Toxicity and bioaccumulation kinetics of cadmium and potassium permanganate in two clades within the *Hyalella azteca* species complex

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The freshwater amphipod *Hyalella azteca* has been frequently used in toxicology since the 1980s due to its general sensitivity, ease of culture, and North America-wide geographic range. Over the past 20 years, molecular methods have revealed considerable genetic diversity underlying minimal phenotypic divergence among *H. azteca* populations, which lead to the recognition of *H. azteca* as a morphologically cryptic species complex comprised of 85 provisional species. Most Canadian and American toxicology laboratories maintain a single species (clade 8), and the Canada Centre for Inland Waters (CCIW) in Burlington, Ontario appears to be the only lab that cultures a different species (clade 1). Only recently has the impact of species-level divergence within this cryptic complex been investigated to determine the impact on toxicity and bioaccumulation. Differences in ionic requirements, bioaccumulation, and tolerance have been previously demonstrated among clades within this complex. Due to their relative prevalence among toxicology laboratories, research from our group has focused on clades 1 and 8, which were identified with genetic barcoding at the mitochondrial cytochrome *c* oxidase I gene. In the present study, the saturation model was used to estimate uptake and depuration kinetics, short-term bioaccumulation, mortality, and growth inhibition responses of these two clades to cadmium and potassium permanganate. Significant differences between tolerances and bioaccumulation could not be determined in this study because of overlapping 95% confidence intervals, likely due to the low tolerance of the clades to both toxicants. Future research may benefit from comparing the acclimation capabilities of these clades. Reporting in toxicology can be improved by genetically identifying clades, particularly as accessibility to genetic sequencing continues to increase.

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Dedication

I am fortunate to the support of my family, and those that have passed I miss dearly.

Bradie Dylan Jones (1984 – 2017)

Marion Elizabeth Gauley (1932 – 2018)

Patricia Elizabeth (Grammie) Sinclair (1944 – 2019)

D. Colleen (Grandma) Hutchinson (1934 – 2019)

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Chapter 1
Introduction

Ecotoxicology is the study of toxic effects in living organisms due to pollution, and how such impacts relate to environmental conditions (McIntyre & Mills, 1975). The goal of this field is to recognize, predict and thereby mitigate effects of contamination, in an ecological context. Applications of toxicological data may result in the development of water quality metrics, control effluent release, or interpret environmental assessments and biomonitoring (EC, 1999).

Laboratory investigations are used to determine the inherent toxicity of a substance. These tests are often single-species, whole organism assays. Model organisms in ecotoxicology are selected for a number of qualities, including general toxicant sensitivity, regional or widespread occurrence, importance to their ecosystem, and the ease of collection, maintenance, and testing (Segner & Baumann, 2016). Some species have become popular subjects for these reasons, including zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), mummichog (*Fundulus heteroclitus*), water fleas (*Daphnia magna* and *Ceriodaphnia dubia*) and *Hyalella azteca*.

Hyalella azteca is a freshwater macroinvertebrate that is commonly used in aquatic toxicology assays and biomonitoring (EC, 2013). Similar to other model organisms, this amphipod's prevalence in toxicology is due to its high sensitivity across toxicant classes, amenability to lab culture conditions, and its North America-wide distribution (EC, 2013). However, recent molecular studies have indicated that *Hyalella azteca*, which was once thought to be a single species with a geographic range across North America, is actually a

complex of morphologically cryptic species (Hrycyshyn, 2016; Weston *et al.*, 2013; Witt & Hebert, 2000; Witt *et al*., 2003, 2006, 2008). This molecular diversity had previously been underestimated because of the morphological similarity shared by the constituent individuals.

Species are used as fundamental units in biological research and the relatively recent revelation of the genetic diversity within the *Hyalella azteca* complex could impact the interpretation and practical application of endpoints from toxicological testing with its members. Toxicant sensitivity associations among members of this complex may be connected to genetically identified species-level groupings and support for clade-level divergence of toxicant tolerance has already been established (Leung *et al*., 2016; Pathammovong, 2016; Soucek *et al*., 2013).

1.1 Application of single species toxicological data

The design of a generic toxicological experiment is simple: a single species is exposed to multiple levels of a substance over a certain duration, and the data are used to determine contaminant levels that cause an *a priori* determined level of effect, often 50% mortality. This protocol provides information about the reaction of a single species to a particular chemical in certain conditions over an exposure period (Blaise *et al*., 1988). Although this "fingerprint-type" testing has incurred much criticism due to the limited scope of each test (Blaise *et al*., 1988), the legacy of this design continues to impact many laboratory and field-based investigations because it is inexpensive, simple to run, and the results are comparable with historical data.

In Canada, water quality guidelines for the protection of aquatic life (CWQG-PAL) are developed by the Canadian Council of Ministers of the Environment (CCME) to prevent adverse outcomes due to anthropogenic impacts (CCME, 2007). Guideline derivation is an extensive process that requires published studies to be evaluated for scientific defensibility. Ideally, the quality and quantity of data would be sufficient for a species sensitivity distribution curve, which is the current gold standard statistical approach to guideline development (CCME, 2007). The guidelines developed by the CCME through this protocol are national benchmarks, and alternative site-specific guidelines (SS-WQG-PAL) can be determined, as appropriate. Similarly, ecological risk assessments are conducted to assess the possibility and potentiality of adverse effects for decision making (Newman, 2014). Water quality monitoring in Canada is a broadly applied method design that considers spatial and temporal scope in the measurement and evaluation of physical, chemical, and biological parameters for safety and other more specific objectives (CCME, 2007). Complementarily, biomonitoring can be used to evaluate biological communities at sites to gauge the health of water bodies (Karr, 1993). Jones (2008) demonstrated that higher taxonomic groups are sufficient to provide robust measures for many biomonitoring programs, but some species-level tolerances differ drastically and even site (population)-specific analysis continues to be necessary.

1.2 Hyalella azteca

1.2.1 A sensitive species in toxicology

Advancement in the field of toxicology accelerated in the early 1970s due to newly elevated concerns over pollution (Burton, 1991). New mandates in the United States stipulated the development of criteria to evaluate the toxicity of contaminated sediment. Recognition that pelagic and sediment-associated species may differ in their responses to contaminated sediment, and that no one species is most sensitive to every toxicant, sparked research to identify benthic species that could be used in standardized toxicity assays (Burton, 1991; De Cooman *et al*., 2015). Nebeker *et al*. (1984) recommended the benthic invertebrates *Hyalella azteca* and *Chironomus tentans* as test species because both are easy to culture and test, exhibit high control survival, are closely associated with the sediment, and are highly sensitive to organic toxicants (De Cooman *et al*., 2015). Research by Environment Canada at the Canada Centre for Inland Waters (CCIW) in Burlington, Ontario resulted in the first standardized *H. azteca* contaminated sediment assays (Borgmann & Munawar, 1989). Over the next decade, Environment Canada (1997), the American Society for Testing and Materials (ASTM) International (1995) and the United States Environmental Protection Agency (USEPA, 1994) released standardized protocols for sediment tests with *H. azteca* (De Cooman *et al*., 2015), and updated versions have since been published (ASTM, 2010; EC, 2013; USEPA, 2000).

1.2.2 Predominant species concepts

Species are the fundamental unit in toxicological research, and they are identified by use of many different concepts, which are often contradictory. Three species concepts predominate,

namely the morphological, biological, and phylogenetic species concepts. Under the morphological species concept individuals are considered to be the same species if their physical characteristics are visually similar. The morphological species concept is easily applied and can be used without extensive equipment requirements. Although the physical characteristics used for identification are chosen in an attempt to be representative of underlying genetics, this species concept does fail to account for molecular diversity that may underlie similar phenotypes. Morphological stasis despite genetic divergence is widely documented, often attributed to strict environmental constraints (Bickford *et al*., 2007; Schönrogge *et al*., 2002).

