# EFFECTS OF POLYETHYLENE MICROPLASTICS AND CADMIUM ON FRESHWATER ANIMALS: A MODELLING FRAMEWORK

**LAUREN ZINK**

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Department of Biological Sciences
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LAUREN ZINK

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| | | |
|---|---|---|
| Dr. Gregory G. Pyle | Professor Emeritus | Ph.D. |
| Dr. Steve Wiseman | Associate Professor | Ph.D. |
| Thesis Co-Supervisors | | |
| | | |
| Dr. Robert Laird | Professor | Ph.D. |
| Thesis Examination Committee Member | | |
| | | |
| Dr. Stacey Wetmore | Professor | Ph.D. |
| Thesis Examination Committee Member | | |
| | | |
| Dr. Tamzin Blewett | Assistant Professor | Ph.D. |
| External Examiner | | |
| Department of Biological Sciences | | |
| Faculty of Science | | |
| University of Alberta | | |
| | | |
| Dr. Dmytro Yevtushenko | Associate Professor | Ph.D. |
| Chair, Thesis Examination Committee | | |

## DEDICATION

To Ryland and Fritter – from whom I am continually reminded that some of the best stories stem from the outliers.

**ABSTRACT**

The emerging field of microplastic-metal mixtures toxicity is riddled with contradictory conclusions relating to the uptake, accumulation, and toxicity of the mixture and each constituent. After initially setting out to fill knowledge gaps through several studies with a phylogenetically diverse set of animal models and exposure scenarios, I recognized that this work contradicted other studies in the field. This thesis draws on our developed understanding of metal behaviour and toxicity in an effort to resolve the confusion relating to microplastic-metal mixtures toxicity. Despite the well-established knowledge that water quality characteristics govern the behaviour of metals in freshwater systems, there has not been adequate water quality reporting in the field of microplastic-metal mixtures toxicity. To address the reality that providing a full suite of water quality characterization is not often feasible for each experiment, we created a model to predict which aspects of water quality primarily govern the association of cadmium and polyethylene, a representative microplastic. The model, combined with understanding the bioavailability of metal-microplastic complexes, provides insight into the hazards of metal toxicity in a given system. This model promotes the harmonization across research in the field which can then collectively be used in policy development to protect aquatic life.

**ACKNOWLEDGEMENTS**

As evident by the length of this thesis, I have been trained that writing scientifically and writing succinctly are synonymous – that is, to convey my message directly and with clarity. While one may be able to sustain this approach conveying scientific results, the same approach fails miserably when trying to express appreciation for others.

The mentorship I have received throughout this journey has been thought-provoking and inspiring. From my mentors near (Greg Pyle, Matt Bogard, Ran Barley, Rob Laird, Stacey Wetmore, Steve Wiseman) and far (Jim McGeer, Kurt Maier, Sarah-Ellen Johnston), though even those geographically near had to operate from a distance during the pandemic, an additional effort for which I am grateful each of them embraced.

Not only was I mentored throughout the program, but I was given opportunities to mentor others. These experiences nurtured my relationship with the scientific research process and challenged my ability to teach skills that had become second nature. My research supervision skills have been initially shaped by my work with Alyssa Hovelling, Anna Shearer, Emily Mertens, Julia Stroud, Karen Shamash, Mikaila Meslo – I hope each of you learned as much from me as I learned from you and will continue to carry forward with me to future positions.

I have learned that science should not, and frankly can not, be done in isolation. I am grateful to my collaborators (Andreas Eriksson, Carolyn Simonis, Chloe Devoy, Jaimie Klemish, Jon Doering, Justin Dubiel, Justin Miller, Parastoo Razmara, Raegan Plomp, Suzanne Chmilar, Xingzi Zhou, Yamin Raza, and the GLEON GALACTIC team) for sharing their comradery, perspective, skills, and in many cases subsequent friendship.

One of the most memorable bits of advice I received as a graduate student was "don't only have other scientists as your friends" – given how memorable it was, it would be helpful if I could remember who said it to me. Regardless, I put it into practice. I have spent countless hours working alongside other doctoral candidates studying World War 1, philosophy, religion on social media, economic business strategies, urban planning, and many other areas that I would never be exposed to if not for our happenstance co-working community – Adriana, Andrea, Anna, Dhvani, Katrina, Linette, Liz, Tanner, and Zabeen: thank you for the late nights, the early mornings, and the slight but modest peer pressure to persevere through the tough times.

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I would not have had the courage or drive to complete this program had it not been for the way in which I was raised by my family. I am forever grateful to my parents for their support and guidance, to my sister and brother-in-law for their encouragement, and to the collection of cousins, aunts, uncles, grandparents, and friends who have provided words of support and reassuring (though sometimes admittedly clueless) nods and smiles when listening to my research updates.

## PREFACE

This thesis is a manuscript-style thesis which is organized based on the University of Lethbridge thesis submission regulations. Inevitably, there is some repetition of content between sections, particularly in the introduction and methods sections of research chapters. Chapters 2, 3, 4, 5, and 6 have all been published in various journals, with author contributions summarized below:

Chapter 2 has been published in *Aquatic Toxicology*: Zink L, Wiseman S, Pyle GG. 2023. Single and combined effects of cadmium, microplastics, and their mixture on whole-body serotonin and feeding behaviour following chronic exposure and subsequent recovery in the freshwater leech, *Nephelopsis obscura*. Aquat Toxicol 259:106538. https://doi.org/10.1016/j.aquatox.2023.106538. The author contributions for this manuscript are as follows, in accordance with the CRediT Author Contributions system: Lauren Zink: conceptualization, methodology, investigation, writing – original draft, writing – review & editing, visualization, funding acquisition; Steve Wiseman: supervision, writing – review & editing; Gregory G. Pyle: conceptualization, supervision, writing – review & editing, funding acquisition.

Chapter 3 has been published in *Environmental Toxicology and Pharmacology* as part of the Special Issue *Microplastics and their Environmental Effects*: Zink L, Simonis C, Wiseman S, Pyle GG. 2023. In vitro characterization of cadmium transport across the gastro-intestinal membrane of the fathead minnow (*Pimephales promelas*) in the presence and absence of microplastics. *Environ Toxicol Pharmacol* 101:104195. https://doi.org/10.1016/j.etap.2023.104195. The author contributions for this manuscript are as follows, in accordance with the CRediT Author Contributions system: Lauren Zink:

conceptualization, methodology, validation, formal analysis, investigation, writing – original draft, writing – review and editing; Carolyn Simonis: investigation, writing – review and editing; Steve Wiseman: resources, writing – review and editing, supervision, funding acquisition; Gregory G. Pyle: resources, writing – review and editing, supervision, funding acquisition.

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Chapter 6 has been submitted for publication: Zink, L., Mertens, E., Zhou, X., Johnston, SE., Bogard, M., Wiseman, S., and Pyle, G.G. Reframing microplastics as a ligand for metals reveals that water quality characteristics govern the association of cadmium to polyethylene. The author contributions for this manuscript are as follows, in accordance with the CRediT Author Contributions system: Lauren Zink: conceptualization, methodology, investigation, funding acquisition, writing – original draft, writing – review and editing; Emily Mertens: investigation, data curation, writing – review and editing; Xingzi Zhou: software, formal analysis, data curation, writing – review and editing; Sarah Ellen Johnston: conceptualization, software, writing – review and editing; Matthew Bogard: resources, writing – review and editing; Steve Wiseman: conceptualization, resources, supervision, writing – review and editing; Gregory G. Pyle: supervision, writing – review & editing, funding acquisition.

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| | |
|---|---|
| ARF | Aquatic research facility |
| bix | Biological index |
| BLM | Biotic Ligand Model |
| Cd/CD | Cadmium |
| CDMP | Cadmium-microplastic mixture |
| CRM | Certified reference material |
| DOC | Dissolved organic carbon |
| DOM | Dissolved organic matter |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| fi | fluorescence index |
| FIAM | Free Ion Activity Model |
| Fmax | Maximum fluorescence |
| GFAAS | Graphite furnace atomic absorption spectroscopy |
| hix | humification index |
| LC | Lab control |
| MP | Microplastic |
| ND | Non-detectable/not detected |
| NOM | Natural organic matter |
| ORP | Oxidation reduction potential |
| PMT | Persistent, mobile, and toxic |
| $SUVA_{254}$ | Specific ultraviolet absorbance at 254 nm |
| TDTK | Toxicodynamic-toxicokinetic Model |
| VIF | Variable importance factor |
| vPvM | Very persistent and very mobile |
| WQ | Water quality |
| 5HT | Serotonin |

# CHAPTER 1: INTRODUCTION

## Plastics contaminating freshwater systems

Research into understanding the role that plastics play in society has been steadily increasing in recent decades and exponentially within the last five years (Nielsen et al. 2020). Research into the consumer trends of plastic products have tended to center around specific plastic products such as plastics bags and single-use plastic packaging, often with a goal of informing policy and implementing regulations around the manufacturing and use of these products (Nielsen et al. 2020). Broader approaches to understanding plastic's role in society have gained traction in recent years with the formation of "the plastic cycle" – describing the (theoretically) circular economy life cycle of plastics (Nielsen et al. 2020; Daniel Tang 2023).

While, in theory, the life cycle of plastics – that is manufacturing/production, consumption, and waste management (recycling, repurposing, etc.) – is expected to be circular, the high production of plastics and a lack of proper waste management strategies have resulted in the plastics cycle being far from circular. The increased production of plastic products, driven primarily by the cost and versatility of plastic materials, has increased exponentially and is forecasted to continually increase into the future despite acts to decrease plastic usage (such as legislation around single-use plastics and plastic bags) (MacLeod et al. 2021; Legislative Services Branch of Canada 2023). Further, the current lack of infrastructure and policy to properly manage plastic waste has led to a greater production of plastic waste, resulting in plastics polluting environments globally (Borrelle et al. 2020).

An emerging area of plastic research surrounds the fate of plastics once introduced (either intentionally, such as through landfill-type disposal mechanisms, or unintentionally) to the environment. Plastics in the environment are constantly degrading due to mechanical, ultraviolet, chemical, and biological processes. Mechanical degradation involves the physical abrasion to the plastic surface, often by particulates such as sediment and rock. Mechanical degradation of plastics is rapid in coastal areas (Mekaru 2020; Sun et al. 2020). Ultraviolet degradation occurs most prominently in plastics exposed to long days of direct sunlight (Campanale et al. 2023; Duan et al. 2023). Chemical degradation describes a multitude of processes such as oxidation and acidification on the plastic surface (Liu et al. 2019b). Finally, biological degradation of plastics refers to the actions of microorganisms to break down plastic material, for example through the utilization of carbon from the plastics for use in cellular structures such as membranes (Shah et al. 2008). Degradation of microplastics results in fragmentation, continually producing smaller and smaller plastics in the environment to the point of creating microplastics (fragments 1 μm– 5 mm) and nanoplastics (fragments < 1 μm); as plastic fragments become smaller, the risks associated with them differs. Larger plastics tend to be associated with external risks such as entanglement and entrapment of organisms whereas smaller (and potentially ingestible) plastics pose internal risks such as digestive blockage and toxicity.

The perpetual production of microplastics has sparked an increase in research to better understand the environmental consequences of microplastic pollution. Research in this area has grown rapidly in recent years, owing to technological and analytical advances increasing the ability to detect plastics in different media such as soil, water, and biota. Advanced detection abilities are of particular importance for microplastics due to their small size. Additionally, the application of techniques such as Fourier-Transform Infrared Spectroscopy allow for the determination of what type(s) of plastic (polyethylene, polypropylene, polystyrene, etc.) is (are) present (D’Amelia et al. 2016; Hanvey et al. 2017).

A variety of microplastic types are detected in freshwater systems. The types and amount of plastic found in lakes and reservoirs relates to the level of urbanization in surrounding areas and the limnological characteristics, particularly surface area, of the waterbody (Nava et al. 2023). The most common types of plastics found in aquatic systems are polyethylenes, polypropylenes, and polyacrylates, which are all commonly used in consumer goods and packaging (Au et al. 2015; Vedolin et al. 2018).

To meet consumer needs, the production of plastic products often involves different manufacturing techniques to achieve the desired characteristics of the product. These differences can include the type of plastic used; for example, using polystyrene to craft insulated containers as the structure of polystyrene creates an effective temperature barrier, whereas polyethylene is often used in packaging in its thin and transparent form. The differences can also be driven by the additives incorporated into plastics at manufacturing; organic compounds such as alkylphenols are incorporated into plastics to provide increased UV stabilization while metals, such as cadmium, are used as plastic stabilizers and as a component of certain pigments (Wang et al. 2019b). With virtually boundless manufacturing variability, there exists an nearly incalculable suite of plastic compositions.

The composition of plastics is a determining factor of their fate and distribution in freshwater systems. The density of plastics determines whether a plastic fragment will exist suspended in the water column or sink to the sediment. Through biological degradation, the composition of plastics can change, which in turn alters their distribution. Biological degradation of plastics often facilitates the sinking of plastics previously found suspended in the water column, due to the colonization of microorganisms on the plastic surface adding weight to the plastic (Shah et al. 2008). Plastics suspended in the water column have a higher transportation rate than those embedded in sediments; however, seasonal hydrodynamic parameters such as changes in water flow can result in the settling of suspended microplastics, or conversely the resuspension of sunken microplastics (Besseling et al. 2016, p.; Nel et al. 2018). The dynamic existence of plastics moving through freshwater systems prompts their interaction with both pelagic and benthic organisms.

**Microplastic interactions with freshwater organisms**

The small size of microplastics combined with their deposition into the environment has resulted in their ingestion by many freshwater species (Scherer et al. 2017; Windsor et al. 2018). Microplastics were found in 50% of riverine macroinvertebrates sampled over approximately 50 km of river in South Wales; microplastics were measured in individuals from all sites (Windsor et al. 2018). Factors that correlated with an increased concentration of microplastics (up to 0.14 MP particles/ mg dry tissue) were macroinvertebrate biomass and taxonomic family (Windsor et al. 2018). Contrarily, other studies have found the driving factors of microplastic detection within invertebrates to be food availability, feeding type, and trophic level (Setälä et al. 2016; Scherer et al. 2017; Windsor et al. 2018; Garcia et al. 2021).

Once ingested, microplastics may accumulate within the organism if unable to be excreted, or if able to cross biological barriers into internal spaces. While many studies speak to the "bioaccumulation of microplastics", most of these assessed whole-body concentrations of plastic and failed to differentiate whether the plastics crossed the gut membrane, for example, and were internalized into tissues. For example, when exposed to suspended polystyrene, snails accumulated polystyrene; however, there was no depuration period to allow clearance of ingested plastics, nor was the digestive tract separated in sample analysis (Roy et al. 2023). This type of experimental approach, which makes up the vast majority of published studies, cannot provide differentiation between plastics that have been ingested (meaning those within the digestive tract that may be excreted later), bioaccumulated (meaning those that are unable to be excreted) and internalized (meaning those that have crossed a biological barrier into internal tissues), leaving a glaring knowledge gap from which we lack an understanding of microplastic-biota interactions post-ingestion.

**Interactions of microplastics with other contaminants**

In addition to existing in multiple compartments (water, sediment, and biota) of freshwater systems, microplastics can also interact with other contaminants, primarily through adsorption to the plastic surface. Plastic characteristics such as shape, size, and composition play a major role in microplastic-contaminant interactions. Plastics with a larger surface area and low degree of crystallinity (poor linearity and an amorphous primary polymeric chain) allow for increased adsorption of contaminants, potentially to a toxic level (Tourinho et al. 2019). The possible sorption and subsequent release of harmful inorganic and organic substances may enable microplastics to serve as vectors for the uptake of these toxicants via a novel pathway (Besseling et al. 2013; Rochman et al. 2013; Brennecke et al. 2016).

In addition to adsorption, microplastics have the ability to release chemical additives from within the manufactured plastic (Anderson et al. 2016). While the associations between plastic and additives may initially be intentional, the conditions under which additives and plastic will dissociate are unclear, though under simulated physiological conditions (hypothesized to be driven by the acidic digestive environment) certain organic pollutants dissociate from microplastics to which they were previously bound (Bakir et al. 2014). As many plastic additives, such as metals, can be toxic to aquatic life, their potential dissociation poses risk to the health of aquatic organisms. Further, the leaching of additives from plastics creates environments in which organisms are co-exposed to the additive and plastic.

The physical properties of microplastics, namely their large surface area-volume ratio and hydrophobicity enable microplastics to serve as a substrate for waterborne contaminants, often resulting in higher contaminant concentrations on the surface of the microplastic than in the surrounding environment (Holmes et al. 2014; Seidensticker et al. 2019; Li et al. 2019). In instances of environments co-contaminated with plastics and toxicants, the effects to aquatic organisms may be heightened due to the toxicant being concentrated to the (potentially ingestible) microplastic.

**Cadmium as a contaminant in freshwater systems**

Metals are ubiquitous in freshwater environments and pose potential risks to the health of aquatic organisms. A metal of particular interest is cadmium, as Canada is one of the leading producers of consumer goods containing cadmium such as rechargeable batteries, pigments, plastic stabilizers, and electroplated coatings, all of which have increased in production in recent decades (Phelps 2020). Cadmium's industrial uses have resulted in extremely varied levels of cadmium being detected in surface waters of urban and industrialized areas (Street 2002; Phelps 2020; Irfan et al. 2023). From 2000 to 2020, nearly 10,000 research articles investigating cadmium in freshwater have been published, highlighting the importance in understanding the behaviour and risk of cadmium in freshwater environments (Irfan et al. 2023). Cadmium persists in water, sediment, and biotic compartments of freshwater environments, the partitioning among which is driven by characteristics of the surrounding environment (Phelps 2020; Wang et al. 2022).

The physico-chemical characteristics of the ambient environment play a major role in how a metal behaves within that environment, including the speciation of that metal and how it partitions in the environment between water, sediment, and biota. The speciation and partitioning of a metal within an environment corresponds to the bioavailability and potential subsequent toxicity of that metal to a particular organism.

The temperature of an environment has been shown to influence metal speciation. With increasing temperatures, the solubility of metal-containing salts increases, releasing more free-ionic metal into the environment (Namieśnik and Rabajczyk 2010). In a similar manner that the solubility of metals can change with temperature, the solubility of other ions, such as the hydrogen ion, behave in the same way, altering the pH of the environment as well (Akiya and Savage 2002).

The acidity of an environment, as measured by pH, has been shown to alter the speciation and subsequent toxicity of metals. As pH decreases, the solubility of metals increases, creating a greater amount of free-ionic form of metal, which is the most bioavailable form of metals (Wren and Stephenson 1991). The increase of hydrogen ions in more acidic solutions has also been found to cause toxicity to some species, even without the presence of a metal (Wang et al. 2016). It is often difficult to isolate the effect of pH changes due to other water quality characteristics that vary alongside pH changes, such as water hardness and alkalinity (Long et al. 2004).

Hardness, the amount of dissolved minerals, largely calcium (Ca) and magnesium (Mg), and alkalinity, the total amount of bases capable of releasing hydroxyl ions in the water, can affect a metal's behaviour through two proposed mechanisms; (1) the complexation of the metal to a form that is less toxic, such as the complexation of copper with calcium to form cupric carbonate (Andrew et al. 1977; Wurts and Perschbacher 1994), and (2) competition between the metal and mineral cations (Ca, Mg) for binding sites (Zitko and Carson 1976). The binding sites may be biotic ligands, competition at which directly influences the bioavailability of the metal to the organism (Pagenkopf 1983), or the binding sites may refer to other ligands such as those of organic matter (Nelson et al. 1986; Playle et al. 1993).

Organic matter plays an important role in freshwater ecosystems including providing nutrition and temporal-spatial information to aquatic organisms (Thomas 1997). Organic matter is a broad term used to refer to a complex and dynamic suite of carbon-based compounds. In a freshwater environment, these molecules can differ in size, aromaticity, molecular weight, and functional groups present (Lam et al. 2007). The characteristics (often referred to as quality) of organic matter determine the ability for that organic matter to bind metals, changing their speciation and potentially altering bioavailability (Playle et al. 1993; Chen et al. 2018). For example, the aromaticity of dissolved organic matter has been shown to explain differential metal binding capacity (Baken et al. 2011). Organic matter can exist both within the water and sediment components of an environment.

The electrical properties of an environment are also important considerations in addressing metal partitioning between different compartments of freshwater environments. The presence of charged ions and salts in the water contribute to an environment's salinity, which is inversely related to the amount of free metal ions within the environment (Hall and Anderson 1995). As the free-ion form of metals has been found to be the most bioavailable and therefore have an increased potential to induce toxicity, environments with a lower salinity are more likely to have a greater prevalence of toxic forms of metals. In addition to salinity, the oxidation-reduction potential (ORP, also commonly called redox potential) is another electrical characteristic of an environment that refers to the overall tendency of a water sample to either seek electrons (oxidizing) or donate electrons (reducing). In most instances, we discuss the redox potential of sediments rather than overlying waters in aquatic environments, as a large redox potential can exist within the sediment; however, the nitrogen cycle, an important chemical process that transforms nitrogenous wastes to less toxic forms of nitrogen, is partly governed by the redox potential of an environment (Shanmugam et al. 1981).

The consideration of sediments when assessing the fate of metals within an aquatic environment is important. Many of the aforementioned substances used to characterize an aquatic environment, such as organic matter, carbonate, redox compounds, and nitrogen exist in both the water and sediment of an environment. Sediment has a high storage capacity for these substances and is therefore often referred to as either a sink (sequestering the substances) or source (liberating the substances). The sequestration to, or liberation of metals from the sediment component of an environment can affect that metal's speciation and bioavailability.

Previously described water quality characteristics including salinity (Jia et al. 2021), redox potential (Moore et al. 1988; Yu et al. 2001; Aigberua et al. 2018), and pH (Hong et al. 2011) have been found to govern the partitioning of metals between the water and sediment components of an environment. In the same manner that metals can be sequestered and liberated from sediments, nutrients also transfer between environmental compartments, introducing changes in available nitrogen and organic matter concentrations (DeLaune and Smith 1985; Miao et al. 2006). Thus far, 'sediment' has referred to settled sediment; however, suspended solids are another important consideration in assessing metal fate and bioavailability. In turbid environments, that have a considerable amount of total dissolved and total suspended solids, there is an increased potential for pelagic organisms to interact with sediment-associated substances (Nasrabadi et al. 2016).

**Cadmium interactions with freshwater organisms**

Cadmium can be taken up by aquatic organisms through two major routes: waterborne or dietary. A greater proportion of toxicology studies have focussed on uptake of waterborne metals, resulting in a less developed understanding of processes of absorption of metals from dietary sources (Dallinger and Kautzky 1985; Wang 2013). Dietary uptake of metals can result from the ingestion of metal-contaminated food or other metal-bound suspended sediments (Besseling et al. 2016; Nel et al. 2018). Studies investigating the toxicity of metals bound to suspended sediments have found that in some cases, the metals are accumulated within organisms and cause adverse effects similar to those observed with the uptake of waterborne metals (Bonnet et al. 2000; Weltens et al. 2000; Fetters et al. 2016).

Cadmium does not serve a biological function and very few organisms have evolved systems for regulating cadmium, resulting in cadmium bioaccumulation. Many animals respond to the presence of heavy metals, including cadmium, through the increased expression of the protein metallothionein. Metallothionein is involved in the transport and storage of metals, sequestering the metal ions (the more toxic form) by binding with cysteine-rich motifs of the protein. Cadmium has been detected in muscle, liver, and brain tissues, demonstrating the ability of cadmium to cross a multitude of biological membranes (Espinoza et al. 2012; Zhang et al. 2021; Drąg-Kozak et al. 2021; Soegianto et al. 2022). The bioaccumulation rates of cadmium are affected by changes in ambient environmental conditions; seasonal shifts of overwintering result in less cadmium bioaccumulation (Suominen et al. 2023).

Exposure to cadmium causes adverse effects in many freshwater organisms. In invertebrates, cadmium has been shown to cause reproductive impairment, decreased activity, and impairment to the function of neurotransmitters, including serotonin (Salánki and Hiripi

1990; Gill and Epple 1992; Almeida et al. 2003; Wu et al. 2015). Serotonin plays many roles in aquatic invertebrates including the regulation of muscle contraction and feeding behaviour (Salánki and Hiripi 1990; Almeida et al. 2003). The mechanism by which cadmium causes a reduction in serotonin is not fully understood, though it appears to vary among species (Salánki and Hiripi 1990; Almeida et al. 2003; Wu et al. 2015). A study of the serotoninergic pathway in planarians, an aquatic flatworm, revealed that cadmium appears to alter the activity of monoamine oxidase, an enzyme that metabolizes serotonin (Wu et al. 2015). Experiments using freshwater mussels suggested that cadmium may interfere with neurotransmission by prematurely releasing serotonin from its storage location within the ganglia (Salánki and Hiripi 1990; Almeida et al. 2003). In freshwater leeches, serotonin is associated with feeding behaviour (Lent and Dickinson 1984, 1988; Lent et al. 1991). Serotonin is found within the ganglia of leeches and has been proposed as a biomarker for satiation state, as hungry leeches consistently have higher levels of ganglionic serotonin than their satiated equivalents (Lent et al. 1991). As the leech ingests food and its gut becomes full, the body wall distends and the stretching of these muscles, presumably signaling that the leech is satiated, results in the downregulation of serotonin synthesis (Lent et al. 1991). Further, cadmium acts as a calcium analog, which in many instances is the grounding mechanistic hypothesis of cadmium toxicity at tissue and cellular level (Lee and Thévenod 2020; Bhattacharya 2022).

**Microplastic-metal mixtures toxicity**

Microplastics are often referred to as contaminants of emerging concern (CEC). Subsets of microplastic research, including microplastic-metal mixtures research are beginning to emerge in this field. In navigating the field of microplastic and metal research during the early stages of this research journey, I conducted a meta-analysis of all microplastic-metal toxicity studies through June 15, 2021. This meta-analysis provided a basis to understand the research areas that remained understudied and allowed me to identify knowledge gaps and design research studies to address those. The first steps in navigating the field were to understand the size and scope of the field.

Publication of microplastic-metals research increased steadily from 2015 to 2021 (**Figure 1)**. The very few studies published prior to 2018 demonstrate the infancy of the field of microplastic-metal research. As this analysis concluded data collection as of June 15, 2021, a projected estimation was calculated utilizing a prorated forecast based on the actual number of articles published during the portion of 2021 for which data was collected (**Figure 1**). A literature search performed on October 24, 2023 indicated an additional 1770 publications since 2022 using the same search criteria, albeit unfiltered for relevance, demonstrating the continued rapid growth in this area of research.

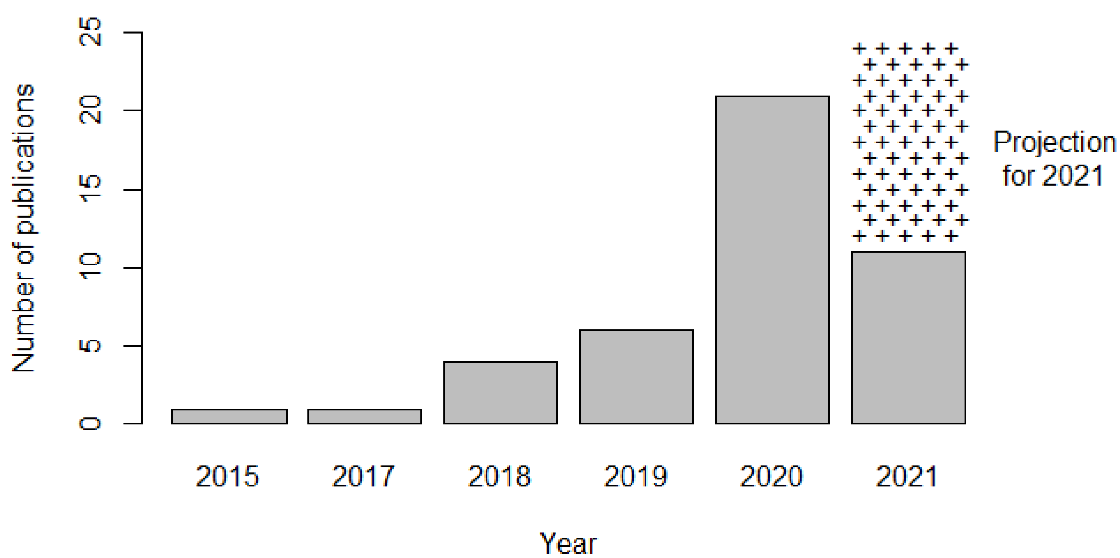


**Figure 1.** Temporal trends of microplastic-metal mixtures toxicity articles published to June 15, 2021.

There are three main areas in the field of microplastic-metal research: (1) characterizing chemical interaction, referring to developing understanding of how metals and microplastics interact; (2) field-collected interactions, referring to field collected and analyzed samples of microplastic-metal mixtures; and (3) biological effects analysis, referring to understanding the effects of exposure to microplastics and metals on biota. The majority (66%) of research in this field fell into biological effects analysis, while 25% of research fell into characterizing chemical interactions and the remaining 9% were of field-collected interactions (**Figure 2**). All the field-collected interactions studies were classified as being field studies (qualified as at least one environmental component – microplastics, water, sediment, or biota being included within the experiment) while zero of the characterizing chemical interactions were classified as a field study type. Of the biological effects analysis studies, only one of the 28 were classified as a field study type.

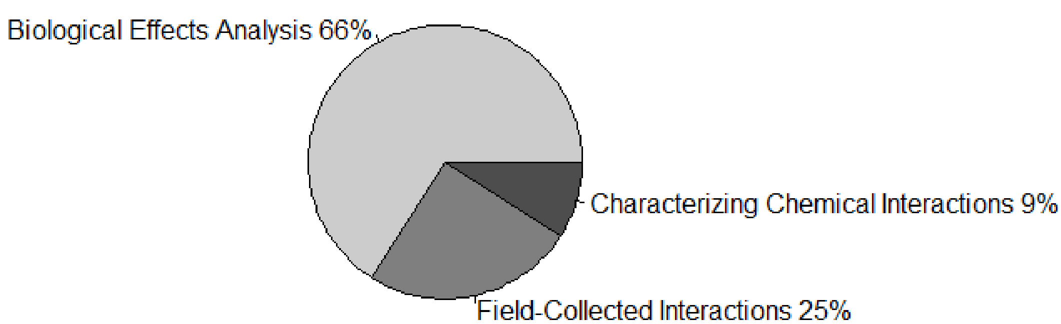


**Figure 2.** Relative proportions of microplastic-metal research studies (to June 15, 2021) classified into three broad research areas: Biological Effects Analysis, Characterizing chemical interactions, and Field-Collected Interactions (n=44 studies).

As is seen in many fields centered around pollutant-based and contaminant-based research, there is a strong pressure to relate the scientific advances to realistic ecological scenarios. As such, assessing the ecological relevance of the curated studies was another aim of my assessment of the field. As discussed with metals previously, the behaviour of contaminants is highly dependent on the environment in which that contaminant exists; therefore, the inclusion of environmental components such as sediment and biota allows for a more realistic environmental simulation. The field of microplastic-metal research was dominated by studies that had not included sediment in the experimental design. Of the studies that excluded sediment, one third of them also did not include biota – meaning that 27% of the microplastic-metals research did not include two major environmental components. The remaining two-thirds of sediment-excluded studies did include biota, all of which were "water-only" exposure routes to assess uptake and potential toxicity (**Figure 3**). Only 13% of studies included sediment in their experimental design (**Figure 3**). Of the studies including sediment, half of them also included biota – this subset comprised 7% of the total dataset, emphasizing that only a small portion of the current research included the two major environmental compartments of sediment and biota (**Figure 3**).

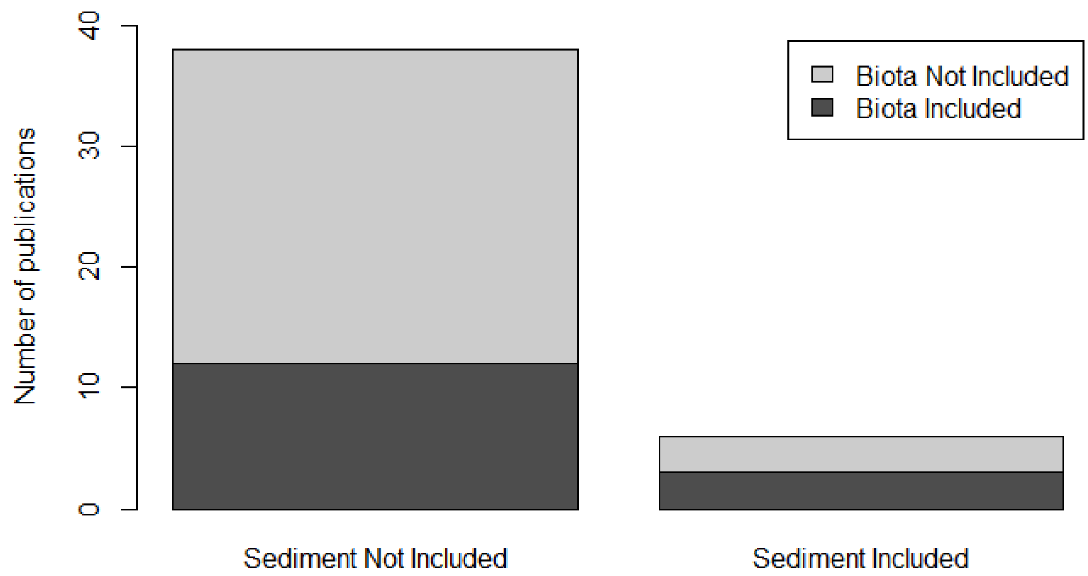


**Figure 3.** The number of microplastic-metal mixtures toxicity studies that included sediment (bars) and biota (colour) within their experimental design (n=44 studies).

