram of the variation partitioning of the response matrix of metal toxicity explained by (a) soil properties and Fulvic acid bound metals (FA-Metal (%)) on toxicity (b) soil properties and free metal ions on toxicity (c) soil properties and total metal on toxicity at 10% effect levels. Residuals show the variations not explained by any of the explanatory variables (soil properties, fulvic acid bound metals, free metals, and total metals).

Table 3- 4. The test of significance of the variations explained by explanatory variables (soil properties, percentage of metal bound to Fulvic acid (% FA-Metal), free metal ions, total metals) and covariation of the explanatory variables on the toxicity of metal mixtures to *Oppia nitens*.

Metal measure	**Matrix**	**EC10**	**EC50**
		p value	*p* value
% Fulvic acid bound metal (% FA-Metal)	Soil properties	0.003**	0.002**
	% FA-Metal	0.014*	0.038*
	Covariation	0.003**	0.011*
	Soil properties + % FA-metal as covariate	0.01**	0.07
	% FA-Metal + Soil properties as covariates	0.082	0.38*
Free metal ion concentration	Soil properties	0.002**	0.004**
	Free metals	0.28	0.33
	Covariation	0.051	0.033*
	Soil properties + Free metals as covariates	0.032*	0.023*
	Free metals + Soil properties as covariates	0.91	0.77
Total metal concentration	Soil properties	0.001**	0.001**
	Total metals	0.65	0.54
	Covariation	0.035*	0.003**
	Soil properties + Total metals as covariates	0.004*	0.001**
	Total metals + Soil properties as covariates	0.77	0.13

**$p < 0.01$, *$p < 0.05$

3.6 Discussion

3.6.1 Soil Properties that Protect, also Hurt

Soil properties that protect *O. nitens* from single metal toxicity, e.g. CEC, also make *O. nitens* in the same soil very sensitive to mixture toxicity. For example, in Loamy Sand, the EC10 for the five combined singles was 30 ± 22 mmol/kg but these same metals present as a mixture had an EC10 of 13 ± 8 mmol/kg. The Loamy soil where *O. nitens* was least sensitive to single metals had the highest CEC and the lowest clay. Although *O. nitens* in the Loamy soil was not the most sensitive to metal mixtures, there were more synergistic interactions in the Loamy soil, which may have been responsible for the increased sensitivity of *O. nitens* in mixtures, compared to its single metal sensitivity. Interactions leading to synergism tend to be higher in soils with higher CEC (Qui et al., 2015). Thus, the protective effect that CEC has on an organism's health by increasing its available energy to detoxify metals is partially lost by the increasingly synergistic effects of metals on the organism's health (Jegede et al., 2019b).

3.6.2 Soil Properties and Metal Speciation Explains Toxicity

Soil properties control on toxicity was not explained by the influences of soil properties on metal speciation. Whereas the percentage of metals bound to fulvic acid explained some of the differences in the toxic response of *O. nitens* at EC10 in the soils, differences in soil properties played a much larger role. For example, Loamy-Alluvial soil had the highest clay and was the soil where *O. nitens* was most sensitive to mixtures at EC10 level. The Acid-Sandy-Arable soil where *O. nitens* was least sensitive to mixtures at both EC50 and EC10 levels, had intermediate soil property values (pH, CEC), although it had the second highest clay and organic carbon of all the soils. Therefore, an interplay between clay and organic carbon may be responsible for the response. Properties such as clay (Owojori et al., 2009) and organic matter (Princz et al., 2010) have been

linked to toxicity, but this is largely seen as an influence on speciation (Hasegewa et al., 2016). The CEC of a soil is a function of the clay (clay type and % clay) and organic matter (St. Arnaud and Sephton, 1972). Together with soil pH, the CEC influenced metal mixture toxicity to the mites at low effect levels (EC10). This work suggests that the influence of clay and organic matter or CEC on fluxes of metal mixtures into organisms may be more substantial than their effect on quasi-equilibrium estimates of metal speciation.

The fulvic-acid bound metal may be the metal fraction that is crucial to regulating uptake into the organism. For mixtures in stream macroinvertebrates, metals bound to humic acid correlated to metal-mixture body burdens causing toxicity (Stockdale et al., 2010). Similarly, we found that fulvic-acid bound metals predicted 34% of the toxicity. For example, Loamy-Alluvial soil had the highest percentage metal bound to Fulvic acid (> 60% for zinc and copper, 25% for the nickel) and was the soil where *O. nitens* was most sensitive to metal mixtures. Mites had higher body burdens of metals when exposed in soils of higher fulvic-acid bound metals (Jegede et al., 2019b). Although this present study did not assess the mite's body burden, increased body burden may be the reason why the mites were more sensitive to the metal mixtures.

3.6.3 Single Metal Toxicity

Nickel was the most toxic metal to *Oppia nitens* out of the five tested metals. This study is the first report on the toxicity of nickel (EC50 = 25.0 ± 11.6 mmol of Ni /kg of soil or 1471.8 ± 677.4 mg/kg of soil) to *O. nitens.* The high nickel toxicity relative to the other metals in this study is consistent with the literature on nickel toxicity to *Folsomia candida*, which is a standard soil invertebrate species. For example, EC50 of nickel to *F. candida* was 475 mg/kg (Lock and Janssen, 2002a) compared to ≥ 700 mg/kg recorded for the other metals (Sandifer and Hopkin, 1996; Sandifer and Hopkin, 1997; Lock et al., 2004). The present study was similar in that nickel toxicity

was 1.4 times greater on a molar level than the nearest other metal. Although, *O. nitens* seemed to be less sensitive to nickel than other soil invertebrates like *F. candida*, it should be noted that the present study could not be directly compared due to differences in soils and more importantly due to differences in metal dosing method (Awuah et al., 2019).

3.6.4 Metal Mixture Toxicity

Metal mixture toxicity did not follow concentration addition (CA), which is a regulatory default method of estimating mixtures (Bunke et al., 2014). Similarly, a meta-analysis of about 91 metal mixtures from the soil, freshwater and marine studies showed that most of the responses were either more or less than additivity and only 13% were additive (Vijver et al., 2010). Across 10 different mixtures, metal interactions were consistently not CA based.

Antagonism and synergism are biological responses to metal mixtures that suggest interaction among metals either in the exposure medium or in the organism (Nys et al., 2017). The metal interactions might be responsible for the differences in sensitivities of *O. nitens* to metals in the soils when comparing single and mixture responses. Single metal toxicity was used to mathematically identify antagonism and synergism in metal mixture toxicity. The soil where *O. nitens* was least sensitive soil to single metals (EC10 = 55 ± 40 mmol/kg) had the highest synergistic responses of metal mixtures and might be responsible for the increased sensitivity (EC10 = 23 ± 8 mmol/kg) of *O. nitens* to metal mixtures. The Acid-Sandy-Arable soil had the second highest antagonistic responses (56%) and was the soil where *O. nitens was* least sensitive to metal mixtures. In the same vein, antagonism was predominantly observed at EC50 level in the Loamy-Alluvial soil and might be responsible for the shift from the increased sensitivity of *O. nitens* at EC10 level to less sensitivity at EC50 level. Using the MIF, all the mixtures deviated from CA in all the soils and CA was not protective of the mite species for most of the soils.

Although, in aquatic systems, Nys et al. (2018) found that CA overestimated mixture effects on invertebrates at 10% effect levels, thus more protective of the species. However, our study showed that CA was more protective at EC50 levels than at EC10 levels in the soils which implies that metal risk assessment at least for Canadian soils may need to be focused on how to ensure that the terrestrial ecosystem is protected from low level metal mixture effects. Mechanistically, single metals' influence on metal mixture interactions is a possibility. Zinc, being the least toxic single metal and maintaining the same pattern in single and in mixtures, may be playing a protective role by influencing interactions. For example, Nys et al. (2017) reported that Zn protected against mixture toxicity of Cd, Cu, Pb, Zn and Ni to the plant, *Hordeum vulgare,* by shifting toxicity more to antagonism. Zinc can also influence organismal response to metal contamination by influencing increased production of antioxidant enzymes to counteract oxidative stress (Cakmak, 2000).

3.6.5 Implications of Study

This study and others showed that metal mixtures do not typically follow concentration addition (Vijver et al., 2010; Heys et al., 2016). In this study, concentration addition was wrong 90% of the time. Further, in a soil where *O. nitens* is sensitive to single metals, it does not mean the mite will be sensitive to metal mixtures in such soil and vice versa. Low level single metal effects in soils, grossly underestimates mixture effects assuming concentration addition. However, differences among soils were not driven by speciation, or put differently, toxicokinetics. Instead, soil properties such as CEC were driving mixture response, likely by altering the toxicodynamics of metals in the organism (Jegede et al., 2019b). Mixture composition did not have a large influence on toxicity, except for protection by zinc. The protective ability of zinc is likely linked to its influence on toxicodynamics and toxicokinetics. Increased zinc reduces impact of oxidative stress (Cakmak, 2000) and zinc often outcompetes other metals for uptake (Posthuma et al., 1997). The

combination of the improved habitat quality (organism energetics) by CEC, with CEC's promotion of synergistic metal toxicity, as well as the protective effect of Zn in metal mixtures, suggests to these authors that a simple assumption of concentration addition in risk assessment is not defensible. Furthermore, differences in metal speciation alone are unlikely to be sufficiently predictive of metal mixture impacts.

4. Manuscript 2: Multigenerational Exposure of Populations of *Oppia nitens* to Zinc Under Pulse and Continuous Exposure Scenarios

4.1 Preface

The influence of zinc on parents and subsequent generations of *Oppia nitens* when exposed once and continuously was assessed. Survival and reproduction of *O. nitens* were assessed as endpoints. Populations of parents and offspring were modelled as endpoints and the effect of zinc was assessed on the populations.

Jegede OO, Hale BA, Siciliano SD. 2019a. Multigenerational Exposure of Populations of *Oppia nitens* to Zinc under Pulse and Continuous Exposure Scenarios. *Environmental Toxicology and Chemistry* 38:896-904

Olukayode Jegede: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing (original draft), visualization
Beverley Hale: Conceptualization, resources, writing (review and editing), Supervision, project administration, funding acquisition
Steven Siciliano: Conceptualization, resources, writing (review and editing), Supervision, project administration, funding acquisition

4.2 Abstract

Current soil remediation guidelines for metals reflect single generation laboratory studies, however in the field, organisms are exposed to metals for more than one generation. This study assessed the multigenerational effect of zinc on *Oppia nitens* under a pulse or continuous exposure scenario. Synchronized adult mites (parents) were exposed to six concentrations of zinc in a field soil (105, 158, 237, 335, 553, and 800 mg/kg). For the pulse exposure, juveniles of parent mites from three of the six concentrations were kept in clean media and reared until the third generation. At every generation, the sensitivity of the mites to zinc was tested in a dose response manner. For the continuous exposure, the mites produced from the parents were re-exposed to the same concentration as their parents. Using critical level estimates like EC50, all populations of the F2 and F3 generation mites in the pulse exposure were less sensitive to zinc than the parents and were protected at 250 mg/kg of zinc (CCME soil quality guideline). However, the mite generations of the continuous exposure remained as sensitive as the parent generation and were not protected by the zinc guideline level. The zinc niche width narrowed considerably for all continuously exposed mite populations indicating that they were more sensitive than the parent. The results of this study showed that zinc has a deleterious multigenerational effect to continuously exposed populations of mites.

4.3 Introduction

Ecotoxicology aims to understand the long term effects of pollutants on organisms (Van Gestel, 2012); however, current guidelines use data collected from single generation laboratory toxicity studies that are often short term, typically 28 d (Amorim et al., 2017; Pereira et al., 2018). Long-term exposure to contaminants like metals cause adverse multigenerational effects (Amorim et al., 2017). For example, continuous exposure to cadmium, copper, lead, and zinc inhibited the growth,

reproduction, and feeding of the nematode *Caenorhabditis elegans* in the third and fourth generation (Yu et al., 2016). Another study showed that mercuric chloride inhibited reproduction in the third generation of the copepod, *Tigriopus japonicus* (Li et al., 2015). However, other studies showed increased tolerance after exposure to metals. For example, the earthworm *Eisenia fetida* tolerated copper and zinc better after two generations of exposure (Spurgeon and Hopkin, 2000). Similarly, the earthworm *E. fetida* and enchytraeid, *Enchytraeus albidus* laboratory cultures exposed to cadmium for more than 12 months showed increased tolerance to cadmium in subsequent toxicity tests (Reinecke et al., 1999, Lock and Janssen, 2001b). Finally, sometimes there are no long term effects. In a study with the mite, *Archegozetes longisetosus* exposed to cadmium, toxicity was not different after two generations (Seniczak et al., 2006).

Tolerance or susceptibility to metals is attributed to different mechanisms associated with the transgenerational effect of metals. One such mechanism is epigenesis. Epigenesis results from the transfer of non-genetic factors from parents to offspring rather than genetic inheritance to offspring (Youngson and Whitelaw, 2008; Polkki et al., 2012) such as DNA methylation (Nilsson and Skinner, 2015); however, the actual DNA sequence is unaltered. For example, the earthworm, *Lumbricus rubellus* from an arsenic-contaminated field soil tolerated arsenic exposure because of its altered DNA methylation transferred from the parents (Kille et al., 2013). Histome modification, another epigenetic mechanism, caused inhibition of vulval development in *C. elegans* (Schultz et al., 2016; Andersen and Horvitz, 2007). In addition to epigenesis, multi-generational exposure can cause teratogenesis in which the embryo or foetus is malformed due to embryo exposure to chemicals (Vargesson and Fraga, 2017). Teratogenesis results in physical deformations (Hovland et al., 2000) and in some cases behavioural aberrations (Weis, 2014) that manifests later in life. For example, exposure to lead caused leg malformation in the larval and

nymphal stages of the mite, *Archegozetes longisetosus* (Kohler et al., 2005). There can also be a direct maternal transfer of metals to other generations (Kim et al., 2013; Schultz et al., 2016). A generational transfer of silver nanoparticles from the parent was detected in the unexposed F1 generation of *C. elegans* (Luo et al., 2016). Based on these examples, the fitness of offspring and/or subsequent generations of the germline can be compromised.

Many multigenerational toxicity studies usually assume continuous or chronic exposure of organisms to persistent contaminants. In that case, both parents and offspring are exposed to these contaminants at the same time. But apart from the continuous exposure of organisms, persistent contaminants can have a long lasting effect and alter normal cell functions even when the contaminants are no longer present (Schug et al., 2011). Unexposed multigenerations of organisms can indirectly be exposed to contaminants through the exposure of their parents. These short-term exposures could be in a pulse-like manner. Pulse exposures are often short duration discharges of pollutants and discontinuous (Andersen et al., 2006). Pulse-like contaminations or discharges of pollutants can occur in the environment from activities such as sludge amendment applications, during mining or industrial and wastewater discharges (Mendes et al., 2018). It is important to evaluate the effect of persistence of metals when metals are present (continuous) and the persistent effect of metals when metals are absent (pulse).

Common critical effect (EC_X, LC_X) estimates may not be adequate to represent effects at the population level because of their focus on individual health. There is a need to use parameters that may represent pollutant effects on populations. One of the parameters that can be used effectively is the instantaneous population growth rate (r) that integrates survival and reproduction in order to measure population growth rate (Stark and Banks, 2000; Herbert et al., 2004). When evaluating the performance of pesticides on pests, the instantaneous population growth rate consistently

predicts deleterious effects of pesticides on pests better than other techniques (Andrade et al., 2012). Thus, we also used instantaneous population growth rate in the form of a niche width to evaluate pulsed and continuous metal exposure. The niche width in this context is a term borrowed from Grimaud et al. (2017) who defined thermal niche width as the "thermal range on which a given species can thrive." Therefore, we defined zinc niche width as a zinc exposure range in which population of organisms can thrive.

We hypothesize that both pulse-like exposures and continuous exposures of a metal to the oribatid mite, *Oppia nitens* will cause an increase in sensitivity in successive generations and that the effect will be more severe in the continuously exposed mites. To test our hypotheses, we assessed the multigenerational toxic effect of zinc exposure to the adult mite populations by (a) evaluating the effect of zinc on subsequent generations of *O. nitens* offspring after one-time exposure to the parents, and (b) evaluating the continuous exposure of mite generations to zinc.

4.4 Materials and Methods

4.41 Test Soil

The soil used was a natural soil (code name of soil = 3.22) collected from the Flin-Flon mining area, Manitoba, Canada. The soils were air-dried, sieved with a 2 mm mesh-sized sieve and were stored dry for several weeks before use. Details of this soil can be found in Hamilton et al. (2016). The properties of the soil are summarized as follows: pH = 3.4, organic carbon = 2.7%, cation exchange capacity = 8 meq/100g, grain size distribution: 4.5% clay; 25.6% silt; 69.9% sand.

4.4.2 Test Species

Oppia nitens are fungivorous oribatid mites, important nutrient recyclers in the soil, and are the most abundant microarthropod in boreal forest soils (Princz et al., 2010). The *O. nitens* specimens used for this study were taken from established laboratory cultures in the Soil Toxicology

Laboratory at the Department of Soil Science, University of Saskatchewan, Canada. Adult mites were cultured on a medium made of plaster of Paris (POP) and activated charcoal in an 8:1 w/w ratio. The POP was moistened twice a week and the mites were fed with bread yeast ad-libitum. The mites were age-synchronized; after about 5-6 weeks, newly emerged amber-coloured adult mites were selected and placed in a new medium of POP and allowed to fully mature. Fully matured mites had dark brown sclerotized pigments and were used for tests.

4.4.3 Test Metal

Zinc oxide was used as the test metal (Sigma Aldrich, puriss p.a ACS (American Chemical Society) reagent ≥ 99%). The zinc oxide was ground to finer particles with a mortar and pestle. The oxide was weighed out into dry soil to make 105, 158, 237, 335, 553, and 800 mg Zn/kg dry soil concentrations for the parent generation test. The oxide was added into the soil and mixed thoroughly. For the control soil, no oxide was added. The test soils were equilibrated for 18 to 24 h before the test organisms were added.

4.4.4 Multigenerational Test

4.4.4.1 Parent (F0) Generation Test

All the zinc-spiked and control soils were moistened with deionised water to 50% water holding capacity of the soil. Twenty-five grams of the spiked and control soils were weighed into a 2 cm diameter glass vial. Fifteen synchronized adult mites were then introduced into the vials in six replicates for control and for spiked soils. The mites were fed with yeast once every week starting from week 0. The experiment was run under constant conditions for 28 d; 21^{o}C, 50 - 60% humidity, > 800 Lux, day to light; 16: 8h regime (Princz et al., 2010). The loss in moisture was replenished by adding commensurate amount of water during feeding. After 28 d, the mites were extracted using a modified Berlese-tullgren extractor for 48 h. The number of surviving adults and the

juveniles produced were counted and recorded against each concentration as populations per concentration. The juveniles were reared to adulthood on POP and then used for the multigenerational tests.

4.4.4.2 Pulse Exposure

Two, three or four replicates (depending on the number of mites produced from previous generation) of fifteen (n=15) adult mites each were exposed to zinc in the spiked soils in increasing concentrations. After 28 d, survival and reproduction were assessed. Juveniles were selected from soils from the control (0 mg/kg) and three concentrations (158 mg/kg – low dose, 335 mg/kg – medium dose, 553 mg/kg – high dose). All the mite generations exposed to the same initial zinc dose were regarded as the same population. For example, all mite generations F1, F2, and F3 that were exposed to the low dose were called population 1. Generations exposed to the medium dose were called population 2, and those exposed to the high dose were population 3. The juveniles from these four populations (including population 0; mites in control soils) were reared on a POP medium until they became adults (F_1). The F_1 mites were then exposed to the four concentrations (including control) for 28 d. The adult survival and reproduction were again assessed. The juveniles from the control of this second range of exposure were reared on POP until adult (F_2 control) and juveniles from the other concentrations were also reared on POP until they became adults (F_2 continuous). The F_2 (control) adults were also exposed to the four concentrations for 28 d. The adult survival and reproduction were assessed. The juveniles from the control of this third range of exposure were reared on POP till they became adults (F_3 control). The F_3 control adults were then exposed to the four concentrations for 28 d. After the 28 d, adult survival and reproduction were assessed. The schematic representation of the experimental design is shown in Figure 4-1.