The second, the biological species concept (BSC), defines a species as an interbreeding population reproductively isolated from other such populations. The BSC is simple in concept, but its application is constrained to cases of sympatry (Coyne & Orr, 2004) and sexually reproducing organisms. In addition, its application is complicated by the different mechanisms that can reproductively isolate organisms (Bickford *et al.*, 2007).

The third predominant species concept is the phylogenetic species concept. This concept considers a species to be a monophyletic clade in which all members are descendants of the most recent common ancestor. This concept is valid for allopatric populations, is capable of considering multiple genotypic and phenotypic traits, and it not limited by the requirement to directly consider reproductive barriers (Bickford *et al.*, 2007).

1.2.3 Taxonomic status of *Hyalella azteca*

Hyalella azteca is a benthic amphipod crustacean that occurs in freshwater habitats, reproduces sexually, and is omnivorous (Cooper, 1965). This species was first described by

Saussure (1858) from a sample obtained in Vera Cruz, Mexico. Research over the next century grew to consider *H. azteca* as a single species distributed across North America, from Mexico to the arctic, and with members on many Caribbean islands (Bousfield, 1973, 1996). However, more recent genetic analyses have determined that *H. azteca* is a complex of morphologically cryptic species and other very divergent, but endemic, taxa (Hrycyshyn, 2016; Wellborn & Cothran, 2004; Wellborn *et al*., 2005; Witt & Hebert, 2000; Witt *et al.*, 2003, 2006, 2008). Although ecological investigations of life history (Strong 1972, 1973; Wellborn 1994a, 1994b) already hinted at life-history and body-size divergence among populations of this species, it was the molecular method of DNA sequencing that successfully delineated these cryptic species.

Many species, or clades, within the complex are considered to be one of two morphotypes: large- or small-bodied. The survival of either morphotype is correlated with the predation regime of the habitat. The small-bodied morphotype is adapted to avoid visual predation by fish species in lake populations (Wellborn, 1994b). Conversely in ponds where the fish species are absent, a large-bodied population will predominate as members are adapted to reach a size refuge from predation by larval dragonflies (Wellborn, 1994b). The time to mature body size, final body size, reproductive investment, and competitive ability is morphotype-specific. Large-bodied clades grow quickly to achieve a larger at-maturity body size, are better competitors, but exhibit comparatively lower reproductive investment than small-bodied clades (Wellborn, 1994a, 1994b, 2002).

Currently, 85 different species have been genetically delineated within *Hyalella azteca* by sequencing at the mitochondrial gene cytochrome *c* oxidase subunit I (COI) (Witt

& Hebert, 2000; Witt *et al*., 2003, 2006, 2008; Witt, unpublished data). Many of these species are endemic to the Great Basin in the United States (Witt *et al.*, 2008; Witt, unpublished data), though a wider geographic range is characteristic of others. *Hyalella* have only recently recolonized previously glaciated regions of North America since the last glacial maximum (Hrycyshyn, 2016). A few species of this complex have recently been described, namely *Hyalella spinicauda* and *Hyalella wellborni* (Soucek *et al.*, 2015); however, most remain provisional species.

1.2.4 *Hyalella azteca* and toxicology

Many model organisms are used for toxicant testing and biomonitoring due to their sensitivity, ease of use, and taxonomic status. One of the species that has been consistently used in toxicological investigation since the mid-1980s is *Hyalella azteca*. This freshwater amphipod is used extensively in laboratory and field studies because it is abundant in lakes, ponds, and streams across North America, an important constituent of the food web, easy to culture, matures quickly, and is a benthic organism that can be used for both water-only and sediment toxicity tests (EC, 2013). Canadian and American guidelines for the use of *H. azteca* in toxicity studies are published by Environment Canada (2013) and the USEPA (2000). These reports summarize physicochemical conditions, food options, and other methods that are useful to guide study designs for standardized endpoints.

Hyalella azteca is routinely recognized for use in toxicological studies with a taxonomic key, which is the recommended method by guidance documents (EC, 2013; USEPA, 2000). Specifically, Environment Canada (2013) provides a key updated from Bousfield (1973) for species identification.

In a laboratory population, Duan *et al*. (2000a, 2000b, 2001) detected allozyme associations with susceptibility to metals (cadmium, zinc, and lead), low pH, and an organic pollutant, fluoranthene. Soucek *et al.* (2013) used laboratory clades 1 and 8, and a small-bodied wild clade that was acclimated to laboratory conditions for two years prior to testing. Significantly different 48- and 96-h LC50 (concentration causing 50% mortality) values among the three clades in nitrate and chloride tests were observed by Soucek *et al*. (2013). Clade 8 has also been demonstrated to have lower growth and reproduction in low-chloride water (below 15-20 $mg \cdot L^{-1}$), a trait not shared by clade 1 (Soucek *et al*., 2015).

Weston *et al.* (2013) contrasted responses of seven wild populations and three laboratory populations to pyrethroid, an insecticide (*Hyalella azteca* are non-target species). Wild populations from high pyrethroid-contaminated sites exhibited the greatest resistance, and the most tolerant among these populations were all assigned to a single clade. Laboratory organisms, all assigned to the same clade, had LC50 values similar to the most sensitive wild populations in the study.

Significantly different LC50s were observed by Leung *et al*. (2016) and Pathammovong (2016) in two- and four-week exposures of clades 1 and 8 to copper and nickel. In two-week tests, clade 1 was significantly more sensitive in both the copper and nickel exposures, according to the LC50 values; however, neither the LC25 values nor the IC25 (concentration causing 25% inhibition) values for growth inhibition were significantly different between the two clades (Leung *et al*., 2016). Significant differences in both the LC50 and LC25 values were observed by Pathammovong (2016) in four-week exposures. A difference in IC25 values was not detected in the four-week study.

Body concentration can be used as a measure of bioavailability because it often provides a better estimate of the bioavailable fraction than water concentration (Borgmann *et al*., 1991, 1998; Borgmann & Norwood, 1997; Niimi & Kissoon, 1994). Although LC50 concentrations were significantly different, LBC50 (lethal body concentration causing 50% mortality) values calculated by Leung *et al.* (2016) and Pathammovong (2016) were not significantly different between the two clades that were tested under the same conditions.

Only two species were detected by COI sequencing in samples from 15 Canadian and American laboratory-maintained populations: clade 1 and clade 8 (Major *et al.*, 2013). All sampled laboratories in the United States and four out of five in Canada used clade 8; the Canada Centre for Inland Waters (CCIW in Burlington, Ontario) was the only laboratory to culture clade 1 (Major *et al*., 2013). These clades are both large-bodied morphotypes (Hrycyshyn, 2016). The wild distribution of clade 1 has been documented across previously glaciated North America (Hrycyshyn, 2016; Major *et al*., 2013; Witt & Hebert, 2000); whereas, clade 8 has only been detected south of the last glacial maximum (Major *et al*., 2013; Hrycyshyn, 2016).

The toxicogenome of *H. azteca* clade 8 was recently sequenced and annotated (Poynton *et al*., 2018). Analysis of the genome revealed evolution of this laboratory species, particularly gene loss related to light sensing, but an increase in gene families for chemoreceptors and stress proteins. There were also determined to be recent duplications in metallothionein proteins, which are used to sequester metals. A similar sequencing project has not been published for other species of *H. azteca*.