In addition to the exclusion of environmental compartments, the water quality of exposure environments was not adequately characterized. Water quality characteristics are key determinants of metal speciation, partitioning (including to microplastics), and subsequent toxicity, which is elaborated on and explained in Chapter 5. I hypothesize that these aspects of experimental design were disregarded due to the complexity in their proper inclusion to the study. The difficulty in characterizing the environment is brought about due to its dynamic nature in that it is constantly changing. Many natural processes can result in altered water quality characteristics including precipitation, the changing of seasons, and alterations in flow (Ueda et al. 2000; Kawagoshi et al. 2019). These fluctuations are often exaggerated in water bodies that are influenced by anthropogenic activities such as agricultural practices and discharge of effluents (Aubert et al. 2014; Tavakoly Sany et al. 2019; Han et al. 2020). Conveniently, water bodies affected by anthropogenic inputs are also where microplastic pollution is most prevalent (Frère et al. 2017; Dai et al. 2018; Tanentzap et al. 2021).

I conducted an additional analysis, herein referred to as the Toxicity Analysis, only on the articles categorized as belonging to the biological effects analysis area of microplastic-metals research (**Figure 2**). From the 29 articles that were identified, 49 unique experimental scenarios (pairings of microplastic and metal) were assessed. The Toxicity Analysis had 4 main objectives, the first of which was to assess the characteristics of the microplastics being used. Four types of microplastics were identified: polyethylene (PE), polyethylene terephthalate (PET), polystyrene (PS), and polyvinyl chloride (PVC) (**Figure 4**). Some studies did not report the type of microplastic, in most cases this was due to the proprietary polymer composition not being released by the manufacturer. For each type of microplastic, multiple shapes of plastic were also identified, including sphere, fibre, and irregularly shaped, as shown in **Figure 4**. Polystyrene was the predominant plastic of choice in microplastic-metals experiments, comprising 39% of studies, while 63% of those studies used polystyrene spheres (**Figure 4**). This is particularly interesting as the most common types of plastics found in aquatic systems are polyethylenes, polypropylenes, and polyacrylates (Au et al. 2015; Vedolin et al. 2018). This disparity may be due to the ease of obtaining primary polystyrene microplastics compared to field-detected microplastics, which are often secondary (degraded) microplastics. The least represented of the microplastics in the analysis was polyethylene terephthalate while the least represented shape was fibrous microplastics.

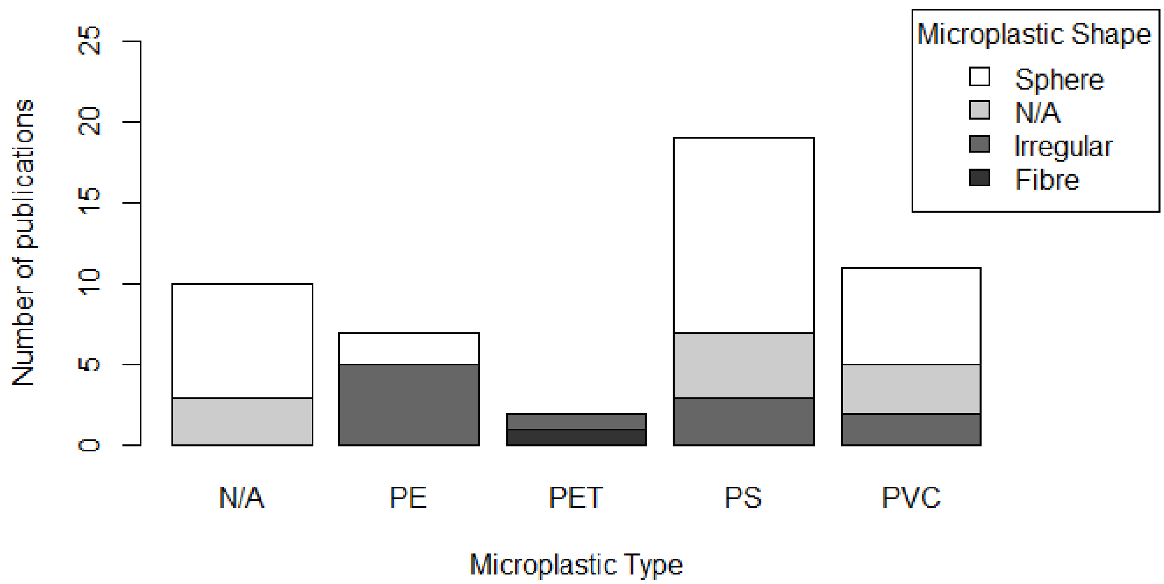


**Figure 4.** Types (bars) and shapes (colour) of microplastics used in microplastic-metal mixtures toxicity studies to June 15, 2021 (n=29). Not applicable/not listed (N/A), polyethylene (PE), polyethylene terephthalate (PET), polystyrene (PS), and polyvinyl chloride (PVC).

The size of microplastics utilized in studies included in the Toxicity Analysis ranged from 1 to 5000 µm (the entire range of the widely accepted defined range of microplastics before being classified as macroplastics (greater than 5 mm) or nanoplastics (less than 1 µm). The average microplastic size was 279.32 ± 869.22 µm (mean ± standard deviation). For some aquatic species, this size of microplastics may not be ingestible and therefore microplastic-metal complexes may not be bioavailable to those organisms.

The Toxicity Analysis also aimed to assess the metals being used in microplastic-metals research. The most used metal was cadmium (35%) followed by copper (29%) **(Figure 5A)**. Silver and lead each comprised 10% of the body of research while iron and nickel comprised 4%

each (**Figure 5A**). The remaining metals (gold, mercury, manganese, zinc) each comprised 2% of the experimental scenarios (**Figure 5A**). The concentrations of each metal used are described in **Figure 5B**. The study using iron did not report a metal concentration. More studies quantified metal presence in environmental compartments than microplastics, though 36% of studies still did not report metal concentrations in any compartment. Twenty-seven percent of studies quantified metals in both water and biota, 12% measured only sediment, 8% measured only water, and 6% measured only biota. Additionally, plastics were considered as an environmental compartment in some studies, as 8% of studies quantified metals on plastics and one study (2%) quantified metal associated with both water and plastic components.

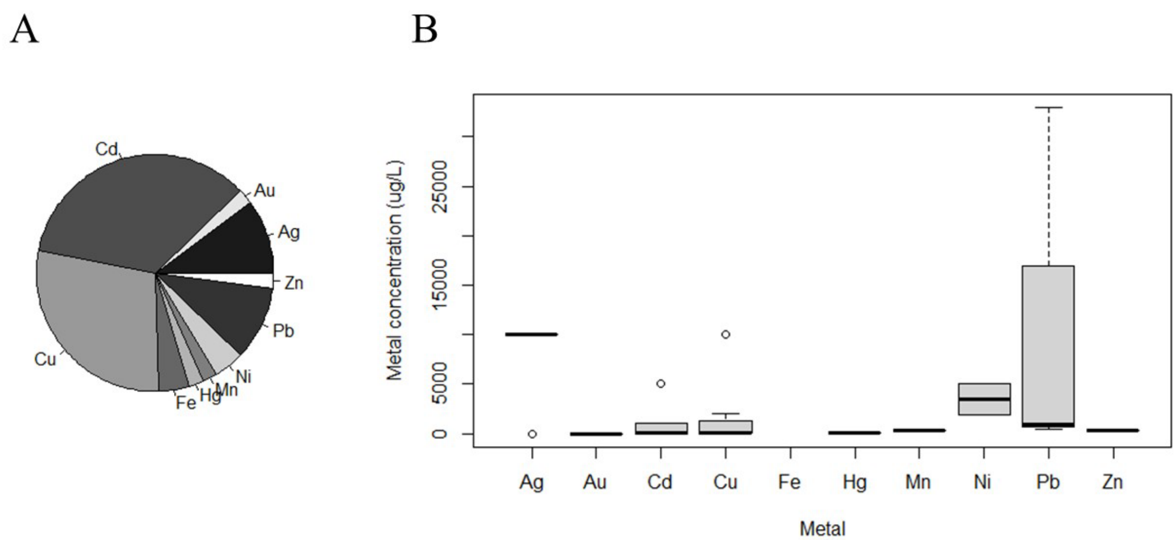


**Figure 5.** A. Relative distribution of metals used in microplastic-metal mixtures toxicity studies to June 15, 2021. B. Concentration (line represents median, box represents interquartile range, whiskers represent range, and circles represent outliers) of metal used in microplastic-metal mixtures toxicity studies to June 15, 2021. Note that iron (Fe) in Panel B did not have a reported concentration (n=29 total studies).

The third objective of the Toxicity Analysis was to determine what organisms were being used to understand the biological effects of microplastic-metal mixtures. The most utilized taxonomic group of organisms were invertebrates (38%) followed by fish and algae, each representing 27%. The least represented groups were plants (6%) and microorganisms (2%) (**Figure 6**). All but one study (utilizing microorganisms) reported the full Latin name (genus and specific epithet) of the organism used. The greatest diversity of organisms was observed in algae and invertebrates, each with five unique species represented; both fish and plant comprised two species (**Table 1**). Of the experimental scenarios included in this analysis, 27 reported a life stage for the organisms used; of these, 41% of organisms were adults, 33% were juvenile, 19% were embryos, and 7% were of a larval life stage.

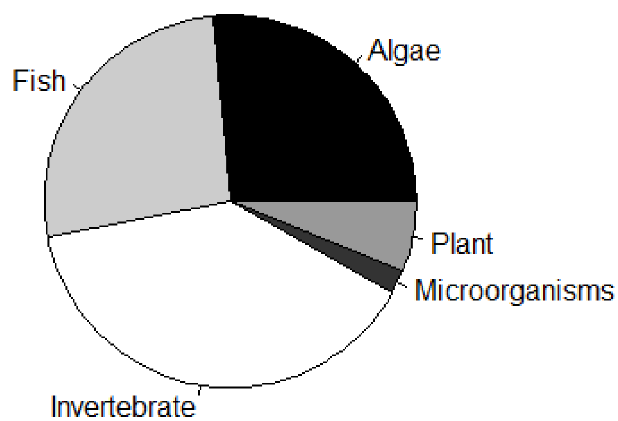


**Figure 6.** The relative proportion of taxonomic groups utilized in microplastic-metal toxicity research studies through June 15, 2021.

**Table 1.** List of species within each taxonomic groups utilized in microplastic-metal toxicity research studies through June 15, 2021.

| Taxonomic Group | Species |
|---|---|
| Algae | *Chlorella pyrenoidosa*<br>*Chlorella sp. TJ6-5*<br>*Chlorella vulgaris*<br>*Microcystis aeruginosa*<br>*Pseudokirchneriella subcapitata NIES-35* |
| Invertebrate | *Corbicula fluminea*<br>*Daphnia magna*<br>*Lymnaea stagnalis*<br>*Moina monogolica* |
| Fish | *Danio rerio*<br>*Symphysodon aequifasciatus* |
| Plant | *Lemna minor*<br>*Vallisneria natans* |

The final aim of the Toxicity Analysis was to assess the types of endpoints investigated in microplastic-metals research. Of the experimental scenarios analyzed, 82% of experiments were sublethal, meaning that the sole goal of the study was not to assess lethality. For this analysis, sublethal endpoints, herein referred to as 'endpoints' were classified into two broad categories: Contaminant Quantification and Resultant Effects. Contaminant Quantification comprised five classes of endpoints: metal uptake and bioaccumulation, microplastic uptake, microplastic retention, metal depuration, and microplastic depuration. Resultant Effects consisted of six classes: population level effects, life history effects, genetic effects, biomolecular effects, physiological effects, and behavioural effects (**Table 2**). On average, studies analysed 1.84 ± 1.21 (mean ± standard deviation) classes of endpoints in total. Within the Contaminant Quantification category, only one study assessed depuration and did so for both metal and microplastic. The maximum number of classes of endpoints measured within the Contaminant Quantification category was three (metal uptake and bioaccumulation, microplastic update, microplastic retention), completed by a single study. The remainder of the studies only analyzed contaminant uptake, with the majority (64%) of those only assessing metal uptake. The maximum number of classes of endpoints measured within the Resultant Effects category was three, which was completed by five studies (10%); ten studies (20%) reported two classes of endpoints, but the majority (65%) of reported on a single class of endpoint. Twenty percent of studies did not report any Resultant Effects endpoints.

**Table 2.** Percent of microplastic-metal toxicity studies to June 15, 2021 that measured various classes of endpoints broadly categorized into categories of contaminant quantification and resultant effects (n=29).

| Category | Class of Endpoint | Percent of studies for which this class of endpoint was measured |
|---|---|---|
| **Contaminant Quantification** | Metal Uptake and Bioaccumulation | 31 |
| | Microplastic Uptake | 12 |
| | Microplastic Retention | 2 |
| | Metal Depuration | 2 |
| | Microplastic Depuration | 2 |
| **Resultant Effects** | Population-Level Effects | 14 |
| | Life History Effects | 10 |
| | Genetic Effects | 10 |
| | Biomolecular Effects | 57 |
| | Physiological Effects | 39 |
| | Behavioural Effects | 4 |

**Study species**

The species utilized throughout this thesis encompass both model and non-model organisms spanning multiple trophic levels and differing feeding types. The species were chosen due to their ease of maintenance and/or culturing in the laboratory allowing for large enough population sizes, tolerance to varying environmental conditions, their widespread abundance and ecological relevance, and for unique physiological characteristics to address a specific research question.

***Daphnia magna***

The water flea, *Daphnia magna*, is a species widespread across the northern hemisphere, which has led to its utilization as a model organism in the fields of ecology, evolution, and toxicology (Adema 1978; Altshuler et al. 2011). There is robust knowledge of the acute and chronic toxicity of cadmium to *D. magna* (van Leeuwen et al. 1985; Bodar et al. 1990; Muyssen and Janssen 2004). The resilience of *D. magna* to a wide range of water chemistry characteristics allows for the investigation of potential toxicity under simulation conditions spanning multiple natural habitats. Previous work found that levels of dissolved oxygen (Ferreira et al. 2008), temperature (Heugens et al. 2003a), water hardness (Winner 1986), dissolved organic carbon (Cloran et al. 2010), and pH (Qu et al. 2013) all mediate cadmium toxicity to *D. magna*. Cadmium has been shown to result in lethality and altered phototactic response in *D. magna* (Semsari and Megateli 2007). As *D. magna* reside in the pelagic zone, they are an ideal species to analyze the potential toxicity of microplastics and microplastic-cadmium complexes that remain suspended in the water column, contributing to increased turbidity.

Feeding type has an impact on the potential toxicity of microplastics to aquatic organisms (Scherer et al. 2017). As *D. magna* are filter feeders, they routinely ingest suspended particles. Algae, the primary food of *D. magna*, can interact with and bind contaminants within the water column such as metals, resulting in a dietary exposure route to cadmium (Taylor et al. 1998). In the same way that contaminated algal cells can be ingested by *D. magna*, cadmium-microplastic complexes may also be taken up, potentially resulting in adverse effects on growth, survival, or reproduction.

***Nephelopsis obscura***

The predatory leech, *Nephelopsis obscura*, is widespread and abundant in North America, inhabiting a wide range of environments with varying water quality (pH, hardness, temperature) parameters. The leech shows high sensitivity to many classes of contaminants, including metals (Scrimgeour et al. 1998). Compared to other, smaller model benthic invertebrates (e.g., *Hyalella azteca*, *Daphnia* spp.), the biomass of *N. obscura* allows for the use of a single individual for each analytical sample for metal bioaccumulation and biochemical analyses, decreasing sample variability as a result of individual differences. *Nephelopsis obscura* resides in the benthic zone, interacting with both water and sediment, which are two sources from which cadmium and microplastics can be taken up.

The biology of *N. obscura* has been well described, including the mapping of serotonin-containing cells within discrete ganglia, allowing for precise measurements of serotonin (Davies and Everett 1977). In addition, the release and sequestration of serotonin in leeches has also been described, which provides an opportunity for insight as to the mechanism by which cadmium may deplete serotonin in this species (Lent et al. 1989, 1991; Glover and Lent 1991). The previously described role of body wall distention in serotonin signaling introduces an additional dimension in regards to the combined effects of cadmium and microplastics, as the accumulation of microplastics within the gut of the leech may lead to false satiation and premature downregulation of serotonin (Lent et al. 1991).

***Pimephales promelas***

The fathead minnow (*Pimephales promelas*) is an ecologically important and physiologically well-understood species that inhabits freshwater ecosystems across North America. The fathead minnow is a model organism in aquatic toxicity testing (Ankley and Villeneuve 2006). Further, metallothionein expression in this species has been characterized

(Benson and Birge 1985). Adults of this species are of sufficient size to allow for the isolation of specific internal tissues and structures, including their gills and livers in which metallothionein is expressed and their digestive tracts to allow for post-ingestion assessment of cadmium and microplastic bioaccumulation.

Fathead minnows are highly tolerant to changes in water quality conditions and can live in a wider range of water quality conditions than either previously mentioned species (Ankley and Villeneuve 2006; Rozon-Ramilo et al. 2011; Zhang et al. 2015). The versatility of this species makes them suitable to acclimate to environments of varying water quality to better understand the influence of environmental parameters on cadmium-microplastic mixtures exposure.

**Thesis objectives**

There is a great interest in understanding the effects of microplastics in aquatic systems, evident by the emerging research trends in the past decade. A small subset of this field, metal-microplastic mixtures effects in freshwater systems, has received less attention than microplastics alone but is starting to garner more interest. The objective of and approach to my research has evolved alongside the growing body of literature investigating microplastic-metal mixtures toxicity. The initial objectives of my research, included in Part 1 of this thesis, were centered around addressing knowledge gaps in the field of microplastic-metal mixtures toxicity utilizing a traditional mixtures toxicology framework: assess the effect(s) of Constituent 1 (cadmium, for example), assess the effect(s) of Constituent 2 (microplastics, for example), and assess the effect(s) of Constituents 1 and 2 together. The outcomes of this approach can broadly be categorized as: (1) addition, in which the toxic effects of a mixture is equal to the toxic effects of each substance comprising the mixture, (2) synergism, in which the exposure to the mixture results in toxic effects that are greater than the sum of each individual substance's effects (i.e., a more-than-additive effect), (3) antagonism, scenarios in which the mixture causes less-than-additive toxic effects, and (4) potentiation, in which one substance increases the toxicity of another. This approach has been taken in the vast majority of studies published in the field of microplastic-metal mixtures toxicity. Three study scenarios were designed that address knowledge gaps determined by my initial assessment of the field.

Firstly, as cadmium and microplastics both exist in multiple environmental compartments including water, sediment, and biota; but the latter two compartments have been broadly disregarded in the field thus far, my first research objective was to discern whether cadmium was more readily taken up from water or sediment when microplastics were present. By discerning the primary route of cadmium uptake, I could then perform an exposure to cadmium, microplastic, and their mixture from the primary source (water- or sediment-borne) to assess bioaccumulation (addressing the third environmental compartment – biota) and subsequent recovery. For this series of experiments, it was important to utilize a species that interacts with both the water and sediment; as such, the predatory leech *Nephelopsis obscura* was selected.

Secondly, based on the observed ingestion of microplastics by *N. obscura* (Chapter 2) and information from the primary literature, it became apparent that understanding the cadmium and microplastic ingestion and potential internalization and bioaccumulation was a priority. As the body of literature to date lacked the ability to differentiate between bioaccumulation and internalization, I prioritized using tissue isolation techniques to address this. I utilized isolated digestive tracts of fathead minnows (*Pimephales promelas*) in an *in vitro* technique to investigate the transport of cadmium across the gut barrier following the simulated ingestion of cadmium, microplastics, and cadmium bound to microplastics. As part of this objective, I assessed whether any cadmium that crossed the gut membrane would adsorb to plastics in the serosal space by simulating the pre-existence of microplastics in the serosal space. A secondary objective was to assess the movement of microplastics across the gut membrane to determine if internalized plastics observed by other researchers may have occurred post-ingestion.

Once the route of uptake and internalization of ingested contaminants had been investigated, my next objective was to understand the multigenerational effects of exposure to cadmium, microplastic, and their mixture as both cadmium and microplastics are persistent pollutants of aquatic environments and chronic exposures provide a more ecologically relevant scenario. As the water flea, *Daphnia magna*, has a short generation time and is pelagic, it was selected as a suitable species for this investigation. Alterations in feeding, growth, and life history traits were assessed for daphnids exposed to cadmium, microplastics, or their mixture.

When the results of the aforementioned studies were situated into the growing body of literature surrounding microplastic-metal mixtures toxicity, it became apparent that there were a multitude of contradictions in the field. Specifically, conclusions about whether microplastic-metal mixtures (primarily the microplastic component, as the metals utilized were generally known toxicants) were toxic – including between the experiments I had performed and the works of other researchers. Not all instances of research quantified the bioaccumulation of microplastics; however, many articles in this field hypothesize that bioavailability (or lack thereof) may be the reasoning behind observing (or not observing) toxicity. In contrast, when assessing the bioavailability of the metal component in a microplastic-metal complex, the bioavailability is partially determined by the amount of metal bound to plastic and the stability of that association. Rather than continuing to contribute to an emerging field of research that lacked harmonization and was already riddled with contradicting conclusions, my research objectives shifted to attempting to resolve these contradictions, outlined in Part 2 of this thesis.

The overwhelming majority of microplastic-metals toxicity research had been performed by researchers with expertise in working with microplastics rather than metals, and appeared to lack an understanding of water quality effects in their experimental design; therefore, I leveraged my understanding of how water quality affects metal behaviour to assess whether contradictions may be due to the speciation and partitioning of the metal. This is outlined in Chapter 5. By performing a meta-analysis of microplastic-metals toxicity research, it became apparent that water quality was not being adequately characterized or reported in this research field. As changes in water quality have immense influence on metal speciation, partitioning, and toxicity, I hypothesized that differences in water quality may be a potential explanation for the observed contradictory conclusions in the field.

To test this hypothesis, I had to change the framework with which I approached the field. Rather than utilizing a traditional mixtures toxicology approach with microplastic-metal mixtures, I shifted to consider microplastics as a ligand for metals. As with other ligands (sediment or organic matter, for example), the association of metal-ligand complexes is governed by ambient water quality characteristics.

As full water quality characterization (including measuring the ionic composition of surface water for all major ions, quantifying and characterizing organic matter and sediment) is not feasible in many instances, the goal of the fifth, and final, study was to discern which water quality parameters were of greatest priority to be measured. I utilized a mixture of traditional laboratory experiments and machine learning techniques to develop a predictive model that ascertained the relative importance of each measured water quality variable in determining the amount of cadmium bound to microplastic. This model was then tested using fathead minnow (*Pimephales promelas*) to relate the amount of associated cadmium with bioaccumulation of cadmium and metal-induced alterations in expression of metallothionein.

Collectively, the studies presented in this thesis represent the identification of a disparity in an emerging field of research – that conclusions around toxicity of microplastic-metal mixtures are contradictory – and subsequently proposes a solution to these contradictions: reframing from a traditional mixtures toxicity approach to a ligand approach and leveraging our understanding of water quality to better characterize and predict the effects of microplastic-metal mixtures in freshwater systems. Through harmonization in reporting requirements, this work promotes metal-microplastic mixtures toxicity research to progress in a way in which results can be corroborated and, with continued growth of the field, drive policy development and inform environmental remediation efforts.

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## PART 1: ADDRESSING KNOWLEDGE GAPS IN METAL-MICROPLASTICS TOXICITY RESEARCH WITH A TRADITIONAL MIXTURES TOXICITY APPROACH

Mixtures toxicology aims to understand the biological effects of multiple chemicals present simultaneously in an exposure scenario. Mixtures toxicity research traditionally takes on a basic framework of assessing the effect(s) of Constituent 1 alone, the effect(s) of Constituent 2 alone, and then the effect(s) of Constituent 1 and 2 together. The current theories and practices used in mixtures toxicology vary depending on the behaviour of the substances comprising the mixture, including their partitioning and persistence within an environment. Both metals and microplastics are found in multiple environmental components, such as sediment, water, and biota. Metals and microplastics have been found to interact with each other through the sorption of metals to microplastics in the environment.

Part 1 of my thesis is composed of 3 chapters, each a separate experimental study aimed to address a knowledge gap in the field of microplastic-metal mixtures toxicity research using a traditional mixtures toxicity framework.

## CHAPTER 2: Single and combined effects of cadmium, microplastics, and their mixture on whole-body serotonin and feeding behaviour following chronic exposure and subsequent recovery in the freshwater leech, *Nephelopsis obscura*

In this chapter, I define three research objectives: (1) to determine whether cadmium is more readily taken up from water or sediment, (2) to utilize that primary source (either water- or sediment-borne) in a 21-day exposure to assess uptake and subsequent toxicity (measured through feeding behaviour and associated biochemical changes), and (3) to determine if recovery is observed following transfer to a non-contaminated environment for 7-days. The predatory leech, *Nephelopsis obscura* was utilized in this study.

We determined that leeches more readily accumulate Cd from water than from sediment. Behaviourally, we observed that the amount of accumulated cadmium shows inverse trends in relation to total distance moved, suggesting that the amount of bioaccumulated cadmium influences individual activity. The same trend was observed in relation to the amount of whole-body serotonin, suggesting cadmium is likely acting as a direct serotonin inhibitor. Microplastics also inhibited serotonin and it is hypothesized microplastic ingestion is likely causing distention of the body wall, which is a mechanical feedback mechanism to decrease serotonin.

This thesis is a manuscript-style thesis which is organized based on the University of Lethbridge thesis submission regulations. Inevitably, there is some repetition of content between sections, particularly in the introduction and methods sections of research chapters. A version of this chapter has been published in *Aquatic Toxicology*:

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The author contributions for this manuscript are as follows, in accordance with the CRediT Author Contributions system:

Lauren Zink: conceptualization, methodology, investigation, writing – original draft, writing – review & editing, visualization, funding acquisition

Steve Wiseman: supervision, writing – review & editing

Gregory G. Pyle: conceptualization, supervision, writing – review & editing, funding acquisition

**Abstract**

Microplastics and metals are contaminants detected in many freshwater systems globally. Interactions of microplastics with other contaminants including cadmium poses potential threats to the health of aquatic organisms including *Nephelopsis obscura*, a predatory leech species that is widespread and serves important ecological and economic roles. The feeding biology of *N. obscura* has been well-described, including that serotonin regulates feeding behaviour. Further, exposure to cadmium has been found to cause decrease whole-body concentrations of serotonin. The influence that microplastic contamination and co-contamination of cadmium and microplastics has on *N. obscura* is unknown. The present study had three objectives: (1) to determine if water or sediment contaminated with cadmium, microplastics, or their mixture resulted in greater cadmium uptake by *N. obscura*, (2) to assess effects of chronic (21-day) exposure of *N. obscura* to waterborne cadmium, microplastics, and their mixture on bioaccumulation of cadmium, concentrations of serotonin, and feeding behaviour (latency to feeding, time spent feeding, and distance moved), and (3) to reassess the bioaccumulation of cadmium, concentrations of serotonin, and feeding behaviour following transfer to an uncontaminated environment for a one-week recovery period. This study revealed that access to and presence of sediment is protective against cadmium uptake and that cadmium is more readily accumulated from waterborne sources, even in environments where both sediment and surface water are contaminated. After 21-days of exposure to waterborne cadmium, microplastics, and their mixture, accumulation of cadmium, decreased concentrations of serotonin, and impaired feeding behaviours were greatest in leeches from the co-exposures compared to leeches from either single contaminant exposure group. Finally, after one week of depuration and recovery in freshwater following the 21-day exposures, concentrations of serotonin and feeding behaviour were restored in individuals from the microplastic exposure; however, cadmium-exposed

individuals continued to show decreased concentrations of serotonin and behavioural deficits. The co-exposure of leeches to cadmium and microplastics resulted in additive effects to serotonin synthesis and feeding behaviour; however, this study demonstrated that leeches were able to recover from microplastic toxicity within a week whereas cadmium toxicity persisted.

**Introduction**

In recent years, there has been an increase in amounts of microplastics detected in aquatic ecosystems, driven by the expansion of plastic usage in everyday products (Li et al. 2018; Wang et al. 2021a; Lofty et al. 2022). The diverse composition of microplastics results in their occurrence in both the water and sediment components of freshwater systems, prompting potential interactions with pelagic and benthic organisms. Plastics suspended in the water column have a higher rate of transportation than those embedded in sediments; however, seasonal hydrodynamic parameters such as changes in water flow can result in the settling of suspended microplastics and the resuspension of sunken microplastics (Besseling et al. 2016, Nel et al. 2018).

While ingested microplastics alone might not induce toxicity, the possible sorption and subsequent release of harmful substances such as metals may enable microplastics to serve as vectors for the uptake of known toxicants (Besseling et al. 2013; Brennecke et al. 2016). The physical properties of microplastics, namely their large surface area-volume ratio and hydrophobicity enable microplastics to serve as a substrate for waterborne contaminants, often resulting in higher contaminant concentrations on the surface of the microplastic than in the surrounding environment (Holmes et al. 2014; Seidensticker et al. 2019; Li et al. 2019).

Metals are ubiquitous in freshwater environments and pose potential risks to the health of aquatic organisms. Cadmium is a metal of interest in toxicology and regulatory fields and has been listed as a contaminant of concern by many agencies (National Research Council 1997; Brown 2016; Environment Programme 2017). Widespread use of cadmium in industrial applications has resulted in varied levels of cadmium being detected in surface waters and sediments of urban and industrialized areas (Street 2002). Cadmium does not serve a biological function and very few organisms have evolved systems for regulating cadmium ions, resulting in the potential for bioaccumulation, particularly when individuals are chronically exposed (Borgmann et al. 1991; Eimers et al. 2002; Mebane et al. 2020).

Exposure to cadmium causes adverse effects in freshwater invertebrates, including reproductive impairment, decreased activity, and impairment to the function of neurotransmitters (Salánki and Hiripi 1990; Gill and Epple 1992; Almeida et al. 2003; Wu et al. 2015). Serotonin, or 5-hydroxytryptamine, is a neurotransmitter that has many functions in aquatic invertebrates including the regulation of muscle contraction and feeding behaviour (Salánki and Hiripi 1990; Almeida et al. 2003). Studies have demonstrated that cadmium can decrease concentrations of serotonin, although the mechanism is not fully understood and appears to vary among species (Salánki and Hiripi 1990; Almeida et al. 2003; Wu et al. 2015). A study of the serotoninergic pathway of planarians demonstrated that cadmium appears to alter the activity of monoamine oxidase, an enzyme that metabolizes serotonin (Wu et al. 2015). Experiments using mussels suggest that cadmium might interfere with neurotransmission by stimulating premature release of serotonin from its storage location within the ganglia (Salánki and Hiripi 1990; Almeida et al. 2003). In freshwater leeches, serotonin is associated with feeding behaviour through inhibiting serotonin synthesis (Lent and Dickinson 1984, 1988; Lent et al. 1991).

Leeches are globally abundant and inhabit a wide range of environments, including those that are polluted (Phillips et al. 2020). Leeches have been shown to bioaccumulate cadmium at concentrations orders of magnitude greater than those measured in the surrounding surface water and sediment (Alaama et al. 2021). For example, leeches living in a lake with surface water cadmium levels around 2 µg Cd/L had tissue concentrations of 150 µg Cd/L (Alaama et al. 2021). Leeches have been shown to survive and reproduce in waters containing 50 µg Cd/L and have been proposed to be used as indicators of toxicity due to their high tolerance to contaminants, including cadmium (Davies et al. 1995; Scrimgeour et al. 1998). Predatory leeches utilize mechanoreception to detect prey, typically detreital material (Sawyer 1986). Leeches do not demonstrate the ability to discriminate between types of prey and are therefore susceptible to mistakenly ingest unintended particulates, such as microplastics (Blinn and Davies 1989).

*Nephelopsis obscura,* a North American predatory leech, resides in the benthic zone of freshwater bodies, interacting with both water and sediment, which are two sources from which cadmium and microplastics can be taken up. The biology of *N. obscura* has been well described, including understanding feeding behaviour and the mapping of serotonin-containing cells (Davies and Everett 1977). Leeches are intermittent feeders that can go for periods of three to five months between gorge meals (Sawyer 1986). Serotonin is found within the ganglia of leeches and is used as a biomarker for satiation state, with hungry leeches consistently having higher concentrations of serotonin than their satiated equivalents (Lent et al. 1991). As the leech ingests food and its gut becomes full, the body wall distends and the stretching of these muscles, signaling that the leech is satiated, results in the downregulation of serotonin synthesis (Lent et al. 1991). The role of body wall distention in regulation of serotonin signaling introduces an additional dimension in regards to the combined effects of cadmium and microplastics, as the accumulation of microplastics within the gut of the leech may lead to false satiation and premature downregulation of serotonin synthesis (Lent et al. 1991).

The first objective of this study was to determine if water or sediment contaminated with cadmium, microplastics, or their mixture resulted in greater uptake of cadmium by *N. obscura*. The second objective was to perform a chronic (21-day) contaminant exposure to the primary source (waterborne or sediment-borne) and assess changes in bioaccumulation of cadmium, concentrations of serotonin, and feeding behaviour. The third and final objective of this study was to determine if *N. obscura* showed signs of recovery one-week after concluding contaminant exposure as it relates to bioaccumulation of cadmium, concentrations of serotonin, and feeding behaviour following transfer to an uncontaminated environment.