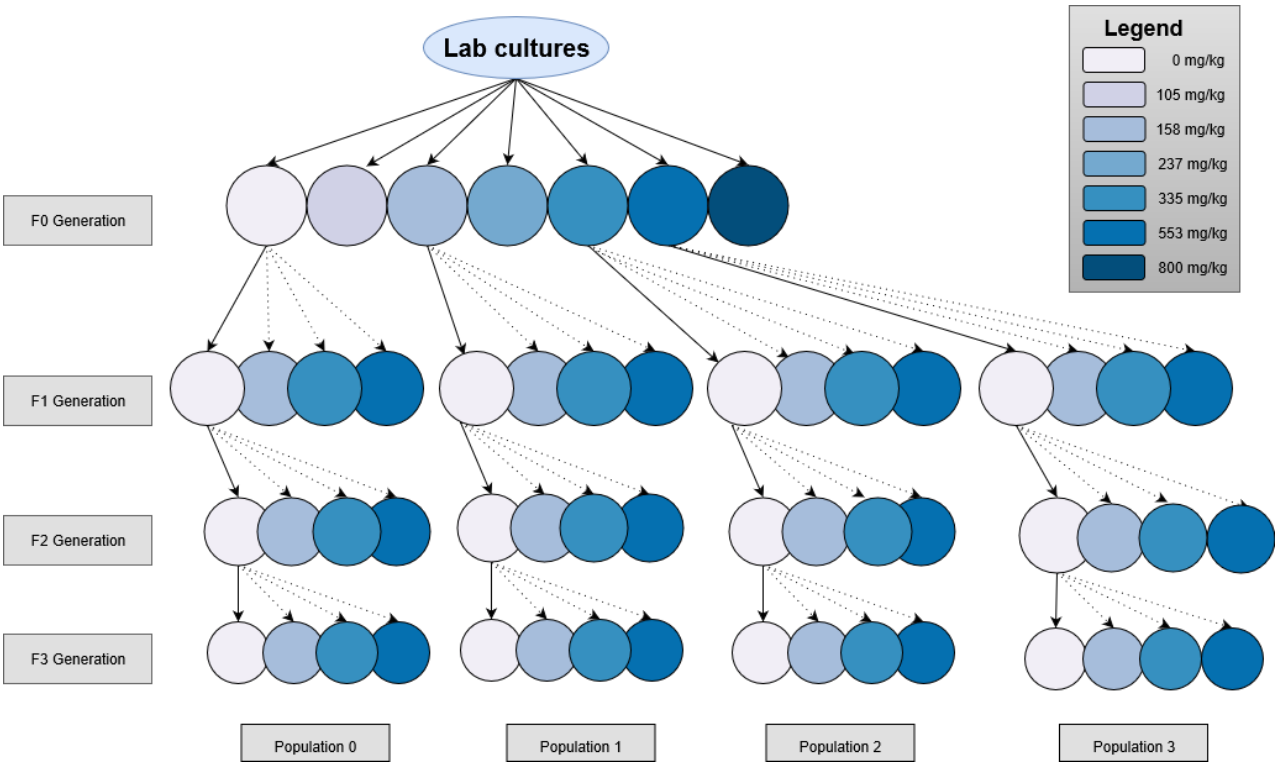


Figure 4- 1. Pulse multigenerational study showing exposure of mites to different nominal soil concentrations (mg/kg) of zinc (0, 105, 158, 237, 335, 553, and 800) in the F0 generation from which offspring from four nominal concentrations of zinc (0, 158, 335, and 553 mg/kg) were collected as four different mite populations. The fitness of the unexposed subsequent three generations (F1, F2, and F3) from the four populations (0, 1, 2, and 3) was tested against four nominal concentrations of zinc (0, 158, 335, and 553 mg/kg). Dashed lines indicate dose-response testing for each generation, whereas solid lines indicate rearing of a new generation.

4.4.4.3 Continuous Exposure

Mites from the F_2 continuous were used for this test. The mites were exposed to the four Zn concentrations (0, 158, 335, and 553 mg/kg) for 28 d. After 28 d, the adult survival and reproduction were assessed. The schematic experimental design for this continuous exposure is in Figure 2.

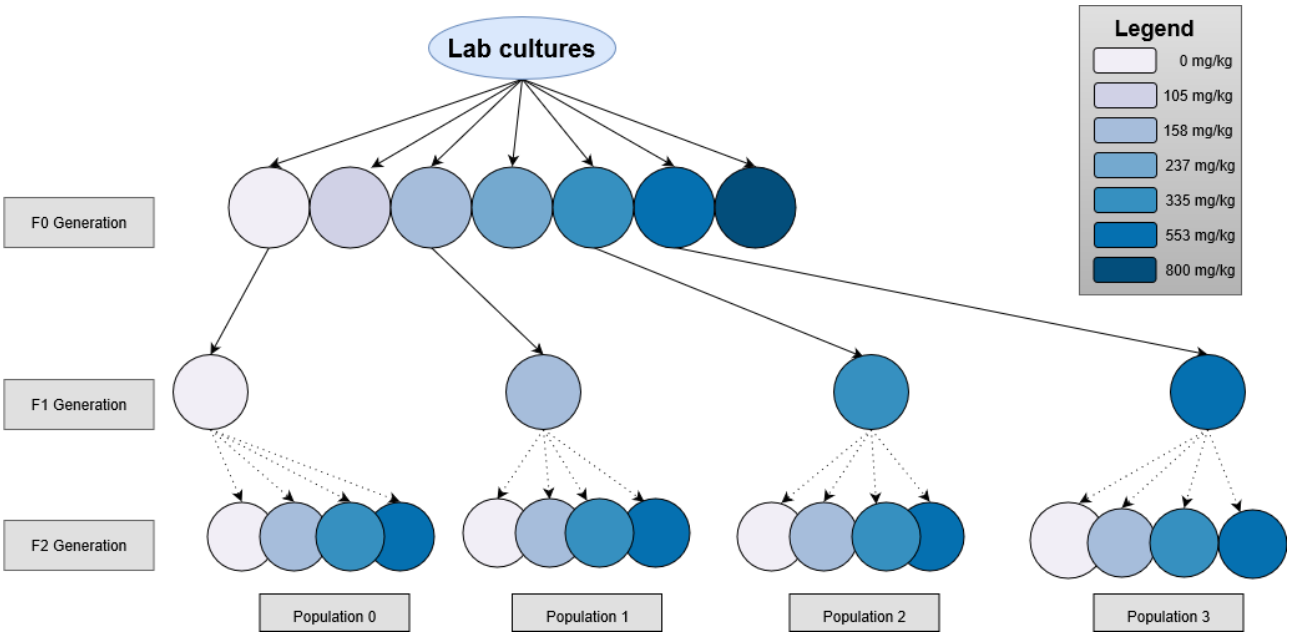


Figure 4- 2. Continuous dosing multigenerational study showing continuous exposure of mites to different nominal soil concentrations (mg/kg) of zinc (0, 105, 158, 237, 335, 553, and 800) in the F0 generation from which offspring from four nominal concentrations of zinc (0, 158, 335, and 553 mg/kg) were collected as four different mite populations. The fitness of the offspring from the exposed F1 generation mites were tested by exposing the mites to four nominal concentrations of zinc (0, 158, 335, and 553 mg/kg) in a dose response manner in the F2 generation. Dashed lines indicate dose-response testing for each generation, whereas solid lines indicate rearing of a new generation.

4.4.5 Statistics

All toxicity estimates were based on nominal zinc concentrations, as there was insufficient soil for total metal measurements. The dose-response relationship of zinc with mite reproduction was fitted with the drc function in the drm package in R (Ritz, 2016). The median effect concentration (EC50) causing 50% reduction in reproduction was estimated with non-linear regression models (3 or 4-parameter log-logistic models) using R. The instantaneous population growth rate (PGR), r_i was calculated thus

$$r_i = In\ (n_f/n_o)/\Delta T,$$

where n_f = final number of animals (total number of mite survival and reproduction), n_o = original number of animals, ΔT is the difference in time (28 d). Logistic regression least-squared fitting of each replicate of r_i were plotted against zinc concentration. The zinc concentration at which $r_i = 0$ (or PGR_{conc}) was calculated as the concentration of zinc at which population is stable. Where PGR_{conc} > highest zinc concentration (553 mg/kg), we assumed the PGR = highest concentration for analysis. The mite generations, F1, F2, F3 from pulse exposure and F2 from the continuous exposure were normalized to 100% of the parent (F0) at each dose. The normalization was calculated thus

(Mite reproduction at *Y* mg/kg of zinc in *F0* / Mite reproduction at *Y* mg/kg of zinc in *FX*) x 100%

and analysis of variance (ANOVA) interaction plot of their simple effect means at $p < 0.05$ was determined using Sigmaplot Systat 12.5. A Student's *t*-test was used to check differences between each generation and the parent (F0 generation) at each dose, and the power of the test was adjusted for Bonferroni correction. Niche width was determined for the naïve (parent), pulse and continuous exposed mite populations as the average of the PGR_{conc} values of all generations within the populations. Analysis of variance (ANOVA) was used to check the difference in means of EC50s and PGR between the generations in the pulse and continuous exposures, and a Tukey post hoc test was used to know where the differences lie.

4.5 Results

4.5.1 Pulse Exposure

Zinc influenced mutigenerational mite reproduction (Table 4-1). Reproduction was not completely inhibited by zinc in any of the generations (Figure 4-3). Non-exposed F1 generation from population 3 displayed the greatest reproduction, with an average exceeding 100 juveniles. No other population's reproduction reached 100 juveniles. There was a statistical interaction between

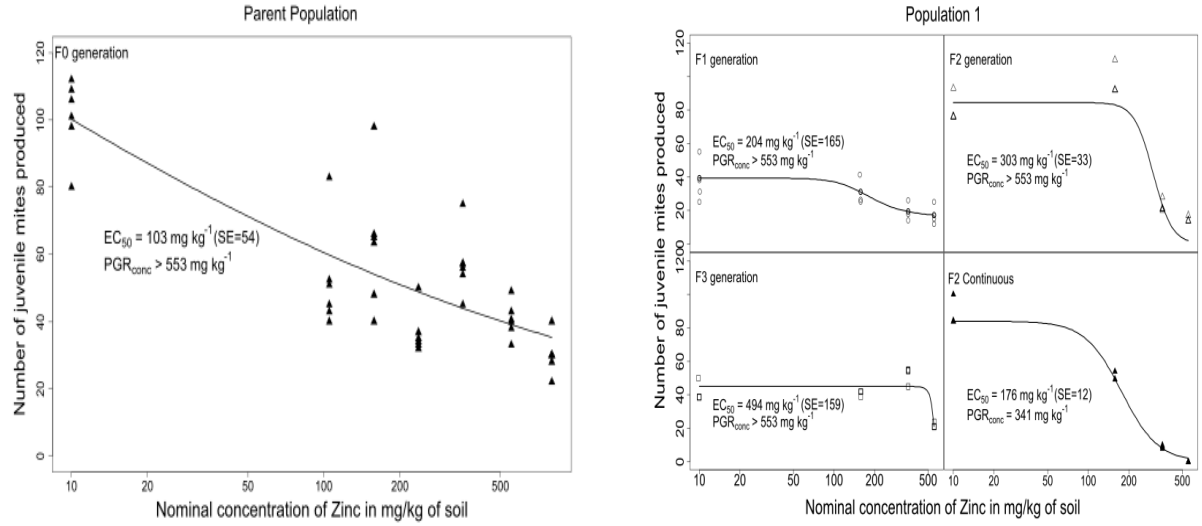
dose and generation in the ANOVA, thus simple effect means (Figure 4-4) were presented. At the highest dose, there was a significant spike in reproduction across all three generations compared to the parent generation (Figure 4-4, Table 4-1). The toxicity was less in the F1, F2, and F3 compared to the parents by about two orders of magnitude (Figure 4-5). However, the Canadian Council of Ministers of Environment (CCME) soil quality guideline (2018) for zinc (250 mg/kg) did not protect the parent and did not protect two populations of the F1 generation. The CCME zinc SQG protected the F2 and F3 populations in all cases (Figure 4-5).

Table 4- 1. The parent population (F0) reproduction compared with the reproduction of mite generations (F1, F2, F3, F2_Continuous) using a student's t-test. F1, F2, F3 are generations from the pulse Zn exposure and F2_Continuous is the second generation of continuous Zn exposed mites. Significant difference of mite generations with F0 was determined when $p < 0.05$. The power of the test was adjusted for Bonferroni correction

Dose	Comparison	p value
Control	F0 - F1	1.00
	F0 - F2	1.00
	F0 - F3	0.85
	F0 - F2_Continuous	1.00
Low (158 ppm)	F0 - F1	1.00
	F0 - F2	0.001*
	F0 - F3	0.913
	F0 - F2_Continuous	1.00
Medium (335 ppm)	F0 - F1	1.00
	F0 - F2	0.89
	F0 - F3	0.40
	F0 - F2_Continuous	0.11
High (553 ppm)	F0 - F1	0.01*
	F0 - F2	0.003*
	F0 - F3	0.001*
	F0 - F2_Continuous	1.00

(a)

(b)



(c)

(d)

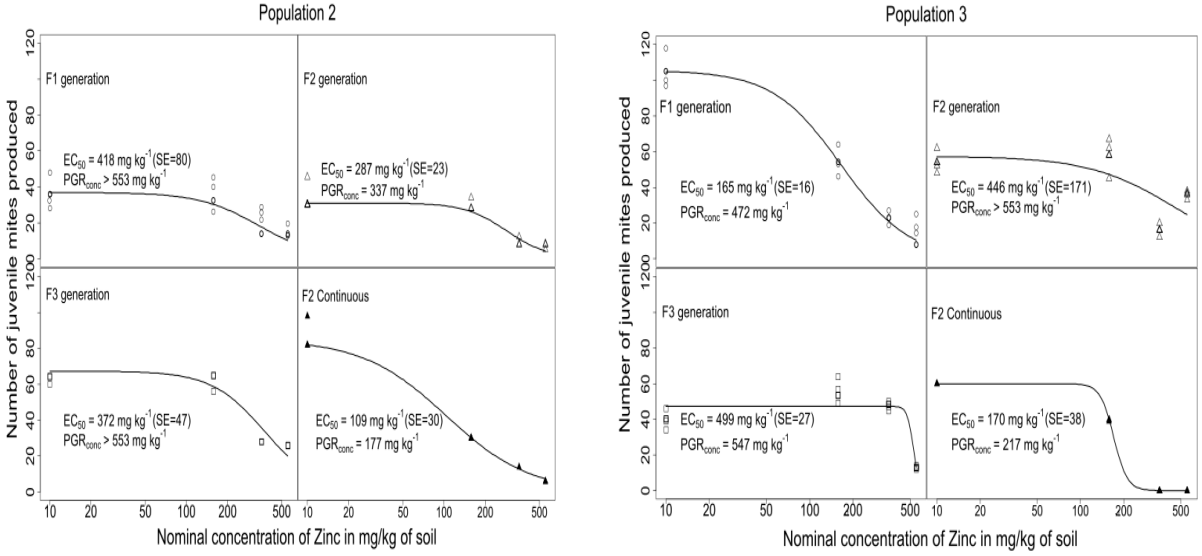


Figure 4- 3. The dose response of *Oppia nitens* exposed to zinc in a natural soil (3.22) for populations F0 generation (a), population 1 (b), population 2 (c), population 3 (d). The F0, F1, F2, F3 generations are the parent, first, second and third filial generations, respectively from the pulse-exposed mites. The F2 continuous are the F2 generations of the continuously exposed mites. The EC_{50} and the population growth rate concentration (PGR_{conc}) are calculated for each generation within each population and printed on each dose response curve.

4.5.2 Continuous Exposure

The median effective concentration showed that the continuously exposed mites were generally more sensitive to metals than the pulse-exposed mites (Figure 4-3) were. The controls of populations 1 and 2 reproduced more than the control group of the parent mites. In some cases, the highest concentration of zinc completely inhibited reproduction of mites. For example, zinc completely inhibited mite reproduction in two populations (populations 1 and 3) when exposed to 553 mg/kg of zinc. Compared to the F0 and other generations, mite reproduction in the continuous exposed mites was lower except in the low dose (158 mg/kg) (Figure 4-4). Toxicity in the continuous population was similar to the parent because the EC50s were in the same order of magnitude. The CCME soil quality guideline for zinc did not protect the parents and the mites exposed to continuous zinc. (Figure 4-5).

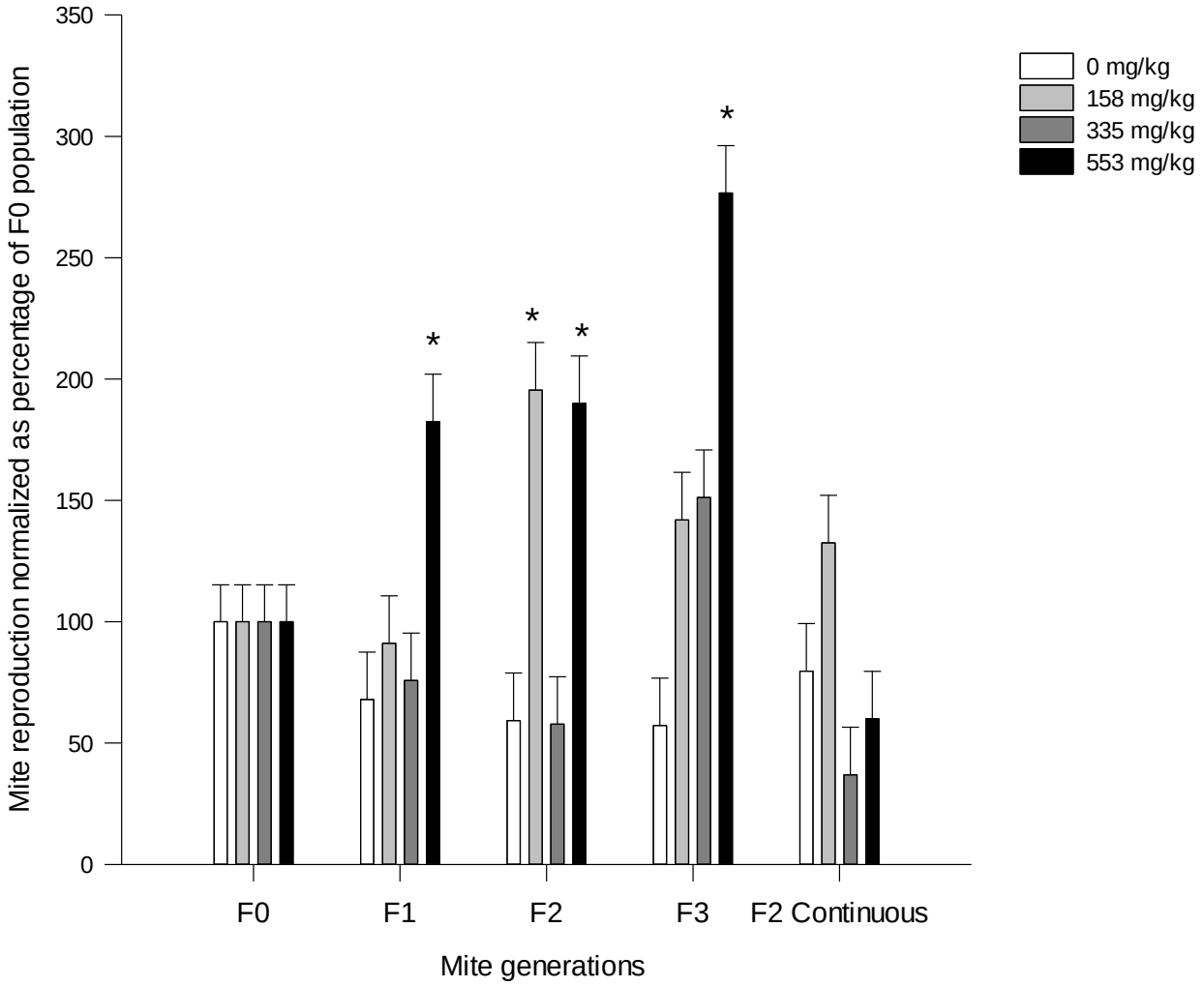


Figure 4- 4. Simple effect means of zinc exposure of F0 on mite reproduction in subsequent generations. The zinc pre-exposure was either 0, 158, 335 or 553 mg/kg. F0 = parent, F1, F2 and F3 are the first, second and third generations in the pulse experiment, respectively. F2 Continuous is the F2 generation of the continuously exposure experiment. The mite reproduction for each dosing group was normalized to the F0 generation's reproduction after exposure to either 0, 158, 335 or 553 mg/kg, respectively. A Student's *t*-test was used to determine the significant differences between F0 and other generations at corresponding zinc doses. The bar height represents the fitted values of the normalized mite reproductions from an ANOVA interaction plot, hence the error estimates are similar across all the plots

* $p < 0.05$.

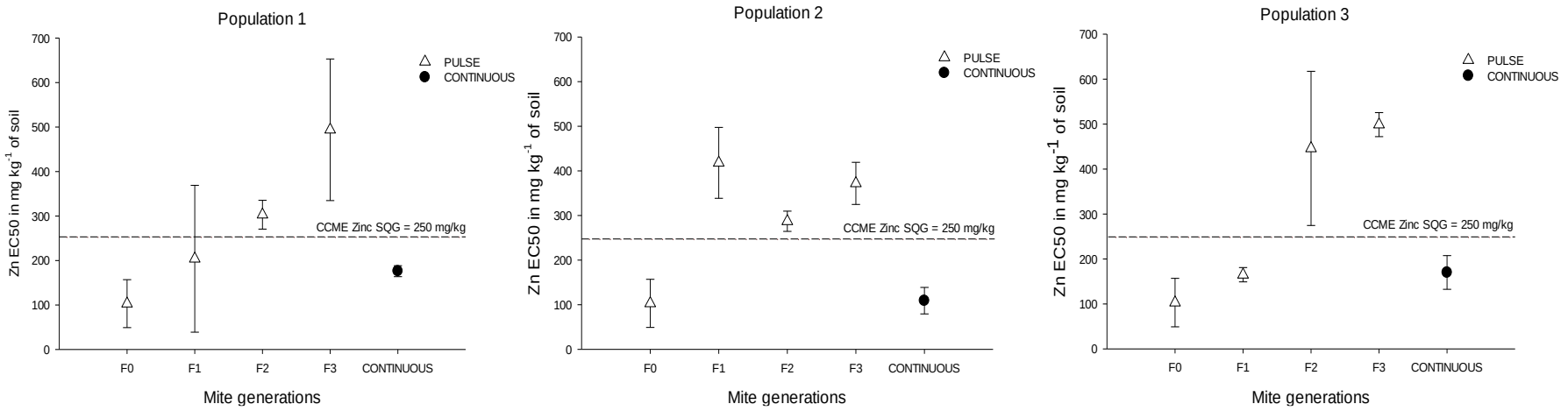


Figure 4- 5. EC50s ± SE of multi-generations of pulse and continuously exposed mites in populations initially exposed to 158, 335 and 553 mg Zn / kg soil (populations 1, 2 and 3). F0 = parent, F1, F2, and F3 = first, second, and third generations of the pulse-exposed mites respectively. Continuous = F2 generation of the continuously exposed mites. The CCME zinc SQG = CCME zinc soil quality guideline value (CCME, 2018). All points above the line are protected *Oppia nitens* populations but all points below the line are unprotected *O. nitens* populations.