1.2.5 Other cryptic species in toxicology

The revelation that *Hyalella azteca* is a complex of cryptic species is not exceptional. With the advance of molecular methods over the last twenty years, many morphological species have been recognized as complexes of cryptic species. In these cases, environmental constraints are posited to retain morphological similarity despite remarkable underlying genetic diversity. Some of these former species are also commonly used in toxicology. For example, in studies with cryptic species of the *Gammarus* complex, one species (lineage B) demonstrated significantly greater stress tolerance over another (lineage A) to two pesticides (Feckler *et al.*, 2012) and ammonia (Feckler *et al.*, 2014), but higher infection rates by acanthocephalan parasites (Westram *et al.*, 2011). In addition, other distinct biological traits were characterized for two of the three *Gammarus* cryptic species (Eisenring *et al.*, 2016). Other examples are the nominal species *Daphnia pulex* and *Cletocamptus deitersi*, which are both used in toxicology, but have also been identified as species complexes. Two wild, co-occurring species of the *C. deitersi* complex, redescribed as *C. stimpsoni* and *C. fourchensis* and identifiable by sequencing the COI gene, displayed significantly different survival curves and endpoint survival over 96-h metal exposures (Rocha-Olivares *et al.*, 2009).

1.3 Cadmium and potassium permanganate

1.3.1 Metals in the environment

Metals are ubiquitous in the environment. Their behaviour and fate are often difficult to predict due to complex environmental chemistry. The natural conditions of an area may have a high concentration of metals due to the mineral composition of the environment,

weathering, climate, pH, redox potential, and dilution from precipitation or snowmelt (CCME, 2014; NRCan, 2004). In addition, anthropogenic activities have increased the flux of metals into the environment (UNEP, 2013).

Over time, populations have adapted and acclimated to metals in the environment. Some metals are used across many species in essential biological functions, while other metals are known to predominately be nonessential and toxic even at low doses. Naturally elevated levels of a metal may result in a locally-adapted ecological community, which may also respond differently to anthropogenically increased concentrations. Local adaptations are not accounted for by national guideline values (CCME, 2014) and may then benefit from a site-specific guideline (CCME, 2007). Some mechanisms for metal detoxification and acclimation are changing uptake channel expression, low molecular weight proteins that bind metals internally, and metal sequestration in inert forms (Peijnenburg & Vijver, 2007).

1.3.2 Cadmium

Cadmium is a transition metal with a molecular weight of 112.40 $g \cdot mol^{-1}$, a density of 8.642 $g \cdot cm^{-3}$, and a Chemical Abstract Service (CAS) number of 7440-43-9 (CCME, 2016). Cadmium has two oxidation states, metallic (Cd^0) and, more commonly, divalent (Cd^{2+}), (CCME, 2014). Cadmium is a relatively rare, naturally occurring element often associated with mineral sulphides, particularly zinc sulphides, in rock deposits (CCME, 2014; Nriagu, 1980). This metal is released as a by-product from zinc, copper, and nickel mining and refining, combustion of fossil fuels, and industrial boilers (NRCan, 2005; Nriagu & Pacyna, 1988). Cadmium itself is used in batteries, metal coatings and plating, nonferrous alloys, electronics, pigments, and plastic stabilizers (Fulkerson & Goeller, 1973; Hutton, 1983;

Pickering & Gast, 1972; Wilson, 1988). Canada was the fourth-largest producer of cadmium in 1994 (NRCan, 2005; Nriagu, 1980), though production in Canada was estimated to have decreased by 50-70% as of 2005 and is likely even lower today (CCME, 2014).

Anthropogenic processes have globally caused a seven-fold increase of cadmium flux into the environment (Wright & Welborn, 1994). Freshwater aquatic environments primarily receive cadmium through atmospheric deposition and industrial waste discharge (Nriagu & Pacyna, 1988; WHO, 1992). Globally, cadmium measured in unpolluted freshwaters can range from under 0.01 to 0.06 μg/L, while contaminated sediments have been reported to contain 5 mg/kg (WHO, 1992). Across Canada, cadmium levels of surface waters were measured from below detection limits (<0.001 μg/L) to, in an extreme case, 112 μg/L in Alberta (CCME, 2014). Measurements from the Great Lakes documented between 2003 and 2006 ranged from below detection limit to 0.098 μg/L (CCME, 2014; Rossmann & Barres, 1992). However, surface water cadmium concentrations in Canadian freshwater bodies are insufficient for inferences of general geographical variation (CCME, 2014). American surface waters are estimated to naturally range between 0.002 and 0.08 μg/L, but polluted waters are generally expected to exceed 2 μg/L (Stephan *et al*., 1994; USEPA, 2016).

Cadmium is one of the most toxic elements, nonessential to almost all organisms, and is readily bioaccumulated by biota, including humans, though it does not biomagnify (Paasivirta, 1991; WHO, 1992). In aquatic systems, the fate and behaviour of cadmium is determined by abiotic conditions, including pH, alkalinity, hardness, temperature, and organic content (CCME, 2014; WHO, 1992). These factors determine the speciation of cadmium, which influences its toxicity (CCME, 2014). Dissolved cadmium may occur as a

hydrated ion, salt, or complexed with organic or inorganic ligands (CCME, 2014). The free metal ion, Cd^{2+}, is the most bioavailable (WHO, 1992; CCME, 2014).

Water hardness is the abiotic factor most strongly inversely correlated with cadmium uptake, bioaccumulation, and toxicity, due to competition between cadmium and calcium ions (Borgmann *et al*., 2010; CCME, 2014). This ion-ion (Ca^{2+}-Cd^{2+}) competition can cause hypocalcaemia, and is thus a short-term mode of toxic action in aquatic organisms (CCME, 2014). Due to the relationship between calcium and cadmium toxicity, CWQGs-PAL benchmark values for cadmium depend on the calcium carbonate ($CaCO_3$) concentrations of the surface waters, and can be adapted for a range of hardness values (CCME, 2014). The short- and long-term CWQGs-PAL benchmark values for cadmium in freshwater are 1.0 and 0.09 µg/L, respectively, at 50 mg $CaCO_3$/L, and indicate total (unfiltered) cadmium concentrations (CCME, 2014). These benchmark values were derived from species sensitivity distributions that included data from studies with *Hyalella azteca*, which was included as one of the most sensitive invertebrate species to cadmium (CCME, 2014).

1.3.3 Manganese and potassium permanganate

Manganese is an abundant naturally occurring metal of atomic mass of 54.9 g/mol, a density of 7.43 g/cm, and a CAS number of 7439-96-5 (‘Manganese’, 2019). Abundant in sedimentary and metamorphic rocks, manganese is released to the aquatic environment through natural geological processes. Anthropogenic processes have increased manganese flux into the environment through mining and mineral processing, steelmaking and ironmaking, bitumen extraction, and pulp and paper mill processes (Napgal, 2001; WHO, 2004). Manganese itself is used in metallurgy, in dry-cell alkaline batteries, electrical coils,

as a dryer and pigment in paints, matches, oil, disinfectants, fungicides, fertilizers, artificial flavouring, animal foods, glass, fireworks, and leather and other textiles (ATSDR, 2012; IMnI, 2015; Nagpal, 2001; Webb, 2008). All manganese used in Canada is imported due to economics, though there are known deposits in east and west coast provinces (Webb, 2008).