## Methods

### *Organism husbandry*

Adult *Nephelopsis obscura* (species confirmed using a dichotomous key (Klemm 1982)) were wild-caught throughout Southern Alberta and housed in holding tanks (16 hour light: 8 hour dark photoperiod, 20 °C) at the University of Lethbridge Aquatic Research Facility (ARF). Leeches were maintained in ARF culture water, the water quality for which is shown in . Conductivity and pH were measured using an Oakton Pocket Tester (Oakton PCTSTestr5). Alkalinity and hardness were measured using the titration method described by the American Public Health Association (American Public Health Association 1992). Dissolved organic carbon was quantified via high temperature catalytic oxidation on a Shimadzu TOC-L CHP (Kyoto, Kyoto, Japan). The water supply to the ARF is piped directly from the City of Lethbridge then dechlorinated using activated carbon filters. Next, large particles are removed by filtration through cartridge filters. An aragonite filter is then used to buffer and maintain the pH of the water at approximately 8. Finally, water is sterilized using a UV lamp before use in culturing and experimentation.

**Table 3.** Water quality characteristics of Aquatic Research Facility (ARF) culture water. Ranges presented are of weekly measurements of pH, conductivity, hardness, and alkalinity (n = 40) and biweekly measures of dissolved organic carbon (DOC, n = 20) taken over the course of the current study.

| **Parameter** | **Value (range)** |
|---|---|
| pH | 7.98 – 8.10 |
| Conductivity at 20 °C | 315 - 360 µS/cm |
| Hardness | 127 - 135 mg/L as $CaCO_3$ |
| Total Alkalinity | 118 - 127 mg/L as $CaCO_3$ |
| DOC | 2.32 – 2.56 mg/L |

Leeches were acclimated in the ARF for at least three months prior to use in experimentation. Acclimation tanks consisted of approximately 50 leeches held in 60 L of culture water (approximate stocking density of 0.03 g/L) in a 75 L tank with a 4 cm deep substrate bed of aquarium sand. Tanks were aerated and water was filtered using two corner filters (Marina, #10894, Mansfield, Massachusetts, USA) layered with activated carbon (Marina, #11293, Mansfield, Massachusetts, USA), polywool (Marina, #11313, Mansfield, Massachusetts, USA), and quartz beads (Top Fin, TF-17-170370, Atlanta, Georgia, USA) that were connected to a compressed air supply. An 80% water change was completed twice weekly. Upon arrival, leeches were fasted for four days after which they were fed a mixture of frozen *Daphnia magna* (from in-house laboratory cultures), frozen bloodworms (Omega One, #5210921, Blacksburg, Virginia, USA), and insect larvae pellets (Fluval Bug Bites, Bottom Feeder Formula, S/M Sinking Granules, Mansfield, Massachusetts, USA) *ad libitum* weekly and allowed to feed for six hours at which point a water change was completed to remove uneaten food and maintain water quality.

***Preparation and analysis of test chemicals***

A stock solution of cadmium (2 mg/L Cd) was prepared by dissolving cadmium sulfate octahydrate (CAS 7790-84-3) in Milli-Q (EMD Millipore, Burlington, Massachusetts, United States) water. The stock solution was used to dose the surface water exposure solutions to 50 μg/L Cd in processed water. The concentration of cadmium was selected based on preliminary studies (data not shown) in which indications of sublethal toxicity was observed through behavioural analysis. Sediment was spiked with Cd for use in the sediment exclusion study by adding surface water exposure solution to sieved sand (particle size 710 – 1000 μm) in a ratio of 1 gram of sediment: 1.5 mL of surface water exposure solution. This ratio allowed for saturation of the sediment with minimal overlying water. The sediment-water mixture was then mixed continuously in a sealed vessel for 24 hours to allow for equilibrium, the time for which had been ascertained from preliminary studies (data not shown). This method allowed for the dissolved porewater concentration of cadmium in the sediment to be approximately equal to the cadmium concentration in the overlying surface water (Selck et al. 1998).

Concentrations of cadmium in the stock solution, exposure solutions, and sediment were confirmed using graphite furnace atomic absorption spectroscopy (GTA 120, Agilent Technologies) utilizing manufacturers specifications outlined in SpectrAA software (Agilent Technologies), with minor modifications of a 90 second ash time, and an additional rinse step added between samples. A certified reference material (CRM, custom-made cadmium standard, Delta Scientific, Mississauga, Ontario, Canada) was run every ten samples to ensure the accuracy of the analysis was maintained over 90%. Each analytical sample and CRM was run in duplicate.

The detection limit for Cd previously established using this method was estimated according to established protocols to be 0.07 μg/L; samples below the detection limit were taken as zero (Royal Society of Chemistry 1987).

High-density polyethylene microplastics (MP) were utilized in this experiment (MPP-620VF, MicroPowders, Inc., Tarrytown, New York, United States of America). The reported characteristics of this product from the manufacturer are: density of 0.96 g/mL at 25°C, mean particle size 5.0-7.0 μm, National Printing Inks Research Institute grind 2.0-3.0, melting point 114-116 °C. To simulate microplastic distribution observed in freshwater systems, microplastics were artificially weathered by agitating 5 g of MP and 25 mL of Tween20® (CAS 9005-64-5) in 5 L of Milli-Q (EMD Millipore) water for 24 hours on a magnetic stir plate at 1200 rpm at room temperature, as has been previously described (Balakrishnan et al. 2019). Microplastics were isolated by vacuum filtration through a 0.45 μm nitrocellulose filter and subsequently rinsed twice with 2 N trace-metal grade nitric acid (CAS 7697-37-2) and three times with Milli-Q (EMD Millipore) water. Microplastics were dried in an oven at 60 °C to constant weight prior to being resuspended in culture water.

***Experimental design: Sediment exclusion study***

As leeches come in contact with both surface water and sediment, a sediment exclusion assay was designed to determine the relative contribution of water and sediment (and associated porewater) as sources of cadmium accumulation in the presence and absence of co-exposure with microplastics. *Nephelopsis obscura* adults were randomly assigned to one of four treatments: laboratory control (LC, no contaminants), 50 μg Cd/L surface water and 8.7 μg Cd/g sediment (translating to ~50 μg Cd/L in porewater to establish surface- and pore-water cadmium equilibrium) (CD), 0.06 g microplastics /L water (MP), or the mixture treatment of 50 μg Cd/L surface and porewater cadmium and 0.06 g microplastics/L water (CDMP). Each treatment had three groups where leeches either had access to sediment (access), did not have access to sediment (blocked), or sediment was not present (no sediment). Leeches in the blocked groups were contained in a 1.22 mm fibreglass mesh basket that allowed the leeches to interact only with contaminants dissolved in the overlying water (**Figure 7**). It was confirmed prior to the experiment that the mesh barrier did not alter contaminant concentrations or distribution within the exposure system. Individuals in the access groups did not have a barrier and were able to interact with the sediment.

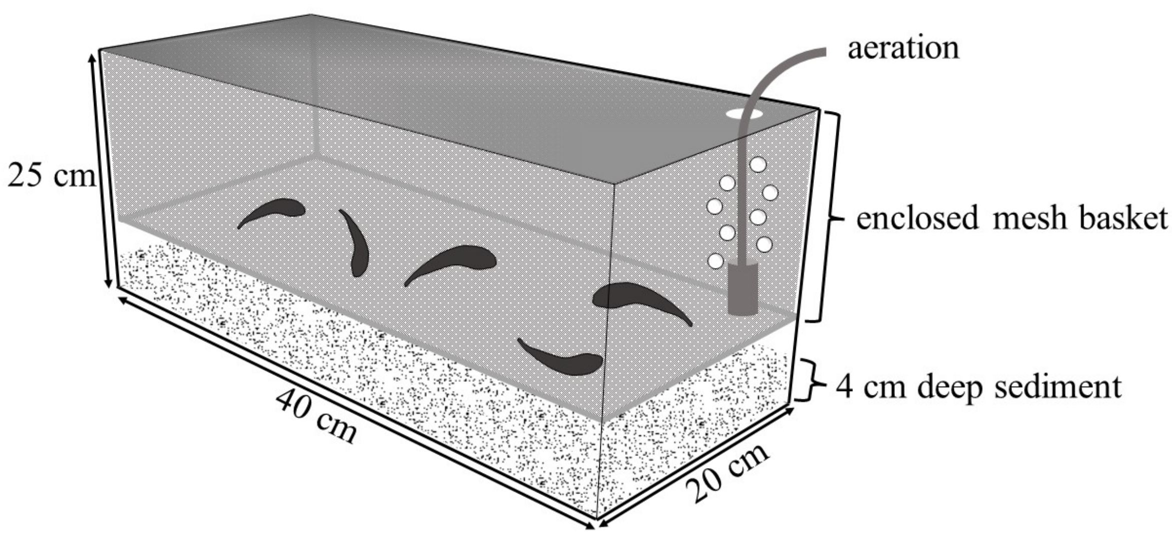


**Figure 7.** Test vessel used in the sediment-exclusion study. *Nephelopsis obscura* were contained within a fibreglass mesh basket which did not come in contact with the underlying sediment. The vessel remained gently aerated.

Leeches were starved for four weeks prior to the start of the experiment at which point they were transferred from acclimation tanks (as previously described) to sediment exclusion test vessels (21 L glass aquaria). Five leeches were transferred to each vessel all filled with culture water and maintained on a 16:8 light cycle. Leeches were acclimated to sediment exclusion vessels for 48-hours prior to the addition of contaminant(s). Three replicates of each of the treatment groups (a full factorial design of the four treatments – LC, CD, MP, CDMP and three groups – access, blocked, no sediment) were established, totalling 180 leeches used in the sediment-exclusion study. Leeches were not fed for the duration of the 21-day exposure. Ammonia was measured twice per week and remained below 0.05 mg/L. Twenty percent water changes were completed twice per week throughout the exposure. Immediately before and approximately two hours after each water change, water samples were taken to confirm stability of waterborne cadmium concentration in the experiment, which was maintained within 5% of the nominal concentration at all times.

After the exposure, all leeches were rinsed in 50 μM ethylenediaminetetraacetic acid (EDTA) solution for one minute to remove surface-bound metals (Norwood et al. 2007). Immediately following EDTA rinse, leeches were dried to constant weight at 60 °C. The dry weight of each individual was measured and recorded to be used in cadmium bioaccumulation analysis. Whole-body cadmium concentration analysis was determined using the same GFAAS method previously described using DOLT-4 (Government of Canada) as a certified reference material.

***Experimental design: Chronic exposure***

One hundred leeches were starved for four weeks then transferred from acclimation tanks (as previously described) to 21 L glass aquaria filled with 20 L of culture water in groups of five per aquarium. No sediment was utilized in this study. Aquaria were maintained on a 16:8 light cycle and gently aerated. Leeches were acclimated to the aquaria for 48-hours prior to the addition of contaminant(s). Five replicate aquaria were established for each treatment (LC, CD, MP, CDMP). Leeches were not fed for the duration of the 21-day exposure. Twenty percent water changes were completed twice per week throughout the exposure. Immediately before and approximately two hours after each water change, water samples were taken to confirm stability of cadmium concentration in the experiment, which was maintained within 5% of the nominal concentration at all times. Turbidity measurements were taken weekly and maintained within 5 Nephelometric Turbidity Units from the last measurement for each tank as an indirect measure of stability of microplastic concentration throughout the experiment. Additionally, pH, conductivity, hardness, and alkalinity were measured weekly and DOC measured biweekly to ensure there were no shifts outside the normal variation of culture water as shown in **Table 3**. Ammonia was measured twice per week and maintained below 0.05 mg/L.

After exposure, four leeches from each treatment were randomly selected and immediately frozen to -80 °C for serotonin quantification analysis. Whole body serotonin was quantified using the General Serotonin ELISA Kit (MyBioSource, Catalogue No. MBS288208 Competitive) following the manufacturer's instructions for whole tissue analysis. Then, four leeches from each tank were transferred from their exposure tank to a behavioural arena (**Figure 8**). The behavioural arena was constructed from smooth acrylic and filled with 30 L of 20 °C ARF culture water prior to each behavioural trial. Leeches were allowed to acclimate to the arena for one hour prior to food (insect larvae pellets, as previously described) being added to the feeding target via the food delivery tube (**Figure 8**) to eliminate human interference and minimize water disturbance. Acclimation time was determined through preliminary studies and established by when the velocity of the leeches remained stable for five consecutive minutes (unpublished), indicating the subsiding of darting behaviour. The entire behavioural arena was video recorded for one hour after food introduction. This process was repeated for each of the five tanks per treatment. Video files were analyzed using EthoVision XT15 (Noldus Information Technology, Wagenigen, Gelderland, Netherlands) video analysis software to determine the latency of leeches to visit the feeding target, the number of visits to the feeding target, the amount of time spent at the feeding target, and total distance moved.

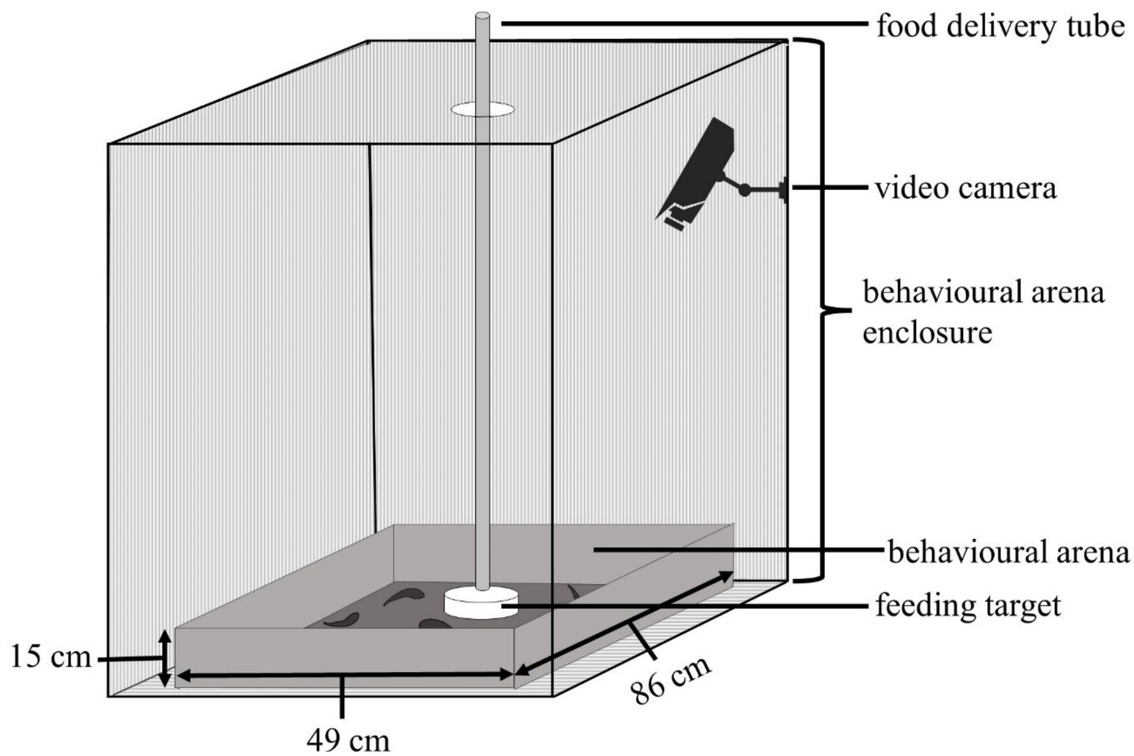


**Figure 8.** Behavioural arena used to assess foraging behaviour of leeches through introduction of insect larvae pellets through the food delivery tube to the feeding target (glass dish).

Following the behavioural assay, eight individuals from each treatment were randomly selected to be used in cadmium bioaccumulation analyses (due to the limited number of leeches available, microplastic accumulation was not assessed). The selected leeches were rinsed in 50 μM EDTA and dried to constant weight at 60 °C. The dry weight of each individual was measured and recorded. Whole-body cadmium concentration was determined using the same

GFAAS method previously described. The remaining twelve individuals in each treatment were then transferred to new tanks for use in a one-week recovery experiment.

***Experimental design: Recovery***

The aforementioned twelve individuals from each treatment were divided into three groups of four and transferred to clean 21 L glass aquaria filled with 16 L of culture water to maintain the same stocking density of previous experiments. Aquaria were held on a 16:8 light cycle. An 80% water change was completed on days 2, 4, and 6 of the one-week recovery period.

At the end of one week, four individuals randomly selected from each treatment were immediately frozen to -80 °C for quantification of serotonin as previously described. The remaining eight individuals from each treatment were transferred to the behavioural arena and the same behavioural assay protocol previously described was carried out, but with two groups of four leeches per treatment. Following the behavioural assays, all leeches from the behavioural arena were dried to a constant weight at 60 °C, which was recorded, and whole body concentrations of cadmium were quantified using GFAAS as previously described.

***Statistical analysis***

Statistical analyses were conducted in R, version 4.2.1 (R Core Team, 2022). Parametric assumption tests for normality and for homogeneity of variance were completed using a Shapiro-Wilks test and a Bartlett Test, respectively. For the sediment exclusion study, a two-way Analysis of Variance (ANOVA) was completed. For the chronic exposure and recovery experiment, the same parametric assumption tests were run. In instances where assumptions were met, a one-way Analysis of Variance (ANOVA) was run with a Tukey's post host test. In instances where parametric assumptions were not met, even after data transformation attempts, Kruskal-Wallis tests were used with a Dunn's post hoc test.

## Results

### *Sediment exclusion study*

The presence of sediment and whether leeches had the ability to interact with that sediment significantly affected the amount of cadmium accumulated by leeches ($F(2, 42) = 31.08$, $p < 0.001$, **Figure 9**). Leeches that had the ability to contact with and burrow into sediment (access group) accumulated the least amount of cadmium (2.3 ± 1.4 mg/kg dry weight, mean ± standard deviation (sd)). Leeches that were blocked from accessing the sediment accumulated 141% more cadmium than the access group, and leeches from exposures that that did not include sediment accumulated the most cadmium, an additional 44% increase in cadmium compared to the blocked group.

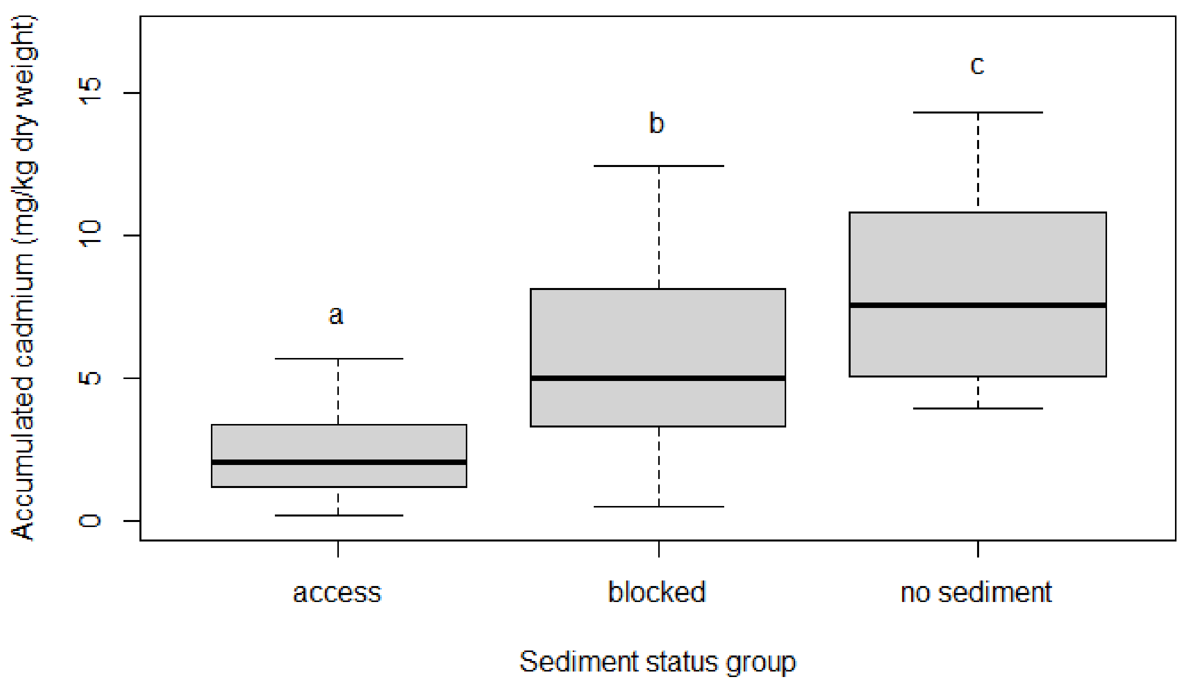


**Figure 9.** Changes in cadmium uptake by *N. obscura* when sediment was present and accessible (access), present but inaccessible (blocked), and in the absence of sediment (no sediment). Differences among treatments were analyzed by using a one-way Analysis of Variance test with a Tukey's post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the median, and the whiskers represent the minimum and maximum values.

Treatments sharing the same letter designation were not significantly different from one another ($p > 0.05$; $n = 15$).

Concentrations of cadmium in leeches from treatment groups that did not contain cadmium (LC and MP) were less than the limit of detection. There was no significant difference in the amount of cadmium accumulated between the cadmium-only (CD) or mixture of cadmium and microplastic (CDMP) treatments ($F(1, 87) = 3.9$, $p = 0.1$). The interaction of sediment status and treatment was insignificant.

***Chronic exposure***

Individuals in the LC and MP treatments did not accumulate any detectable cadmium over the course of the chronic exposure. The amount of cadmium accumulated by individuals in CD and CDMP treatments differed significantly ($H(1) = 7.38$, $p = 0.01$, **Figure 10**). Leeches exposed to the mixture of cadmium and microplastics (CDMP) accumulated 63% more cadmium than those exposed only to cadmium (CD) (**Figure 10**).

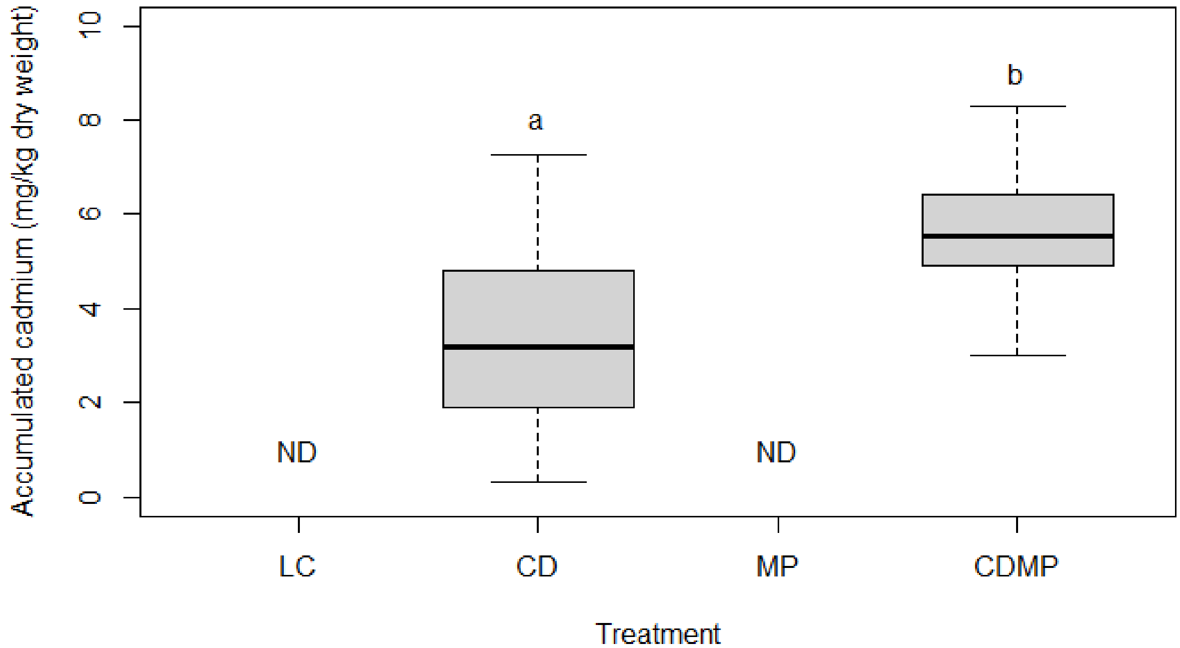


**Figure 10.** The amount of cadmium accumulated by *N. obscura* during a 21-day exposure to cadmium (CD) and cadmium-microplastic mixture (CDMP). No detectable cadmium (ND) was reported in the control (LC, no contaminants) or microplastics-only exposed individuals (MP). Differences among treatments were analyzed by using a Kruskal-Wallace test with a Dunn's post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the media, and the whiskers represent the minimum and maximum values. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; n=8).

The treatment to which an individual was exposed significantly affected the concentration of serotonin in their body ($F(3, 12) = 21.37$, $p < 0.001$). Leeches in the control group (LC) had the highest serotonin concentrations (22.4 ± 2.7 ng/L serotonin, mean ± sd, **Figure 11**). Concentrations of serotonin in microplastic-exposed individuals (MP) was 44% less than in control (LC) animals, but this difference was not statistically significant. Cadmium-exposed (CD) individuals had 75% less serotonin than LC and 55% less serotonin than MP (**Figure 11**). Individuals co-exposed to both cadmium and microplastics (CDMP) had the least amount of serotonin, 95% less than LC.

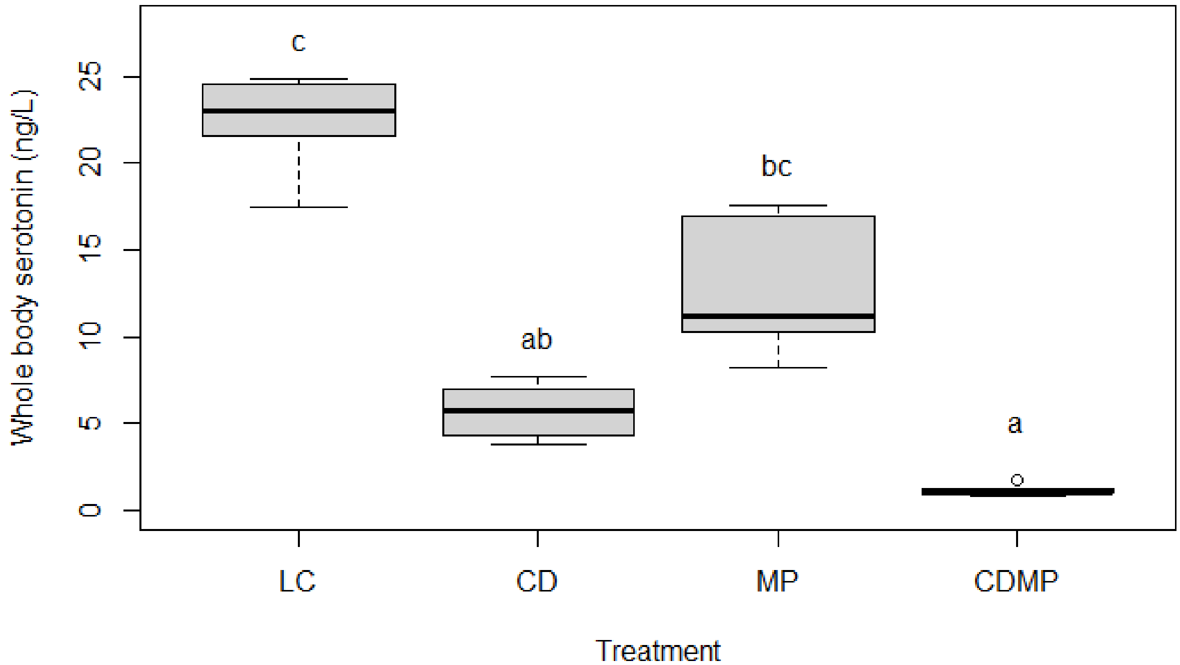


**Figure 11.** Whole-body serotonin concentrations following a 21-day exposure to cadmium (CD), microplastics (MP), cadmium and microplastics (CDMP), or not exposed to any contaminants (LC). Differences among treatments were analyzed by using a one-way Analysis of Variance test with a Tukey's post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the media, and the whiskers represent the minimum and maximum values. Circles represent outliers. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; $n = 4$).

There were differences in certain behaviours as a function of the treatment to which individuals were exposed. The exposure treatment did not affect the latency of leeches to first visit the feeding target ($H(3) = 0.29$, $p = 0.9$). The number of visits to the feeding target was affected by the treatment to which an individual was exposed ($H(3) = 67.7$, $p < 0.001$). Leeches not exposed to any contaminants (LC) visited the feeding target 205% more than leeches from the MP treatment, 1029% more than leeches exposed to CD, and 2533% more than leeches exposed to CDMP (**Figure 12A**). There was not difference in the effect of the two cadmium-containing treatments, CD and CDMP ($p = 0.2$, **Figure 12A**).

The treatment to which leeches were exposed affected the total amount of time spent feeding during the behavioural assays ($H(3) = 34.24$, $p < 0.001$). Leeches not exposed to contaminants (LC) spent 1767% more time feeding than leeches exposed to MP. Leeches exposed to CD and CDMP showed a 64% and a 91% decrease, respectively, in time spent feeding compared to leeches exposed to MP (**Figure 12B**).

The total distanced moved by individuals during the length of the behavioural assay was influenced by the treatment that leeches were exposed to ($H(3) = 25.31$, $p < 0.001$). Individuals not exposed to contaminants and those exposed to only MP moved the same distance throughout the trials ($p = 0.11$). Leeches exposed to CD and CDMP showed a 35% and 74% decrease in total distance moved relative to LC, respectively (**Figure 12C**).

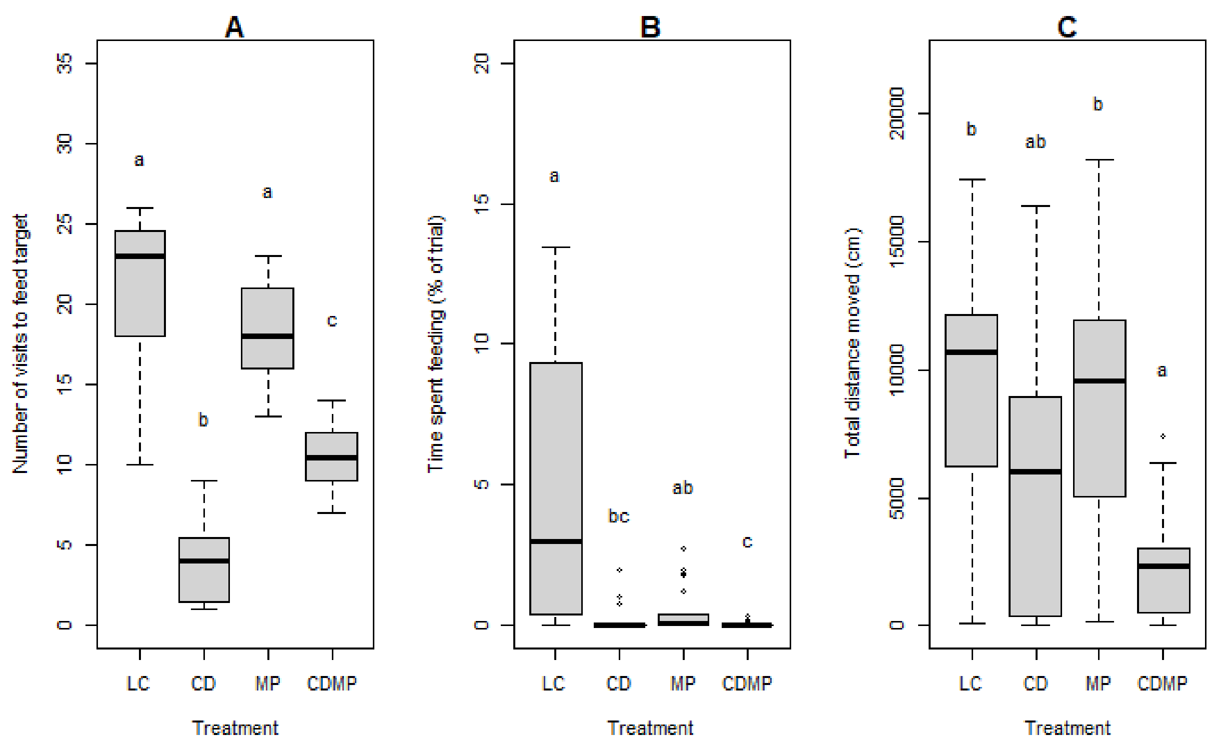


**Figure 12.** The frequency of visits to the feeding target (A), amount of time spent feeding (B), and total distance moved (C) by individuals during the behavioural trial following exposure to no contaminants (LC), cadmium (CD), microplastics (MP), or a mixture of cadmium and

microplastics (CDMP). Differences among treatments were analyzed by using a Kruskal-Wallace test with a Dunn's post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the media, and the whiskers represent the minimum and maximum values. Circles represent outliers. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; $n = 20$).

***Recovery***

After one-week of recovery in freshwater, there remained no detectable cadmium in individuals exposed to LC and MP. The amount of cadmium retained in individuals following the one-week recovery period was 56% greater in individuals previously exposed to only CD than those exposed to CDMP ($F(3, 28) = 15.25$, $p < 0.001$, **Figure 13**). On average, leeches exposed to CD depurated 25% of cadmium during recovery whereas leeches exposed to CDMP depurated 58% of cadmium during the one-week recovery period.