4.5.3 Niche Width

Continuous zinc exposure significantly narrowed down the zinc niche of the mite populations relative to the naive population (Figure 4-6). Zinc pulses also caused smaller decreases in niche width compared to the naive population. Niche width differed between population 1 and the other populations by almost two orders of magnitude in the continuous Zn exposure. ANOVA showed that PGR_{conc} was significant ($p = 0.0053$) across mite generations. The Tukey post-hoc analysis showed that PGR_{conc} significantly differed ($p < 0.05$) between the continuous zinc exposed mites and each of the F1, F2, and F3 generations of the pulse-exposed mites (Table 4-2). See PGR_{conc} values (Appendix B, Figures B1-B4).

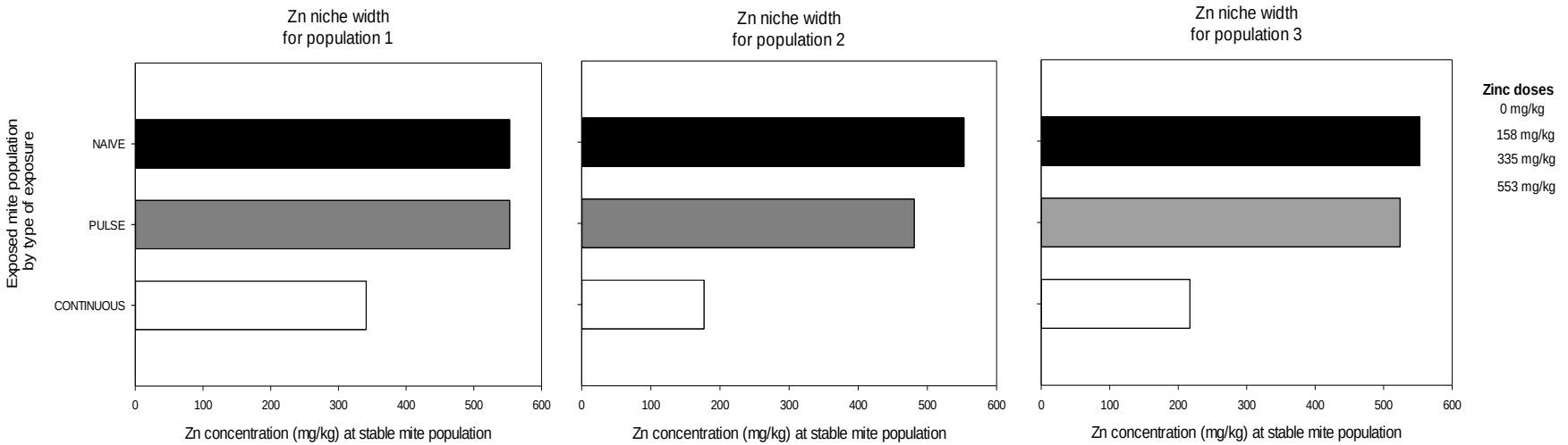


Figure 4- 6. Zinc niche width for population 1, 2 and 3 mites by type of exposure. The naive are the parent population, pulse are the pulse-exposed mites and continuous are the continuously exposed mites. When concentration is above the tolerance range for the population, the population tends towards extinction. Zinc niche width is the zinc-contamination tolerance range for each of the mite populations. All mite populations were exposed to 0, 158, 335, and 553 mg/kg doses of zinc.

4.5.4 EC50 and PGR Differences across Generations.

EC50s in a similar way to mite reproduction, differed (P = 0.017) between mite generations (Table 4-2). The tukey post-hoc analysis showed that there were no significant differences ($p > 0.05$) between the EC50s of the continuously exposed mites and the F1, F2 generations of the pulse-exposed mites. However, the EC50 in the F3 generation was high and differed significantly ($p = 0.013$) from the EC50 in the continuous exposed mites. The continuous zinc exposure increased the mites' sensitivity more than the pulse zinc exposure did. The PGR differed (P = 0.0053) between generations. The tukey post-hoc analysis showed that continuous differed from all the pulse exposed generations. However, F1, F2 and F3 pulse did not differ (Table 4-2).

Table 4- 2. Analysis of variance (ANOVA) of the EC50s and PGR per generation of mites. EC50 is the median effective concentration of zinc on mite reproduction in mg/kg of soil. PGR is the population growth rate.

	Analysis of variance	Tukey
EC50	P = 0.017* F = 6.26	F1 – Continuous = 0.47 F2 – Continuous = 0.13 F3 – Continuous = 0.013* F1 – F2 = 0.74 F1 – F3 = 0.10 F2 – F3 = 0.40
PGR	P = 0.00531** F = 9.41	F1 – Continuous = 0.0105* F2 – Continuous = 0.0267* F3 – Continuous = 0.00642** F1 – F2 = 0.895 F1 – F3 = 0.979 F2 – F3 = 0.709

* $p < 0.05$
** $p < 0.01$

4.6 Discussion

4.6.1 Sensitivity of Mite Generations

Multigenerational exposures of *Oppia nitens* to zinc manifested toxic effects that were not detected in single generation tests. Effects not seen in a single generation test can be observed in multigenerational exposures. When *O. nitens* are exposed to pulse of metals in the soil, unexposed offspring develop tolerance (Figure 4-4, Table 4-1). Yet continuously exposed mites become more sensitive with some populations becoming extinct (Figure 4-3). Similarly, the nematode, *C. elegans* became more sensitive in the third and fourth generation after continuous exposure to metals (Yu et al., 2016). In contrast, the earthworm, *Eisenia fetida* developed tolerance to zinc after two generations of exposure (Spurgeon and Hopkin, 2000). Typically, soil quality guidelines assume a continuous exposure, and predictive models are needed to link the traditional single generation toxicity test to the multigenerational reality of soil pollution.

4.6.2 Mite Offspring Tolerance

Once metal stress was removed, reproduction immediately rebounded. After pulse exposing mites at 553 mg/kg, mite reproduction increased (Figure 4-4) and after continuously exposing mites for two generations and then transferring to a clean soil, mite reproduction increased. Similarly, *Folsomia candida* exposed to silver increased its juvenile production after transferring them to clean soil (Mendes et al., 2018). These authors attributed the phenomenon to activation of the anti-oxidant defenses when exposed to high levels of metal. The authors also found that when the stressor was removed, it must have induced a compensatory effect in reproduction. Another potential mechanism could be that adaptations can be transferred to subsequent generations by epigenetic mechanisms that can be observed under unstressed conditions (Calabrese and Mattson, 2017). Alternatively, k-strategists like *O. nitens* may conserve energy under metal stress by reducing reproduction, and once this stress is removed, *O. nitens* diverts energy reserves into reproduction. More work is needed to understand the dynamic energy budgets of invertebrates exposed to pollutants to disentangle physiological compensation to exposure from epigenetic influences.

4.6.3 Stress Induced by Low Exposure Concentrations

In *O. nitens,* a low pulse of zinc reduced the rebound in reproduction suggesting that there is a fundamental difference in response type between low and high doses of zinc. The toxicity of copper oxide nanoparticles (CuONMs) and copper chloride ($CuCl_2$) to *Enchytraeus crypticus* was more severe when concentrations of CuONMs were 20 mg/kg and $CuCl_2$ was 500 mg/kg than at 180 mg/kg CuONMs and 1400 mg/kg of $CuCl_2$ (Bicho et al., 2017). Low (32 mg/kg) cadmium concentrations inhibited *F. candida* reproduction more than 60 mg/kg of cadmium in a long duration study (Amorim et al., 2017). In the same study, after the 13^{th} generation, the collembolans exposed to the lower cadmium concentration became extinct. In contrast, the collembolans recovered from the highest

concentrations did not go extinct until the experiment ended after 41 generations. The authors suggested that the organisms developed adaptive mechanisms at higher concentrations of metal exposure. For example, *Daphnia magna* reduced mercury uptake in response to high, lethal concentrations of mercury (Tsui and Wang, 2006). Epigenetic mechanisms also help to induce tolerance to stressors in organisms. For instance, F1 generations of zebrafish through transgenerational epigenetic resistance developed hypoxia resistance (Ho and Burggren, 2010). Organisms continuously exposed to metals must trade off using energy for reproduction versus coping with the metal by transferring toxic metal loads to offspring. The outcomes of this trade-off will likely determine the ability of populations to survive in metal contaminated soils.

Continuous multigenerational exposure tends to have more severe effects than single generation. Continuous zinc exposure reduced mite reproduction considerably and completely inhibited reproduction in some of the mite populations. For example, zinc completely inhibited *O. nitens* reproduction in populations 1 and 3 when exposed to 553 mg/kg of zinc (Figure 4-3b and 4-3d). In a continuous exposure of *Caenorhabditis elegans* to gold nanoparticles, sensitivity increased in the third generation (Kim et al., 2013). Similarly, exposure to cadmium, copper, lead, and zinc increased the sensitivity of *C. elegans* in the third and fourth generations (Yu et al., 2016). Because metals persist in the soil, soil dwelling organisms are exposed to metals continually, which adversely affect their populations.

4.6.4 Population Growth Rate

Population growth rate (PGR) predicts the performance of populations in metal contaminated soils better than critical level estimates like EC50. PGR is a sensitive endpoint that improves the overall ecological relevance of a test (Forbes and Calow, 1999). Despite higher EC50 values indicative of less toxicity in the pulse and continuous zinc exposure, PGR through the use of niche width showed

that the pulse and continuous zinc exposure impacted the offspring more than the parents (Figure 4-6). The niche width of the pulse and continuous zinc exposed mite populations narrowed as the concentration of zinc increased to as low as 177 mg/kg, below the putative EC50.

Classically, toxicity of a stressor depends on the duration of exposure (Heckmann et al., 2010) and magnitude of the stressor (Campbell et al., 2006). Currently, data used for metal risk assessment are from single-generational tests (Amorim et al., 2017). However, ecological receptors are continuously exposed to metals. Aging or adjustment factors commonly used in risk assessments are typically meant to adjust for toxicity differences between freshly spiked and field polluted soils. Therefore, our study suggests uncertainty factors or predictive models correcting for multigenerational test data gaps should be developed. To the best of our knowledge, there are no uncertainty factors being used in soil risk assessment to adjust single generation to multigenerational values.

4.6.5 Implications of Study on Tests for Metal Risk Assessment

The toxic effects of zinc on populations of mites that were not seen in a single generation test were observed in a multigenerational test. In a multigeneration scenario, the toxic effect of zinc was more severe in the mites that were continuously exposed to zinc than the pulse exposed mites. In some instances, the mites that were pulse-exposed to high zinc concentrations developed tolerance relative to low pulse of zinc. Compared to the parents, pulse and continuous exposed mites had higher EC50 values indicating less toxicity. However, the zinc niche width determined from the PGR, showed that the populations of both pulse and continuous exposed mites were impacted by the zinc contamination relative to the parents. The findings from this study implies that using results from a one-generational standard toxicity tests might not be protective enough of population effects. Therefore, this study suggests the development of uncertainty factors or predictive models correcting for multigenerational test data gaps.

5. Manuscript 3: The Forgotten Role of Toxicodynamics: How Habitat Quality Alters the Mite, *Oppia nitens,* Susceptibility to Zinc, Independent of Toxicokinetics.

5.1 Preface

The influence of soil habitat quality on toxicodynamics and toxicokinetics of zinc was investigated. Survival, reproduction of mites, biochemical responses of mites, Zn speciation and Zn bioavailability were assessed in low, medium and high habitat quality soils.

Jegede OO, Awuah KF, Fajana HO, Owojori OJ, Hale BA, Siciliano SD. 2019b. The forgotten role of toxicodynamics: How habitat quality alters the mite, *Oppia nitens*, susceptibility to zinc, independent of toxicokinetics. *Chemosphere* 227: 444-454

Olukayode Jegede: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing (original draft), visualization
Kobby Awuah: Investigation, methodology, writing (review and editing),
Hamzat Fajana: Investigation, methodology, writing (review and editing),
Olugbenga Owojori: Investigation, methodology, writing (review and editing),
Beverley Hale: Conceptualization, resources, writing (review and editing), supervision, project administration, funding acquisition
Steven Siciliano: Conceptualization, resources, writing (review and editing), supervision, project administration, funding acquisition

5.2 Abstract

Soil habitat quality is thought to influence metal toxicity via changes in speciation and thereby toxicokinetics. Here, we assessed the toxicokinetic and toxicodynamic effects of habitat quality on mite, *Oppia nitens* when exposed to zinc (Zn) contaminated soils. Forty-seven soils were ranked into three habitat qualities; high, medium, and low based on biological reproduction of *Folsomia candida*, *Enchytraeus crypticus*, and *Elymus lanceolatus*. From the 47 soils, eighteen soils (comprising of six soils from each habitat quality) were randomly selected, and dosed with field relevant concentrations of Zn. Mite survival and reproduction were assessed after 28 days. Total Zn, bioaccessible Zn, Zn bioavailability, Zn body burden, lactate dehydrogenase activity (LDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities of the mites were determined. Zinc toxicity and potency were much less in the high compared to low quality soils and the mites in the high habitat quality soils tolerated higher zinc body burdens (2040 ± 130 µg/g b.w) than the lower habitat quality (1180 ± 310 µg/g b.w). Lower LDH activity (20 ± 2 µU mg^{-1}) in the high quality soils compared to lower quality soils (50 ± 8 µU mg^{-1}) suggested that there was less stress in the high habitat quality mites. Despite changes in speciation across habitat qualities, bioavailability of zinc was similar (~ 20%) irrespective of habitat quality. Our results suggest that the influence of soil properties on survival is modulated by toxicodynamics rather than toxicokinetics. Restoring habitat quality may be more important for soil invertebrate protection than metal concentration at contaminated sites.

5.3 Introduction

Toxicokinetics are how an organism influences chemical uptake, metabolism and excretion (Zhang et al., 2019). Metal speciation, influenced by environmental parameters such as pH and organic matter in the exposure medium, drives toxicokinetics (Constantino et al., 2011). Biotic ligand and fugacity

models characterize the influence of environmental and organismal factors on the toxicokinetics of metals and organic chemicals respectively (Le et al., 2012; Celsie et al., 2016). In contrast, toxicodynamics is how a chemical influences an organism (Ardestani et al., 2014). It is widely assumed that the majority of the environment's influence on metal toxicity is through toxicokinetics. However, soil modifies metal speciation and is the habitat for many organisms, and its influence on metal toxicodynamics may outweigh its toxicokinetic effects.

Soils' alteration of metal toxicokinetics are well established. For example, soil factors decrease metal toxicity by reducing cationic metals availability to an organism (Kuperman et al., 2009; Van Gestel, 2012) i.e. by altering toxicokinetics. Soil factors also influence survival and reproduction of soil organisms independently of their effects on metal speciation. For example, soil organic matter influences the reproduction of oribatid mite, *Oppia nitens* with peak reproduction occurring at 7% organic matter content (Princz et al., 2010). Habitat quality is the ability of an ecosystem to sustain individuals and populations by providing appropriate ecological conditions (Hall et al., 1997). It is with this view that the soil habitat function was recommended to be tested (ISO, 2019a). The conditions necessary to sustain organisms is linked to the resources available for survival and suitability of environmental conditions (Johnson, 2007; Pulliam, 2000).

Soil pH, texture and organic matter are some of the dominant drivers of soil habitat quality. For example, optimal reproduction of the collembolan *Folsomia candida* occurs between pH 5.4 and 6.6, is reduced by 50% when pH is 3.5 and is completely inhibited at soil pH above 7.7 (Jansch et al., 2005). Similarly, the reproduction of the enchytraeid, *Enchytraeus albidus* is inhibited in soils below pH 5 (Kuperman et al., 2009). Texture has a strong influence on earthworm (*Eisenia andrei)* reproduction, which was less in clayey agricultural and sandy soils compared to organic, clay loam, or forest organic soils (Jansch et al., 2005). As noted above, high organic matter in soil leads to greater

reproduction and survival of organisms in soils (Princz et al., 2010; Son et al., 2007). Soil organic matter acts as a preferred location for mites to lay eggs (Princz et al., 2010), alters soil texture and can also serve as a surrogate food source. For example, after 50 days in soils with high organic matter content (16.5%) and without food, Collembola juvenile and adult survival did not differ from low organic matter soils (ca 2%) with food (Bur et al., 2010). Organic matter also influences soil structure, increasing pores and channels which was ascribed to increasing the collembolan numbers (Son et al., 2007). Thus, the interaction among soil pH, texture and organic matter content is a potentially strong predictor of habitat quality.

Habitat quality is proportional to an organism's energy balance, with increased habitat quality increasing energy supply, in the form of food, or limiting energy expenditure by reducing environmental stress (Hope, 2001). For example, organic matter is an indirect source of food and energy for many soil invertebrates. Increased organic matter in litter and humus layers leads to an increase in fungal biomass, which is a primary food source for the mites (Princz et al., 2010). The increased fungal biomass leads to an increase in energy for the mites. Alternatively, habitat characteristics that reduce energy expenditures increase fitness because an invertebrate's energy budget is critical for maintenance, growth and reproduction (Hope, 2001). For example, the earthworm *E. fetida* produced significantly more cocoons in soils with 40% clay than with 5% clay (Owojori et al., 2010). The authors attributed the higher cocoon production in soils with 40% clay to less movement of earthworms, thereby conserving energy that was used for reproduction. In the event of pollution, organisms will spend energy resisting contaminants by avoidance, exclusion or removal from the body thereby depleting the energy budget (Sibly and Calow, 1989; Donker, 1992). Energy budget depletion has a detrimental effect on survival, growth rate and reproduction of organisms

(Widdows and Donkin, 1991; Donker et al., 1993). Hence habitat quality is crucial to optimizing energy resources for invertebrates which could alter the toxicodynamics of metals.

Biochemical markers of altered invertebrate energy regulation such as lactase dehydrogenase (LDH) and glucose-6-phosphate-dehydrogenase (G6PDH) are used in toxicology studies (Diamantino et al., 2001), and can provide a measure of habitat quality's influence on mite energy balance. LDH is a glycolytic enzyme that indicates glycogen breakdown to lactate and implies that aerobic energy was not enough to sustain an organism (Yallappa and Nuzhat, 2018). Increased LDH activity has been associated with chemical toxicity in a number of studies and it is indicative of organism stress. For example, LDH and G6PDH activities increased in response to mercury toxicity in *Daphnia magna* (De Coen et al., 2001). In earthworm (*Glyphidrillus tuberosus*), LDH activity increased with increasing solid waste (phosphogypsum) concentrations (Nayak et al., 2018). Zn oxide nanoparticles induced cytotoxicity causing cell lysis, thus releasing cellular LDH into the cytoplasm (Saptarshi et al., 2015). The rate-limiting enzyme G6PDH is a biomarker of oxidative stress (Liu et al., 2007) and is involved in metabolising glucose through the oxidative pentose phosphate pathway (Liu et al., 2007. Overall, G6PDH and LDH are two promising biomarkers for toxicodynamics responses and in studying the subcellular stress response of soil invertebrates.

Here, we wished to differentiate the effects of soil properties on toxicokinetics from those of toxicodynamics. We hypothesize that soil habitat quality will dominate toxicodynamics by increasing energy available to mites after correcting for soil's influence on metal speciation and bioavailability. To test our hypothesis, we categorized forty-seven soils spanning Western Canada into high, medium and low habitat quality categories using reproduction of *F. candida, E. crypticus, and E. lanceolatus*. Three soils from each habitat quality were chosen, and then for these nine soils, we exposed the mite, *Oppia nitens* to increasing Zn concentrations. After 28 days of exposure, we estimated metal

speciation and bio-accessibility (calcium chloride extracted metal concentration), dissolved organic carbon (DOC) assessed mite survival, reproduction, metal bioavailability and energy stores. We then repeated this entire experiment with another nine soils spanning all three habitat categories from the remaining 38 soils. Thus, in total we assessed the toxicodynamics and toxicokinetics of Zn in 18 soils in two independent experiments.

5.4 Materials and Methods

5.4.1 Soil Collection

Forty-seven (n=47) soils were collected from agricultural and forested locations in Canada (Saskatchewan, Alberta, Manitoba and Western Ontario). The soils were air dried and sieved with a 2 mm mesh-sized sieve to remove debris and rocks. The physicochemical parameters of the soils were determined (Appendix C, Table C-1).