Abiotic factors influence manganese environmental behaviour and fate. The strongest influence is exerted by pH and redox potential. Manganese has eleven oxidation states, though Mn^{2+}, Mn^{4+}, and Mn^{7+} are the most common. In natural freshwater environments, manganese primarily occurs as insoluble manganese dioxide which is biologically unavailable (WHO, 2004). However, this precipitation of manganese is slow in waters below pH 8.5, and can take years in some synthetic waters (Stokes *et al*., 1988). Unlike many metals, organic complexation plays only a small role in manganese speciation in natural waters, as DOC only weakly binds manganese (WHO, 2004). Aqueous Mn^{2+} is considered the most bioavailable in aquatic systems, and is taken up by organisms via the gills (Baden *et al*., 1995, 1999; WHO, 2004).

Manganese is used as an essential element by most organisms in enzyme pathways to metabolize protein and carbohydrates, and is under homeostatic control (Santamaria & Sulsky, 2010). In fish, excess manganese enhances production of reactive oxygen species (ROS) in, likely, specific organs, including the brain (Dolci *et al.*, 2013; Gabriel *et al*., 2013; Vieira *et al*., 2012). However, most manganese mode-of-action studies have been conducted with mammals and information about toxic processes in aquatic organisms is limited.

Water hardness will modify manganese uptake in fish and invertebrates. Elevated hardness decreases manganese uptake through competition for gill transport sites among Mn^{2+} and Ca^{2+}, and Mg^{2+} (Davies *et al.*, 1998; Peters *et al*., 2011; Reimer, 1999). The effect of calcium carbonate ($CaCO_3$) on short-term toxicity was additionally demonstrated by Lasier *et al.* (2000) in *H. azteca*. Neither dissolved oxygen nor dissolved organic matter appear to impact the rate of manganese uptake. In addition, there is no evidence for the effect of pH on toxicity in fish and invertebrates, though H^+ ions have been shown to compete for binding sites in algae (CCME, in review; Peters *et al*., 2011).

The CCME is in the CWQG-PAL development process for manganese, and released a draft for review in 2018. In the species sensitivity distributions that were used for short- and long-term guidelines, the most sensitive invertebrate species was *H. azteca*. The 96-h LC50 value for *H. azteca* was 8.6 mg/L, and for contrast, 14.5 mg/L for *Ceriodaphnia dubia*, which is another commonly used invertebrate in toxicology (Lasier *et al*., 2000).

Potassium permanganate ($KMnO_4$) is an inorganic compound of manganese commonly used to treat drinking water and wastewater (IMnI, 2015). This substance is a dark purple or bronze odorless compound with a molecular weight of 158.032 $g \cdot mol^{-1}$, a density of 2.7 $g \cdot cm^{-3}$, and a CAS number of 7722-64-7 ('Potassium permanganate', 2019). This compound is highly oxidative due to the permanganate ion, and water soluble. In aquaculture, the use of antibiotics is decreased through proactive potassium permanganate treatments for gill pathogens, parasites, and external bacterial and fungal infections (Lazur & Yanong, 2013). The suggested treatment concentration in aquaculture for most fish is 2 $mg \cdot L^{-1}$, though consideration for particularly sensitive aquaculture species or presence of

reductive material in the pond can cause the user to recalculate the potassium permanganate demand (PPD) (Lazur & Yanong, 2013). The suggested dose requires a visible colour change for 8 to 12 hours, and is then repeated every other day up to three times (Tucker &Robinson, 1990).

In water, potassium permanganate completely dissociates to potassium (K^+) and permanganate (MnO_4^-) ions, the latter of which can then be further reduced to insoluble manganese dioxide (MnO_4) and finally the manganous ion (Mn^{2+}), though the reaction is dependent on the pH (Hobbs *et al*., 2006). The toxic effect is rapid and likely predominantly due to the high oxidative potential of MnO_4^- ions (Hobbs *et al*., 2006). Organic material is a powerful potassium permanganate toxicity modifying factor, as is pH.

Toxicity values of potassium permanganate to several species are given in Table 1.1. *Hyalella azteca* was determined to have a nominal 96-h LC50 values of 4.74 $mg \cdot L^{-1}$ and 12.30 $mg \cdot L^{-1}$ in synthetic moderately hard water and pond water, respectively (Hobbs *et al.*, 2006). The synthetic moderately hard water PPD was 0.329 $mg \cdot L^{-1}$ and the pond water PPD was 5.357 $mg \cdot L^{-1}$ (Hobbs *et al.*, 2006). The 16-fold difference is due to the additional organic material in the pond water. Notably, the laboratory-tested *H. azteca* 96-h LC50 values were below the PPD.

Table 1.1: Endpoints of species exposed to potassium permanganate in moderately hard water

Test organism	Endpoint	$KMnO_4$ ± standard deviation (mg/L)
Daphnia magna	96-h LC50	0.053 ± 0.009
Ceriodaphnia dubia	96-h LC50	0.058 ± 0.006
Pimephales promelas	96-h LC50	2.13 ± 0.07
Chironomus tentans	96-h LC50	4.43 ± 0.79
Hyalella azteca	96-h LC50	4.74 ± 1.05

Data from Hobbs *et al*. (2006). Potassium permanganate nominal concentrations are listed in the table. Tests were conducted in moderately hard water, prepared according to American Public Health Association (1998).

1.4 Toxicity and bioaccumulation models

There are many different bioaccumulation models that can be used to model metal uptake. These models can be mechanistic, but are limited by the information attainable in a typical study. In the past, bioaccumulation factors and bioconcentration factors have been used to describe toxicant accumulation from all sources and only from water, respectively, but they do not adequately describe the relationship between metal concentrations in water and tissues (McGeer *et al*., 2003). The biotic ligand model (BLM) (Di Toro *et al*., 2001; Santore *et al*., 2001; Paquin *et al*., 2002), biodynamic model (Luoma & Rainbow, 2005; Rainbow, 2002), and saturation models (Borgmann *et al.*, 2004) are three models that can be developed to describe the relationship between external metal sources and metal in organisms. The BLM was developed for acute toxicity and uses a variety of water parameters and saturation kinetics to predict metal bioaccumulation and toxicity. Although the BLM has been used in a

plethora of studies and provides adequate predictions, it is cumbersome due to its extensive data requirements.

Similar to the BLM, the saturation model (SM) uses the concept of saturation to model bioaccumulation across a range of water concentrations. This model has been used in *H. azteca* studies in the past, including work done by Borgmann *et al*. (2004), Norwood *et al*. (2006, 2007), Leung *et al.* (2016), and Pathammovong (2016). This model is easily applied and compared among similar water conditions (e.g., water hardness, pH, temperature, DOC). A strength of the SM is its ability to provide a meaningful, mechanistically-based link between water concentration-based mortality (e.g., LC50) and body concentration-based mortality (e.g., LC50).

1.5 Objectives

Hyalella azteca species are important in toxicological science due to their general high sensitivity and prevalence in the environment and research. The recognition of *H. azteca* as a cryptic species complex has left questions about whether there exist detectable species-level (rather than genus-level) differences in toxicant sensitivity among members of the complex.

Past studies (Soucek *et al.*, 2013; Weston *et al.*, 2013; Leung *et al.*, 2016, Pathammavong, 2016) have detected dissimilar responses to organics, metals, and media manipulations among clades in the *H. azteca* species complex. Two clades (1 & 8) are of particular interest because of their prevalence as laboratory-maintained populations across North America and the central literature based on experiments with them. Clade 8 is the most common laboratory species, but wild populations have only been detected south of the

last glacial maximum (Major *et al.* 2013; Hyrcyshyn, 2016). On the contrary, clade 1 is far less common in North American laboratories but has been detected across most of previously-glaciated regions of North America (Hrycyshyn, 2016). These two species are morphologically similar large-bodied species, but are distinguishable at the COI gene. Thus, the objective of this Masters project is to determine whether there are detectable differences between the two clades in their bioaccumulation and toxic responses to a metal (cadmium) and a permanganate salt (potassium permanganate).