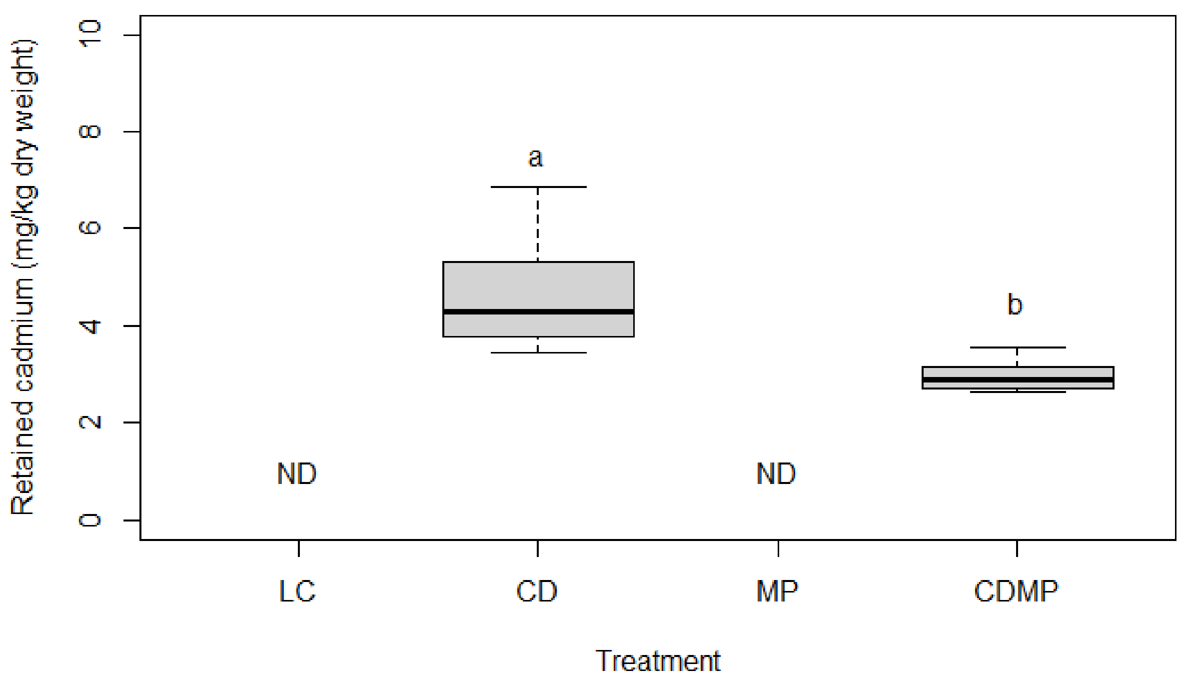


**Figure 13.** The amount of cadmium retained by *N. obscura* after a one-week recovery period following exposure to cadmium (CD) and cadmium-microplastic mixture (CDMP). No detectable cadmium (ND) was reported in the control (LC, no contaminants) or microplastics-only exposed individuals (MP). Differences among treatments were analyzed by using a one-way

Analysis of Variance with a Tukey's post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the media, the whiskers represent the minimum and maximum values. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; n=8).

Following a one-week recovery period in non-contaminated water, the whole-body serotonin levels in leeches were still affected by the treatment they were exposed to prior to recovery ($H(3) = 16.75$, $p < 0.001$, **Figure 14**). Individuals previously exposed to CD had 50% less serotonin than both LC and MP (**Figure 14**). Leeches exposed to CD had 38% less serotonin compared to leeches previously exposed to CDMP (**Figure 14**).

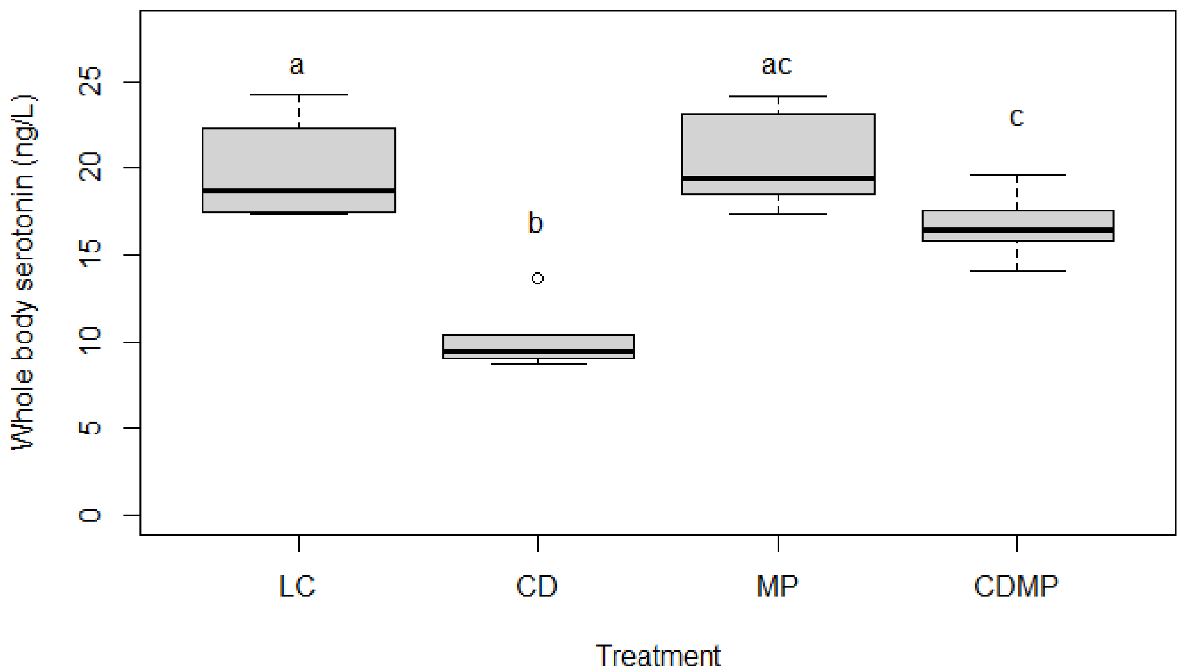


**Figure 14.** Concentrations of serotonin following a 7-day recovery period from exposure to no contaminants (LC), cadmium (CD), microplastics (MP), or co-exposure to both cadmium and microplastics (CDMP). Differences among treatments were analyzed by using a Kruskal-Wallace test with a Dunn's post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the media, and the whiskers represent the minimum and maximum

values, and circles represent outliers. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$, n=4).

The same four behavioural endpoints that were assessed following the 21 day exposure exposure were assessed in the post-recovery behavioural trials. The latency of leeches to first visit the feeding target remained unaffected by treatment ($H(3) = 5.21$, $p = 0.16$). The number of visits to the feeding target remained affected by the treatment to which an individual had been exposed, even after the recovery period ($F(3, 28) = 32.69$, $p < 0.001$). Leeches previously not exposed to any contaminants (LC) or previously exposed only to microplastics (MP) visited the feeding target with the same frequency ($p = 0.52$). Individuals previously exposed to CDMP and CD treatments showed a 50% and 81% decrease in visits to the feeding target relative to LC, respectively (**Figure 15A**).

The treatment to which leeches were previously exposed still affected the total amount of time spent feeding following the one week recovery period ($F(3, 28) = 7.76$, $p < 0.001$). Leeches not previously exposed to contaminants (LC) and those previously exposed to only microplastics (MP) did not differ in their time spent feeding ($p = 0.81$). Leeches previously exposed to cadmium-containing treatments CD and CDMP showed 53% and 28% less time spent feeding relative to LC, respectively (**Figure 15B**).

The total distanced moved by individuals during the length of the behavioural assay remained influenced by the treatment that leeches were exposed to even after the recovery period ($H(3) = 23.49$, $p < 0.001$). Individuals not exposed to contaminants and those exposed to only microplastics moved the same distance throughout the trials ($p = 0.98$) which was a greater distance than leeches from cadmium-containing treatments, which also did not differ from each other ($p = 0.13$, **Figure 15C**). Leeches in CDMP and CD moved 50% and 83% less distance than LC, respectively (**Figure 15C**).

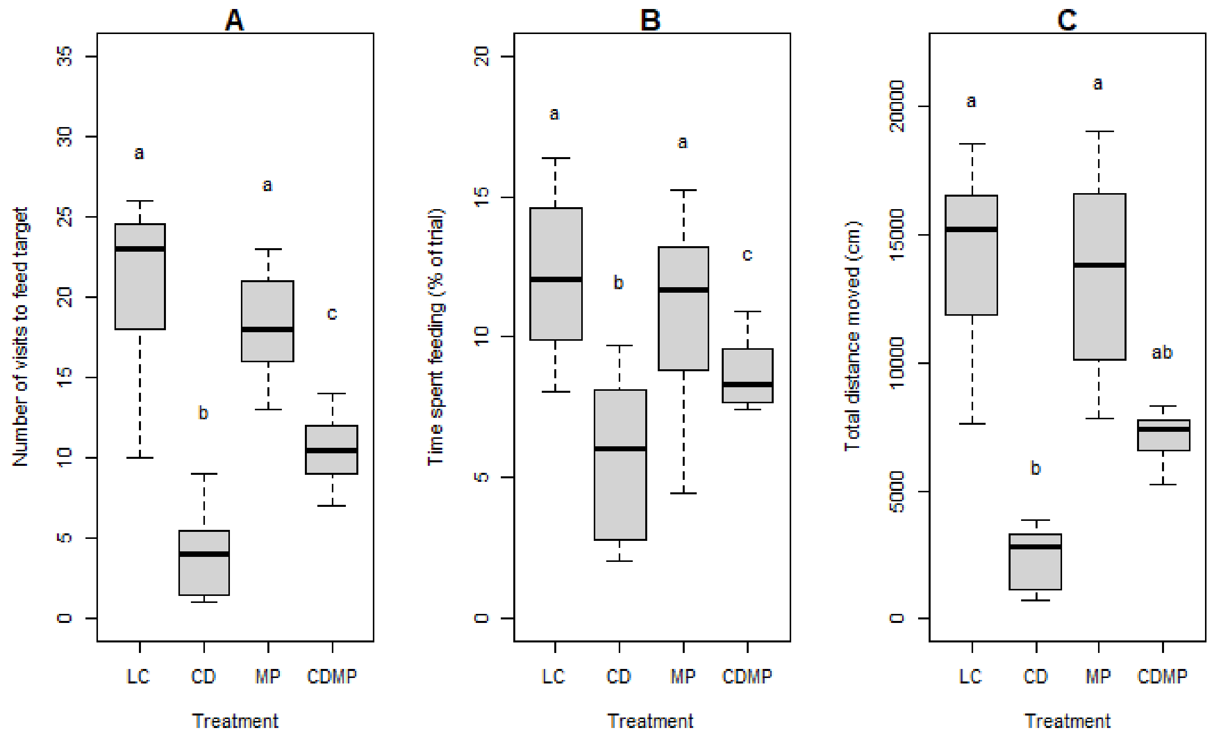


**Figure 15.** The frequency of visits to the feeding target (A), amount of time spent feeding (B), and total distance moved (C) by individuals during the behavioural trial following a 7-day recovery period from exposure to no contaminants (LC), cadmium (CD), microplastics (MP), or a mixture of cadmium and microplastics (CDMP). Differences among treatments were analyzed by using a one-way Analysis of Variance with a Tukey's post-hoc test (A) or Kruskal-Wallace test with a Dunn's post-hoc test (B,C). The shaded box area represents the interquartile range, the line within the box represents the median, and the whiskers represent the minimum and maximum values. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; n=8).

## Discussion

### *Sediment exclusion study*

The sediment-exclusion study demonstrated that leeches exposed to cadmium-contaminated environments accumulated cadmium during a 21-day exposure. The accumulation of cadmium was unaltered by co-exposure with microplastics. *Nephelopsis obscura* that were allowed access to sediment accumulated less cadmium than leeches without access to the sediment. This is corroborated by the observation that the most cadmium was accumulated by leeches in exposures where sediment was absent, indicating that presence of the sediment reduces cadmium bioaccumulation over the exposure period. Benthic organisms are particularly susceptible to adverse effects caused by uptake of contaminants, including metals, from both sediment and overlying water sources. The uptake of metals by many benthic organisms can be partially attributed to the ingestion of contaminated sediment (Ingersoll et al. 1994). However, leeches are known not to ingest sediment (Sawyer 1986). Consistent with this, it has previously been observed that leeches accumulated less cadmium from contaminated sediments than other aquatic invertebrates in the same environment (Keser et al. 2020). The leech integument is the primary structure responsible for ionoregulation and uptake of metals (Sawyer 1986; Weber et al. 1993). Exposure to some substances, such as crude oil, have been shown to alter leech integument; however, it is unknown whether the presence of sediment alters the integument, thereby altering ionoregulation (Weber et al. 1993; Petrauskienë 2005). It has been proposed that the change in ion concentrations in porewater relative to overlying surface water may alter the uptake of ions, such as cadmium, through the integument (Keser et al. 2020).

***Chronic exposure***

When exposed only to waterborne contaminants, the leeches accumulated cadmium to a greater degree when co-exposed to cadmium and microplastics than when exposed to cadmium alone. Cadmium is a direct inhibitor of serotonin synthesis in leeches (Jovanovic et al. 2016; Jovanovic 2021). In the present study, whole-body concentrations of cadmium and whole-body concentrations of serotonin were inversely related, with individuals exposed to the cadmium-microplastic mixture accumulating the most cadmium and having the least amount of serotonin. Individuals exposed only to cadmium had slightly higher concentrations of serotonin than those exposed to the mixture. Despite not being exposed to cadmium, individuals exposed to microplastics also showed decreased whole body concentrations of serotonin compared to those not exposed to any contaminants. In leeches, distension of the body wall as is caused when materials in the gut cause expansion of digestive muscles, decreases synthesis of serotonin (Lent et al. 1991). In preliminary studies using the same concentration of microplastics used in the present study, leeches accumulated microplastic aggregations in the gut that were large enough to stretch the gut wall. Thus, decreased concentrations of serotonin in leeches exposed only to microplastics might have been caused by ingestion of microplastics causing distention of the body wall. The further decrease in serotonin concentrations in leeches exposed to the mixture of cadmium and microplastics compared to the single contaminant exposure groups is indicative of an additive effect on inhibition of synthesis of serotonin.

Cadmium has been found to decrease activity of *N. obscura* (Wicklum et al. 1997). The amount of accumulated cadmium shows inverse trends in relation to total distance moved (used as a surrogate measure for activity in this study), suggesting that the amount of bioaccumulated cadmium influences activity. When serotonin levels are low, the exploratory and searching behaviour of leeches decreases (Sawyer 1986). As leeches primarily exhibit exploratory and searching behaviour when searching for food, the decrease in these behaviours might be due to a sense of satiation (Sawyer 1986; Wicklum et al. 1997). Individuals exposed to the cadmium and microplastic mixture showed the least activity and also showed the lowest concentrations of serotonin. Individuals in this treatment, with extremely low serotonin levels, would not have a sense of hunger and therefore were not as active in foraging (Sawyer 1986).

The feeding behaviour of predatory leeches is composed of actions of searching and latching to food and is not affected by water movement, shadows, or conspecifics (Sawyer 1986). Individuals not exposed to contaminants spent the most time feeding during the trials. These individuals did not accumulate any cadmium or ingest microplastics that could inhibit serotonin synthesis. Time spent feeding shows the same relative trends as serotonin levels across all treatments, with the mixture treatment showing the least amount of time spent feeding. Cadmium is likely acting as an inhibitor to serotonin synthesis, as has been previously observed in leeches (Jovanovic 2021), while microplastic ingestion is likely causing distention of the body wall, both of which decrease serotonin, resulting in additive toxicity of microplastics and metals.

***Recovery***

Following the one-week recovery period, serotonin concentrations in leeches exposed to microplastics returned to those of control individuals, indicating that the microplastics hypothesised to have signalled serotonin downregulation via distention of the body wall were depurated during the recovery period. Depuration of microplastics has been observed in freshwater invertebrates and fish, but has not been quantified in leeches (Elizalde-Velázquez et al. 2020). Individuals co-exposed to cadmium and microplastics showed partial recovery of serotonin levels whereas individuals exposed only to cadmium still had significantly less serotonin than any other treatment following the recovery period. There is no evidence that *N. obscura* has evolved systems for depuration of bioaccumulated cadmium (Sawyer 1986). The significantly lower concentrations of serotonin in cadmium-exposed individuals coupled with the observed recovery of concentrations in microplastic-exposed individuals indicates that cadmium bound to microplastics likely depurated from individuals in the mixture treatment but that unbound cadmium did not depurate.

Transepithelial exchange is the primary mode of ion exchange in leeches (Sawyer 1986). The skin is permeable to calcium ions, but only from interior to exterior (Sawyer 1986). Cationic cadmium structurally resembles calcium ions and in many instances has been found to participate in calcium-dependent pathways and processes (Choong et al. 2014). Therefore, despite the fact that cadmium does not serve a biological function, the observed depuration of cadmium might be occurring in part through cadmium acting as a calcium analog (Sawyer 1986). Additional routes of cadmium transport that were not assessed in this study but should be investigated in the future include dietary and urinary excretions, ion transport through the extcretion of mucus and mucosal sloughing to better characterize the depuration observed in this study.

The changes in behaviour after one-week of recovery in clean water mirror the amount of cadmium retained and concentrations of serotonin levels. Individuals from the microplastics-only treatment showed the same feeding behaviour (time spent feeding and number of visits to feed target) and activity (total distance moved) as individuals who had not been exposed to any contaminants. Individuals previously exposed only to cadmium still had lower feeding and activity measures following the recovery period. Individuals previously co-exposed to cadmium and microplastics showed partial recovery of feeding and activity behaviours. The trends observed in the behavioural endpoints further corroborate the notion that there is a relationship between the amount of cadmium accumulated by leeches and concentrations of serotonin which affects feeding behaviour and activity.

**Conclusion**

This study investigated the effect of exposure to sublethal concentrations of cadmium, microplastics, and their mixture on whole-body serotonin and feeding behaviour of *Nephelopsis obscura*. Leeches more readily accumulated waterborne cadmium compared to sediment-borne cadmium. The ability of an organism to feed directly relates to an individual's biological fitness. In this study, cadmium and microplastics altered leech feeding behaviour. The observed changes in feeding behaviours directly relate to the amount of cadmium accumulated by individuals and to whole body concentrations of serotonin. Cadmium and microplastics alter serotonin levels in *N. obscura* via different mechanisms: cadmium as a direct inhibitor of serotonin synthesis and microplastics in the gut causing distention of the body wall which in turn downregulates synthesis of serotonin. Together, exposure to cadmium and microplastics result in additive adverse effects on behaviour via independent actions on serotonin. When transferred to uncontaminated environments, alterations to serotonin and feeding behaviour caused by microplastics were alleviated but cadmium toxicity persisted.

Cadmium and microplastics are two contaminants found to co-occur in freshwater ecosystems. Both cadmium and microplastics exert adverse effects on *N. obscura* feeding behaviour by decreasing serotonin. Within one week in a clean environment, the microplastic-induced changes in serotonin levels and feeding behaviour were alleviated; however, cadmium toxicity persisted, suggesting that in the long-term, and even for recently remediated sites, that cadmium poses a threat to *N. obscura*.

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## CHAPTER 3: *In vitro* characterization of cadmium transport across the gastro-intestinal membrane of the fathead minnow (*Pimephales promelas*) in the presence and absence of microplastics

Chapters 2, 3, and 4 of my thesis represent the initial research objectives in which we aimed to address knowledge gaps in the field of microplastic-metal toxicity research. In the previous chapter, we determined that waterborne cadmium was more readily taken up from water than sediment, that cadmium did bioaccumulate, and that both microplastics and cadmium altered feeding behaviour and serotonin levels in leeches. With this knowledge, our next objective (explored in this chapter) was to investigate the mechanism of bioaccumulation of cadmium from ingested microplastic-cadmium complexes. An *in vitro* gut sac technique in fathead minnow (*Pimephales promelas*) was utilized to investigate the transport of cadmium across the gut barrier following the simulated ingestion of cadmium, microplastics, and cadmium bound to microplastics. As part of this objective, we assessed whether any cadmium that crossed the gut membrane would adsorb to plastics in the serosal space by simulating the pre-existence of microplastics in the serosal space and the movement of microplastics across the gut membrane.

This study revealed that microplastics did not translocate across the gut barrier. Cadmium was able to cross the gut barrier and be internalized. Microplastics were protective against cadmium internalization to the serosal space when compared to the same amount of cadmium introduced in the absence of microplastics. The presence of serosal microplastics did not alter the movement of cadmium.

This thesis is a manuscript-style thesis which is organized based on the University of Lethbridge thesis submission regulations. Inevitably, there is some repetition of content between sections, particularly in the introduction and methods sections of research chapters. A version of this chapter has been published in *Environmental Toxicology and Pharmacology* as part of the Special Issue *Microplastics and their Environmental Effects*:

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The author contributions for this manuscript are as follows, in accordance with the CRediT Author Contributions system:

Lauren Zink: conceptualization, methodology, validation, formal analysis, investigation, writing – original draft, writing – review and editing

Carolyn Simonis: investigation, writing – review and editing

Steve Wiseman: resources, writing – review and editing, supervision, funding acquisition

Gregory G. Pyle: resources, writing – review and editing, supervision, funding acquisition

**Abstract**

There is concern that microplastics can act as a vector for cadmium through adsorption and desorption of free-ionic cadmium. Little is known about the uptake of cadmium following ingestion of cadmium-microplastic complexes. This study used an *in vitro* gut sac technique to investigate the translocation of cadmium across the gut barrier of fathead minnows following the simulated ingestion of cadmium, microplastics, or their complexed mixture. Microplastics did not cross the gut membrane, nor did microplastics alter the rate of cadmium translocation, which was estimated to be 1.2 ± 0.04 ng Cd / hour. Less cadmium translocated when cadmium-microplastic complexes were injected than the equivalent dose of only cadmium, indicating that the presence of microplastics was protective of dietary cadmium uptake. This work highlights the importance of considering dietary uptake and the role of microplastics acting as a vector for cadmium in aquatic environments and stresses the need to understand how environmental (digestive or ambient) characteristics govern cadmium-microplastic interactions.

**Introduction**

The gill and the gut are the two major routes through which fish take up metals from the environment. Toxicology studies have tended to focus on gill-based uptake of waterborne metals, resulting in a less developed understanding of processes of absorption of metals from the gastro-intestinal tract (Dallinger and Kautzky 1985; Wang 2013). Dietary uptake of metals can result from the ingestion of metal-contaminated food or other suspended particulates, such as microplastics, to which metals are bound (Besseling et al. 2016; Nel et al. 2018). Studies investigating the toxicity of metals bound to suspended sediments have found that in some cases, the metals are accumulated within organisms and cause adverse effects similar to those observed with metals dissolved in water (Bonnet et al. 2000; Weltens et al. 2000; Fetters et al. 2016).

Speciation, uptake, and toxicity of metals in aquatic environments are dependent on water chemistry, including water hardness, dissolved organic carbon, and pH, which are all well-studied; however, less is known about the role of suspended particulates (commonly measured as turbidity) as an ingestible vector for dietary metal uptake (Renner 1997). Among metals of concern, cadmium serves no known biological function, and most organisms do not have systems for regulating it, which increases the risk of cadmium bioaccumulation and adverse effects. Few studies have examined the role of dietary exposure in overall cadmium toxicity (Outridge et al. 1994; Street 2002).

Ingestion and bioaccumulation of microplastics (and any contaminants adsorbed to them) in fish is a growing area of research. Ingestion of microplastics by fish, and retention of microplastics within the gut, have been reported for both field and laboratory exposed fish (Dantas et al. 2020; Maaghloud et al. 2020). Additionally, the accumulation of microplastics in internal tissues such as in liver and muscle tissue have prompted questions as to the translocation potential of microplastics; however, routes of translocation are unknown (McIlwraith et al. 2021). Cadmium has the ability to rapidly adsorb to and desorb from a variety of particulate types in response to changes in water quality characteristics (Gardiner 1974). Multiple studies have demonstrated the adsorption of cadmium to microplastics in both field and laboratory settings (Wang et al. 2019a, 2020b; Zhou et al. 2020; Liu et al. 2021; Zink et al. 2023). Changes in pH have been shown to alter cadmium adsorption to microplastics, with more acidic conditions favouring dissociation of cadmium from microplastics (Wang et al. 2019a). As the gut environment has a lower pH and hardness compared to the ambient (surface water) environment, it remains unknown whether microplastic-cadmium complexes remain stable within the digestive environment. As cadmium ions are able to cross the gut membrane, the ingestion of cadmium-microplastic complexes could serve as a route of dietary cadmium uptake if cadmium desorbs from plastics in the acidic gut environment.

The fathead minnow (*Pimephales promelas*) is an ecologically important and physiologically well-understood species that inhabits lentic and lotic systems across North America, often serving as a model organism in aquatic toxicity testing (Ankley and Villeneuve 2006). Fathead minnows are highly tolerant to changes in water quality conditions and as such are widely used to assess the potential toxicity of chemicals and mixtures including those derived from agriculture, industry, commerce, and medicine (Ankley and Villeneuve 2006; Rozon-Ramilo et al. 2011; Zhang et al. 2015). An *in vitro* gut sac technique has been previously established to assess the transportation of substances across the gut membrane (Ojo and Wood 2008; Khan et al. 2017). The primary objective of the present study was to investigate the transport of cadmium across the gut barrier following the simulated ingestion of cadmium, microplastics, and cadmium bound to microplastics. As part of this objective, we assessed whether any cadmium that crossed the gut membrane would adsorb to plastics in the serosal space by simulating the pre-existence of microplastics in the serosal space. A secondary objective was to assess the movement of microplastics across the gut membrane.

## Methods

### *Experimental Animals*

Adult (> 1 year old, approximately 4 grams, N = 36) male fathead minnows were obtained from long-standing breeding cultures from the Aquatic Research Facility at the University of Lethbridge (ARF). Fish were maintained in a 336 L tank of aerated water with a 30% water change occurring daily on 16 hour light: 8 hour dark photoperiod at 22 ± 1 °C. The water supply to the ARF is piped directly from the City of Lethbridge and dechlorinated using activated carbon, filtered through particulate cartridge filters, and pH buffered using aragonite. Water is UV-sterilized before entering culture tanks. Water samples were regularly collected from the holding tank and measured for water quality (**Table 4**). Conductivity and pH were measured using an Oakton Pocket Tester (Oakton PCTSTestr5). Alkalinity and hardness were measured using the titration method described by the American Public Health Association (American Public Health Association 1992). Dissolved organic carbon was quantified via high temperature catalytic oxidation on a Shimadzu TOC-L CHP (Kyoto, Kyoto, Japan). Additionally, neither cadmium or polyethene was detected in the holding tank, utilizing the methods described in subsequent sections. Fish were fasted for two days prior to the start of the experiment at which time fathead minnows were euthanized with an overdose of MS-222 (CAS: 886-86-2) (0.25 g/L). All fish utilized in this experiment showed no apparent health concerns and were randomly selected from the population.

**Table 4**.Water quality characteristics of Aquatic Research Facility (ARF) culture water. Values presented as a range (n=4).

| Parameter | Value |
|---|---|
| pH | 8.03 - 8.07 |
| Conductivity | 321 - 355 µS/cm |
| Hardness | 118 - 130 mg/L as $CaCO_3$ |
| Total Alkalinity | 114 - 126 mg/L as $CaCO_3$ |
| Dissolved organic carbon | 2.02 - 2.06 mg/L |

***Experimental design and contaminant solutions***

Modified Cortland saline, as described by Ojo and Wood (Ojo and Wood 2008), was prepared and consisted of: 133.0 mmol NaCl /L (CAS: 7647-14-5), 1.0 mmol $Ca(NO_3)_2 \cdot 4H_2O$/L (CAS 13477-34-4), 1.9 mmol $MgSO_4 \cdot 7H_2O$/L (CAS: 10034-99-8), 5.0 mmol KCl/L (CAS: 7447-40-7), and 5.5 mmol glucose/L (CAS: 50-99-7). All reagents utilized exceeded 97% purity. Herein, this modified Cortland saline is referred to as ‘saline’.

Cadmium solutions were prepared by dissolving cadmium sulfate octahydrate (CAS 7790-84-3) in saline at a concentration of 50 µg Cd/L. The cadmium concentration was confirmed prior to experimentation by use of graphite furnace atomic absorption spectroscopy as described later in this chapter. The concentration of cadmium, 50 µg Cd/L, was chosen as it has been observed to cross the gut barrier in detectable quantities following the experimental design (time of exposure, saline solution) used in the present study (Ojo and Wood 2008).

High-density polyethylene microplastics (MP) were utilized in this experiment (MPP-620VF, MicroPowders, Inc., Tarrytown, New York, United States of America). As reported from the manufacturer, MP characteristics were: density of 0.96 g/mL at 25 °C, mean particle size 5.0-7.0 µm, National Printing Inks Research Institute grind 2.0-3.0, melting point 114-116 °C. Microplastics were artificially weathered by agitating 5 g of MP and 25 mL of Tween20® (CAS 9005-64-5) in 5 L of Milli-Q (EMD Millipore, Burlington, Massachusetts, United States) water for 24 h on a magnetic stir plate at 1200 rpm at room temperature (Balakrishnan et al. 2019). The microplastics were isolated by vacuum filtration through a 0.45 µm nitrocellulose filter and subsequently rinsed twice with 2 N trace-metal grade nitric acid (CAS 7697-37-2) and three times with Milli-Q (EMD Millipore) water. Microplastics were dried in an oven at 30 °C to constant weight prior to being resuspended in saline.

To determine the concentration of microplastics used in the present study, a preliminary experiment was performed in which a geometric dilution series of artificially weathered (as described above) microplastics were added to 100 mL 50 µg Cd/L saline in 300 mL beakers. The concentration of microplastics added ranged from 0.25 g MP/L to 5 g MP/L. The solutions were covered and aerated for 48 h to exceed reported equilibrium times for metal-microplastic adsorption (Wang et al. 2019a). Following equilibrium, the solutions were filtered through a 0.45

μm nitrocellulose filter to remove all microplastics. The amount of cadmium in the filtrate was determined by GFAAS as described later in the chapter. The concentration of microplastics used in this experiment was 2 g MP/L as it was the lowest concentration of microplastics in which the amount of cadmium in the filtrate fell below the detection limit of GFAAS.

***In vitro gut sac exposure***

The gut sac isolation protocol followed that previously described by Ojo and Wood (Ojo and Wood 2008). In short, immediately following fish being euthanized in MS-222, a ventral incision was made from the gills to the vent to remove the entire digestive tract. The gut sac was made by ligating with a suture between the esophagus and stomach and at the posterior intestine. To maintain the integrity of the mucosal membrane and maintain the gut environment, the guts were not flushed (Boyle et al. 2020). Each gut sac was then filled via syringe with 0.2 mL of the assigned luminal saline solution: LC – saline; CD – 50 μg Cd/L in saline; MP – 2 g MP/L in saline; CDMP - 50 μg Cd/L and 2 g MP/L in saline. The sacs were then suspended in 15 mL aerated Falcon™ tubes (Product Number: 352096, Corning, New York, USA) by the anterior suture thread secured to a rod set across the top of the tube. The tubes, which represent the serosol space, were prefilled with 10 mL of one of two saline solutions: LC – saline or MP – 2 g MP/L in saline. The time from euthanasia to gut sac suspension was less than ten minutes to minimize the risk of tissue deterioration. Six treatment groups were established to assess luminal to serosal movement of cadmium, denoted by luminal/serosal solution: LC/LC, MP/LC, CD/LC, CDMP/LC, CD/MP, CDMP/MP. Six gut sacs were prepared and randomly assigned to each treatment group.

After incubation for 4 h, gut sacs were removed from the serosal solution and the exterior of the sacs were rinsed in 50 μM ethylenediaminetetraacetic acid (EDTA, CAS: 60-00-4) solution for one minute to remove surface-bound metals and for 5 seconds with 10% Tween20 to remove adhered plastics (Norwood et al. 2007). Immediately following the rinses, each gut, inclusive of its contents, was dried to constant weight in an oven set to 30 °C. Due to the small volume of luminal solution used in this study, gut contents were not separated from the tissue; therefore, samples defined as 'gut' are inclusive of tissue and contents. The dry weight of each gut was measured using an analytical microscale (AT21 Comparator, Mettler Toledo, Columbus, Ohio, USA) and recorded. Only when three consecutive daily weight measurements matched (± 2%) was a constant weight recorded to have been achieved.

***Microplastic analysis by Fourier-Transform Infrared Spectroscopy***

The presence or absence of microplastics in samples of the gut (tissue and contents) and post-exposure serosal solutions were determined by Fourier-Transform Infrared Spectroscopy (FTIR; Tensor 37, Bruker, Billerica, Massachusetts, USA). Samples were run according to manufacturer specifications in the OPUS software. In short, sixty absorbance scans of both sample and background were taken with an interferogram size of 28440 at a resolution of 2 $cm^{-1}$. The characteristic peaks used to establish the presence of MP were at 719 and 1472 $cm^{-1}$, which aligns with other studies that identified characteristic peaks of other polyethylene samples (D'Amelia et al. 2016).

***Cadmium analysis by Graphite Furnace Atomic Absorption Spectroscopy***

Dried gut samples were digested using a previously established and validated tissue digest protocol (Lindh et al. 2019). In short, guts were digested in concentrated, trace metal grade nitric acid (CAS: 7697-37-2) at a 1:10 ratio (tissue mass (mg): acid (µL)) for 3 h at 80°C. Acid-only blanks and certified reference material (acting as procedural controls) were digested simultaneously (Product Identifier: DOLT-4, National Research Council of Canada, Ottawa, Ontario, Canada). Following digestion, samples were cooled to room temperature and diluted as required to fall within the calibrated range of the instrument.