5.4.2 Test Species

Oppia nitens is an oribatid mite which feeds mainly on fungi and the mites are important in soil nutrient cycling. Oribatid mites are the most abundant microarthropod in boreal forest soils (Princz et al., 2010). The specimens of *O. nitens* used for this study were supplied from the already established laboratory cultures in the Soil Toxicology Laboratory at the Department of Soil Science, University of Saskatchewan, Canada. The mites were cultured and used for the test as described in Jegede et al. (2019a).

Folsomia candida is a springtail of the order Collembola. It is one of the most commonly used microarthropod species in the ecotoxicological literature and protocols (Environment Canada, 2014; OECD, 2009, ISO, 1999) are available for their use. The *F. candida* specimens used for this study were obtained from cultures maintained in the Soil Toxicology Laboratory at the Department of Soil Science, University of Saskatchewan, Canada. The cultures were maintained on a medium made of a

mixture of POP and activated charcoal in a 8:1 w/w ratio. The medium was moistened once a week and the collembolans were fed with bread yeast once in a week. After about 10 days, the eggs laid by the adult collembolans were picked up with a wet brush into a new medium where they were placed until hatching.

Enchytraeus crypticus is an oligochaete annelid. Enchytraeids are actively involved in soil nutrient cycling, organic matter decomposition, soil aeration and stabilization by creating a soil fine-grained crumb structure after feeding (Jansch et al., 2005). The specimens of *E. crypticus* were obtained from cultures maintained in the Soil Toxicology Laboratory at the Department of Soil Science, University of Saskatchewan, Canada on artificial soils moistened to 50% water holding capacity (WHC) and fed once a week with oats.

Elymus lanceolatus or northern wheatgrass, is widely distributed in North America (Scher, 2002). It is a perennial monocotyledon belonging to the family Poaeceae (Environment Canada, 2007). Northern wheatgrass is a common forage for livestock and is often applied as erosion control because of its deep root system (Scher, 2002). Northern wheatgrass grows very fast, has high seed vigour and is extensively used in plant toxicity testing (Anaka et al., 2008). The seedlings of the Northern wheatgrass used for these tests were donated by BrettYoung Seeds (Winnipeg, Manitoba).

5.4.3 Habitat Quality Determination

Habitat quality was determined from the survival and reproduction of the invertebrates (*F. candida* and *E. crypticus*), and plant (*E. lanceolatus*) biomass in the forty-seven soils with no Zn amendment.

5.4.3.1 Survival and Reproduction of F. candida

Soils were moistened to 50% of the WHC with distilled water. Ten age-synchronized (9-12 day old) collembolan juveniles were introduced into each replicate of soil. The springtails were fed once a

week with baker's yeast and the moisture lost from the soil was replaced at the time of feeding. The springtails were kept in the soil for 28 days at a constant temperature of 21ºC, 50 to 60% humidity, 400 to 800 Lux, 16h light:8h dark (OECD, 2009). The surviving adult and juvenile springtails produced were counted after 28 days by adding water to the soil thereby causing the springtails to float. The counts were used to determine adult survival and reproduction (juveniles produced) per soil.

5.4.3.2 Survival and Reproduction of E. crypticus

For this test, the soils were moistened with distilled water to 50% of the WHC. Ten adult enchytraeids (selected based on their larger sizes and developed clitellum) were introduced into each replicate of soil. The enchytraeids were fed once a week with ground-rolled oats and moisture lost from the soil was replaced on each day of feeding. The enchytraeids were kept for 28 days at a constant temperature of 21°C, 50 to 60% humidity, 400 to 800 Lux, 16h light: 8h dark, following the standard protocol (ISO, 2014). After 28 days, the surviving adults and juveniles were stained with Bengal red dye and were removed from the soil by wet sieving before counting under a microscope.

5.4.3.3 Determination of E. lanceolatus Biomass Production

Five seeds of Northern wheatgrass, *E. lanceolatus* were planted per 350g of dry soil, moistened to 70% WHC. The soils were then kept in a chamber with full spectrum fluorescent light of 300 ± 100 µmol/m^2/s, constant temperature of 21°C, 50 to 60% humidity (Environment Canada, 2007). Plants were harvested after thirty-five days of growth to maximize yield and biomass, oven-dried at 70°C for 24 hours and then weighed with a Mettler Toledo balance to determine the biomass.

5.4.3.4 Calculation of Habitat Quality Scores

Habitat quality was calculated by combining plant biomass, and enchytraeid and springtail reproduction tests into a single index as follows: Plant biomass, enchytraeid reproduction and springtail reproduction in each of the forty-seven (n=47) soils were normalized by dividing by their averages and multiplying by 100%. The normalized scores for each of the plant biomass, enchytraeid and collembolan reproduction were summed together to give a total score per soil. The soils with scores from 400 and above were indexed as 1 (high habitat quality), while those with score range of ≥ 200 < 400 were indexed as 2 (medium habitat quality) and soils of < 200 were indexed as 3 (low habitat quality).

How habitat quality was calculated:

Given 'n' number of soils,

$$\frac{\sum \text{Plant biomass in 'n' soils}}{\text{n}} = \text{Average plant biomass in 'n' soils (Average P)}$$

$$\frac{\sum \text{Sprintail reproduction in 'n' soils}}{\text{n}} = \text{Average springtail reproduction in 'n' soils (Average S)}$$

$$\frac{\sum \text{Enchytraeid reproduction in 'n' soils}}{\text{n}} = \text{Average enchytraeid reproduction in 'n' soils (Average E)}$$

Given a particular soil A,

To normalize for plant biomass, $\frac{\text{Plant biomass in soil A}}{\text{Average P}}$ x 100% = PA

To normalize for springtail reproduction, $\frac{\text{Springtail reproduction in soil A}}{\text{Average S}}$ x 100% = SA

To normalize for enchytraeid reproduction, $\frac{\text{Enchytraeid reproduction in soil A}}{\text{Average E}}$ x 100% = EA

Total score for soil A = PA + SA + EA.

5.4.4 Metal Toxicity Test.

To assess the influence of these soils on *O. nitens*, the subset of nine soils chosen from the calculation of habitat quality score were dosed at 50% WHC with ZnO (Sigma Aldrich, puriss p.a ACS reagent ≥ 99%) at increasing nominal concentrations of 0, 100, 200, 500, 1500, 4500, and 14000 mg of Zn per kg of soil. Fifteen (n=15) *O. nitens* were introduced to each of the four dosed soil replicates, baker's yeast was added as food and the mites were exposed for 28 days. The exposed mites were fed once a week with baker's yeast and the moisture loss was replaced at feeding time. After 28 days of exposure, the surviving adult and juveniles produced were extracted into a plastic cup with a modified Mcfayden apparatus for 48 hours. The surviving adults and juveniles in the plastic cups were counted to determine the survival and reproduction of the mites. The experiment was repeated with another subset of nine soils chosen from the remaining 38 soils, making a total of eighteen soils used for the both tests.

5.4.5 Chemical Analysis.

5.4.5.1 Total Zn Concentrations

The total Zn concentrations in the dosed and control soils were determined by X-Ray Fluorescence (XRF) (Margui et al., 2016). Four (4) g of dry soils were weighed and ground. The ground soils were homogenized with 0.8 g of 44 µm powdered Chemplex spectroblend acting as adhesive to hold the soils together. The homogenized samples were transferred into Chemplex pellet cups, covered with polypropylene thin-films and vacuum-sucked into a pellet die set. The pellet set was mounted on a hydraulic press and the samples were pressed with a force of about 10,000 psi for 5 minutes to form soil discs. The soil discs were analyzed on the Thermofisher ARL Optim-X X-ray analyzer for total

metal concentrations. Recoveries of Zn from a certified reference material (Montana 2710a) were 95-96.5%.

5.4.5.2 Calcium Chloride Extracted Zn Concentrations

The extracted Zn, otherwise called the bio-accessible Zn concentration, was determined by the calcium chloride extraction method (Quevauviller, 1998). Soil (2.5g) was weighed into a 50 ml centrifuge tube and 25 ml of 0.01M $CaCl_2$ added. The $CaCl_2$ and soil mixture was shaken for 3h at 15 rpm using the rotary shaker. A subsample of the solution was used for pH and the remaining sample centrifuged for 10 minutes at 5000*g*, filtered through a 0.45 µm filter, and refrigerated prior to analysis. The filtered samples were then analyzed using an Agilent microwave plasma atomic emission spectrometer (MP-AES) at the Department of Soil Science, University of Saskatchewan, Canada. Standard Zn solution (VWR atomic absorption standard) was diluted with 0.01M calcium chloride serially from 0, 1, 5, 15, 30 and 50 mg/L as standards. The quality control included blanks, duplicates and calibration standards every 21 samples.

5.4.5.3 Anions and Cations

The anions and cations in the soil were determined using the method described by Quevauviller (1998). However, the method was modified by using water instead of calcium chloride. The filtered extracted-samples were divided into two and one part was analyzed for anions and the other part was analyzed for base cation concentrations. The anions were analyzed by ion chromatography (IC) with a Dionex ICS-2000 using the Chromeleon 7 software at the Department of Soil Science, University of Saskatchewan, Canada. The base cations (Ca^{2+}, K^{+}, Mg^{2+}) were analyzed with an Agilent MP-AES at the Department of Soil Science, University of Saskatchewan, Canada. Standards for the cations were run randomly in the MP-AES and the calibration curve of the absorbance (Ca^{2+} = 616.21 nm,

K^+ = 769.89 nm, Mg^{2+} = 383.80 nm) at different concentrations was determined. The quality control included blanks, duplicates and calibration standards every 21 samples.

5.4.5.4 Dissolved Organic Carbon

Dissolved organic carbon (DOC) was determined by a method described by Chantigny et al. (2008). Soil (15 g) was mixed with 30 ml of 0.005M $CaCl_2$ in a 50 mL centrifuge tube. The soil and the $CaCl_2$ were mixed gently for a minute with a glass rod. After this, the soil-water mixture was centrifuged at 12000g for 10 minutes. The supernatants from the centrifuged samples were filtered with 0.4 µm polycarbonate through vacuum suction into 30 mL dram vials. The filtered samples were immediately analyzed for DOC using a Mandel Total organic carbon analyzer at the Department of Soil Science, University of Saskatchewan, Canada. The precision criteria was met because the percent coefficient of variation for replicate injections was less than 2%.

5.4.5.5 Speciation Calculations

The Windermere Humic Aqueous Model version 7 (WHAM 7) (Tipping et al., 2011) was used to determine how Zn speciation differs in the different habitat quality soils. The input parameters were dissolved organic carbon (DOC), cations (Ca^{2+}, Mg^{2+}, K^+, Zn^{2+}) and the anions (Cl^-, NO_3^-, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}). The reaction conditions were temperature = 298K, partial pressure of CO_2 = 0.00038 atm, and pH (Peng et al., 2018). Fulvic acid (FA) was estimated from the DOC by assuming 65% of the DOC is the active FA and that DOC is 50% of dissolved organic matter in soil (Tipping et al., 2003; Rooney et al., 2007). The output parameters were the free Zn ion species (Zn^{2+}) and other Zn species ($ZnSO_4$, Zn $(OH)^+$, $Zn(OH)_2$, $ZnCO_3$, $ZnCl_2$, $Zn(HCO_3)_2$, Zn-bound to FA).

5.4.5.6 Mite Tissue Zn Concentration

The mite tissue Zn concentrations (body burden) were determined after acid digestion (Owojori and Siciliano, 2012). Briefly, mite replicates per concentration were pooled together and frozen (-80°C),

and then digested with a mixture of 5 ml of ultrapure 69% nitric acid and 1.5 ml of suprapur hydrogen peroxide. The fully digested samples were heated at 70°C on a hotplate and were evaporated to about 1cm^3. The evaporated samples were then diluted with a 2% nitric acid and filtered using a 25 µm syringe filter before metal analysis. For every batch of digested mites, blanks were prepared in order to eliminate contamination effect during the digestion. The Zn concentrations per gram (g) body weight of mite were determined in the filtered samples using a multi-element inductively-coupled plasma mass spectrometer (ICP-MS) triple quad at the Toxicology Centre, University of Saskatchewan, Canada. The detection limit of Zn was estimated from the mean and standard deviation of the procedural blanks. For the quality control, the Zn content was determined in the certified reference material named TORT-3 (lobster hepatopancreas) obtained from the Natural Research Council, Canada using the same digestion procedures. The average recovery ranged from 86% to 100%.

5.4.5.7 Biochemical Analysis (Stress Biomarker Assays)

Mites were recovered from 0, 1500 and 14,000 mg of Zn/kg of soil and their protein concentrations were determined using Coomassie reagent (Bradford, 1976) at an absorbance of 590 nm using Bovine Serum Albumin (BSA) as standard. All assays were carried out on 5µL of the sample supernatant. The supernatant was prepared by homogenizing 30 adult mites/test concentration/habitat quality soils in 100µL of Phosphate-buffered Saline (PBS) buffer (0.01M, pH=7.4). The homogenate was centrifuged at 10,000 g for 15 mins under 4℃ to obtain a clear supernatant. Lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PDH) were measured as described by De Coen et al. (2001) but adapted for a 96-well microplate using commercial kits (G6PDH assay kit, Cat. No. MAK015; LDH assay kit, Cat. No. MAK066) from Sigma-Aldrich, Canada. The activity of the enzymes was measured at an absorbance of 450 nm in a spectrophotometer and the relative activity

(mU/mL) was corrected by the protein concentration for each sample to obtain the specific activity (mU/mg of protein) of the enzymes.

5.4.6 Statistical Analysis

To check the differences in mite reproduction at 95% significant level across the different habitat qualities, a one-way analysis of variance (ANOVA) was used. Relationships between habitat quality and soil properties were assessed using an ordered logistic regression and significance was tested at $p < 0.05$ level. For the experimental replication, a mixed effect model was used to assess the fixed effects of nominal Zn concentration and habitat quality on mite reproduction and random effects of soils nested within the experiment. Because the experiments were performed at different times, a ratio of variance test was also used to see if there were time effects on experiments. For the toxic concentration of Zn, three or four parameter (depending on which best fit the model) non-linear log-logistics was used to determine the effective median concentration (EC50) using the drm package in R (Ritz, 2016). The Trimmed Spearman-Karber (TSK) method (Hamilton et al., 1997) was used to determine the median lethal concentration (LC50) of Zn. The average slope of the three dose response curves per each of the three habitat qualities was determined to show the rate of change of the mite reproduction inhibition with Zn concentration. For all the regression analyses, the total, $CaCl_2$ extracted, and free Zn concentrations were expressed as moles of Zn per kg of soil. The body burden was expressed as moles of Zn per g body weight of mite. The bioavailability of Zn was calculated as the slope of the log-linear regression of Zn body burden after 28 days relative to the total Zn concentration in the soil. From the regression line, the EC50 for Zn tissue concentration was determined at the point where Zn body burden equals to the total Zn EC50. Generalized linear model with ANOVA was used to check if there were significant interactions at 95% level, between habitat quality and Zn doses on Zn speciation with soils as random effect. For the stress biomarkers, a two-

way ANOVA with dose and habitat qualities as independent variable, was used to check if there were significant differences ($p < 0.05$) between LDH and G6PDH activities in different habitat qualities control and dosed soils.

5.5 Results

5.5.1 Habitat Quality Influences Mite Fitness.

Mite reproduction was highest (175±11 juvenile mites) in soils with the greatest habitat quality (HQ) as defined by plant biomass, and enchytraeid and springtail reproduction. Mite reproduction significantly differed ($p < 0.05$) between high HQ (175±11) and low HQ (95±9) but there was no difference between high HQ and medium HQ (129±12) (Figure 5-1b). Across the 47 soils, soils (n=9) of high HQ values were linked with high CEC and % OC. If the HQs were based on enchytraeid and springtail reproduction only, more than 21 of the soils were classified as medium quality (Habitat quality 2) but if HQ was based on plant biomass only, then 18 soils were high quality (Habitat quality 1) classification (Figure 5-1). When all the variables were factored together, both CEC and % OC were the significant ($p < 0.05$) HQ determinants but CEC was a stronger determinant (coefficient = 0.66) of HQ compared to % OC (coefficient = 0.28) (Appendix C, Table C-2). Although, pH was not a significant ($p > 0.05$) HQ determinant when factored with other variables, it had the highest coefficient (0.78) when keeping other variables constant (Appendix C, Table C-2).

(a) (b)

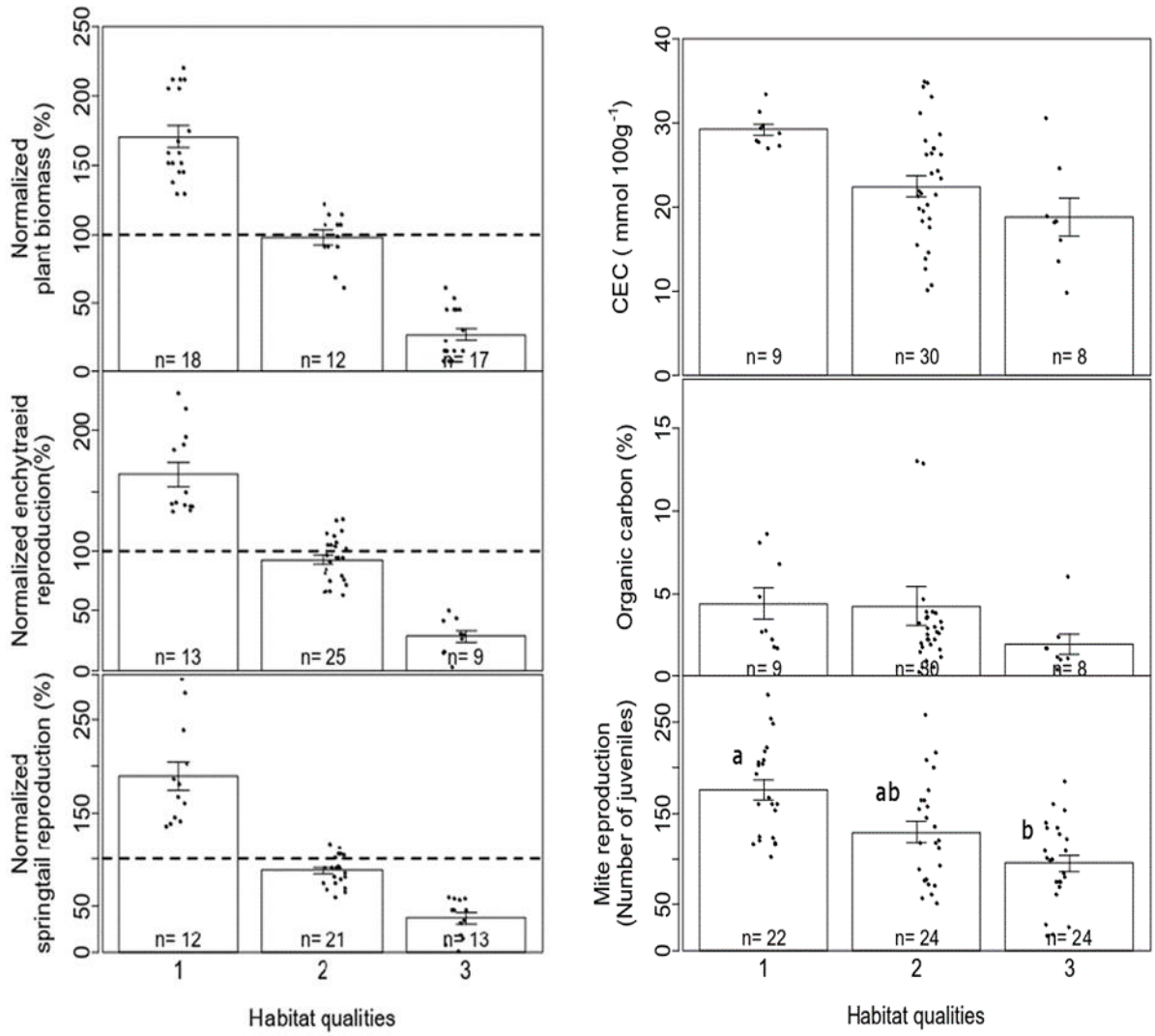


Figure 5- 1. Jitter plot of normalized plant biomass, enchytraeid reproduction and collembola (springtail) reproduction according to their habitat qualities (Panel a). The dotted lines indicate the average normalized score which is 100%. Jitter plots of habitat quality (HQ) determinants, namely cation exchange capacity (CEC: mmol/100g soil) and organic carbon (%OC) in 47 soils, and mite reproduction in each HQ (Panel b). Habitat quality (HQ) 1 is the high HQ, habitat quality two is the medium HQ and habitat quality three is the low HQ. Letters "a" and "b" represents significant differences ($p < 0.01$) and "ab" represent no significant differences ($p > 0.05$).