In single-toxicant short-term (7-day) exposures, the two *H. azteca* species were evaluated and contrasted for mortality, growth inhibition, and bioaccumulation. Four-week uptake and depuration experiments were also conducted to determine uptake and elimination kinetics of each clade. Metal bioaccumulated at the site of action is a better predictor of toxicity than water concentration (Connolly, 1985; McCarty, 1991). In *H. azteca*, total body concentration has been used as a surrogate for toxicant concentration at the target site (Norwood *et al*., 2007). Thus, mortality and growth inhibition were evaluated both on the basis of water concentration and bioaccumulation in the toxicity experiments. In addition, if the clades differ in their toxic responses, a contrast of their uptake and depuration kinetics may provide a mechanistic explanation.

Data were collected and modelled to determine the EC_x (the concentration at which *x%* effect is observed) values by following protocols used in similar studies with *Hyalella*. The results are considered for potential implications in the use *H. azteca* species in toxicology and informing regulatory decisions.

Chapter 2
Materials and Methods

2.1 Stock cultures of *Hyalella* species

The laboratory *Hyalella* stock culture of clade 1 was established from donations by Warren Norwood at Environment Canada, Centre for Inland Waters (CCIW) in Burlington, Ontario. This population was originally collected in 1986/87 from Valens Lake, Hamilton Conservation Authority, Ontario (Warren Norwood, personal communication) and has since been maintained in isolation at CCIW. The clade 8 stock culture was obtained from Bruce Greenberg at the University of Waterloo, Waterloo, Ontario. The original source of this population is unknown.

Hyalella were maintained, cultured, and tested in a five-salt standard artificial media (SAM-5S, or SAM), originally developed by Borgmann (1996). This medium was selected because it is the recommended reconstituted water for *Hyalella* cultures (EC, 2013). The key contents of this media are bromide (Br), calcium (Ca), sodium (Na), bicarbonate (HCO_3), magnesium (Mg), and potassium (K). The recipe contains an optimum ratio of Ca to Br for Ca use by the amphipods (Borgmann, 1996). In addition, Mg and K are crucial for *H. azteca* growth and reproduction, and Na and HCO_3 are the most essential ions for survival (Borgmann, 1996). Batches of SAM were prepared and aerated until transfer to culture or test containers. The recipe for SAM is given in Table 2.1.

Table 2.1: Recipe for SAM-5S reconstituted water (Borgmann, 1996) used as culutre and test media

Salt	mg/L
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_20$)	147.01
Sodium hydrogen carbonate ($NaHCO_3$)	84.01
Sodium bromide (NaBr)	1.02
Potassium chloride (KCl)	3.73
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	61.6

Analysis of major ions was conducted by use of inductively coupled plasma optical emission spectrometry (specifically, a Perkin Elmer Optima 8000 ICP-OES). Media were acidified to 20% HNO_3 with 70% ultrapure nitric acid for ICP-OES analysis. A total of four test batches of test SAM (made and held separately from culture SAM) were prepared. The characteristics of SAM were analysed by Gena Braun at the Centre for Cold Regions and Water Science at Wilfred Laurier University, and are recorded in Table 2.2. An earlier preparation SAM-5S in our lab was evaluated by the National Laboratory for Environmental Testing (NLET, Environment Canada) in Burlington, Ontario. These results, reported by Jessica Leung in her Masters of Science thesis (2014) are also included in the Table 2.2.

Table 2.2: Measured constituents of culture and test media

	Mean (± SD), (mg/L)	NLET (Leung, 2014), (mg/L)
Mg	6.765 (± 0.295)	5.9
Na	24.642 (± 1.652)	22.2
K	2.080 (± 0.134)	2.0
Ca	45.963 (± 1.729)	Not reported
S	10.292 (± 0.551)	Not reported
Chloride	0[1]	67.1
Alkalinity	51.25 (± 3.620)	41.5
Total organic carbon (TOC)	1.287 (± 0.416)	0.3
Total inorganic carbon (TIC)	13.46 (± 0.901)	9.43
Hardness ($CaCO_3$)	Not measured	89.9
SO_4	Not measured	23.3

[1]The lower limit of the chloride probe was 34 mg/L (1 mM).

Stock populations of *H. azteca* species were maintained in aerated 36-L aquaria under a light and dark cycle of, respectively, 16 and 8 hours. Aquaria were filled with SAM, and cotton balls were added as a substitute for substrate. Clades 1 and 8 were maintained in segregated areas to minimize cross-contamination risk. Ambient temperature ranged between 23 and 28°C, with some seasonal fluctuation. All tanks were fed 5 to 10 mg of TetraMin® three to five times each week. TetraMin® is a commercial fish food that is widely available. For use in *Hyalella* feeding, TetraMin® flakes were ground by use of a mortar and pestle and sifted through 750 µm mesh. Ground TetraMin® that passed through the sieve was then collected and stored dry in an acid-washed container for use. The same food was used for stock populations, cultures, and experiments.

Previous work in this laboratory by Jessica Leung (2014; Leung *et al.*, 2016) and Patrick Pathammovong (2016) determined the genetic identity of the *Hyalella* cultures. The

method outlined by Witt and Hebert (2000) was followed to amplify a region (637 base pairs) of the cytochrome *c* oxidase I mitochondrial gene. The genetic identity of all individuals matched the stock culture from which they were obtained.

2.2 *Hyalella* culturing

The culture procedure for *Hyalella* species was adapted from Borgmann and Munwar (1989). Culture containers were 2-L high density polyethylene vessels, containing 1-1.5 L of SAM, and a 7 cm^2 piece of cotton gauze. Five to fifteen culture containers were maintained per clade at any time and were replaced as necessary (i.e., in the case of multiple weeks of low reproduction or a decline in the number of individuals in a container). To begin a new culture container, ten to fifteen mating pairs (amplexed pairs) were pipetted from a stock aquarium into a tub prepared with SAM and gauze. These containers were then maintained under the same lighting and temperature conditions as the stock tanks. Each culture container was fed 2.5 to 5 mg of ground TetraMin® three times each week on non-consecutive days. To limit evaporation, culture containers were covered with a translucent plastic sheet. The purpose of culturing the *Hyalella* populations was to obtain groups of juveniles sorted by age. The short-term toxicity tests used juveniles aged 2-9 days, and the uptake and depuration (UD) tests used individuals aged 3 – 8 weeks. Thus, both clades were renewed once every seven days.

To renew a single container, the gauze was gently held with tweezers and sprayed with SAM to dislodge from it any amphipods. The container's contents were then poured through two mesh sizes, 700 μm and 300 μm. Adult *Hyalella* were collected on the larger

mesh size, and juveniles were able to pass through and collect on the smaller mesh. Each collection was then moved to separate petri dishes from where individuals were enumerated as they were pipetted into appropriate containers. The original culture container was refilled and restocked with the same adult amphipods. Juveniles of the same clade were all transferred to a newly prepared container that was distinguished by date of culturing, to a limit of 300 amphipods per vessel. Juveniles' containers were maintained for two days before they were either used in a short-term toxicity test or transferred to a stock aquarium. The exception to this was in preparation for an uptake-depuration test for which juveniles were maintained for the appropriate amount of weeks, with weekly water changes and sorting individuals into a greater number of containers (to decrease mortality due to overcrowding). In some cases, juveniles maintained in this fashion had to be discarded due to high mortality. If these containers were maintained for more than four weeks, the weekly water changes included removal of offspring of the now-mature juveniles.