Concentrations of cadmium in gut and serosal solutions were analyzed using graphite furnace atomic absorption spectroscopy (GTA 120, Agilent Technologies) utilizing manufacturers specifications outlined in SpectrAA software (Agilent Technologies), with modifications: a hot inject of 25 µL at an injection speed of 30 seconds at 95 °C, an extended dry time to 45 seconds at 120 °C, and a 90 second ash time. A certified reference material (CRM, custom-made cadmium standard, Delta Scientific, Mississauga, Ontario, Canada) was run every ten samples to evaluate the accuracy of the analysis which was maintained over 90%. Each analytical sample and CRM was run in duplicate. The detection limit for Cd previously established using this method was estimated to be 0.02 µg/L; readings below the detection limit were taken as zero.

***Calculations and Statistical Analysis***

Cadmium translocation rates across the gut barrier and into the serosal space were calculated according to Equation 3.1. For purposes of this study, it was assumed that cadmium transportation across the gut was at a constant rate over the entire experiment and that net fluid movement across the gut membrane was negligible. Further, the amount of cadmium adhered to the outer lining of the gut (which was rinsed away with EDTA) was not quantified; therefore, serosal cadmium is only cadmium suspended in the serosal space.

Equation 3.1. Calculation of cadmium translocation rate from the gut to the serosal space.

$$\begin{gathered} Cd\ translocation\ rate \\ (ng\ Cd_{gut \rightarrow serosal}\ /\ g\ dry\ gut\ tissue\ /\ hour) \end{gathered} = \frac{\left(\frac{[Cd]_{serosal,after\ experiment}}{[Cd]_{gut,start\ of\ experiment}}\right) \div dry\ mass\ of\ gut}{4\ hours}$$

Statistical analyses were conducted in R, version 4.2.1 (R Core Team, 2022). A Shapiro-Wilks test for normality and a Bartlett test for homogeneity of variance were performed to determine if data met assumptions for parametric analyses. Data in this study were not normally distributed and exhibited heterogeneous variance, even after attempted data transformations; therefore, Kruskal-Wallis tests were used with a Dunn's post hoc test.

**Results**

The gut (tissue and contents) and serosal (external solution in which gut was suspended) were analyzed for the presence of microplastics. Microplastics were detected in solutions in which they were originally introduced, but no microplastics translocated across the gut barrier (**Table 5**).

**Table 5**. Presence or absence of microplastics in gut and serosal solutions determined by use of FTIR. Detection of polyethylene was determined by the presence of characteristic peaks at 719 and 1472 $cm^{-1}$. Results were homogenous for all samples (triplicate samples run for each solution) within a single treatment and solution type (n=6).

| Treatment (gut/serosal) | Solution Type | Detection of polyethylene |
|---|---|---|
| LC/LC | Gut | Absent |
| | Serosal | Absent |
| MP/LC | Gut | Present |
| | Serosal | Absent |
| CD/LC | Gut | Absent |
| | Serosal | Absent |
| CDMP/LC | Gut | Present |
| | Serosal | Absent |
| CD/MP | Gut | Absent |
| | Serosal | Present |
| CDMP/MP | Gut | Present |
| | Serosal | Present |

LC: lab control, no contaminants
MP: microplastics only
CD: cadmium only
CDMP: cadmium-microplastic mixture

The amount of cadmium in gut samples differed between treatments ($H(5) = 26.90$, $p < 0.001$, **Figure 16**). Cadmium was not detected in the LC/LC or MP/LC treatments (**Figure 16**). The concentration of Cd in gut sacs injected with the CDMP mixture, regardless of the serosal solution (both CDMP/LC and CDMP/MP) did not differ from each other (**Figure 16**). The CD/LC treatment showed an average of 14% and 20% decrease in gut cadmium concentration relative to CDMP/MP and CDMP/LC, respectively, but these differences were not statistically significant (**Figure 16**). The CD/MP treatment showed a 15% and 21% decrease in mean gut cadmium concentration relative to CDMP/MP and CDMP/LC, respectively, but the differences were statistically insignificant (**Figure 16**). The gut cadmium concentration of CD/LC and CD/MP did not differ statistically from treatments that did not include any cadmium (LC/LC and MP/LC, **Figure 16**).

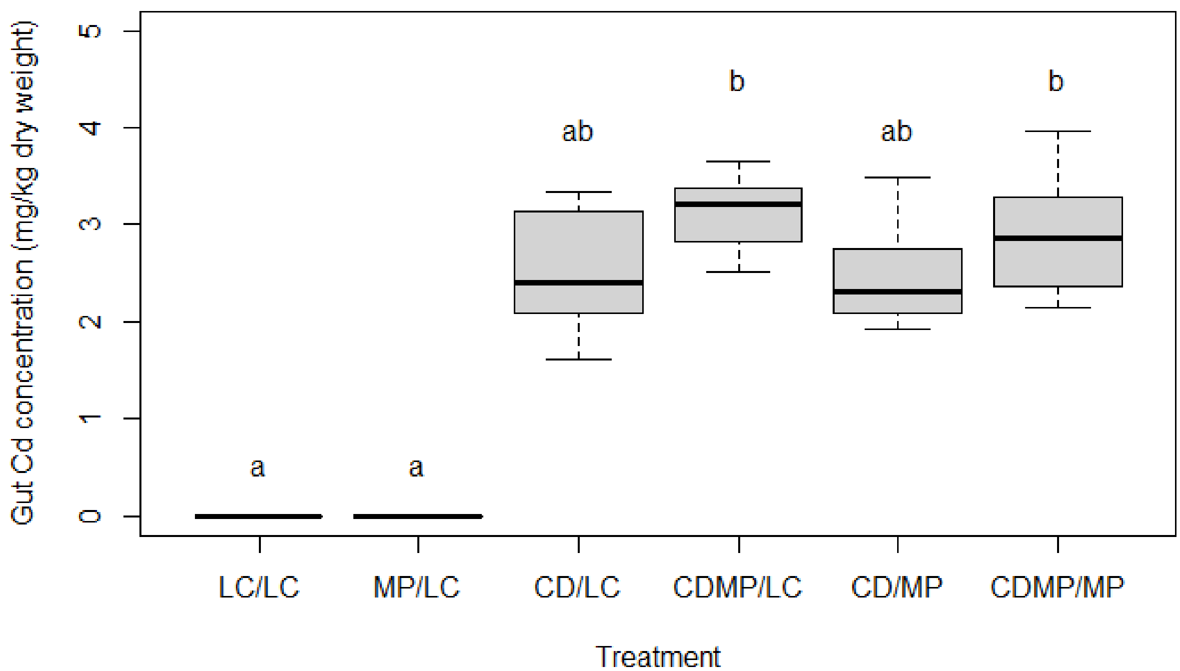


**Figure 16**. Concentrations of cadmium in guts following *in vitro* gut sac exposure. Differences among treatments were analyzed by using a Kruskal-Wallis test with a Dunn’s post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the median, the whiskers represent the minimum and maximum values. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; $n = 6$).

The amount of cadmium in the serosal solution differed among treatments (H(5) = 26.40, $p < 0.001$, **Figure 17**). No cadmium was detected in the LC/LC or MP/LC treatments (**Figure 17**). The serosal concentration of cadmium in the serosol of the CDMP/LC and CDMP/MP treatments did not differ from each other or from LC/LC and MP/LC (**Figure 17**).

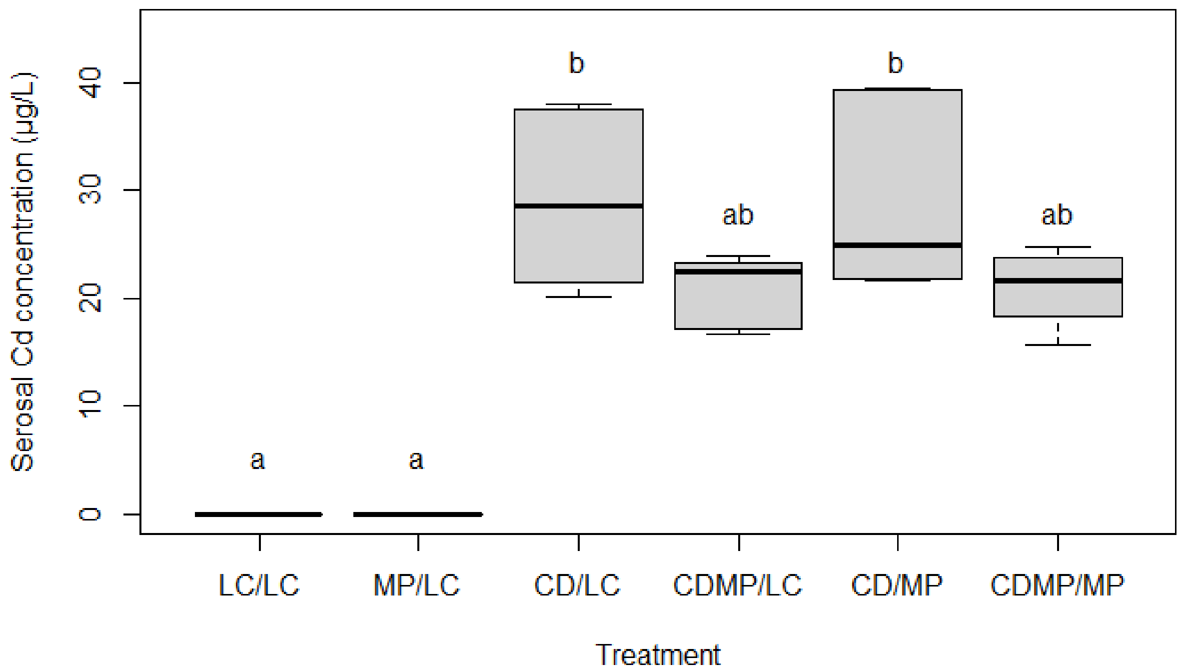


**Figure 17.** Concentrations of cadmium in serosal following *in vitro* gut sac exposure. Differences among treatments were analyzed by using a Kruskal-Wallis test with a Dunn's post-hoc test. The shaded box area represents the interquartile range, the line within the box represents the median, the whiskers represent the minimum and maximum values. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; n = 6).

The rate of cadmium translocation from the gut to the serosal space differed by treatment (H(5) = 16.75, $p = 0.002$, **Figure 18**). Cadmium was not detected in the serosal solution following the LC/LC or MP/LC treatments (**Figure 18**). Translocation rates in CD/LC and CDMP/LC did not differ from each other (**Figure 18**). The average translocation rate in cadmium-inclusive treatments (CD/LC, CD/MP, CDMP/LC, CDMP/MP) was 1.2 ± 0.04 ng Cd / hour (mean ± standard deviation, n=24).

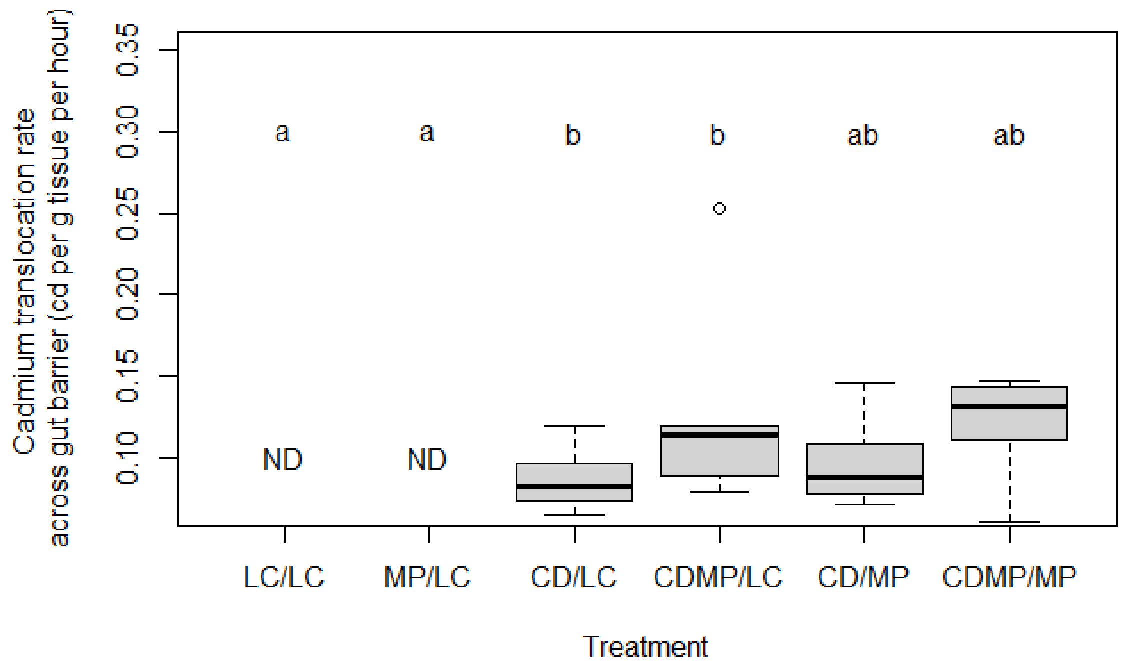


**Figure 18.** Cadmium transportation rate during *in vitro* gut sac exposure. Differences among treatments were analyzed by using a Kruskal-Wallis test with a Dunn's post-hoc test. No detectable cadmium (ND) was reported in LC/LC and MP/LC. The shaded box area represents the interquartile range, the line within the box represents the median, the whiskers represent the minimum and maximum values. Circles represent outliers. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$; $n = 6$).

**Discussion**

The physical properties of microplastics, primarily their large surface area-volume ratio, enable them to serve as a substrate for contaminants, resulting in higher contaminant concentrations on the surface of the microplastic than found in other environmental compartments such as water, sediment, and biota (Holmes et al. 2014; Seidensticker et al. 2019; Li et al. 2019). Should the microplastic-metal complexes be ingested, more cadmium might be internalized from the gut than would be following ingestion of cadmium-contaminated prey or other food. This study demonstrated that within the digestive environment, cadmium does dissociate from microplastics and translocate across the gut into the serosal space, but not entirely.

There have been recent advances in the field of microplastic-metal mixtures to better characterize the absorptive association of metals to microplastics under varying environmental conditions. Changes in pH alter the amount of cadmium that desorbs from plastic (Boucher et al. 2016; Liu et al. 2020; Xu et al. 2023). As the pH of the gut tends to be more acidic than that of the ambient environment, this study used an *in vitro* gut sac model to understand the behaviour of cadmium-microplastic complexes in the digestive environment. Cadmium, whether free in solution or initially bound to microplastics, translocated across the gut membrane at the same rate. No difference in cadmium translocation was observed under simulated conditions of microplastics previously accumulated in internal tissues (MP serosal solution treatments), indicating that the presence of microplastics in the serosal does not alter cadmium movement or attract cadmium across the gut barrier. Further research should aim to better understand the factors that govern the attraction and stability of metal-microplastic complexes to determine under what conditions microplastics are acting as vectors for metals.

While luminal microplastics did prevent some of the adsorbed cadmium from entering the serosal space, it is important to note that at the observed rate of cadmium translocation, the amount of bioaccessible cadmium could exceed that of the lethal dose to some aquatic species (Hollis et al. 1999; Miranda et al. 2019). The concentration of cadmium and microplastic used in this study were chosen based on the microplastic adsorption capacity rather than by environmental relevance. The cadmium concentration used in this simulation is two orders of magnitude higher than the water quality guidelines for the protection of aquatic life set by many jurisdictions including Canada, the United States of America, and Europe (Nugegoda and Kibria 2013). Further research at environmentally relevant concentrations of cadmium is required to understand how this translocation rate shifts when less cadmium is ingested.

This study brings attention to the need to consider dietary metal uptake when assessing the capacity to which microplastics act as a vector for metals. This study indicated that microplastics serve a protective role when co-ingested with cadmium by decreasing the amount of cadmium that crosses the gut barrier compared to if the same amount of cadmium, not bound to microplastics, was ingested. *In vivo*, the uptake of cadmium across the gut barrier could result in increased cadmium concentrations in the blood stream and subsequent toxicity in organs including the gill, liver, and kidney (Kay et al. 1986; Gill et al. 1991). Dietary exposure of fishes to cadmium has been observed to result in cellular, physiological, and behavioural toxicity (Lee et al. 2000; Croteau et al. 2005; Croteau and Luoma 2009; Defo et al. 2019).

Microplastics larger than those used in the present study have been observed in the digestive tract of field-collected and laboratory-exposed fish (Lu et al. 2016; Kim et al. 2022) as well as in other tissues such as the liver and muscle (Collard et al. 2017; McIlwraith et al. 2021). How microplastics are internalized in these tissues is currently not known. This study was an acute scenario; further research is required to determine if the movement of microplastics differs under chronic conditions under which pathologies may be introduced. No translocation of microplastics across the gut membrane was observed in this study, which aligns with previous assessments of the accumulation and depuration of natural particles in *P. promelas* (Elizalde-Velázquez et al. 2020). Additional research is required to identify and characterize the routes by which microplastics accumulate within internal tissues and organs.

## Conclusion

This study utilized an in vitro gut sac technique to investigate the transport of cadmium across the gut barrier under multiple exposure scenarios including the ingestion of cadmium-microplastic complexes and the ingestion of cadmium alone. Microplastics (5.0 – 7.0 μm) did not translocate across the gut barrier. The presence of serosal microplastics did not alter the movement of cadmium, suggesting that previous hypotheses of microplastics adsorbing metals in internal tissues is not supported across the gut barrier. When cadmium-microplastic complexes were simulated to have been ingested, only a portion of the cadmium previously adsorbed to the plastics desorbed and translocated across the gut membrane, indicating that microplastics were protective against cadmium internalization to the serosal space when compared to the same amount of cadmium ingested in the absence of microplastics. As the gut environment is more acidic and has a different ionic profile than ambient water, this work suggests that the interaction of cadmium and microplastics change in digestive conditions. Further research is needed to strengthen our understanding of the stability of metal-microplastic associations and develop our understanding of the risk of co-ingestion of metals and microplastics in the natural environment at more realistic concentrations.

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## CHAPTER 4: Effects of exposure to cadmium, microplastics, and their mixture on survival, growth, feeding, and life history of *Daphnia magna*

Chapters 2, 3, and 4 of my thesis represent the initial research objectives in which we aimed to address knowledge gaps in the field of microplastic-metal toxicity research. In this chapter, we determine if exposure to a sublethal concentration of Cd, an environmentally relevant concentration of polyethylene microplastic, or their mixture affects life-history traits (as measured by survival, time to first brood, the number of neonates released at each reproductive event, and the size of neonates produced), growth, and feeding of the water flea, *Daphnia magna*.

In this chapter, we determined that microplastics and cadmium exerted additive toxicity to daphnids through independent mechanisms of action. Microplastics inhibited early-life growth whereas cadmium decreased feeding efficiency and when co-exposed to both microplastics and cadmium, both growth rate and feeding were decreased. Neither microplastics nor cadmium altered life history traits.

This thesis is a manuscript-style thesis which is organized based on the University of Lethbridge thesis submission regulations. Inevitably, there is some repetition of content between sections, particularly in the introduction and methods sections of research chapters. A version of this chapter has been published in *Environmental Toxicology and Chemistry*:

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The author contributions for this manuscript are as follows, in accordance with the CRediT Author Contributions system:

Lauren Zink: conceptualization, investigation, formal analysis, methodology, visualization, writing – original draft, writing – review & editing

Anna Shearer: investigation, writing – review & editing

Steve Wiseman: funding acquisition, supervision, writing – review & editing

Gregory G. Pyle: conceptualization, funding acquisition, resources, supervision, writing – review & editing

## Abstract

There is concern that microplastics can act as a vector for cadmium, altering the bioavailability and subsequent toxicity of cadmium to ecologically important species such as *Daphnia magna*. The toxicity of cadmium to *D. magna* has been well-described; however, what is not known, and what this study aimed to address was how the addition of polyethylene microplastics altered cadmium toxicity. Using high-throughput feeding assays and size assessments, this study quantified effects of exposure to cadmium, microplastics, or their mixture on daphnids from neonate to adult. Exposure to cadmium inhibited feeding efficiency while exposure to microplastics inhibited growth rates of juveniles. *Daphnia magna* co-exposed to cadmium and microplastics showed significant decreases in both feeding and pre-reproductive growth rate. There were no differences in life history traits across any treatments. The alterations of feeding and growth while maintaining reproductive endpoints (time to first brood, reproductive frequency, the number of neonates released at each reproductive event, and the size of neonates produced) might be the result of a shift in energy allocation away from somatic growth, allowing individuals to maintain reproductive output despite lower nutritional reserves. Our findings suggest that co-contamination of microplastics and cadmium has additive effects on feeding and growth rates, resulting in a greater energy allocation shift.


## Introduction

The increased use of plastics in everyday life has resulted in their infiltration into aquatic environments, primarily by household and industrial effluent (Barnes et al. 2009; Rezania et al. 2018). The most common types of plastics found in aquatic systems are polyethylene, polypropylene, and polyacrylate (Au et al. 2015; Vedolin et al. 2018). The diverse composition of plastics at manufacturing and their subsequent degradation following introduction to aquatic systems results in the distribution of microplastics in multiple environmental compartments including water, sediment, and biota (Du et al. 2020). Microplastics suspended in the water column are more readily transported than those embedded in sediments; however, seasonal changes in hydrodynamic flow can result in the settling of suspended microplastics or the resuspension of sediment-bound microplastics (Besseling et al. 2016, p.; Nel et al. 2018). The resuspension and transport of microplastics in aquatic systems increases their interaction with pelagic biota such as daphnids.

There is debate as to whether microplastics on their own are toxic to aquatic life; however, interactions of microplastics with other pollutants has raised concerns about the potential for microplastics to act as a vector for toxicants (Backhaus and Wagner 2020). Microplastics are inadvertently ingested by many freshwater invertebrates, particularly filter feeders (Scherer et al. 2017; Windsor et al. 2018). Daphnids are pelagic filter feeders that routinely ingest suspended particles, including microplastics (Ogonowski et al. 2016; Rist et al. 2017; Scherer et al. 2017). Exposure to microplastics can cause immobilization, oxidative stress, and mortality in *Daphnia magna,* an ecologically important filter feeder (Miloloža et al. 2021). The physical properties of microplastics, namely their large surface area to volume ratio and negative surface charge attract cationic contaminants, often resulting in higher contaminant concentrations on the surface of the microplastic than in the surrounding environment (Holmes et al. 2014; Seidensticker et al. 2019; Li et al. 2019). The stability of the associations between microplastics and other contaminants remains unknown, though under simulated physiological conditions certain organic pollutants (phenanthrene, DDT, and perfluorooctanoic acid) have been found to dissociate from microplastics (Bakir et al. 2014).

Metal bioavailability is dependent on the partitioning of the metal in a given environment (i.e., the metal must have the opportunity to interact with an organism) and the speciation of the metal (i.e., the metal is in a form that is bioavailable to that organism). The free ionic form of a metal is generally considered to be more toxic to organisms due to the small size and charge enabling direct interaction with many biotic ligands (i.e., bioavailable); however, metals in the form of complexes (e.g., organometallic compounds, metals bound to microplastics or food) might also be bioavailable through less direct routes such as ingestion and subsequent dissociation of the complex (Kamunde and Wood 2004; Sappal and Kamunde 2009). The association of metals with microplastics might pose risk to the health of aquatic organisms should a metal-microplastic complex be ingested and the metal subsequently dissociates from the plastic. Cadmium has been found to bind to microplastics in many environments (Banaee et al. 2019; Godoy et al. 2019; Almeida et al. 2020). Cadmium is a metal of interest because exposure to cadmium causes adverse effects in many freshwater invertebrates and is ubiquitous in freshwater environments, particularly in industrial zones (Salánki and Hiripi 1990; Gill and Epple 1992; Outridge et al. 1994; Almeida et al. 2003; Wu et al. 2015).

Currently proposed mechanisms of toxicity of microplastics and cadmium to daphnids differ. Microplastics are thought to induce their toxicity through the induction of oxidative stress and have been observed to result in altered swimming behaviour, though no mechanism has been hypothesized (Rehse et al. 2016; Canniff and Hoang 2018; Aljaibachi et al. 2020). Cadmium causes lethality, altered metabolic activity, and shifts in reproduction in *D. magna* (Bodar et al. 1988a, b; Semsari and Megateli 2007). In a mixture, cadmium and microplastics acting independently, following a Response Addition mixture model, would result in additive toxicity. If cadmium-microplastic complexes are ingested by *D. magna* and the adsorbed cadmium dissociates from the microplastic and is bioavailable, microplastics could attenuate cadmium toxicity by increasing the amount of cadmium uptake to *D. magna.*

The objective of this study was to determine if exposure to a sublethal concentration of cadmium, an environmentally relevant concentration of polyethylene microplastic, and their mixture, affect life history traits (as measured by survival, time to first brood, the number of neonates released at each reproductive event, and the size of neonates produced), growth, and feeding of *D. magna.*

## Methods

### *Test chemicals*

A stock solution of cadmium (2 mg/L Cd) was prepared by dissolving cadmium sulfate octahydrate (CAS 7790-84-3) in Milli-Q (EMD Millipore Burlington, Massachusetts, United States of America) water. The stock solution was used to dose the exposure solutions (5 µg/L Cd) in moderately-hard reconstituted water (see below). Concentrations of cadmium in the stock and exposure solutions were confirmed using graphite furnace atomic absorption spectroscopy (GTA 120, Agilent Technologies, Santa Clara, California, USA) utilizing manufacturers specifications outlined in SpectrAA software (Agilent Technologies). A certified reference material (SLRS-6) (Government of Canada) was run every ten samples to evaluate the accuracy of the analysis which was maintained over 90%. Each analytical sample and certified reference material was run in duplicate. The detection limit for Cd previously established using this method was estimated to be 0.7 µg/L.

High-density polyethylene microplastics (MP) were utilized in this experiment (MPP-620VF, MicroPowders, Inc., Tarrytown, New York, United States of America). The reported characteristics of this product from the manufacturer are: density of 0.96 g/mL at 25°C, mean particle size 5.0-7.0 µm, National Printing Inks Research Institute grind 2.0-3.0, melting point 114-116 °C. Microplastics were artificially weathered by agitating 1 g of MP and 5 mL of

Tween20® (CAS 9005-64-5) in 1 L of Milli-Q (EMD Millipore, Burlington, Massachusetts, United States) water for 24 hours on a magnetic stir plate at 1200 rpm at room temperature in darkness. Following agitation, the solution was filtered through a 0.45 µm nitrocellulose filter and subsequently rinsed twice with 2 N trace-metal grade nitric acid (CAS 7697-37-2) and three times with Milli-Q (EMD Millipore, Burlington, Massachusetts, United States) water. Microplastics were dried in an oven at 60 °C to constant weight prior to being resuspended in reconstituted culture water (see 2.2 below). This weathering protocol degrades the surface of the microplastics to more closely mirror the condition and dispersion of plastics found in the environment without altering the uptake of microplastics by *D. magna* (Balakrishnan et al. 2019). Though not quantified in this study, the degradation of plastics increases the amount of other contaminants (including metals) that can adsorb to the surface (Sun et al. 2020).

***Organism culture conditions***

*Daphnia magna* utilized in this study were reared from long-standing cultures maintained at the University of Lethbridge (Lethbridge, AB, Canada). Cultures were maintained in 19 L tanks with 15 L of moderately hard water (90 mg/L as $CaCO_3$) reconstituted from Milli-Q (EMD Millipore, Burlington, Massachusetts, United States) water by adding 0.096 mg/L $NaHCO_3$, 0.06 mg/L $CaSO_4 \cdot H_2O$, 0.06 mg/L $MgSO_4$, 0.012 mg/L KCl, 2.4 µg/L $Na_2SeO_4$ and 3.2 µg/L vitamin $B_{12}$. A 50% water change was performed once per week and cultures were fed daily with an algae mixture of *Raphidocelis subcapitata, Ankistrodesmus, Chlorella, Scenedesmus,* and *Selenastrum* at a cell density of $10^6$ cells per mL. Tanks were kept at 20 ± 1 °C and held on a 16:8 h (light:dark) cycle. Sensitivity of the culture was monitored using sodium chloride reference toxicity testing, as described in Environment Canada - Environmental Protection Series Protocol

RM-11 (Government of Canada). Neonates (i.e., ≤ 24 h old) were pooled from all culture tanks to serve as the founding individuals in the present study.

***Experimental design***

Two-hundred neonates were randomly assigned and individually placed into 20 mL glass scintillation vials with 20 mL of culture water to serve as the founding population. Each of the 200 vials was aerated by pipette, generating one bubble per second. When an individual of the founding population produced offspring, each neonate was removed from the parent vial and randomly assigned to 20 mL scintillation vials containing 20 ml one of the four exposure solutions: either 5 μg/L cadmium (CD), 0.06 g/L (which corresponds to 120 ± 6 particles/L) microplastics (MP), 5 μg/L Cd and 0.06 g/L MP (CDMP), or culture water without Cd or MP, that served as a control solution (LC). Complete exposure solution replacements were performed twice per week. All vials were fed daily with the same algae mixture as the cultures. Vials were kept at 20 ± 1 °C and held on a 16:8 h (light:dark) cycle.

In preliminary work (unpublished), 5 μg/L Cd in culture water was the maximum concentration in which reproduction still occurred by all tested daphnids and was therefore chosen as the concentration for use in the present study. The concentration of microplastics chosen reflect those measured in habitats in which daphnids reside (Li et al. 2018). Previous work assessing the toxicity of microplastics to *D. magna* found that 0.06 g MP/L induced immobilization, mortality, and population decline (Aljaibachi et al. 2020; Miloloža et al. 2021). In preliminary exploration (unpublished) for the present study, the microplastics utilized in this study were observed to be ingested and excreted by *D. magna.* The ingestion and excretion of microplastics was observed visually using an apparatus that allowed for live viewing of individual daphnids mounted within a test chamber (Lari et al. 2017). Both juvenile and adult daphnids were observed to ingest and excrete microplastics in this chamber. Due to the short amount of time in which daphnids can be mounted in this chamber without showing signs of stress, efforts were not made to calculate ingestion or excretion rates.

***Experimental Design - Life History***

Each vial was checked for mortality/immobilization daily using standardized methods in which absence of movement and heartbeat are used to indicate death (Government of Canada). Daphnids were also checked daily to determine 24-hour reproductive output and the date of reproductive events was recorded for each individual. If an individual produced offspring, the offspring were immediately counted and then removed from the vial to measure body length, as described below, and removed from the experiment.

***Experimental Design - Growth***

To establish growth curves, the length of each individual was measured once during the pre-reproductive phase and once while reproductive, if the individual survived to such time. Gently, individuals were transferred from their scintillation vials to a glass microscope slide while remaining submerged in a drop of the exposure solution and photographed using a EP50 camera mounted to a CX43 light microscope (Olympus, Toronto, ON, Canada). Immediately after the image was taken, the individual was returned to their scintillation vial. Length was determined from a single straight line drawn from the centre of the eye to the base of the tail as described previously (Duckworth et al. 2019), which was measured using integrated image analysis software (EPView, Olympus) calibrated in accordance with manufacturer’s specifications.

***Experimental Design - Feeding***

Feeding assays were performed once per week throughout the experiment to determine the 24-hour feeding rate of each individual. Immediately following a complete exposure solution change of the scintillation vials (to remove uneaten algae fed throughout the week), each individual was fed 500 µL of the same algae mixture described previously. Additionally, three vials (the same 20 mL scintillation vials used for daphnids) for each of LC, CD, MP, CDMP treatments, but without a daphnid, were established and hereafter referred to collectively as "fluorescence control vials". All vials were left undisturbed for 24 hours after which each vial was mixed by inversion to ensure even suspension of remaining algae and 200 µL was pipetted from the middle of the vial into one well of a black 96-well plate. This process was repeated in triplicate for each vial. Samples were analyzed on a fluorescence microplate reader (Varioskan Flash, ThermoScientific, Ottawa, ON, Canada) to assess the amount of fluorescence (i.e., uneaten algae) in each sample utilizing previously established methods (Hite et al. 2020). An excitation wavelength of 485 nm (20 nm bandwidth) and emission wavelength of 665 nm (10 nm bandwidth) were used to quantify chlorophyll-*a* content (Hite et al. 2020). Fluorescence readings of the fluorescence control vials for each treatment were used to correct for any changes in fluorescence not caused by consumption by the daphnid, such as increasing fluorescence by algal reproduction or decreasing fluorescence due to algal death.

***Statistical analyses***

Statistical analyses were conducted in R, version 4.2.1 (R Core Team, 2022). For comparison of means (lifespan, sizes of individuals at individual time points), parametric assumption tests for normality (Shapiro-Wilks) and homogeneity of variance (Bartlett Test) were completed. In instances where assumptions were met, analysis of variance (ANOVA) was run with a Tukey's HSD post-hoc test. In instances where parametric assumptions were not met, even after data transformations (square root, log, inverse, or trigonometry functions), Kruskal-Wallis tests were used with a Dunn's post hoc test. Growth and feeding rates were analysed by regression analyses and compared using analysis of covariance (ANCOVA).