5.5.2 Low Habitat Quality Potentiates Zn Effects on Mite Reproduction

Increasing Zn doses from 500 to 14,000 mg/kg of soil reduced ($p < 0.05$) mite reproduction by 50 % (Appendix C, Table C-3). The EC20s expressed as nominal Zn in the high HQ was 3273 ± 1751, medium HQ was 2594 ± 1066 and the low HQ was 2038 ± 1194 (Appendix C, Table C-4). The highest nominal dose of 14,000 mg/kg completely inhibited mite reproduction in three of the six low HQ soils but mite reproduction was not completely inhibited in any of the six medium or six high HQ soils. For one of the low HQ soils, 4500 mg/kg completely inhibited mite reproduction; this soil had the lowest CEC and OC (9.9 mmol/100g and 0.4%, respectively). A linear mixed effect model of these data indicated that the difference between the experiments was negligible with a standard deviation of 0.0041 (Appendix C, Table C-3). The variance ratio test indicated no difference in variances between the two experiments ($p = 0.93$) and the ratio of the variances was 0.98 (Appendix C, Table C-5).

5.5.3 Habitat Quality Influences Zn Speciation

As expected, soils with high CEC and % OC had less free Zn because more soil Zn was bound to fulvic acid (FA-Zn) suggesting that high HQ soils would have lower Zn bioavailability. At the nominal Zn dose of 4,500 mg/kg (the closest concentration to the nominal Zn EC20 in the high HQ), the free Zn ion concentration in the high HQ soil (43 ± 2.8%) was less ($p < 0.05$) than in the low HQ (76.9 ± 2.4%). Conversely, the FA-Zn (40.9 ± 3.6%) in the high HQ soil was higher ($p < 0.05$) than in the low HQ soil (15.5 ± 1.8%). At nominal Zn doses of 14,000 mg/kg, free Zn ion and FA-Zn followed the pattern observed at 4,500 mg/kg for the HQs but there were no differences ($p > 0.05$) (Figure 5-2). Other Zn species were similar across all the HQs, except for Zn sulphate at 14,000 mg/kg, which was 1.3 ± 0.3% in the high HQ and more ($p < 0.05$) than in the low HQ (0.6 ± 0.7%).

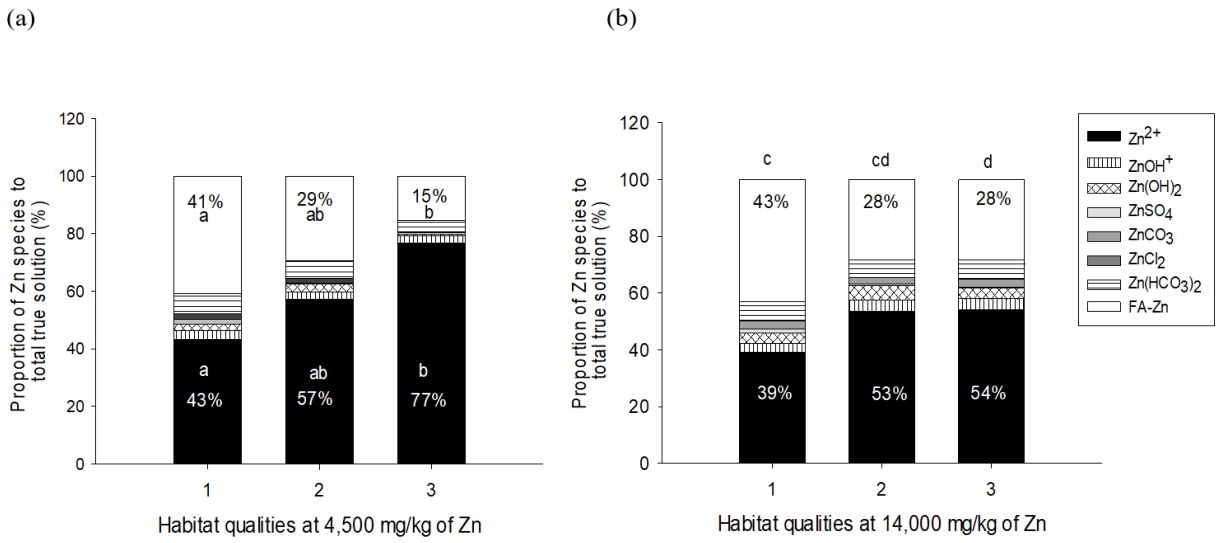


Figure 5- 2. The proportion of Zn species in different habitat qualities at (Panel a) 4,500 and (Panel b) 14,000 mg/kg nominal Zn concentrations, separately for HQ = 1, 2 or 3. The 4500 mg/kg of Zn is the low HQ EC50 and 14,000 mg/kg is the high HQ EC50. The proportion of Zn species in each HQ were calculated as averages in the six soils that made up each HQ. The letters "a" and "b" represents significant differences ($p < 0.05$) and "ab" represents no significant differences ($p > 0.05$). The letters "c" and "d" represents significant differences ($p < 0.05$) in $ZnSO_4$ and "cd" represents no significant differences ($p > 0.05$) in $ZnSO_4$

5.5.4 Total Zn Predicted Body Burden and Toxicity Better than $CaCl_2$ Extracted Zn or Free Zn

Based on log concentrations, all the measured external Zn concentrations in the soils positively correlated with the internal Zn concentrations (body burden) in the mites. Total soil Zn concentration was most strongly correlated (r=0.76) with body burden of Zn in the mite whereas the free Zn concentration was most weakly correlated (r=0.60) to body Zn burdens and the calcium chloride extracted Zn was moderately correlated (r=0.71) (Appendix C, Figure C-1 & C-2). Therefore, total Zn was selected as best representing measured external Zn concentrations exposed to the mites.

5.5.5 Toxicity of Zn Across the Three Habitat Qualities

Using the EC50s expressed as total Zn concentrations measured, the toxicity of Zn in the high HQ is about one quarter or one half that in the medium and low HQs, respectively. The Zn EC50 was

on average 16,000 ± 970 mg/kg for the high HQ compared to 7800 ± 2500 mg/kg for medium HQ and 4450 ± 2300 mg/kg for the low HQ (Figure 5-3). Mite reproduction was completely inhibited in three low HQ soils (Black spruce, Carrot river and PRT). Specifically, total Zn concentration of 3,215 mg/kg completely inhibited mite reproduction in Black spruce (CEC = 9.9 mmol/100g, % OC = 0.4, EC50 = 139 mg/kg). Total Zn concentration of 14,000 mg/kg completely inhibited mite reproduction in Carrot river (CEC = 13.6 mmol/100g, % OC = 1.7, EC50 = 982 mg/kg) and PRT (CEC = 16.1 mmol/100g, and % OC = 1.7, EC50 = 1,100 mg/kg). Lethal concentration of Zn causing 50% mortality (LC50) was determined in two low HQ soils (Black spruce LC50 = 1805 (1,380 - 2,361) mg/kg and Sarah LC50 = 11,076 (7,123 -17,223) mg/kg). The LC50 could not be determined in any of the high or medium HQ soils.

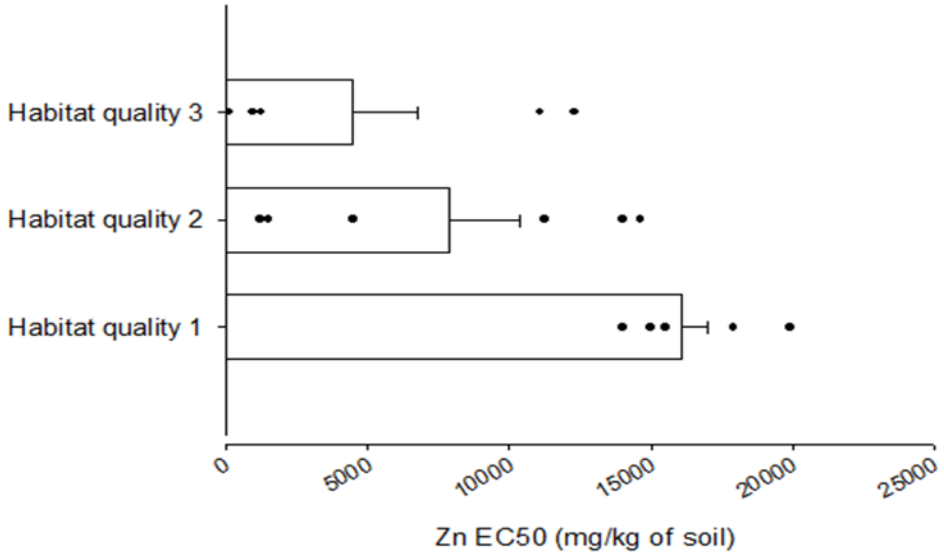


Figure 5- 3. Average EC50 for total soil Zn concentration for three habitat qualities (Habitat quality 1 - high HQ, Habitat quality 2 - medium HQ, Habitat quality 3 - low HQ).

5.5.6 Zn Bioavailability Did Not Depend on Habitat Quality or Zn Speciation

We calculated bioavailability as the slope of internal body burden versus total measured Zn in soil. Bioavailability did not differ ($p > 0.05$) between different quality soils averaging about 20% and mites

living in high HQ soils could tolerate higher internal Zn concentration (measured at external EC50 level) compared to mites living in low HQ soils (Figure 5-4). The internal Zn concentration in the mites living in the high HQ soils (2040 ± 130 µg/g b.w) was not significantly greater ($p < 0.05$) than the internal Zn concentrations in the low HQ soils (1180 ± 310 µg/g b.w). Using the dose response slope as a metric, the change in mite reproduction relative to Zn was less ($p < 0.05$) in the high HQ (0.63 inhibition of mite reproduction per µg/g of Zn) compared to 5.05 inhibition of mite reproduction per µg/g of Zn for the low HQ. The slope from the medium HQ soils (1.35 inhibition of mite reproduction per g/g of Zn) was not different from that from the high HQ soils.

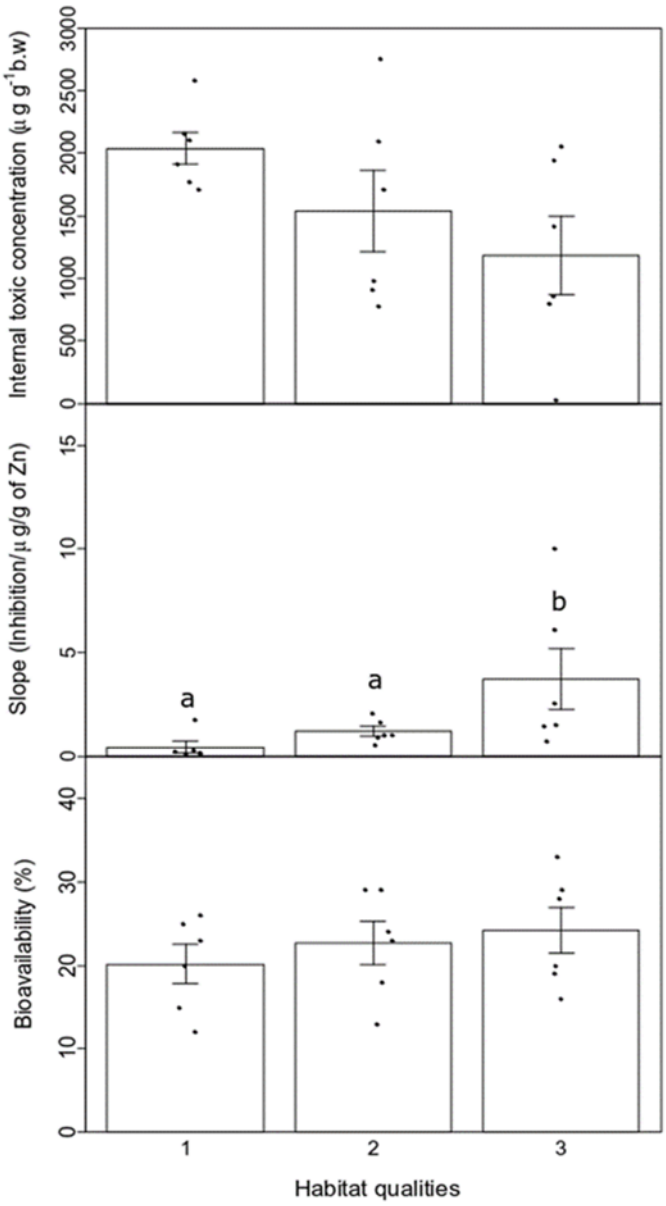


Figure 5- 4. The distribution of the internal EC50 concentration of Zn (µg g^{-1} b.w) in mites, slope of the dose response curves and bioavailability of Zn in three different soil habitat qualities. The slope was measured as mite reproduction inhibition per concentration of exposed cadmium (Inhibition/ µg/g Zn). Bioavailability of Zn was calculated as the slope of the regression of the Zn body burden with total Zn concentration. Habitat quality 1 is the high HQ, habitat quality 2 is the medium HQ and habitat quality 3 is the low HQ. Body weight = b.w. Letters "a" and "b" represents significant differences ($p < 0.05$). No significant differences ($p > 0.05$) where there are no letters.

5.5.7 Habitat Quality Influences Cellular Responses

When stressed with Zn, mites living in soils of low and medium HQ increased their use of anaerobic reserves. The LDH enzyme activity increased for mites living in soils of both medium and low HQ when exposed to the high concentration of Zn (14,000 mg/kg) (Figure 5-5a). The activity of G6PDH increased in mites living in the soils of medium and low HQ at 1,500 mg/kg of Zn exposure but was regulated back to control levels at 14,000 mg/kg of Zn in the medium HQ. However, in the lower HQ soils, G6PDH activity increased at all tested Zn concentrations in a dose dependent pattern (Figure 5-5b). There was no change in LDH and G6PDH activities in the mites living in high HQ soils at the moderate to high Zn concentrations tested (Figure 5-5a, 5-5b).

(a) (b)

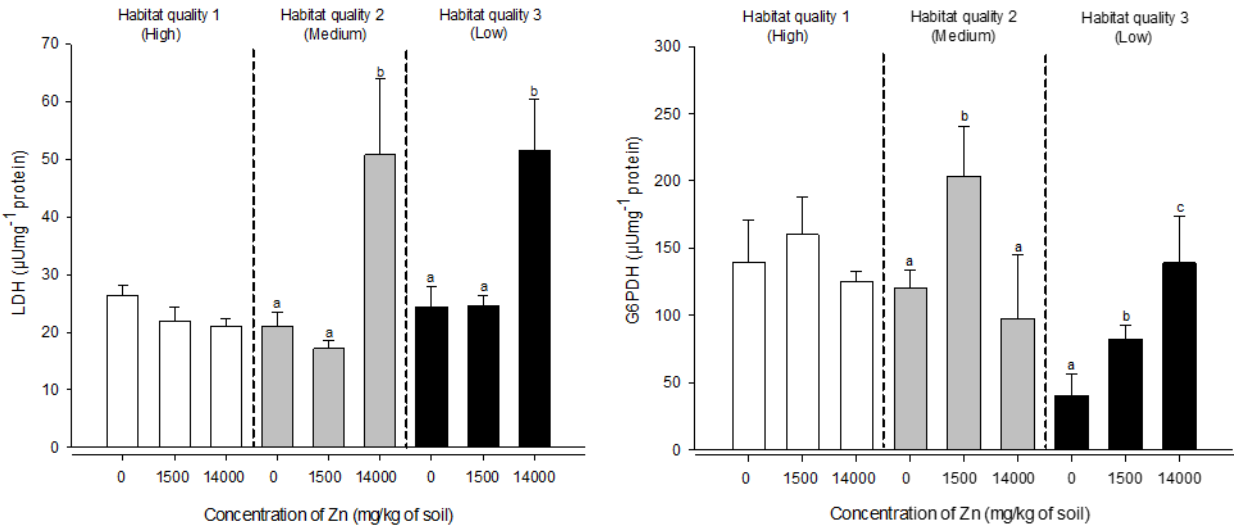


Figure 5- 5. Activities of LDH (a) and Glucose 6-phosphate dehydrogenase (G6PDH) (b) in mites from three HQ soils exposed to nominal 1500 mg/kg and 14,000 mg/kg of Zn in three HQ soils. Habitat qualities are 1, 2, 3 high, medium and low HQ soil mites respectively. The letters "a" and "b" represents significant differences ($p < 0.05$). Bars within an HQ with the same letter represents no significant difference ($p > 0.05$). No letters in Habitat quality 1 for LDH and G6PDH because there were no significant differences ($p > 0.05$).

5.6 Discussion

5.6.1 Habitat Quality Influences Toxicodynamics

Independent of toxicokinetic effects, greater HQ increases the resilience of *Oppia nitens* by altering the toxicodynamics of Zn (Figure 5-4). Increased resilience to Zn was indicated by less energy stress in higher quality soils that enables organisms to tolerate higher internal Zn concentrations before adverse reproductive or survival effects occurred. As expected, soil quality altered metal speciation, but this change in metal speciation did not alter Zn bioavailability, with a slope of approximately 0.2 mol of Zn kg^{-1} mite / mol of Zn kg^{-1} soil.

5.6.2 Soil Properties Determines Habitat Quality

As expected, CEC and OC were soil properties that determined habitat quality of soils. However, other properties commonly found to determine habitat quality, i.e. pH and soil texture (Kuperman et al., 2009) were not significant predictors in our study. This may be because our soil library was focussed on the Prairie region with less variability in pH whereas other studies using forest and agricultural soils found that *Folsomia candida* reproduction varied based on organic matter, pH and soil texture (Rombke et al., 2006). *Oppia nitens* performed better (increased reproduction) in soils that were amended with increased organic matter (Princz et al., 2010). This is consistent with the present study where *Oppia nitens* reproduced more in the high HQ soils than other soils because of the high CEC and OC associated with the high-quality soils. Apart from determining the performance of soil organisms, CEC and OC influence toxicokinetics of metals by modifying metal concentration in soils (Lock and Janssen, 2001c; Bur et al., 2012; He et al., 2015; Li et al., 2016).

5.6.3 Metal Bioavailability

In order for metals to cause toxicity, they have to be bioavailable and bioavailability is thought to be dependent on metal speciation (McLean and Bledsoe, 1992; Sauve, 2002; Cances et al., 2003). Free metal ions are readily available for uptake by organisms (Qui et al., 2014) and sometimes Zn toxicity is linked to Zn free ion concentration (Hooper et al., 2011; Tourinho et al., 2013). In contrast, a number of studies reported total metal concentrations to be a good predictor of metal toxicity to soil invertebrates (Dai et al., 2004; Veltman et al., 2007; Gonzalez et al., 2013). For example, total metal (Zn, Cd, and Pb) content best predicted metal toxicity to the earthworm *Eisenia fetida* probably due to soil ingestion by the earthworm (Gonzalez et al., 2013). In our study, speciation followed expectations with free Zn ion concentration lower in high quality soils than in the medium or poor-quality soils due to changes in CEC and OC. Despite this, Zn toxicity to *Oppia nitens* was best explained by the total Zn concentration and not $CaCl_2$ extracted Zn concentration or the free Zn ion concentration. We base this assertion on better dose-response models when total Zn was used (data not-shown), a stronger relationship to body burden across dose responses (Appendix C, Figure C-1) and at EC50's (Supplemental Data Figure S2). Total Zn may be a better predictor for *Oppia nitens* toxicity because Zn exposure may be primarily occurring via the organic matter upon which *Oppia nitens* feeds or it may be linked to Zn's role as an essential micronutrient.

Despite changes in metal speciation across habitat qualities, Zn bioavailability was similar at about 0.2 mol of Zn kg^{-1} mite / mol of Zn kg^{-1} soil across all habitat qualities. The similar concentrations of Zn may be due to the essentiality of Zn and homeostatic control of Zn by organisms. However, the internal concentration of Zn that caused impairment of reproduction and survival was higher in the mites of the high HQ soils than other HQ soils. Further, the slope of the dose response curve at EC50 was smaller in high HQ compared to lower HQ levels. Thus, it appears that despite similar Zn

accumulation rates, i.e. toxicokinetics, Zn's potency was lower in higher HQ soils. The highest internal toxic concentration of Zn causing 50% reproduction was 2,040 µg/g in the high HQ soil, similar to about 2000 µg/g Zn reported by Owojori and Siciliano (2012). We suggest that habitat quality was modifying Zn toxicodynamics in mites by altering the dynamic energy budget of mites. This allowed the mites to tolerate higher metal contents before becoming physiologically impaired.

5.6.4 Stress Biomarkers

Two markers of mite energy systems, LDH and G6PDH (Firat et al., 2009; Yallappa and Nuzhat, 2018) demonstrated that mites in the high HQ soils were more resilient. There were no changes in the LDH and G6PDH levels in the mites in high HQ soils when challenged with Zn. In contrast, in low HQ soils, both LDH and G6PDH increased in response to Zn challenge, indicating stress. High HQ is associated with soils that have more food resources and thus provide more energy compared to low HQ sites (Hope, 2001). On exposure to metals, the metabolic activity of organisms increases, due to increased energy demand to combat stress (Gomes et al., 2015b). The increase in LDH and G6PDH during Zn challenge suggests that mites used energy reserves to combat Zn stress. In the case of the lower HQ soils, the mites depended on an additional increase in G6PDH to fight off stress. Alternatively, G6PDH increases may be linked to oxidative stress (Azevedo et al., 2007; Firat et al., 2009) and not to increased demand for energy. Metals are classically known to cause or promote oxidative stress as a mechanism of toxicity (Templeton, 2015) and habitat quality is one of the factors that contribute to oxidative stress levels (Adams et al., 2009; Theodokratis et al., 2017). Dynamic energy budgets models are needed for *Oppia nitens* to disentangle if enzymes are increasing energy available for organism to repair damaged proteins or if the enzymatic systems are directly combating primary or secondary toxicants, e.g. reactive oxygen species.