2.3 Experiments

2.3.1 Short-term experiments (1 week)

The short-term exposures were selected and designed to compare the toxicity of single contaminants to *Hyalella* clades 1 and 8. The two toxicants were cadmium and potassium permanganate.

The methods for the exposure tests are based on and modified from tests conducted with *Hyalella* species described in "Biological Test Method: Test for Survival and Growth in

Sediment and Water Using the Freshwater Amphipod *Hyalella azteca*, Second Edition" (EC, 2013), Norwood *et al.* (2006), Leung *et al.* (2016), and Pathammovong (2016).

In short-term exposures, amphipods were exposed to one of a series of treatment levels in a single-metal, non-renewal, one-week experiment. The experimental design for both metals was the same, and any deviations are noted below. Each experiment was designed to have three concurrent controls in which amphipods were exposed to no, or background, metal concentrations. The above-background series of concentrations each had two replicates. Clades were tested simultaneously and identically. Experiments were repeated for a total of three times.

Before the tests were started, 200 L of SAM-5S was prepared to ensure media consistency throughout a test. These batches of prepared media were analyzed for major ions by Gena Braun at the Centre for Cold Regions and Water Science (CCRWS) at Wilfred Laurier University (Table 2.2).

Aqueous potassium permanganate stock of 0.05 M was prepared by dissolving its salt (purchased from MilliporeSigma) in deionized water and then maintained in the dark. Cadmium stock was prepared by diluting aqueous standard (ACROS Organics, Cadmium standard solution for AAS) in deionized water and acidified to 2% HNO_3. Stock concentrations were prepared in acid-washed glassware.

Short-term tests were run in 500 mL acid-washed high-density polyethelene containers. Each container was filled with 100 mL of SAM, spiked for the final desired concentration, and then filled to the total volume of 250 mL. The cadmium and manganese nominal exposure concentrations are displayed in Table 2.3. A single piece of 5 cm^2 pre-

soaked cotton gauze, serving as a substrate surrogate, was added to each vessel. Concentrations were then left to equilibrate for 4-24 hours before adding amphipods.

After the equilibration period, water samples were obtained (refer below for the water sampling procedure) from one repetition of each control and treatment level, from each clade. These samples constitute the first or two sets filtered and unfiltered water samples taken from each experiment: the initial (day 0) samples. From the same vessels, 60 mL aliquots were poured into small polyethelyene cups to evaluate water quality (refer below for the water quality protocol).

Following water sampling, juveniles aged 2-9 days were haphazardly (cannot be considered technically random selection) sorted into sets of 15 and added to one of the test vessels. Each vessel was allotted 2.5 mg of prepared TetraMin® food. A fine spray of media was used to sink the food into the media.

Test vessels were randomly placed in the incubator and maintained at 25 ± 2 ^{o}C, with overhead full-spectrum lighting and a photoperiod of 16-h light and 8-h dark. The test vessels were loosely covered with a clear plastic sheet to minimize evaporation, and fed as previously described on days zero, two, and five. Environment Canada (2013) recommends a temperature daily average of 23 ± 1^{o}C. Temperature was recorded on five of the seven test days.

Table 2.3: Nominal concentrations in short-term toxicity tests

Series Number	Cadmium (nM)	Potassium permanganate (nM)
Control	0	0
1	10	500
2	16	900
3	22	1600
4	34	2800
5	50	5000
6	76	9000
7	114	16000
8	-	28000
9	-	50000

On day seven, test vessels were removed from the incubator. Final (day 7) water filtered and unfiltered water samples were collected. Sixty-millilitre aliquots of test media were allocated for water quality. Juveniles were decanted into a clean dish for survival enumeration, and subsequently transferred, by pipette, to a cup containing 60 mL of 50 uM Ethylenediaminetetraacetic acid (EDTA), fresh gauze, and 2.5 mg of TetraMin®. These cups were incubated for an additional 24 hours for gut clearance (Neumann *et al*., 1999). Refer to Tissue Samples, below, for the tissue sample protocol, which includes the procedures for data collection to determine growth inhibition and bioaccumulation.

The cadmium toxicity exposure was conducted by Evelyn Worthington. All further analyses and other tests were conducted by the author of this thesis.

2.3.2 Short-term test observations, measurements, and endpoints

The endpoints for the short-term toxicity tests were survival and mean dry weight. The data were fitted to saturation bioaccumulation and mortality models, and an empirical growth inhibition model. Point estimates from the data are based on lethal water concentration (LC25 and LC50), lethal body concentrations (LBC25 and LBC50), growth inhibition water concentrations (IC25), and growth inhibition body concentrations (IBC25). The fitted models and point estimates could then be used to compare the bioaccumulation and toxic effects on clade 1 and 8.

2.3.3 Uptake and depuration tests

Uptake and depuration tests (UD) were designed to examine the bioaccumulation and depuration of two metals in single-concentration tests with the two *Hyalella* clades in this study. The methods for estimating the kinetics of bioaccumulation are outlined in Modeling (Section 2.4), below.

The test design included two weeks of uptake, over which exposure containers were renewed and spiked weekly with the toxicant of study, followed by two weeks of depuration, in which both control and exposure containers were not spiked. The test containers used were larger (2-L high density polyethylene tubs) than those used in the short-term experiment. The source and culturing of animals, feeding regime, lighting, and aqueous metal stock preparation were also similar, but did require modifications. Each vessel contained 80 to 100 *Hyalella* aged 3-8 weeks, and containers were fed 5 mg instead of 2.5 mg per feeding event. Instead of a concentration series, there was only one concentration to which *Hyalella* were exposed, and its number of concurrent repetitions matched that of the

control. The nominal concentration in the manganese uptake exposure was 3000 nM; the nominal concentration in the cadmium uptake exposure was 27 nM. The manganese experiment was conducted only once, with three concurrent control and exposure repetitions; the cadmium experiment was conducted twice, each with two concurrent repetitions. The single exposure level for each metal was chosen based on short-term exposure LC12.5 and LC25 values calculated from nominal concentrations of each clade. If one clade appeared to be more sensitive, the concentration was chosen to accommodate that clade. For example, if the estimated nominal clade 1 LC12.5 value was lower than that for clade 8, both clades would be exposed to a concentration of approximately the clade 1 LC12.5 value.

In preparation for an experiment, exposure vessels were filled with 500 mL of SAM, spiked, and then were filled to 1.5 L. A 25 cm^2 piece of gauze was added. Control and exposure vessels were prepared simultaneously. These containers were then left for 4 to 24 hours to equilibrate. After this equilibration period, but before the addition of amphipods, water quality was measured and filtered and unfiltered water samples were obtained from each vessel, for metal analysis (refer to Section 2.3.4 Water Samples). Finally, *Hyalella* were haphazardly sorted and added to each vessel, followed by 5 mg of TetraMin®. Test containers were then randomly placed into an incubator (25 ± 2°C) and covered with a clear plastic sheet.