## Results

### *Life History*

Exposure to cadmium shortened the lifespan of individuals, but the presence of microplastics had no effect on lifespan, nor did microplastics alter the toxicity of cadmium ($H(3) = 84.93$, $p < 0.001$, $n = 159$-$164$, **Figure 19**). The lifespan of individuals in the CD and CDMP treatments averaged half (mean lifespan 5 days in both treatments) that of those in LC and MP treatments (mean lifespan 11 and 10 days, respectively). Most of the mortality in the cadmium-containing treatments (CD and CDMP) occurred within the first 48 hours of exposure.

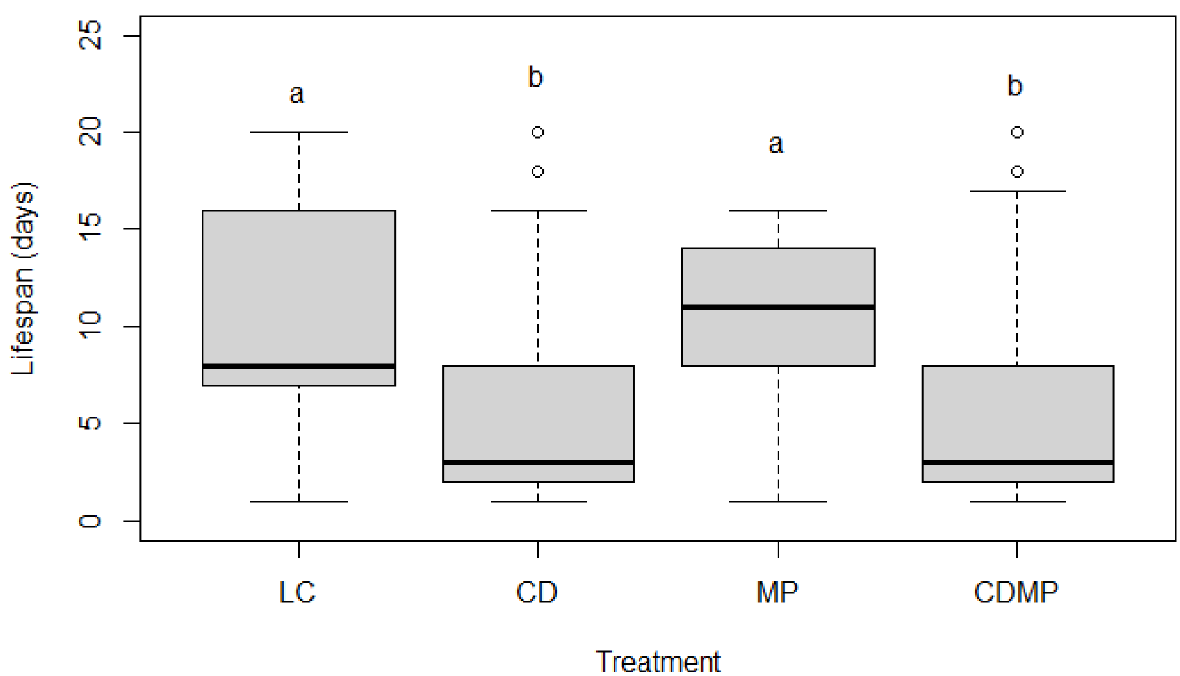


**Figure 19**. Changes in lifespan of *D. magna* exposed to no contaminants (LC, n=159), cadmium (CD, n=161), microplastics (MP, n=164), and their mixture (CDMP, n=163). Differences among treatments were analyzed by using a Kruskal-Wallis test with a Dunn's post-hoc test. Letters

above each treatment indicate significant differences from each other using a significance threshold of 0.05. The shaded box area represents the interquartile range, the horizontal line within the box represents the median, the whiskers represent the minimum and maximum values. Circles represent outliers.

Neither treatment had an effect on any of the other life history traits measured: time to first brood, the number of neonates released at each reproductive event, or the size of neonates. Individuals across all treatments released their first brood of neonates at $11 \pm 2$ days (mean ± standard deviation) and individuals from all treatments averaged $4 \pm 3$ neonates (mean ± standard deviation) per brood.

***Growth***

Individuals from each treatment began reproducing at nine days old; as such, the pre-reproductive stage was defined from days zero through eight. The size of neonates at the start of exposure (zero days old) showed no differences between treatments ($H(3) = 0.89$, $p = 0.72$, **Figure 20**); however the growth curves (slopes) differed between treatments ($F (3, 687) = 13.9$, $p < 0.001$). Post hoc comparisons using the Tukey's HSD test indicated that the pre-reproductive growth curves for the LC and CD exposures were the same ($p = 0.91$, Figure 14) but differed from the MP treatment (LC – MP $p = 0.0021$, eta-squared = 0.81; CD – MP $p = 0.044$, eta-squared = 0.80, **Figure 20**) which also differed from the CDMP treatment (LC – CDMP $p = 0.025$, eta-squared = 0.82, **Figure 20**). Individuals exposed to microplastics showed a slower pre-reproductive growth rate than LC or CD exposed individuals and individuals in the CDMP treatment showed the slowest pre-reproductive growth rate.

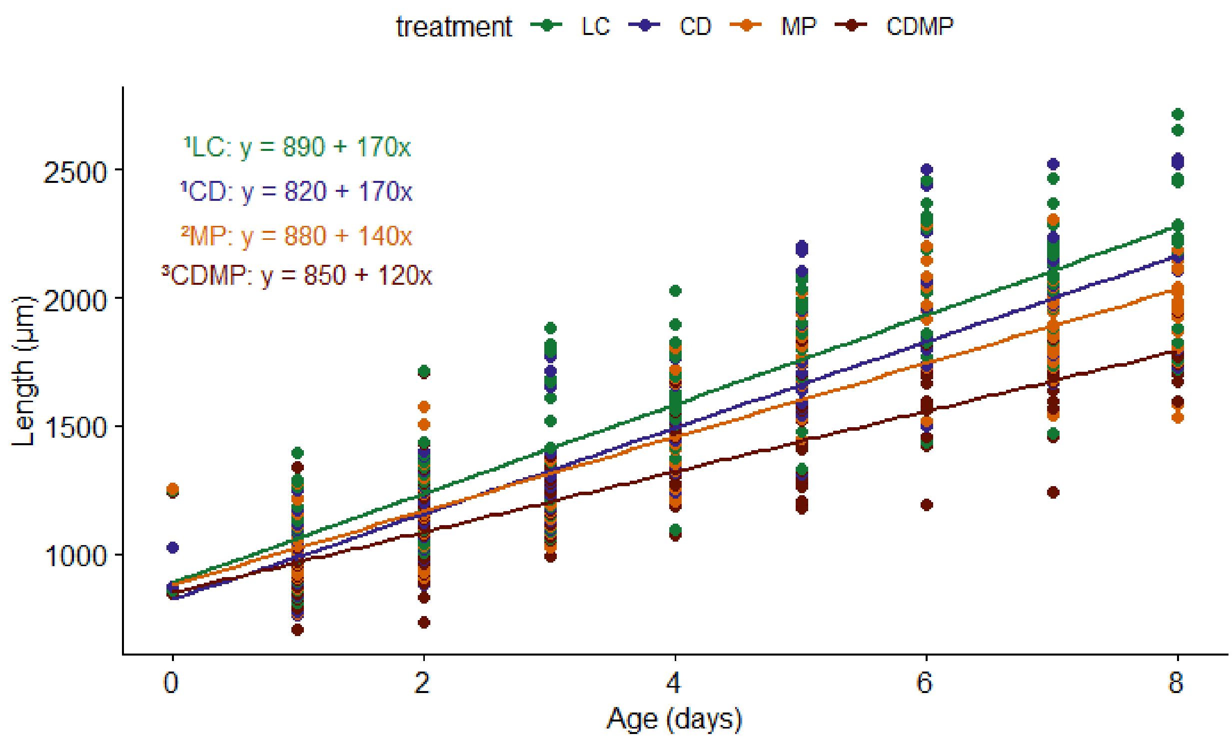


**Figure 20.** Growth curves of D. magna exposed to no contaminants (LC, n=194), cadmium (CD, n=146), microplastics (MP, n=205), and co-exposed to cadmium and microplastics (CDMP, n=150). Regressions among treatments were compared using Analysis of Covariance. Different superscript numbers preceding the treatment label and regression equation indicate significant differences in slope at a significance threshold of 0.05.

The maximum pre-reproductive size (at day eight) of individuals differed between treatments ($H(3) = 17.1$, $p < 0.001$, $n = 10 – 19$, **Figure 21**). Individuals in the LC treatment were significantly larger at day eight than CDMP exposed individuals (Cohen's D -1.84). Individuals in CD and MP did not differ (**Figure 21**) from each other, but CD shows a medium effect (-0.60 Cohen's D) and MP a large effect (-1.06 Cohen's D) relative to the control.

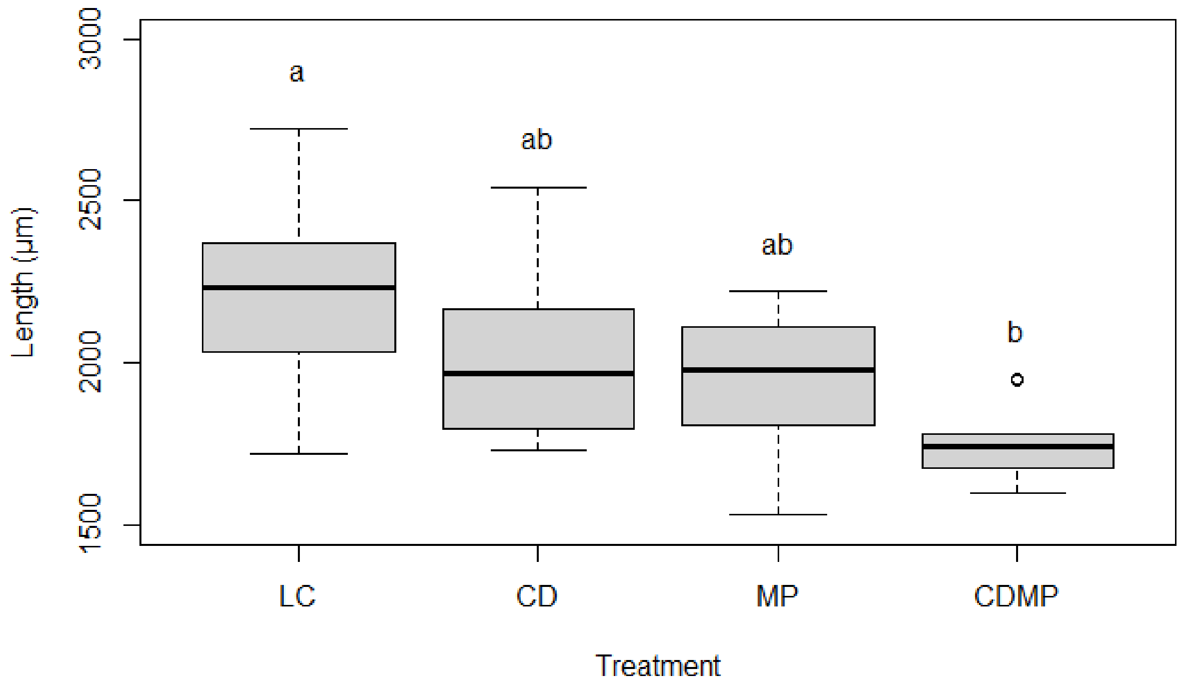


**Figure 21.** Changes in size (length) of D. magna exposed to no contaminants (LC, n=19), cadmium (CD, n=19), microplastics (MP, n=18), and their mixture (CDMP, n=10). Differences among treatments were analyzed by using a Kruskal-Wallis test with a Dunn's post-hoc test. Letters above each treatment indicate significant differences from each other using a significance threshold of 0.05. The shaded box area represents the interquartile range, the line within the box represents the media, the whiskers represent the minimum and maximum values. Circles represent outliers.

***Feeding***

The amount of algae consumed over a 24-hour period was measured weekly for individuals from each treatment. Each feeding assay included individuals ranging from neonate to adult. Treatment had a significant effect on feeding rate ($F(3, 2331) = 0.072$, $p < 0.001$, n = 567-600, **Figure 22**). Post hoc comparisons indicated that individuals exposed to LC and MP showed equal feeding efficiency ($p = 0.18$) which significantly differed from the CDMP treatment ($p < 0.001$, **Figure 22**). The feeding efficiency of individuals exposed to CD did not differ from those exposed to MP or CDMP (CD – MP $p = 0.48$, eta-squared = 0.38; CD – CDMP $p = 0.29$, eta-squared = 0.41, **Figure 22**).

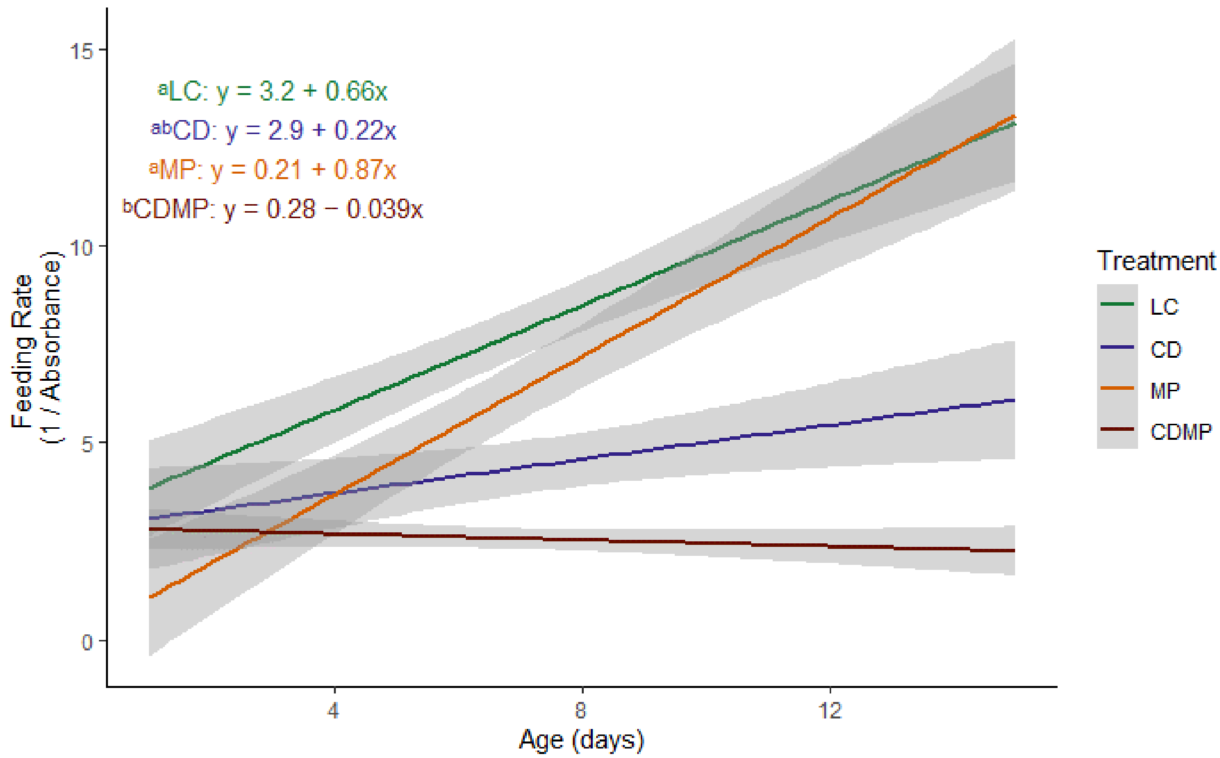


**Figure 22.** 24-hour feeding rates of D. magna, based on change in algal fluorescence, exposed to no contaminants (LC, n=600), cadmium (CD, n=576), microplastics (MP, n=591), and co-exposed to cadmium and microplastics (CDMP, n=567). Shading represents one standard deviation. Regressions among treatments were compared using Analysis of Covariance. Superscript letters preceding the treatment label and regression equation indicate significant differences, shared superscripts indicate no statistical difference ($p > 0.05$).

**Discussion**

The toxicity of cadmium to daphnids is dependent on environmental characteristics including water hardness, which is used to adjust site-specific water quality guidelines (Winner 1986; Heugens et al. 2003b). In soft water, concentrations of Cd as low as 1.5 µg/L causes mortality of *D. magna* (Pérez and Hoang 2018). As this study utilized moderately hard water, the concentration of 5 µg Cd/L was the maximum concentration in which reproduction still occurred by all tested daphnids and was therefore chosen as the concentration for use in the present study. . Cadmium-exposed individuals showed the same early-life growth rate as daphnids not exposed to contaminants; however, the feeding efficiency of cadmium-exposed individuals was reduced relative to the control. Though not assessed in the present study, the disparity in cadmium-exposed and control individuals might be due to the control individuals storing excess nutrients as lipid bodies. Further research should assess lipid body characteristics in daphnids under these exposure conditions.

Complexation of metals and microplastics has been observed under a variety of water quality conditions, and previous work has suggested that microplastics might be protective against toxicity caused by free-ionic metals by acting as a ligand (Turner and Holmes 2015; Wang et al. 2017; Guan et al. 2020). However, this protective effect was not evident in the present study. Microplastics did not alter the lifespan of daphnids from the control and daphnids co-exposed to microplastics and cadmium had the same decrease in lifespan as those only exposed to cadmium, indicating that microplastics were not lethal to daphnids and did not enhance or attenuate the lethality of cadmium. The equal lifespan of CD and CDMP treatments indicates that the ingestion of cadmium-microplastic complexes results in uptake of the same amount of cadmium that daphnids take up from waterborne cadmium exposures.

Growth rate of juvenile (pre-reproductive) daphnids is a well-established apical endpoint in tests of chronic toxicity and follows a linear trend, which was observed in this study (Hanazato 1998; Smirnov 2017). In our study, there were significant changes in early life growth rates among treatments. The growth rate for individuals exposed only to cadmium closely resembled that of the control group, indicating that cadmium exposure did not alter growth. Exposure to cadmium at sublethal concentration can alter the development of *D. magna,* which has previously been described using changes in daphnid dry weight; however, this was not corroborated by the present study using a length-based measure of growth (Pérez and Hoang 2018). Individuals exposed to microplastics had a slower growth rate than control and cadmium-exposed individuals, and when co-exposed to cadmium and microplastics, the growth rate of daphnids slowed markedly, differing from both contaminants exposed individually, indicating that exposure to cadmium-microplastic mixtures result in additive effects on early-life growth.

Our results indicate that for *Daphnia magna*, exposure to microplastics did not alter lifespan but did influence growth. Despite the additional suspended microplastic particles in the MP treatment, daphnids maintained feeding efficiency the same as individuals not exposed to contaminants. However, microplastic-exposed individuals showed lower pre-reproductive growth rates, potentially because the additional energy expenditure required to excrete ingested plastics limited the amount of energy available for somatic growth. Similar observations of decreased growth rates have been made with daphnids exposed to natural suspended particles such as clay and sediment (Hart 1992; Robinson et al. 2010).

Daphnia exposed to the cadmium-microplastic mixture had both the lowest growth rate and the lowest feeding rate. Based on our observations, microplastics did not inhibit the feeding ability or efficiency of *D. magna.* The decrease in juvenile growth rate suggests an additional energetic expense to excrete ingested microplastics resulting in decreased energy allocation to somatic growth. Daphnids rapidly respond to environmental stressors and have been shown to alter their energy allocation and life history patterns when under stress toward reproduction, such as exposure to toxicants, limited food availability, and shifts in water quality (Bodar et al. 1988b; Chandini 1989). It is likely that the effects we observed on growth rates are indicative of a shift in energy allocation away from somatic growth and towards reproduction, as exposure to microplastics did not alter any life history traits.

In our study, there were no differences in any of the life history traits measured (time to first brood, reproductive frequency, the number of neonates released at each reproductive event, and the size of neonates produced) between treatment groups. This indicates that even though daphnids exposed to the cadmium-microplastic mixture might not have acquired the same amount of nutrients through feeding as the other treatment groups, those individuals dedicated a greater proportion of their available energy towards reproduction. This is also true for the cadmium-only group, albeit to a lesser magnitude.

*Daphnia magna* have been observed to undergo energy allocation shifts, such as the one proposed by this study, when under stress. Under a nutrient deficit (as measured by feeding efficiency), daphnids in this study showed a decrease in early-life growth but maintenance of reproductive output, which would translate to maintenance of population levels but an overall decrease in size of individuals in the population (Enserink et al. 1995). This shift could have cascading effects such as a restriction in suitable food sources due to smaller mouthparts, a decreased space capacity for energy reserves, or shifts in metabolism (McMahon 1965; Glazier 1992). Metabolic changes such as detoxification reactions, including expression of metallothioneins that detoxify metals, have been observed in *D. magna* and may also be occurring in this study, though were not measured (Smirnov 2017). Further research should investigate additional concurrent potential shifts in xenobiotic metabolism and energy allocation under stress of contaminant mixtures. In addition to metabolomics, future research should investigate changes in swimming velocity and overall activity as these behaviours have been shown to be influenced by toxicant stress and could be extrapolated to understanding foraging and predator evasion decisions relative to energy allocation (Baillieul and Blust 1999).

**Conclusion**

This study investigated the effect of exposure to sublethal concentrations of cadmium, microplastic, and their mixture on the survival, growth, feeding, and life history of *Daphnia magna*. Microplastics and cadmium exerted additive toxicity to daphnids through independent mechanisms of action. Microplastics inhibited early-life growth whereas cadmium decreased feeding efficiency and when co-exposed to both microplastics and cadmium, both growth rate and feeding were decreased. Neither microplastics nor cadmium altered life history traits as measured in this study by time to first brood, reproductive frequency, the number of neonates released at each reproductive event, and the size of neonates produced. This study demonstrates a prioritization of reproduction over somatic growth in *D. magna* when under toxicant stress and resultant nutrient limitation. Energy allocation shifts, such as the shift towards reproduction in this study, can result in long-term implications to both individual and population health. For example, individuals unable to store excess nutrients in lipid bodies may be unable to survive periods of low food availability. This study indicates that co-exposure to cadmium and microplastics causes a greater energy allocation shift than either contaminant alone and that daphnids in co-contaminated environments are at greater risk of subsequent implications of that shift than those exposed to either contaminant alone.

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# PART 2: EXPLORING METAL-MICROPLASTICS TOXICITY RESEARCH WITH A METAL LIGAND APPROACH

Part 1 (Chapters 2, 3, and 4) of my thesis represents contributions to the emerging field of microplastic-metal mixtures toxicity research using a traditional mixtures toxicity study design framework (assessing the effect(s) of Constituent 1 alone, the effect(s) of Constituent 2 alone, and the effect(s) of Constituents 1 and 2 together). The studies described in these chapters were designed to address specific knowledge gaps in the field: the role that multiple environmental compartments play in cadmium-microplastic uptake, the behaviour of these contaminants post-ingestion, and the multigenerational effects of exposure to cadmium, microplastics, and their mixture.

Generally, we would expect contributions such as in Part 1 to advance the field forward – a primary goal of any doctoral thesis; however, this was not the case. Rather than providing novel theories, supporting evidence, or complimentary knowledge to the existing body of literature, the studies in Part 1 contradicted conclusions drawn by other researchers. For example, the data suggested that microplastics alone did not influence the life history of daphnia, whereas another research group (Schür et al. 2020) with a very similar experimental design found that microplastics impeded reproduction in the same species. In framing my research in the field, not only did contractions arise with my own research, but between other researchers as well.

The next stage of my journey (Part 2) entailed delving into the literature to understand why these contradictions were so prevalent. In reading the literature, it became apparent that the expertise of researchers in this field were in microplastics and other particulate contaminants, and that the majority of microplastic-metal mixtures toxicity researchers had not previously worked with metals. I leveraged my knowledge as a researcher coming from a metals-based laboratory to determine if our knowledge of metals behaviour (which is vast in comparison to our understanding of microplastics) could explain the discrepancies in microplastic-metals toxicity research.

The toxicity of cadmium in aquatic environments is dependent on water chemistry parameters including water hardness, dissolved organic carbon (DOC) and pH (Outridge et al. 1994). When water hardness increases, primarily attributed to calcium and magnesium, the toxicity of cadmium is attenuated due to competition between cadmium and calcium (both divalent cations) at biotic ligand sites, such as the fish gill (Meyer et al. 2007). Similarly, as hydrogen ions increase with a decreasing pH, competition between cadmium and hydrogen at biotic ligand sites decreases cadmium toxicity (Meyer et al. 2007). Further, changes in pH can alter the speciation of metals with lower pH favouring the free bivalent cation form of cadmium, which has been demonstrated to be the most toxic form (Qu et al. 2013). Dissolved organic carbon within surface water acts as a ligand that can bind cadmium and render it no longer bioavailable to aquatic organisms (Meyer et al. 2007). Just as these parameters govern the interaction of cadmium with inorganic anions and organic matter (both of which are natural ligands), I hypothesized that the interactions of microplastics (an anthropogenic ligand) and cadmium may also be dependent on water quality.

To this end, I first aimed to leverage the pre-existing literature to perform a meta-analysis of water quality parameters currently being reported. The assessment of existing literature is Chapter 5 of this thesis, which reveals that water quality is poorly reported. A meta-analytical approach was insufficient in determining whether differences in water quality could explain the contradictions due to the lack of water quality reporting in existing literature. The final research objective of this thesis was to collect the data to test my hypothesis that water quality could explain the contradictions of this field. By utilizing a combination of laboratory experiments and machine learning techniques, Chapter 6 describes the model I developed and tested that outlines which water quality characteristics govern the association of cadmium with microplastics.

## CHAPTER 5: A proposed reporting framework for microplastic-metal mixtures research, with emphasis on environmental considerations known to influence metals

In this chapter, we leverage our knowledge of how water quality characteristics influence the behaviour of metals in aquatic systems. We assess the current body of microplastic-metal mixtures toxicity research to determine the current state of water quality reporting.

This thesis is a manuscript-style thesis which is organized based on the University of Lethbridge thesis submission regulations. Inevitably, there is some repetition of content between sections, particularly in the introduction and methods sections of research chapters. A version of this chapter has been published in *Ecotoxicology*:

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Lauren Zink: conceptualization, methodology, investigation, writing – original draft, writing – review & editing, visualization, funding acquisition

Gregory G. Pyle: supervision, writing – review & editing, funding acquisition

**Abstract**

In recent years, there has been an increase in research to understand the consequences of microplastic contamination. A subset of this research assesses the interaction of microplastics with metals and the subsequent effects of the resulting microplastic-metal complexes in freshwater environments. While our understanding of how microplastics behave in freshwater remains largely unknown, our knowledge of metal behaviour in those same environments is well-established. The behaviour (partitioning, speciation, bioavailability) of metals is highly dependent on environmental characteristics, including water quality variables such as hardness, pH, and dissolved organic matter. This study reveals that despite our understanding of metal behaviour, there is little consideration for these influential factors in the current body of microplastic-metal research. Multiple instances highlighted throughout this study show that even when similar plastic, metal, and biota are utilized, there are conflicting observations as to whether the mixture is toxic; we stress that without adequate reporting of environmental conditions, these contradictions are likely to persist without explanation. Through justification of water quality characteristics known to influence metal behaviour, this study proposes a framework of reporting requirements for all future microplastic-metal research.

**Introduction**

Mixtures toxicology aims to understand the biological effects of multiple chemicals present simultaneously in an exposure scenario. The current theories and practices used in mixtures toxicology vary depending on the behaviour of the substances comprising the mixture, including their partitioning and persistence within an environment. Both metals and microplastics are found in multiple environmental compartments, such as sediment, water, and biota (Sarijan et al. 2020). Metals and microplastics interact with each other through the sorption of metals to microplastics in the environment (Caruso 2019; Leiser et al. 2020). To estimate the potential toxicity of a mixture, it is vital to understand the individual effects of each substance comprising the mixture and understand how they interact with each other and with other confounding variables. For a number of contaminants, the exposure background characteristics are confounding variables that must be considered because varying environmental conditions can alter contaminant bioavailability and contaminant form. In aquatic environments, water quality characteristics such as pH, organic matter, and water hardness influence the speciation and bioavailability of many classes of contaminants including metals (Renner 1997), pharmaceuticals (Alsop and Wilson 2019), and pesticides (TenBrook et al. 2009).

Our understanding of how metals and microplastics behave in the natural environment is very different. While metal behaviour in aquatic systems has been actively investigated for decades, research into the behaviour of microplastics in freshwater systems has only gained widespread research interest within the last five years. The physico-chemical characteristics of the ambient environment alter how a metal partitions by changing solubility and metal speciation. The free-ion form of metals is the most bioavailable and therefore the most toxic. The increasing temperature of an environment increases the solubility of metal-containing salts, releasing more free-ionic metal into the environment (Namieśnik and Rabajczyk 2010).

The acidity of an environment, as measured by pH, alters the speciation of metals. As pH decreases, the solubility of metals increases, creating a greater proportion of the free-ionic form and increasing its bioavailability (Wren and Stephenson 1991). It is often difficult to isolate the effects of pH changes on toxicity due to other water quality characteristics that inherently co-vary with pH, such as water hardness and alkalinity (Long et al. 2004).

The ionic composition of surface waters, primarily measured as hardness and alkalinity can influence metal behaviour. Water hardness represents the amount of divalent metal cations, which are largely calcium (Ca) and magnesium (Mg), expressed as the concentration of calcium carbonate. Water hardness can affect a metal's behaviour through competition between the metal and mineral cations (Ca, Mg) for negatively charged binding sites (Zitko and Carson 1976). The binding sites may be biotic ligands, competition at which directly influences the interaction of the metal to the organism (Pagenkopf 1983); alternately, the binding sites may be other ligands such as those of organic matter (Nelson et al. 1986; Playle et al. 1993) which indirectly influence the bioavailability of the metal by sequestering metal away from an organism. Biotic ligands, such as fish gills, have a decreased permeability to metals in hard water because the permeability of such membranes to divalent metal ions is inversely related to the aqueous calcium due to Ca-Ca cross-bridging on the apical surface of the gill (Varanasi and Gmur 1978; Pärt et al. 1985). Total alkalinity is a measurement of the concentration of all alkaline substances in an environment, primarily carbonates and hydroxides; the presence of these anions enable an environment to resist changes in pH through increased buffering capacity. In alkaline environments free metal cations form inorganic complexes, such as metal carbonates or hydroxides, which reduce metal bioavailability.

Organic matter plays an important role in freshwater ecosystems including providing nutrition and temporal-spatial information to aquatic organisms (Thomas 1997). Organic matter is a broad term used to refer to the complex and dynamic suite of dissolved carbon-based compounds resulting from the decomposition of living matter, either running off from the local landscape (allochthanous) or producedwithin the system (autochthanous). In a freshwater environment, these molecules can differ in size, aromaticity, molecular weight, and functional groups present (Lam et al. 2007). The characteristics of organic matter, predominantly the degree of aromaticity, determine its ability to bind metals and alter their bioavailability (Playle et al.

1993; Chen et al. 2018). For example, minor increases to the aromaticity of dissolved organic matter accounted for up to a 10-fold increase in metal binding capacity for nickel, cadmium, and zinc, subsequently resulting in a decrease in the amount of free-ionic metal (Baken et al. 2011). This not only highlights the need to consider the amount of organic matter present, but also the quality of that organic matter.

Microplastics, generally accepted as plastics ranging from 1 µm to 5 mm, can be introduced to aquatic environments through household and industrial effluent, stormwater and other runoff sources, as well as through atmospheric deposition (Allen et al. 2019; Blair et al. 2019; Gong and Xie 2020; Werbowski et al. 2021). Once introduced, microplastics undergo inevitable degradation. There are many types of degradation that impact microplastics to varying degrees including ultra-violet (UV) degradation (greater significance for floating microplastics), thermal degradation (greater significance in water bodies with higher temperatures and larger temperature fluctuations), mechanical degradation, and biological degradation (Luo et al. 2020; Ding et al. 2020). These modes of degradation demonstrate how the conditions of the exposure background can affect microplastics *in situ*, which likely affects their behaviour. For example, UV-degraded microplastics show greater surface cracking than intact particles, resulting in an increased surface area and, in turn, an increased adsorption capacity for other contaminants, such as metals (Liu et al. 2019a; Wang et al. 2020a).

In addition to surface area, the surface charge of microplastics is an important factor when considering how microplastics and metals interact. Microplastics generally have a negative surface charge whereas free metals are cationic, resulting in an ionic attraction between the opposite charges of microplastics and metals (Mammo et al. 2020). We continue to develop our understanding of how ionizable compounds (such as organic compounds, pharmaceuticals, and metals) bind to various ligands (including sediment, biota, or microplastics). Water quality characteristics influence the loading of ionizable compounds to ligands (Barber et al. 2006; Fu et al. 2009; Seidensticker et al. 2018). The ionic attraction between metal ions and microplastics can result in the concentration of metal adsorbed to the plastic surface being higher than that of the ambient environment.

Currently, conclusions in the field of microplastic-metal mixtures research remain highly divided on whether microplastic-metal complexes are toxic to aquatic life. Even in instances where many components of experimental design are similar, opposite conclusions are reported. For example, two studies analyzing the effects of chronic exposure of *Chorella vulgaris* to varying amounts of copper (Cu) and polystyrene (PS) found that exposure to Cu-PS resulted in antagonism (Wang et al. 2021b) while the other reported synergism (Tunali et al. 2020). Despite the suite of similar aspects of experimental design, these studies failed to adequately report the exposure conditions (neither study reported water hardness or organic matter, and only Tunali et al. (2020) reported pH) or assessment of bioavailability of either microplastics or metals. Another example of this disparity is the comparison of two studies assessing the effects of acute exposure of nickel (Ni) and PS mixtures to *Daphnia magna*; one study reported antagonism at low concentrations of MPs which transformed to synergism with increasing amounts of MP (Yuan et al. 2020), whereas another study using a very similar design reported no change in toxicity across experimental treatments (Kim et al. 2017). While Kim et al. (2017) assessed the adsorption capacity of metals to the surface of microplastics, identifying the presence of carboxyl groups to be an indicative factor of Ni adsorption capacity, neither study characterized exposure conditions. Without adequate reporting of environmental characteristics known to influence metal behaviour, these types of contradictions – which are currently common in the field – are unable to be resolved.