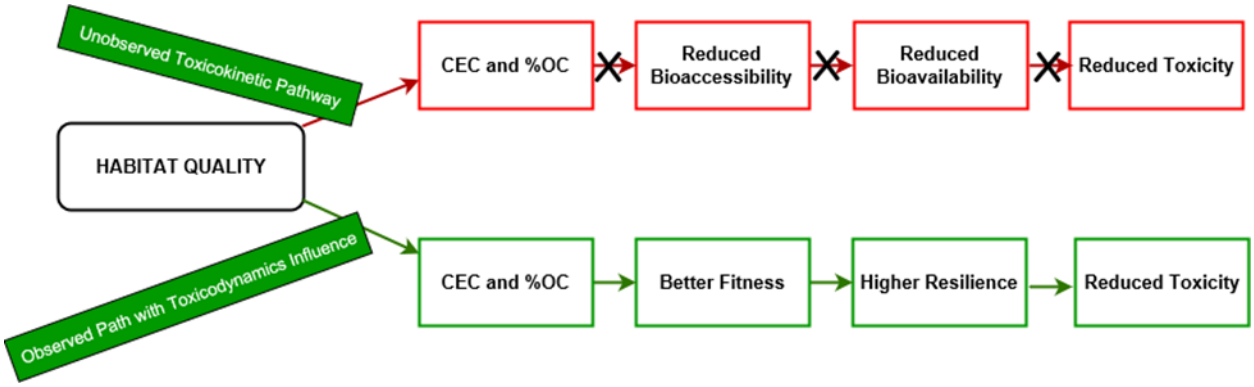


Figure 5- 6. The alternative path to management of metal contaminated sites and protection of terrestrial organisms.

Our work suggests an alternative path for managing metals, or at least Zn contaminated soils (Figure 6). Rather than focus on only reducing or immobilizing Zn, restoration of habitat quality together with reducing or immobilizing Zn may be a sustainable means of reducing Zn impacts on terrestrial organisms. Furthermore, remediation processes such as excavation can significantly degrade soils and reduce soil quality (Lanno, 2003), and based on our work, may exacerbate effects on biota from metal pollutants. Practices that enhance the natural habitat quality of soils, such as cover crops to increase organic matter levels, are therefore encouraged. In some jurisdictions, sites are classified by land use and based on this, metal remedial guidelines are set. Land use is used primarily due to its influence on organism exposure to pollutants. We suggest that land-use should be extended to landscape by combining land-use and soil quality, as sites with higher soil quality will likely be able to tolerate higher Zn concentrations with no ill effect.

5.6.5 Implications of Study on Contaminated Site Remediation

Habitat quality (HQ) modulates Zn toxicity through toxicodynamics rather than toxicokinetics. The bioavailability of Zn was similar across the different HQs despite differences in Zn speciation observed across the different HQs. Total Zn predicted toxicity to *Oppia nitens* better than free Zn and

higher Zn body burdens were found in mites inhabiting high HQ soils than low HQ soils. Energy-based biomarkers (LDH and G6PDH) demonstrated that mites inhabiting high HQ soils were more resilient to metal contamination. The result of this study implies that HQ plays a huge role in protecting organisms directly from metal toxicity rather than through reduction in metal bioavailability as commonly known. Organisms living in high HQs have more energy to combat metal stress than organisms inhabiting low HQs. In order to manage metal contaminated sites efficiently, restoring HQ may be more important than reducing the metal levels. For example, in a case of a zinc contaminated site, our study is suggesting that restoring the HQ may be a better sustainable way of reducing zinc impacts on soil biota than focus on reducing the zinc concentrations.

6 Synthesis, Conclusions and Future Directions

Due to the inevitable use of metals and the consequent increase in metal production to meet human demands, the severity of metal pollution in the environment cannot be over-emphasized. It is against this background that metal risk assessment of terrestrial or aquatic ecosystems is very important. But more important is the robustness of the metal risk assessment, which is borne out of how close to reality the data used is. With the current metal toxicity data used in risk assessment, the reality was not often represented in previous works; rather "conservativeness" has been the prevailing approach. It is necessary however to use realistic data rather than just conservative data to account for over-protection that "conservativeness" presents.

To access metal risk, current risk assessment accounts for only single metals instead of metal mixture, which is more realistic. Furthermore, typically, it is assumed that metal mixture toxicity is just a simple addition of single metals present on site. It is well known that metals cannot be degraded nor destroyed (Mansour, 2014); therefore, their effects linger on for a longer period than is currently accounted for in metal risk assessment. The inherent effect of habitat quality on resilience of organisms to metal stress, in this case soil habitat quality, has not been deliberately investigated. This might have helped to unravel some of the unexplained response of organisms to metal contamination across different sites. Therefore, the primary goal of this research was to initiate and generate realistic site-specific metal toxicity data that can be used for metal risk assessment. The collected data were based on the facts that: (1) contaminated sites are usually polluted with metal mixtures as addressed in Manuscript 1 (2) metals persist in soils, so also its effect on biota as dealt with in Manuscript 2 and that (3) responses of ecological receptors to metal contaminated sites are dependent on the habitat quality regardless of metal bioavailability as addressed in Manuscript 3.

The principal goals of this research were to: (i) generate metal mixture toxicity data that is representative of the environmental occurrence of metal mixtures, taking into account the average mixture ratios of metals in agricultural sites and metal mining/smelting sites that are common to Canada; (ii) assess the effect of metal persistence on soil ecosystem through multigenerational exposure of metals to soil organisms and determine the sensitivity of organisms' populations to metal persistence; and (iii) assess the direct role the site quality plays on organisms' fitness in response to metal contamination.

Fundamental research questions addressing these objectives were:

- How can we move away from single metal toxicity to generate metal mixture toxicity data that is representative of the Canadian agricultural landscape and mining/smelting sites, hence increase confidence in site-specific risk assessment of metals in Canada?
- How does persistence of metals in the soil affect biota exposed to the metals continually, and how will this impact the current way of doing metal risk assessment?
- What is the advantage of soil habitat quality in mitigating metal effects on populations of organisms inhabiting the soil? Can this help to re-align metal risk assessment and contaminated site remediation processes?

6.1 Single and Metal Mixture Toxicity (Manuscript 1)

6.1.1 Synthesis and Conclusions

The traditional way of doing metal risk assessment is using single metal toxicity data to represent effects of metals and when considering metal mixtures, assume that metals do not interact and metal mixture toxicity is the sum of their individual toxic units. However, Manuscript 1 concluded that the pattern of the toxic response of soil organisms such as mites, *Oppia nitens* to single metals in different soils, was different from its response to metal mixtures. Mite sensitivity to metals in soils switched depending on if the exposure was to a single metal or a mixture of metals. The soil, which was the least sensitive to single metals, became more sensitive to metal mixtures. Soil properties like CEC and clay were significant modifiers of this switching. Metals that formed complexes with organic matter were the bioavailable metal species responsible for metal mixture toxicity. Few metals were responsible for driving most toxicity to the mites. Metal interactions were more common than non-interactions of metals in a mixture, and more mixtures were synergistic with a marginally higher rate of 4% than antagonism.

6.1.2 Future Directions

The results generated here showed that CEC was the master soil variable influencing different responses with single and metal mixtures. Moreover, CEC influences organism fitness and susceptibility to metals (Jegede et al., 2019b). Therefore, there could be a link between organism fitness as dictated by CEC and single or metal mixture toxicity. CEC is a surrogate measure of soil fertility because it is the ability of soils to hold on to nutrients in the form of cations. These nutrients can then be made available to soil biota. It is possible that increased fitness of mites in Jegede et al. (2019b) as a function of CEC was due to increased provision of nutrients. In the event of metal mixture contamination of soils, increased CEC could lead to increased ability to hold on to myriads

of metals, consequently increasing the ability to supply such toxic metals to organisms in the soils. Qui et al. (2015) suggested that interactions of metals increased in the high CEC soils because of the more binding sites, hence leading to synergistic effects to *Hordeum vulgare;* this might be the case with *Oppia nitens* in this study. To estimate the influence of CEC on metal mixtures toxicodynamically, the mite body burden of the metals should be assessed. Therefore, it is recommended that future studies on metal mixture toxicity to *Oppia nitens* should include metal body burden in relation to the most accurate soil metal metric (total metal or other metal species) as done by Jegede et al. 2019b.

The metals bound to fulvic acid predicted metal toxicity to *O. nitens* better than total and free metal speciation. This implies that using resins may be more predictive than metal speciation. The assertion that resins may be better aligns with Smolders and McLaughlin's (1996) claim that cadmium loaded on a chelating resin was more phytoavailable than free cadmium species to a plant, *Beta vulgaris*. Resins in the form of DGT (Diffuse gradients in thin films) 98% correlated with cadmium accumulation in earthworm, *Eisenia fetida* (Gu et al., 2017). Moreover, using resins avoids the uncertainties associated with calculating metal speciation (Versieren et al., 2013). Resins can better mimic metals bound to organic ligands, and it will be interesting to see if metal flux on resins can correlate with mite body burden in future metal mixture toxicity studies. Zinc was the least toxic of the metals. The role of zinc in modulating metal toxicity in the presence of other metals needs further investigation. Studies have reported the protective role that zinc plays in metal mixture toxicity (Wu and Zhang, 2002; Cherif et al., 2011; Versieren et al., 2016). One suggestion for this observation was that in an exposed organism, zinc reduces lipid peroxidation caused by other metals by activating protective enzymes such as superoxide dismutase (Versieren et al., 2016). Another suggestion was that, in the presence of other metals, zinc tends to be more soluble, and consequently outcompete

other metals at uptake sites in the organism. For example, Posthuma et al. (1997) observed that zinc did not sorb to soil particles in the presence of copper; instead, uptake of soluble zinc by *E. crypticus* was stimulated. Future studies should be designed to explore these proposed mechanisms as a way to explain how zinc might be protecting mites from metal mixture toxicity.

6.2 Long-term Metal Exposure (Manuscript 2)

6.2.1 Synthesis and Conclusions

Existing toxicity data used for metal risk assessment are based on short-term tests that only cover one generation and may not be protective of multigenerational exposures of organisms to metals. In this regard, Manuscript 2 concluded that prior exposure or continuous exposure of metals had consequent toxic effects that were only seen in a multigenerational test scenario and not in the common single generational tests. Higher exposure concentrations triggered a tolerant response of the mites in subsequent generations. Continuous exposure to lower concentrations of metal (Zinc) was fatal to the mites than high concentrations of the metal. Both long-term persistent effect modelled by pulse exposure scenario and long-term exposure effect modelled by the continuous exposure scenario had detrimental effects on the population of the mites, using a zinc niche width.

6.2.2 Future Directions

The results generated here indicated that offspring developed tolerance to high concentrations of metals and was susceptible at lower concentrations. Metal tolerance in pre-exposed soil invertebrates has been demonstrated in some studies e.g. *E. fetida* developed tolerance to Zn after two generations (Spurgeon and Hopkin, 2000), the springtail, *Orchesella cincta* collected from metal-contaminated sites were more tolerant to cadmium than the ones collected from reference sites (Posthuma et al., 1992). However, only one study (Amorim et al., 2017) has shown that offspring developed tolerance to high concentrations of metals and was susceptible at low concentrations just as was observed in

this present study. Amorim et al. (2017) observed that *F. candida* exposed to Cd at EC10 level became extinct after one year but the ones exposed to Cd at EC50 levels did not go extinct till it was stopped at the 40th generation. Different mechanisms were suggested for this result. For example, there could have been reduced uptake of metals at higher concentrations by the springtails; a phenomenon that was observed in *Daphnia magna* where mercury uptake was reduced at lethal concentrations (Tsui and Wang, 2006). The induction of protective enzymes like metallothionein and other enzymes are concentration-dependent; hence, increased induction is observed when organisms have been pre-exposed to high metal concentrations such as organisms collected from metal-contaminated sites (Van Straalen and Roelofs, 2005). It is also possible that organisms exposed to high concentrations of metals developed higher excretion efficiency than organisms exposed to low metal concentrations; therefore, metal transfer to subsequent generations may be lower for organisms exposed to higher metal concentrations.

In recent times, knowledge about epigenetic memory is increasing. This is the case where a previous stimulus induces a heritable change in gene expression (D'Urso and Brickner, 2014). For example, when *C. elegans* was exposed to 25^{o}C, a daf-21(Hsp90) promoter:: fluorescent protein constructs were highly expressed. These protein constructs were still highly expressed in a single copy transgene in subsequent five generations, but the descendants after the fifth generation did not express these transgenes (Klosin et al., 2017). Therefore, epigenetics might be able to explain why tolerance was induced in mites exposed to the high concentration of metal but not induced in mites exposed to low metal concentration.

To unravel the mechanism behind this phenomenon going forward, multigenerational studies should be designed such that response of protective enzymes can be assessed, body burdens of parents and subsequent generations can be assessed in order to investigate excretion efficiencies and

possible maternal transfers. Although genome sequencing of *O. nitens* has not been done until date, it will be interesting to investigate if epigenetic mechanisms can be assessed in multigenerational studies with *O. nitens* after the full genome of *O. nitens* has been characterized.

6.3 Soil Habitat Quality's Influence on Metal Toxicity (Manuscript 3)

6.3.1 Synthesis and Conclusions

Traditionally, it is known that habitat quality influences the toxicity of metals in contaminated sites by modifying metal bioavailability. In this vein, a high habitat quality soil should protect organisms by reducing metal bioavailability. However, this study showed that habitat quality may not necessarily change the metal bioavailability in contaminated sites, but more importantly, it influences the organism's response directly. The main findings from Manuscript 3 concluded that soil mites, *Oppia nitens* inhabiting soils of high habitat quality were more resilient to metal stress than mites inhabiting soils of lower habitat qualities were. Soil CEC and OC were two soil properties determining soil habitat quality. Alteration of dynamic energy budgeting, when faced with stress, is a function of the habitat quality as demonstrated by LDH and G6DPH activities. High habitat quality does not only protect by reducing metal exposure but it dominantly protects by providing more energy for its inhabitant to cope with the metal exposure.

6.3.2 Future Directions

High habitat quality of soils is a function of high CEC (Jegede et al., 2019b) and high CEC is also related to synergistic effect (increased toxicity) of metal mixtures in soils. However, the increased ability of soil organisms to withstand single metals relates to high habitat quality. It will be interesting to see if high habitat quality will enable organisms to withstand metal mixture better or if organisms in high habitat quality will be susceptible to metal mixtures as a function of its high CEC. Therefore, the effect of habitat quality on the response of organisms to metals should be assessed with metal

mixtures in future studies, which will help our understanding of the roles CEC plays in influencing metal mixture toxicity in soil organisms.

Another question to ask and answer is this: "How will habitat quality influence mite response to metals in a multigenerational metal exposure scenario?" Likely, high habitat quality will still protect organisms and their progenies in subsequent generations; but the reverse could also result. For example, the proposed mechanism of multigenerational effect of metal on *O. nitens* by Jegede et al. (2019a) shows that it is possible that increased induction in energy-based biomarkers like LDH and G6DPH may have a protective effect on subsequent generations in the low quality habitat versus the high quality habitat; and the opposite could be the case. Therefore, it will be interesting to address these concerns in future studies.

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8. Appendix A: Chapter 3 Supplementary Material

Table A-1. The effective concentrations ± SE of five metals inhibiting 10% (EC10), 25% (EC25) and 50% (EC50) mite reproduction expressed as mg/kg of soil in five soils (ASF, LOA, LO, ASA, and LOS). ASF = Acid Sandy Forest, LOA = Loamy Alluvial, LO = Loamy, ASA = Acid Sandy Arable, LOS = Loamy Sand.

Soil	Metal	EC50 (mg/kg)	EC25 (mg/kg)	EC10 (mg/kg)
ASF	Pb	1181 ± 627	1051 ± 311	712 ± 307
	Zn	646	401	248
	Cu	923 ± 491	537 ± 283	268 ± 195
	Ni	133 ± 120	26 ± 24	5 ± 10
	Co	1213 ± 667	567 ± 419	325 ± 307
LOA	Pb	3030 ± 1259	430 ± 107	171 ± 97
	Zn	8689 ± 1209	6797 ± 1384	5134 ± 1781
	Cu	Not toxic	Not toxic	Not toxic
	Ni	159 ± 77	107 ± 36	80 ± 28
	Co	Not toxic	Not toxic	Not toxic
LO	Pb	21625 ± 20813	2830 ± 2267	638 ± 747
	Zn	Not toxic	Not toxic	Not toxic
	Cu	26671	2336	144
	Ni	3606 ± 259	3049 ± 262	2577 ± 344
	Co	14921 ± 1682	12482 ± 2589	10180 ± 3385
ASA	Pb	9165 ± 3866	6193 ± 3731	4649 ± 3908
	Zn	Not toxic	Not toxic	Not toxic
	Cu	3796	1426 ± 2690	536 ± 900
	Ni	2439	1425 ± 908	961 ± 838
	Co	Not toxic	Not toxic	Not toxic
LOS	Pb	1404 ± 380	654 ± 214	271 ± 97
	Zn	8121 ± 247	8022 ± 334	7925 ± 451
	Cu	3466 ± 1130	449 ± 161	56 ± 21
	Ni	1022 ± 78	992 ± 102	670 ± 148
	Co	6373 ± 6034	2320 ± 3361	845 ± 1200

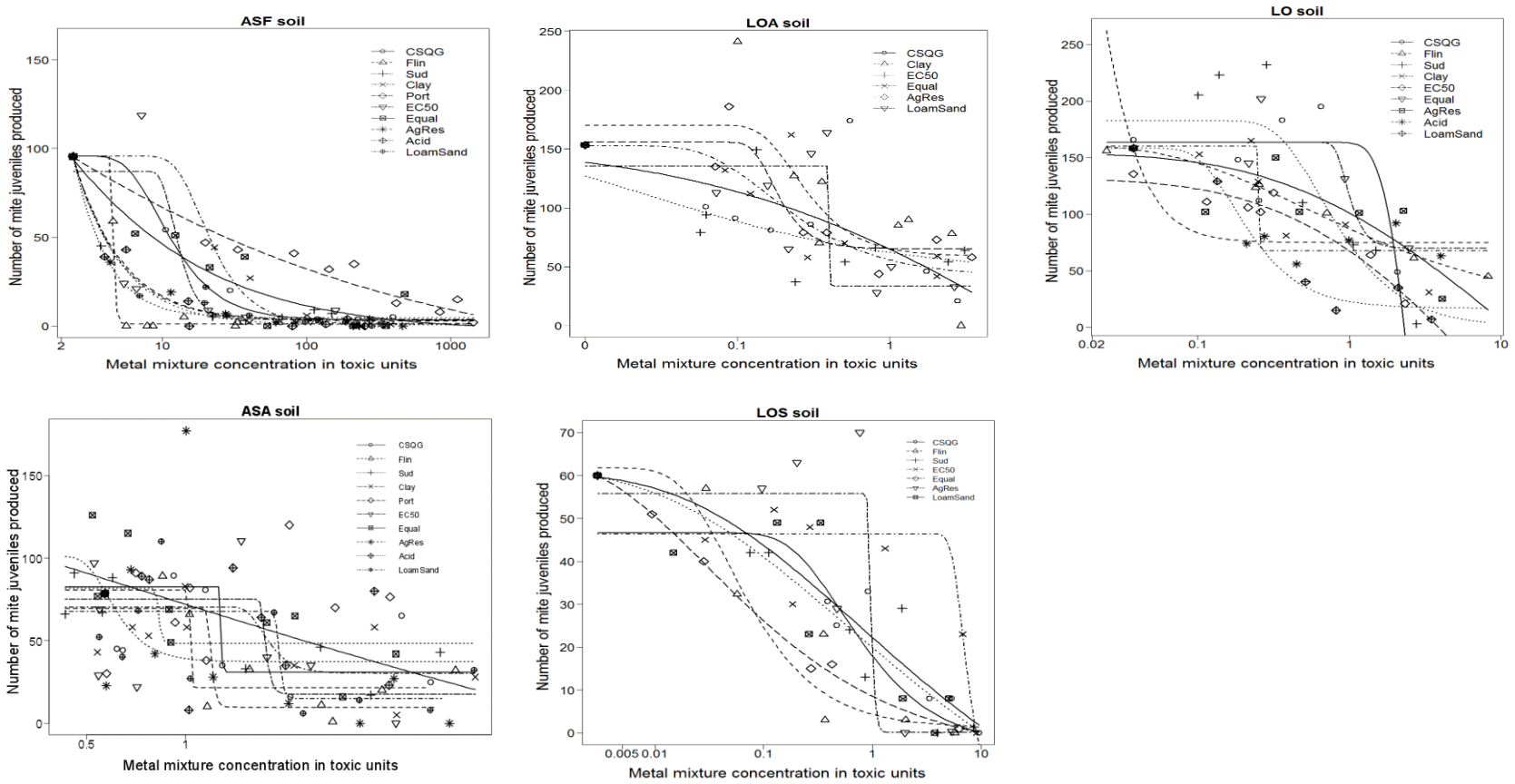


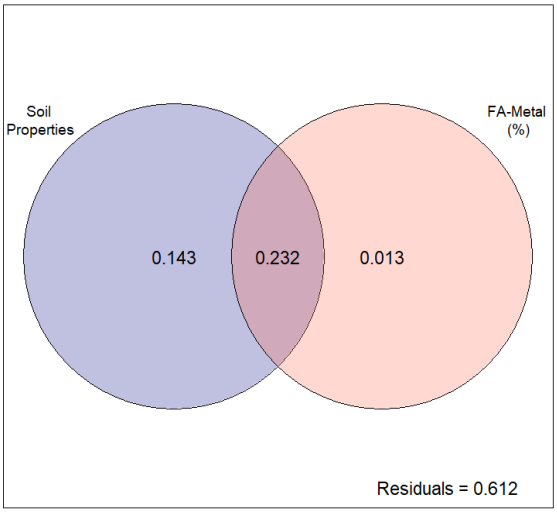
Figure A-1. Dose response curves of ten fixed ratio rays made of five-metal mixtures (Zn, Pb, Cu, Ni, Co) to Oppia nitens in five soils (ASF = Acid Sandy Forest, LOA = Loamy Alluvial, LO = Loamy, ASA = Acid Sandy Arable, LOS = Loamy Sand). Metal mixture toxicity is expressed in toxic units. Fixed Rays: CSQG = Canadian Soil Quality Guideline, Flin = Flin Flon, Sud = Sudbury, Clay = Clay Peat, Port = Port Colborne, EC50, Equal = Equal Ratio, AgRes = Agricultural/Residential landscape + loamy soil, Acid = Acid Sandy, LoamSand = Loam Sandy + Industrial landscape.