Water samples were taken at the beginning and end of each renewal period. At this time, any offspring released during the week were removed. During the uptake period, tissue samples were taken after 1 hour, 4 hours, and then after 1, 2, 5, 7, 9, 12, and 14 days. After 14 days, *Hyalella* were transferred to depuration (unspiked) media. Following this transfer,

samples were taken after 4 hours, and then after 1, 2, 5, 7, 9, 12, and 14 days (for clarity these sampling times are hereafter referred to as the number of days from the beginning of the experiment. E.g., sampling after 14 days of depuration is referred to as 28 days). To obtain a tissue sample, three *Hyalella* were haphazardly (cannot be considered random selection) pipetted from a test vessel, rinsed in deionized water, pipetted onto a pad of Kimwipes® to remove excess moisture, and weighed on a microbalance to obtain the total wet weight. This process was repeated for all vessels at each sampling time. There was no gut clearance. These samples were then dried and processed as tissue samples (Section 2.3.5 Tissue Samples). Upon completion of the final sampling time, the experiment was taken down and all remaining individuals were rinsed, counted, dried, and stored in additional microcentrifuge tubes.

2.3.4 Water samples

At the beginning and conclusion of each test, or week (in the case of uptake and depuration experiments), filtered and unfiltered water samples were obtained by collecting 1 mL from one repetition of each treatment level, for each clade. In preparation for sampling, a plastic 3 mL syringe was rinsed three times with deionized water. A 0.45 μm filter (Millipore® membrane filters) was then attached to the syringe and rinsed with 1 mL of deionized water. The syringe was next rinsed with the treatment water, and the filter was then attached to obtain the filtered sample. The filter was then removed to allow unfiltered collection. Method blanks containing deionized water were taken with each set of water samples collected and were treated identically to the water samples in preparation for graphite furnace atomic absorption spectroscopy (GFAAS) analysis. All water samples were acidified by

addition of 10 µL of 70% ultrapure nitric acid. The filtered samples are used to estimate the dissolved metal concentrations, whereas the unfiltered samples correspond to total metal concentrations. At each level of nominal exposure, average total and dissolved values were determined from the concentrations at the beginning and end of the experiments. Measured filtered concentrations were preferentially used because the dissolved concentration is theoretically a better estimate of the bioavailable toxicant fraction than is the total metal fraction.

2.3.5 Tissue samples

In the short-term experiments, wet weight was obtained after the 24-hour gut clearance by placing juveniles on a pad of Kimwipes® to remove excess moisture, where they were re-counted prior to weighing in a microbalance (Mettler Toledo, accurate to 0.001 mg). Juveniles were then transferred to 2 mL microcentrifuge tubes and dried by placing the tubes in an oven set at 55^{o}C for 72 hours.

Upon removal from the oven, total dry weights of amphipods from each container were obtained by use of the microbalance to determine growth (and bioaccumulation). From each repetition in the manganese experiment, six amphipods were then haphazardly selected for the digest procedure, weighed, and transferred into a new microcentrifuge tube. In cases where fewer than six juveniles survived, all were included. In the cadmium experiment, all amphipods from a treatment replicate were included in the digest. The final dry weight for each replicate was the average weight per individual (total weight divided by number of amphipods in the sample). The digestion protocol employed here was originally described by Stephenson and Mackie (1988) and modified by Borgmann *et al.* (1991) and Norwood *et*

al. (2006). For the digest procedure, tissue samples were binned into four weight categories (Table 2.4). All treatments in the short-term exposures fell within the lowest weight category (below 0.750 mg). Thus, 13 µL of 70% nitric acid was added to each microcentrifuge tube and loosely capped. After six days, 10 µL of 30% hydrogen peroxide was added to the vial, and re-capped for another 24 hours. Finally, 477 µL of MilliQ was added to the vial to bring the total volume to 500 µL. These vials were maintained until they were analyzed by use of GFAAS.

There are a few distinctions between processing tissue samples in the short-term experiments and those in the uptake and depuration experiments. In the latter, tissue samples contained three individuals pipetted directly from the test media, rinsed in deionized water, placed for no more than 30 seconds on a pad of Kimwipes®, weighed in a microcentrifuge tube and then moved immediately to the oven (set at 55ºC) for 72 hours to dry. In certain cases, amphipods were placed back in the oven for an additional 72 hours if, upon visual inspection, they did not appear to have completely dried. Incomplete drying was identified as a dark brown to black colour, which faded to a lighter brown after further time in the oven. Once oven dried, samples were weighed as in the short-term experiments. Because the dry weights of uptake and depuration tissue samples were larger than those of short-term samples, they had to be treated with different volumes of nitric acid, hydrogen peroxide, and MilliQ, according to the digest procedure. Higher volumes of acid, hydrogen peroxide, and MilliQ were added, according to the dry weight. The four weight categories, corresponding to total weight, are included in Table 2.4 (Warren Norwood, personal correspondence).

Table 2.4: Digest treatments for four dry weight categories of tissue samples

Total dry weight range (mg)	70% Nitric acid (µL)	30% Hydrogen peroxide (µL)	MilliQ (µL)
0 – 0.749	13	10	477
0.750 – 1.499	25	20	955
1.500 – 2.25	38	30	1432
>2.250	50	40	1910

Tissue digest procedure from Stephenson and Mackie (1988) modified by Borgmann *et al.* (1991) and Norwood *et al*. (2006). Reproduced from personal correspondence with Warren Norwood.

2.3.6 Acid washing

The purpose of the acid washing procedure was to minimize metal contamination. The acid-washing procedure followed is from Warren Norwood (personal communication). Items were first rinsed with deionized water, then set in a fume hood and filled with 20% reagent grade nitric acid. If containers or microcentrifuge tubes with lids were used, the lids were also subjected to the procedure. After 24 hours in the fume hood, containers were emptied and rinsed thoroughly with deionized water. Microcentrifuge tubes used for water and tissue samples were not acid washed. All glassware and containers used for stock solutions or in the graphite furnace procedure were acid washed following this procedure.

2.3.7 Graphite furnace atomic absorption spectroscopy

A Perkin Elmer PinAAcle 900T AA was used on the graphite furnace setting to measure all samples. A manganese method was developed from the suggested conditions in the AAS software (WinLab). A Zeeman background correction was used. All manganese samples were run without use of a modifier. The cadmium method also used a Zeeman background

correction, but samples were run with a 1.0% ammonium phosphate ($NH_4H_2PO_4$) and 0.06% magnesium nitrate ($Mg(NO_3)_2$) modifier, purchased from PerkinElmer. Standard curves were determined by analysis of a stock solution prepared from a high purity cadmium or manganese AA standard in SAM acidified to 10% with high purity 70% nitric acid. Quality checks and machine blanks were run every ten samples to check for carryover and to correct for drift. Method blanks were used to correct for contamination from the experiment methodology. Certified reference materials (CRM) were analyzed to verify the accuracy and precision of analytic methods. Aqueous reference materials were purchased from PerkinElmer (Quality Control Standard, 21 Elements, Matrix per Volume 5% HNO_3 per 100 mL) and High-Purity Standards Inc. (Drinking Water Reference Material). The tissue digest reference material was TORT-2 (Lobster Hepatopancreas Reference Material for Trace Metals), and was obtained from Environment Canada. In the cadmium method, percent recovery of the aqueous CRM was 85% (95% CI: 80 – 90), recovery of TORT-2 was 112% (95% CI: 96 – 130) and the detection limit was determined to be 0.178 nM. In the manganese method, percent recovery of the aqueous CRM was 115% (95% CI: 111-120), recovery of TORT-2 was 118% (95% CI: 100 – 137), and the detection limit was determined to be 26.2 nM.