Characterizing the exposure environment allows for the prediction of the amount of dissolved metal and its speciation, which in turn determines its bioavailability. As the free-ionic metal is the most toxic and the most likely to interact with microplastics, it seems logical that water quality characteristics known to govern the amount of free-ionic metal present may explain the variation of microplastic-metal associations and subsequent potential toxicity seen across microplastic-metal mixture research. The inconsistent conclusions present in microplastic-metal mixtures must be resolved before we can begin to establish priorities in areas of risk assessment, water quality criteria development, and policy creation; the importance of characterizing water quality characteristics in metals research is well established and as such it is likely to be important in the field of microplastic-metal mixture research. The objectives of this study were to (1) compile microplastic-metal research published to date, (2) perform a literature synthesis to determine the degree to which microplastic surface characterization and water quality reporting are present, and (3) propose minimum reporting requirements for the field of microplastic-metal mixture research.

**Methods**

The compilation of microplastic-metal research was based on the results of a Google Scholar query and followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 checklist where possible (Page et al. 2021). The search included results up to June 15, 2021. Search criteria included microplastics in general, specific plastic types, and metals (including individual metal names and symbols). The initial search resulted in 3631 hits. After censoring results for language and relevance, 44 articles remained.

Each study included in the literature synthesis (those that analyzed microplastic-metal mixtures) was processed for information pertaining to microplastics (size, surface characterization, whether microplastics were degraded), metals (element, concentration, original source compound of the metal, and the environmental component (water, sediment, biota) in which metal was assessed), and water quality characteristics (temperature, pH, hardness, alkalinity, and measures of organic matter). Data were generated from individual articles and corresponding supplementary information quantifying the articles that reported each of the microplastic and water quality characteristics of interest; no attempt was made to contact authors.

**Results**

The size of microplastic particles was reported in 64% of all articles, from which the plastics spanned the entire size range (1 µm – 5 mm). The average microplastic size utilized was 279 ± 869 µm (mean ± standard deviation, n=51). The average size range of microplastics used in a given experiment, was 149 ± 410 µm (mean ± standard deviation, n=51). Most (91%, 40 of 44 studies) studies utilized primary, pristine microplastics; of the remaining (9%, 4 of 44 studies) studies, a majority (59%, 26 of 44 studies) simulated degradation as a controlled experimental variable.

The majority of work to date (89%, 39 of 44 studies) has taken place under controlled laboratory conditions as opposed to the uncontrolled conditions of natural systems. Studies that have been conducted in the field or performed in the laboratory using field-collected environmental components (water, sediment, and/or biota) have included lake, river, wetland, and reservoir environments. All but one study failed to characterize exposure background conditions of field sites.

The exposure conditions in the laboratory experiments were also generally poorly characterized. Temperature was recorded in 73% (32 of 44) of studies (**Figure 23**). Fewer than half (43%, 19 of 44 studies) reported pH, only 11% (5 of 44 studies) reported water hardness and 7% (3 of 44 studies) reported alkalinity. For purposes of this analysis, a variety of measures of organic matter were considered, though very few were reported: total organic carbon (9%, 4 of 44 studies), dissolved organic carbon (0%), dissolved organic matter (5%, 2 of 44 studies), or natural organic matter (5%, 2 of 44 studies; **Figure 23**).


**Figure 23.** Proportional representation of water quality characteristics reported in metal-microplastic mixtures toxicity studies.

## Discussion

By treating microplastics as an additional ligand for metals to interact with in a given environment, we can leverage our understanding of metals in order to establish an informed, logical path forward for microplastic-metals toxicology research. There are established considerations when constructing minimum reporting requirements such as CRED, an evaluation criteria grounded in consistency, transparency, reliability, and relevance (Moermond et al. 2016).

The inclusion of the criteria listed in CRED allow for studies to be compared, compiled, and contribute to risk assessment. Similarly, a clear set of reporting requirements, as suggested in **Table 6** for the field of microplastic-metal research would allow that same comparative ability to better understand this emerging field of research. In addition to the reporting requirements stated below, discretion in frequency of water quality measurements being taken should be used, particularly for chronic and field-based studies in which temporospatial considerations such as those of uptake rates, hydrological residence time, seasonality, and pulse events could influence exposure conditions.

**Table 6.** Water quality characteristics of Aquatic Research Facility (ARF) culture water. Ranges presented are of weekly measurements of pH, conductivity, hardness, and alkalinity (n = 40) and biweekly measures of dissolved organic carbon (DOC, n = 20) taken over the course of the current study.

| | At minimum, must include | To strengthen, should also include | For completeness, ideally also includes |
|---|---|---|---|
| Water Quality Characteristics | Temperature | | |
| | pH<br>Water Hardness | Alkalinity | Ionic composition of surface water for all major ions [a] |
| | Dissolved Organic Matter | Degree of aromaticity of organic matter | Characterization of organic matter [b] |
| Microplastic Bioavailability | Determination of whether ingestion and potential to form blockages is possible and/or plausible<br><br>Assessment of interaction resulting in interference to physiological function (e.g., gill clogging) | Quantification of MP uptake | Tissue-specific accumulation and assessment of membrane crossing capability |
| Metal Bioavailability | Proportion of dissolved metal [c] | Proportion of free-ionic metal [d] | Metal equilibrium constant across all environmental compartments (water, sediment, biota, organic and inorganic particulate, MP, etc.) |
| | AND, IF MP IS BIOAVAILABLE<br>Amount of metal bound to MPs [e] | Degradation and functional group characterization of MP surface [f] | |

[a] Major ions in freshwater systems include but are not limited to calcium, magnesium, sodium, potassium, ammonium, hydrogen, sulfate, chloride, and nitrate (Schlesinger and Bernhardt 2013)

[b] Characterizations of organic matter include age, functional groups present, and fluorophore signatures (Bianchi 2006; Murphy et al. 2010)

[c] Dissolved metals can be quantified using membrane filtration, acid preservation, and instrumental analysis (Environmental Monitoring Systems Laboratory 1996)

[d] Multiple techniques allow for the quantification of free-ionic metals in water including anodic stripping voltammetry, Donnan dialysis, and cation exchange resin (Berggren 1990; Ge et al. 2005; Unsworth et al. 2006)

[e] Metals associated with microplastics can be quantified by acid digestion and subsequent instrumental analysis, though it relies on the ability for a sufficient amount of microplastics to be isolated from the system (Hildebrandt et al. 2020)

[f] Functional groups present on the surface of microplastics can be characterized using scanning electron microscopy/energy-dispersive X-ray spectroscopy, and Fourier transform infrared spectroscopy (Wang et al. 2019a; Yao et al. 2022)

With the understanding of how temperature, pH, water hardness, and dissolved organic matter influence metal behaviour, all microplastic-metal research must report these characteristics. If feasible, alkalinity should also be assessed as alkalinity can influence metal behaviour, though to a lesser degree than the aforementioned characteristics. Ideally, a full ionic profile would be carried out to fully understand the ionic interactions occurring; however, this may not be achievable for all studies.

Our understanding of microplastic-metal toxicity also relies on determining the bioavailability of microplastic-metal complexes to aquatic organisms. As environmental quality guidelines are currently established for water, sediment, and tissue residues; however, considerations of potential dietary exposure of ingested microplastic-metal complexes must be considered by assessing bioavailability. Work in this field must include an assessment of microplastic-organism interactions, including whether a microplastic is of an ingestible size, whether a microplastic or and aggregation of microplastics could cause blockages, or whether microplastics adhere to the organism and interfere with physiological function (e.g., gill clogging). If the microplastic (and therefore microplastic-metal complex, due to the negligible relative size of adsorbed metal) is deemed to be bioavailable, relevant tissue-specific uptake, accumulation, and toxicity should also be assessed, such as digestive absorption and detoxification structures with the overarching goal to develop an understanding of tissue-specific accumulation and migration potential of microplastics across biological membranes.

As the free-ionic form of metal is the most toxic form, we must work to at least quantify the amount of dissolved metal present in the system. If feasible, quantification specifically of free-ionic metal ions would be more indicative of potential toxicity and should be sought when possible. Though costly in both time and required instrumentation, establishing metal equilibrium constants across all environmental compartments (water, sediment, microplastic, organic matter, biota, etc.) would provide additional understanding of metal bioavailability within a system.

In instances where microplastics (and inherently microplastic-metal complexes) are found to be bioavailable, whether through ingestion or adherence, we must quantify the amount of metal associated with the microplastic to understand the potential bioaccumulation of metal which is transported by microplastics acting as a vector. When microplastics are able to be isolated in sufficient quantity from the system, this can be achieved through digestion and instrumental analysis. Where it is not possible to isolate the microplastics, the characterization of the microplastic surface would be a viable alternative, though this requires complex instrumentation and analysis. In many instances throughout our dataset, primary microplastics were purchased directly from a polymer manufacturer; in many of these cases, the polymer composition was not disclosed which also cannot be resolved without spectral analysis. Further, various additives and stains are added to many manufactured polymers for easier visualization in either the visible or fluorescent light spectra, though the influence that the dyes have on surface characteristics and subsequent adsorption capacity is unknown.

The characterization of microplastic surface structure and functional groups would allow deeper understanding of the microplastic-metal association and may provide insight into the stability of that association. Polymer composition is an integral factor contributing to the surface characteristics of that plastic (Burrows et al. 2020). Further, considering the changes to the surface after degradation, the adsorption capacity of microplastics is not easily defined without spectral analysis. In instances where instrumentation does not allow for such analyses, efforts should be taken to quantify the surface area (potentially through mathematical modelling under assumptions of a basic shape) and degree of degradation (through surface image analysis) to allow for a better understanding of adsorption capacity.

The few studies that have investigated how degradation affects the adsorption capacity of microplastics have uniformly found that degradation increases surface area and increases the adsorption capacity of metals to plastics (Luo et al. 2020; Kalčíková et al. 2020; Rozman et al. 2021). One study looking exclusively at how degraded plastic differentially binds metals found that adsorption increased by 51% and 33% in aged plastics compared to pristine for copper and lead, respectively (Wang et al. 2020a). Variability of metal concentration of this magnitude can overwhelm detoxification capabilities of aquatic species should the resulting microplastic-metal complex be ingested and dissociate under digestive conditions. For many metals, waterborne concentrations that remain below toxicity thresholds may not be of concern; however, dietborne exposure to those same concentrations can cause toxicity as ingestion of contaminated particles results in a pulse exposure and metals may be of a different (and potentially more toxic) species when associated with food rather than surface water (DeForest and Meyer 2015).

There is currently little evidence to suggest that water quality characteristics govern the behaviour of microplastics beyond their density determining the positioning of a plastic within the water column; however, there is extensive evidence detailing how water quality characteristics govern metal behaviour in aquatic systems. This review revealed a paucity of measured water quality characteristics in microplastic-metal research. The variable conclusions drawn regarding microplastic and microplastic-metal mixture toxicity could be due to the lack of adequate environmental characterization. As the free-ionic form of metal is the most toxic and the most likely to interact with negatively-charged ligands, such as microplastics, characterizing metal speciation would allow for further understanding of microplastic-metal adsorption capacity and to better understand the relative partitioning co-efficient of a metal to a microplastic. Without the ability to assess and populate required fields for predictive modelling tools such as MINTEQ and the Biotic Ligand Model, this field will continue to rely on individual studies reporting exposure conditions, which is costly and not currently adequately employed. The inability to consolidate studies and subsequently perform meta-analyses that can inform risk assessment and the development of policy limits the ability for this field of research to progress.

The justification for the factors that comprise minimum reporting requirements of microplastic-metal research is vital. While environmental factors that influence metal behaviour are well described, the environmental factors that influence microplastic behaviour remain largely unknown, though research directives and minimum criteria recommendations are beginning to emerge for microplastics studies (de Ruijter et al. 2020; Thornton Hampton et al. 2022). The feasibility of controlled experiments in which environmental parameters are well-characterized are costly and may be unrealistic in many cases; however, work of that nature is required to create a body of research that is conducive to a meta-analysis which can then identify the degree to which environmental characteristics influence microplastic behaviour. In the interim, at minimum, factors that have already been established to govern metal behaviour in aquatic systems (temperature, pH, water hardness, and organic matter) and those which directly establish the bioavailability of microplastics and metals should be reported for all future microplastic-metal mixtures research.

**Conclusion**

The overarching goal of metal and microplastic mixtures research is to perform meaningful risk assessments to determine potential negative effects on aquatic life. To focus microplastic-metal efforts towards achieving this goal, it is vital that all future microplastic-metal research adequately characterize environmental parameters, particularly factors known to influence metal behaviour (pH, water hardness, organic matter, temperature) and microplastic surface characteristics that affect adsorption capacity. The establishment of minimum reporting requirements for microplastic-metals research will enable this field to progress on a strong foundation to investigate the more complex questions in the field of microplastic-metal toxicity.

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## CHAPTER 6: Reframing microplastics as a ligand for metals reveals that water quality characteristics govern the association of cadmium to polyethylene

In the previous chapter, it became apparent that the water quality reporting in the existing body of literature was insufficient to determine if differences in water quality could explain the contradictions in conclusions drawn surrounding microplastic-metal mixtures toxicity. In this chapter, we use laboratory experiments and machine learning to develop and test a model that outlines which environmental characteristics predominantly govern the association of cadmium with polyethylene, a representative microplastic. To increase accessibility and usability, we have formatted this tool into a dichotomous-style key where users determine whether an environment falls above or below the given thresholds for each influential water quality parameter, which collectively determine the proportion of cadmium that is bound to microplastic. Further, using fathead minnows, we assessed how water quality characteristics determined by our model (assessing cadmium-microplastic interactions) works in tandem with biota (assessing cadmium-gill interactions) to provide insight on how cadmium-microplastic ligand interactions relate to cadmium toxicity.

This thesis is a manuscript-style thesis which is organized based on the University of Lethbridge thesis submission regulations. Inevitably, there is some repetition of content between sections, particularly in the introduction and methods sections of research chapters. A version of this chapter has been submitted for publication:

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The author contributions for this manuscript are as follows, in accordance with the CRediT Author Contributions system:

Lauren Zink: conceptualization, methodology, investigation, funding acquisition, writing – original draft, writing – review and editing

Emily Mertens: investigation, data curation, writing – review and editing

Xingzi Zhou: software, formal analysis, data curation, writing – review and editing

Sarah Ellen Johnston: conceptualization, software, writing – review and editing

Matthew Bogard: resources, writing – review and editing

Steve Wiseman: conceptualization, resources, supervision, writing – review and editing

Gregory G. Pyle: supervision, writing – review & editing, funding acquisition

**Abstract**

Environmental characteristics including water quality and sediment properties alter the hazard that metals pose to aquatic systems by governing the speciation and partitioning of metals between water, sediment, and biotic ligand components of aquatic systems; however, alternate ligands are being introduced into aquatic systems through anthropogenic activity. Microplastics are a ligand on which metals interact through adsorption to the plastic surface. It remains unknown what factors determine the amount of metal bound to microplastic. Using a combination of laboratory experiments and machine learning, we tested a suite of twenty-six water quality parameters to understand how they influence association of cadmium to a representative microplastic, polyethylene. From this, we developed and tested a predictive model that outlines the water quality characteristics that favour the association of cadmium to microplastic. Alkalinity, humification index of dissolved organic matter, and pH were the three main factors determining the proportion of cadmium adsorbed to plastics. These results align with other predictive models, such as the Biotic Ligand Model in demonstrating the governance of metal behaviour by water quality characteristics. To assess the relationship of hazard (amount of cadmium bound to microplastic) and toxicity, an exposure assessment was completed. Fathead minnows (*Pimephales promelas*) were acclimated to environments that fell in each of the potential outcomes of the model; however, the uptake of cadmium was not significantly different between groups, indicating that the stress of alterations to water quality may be a confounding factor in determining the exposure risk of microplastics and cadmium.

**Introduction**

The intrinsic properties of substances determine the risk they pose in the environment. Special classifications have been defined for substances that pose particularly high risk. Microplastics an emerging pollutant increasingly being detected in freshwater systems, and are characterized as being vPvM (very persistent and very mobile) because of their high transportation rate and slow degradation (Besseling et al. 2013; Akdogan and Guven 2019; Miloloža et al. 2021; Koutnik et al. 2021). Many metals are characterized as being PMT (persistent in the environment, mobile in the aquatic environment, and toxic) (Nasrabadi et al. 2016; Wang and Tan 2019). To assess the risk that a substance poses in an environment, two steps must be completed: (1) a hazard assessment to describe the behaviour of the substance in an environment, and (2) an exposure assessment to describe the substances' interaction with biota in those environments. Together, the hazard and exposure assessments comprise a risk assessment.

The hazard of a substance is relative to the environment that it is in. The hazard that a metal poses in an aquatic environment is dependent on water quality and sediment characteristics driving changes in metal partitioning and speciation, through precipitation, complexation, and sorption reactions (Allen 1993; Outridge et al. 1994). Water hardness, alkalinity, and pH are measures of ionic composition, with water hardness primarily being attributed to calcium and magnesium, and pH being a measure of hydrogen, which contributes to precipitation and complexation reactions with metals. Dissolved organic matter (DOM) and sediment act as metal ligands, contributing to complexation and sorption reactions (Gardiner 1974; Besser et al. 2003). Interactions between metal cycling and water quality conditions are likely shifting with widespread salinization, alkalization, brownification and increased DOM concentrations, or acidification of surface waters worldwide, with unclear implications for aquatic organisms (Kaushal et al. 2005; Solomon et al. 2015; Kerr 2017; Kritzberg et al. 2020; Rodríguez-Cardona et al. 2022).

In addition to natural ligands, including sediment and DOM, anthropogenic particulates such as microplastics can act as abiotic ligands for metals (Dris et al. 2015; Anderson et al. 2016). Generally speaking, the free-ionic form of a metal poses the greatest hazard risk due to increased mobility and toxicity – two of the three PMT characteristics (Lemly 1995; Skeaff et al. 2002); further, the free-ionic form of metals interacts with microplastic ligands (Turner and Holmes 2015). As microplastics are very mobile, the potential for microplastics to serve as a vector for metals and thereby potentially increasing their mobility through aquatic environments, could result in a greater hazard of metal-microplastic complexes compared to the hazard of either contaminant alone (Teuten et al. 2009; Caruso 2019).

Microplastics serving as a vector for metals may alter the exposure risk of metals to aquatic biota by altering the bioavailability of the metal at biotic ligands. It is well-established that water quality characteristics alter the activity of metals at biotic ligands (Campbell and Fortin 2013). Perhaps the most studied aquatic biotic ligand is the fish gill, as it comes in direct contact with waterborne contaminants (Playle et al. 1993). The gill-based Biotic Ligand Model parameterizes the influence of multiple water quality characteristics including pH, hardness, and DOC to predict how toxic a metal will be at the gill (Morel and Hering 1993; Paquin et al. 2002; Niyogi and Wood 2004). The current body of literature investigating metal-microplastic associations and toxicity cannot be used to inform risk assessment, primarily because most studies do not report water quality and sediment characteristics, making a hazard assessment impossible (Zink and Pyle 2023). A multitude of contradictions exist within metal-microplastic mixtures toxicity, likely stemming from the lack of understanding in how these two classes of contaminants interact (Zink and Pyle 2023). The role that microplastics play as ligands, including the affinity of metals to sorb to microplastics under environmentally relevant water quality conditions, remains unknown. Further, it is unclear how the water quality characteristics that govern metal-microplastic associations relate to those that govern metal-gill interactions.

Cadmium is a non-essential metal, classified as being PMT (National Research Council 1997). As cadmium serves no known biological function, very few organisms can regulate internal cadmium, and low doses of cadmium are toxic to many species (National Research Council 1997). Cadmium exposure can result in reproductive impairment, oxidative stress, and interference with the perception and response to alarm cues (Bodar et al. 1988b; Espinoza et al. 2012; Dew et al. 2016). In fish, the gill is a major route of cadmium uptake, the activity of which is dependent on water quality characteristics of the surrounding environment (Playle et al. 1993). Exposure to cadmium may result in the upregulation of metallothionein, a protein involved in the transport and storage of metals, which can be measured in both external-facing tissues such as the gill and in internal detoxifying tissues such as the liver (Hamilton and Mehrle 1986).

The present study provides both a hazard assessment and exposure assessment of cadmium and a model microplastic (polyethylene). The hazard assessment utilizes laboratory experimentation and machine learning to determine which water quality and sediment characteristics drive cadmium-microplastic associations over a wide range of conditions, producing a predictive model of the proportion of cadmium bound to microplastic ligands. Using the thresholds provided by the hazard assessment model, we then performed an exposure assessment using a highly tolerant model species, the fathead minnow (*Pimphales promelas*), to discern how the model established through the hazard assessment (assessing cadmium-microplastic interactions) translates to cadmium uptake and toxicity (assessing cadmium-biota interactions).

## Methods
### *Hazard Assessment*

To discern how water quality and sediment characteristics influence the amount of cadmium associated to microplastics, we established 137 simulated freshwater environments in glass 300 mL tall-form beakers (PYREX Item #1060-300) that varied in water quality and sediment characteristics, all of which are summarized in **Table 7**. Each environment, less a small subset of environments which contained only water, had a 1 cm layer of acid-rinsed sand. This depth was chosen to represent the surface layer of sediment in which cadmium is most greatly exchanged between sediment and overlying surface water (Ramamoorthy and Rust 1978; Green et al. 1993). The sand fraction was used to allow for subsequent microplastic recovery. As sediment particle size influences the cadmium-sediment interaction, each environment varied in the size fraction of sediment used (Tran et al. 2002; Wang et al. 2022). A series of sediment sieves were used to obtain five size classes of sediment with particle ranges: 106 – 250 µm, 250 – 500 µm, 500 – 710 µm, 710 – 1000 µm, and 1000 – 2000 µm. For each environment, one size class of sediment was used (excluding the water-only environments).

**Table 7**. Sediment and water quality characteristics measured and considered in analysis

| Sediment or water quality characteristic | Description |
|---|---|
| Sediment size class | Particle size class of sediment utilized |
| pH | Acidity of the environment |
| Temperature | Temperature of the environment |
| Water hardness | Amount of divalent metal cations, which are largely calcium and magnesium |
| Alkalinity | The concentration of all alkaline substances in an environment, primarily carbonates and hydroxides |

| | |
|---|---|
| Dissolved organic carbon | Quantity of dissolved organic carbon (DOC) |
| Absorption coefficients | $a_{254}$, $a_{300}$, and $a_{350}$, collectively indicators of chromophoric DOM (dissolved organic matter) (Dobbs et al. 1972) |
| $SUVA_{254}$ | Indicator of aromaticity, calculated by integration of total DOC content at 254nm absorbance (Cory and McKnight 2005; Findlay and Sinsabaugh). |
| Biological index (bix) | Indicator of fresh DOM (Fellman et al. 2010; Hansen et al. 2016) |
| Humification index (hix) | Indicator of degraded DOM (Fellman et al. 2010; Hansen et al. 2016) |
| Fluorescence index (fi) | Indicator of aromatic DOM (Fellman et al. 2010; Hansen et al. 2016) |
| Maximum fluorescence (Fmax) | Maximum fluorescence intensity in the fluorescence spectra |
| Fluorescence and absorbance peak intensities: A, C, M, B, T (Dobbs et al. 1972; McKnight et al. 2001; Fellman et al. 2010; Li and Hur 2017) | A (excitation/emission (ex/em)=260 nm/380 to 410 nm)<br>C (ex/em=350 nm/420 to 480 nm)<br>M (ex/em=312 nm/380 to 420 nm)<br>Collectively, A, C, and M peaks indicate humic-like DOM<br>B (ex/em=270 nm/310 nm)<br>T (ex/em=275 nm/340 nm)<br>Collectively, B and T peaks indicate protein-like DOM |

Each simulated freshwater environment contained 200 mL of overlying surface water. Surface water quality variation was achieved through the creation and modification of various types of freshwater sources including dechlorinated City of Lethbridge (Alberta, Canada) tap water, multiple University of Lethbridge Aquatic Research Facility systems that house fish species including trout, fathead minnows, and zebrafish, and reconstituted (from double-distilled water) freshwater invertebrate culture waters such as those stated in Environment and Climate Change Canada and United States Environmental Protection Agency standardized toxicity testing and culturing documents (US EPA 2015; Canada 2022). Waters were also supplemented with natural organic matter isolates collected from various water bodies in Ontario, Canada (natural organic matter isolates provided courtesy of Prof. Jim McGeer, Wilfrid Laurier University).

We then contaminated each environment with a known amount of cadmium (range: 2 – 10 µg Cd/L, from cadmium sulfate octahydrate (CAS 7790-84-3)) and artificially weathered microplastics (range: 0.025 – 0.1 g). High-density polyethylene microplastics (MP) were utilized in this experiment (MPP-620VF, MicroPowders, Inc., Tarrytown, New York, United States of America). As reported by the manufacturer, these microplastics have a density of 0.96 g/mL at 25°C, mean particle size 5.0-7.0 µm, National Printing Inks Research Institute grind 2.0-3.0, and a melting point of 114-116 °C. To simulate microplastic distribution observed in freshwater systems, microplastics were artificially weathered by agitating 5 g of MP and 25 mL of Tween20® (CAS 9005-64-5) in 5 L of Milli-Q (EMD Millipore, Burlington, Massachusetts, United States) water for 24 hours on a magnetic stir plate at 1200 rpm at room temperature, as has been previously described (Balakrishnan et al. 2019). Microplastics were rinsed by vacuum filtration over a 0.45 µm nitrocellulose filter twice with 2 N trace-metal grade nitric acid (CAS 7697-37-2) and three times with Milli-Q (EMD Millipore) water. Microplastics were then dried in an oven at 60 °C to constant weight prior to use in the present study. Once the contaminated environments were established, each was sealed with parafilm and set to incubate at a constant temperature (ranging from 8 – 27 °C) for 96-hours.

Following equilibration, microplastics were isolated from surface water by filtration through a 0.45 µm nitrocellulose filter and dried to constant weight in an oven at 60 °C. Microplastics were quantified to determine the percent recovery. Dried microplastics were digested using a previously established and validated digest protocol to isolate metals adsorbed onto plastics (Turner and Holmes 2015; Turner et al. 2020). Plastics were suspended in 2.5 mL of 20% aqua regia for 16 hours at 22 °C and subsequently diluted to 10 mL before being analyzed by GFAAS as described in SI – Graphite Furnace Atomic Absorption Spectroscopy. Aqua regia-only blanks and certified reference material (acting as procedural controls) were digested simultaneously (Product Identifier: DOLT-4, National Research Council of Canada, Ottawa, Ontario, Canada). It was assumed that any microplastics not recovered had the same amount of cadmium bound as those captured; therefore, we calculated the percentage of total cadmium in the system bound to microplastics.

. The amount of cadmium adsorbed to the microplastics was quantified using graphite furnace atomic absorption spectroscopy (GFAAS). The concentration of cadmium in solution was confirmed by graphite furnace atomic absorption spectroscopy (GTA 120, Agilent Technologies, Santa Clara, California, USA) utilizing manufacturers specifications outlined in SpectrAA software (Agilent Technologies), with modifications: a hot inject of 25 μL at an injection speed of 30 seconds at 95 °C, an extended dry time to 45 seconds at 120 °C, and a 90 second ash time. A certified reference material (CRM, custom-made cadmium standard, Delta Scientific, Mississauga, Ontario, Canada) was run every ten samples to evaluate the accuracy of the analysis which was maintained over 90%. Each analytical sample and CRM was run in duplicate. The detection limit for Cd previously established using this method was estimated to be 0.02 μg/L.

Water from each environment was analyzed for all water quality characteristics outlined in **Table 77**. Conductivity, pH, total dissolved solids, and salinity were measured using an Oakton Pocket Tester (Oakton PCTSTestr5). Hardness and alkalinity were measured using the titration method described by the American Public Health Association (American Public Health

Association 1992). Organic matter samples were filtered through a 0.45 µm nitrocellulose filter and dissolved organic carbon (DOC) quantified via high temperature catalytic oxidation on a Shimadzu TOC-LCHP (Kyoto, Kyoto, Japan). The optical properties of DOM were characterized by spectrofluorophotometric analysis (excitation wavelength between 230 to 500 nm (5 nm intervals) and emission between 250 to 700 nm (2 nm intervals); Shimadzu RF-6000) and by UV/Visible spectrophotometry (absorbance measured between 230nm and 800nm wavelength; Biochrom Ultrospec 3100 Pro). Before calculating parameters, the fluorescence spectra were corrected for inner-filter effect and scattering and Raman normalized (Murphy et al. 2013; Pucher et al. 2019). The optical metrics used and associated methods of calculation are defined and summarized in **Table 7**, and were calculated in R, version 4.2.1 (R Core Team, 2022) using the *staRdom* package.

We related all measured water quality and sediment characteristics (each acting as a candidate explanatory (or independent) variable) to the amount of microplastic-bound cadmium (the single response (dependent) variable) using a bootstrap aggregating ("bagging") regression tree analysis. Statistical analyses were conducted in R, utilizing the *rpart, rpart.plot, rsample, dplyr, ipred,* and *caret* packages. The data (from the 137 simulated environments) were randomly divided *a priori* so that 80% (112 environments) were used to create (herein referred to as "training data") the model. All candidate explanatory variables in the training data were assessed for collinearity. Due to the integrative nature of water quality characteristics, a variable inflation factor of 5 was selected as the collinearity threshold, above which characteristics were removed prior to construction of the model.

For model exploration and simplification, a variable importance factor (VIF) analysis was completed on the training data. This analysis was done to provide insight as to which variables have relatively low influence on the response (amount of cadmium bound to microplastic). Further, this analysis supplemented the collinearity analysis to determine if interrelations between closely related water quality characteristics was present, as evident by two similarly large VIFs for highly related water quality variables such as salinity and conductivity.

The regression tree was constructed from the bootstrapped training data through successive binary partitioning based on each candidate explanatory variable to minimize the sums of squares residual error. To avoid overfitting of the resultant regression tree, the tree was pruned based on the complexity parameter which assessed the amount by which splitting a node of the regression tree improved the relative error. The minimum leaf size (the minimum number of environments to be included in each leaf – or final bin – of the regression tree) was set to 10. A total of 1000 pruned trees were created and compared to each other to discern common nodes (divisions in the data) among regression tree iterations. The most commonly represented regression tree, indicating the most probable model, was accepted as the resultant regression tree. The resulting regression tree was used to make predictions on the amount of microplastic-bound cadmium inferred from the water quality and sediment characteristics of the simulated freshwater environments with the remaining 20% (25 environments) of the original dataset (herein referred to as "testing data"). Coincidently, at least two instances of testing data fell within each node of the pruned tree.

***Exposure Assessment***

To investigate the relationship between hazard and exposure, an exposure assessment was completed to determine how the established model translates to organismal effects, as measured by cadmium uptake and alterations to gene expression in a model fish species. Sub-adult (4 months old) fathead minnows were randomly selected from a long-standing breeding culture maintained in the Aquatic Research Facility at the University of Lethbridge. Fish were maintained in multiple 9 L flow-through tanks for a total 336 L of aerated water with a 30% water change occurring daily on 14 hour light: 10 hour dark photoperiod at 25 ± 1 °C. Density in each tank was maintained below 1 g/L. The water supply to the ARF is piped directly from the City of Lethbridge and dechlorinated using activated carbon, filtered through particulate cartridge filters, and pH buffered using aragonite. Water is UV-sterilized before entering culture tanks.

Prior to the start of the experiment, fish were fasted for 24-hours at which point 150 minnows were transferred from culturing units to experimental tanks. None of the fish showed visible signs of compromised health. Five fish were transferred to each 4 L experimental tank (30 total experimental tanks) filled with the same water as used in their culturing tanks. Experimental tanks were maintained at 25 ± 0.5 °C, on a 14-hour light: 10-hour dark photoperiod, and constantly aerated. Manual water changes of 50% total volume were completed daily for all experimental tanks. The first two days of water replacement was with Aquatic Research Facility culture water after which water quality manipulation began. The subsequent two weeks of acclimation time allowed for gradual manipulation of water quality parameters to establish environments that fall within all nodes of the regression tree established during the Hazard Assessment. Water quality was manipulated by replacement of removed water with modified freshwater sources, as previously described in the Hazard Assessment. For the entirety of the acclimation period, less the date of transfer to the experimental tanks, fish were fed once daily with Tetramin (Tetra US, USA) fish flake food.

Following the acclimation period, each environment was contaminated with cadmium and microplastics, beginning the exposure period. Microplastics were degraded in the same manner as described in SI - Microplastic Degradation - and the same cadmium source was used as described in the Hazard Assessment. The cadmium-microplastic mixture was introduced within a contained glass filtration thimble (Cytiva Whatman™ Glass Microfiber Extraction Thimbles, Grade 603G, Cytiva Catalog #10371019), the opening of which was secured above the water to eliminate the risk of spillage from the thimble into the surrounding water. The pore size of the thimble was 0.8 µm, allowing for passage of cadmium across the membrane but prevented movement of microplastics out of the thimble, therefore eliminating the direct contact of fish and plastics. From the introduction of the thimble, water changes and feeding ceased to allow for cadmium equilibrium and to eliminate confounding dietary cadmium toxicity, respectively. At the end of the 96-hour exposure period, fish were euthanized in an overdose of MS-222 (0.25 g/L; CAS: 886-86-2).