Table A-2. Metal mixture toxicity expressed as toxic units (TU) ± SE at 10% and 50% effect levels of ten mixtures (CSQG = Canadian Soil Quality Guideline, Flin Flon, Sudbury, Clay Peat, Port Colborne, EC50 = Effective concentration at 50% reproduction inhibition in Collembola, Equal = Equal metal ratio, Agric/Res/Loamy = Agricultural/Residential land use guideline values of Canada + Loamy soil values from EU PNEC reference, Acid Sandy = EU PNEC reference, Loamy Sand Industrial = Loamy Sand values from EU PNEC reference + Industrial land use guidelines of Canada) in five soils (ASF, LOA, LO, ASA, and LOS). ASF = Acid Sandy Forest, LOA = Loamy Alluvial, LO = Loamy, ASA = Acid Sandy Arable, LOS = Loamy Sand. *Not significantly different ($p < 0.05$) from Concentration addition.

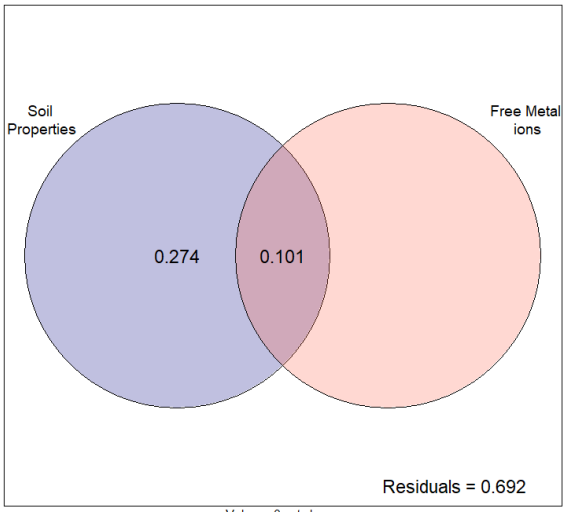
Metal mixture	ASF		LOA		LO		ASA		LOS	
	TU50	TU10	TU50	TU10	TU50	TU10	TU50	TU10	TU50	TU10
CSQG	1.68 ± 0.91	1.70	8.02 ± 2.69	0.12	0.13 ± 0.1	0.75	1.19 ± 0.14	4.20	1.85 ± 0.54	0.07
Flin Flon	1.77 ± 0.28	4.42	Not toxic	Not toxic	0.17 ± 0.003	24	0.24 ± 0.04	0.67	0.30 ± 0.09	0.0075
Sudbury	1.47 ± 0.63	0.30 ± 3.1	Not toxic	Not toxic	0.04 ± 0.005	0.57	0.90*± 0.34	0.89	0.57 ± 0.16	1.47
Clay Peat	2.26 ± 0.54	12.77 ± 11.8	4.96 ± 2.58	3.0 ± 4.9	0.31 ± 0.29	0.1	2.06 ± 0.49	1.3 ± 0.89	N/A	N/A
Port Colborne	1.26*± 0.54	0.033	79	1.19*	Not toxic	Not toxic	4.00 ± 10	11.9	N/A	N/A
EC50	2.03 ± 0.60	10.32	2.18 ± 3	0.0039	0.59 ± 0.09	8.86	0.88 ± 0.24	2.32	7.57 ± 1.14	105
Equal	2.84 ± 0.53	0.17	5.62 ± 7.7	0.23	0.9 ± 0.26	0.71	Not toxic	Not toxic	0.4 ± 0.14	0.55
Agric/Res/Loamy	0.84	0.18	6.26	0.24	0.48 ± 0.19	N/A	0.22 ± 0.1	0.87	1.40*± 2.29	4.3
Acid Sandy	1.02*± 2.07	0.28	Not toxic	Not toxic	0.91*± 1.5	N/A	1.54 ± 0.12	1.80	0.041 ± 0.009	0.01
Loamy Sand Ind	0.97*	0.12 ± 1.3	2.25 ± 3	5.21	0.33 ± 0.082	1.03*	0.55 ± 0.17	1.80	0.68 ± 0.3	0.09

Supplementary

(a)



(b)



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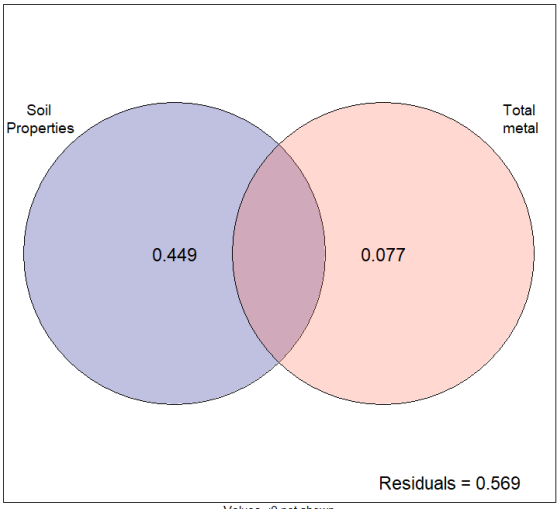

Figure A-2. Venn diagram of the variation partitioning of the response matrix of metal toxicity explained by (a) soil properties and Fulvic acid bound metals (FA-Metal (%)) on toxicity (b) soil properties and free metal ions on toxicity (c) soil properties and total metals at 50% effect levels. Residuals show the variations not explained by any of the explanatory variables (soil properties, fulvic acid bound metals, free metals, and total metals).

Table A-3. Subset of soil properties and metal that explained significant variances in toxic response at metal mixture 10% effect level (EC10) and 50% effect level (EC50) with fulvic acid bound metals, free metals, and total metals.

Metal measure		EC10	EC50		EC10	EC50
	Soil properties	*p* value	*p* value	Metal	*p* value	*p* value
% Fulvic acid bound metal	CEC	0.69	0.17	Cobalt	0.005**	0.03**
	OC	0.69	0.26	Nickel	0.96	0.50
	pH	0.02*	0.28	Copper	0.61	0.39
	Clay	0.13	0.06	Zinc	0.17	0.42
				Lead	0.025*	0.65
Free metal	CEC	0.75	0.14	Cobalt	0.25	0.89
	OC	0.68	0.24	Nickel	0.80	0.71
	pH	0.02*	0.34	Copper	0.91	0.88
	Clay	0.15	0.07	Zinc	0.09	0.42
				Lead	0.75	0.21
Total Metal	CEC	0.71	0.20	Cobalt	0.74	0.17
	OC	0.62	0.98	Nickel	0.40	0.31
	pH	0.005**	0.74	Copper	0.45	0.88
	Clay	0.07	0.035*	Zinc	0.60	0.36
				Lead	0.17	0.45

**$p < 0.01$

*$P < 0.05$

Table A-4. Subset of soil properties and metal that explained significant variances in toxic response at 10% effect level (TU10) and 50% effect level (TU50) with fulvic acid bound metals, free metals, and total metals.

Metal measure		**TU10**	**TU50**		**TU10**	**TU50**
	Soil properties	*p* value	*p* value	Metal	*p* value	*p* value
% Fulvic acid bound metal	CEC	0.92	0.15	Cobalt	0.15	0.35
	OC	0.20	0.09	Nickel	0.94	0.03*
	pH	0.23	0.30	Copper	0.36	0.690
	Clay	0.71	0.01**	Zinc	0.47	0.04*
				Lead	0.16	0.06
Free metal	CEC	0.92	0.035*	Cobalt	0.36	0.72
	OC	0.23	0.12	Nickel	0.59	0.40
	pH	0.23	0.39	Copper	0.58	0.96
	Clay	0.69	0.005**	Zinc	0.38	0.78
				Lead	0.34	0.89
Total Metal	CEC	0.95	0.005**	Cobalt	0.80	0.02*
	OC	0.23	0.15	Nickel	0.66	0.05*
	pH	0.19	0.23	Copper	0.46	0.42
	Clay	0.57	0.31	Zinc	0.19	0.44
				Lead	0.78	0.74

**$p < 0.01$

*$P < 0.05$

9. Appendix B: Chapter 4 Supplementary Material

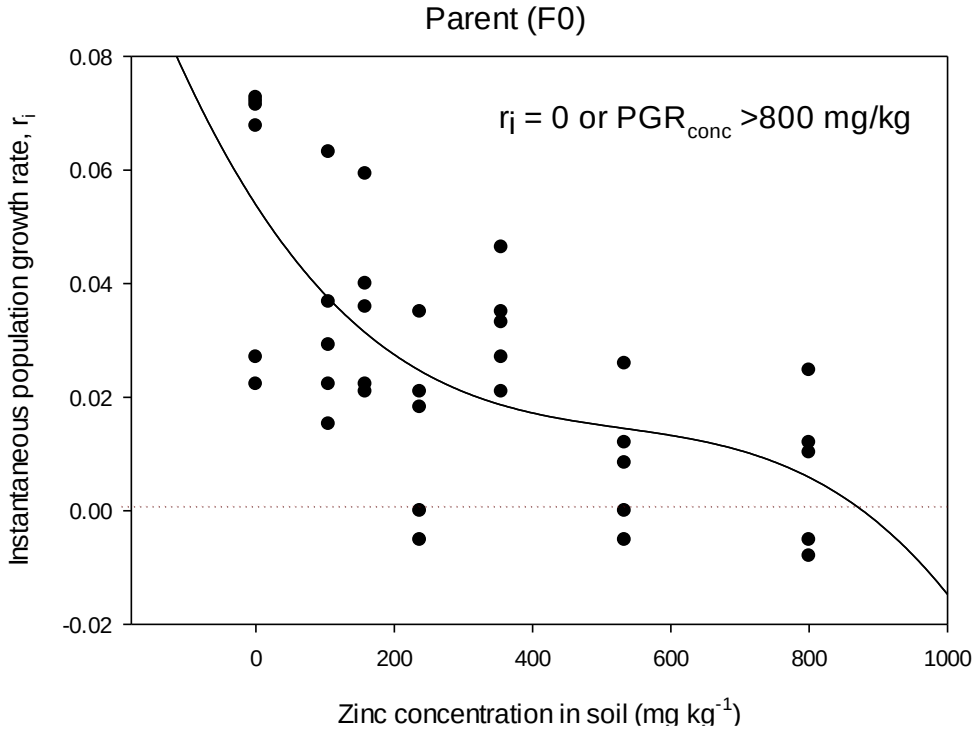

Figure B1. Least-squared fitting logistic regression of the population growth rate (r_i) of the parent mite (F0) with zinc concentration. $r_i = 0 = PGR_{conc}$

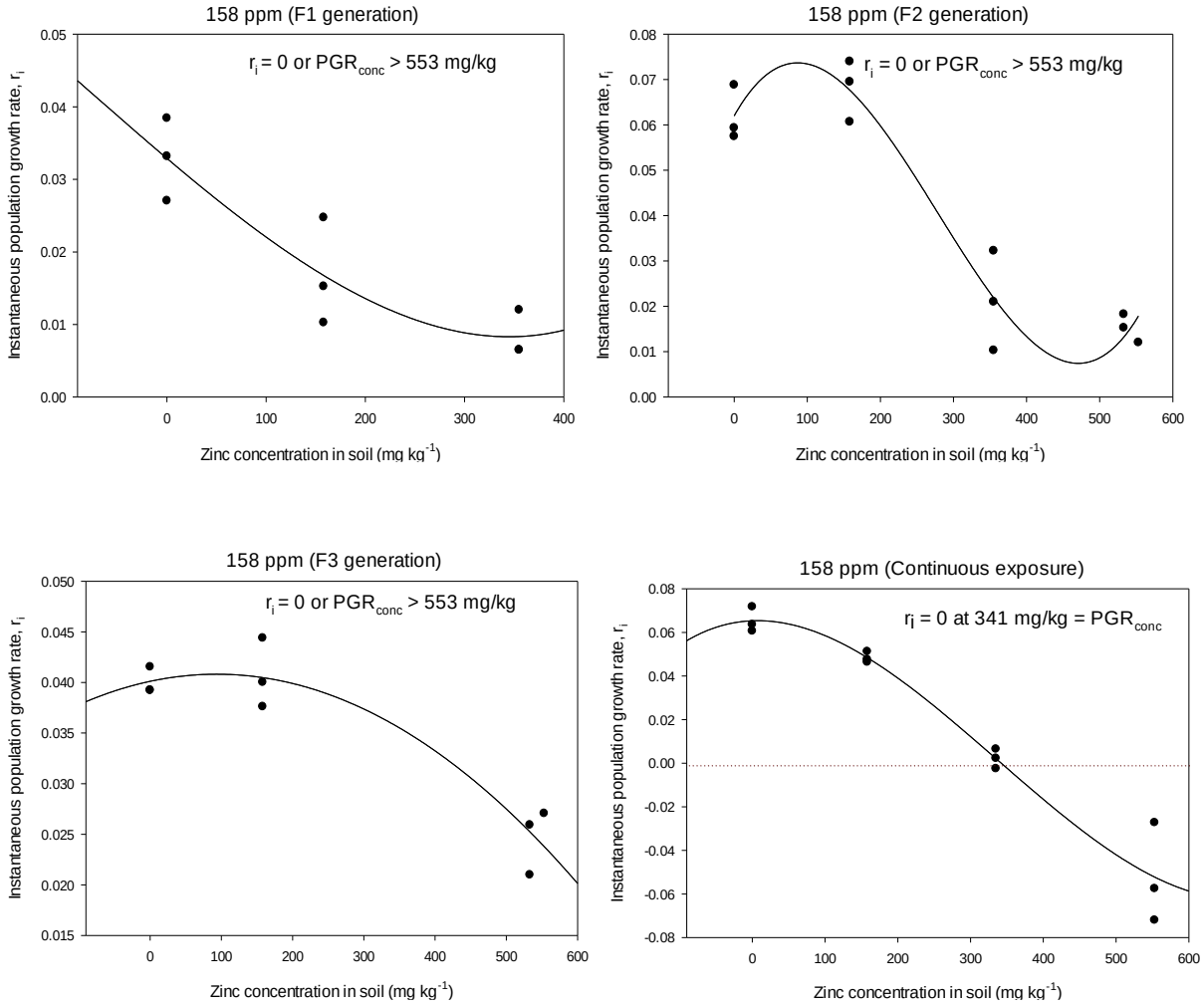

Figure B2. Least-squared fitting of logistic regression of r_i of 158 ppm (or Population 1) against zinc concentration in soil. When ri = 0, the mite populations are stable at the corresponding zinc concentration.

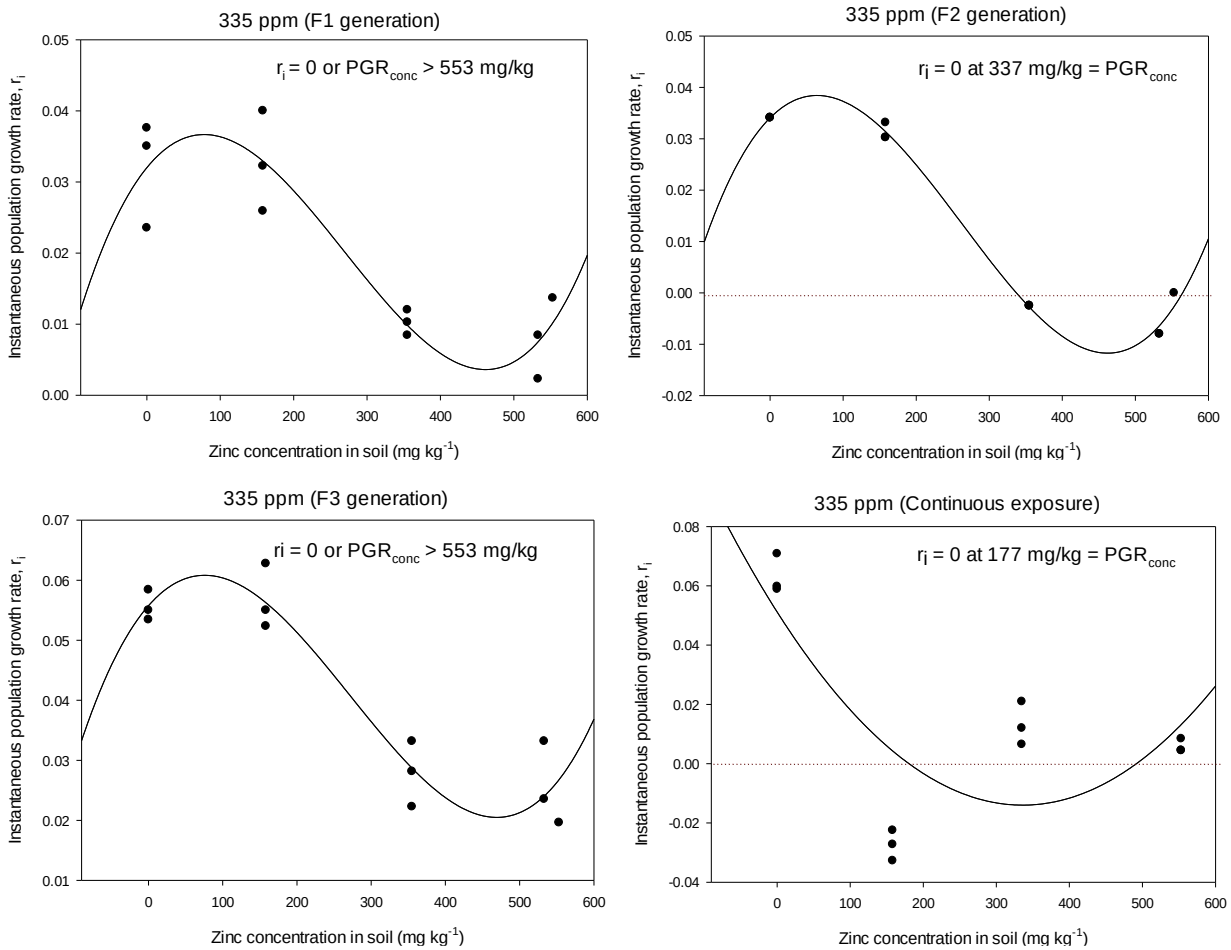

Figure B3. Least-squared fitting of logistic regression of r_i of 335 ppm (or Population 2) against zinc concentration in soil. When ri = 0, the mite populations are stable at the corresponding zinc concentration.

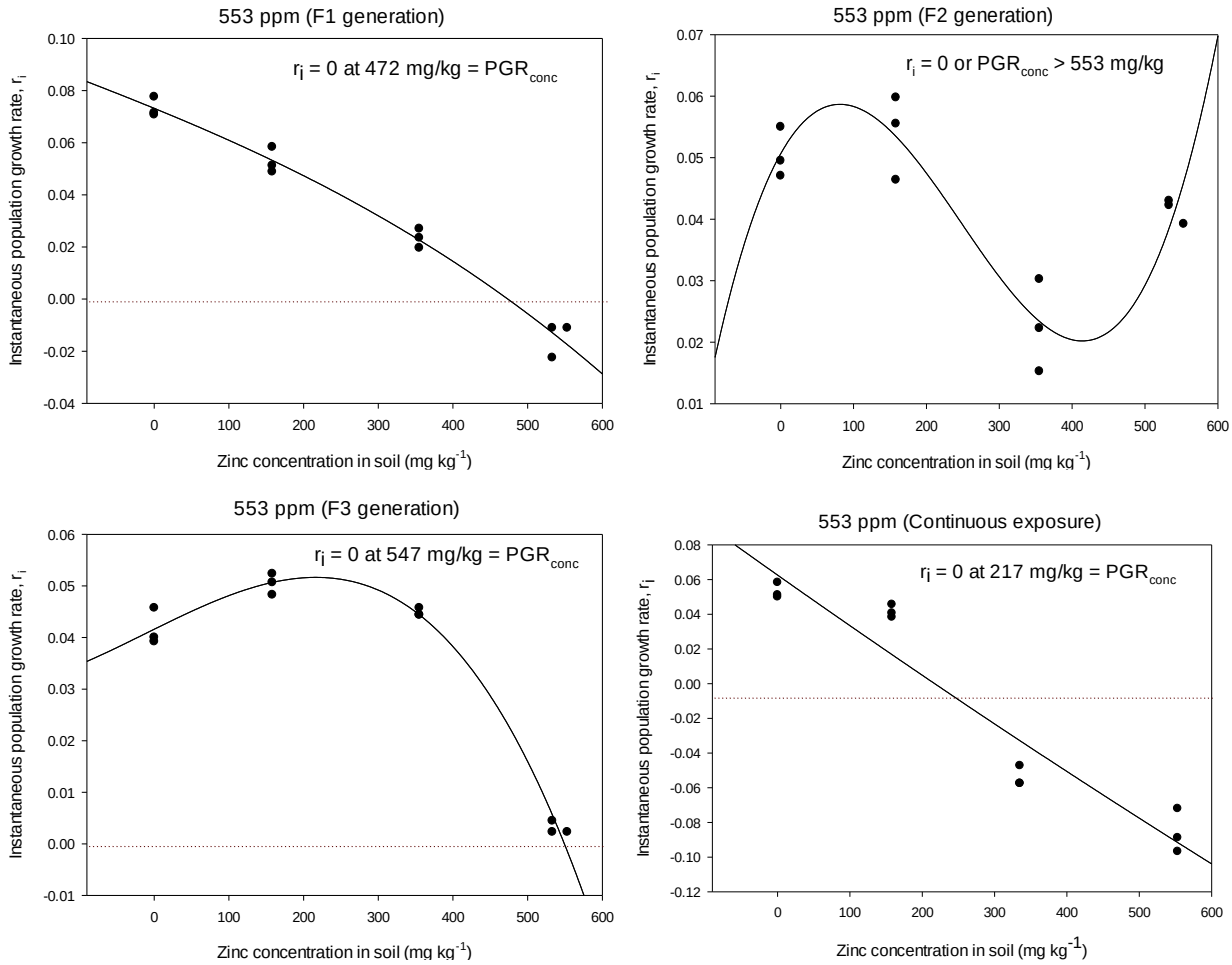

Figure B4: Least-squared fitting of logistic regression of r_i of 553 ppm (or Population 3) against zinc concentration in soil. When ri = 0, the mite populations are stable at the corresponding zinc concentration.

10. Appendix C: Chapter 5 Supplementary Material

Table C-1. Soil properties (pH, EC, WHC, Sand, Silt, Clay, and OC) of forty-seven (47) Canadian soils (pH was determined by 0.01 M $CaCl_2$ in a ratio of 1 : 5 (solid : liquid) (Kamoun Jaabiri et al., 2018), WHC was determined as described by Kamoun Jaabiri et al. (2018), EC was measured by inserting an electrical conductivity meter electrode in soil solution made by adding ionized water to soil, soil texture was measured as described by Kamoun Jaabiri et al. (2018), OC was determined using the LECO SC-623 CNS analyzer (Elikem et al., 2019), CEC was determined as described by Yukelsen and Kaya (2008)).

Soil Name	pH	EC (mS/m)	WHC (ml/g)	Sand (mg/g)	Silt (mg/g)	Clay (mg/g)	OC (g/kg)	CEC (mmol/100g)
Aaron 1	7.6	0.43	32.5	34.0	37.6	28.4	2.5	28.0
Alameda	6.2	0.41	31.8	36.5	41.7	21.8	3.8	24.0
Ardill	7.5	1.24	32.3	44.0	32.0	24.0	2.4	24.6
Babisky	7.7	0.67	31.5	75.1	7.2	17.7	2.7	23.5
Bernie	7.8	0.68	27.4	57.3	26.2	16.5	1.7	19.0
Brewster	7.6	0.74	37.1	14.8	49.1	36.1	4.8	31.4
Brian 1	6.9	0.16	26.8	47.9	30.1	22.0	3.0	21.6
Brian 2	7.4	0.01	42.6	22.3	60.4	17.2	12.9	31.2
Brownlee	7.7	0.40	26.8	47.6	25.0	27.4	1.5	24.4
Carrot River	5.6	0.32	29.1	89.2	2.1	8.6	1.2	13.6
Copeland 1	6.6	0.51	33.1	7.4	42.4	50.2	4.7	33.2
Copeland 2	6.4	1.47	42.0	7.8	60.6	31.6	8.1	28.0
Doelger	6.3	0.14	23.2	94.0	0.0	6.0	0.9	14.7
Donny	7.5	1.18	32.8	44.4	27.5	28.1	3.9	26.3
Echo	6.5	0.66	29.5	46.0	32.0	22	2.0	19.6
Estevan	7.5	0.40	32.2	19.6	49.6	30.8	2.8	29.4
Henry 1	5.5	0.60	31.1	81.2	4.6	14.2	2.6	18.4
Henry 2	7.1	0.74	33.1	42.8	32.0	25.2	2.7	27.4
Jackson	7.3	0.33	33.1	20.3	57.7	22.1	1.8	27.1
Jay	7.4	0.80	32.4	26.3	58.1	15.6	1.9	21.9
Jeremy 1	5.3	0.66	27.8	59.6	20.7	19.7	2.2	18.7
Jeremy 2	5.3	0.74	26.7	71.3	14.4	14.3	1.8	17.7
John	7.4	0.85	33.7	28.4	43.6	28.1	2.2	27.0
Melita	6.7	0.13	25.7	75.9	9.7	14.5	1.6	19.9
Nipawin	5.0	0.53	28.4	49.8	36.0	14.2	2.2	15.6
P. Plain	6.5	0.67	72.6	16.1	21.7	62.2	36.0	34.8
Powerline	6.5	0.66	40.2	64.6	20.8	14.6	8.6	28.8
PRT	6.6	0.33	20.5	88.4	3.9	7.7	1.7	16.1
Randy 1	6.8	0.48	31.8	62.8	15.3	21.9	3.6	26.3
Randy 2	7.5	0.66	35.0	38.2	39.4	22.4	3.9	27.0
Rob	6.0	0.46	29.9	41.0	36.2	22.9	3.2	20.3
Roland	5.6	0.84	25.1	92.2	0.0	7.7	1.2	13.9
Sand Lens	6.8	0.09	19.8	71.8	13.1	15.1	1.0	18.2

Sarah	6.4	0.12	28.6	67.9	16.2	15.9	1.1	18.4
Sceptre	7.6	0.49	37.4	7.6	36.0	56.4	1.7	33.5
Stuart	7.4	0.79	38.0	7.6	33.0	59.4	2.2	27.8
Thomson 2	7.3	0.66	44.0	14.7	68.4	16.9	13.0	34.4
William	7.5	0.86	38.3	25.9	55.3	18.8	6.8	29.7
Yorkton	7.4	0.52	29.4	57.7	27.3	15.0	2.9	21.5
Aaron 3	6.8	0.30	34.6	8.4	39.5	52.1	3.3	28.7
Black Spruce	4.6	0.09	20.1	90.2	7.5	2.3	0.4	9.9
Broad Creek	7.9	-	42.6	50.2	27.5	22.3	6.0	30.6
Sherri	8.2	-	32.1	52.7	25.0	22.3	2.9	26.4
Mixedwood	5.9	0.32	19.2	75.2	20	4.8	0.6	10.1
Burnees	5.6	0.23	15.5	52.8	34.8	12.4	0.6	12.7
Dave 2	7.4	0.20	34.4	29.6	52.1	18.3	3.5	21.4
Sand dunes	6.3	-	17.4	85.1	10.0	4.9	0.3	10.8

Footnote
EC = Electrical conductivity of soil, WHC = Water Holding Capacity of soil, OC = Organic carbon of soil, CEC= Cation Exchange Capacity

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Elikem E, Laird BD, Hamilton JG, Stewart KJ, Siciliano SD, Peak D. 2019. Effects of chemical speciation on the bioaccessibility of zinc in spiked and smelter-affected soils. *Environmental Toxicology and Chemistry* 38, 448-459.

Jaabiri Kamoun I, Jegede OO, Owojori OJ, Bouzid J, Gargouri R, Römbke J. 2018. Effects of deltamethrin, dimethoate, and chlorpyrifos on survival and reproduction of the collembolan *Folsomia candida* and the predatory mite *Hypoaspis aculeifer* in two African and two European soils. *Integrated Environmental Assessment and Management* 14, 92-104.

Yukselen Y, Kaya A. 2008. Suitability of the methylene blue test for surface area, cation exchange capacity and swell potential determination of clayey soils. *Engineering Geol*ogy 102, 38-45.

Table C-2: Ordered logistic regression of habitat quality against CEC (cation exchange capacity), OC (organic carbon), pH, total nitrogen, WHC (water holding capacity) and EC (electrical conductivity) showing the value, standard error (Std. error), t-value and p-value. Delineation within high (high), medium (medium) and low (low) habitat qualities. Odds ratios of significant soil properties (CEC and OC) at 2.5% and 97.5% CI (Confidence interval).

Variable	*Value*	*Std. Error*	*t-value*	*p-value*
CEC	-0.66	0.18	-3.7	0.00020
OC	0.28	0.13	2.2	0.028
pH	0.78	0.89	0.87	0.38
Total Nitrogen	-0.0053	0.0028	-1.9	0.058
WHC	0.012	0.052	0.23	0.82
EC	-0.40	1.5	-0.27	0.79
1 High|1 High	16.2	6.2	-2.6	0.0086
1 High|2 Med	-12.8	5.9	-2.2	0.029
2 Med|3 Low	-6.8	5.4	-1.3	0.20
	Odds Ratio	2.5% CI	97.5% CI	
CEC	0.52	0.34	0.70	
OC	1.3	1.1	1.7	

**Significant at $p < 0.05$*

Table C-3: Parameter estimates from linear mixed effect model (n=20) of the effects of habitat quality and dose on mite reproduction with random effects of experiments nested with soils. Parameters estimated were value, standard error (Std. error), degree of freedom (DF), t-value, p-value

Fixed effects: Mite reproduction ~ Habitat quality (HQ) * Dose					
	Value	Std. Error	DF	t-value	p-value
Intercept	169.69	15.75	90	10.77	0.00
HQ**1:**HQ2	-40.44	22.28	14	-1.82	0.09
HQ**1:**HQ3	-74.44	22.28	14	-3.34	0.0048
Dose **0:**100 ppm	6.64	14.53	90	0.46	0.65
Dose **0:**200 ppm	-3.44	14.53	90	-0.24	0.82
Dose **0:**500 ppm	-30.86	14.53	90	-2.12	0.04
Dose **0:**1500 ppm	-42.40	14.53	90	-2.92	0.0044
Dose **0:**4500 ppm	-46.99	14.53	90	-3.23	0.0017
Dose **0:**14000 ppm	-78.69	14.53	90	-5.42	0.00
HQ**1:**HQ2:Dose 100 ppm	-7.46	20.55	90	-0.36	0.71
HQ**1:**HQ3:Dose 100 ppm	-14.22	20.55	90	0.69	0.49
HQ**1:**HQ2:Dose 200 ppm	1.96	20.55	90	0.10	0.92
HQ**1:**HQ3:Dose 200 ppm	11.78	20.55	90	0.57	0.57
HQ**1:**HQ2:Dose 500 ppm	25.36	20.55	90	1.23	0.22
HQ**1:**HQ3:Dose 500 ppm	8.51	20.55	90	0.41	0.68
HQ**1:**HQ2:Dose 1500 ppm	13.28	20.55	90	0.65	0.52
HQ**1:**HQ3:Dose 1500 ppm	15.24	20.55	90	0.74	0.46
HQ**1:**HQ2:Dose 4500 ppm	-6.68	20.55	90	-0.33	0.75
HQ**1:**HQ3:Dose 4500 ppm	0.07	20.55	90	0.0034	1.00
HQ**1:**HQ2:Dose 14000 ppm	-10.65	20.55	90	-0.52	0.61
HQ**1:**HQ3:Dose 14000 ppm	2.14	20.55	90	0.10	0.92
Random effects:					
Formula: ~1| Experiment					
	Intercept				
Std Dev	0.0041				
Formula: ~1| Soil nested within experiment					
	Intercept	Residual			
Std Dev	29.25	25.16			

Table C-4: The effective concentration inhibiting reproduction at 20% (EC20) and at 50% (EC50) of zinc (mg/kg of soil) in 18 soils and the mean and standard error (SE) of EC20s and EC50s per habitat quality of soils.

Soil	HQ	EC20 (mg/kg)	Mean EC20 (mg/kg)	Standard error (SE)	EC50 (mg/kg)	Mean EC50 (mg/kg)	Standard error (SE)
Henry 2	1	2711	3273	1751	21731	13920	4328
Powerline	1	156			4524		
William	1	165			8816		
Stuart	1	126			3967		
Jackson	1	10796			13605		
Estevan	1	5688			30882		
Melita	2	7416	2594	1066	14499	6816	2102
Rob	2	3091			11527		
Brian 1	2	315			6326		
Jeremy 2	2	394			1443		
Brownlee	2	1837			4267		
Randy 1	2	2512			2838		
PRT	3	476	2038	1194	1140	4523	1948
Sand lens	3	772			5539		
Black spruce	3	102			201		
Sarah	3	7620			13155		
Carrot	3	2964			5339		
Bernie	3	298			1768		

Table C-5: The ratio of variance of two experiments, experiment 1 (Expt 1) and experiment 2 (Expt 2), p-value, degree of freedom (df).

	Ratio of variance	p-value	df
Expt 1:Expt 2	0.98	0.93	-
Expt 1	-	-	62
Expt 2	-	-	62

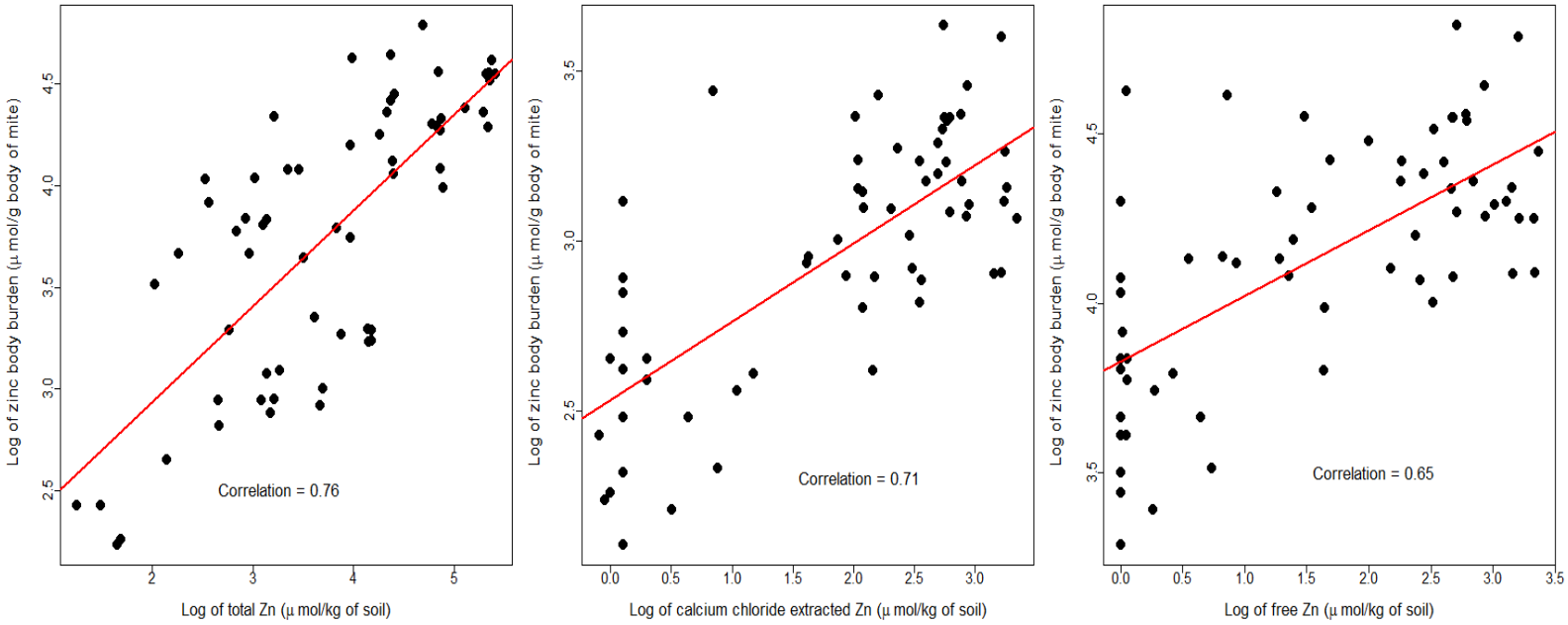

Figure C-1. The log of Zn body burden of adult *Oppia nitens* measured after 28 days of exposure to Zn in 18 soils related to the log of the total, calcium chloride ($CaCl_2$) extracted and Zn concentrations in the soil. The lines show a linear fit for each of the measured external Zn (total, $CaCl_2$, free Zn) and the body burdens. The correlation, r was determined as the Pearson's product moment correlation coefficient.

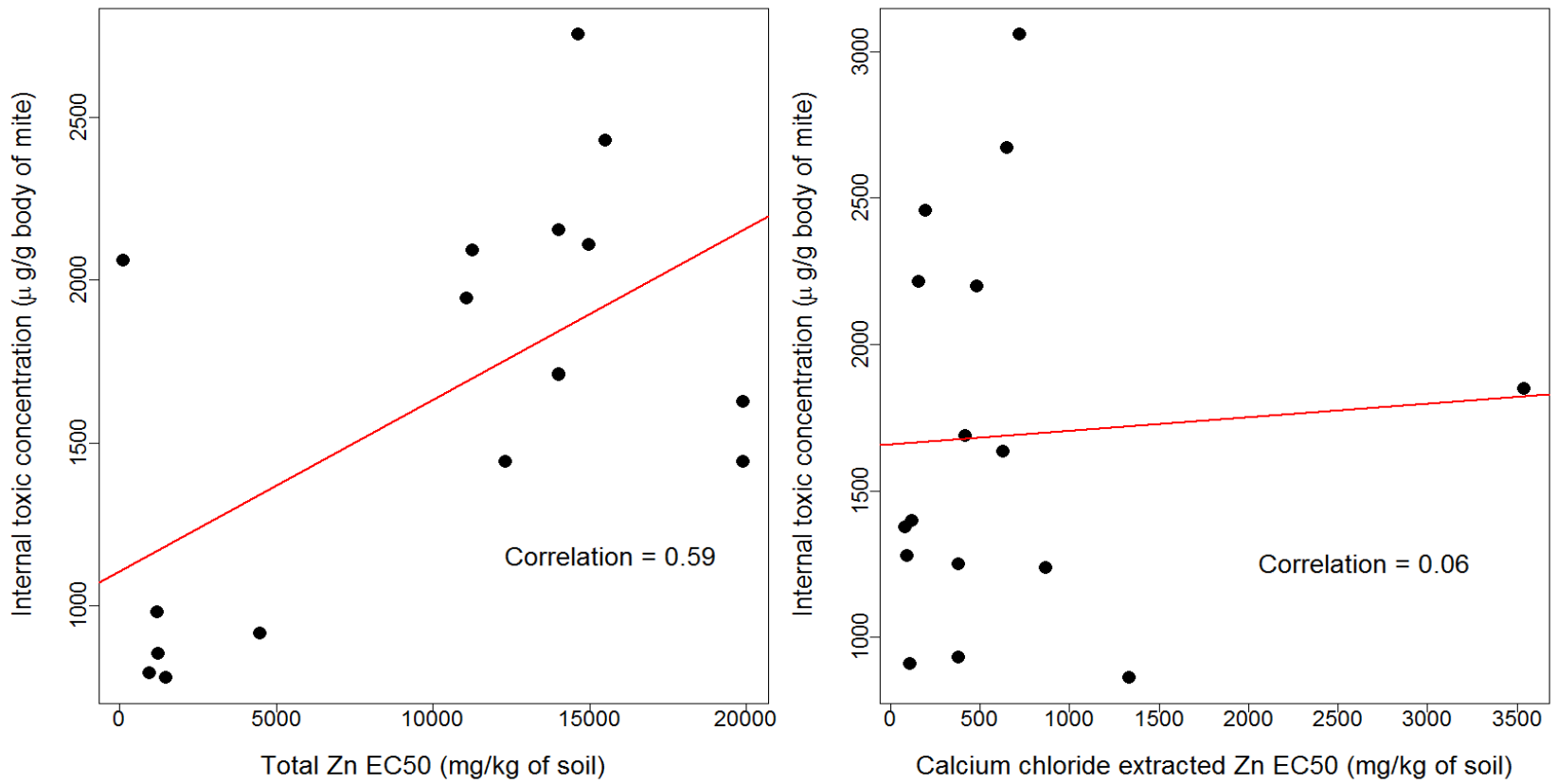

Figure C-2. The internal toxic concentration of Zn (µg/g body of mite) after 28 days of exposure to Zn in 16 soils, related to the total Zn EC50 and the calcium chloride extracted Zn EC50s. The internal toxic concentration of Zn for each soil was derived from the regression line of the external Zn (total and $CaCl_2$ extracted) concentrations and body burden. The internal toxic concentration of Zn was the point where body burden equals to the external Zn EC50. The lines show a linear fit for each of the measured external Zn EC50s and internal toxic concentrations. The correlation, r was determined as the Pearson's product moment correlation coefficient and r^2 as the fit of the regression