Gills and liver were immediately dissected from each individual for analysis of cadmium uptake and metallothionein mRNA abundance by qPCR. For cadmium uptake, tissue samples were rinsed in 50 µM ethylenediaminetetraacetic acid (EDTA) solution for one minute to remove surface-bound metals (Norwood et al. 2007) then dried to a constant weight at 60 °C in pre-weighed tubes. The dry tissue weight was recorded. Dried tissue samples were digested using a previously established and validated tissue digest protocol (Lindh et al. 2019). Samples were digested in concentrated, trace metal grade nitric acid (CAS: 7697-37-2) at a 1:10 ratio (dry tissue mass (mg): acid (µL)) for 3 h at 80°C. Acid-only blanks and certified reference material, acting as procedural controls, were digested simultaneously (Product Identifier: DOLT-4, National Research Council of Canada, Ottawa, Ontario, Canada). Following digestion, samples were cooled to room temperature and analyzed using the previously described GFAAS method. Transcript abundance of metallothionein was quantified to evaluate induction of metallothionein

(*MT*) following exposure to cadmium (Shekh et al., 2019). Abundance of this mRNA was determined relative to abundance of ribosomal protein L8 (*rpl8*). The *rpl8* transcript was chosen as a housekeeping gene to normalize the abundance of *MT* transcripts to for all replicates as it has been demonstrated to be highly stable in gills exposed to metals (Shekh, et al., 2017). Total RNA was isolated from one gill and half of each liver from each fish by using QIAzol™ Lysis Reagent (Qiagen Inc., Mississauga, ON, Canada) according to manufacturer's protocol. Following RNA extractions, total RNA concentration was quantified using a Nanodrop™ One© spectrophotometer (Thermo Scientific, Ottawa, ON, Canada). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA, which includes a genomic DNA elimination step, using a Qiagen QuantiNova™ Reverse Transcription Kit (Qiagen Inc., Mississauga, ON, Canada). To create reaction samples for qPCR, 1.25 μL of cDNA was mixed with 23.75 μL of a master mix containing 1.25 μL of primers (*MT* or *rpl8*), 10 μL RNase free water, and 12.5 μL SensiFAST™SYBR® NO-ROX Kit (Meridan Bioscience, Cincinnati, OH, USA) for each replicate. Reactions were performed in duplicate in a 96-well plate and a CFX96 Touch Real-Time PCR Detection System was used (Bio-Rad, Mississauga, ON, Canada). Reactions were subject to denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds and extension at 60°C for 10 seconds. To confirm successful amplification of qPCR products, melt curves were generated and analyzed to ensure consistency between each duplicate. A no-template control was included to verify qPCR reactions were not contaminated. Alterations in *MT* transcript abundance was assessed and corrected relative to the control by using the efficiency corrected method (Pfaffl, 2001). Before calculating abundance of mRNA, efficiencies of PCR reactions were determined using 5-fold serial dilutions of the cDNA template and were maintained at 110%.

A two-way Analysis of Variance was conducted to assess how the predicted quantity of MP-bound Cd (from the hazard assessment) affected the amount of cadmium accumulated and metallothionein abundance at the gill, a tissue in direct contact with contaminated water, and the liver, a tissue known to accumulate cadmium.

## Results

### *Hazard Assessment*

The ranges of a subset of water quality characteristics are summarized in **Table 8** and the complete water quality profiles of all environments are available as a separate file. When assessed for collinearity, total dissolved solids and conductivity were colinear (above the predetermined variable inflation factor threshold of 5) with salinity. Therefore, total dissolved solids and conductivity were removed from the model and salinity was retained. All other variable inflation factors fell below the threshold.

**Table 8.** Summarized subset of water quality characteristics of environments used in the Hazard Assessment.

| Water quality characteristic | Mean ± standard deviation (*for pH: Median, range) |
|---|---|
| pH | 7.25, 1.37 – 12.12 |
| Water hardness | 196 ± 191 mg/L as $CaCO_3$ |
| Alkalinity | 93 ± 76 mg/L as $CaCO_3$ |
| Dissolved organic carbon | 6.7 ± 9.8 mg/L |

The relative VIFs of the training data were calculated for the remaining variables; the VIFs, scaled to have a maximum value of 100, are presented in **Figure 24**. All sediment-related variables had VIFs of less than 10 and for readability were omitted from **Figure 24** and from further analyses. The most important variable was pH with a scaled VIF of 100, followed by alkalinity (VIF = 78) and humification index (hix; VIF = 57). All other variables had an VIF less than 50.

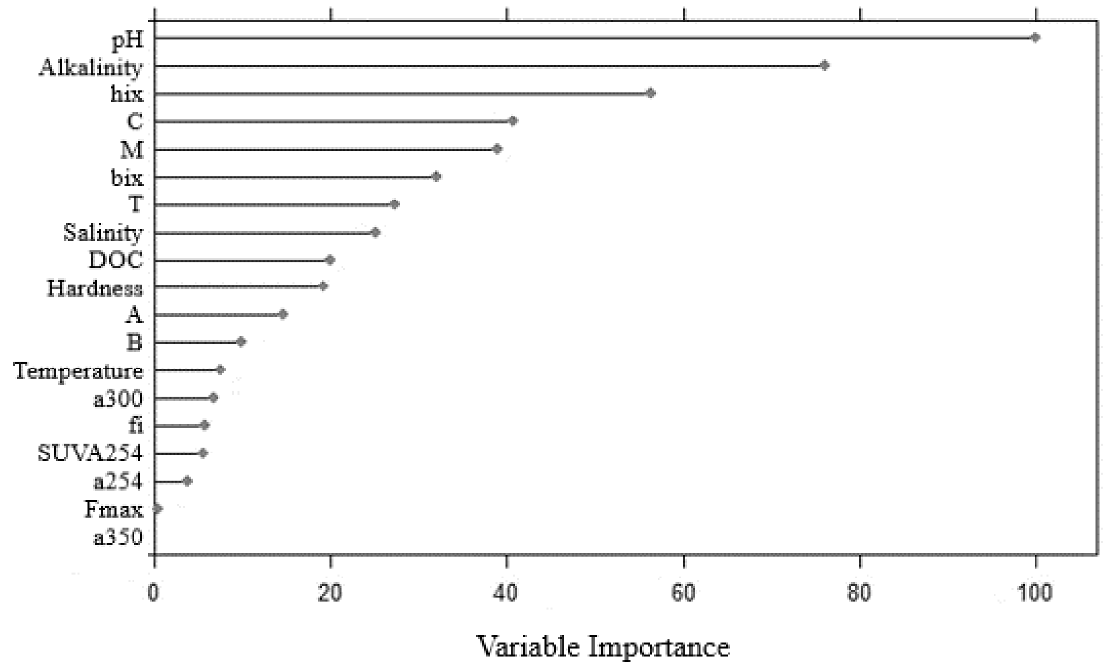


**Figure 24.** Relative, scaled VIF of each water quality variable used in the model. Definitions of each variable listed along the y-axis are available in **Table 7**.

The resultant regression tree (**Figure 25**, read from left to right) contained three splits. These three splits (split once for each of alkalinity, hix, and pH factors) were present in nearly all regression tree iterations. The first determining factor was alkalinity, with a threshold of 197.5 mg/L as $CaCO_3$ – above that threshold, 95 ± 10% (average ± standard deviation) of cadmium in the system was bound to microplastics, and below that the next determining factor was hix (**Figure 25**). Systems with an alkalinity below 197.5 mg/L as $CaCO_3$ and a hix value greater than 0.85 resulted in 36 ± 11% (average ± standard deviation) of cadmium in the system being bound to microplastics. The final node in the tree, used when alkalinity and hix fell below 197.5 mg/L as $CaCO_3$ and 0.85, respectively, was the pH threshold of 8.6 – above this threshold 25 ± 3.4% (average ± standard deviation) of cadmium in the system was bound to microplastics, and below pH 8.6, 10 ± 4% (average ± standard deviation) of cadmium in the system was bound to microplastics. The resultant regression tree corroborates the VIF analysis in that the three most important factors (having the greatest VIFs; pH, alkalinity, and hix; **Figure 24**) were also the three variables determined to be predictors in the resultant regression tree (**Figure 25**).

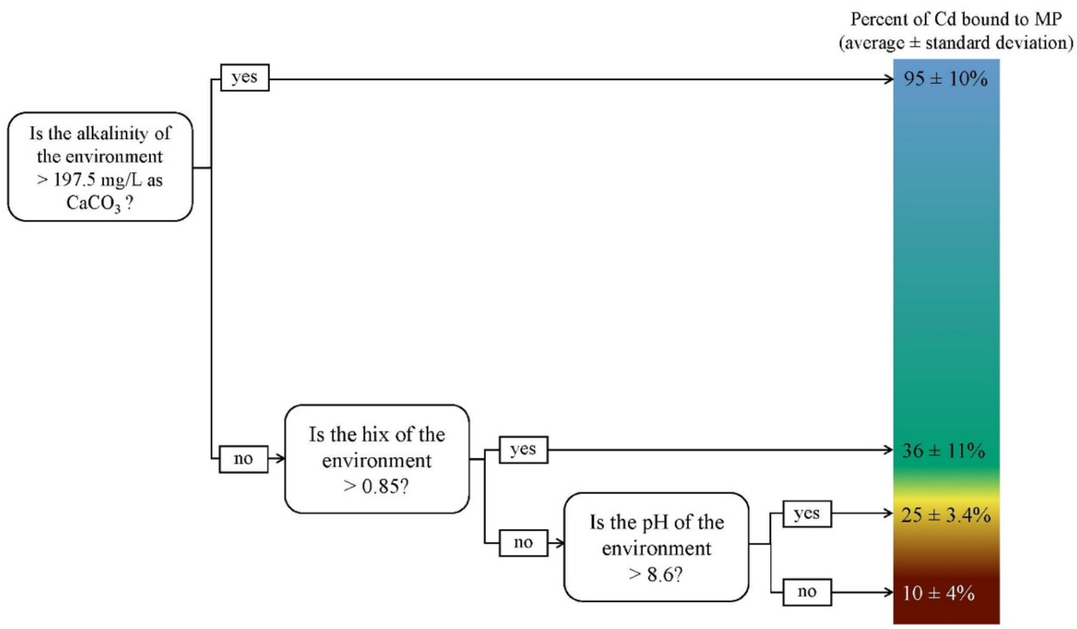


**Figure 25.** Pruned regression tree created through bootstrapped regression tree analysis of the amount of microplastic-bound cadmium in environments of variable water quality. The tree is read from left to right, with each prompt (node of the model) providing a threshold value in which an environment can fall above or below, determining the result (percent of cadmium (Cd) bound to microplastics (MP)) or the next factor to be assessed.

To assess the performance of the model created by the regression tree analysis, the testing dataset was manually sorted to the tree shown in **Figure 25**. All instances of the testing data set but one, assumed to be an outlier, fell within 1.5 standard deviations of the average presented in **Figure 25**.

***Exposure Assessment***

The ranges of a subset of water quality characteristics are summarized in **Table 9** and the complete water quality profiles of all environments are available as a separate file. There were three environments in each of the 95% and 36% average predicted groups, nine environments in the 25% predicted group, and fifteen environments in the 10% predicted group (as predicted by the regression tree in **Figure 25**).

**Table 9.** Summarized subset of water quality characteristics of environments used in the Exposure Assessment.

| Water quality characteristic | Mean ± standard deviation (*for pH: Median, range) |
|---|---|
| pH | 7.25, 1.37 – 12.12 |
| Water hardness | 227 ± 148 mg/L as $CaCO_3$ |
| Alkalinity | 124 ± 55 mg/L as $CaCO_3$ |
| Dissolved organic carbon | 1.95 ± 2.8 mg/L |

The interaction of tissue (gill or liver) and model-predicted MP-bound Cd showed a significant interaction relative to the concentration of accumulated cadmium ($F(3,149) = 2.7$, $p = 0.04$; **Figure 26**). At the gill, the group with the lowest predicted MP-bound Cd (10%) accumulated the most cadmium; 1.3-, 1.6-, and 1.4-fold greater than each of the 25%, 36%, and 95% MP-bound Cd predicted groups, respectively (**Figure 26**). The same trend was not observed in the liver, which accumulated less cadmium than the gill for all but the 36% average group, though this group showed the highest variation (**Figure 26**).

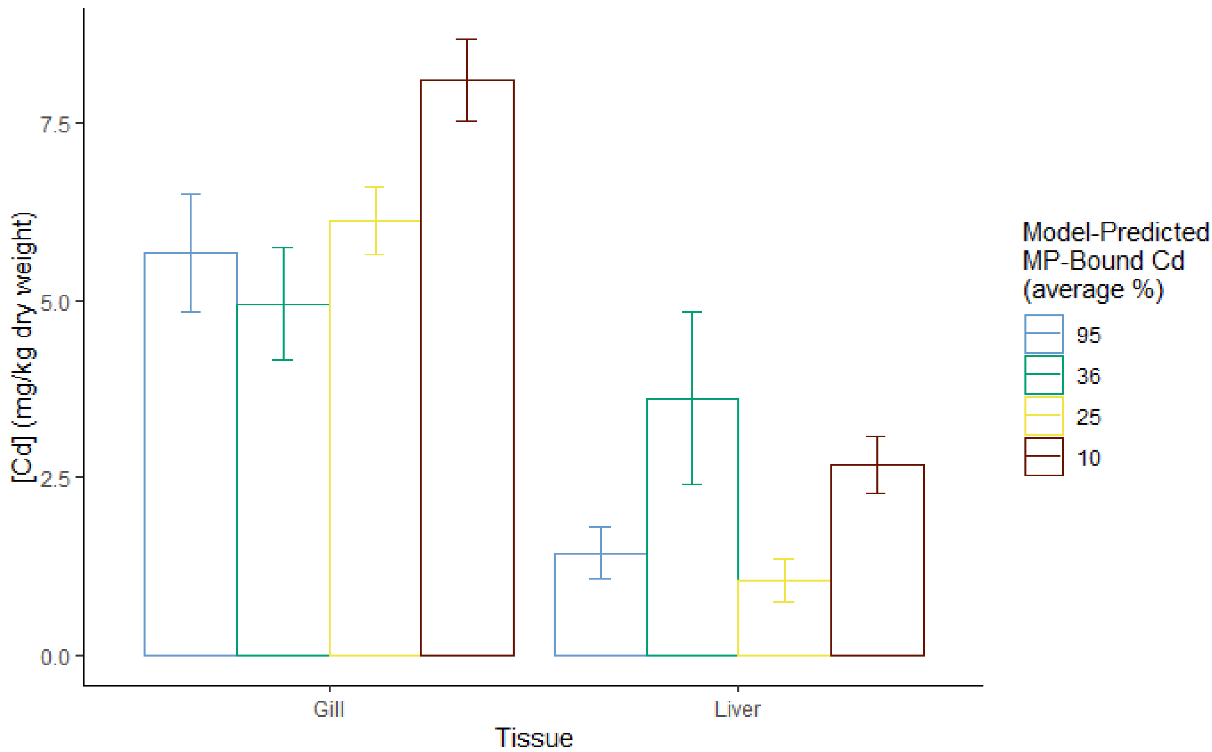


**Figure 26.** Cadmium (Cd) accumulated in the gill and liver of fathead minnows following 96-hour exposure in environments of different water quality characteristics. Each point represents the group average and error bars represent one standard error of the mean. For each environment, the hazard assessment predicted the amount (average %) of microplastic (MP)-bound Cd to be either 95, 36, 25, or 10 % (n= 3, 3, 9, 15 for each group, respectively). Differences among treatments were analyzed by ANOVA with a Tukey's post-hoc test.

The analysis of metallothionein yielded a significant main effect of tissue type ($F(1,29) = 11.59$, $p = 0.001$; **Figure 27**), there was no significant interaction between tissue type or model-predicted MP-bound Cd, nor was the predicted MP-bound Cd significant on its own. Minimal expression of metallothionein was observed at the liver across all groups, whereas more metallothionein was expressed at the gill with increased variability between groups (**Figure 27**). At the gill, the group with the lowest predicted MP-bound Cd (10%) accumulated the most cadmium; 3.25-, 1.8-, and 13-fold greater than each of the 25%, 36%, and 95% MP-bound Cd predicted groups, respectively (**Figure 27**). More metallothionein was expressed in gill of the

10% average group, compared to the liver ($p < 0.001$; **Figure 27**); no other significant results were observed.

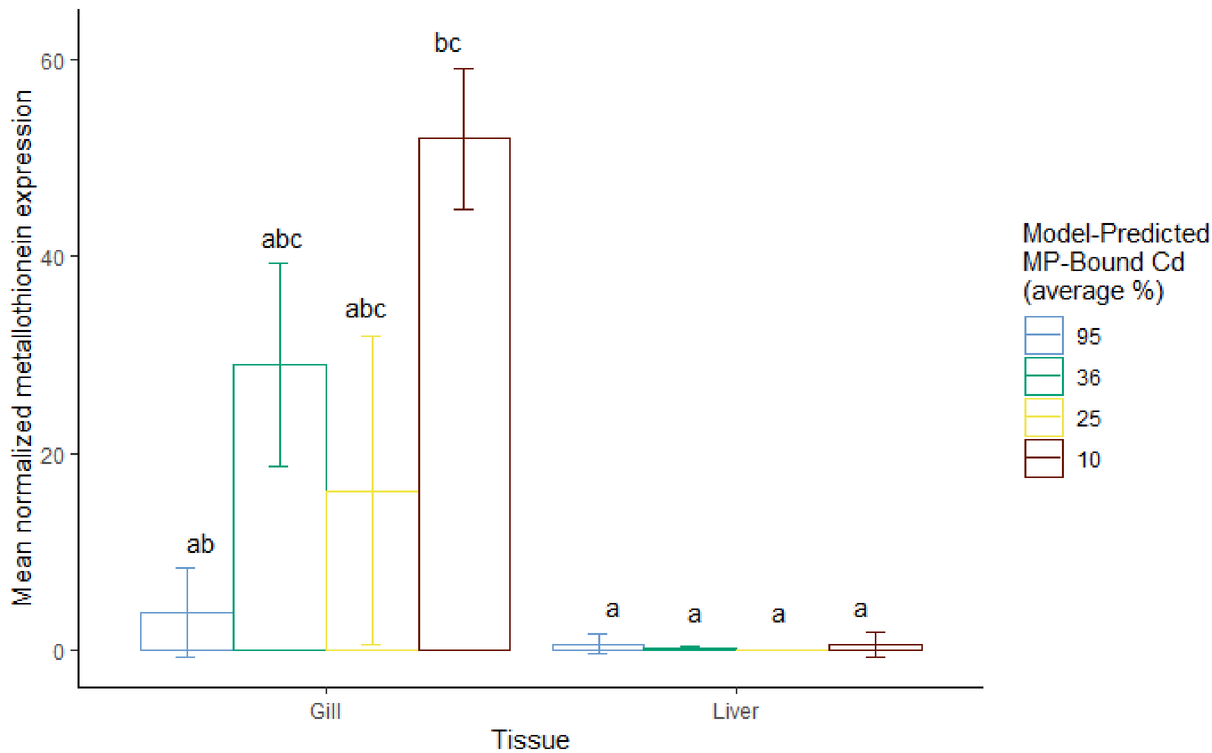


**Figure 27.** Mean normalized metallothionein expression at the gill and liver of fathead minnows following 96-hour exposure in environments of different water quality characteristics. Each point represents the group average and error bars represent one standard error of the mean. For each environment, the hazard assessment predicted the amount (average %) of microplastic (MP)-bound Cd to be either 95, 36, 25, or 10 % (n= 3, 3, 9, 15 for each group, respectively). Differences among treatments were analyzed by ANOVA with a Tukey's post-hoc test. Treatments sharing the same letter designation were not statistically different from one another ($p > 0.05$).

**Discussion**

Metal fate models, such as the one described in the present study, are used to discern how water quality characteristics govern the association of metal to ligands. To date, these models have biotic or natural abiotic ligands (such as sediment or DOM) whereas the present study exemplifies the applicability of this approach to microplastics, an anthropogenic ligand. These models, including the Free Ion Activity Model (FIAM), Biotic Ligand Model (BLM), and

Toxicodynamic-toxicokinetic Model (TDTK), are all governed by water quality characteristics. Similarly, the present model showed a select set of water quality characteristics – alkalinity, pH, and hix - drive the interaction of cadmium to microplastics. Current regional and global trends of freshwater water quality are of particular interest when assessing the applicability of the present model. The global and regional trends are driven by alterations to land use and anthropogenic influence, which are also factors influencing the introduction of microplastics to freshwater systems (Nava et al. 2023).

There are global trends in the ionic composition of freshwaters, primarily salinization of freshwater and an increase in major ions resulting in higher alkalinity and altered pH (Kaushal et al. 2005; Kerr 2017). The most important water quality characteristic defined by the present model is pH. As pH decreased, the solubility of cadmium increased and was more abundant in the free-ionic form (Wren and Stephenson 1991). Alkalinity, which affects a metal's behaviour by acting as an anionic ligand to cationic metals, was an important factor in the present model (Nelson et al. 1986; Laurén and McDonald 1986). In other metal models such as the Biotic Ligand Model, water hardness, which is related to alkalinity, tends to be more of a driving factor than alkalinity (Davies et al. 1993; Niyogi and Wood 2004). Generally, it has been considered that cationic competition between metals and major ions (calcium and magnesium, collectively representing water hardness) governs metal binding at ligands; however, our model showed alkalinity as a driving factor, which suggests that counter ion competition is of greater importance when microplastics are the ligand. Our hazard assessment suggests that carbonate anions are stronger counter ions for cadmium than microplastics, particularly in highly alkaline environments; however, the uptake of cadmium in highly alkaline environments did not differ in our exposure assessment. Freshwater alkalinity generally ranges from 2-200 mg/L as $CaCO_3$ and our model provided a threshold value of 197.5 mg/L as $CaCO_3$, resulting in one group of environments being relatively high alkalinity, which will alter the organisms' physiology and subsequent response to metal exposure (Alberta and Environment and Sustainable Resource Development 2014; Ge et al. 2023). Additional studies into the ionic interactions between metals and abiotic (microplastic) and biotic (gill) ligands are warranted to explore these observations.

The chemical properties of organic matter influences its capacity to bind metals (Playle et al. 1993; Chen et al. 2018). There are widely observed trends of brownification of surface waters in some regions, indicative of shifts in organic matter quantity and characteristics (Solomon et al. 2015; Kritzberg et al. 2020). These shifts in organic matter quality influence the hix of environments: as aromatic organic matter is increased, the hix increases, and according to the present model, the more metal becomes bound to plastic. The aromaticity of DOM explains differential metal binding capacity and is often used as a determining factor in metal fate models such as the Biotic Ligand Model (Baken et al. 2011). The humification index (hix), determined to be a driving factor in the current model, describes the level of degradation of organic matter. The higher the hix ratio (i.e., closer to 1), the more degraded and aromatic the organic matter is, inherently having fewer binding sites available for metals. The proportion of closed ring structures increases with increasing degradation of organic matter, and consequently there is a decrease in the number of sites available for binding of metals (Al-Reasi et al. 2013). In our model, the more degraded the organic matter, the more cadmium was associated with microplastics, presumably because there is less cadmium bound to organic matter (Playle et al. 1993).

The use of predictive modelling to determine how water quality characteristics govern the association of metals with various ligands is a useful tool in the fields of toxicity and risk assessment. While this model used the most common type of plastic found in freshwaters, future studies should test and adapt this model under additional scenarios including using different types of plastics and other metals. This model currently indicates that environments with high alkalinity favour the most cadmium-microplastic complexes, lowering the proportion of free-ionic cadmium in the environment. Additional work should aim to further discern the factors driving the association between metals and microplastics in near neutral pH environments to improve the model's usability in neutral or acidic waterbodies.

The exposure assessment in this study did not reveal significant differences in cadmium accumulation or metallothionein expression between fish in each of the groupings predicted by the hazard assessment. Differences were observed with more cadmium and metallothionein at the gill compared to the liver, which over the course of a 96-hour exposure signifies the gill accumulated and responded to cadmium, likely because it is in direct contact with the water, trends which align with many other observations (Kay et al. 1986; Hollis et al. 1999; Adams et al. 2020). Additional work in which organisms are collected from field sites or long-term laboratory-reared/raised for environments meeting the hazard assessment characteristics should be conducted to resolve whether physiological stress caused by altered water quality confounded the toxicity of cadmium in the present study.

**Conclusion**

The hazard assessment in this study represents the initial steps in providing a clear understanding of what governs interactions between cationic metals and microplastics. The present study provides the initial framework to understand the factors that govern partitioning of cadmium to microplastics. Three water quality characteristics: pH, alkalinity, and DOM humification index are the primary factors that drive the association between cadmium and microplastics. When the exposure effects of cadmium in each of the predicted model scenarios were assessed, there was no difference in the uptake of cadmium, suggesting that the stress of altered water quality to be a confounding factor in discerning the toxicity of cadmium predicted by the model.

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# CHAPTER 7: CONCLUSION

This thesis began by seeking to address discrete knowledge gaps in the field of microplastic-metal mixture toxicity, as outlined in Part 1, utilizing a traditional mixtures toxicity approach; that is, assess the effect(s) of Constituent 1, assess the effect(s) of Constituent 2, and assess the effect(s) of Constituents 1 and 2 together. The outcomes of this approach can broadly be categorized as: (1) addition, in which the toxic effects of a mixture is equal to the toxic effects of each substance comprising the mixture, (2) synergism, in which the exposure to the mixture results in toxic effects that are greater than the sum of each individual substance's effects (i.e., a more-than-additive effect), (3) antagonism, scenarios in which the mixture causes less-than-additive toxic effects, and (4) potentiation, in which one substance increases the toxicity of another. While this approach allows for a relatively straightforward interpretation of mixtures effects, in practice this approach resulted in contradictory conclusions when placed within the field of microplastic-metal research.

Chapter 2 aimed to determine whether waterborne or sediment-borne cadmium was more readily taken up by leeches when co-exposed with microplastics. It was determined that waterborne cadmium was more readily taken up, accumulated over time, resulting in toxicity as evident by hindered foraging and feeding behaviour, hypothesized to be caused by direct inhibition of the movement of serotonin from storage locations. Simultaneously, microplastics also suppressed foraging and feeding behaviours, hypothesized to be by false satiation. In this study, cadmium and microplastics resulted in additive effects through independent action. In contrast, researchers working with a terrestrial annelid concluded microplastics potentiated the toxicity of cadmium (Huang et al. 2021).

Given the observations of both cadmium and microplastics influencing feeding behaviour and regulation in the leech, the research focus shifted to understanding the behaviour of these contaminants following ingestion differentiating between microplastic bioaccumulation and microplastic internalization, which is sorely lacking in this research field. Studies have reported that microplastics accumulate in internal tissues, such as the liver (Lu et al. 2016; Collard et al. 2017; McIlwraith et al. 2021). Using an *in vitro* gut sac technique with fathead minnows (Chapter 3), I determined that microplastics (5 – 7 μm) were unable to cross the gut barrier, suggesting that movement of microplastics across the gut does not contribute to accumulation in tissues. Identification of internalization routes should remain a priority research area as internalization of contaminants increases their risk for trophic transfer and biomagnification, particularly when found in tissues commonly consumed by predators. This study also determined microplastics in the gut were protective against cadmium internalization, which contradicts a study which found gut conditions caused the released of sorbed contaminants (albeit, persistent organic pollutants) from microplastics (Bakir et al. 2014), representing yet another contradiction between my findings and those of other studies.

As both cadmium and microplastics are persistent pollutants, chronic exposures are of more ecological relevance than acute exposures. The final chapter of Part 1 of this thesis (Chapter 4) explored the multigenerational effects of cadmium, microplastics, and their mixture to *Daphnia magna.* Neither cadmium nor microplastics influenced reproduction of daphnids, but cadmium decreased feeding efficiency and microplastics hindered early-life growth rates. When co-exposed, additive effects on both feeding and growth were observed. Mechanisms of feeding and growth inhibition were not explored in this study, though could be a goal of future studies. A study on the same species and a multigenerational design concluded that microplastics alone decreased the survival, reproduction, adult and neonate growth and caused extinction of the third generation (Schür et al. 2020, 2021). The microplastics used in my study were 5 – 7 µm whereas those used by Schür et al. ranged from 6 – 70 µm which may account for the differences in effects seen; however, Schür et al. did not fully quantify water quality characteristics, meaning that water quality differences can not be ruled out as confounding variables.

When placed in the broader scope of microplastic-metal research, studies outlined in Part 1 of this thesis was in contrast to other studies with regards to the effects of exposure to cadmium-microplastic mixtures. While initially disheartening, this realization prompted me to delve deeper into the field to determine if these types of contradictions were common – needless to say, they were aplenty. I returned to the fundamental framework that my research had been built on thus far, that is: assess the effect(s) of cadmium and microplastics alone, and in combination, then conclude whether the mixture caused additive, synergistic, antagonistic, or potentiated effects. This framework being simplistic and prominent in this field made utilizing it the clear choice, initially. However, it was too simple and failed to appreciate the foundational knowledge we already had of metal behaviour.

The vast majority of researchers publishing in the field of microplastic-metal toxicity research had expertise in work with microplastics and other particulates as opposed to metals. This, I hypothesized, was the root of why the traditional mixtures toxicology framework was adopted so widely in this emerging area of research. I leveraged my knowledge of metals toxicity to shift my approach and rather than treating microplastics as another constituent in a mixture, I utilized a ligand framework in which microplastics were another ligand that a metal may bind to. The limitation of this approach is that it fails to appreciate any toxicity caused by microplastics; however, with the multitude of pre-existing contradictions in the field, it remains highly debated whether microplastics alone are toxic. Further research to determine the potential toxicity of microplastics will need to assess the multitude of plastic characteristics that can vary, including manufacturing materials and additives used; therefore, this type of work is best left for those not working with microplastics alongside other contaminants.

The shift to Part 2 of this thesis leveraged the established knowledge that water quality influences the toxicity of metals by altering the speciation of metals and their partitioning, including to ligands, one of which I hypothesized to be microplastics. When I shifted to the ligand approach (Part 2 of this thesis), the first step was to assess the current state of water quality reporting in microplastic-metal toxicity research. The reporting of water quality characteristics was glaringly absent in the field – with less than 50% of studies reporting any water quality beyond temperature, making utilizing pre-existing research for a meta-analysis impossible. This guided my next step which was to create a dataset to model how shifts in water quality characteristics govern the association of cadmium to polyethylene. I determined that water quality characteristics were a useful tool to predict the association of cadmium to microplastics. Specifically, that alkalinity, pH, and humification index of organic matter were the three water quality characteristics that predicted how much cadmium was bound to microplastic. The range of microplastic-bound cadmium was from 10-95% of cadmium – this large of a range in metal partitioning alters the hazard of the metal. While establishing the abiotic model provided a hazard assessment (i.e., how much cadmium was bound to microplastic), the next step towards proposing a solution to the contradictions within the literature was to translate the hazard into exposure scenarios. In using fathead minnows acclimated to environments spanning the model predictions, we found that fish in environments predicted to have less plastic-bound cadmium accumulated more cadmium at the gill. While not directly assessed in this study, accumulation of cadmium at the gill can translate to olfactory inhibition and impede the ability for fish to find food or detect predators. The demonstrated agreement between the model prediction and adverse effects observe in fish indicates that the model is applicable in predicting risk and toxicity.

The model constructed in this thesis represents the first step towards informing policy development for waters co-contaminated with cadmium and microplastics. The outcome of this model demonstrates that water quality characteristics can be used to predict the amount of cadmium associated to microplastics in freshwater systems. By developing our understanding of what site characteristics, in the case of aquatic ecosystems – water quality characteristics – that increase the potential risk of toxicity, we can work to protect aquatic life, with a targeted emphasis on more susceptible ecosystems. To improve the applicability and usability of this model, three future directions need to be taken: (1) microplastic-metal mixtures toxicity research must report water quality adequately (minimally as defined in the model, but ideally as defined in the proposed framework in Chapter 5) so that proper meta-analysis can be done and work can be corroborated, (2) researchers across the field must test the model under additional laboratory and field scenarios, and (3) to increase the number of metals assessed in this manner, such as other metals of concern (e.g. copper). .

My model works toward an overarching goal of ecotoxicity: to proactively protect aquatic life. The development of water quality guidelines for the protection of aquatic life are informed by laboratory experimentation and are revised as our understanding of contaminant behaviour develops. For example, the major ions attributed to water hardness (Ca and Mg) can modify the toxicity of each other and modify the toxicity of other ions, including metals; therefore, additional consideration in measuring water hardness in the development of water quality guidelines is warranted (Bogart et al. 2018). For informative meta-analyses such as that performed by Bogart et al. to be conducted and actionable towards policy development, researchers must report a minimum set of water quality characteristics to establish the data from which we can draw upon. This thesis highlights that as the field of microplastic-metal toxicity research has grown, that minimum reporting requirements for water quality are not being upheld in the same manner as they are for other metals toxicity research, where it is considered an essentially standard practice. The demonstrated agreement between my model predictions and adverse effects observe in fish indicates that the water quality is a viable answer to the observed contradictions in the field of microplastic-metal research.

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