2.4 Modeling

2.4.1 Bioaccumulation

The saturation model (SM) invokes a number of assumptions about metal bioaccumulation. Principally, this model states that metal will bioaccumulate in an organism until it reaches a maximum, producing a curve described by **Equation 1**. This saturation could be due to a number of different mechanisms that can produce the same curve, but details for interpreting *max* and *K* are distinct. Maximum body concentration could be due to (1) saturation of uptake sites (theory similar to the BLM), (2) a maximum amount of metal that the organism can bioaccumulate (Borgmann *et al.*, 2004), and (3) the ratio of uptake and elimination rate constants (Borgmann & Norwood, 1995). Despite the ambiguity of parameter interpretation, the Michaelis-Menten-based saturation kinetics provide a better description of metal bioaccumulation across a range of water concentrations, particularly at elevated metal concentrations than a similarly employed allometric model (Borgmann *et al.*, 2004). **Equation 1** gives the mechanistically-based bioaccumulation saturation model.

$$C_{TB} = \frac{\max \cdot C_W}{C_W + K} + C_{BK} \qquad \text{(Eq. 1)}$$

In this equation, C_{TB} is the whole-body metal concentration, C_W is the metal concentration in the water, C_{BK} is the background metal body concentration, and K is the half-saturation constant. K is a fitted parameter that can be difficult to meaningfully interpret in toxicity models, because due to complexity it can have multiple interpretations (Norwood *et al.*, 2006). The allometric model also describes metal accumulated in tissue, but it is a linear

model and has been shown to be inferior to the saturation model at high metal concentrations. The strength of the saturation model is in the assumption that metal uptake reaches a maximum and body concentration becomes asymptotic (Borgmann *et al*., 2004).

2.4.2 Mortality

Borgmann *et al*. (2004) developed toxicity models to describe mortality based on water concentration and body concentration, respectively **Equation 2** and **Equation 3**.

$$m = m' + \frac{\ln(2)}{t} \cdot \left[C_W \left(\frac{\frac{1}{LC50} + \frac{1}{K''_W}}{1 + \frac{C_W}{K''_W}} \right) \right]^{n_b} \quad \text{(Eq. 2)}$$

$$m = m' + \frac{\ln(2)}{t} \cdot \left[C_{TB} \left(\frac{\frac{1}{LBC50} + \frac{1}{K''_{TBX}}}{1 + \frac{C_{TB}}{K''_{TBX}}} \right) \right]^{n_b} \quad \text{(Eq. 3)}$$

In Equations 2 and 3, m is the overall mortality rate at a concentration, m' is the mortality rate of the control, and the remainder of each equation is a term to describe the above-background mortality. These combinations of terms describe the above-background mortality rate in terms of either water concentration or body concentration, and associated saturation kinetics. The LC50 is the concentration that produces 50% above-background morality of the test organisms, C_W is the water concentration, K''_W is the half-saturation constant of above-background mortality, and n_w is a fitting parameter. Similarly, the LBC50 is the above-background body concentration causing above-background 50% mortality of test organisms, K''_{TBX} is the half-saturation constant (the tissue metal concentration when the

mortality rate is halfway between background and maximum mortality rate) of above-background mortality, and n_b is a fitting parameter. The usefulness of these equations is due to the assumption of mechanistically-based bioaccumulation, which facilitates a connection between water concentration-based mortality and tissue metal (bioaccumulation)-based mortality. A more extensive derivation and contrast of this model against the allometric model is given in Borgmann *et al.* (2004). Furthermore, possible interpretations of *K* are discussed by Borgmann *et al*. (2004) and Norwood *et al.* (2006).

2.4.3 Growth inhibition

The SM is inappropriate for use in modeling growth effects, thus a general growth model was used to predict growth inhibition: **Equation 4** (Norwood *et al.*, 2007).

$$W = W'(1 + aC^n)^{-1} \qquad \text{(Eq. 4)}$$

In Equation 4, *W* is the final body weight at the end of the exposure, *W'* is the control weight, *C* is the water concentration or the background-corrected tissue concentration, and *a* and *n* are constants (Borgmann *et al*., 1998; Norwood *et al*., 2007).

2.4.4 Uptake and depuration

Equation 1 is a mechanistically-based equation to describe bioaccumulation tissue concentrations over a range of water concentrations. It is also useful to model metal uptake rate over time, which can be accomplished with the use of a sing-compartment first order uptake and elimination model. Two differential equations describe the change in body

concentration over an uptake phase given as **Equation 5**, followed by a depuration phase **Equation 6** (MacLean *et al*., 1996).

$$\frac{\mathrm{d}C_{TB}(t)}{\mathrm{d}t} = k_u C_W - k_e C_{TB}(t) \quad \text{(Eq. 5)}$$

$$\frac{\mathrm{d}C_{TB}(t)}{\mathrm{d}t} = k_u C_W - k_e C_{TB}(t) \quad \text{(Eq. 6)}$$

In these equations, $C_{TB}(t)$ is total body concentration as a function of time, k_u and k_e are uptake and elimination rate constants, and C_W is the water concentration of the metal. If rate constants and water concentrations are assumed to be constant, these equations can be solved as shown in **Equations 7** and **8**.

$$C_{TB} = \frac{k_u}{k_e} C_W (1 - e^{-k_e t}) + C_{BK} \quad \text{(Eq. 7)}$$

$$C_{TB} = C_{TB0} \cdot e^{-k_e t} + C_{BK} \quad \text{(Eq. 8)}$$

If metal bioaccumulated reaches a steady state, the rate of change of total body concentration as a function of t is zero, as t approaches infinity, or $\frac{\mathrm{d}C_{TB}(t)}{\mathrm{d}t} = 0$ as $t \rightarrow \infty$. Therefore, the whole-body metal concentration at steady state, C_{SS} is given in **Equation 9**.

$$C_{SS} = \frac{k_u}{k_e} \cdot C_W + C_{BK} \quad \text{(Eq. 9)}$$

2.4.5 Short-term experiment mortality modelling

Data from the short-term toxicity test were modeled with equations 1 – 4. Mortality rates data for equations 2 and 3 can be calculated from survival data using a hazard-based equation, **Equation 10** (Borgmann *et al.*, 1998).

$$m = \frac{-\ln\left(\frac{N}{N_0}\right)}{t} \qquad \text{(Eq. 10)}$$

Mortality rates were calculated for each repetition. The mortality rate, m, is the negative natural logarithm of N, the survival count at the end of the exposure, divided by N_0, the initial number of amphipods. If N/N_0 was 0 or 1 (0% or 100% survival) in both replicates of a concentration, the survival was changed, respectively, to 0.5 and 14.5 to allow the conversion to mortality rate, and provide the model with data points at minimal and maximal mortality. If there was no survival in both replicates of multiple concentrations, the modified survival was applied to only one of the replicates in the lowest of these concentrations.

2.5 Software

All data were preparations and preliminary comparisons and calculations were conducted in Microsoft Office Excel® and MATLAB®. Data were fitted to the saturation bioaccumulation, uptake, and depuration models by use of the "Nonlin" method for regression in SYSTAT 13, and the "Funpar" command was used to obtain the 95% confidence intervals of associated parameters. All boxplots and other statistical analyses

were conducted with IBM SPSS® version 25 software. Finally, MATLAB® was used to plot the data and regressions for display.

Chapter 3
Cadmium

3.1 Results

3.1.1 Short-term toxicity experiment

3.1.1.1 Water Samples

A two-way ANOVA was performed on GFAAS-measured water concentrations to determine whether filtering water samples had a significant effect on metal recovery across the concentration series. Figure 3.1 displays the initial (day 0) dissolved and total cadmium water samples graphed against the nominal concentration. To fit the assumptions of normality and homoscedascity, the cadmium data were logarithmically transformed (transformed data = log(a+min+1), where a is a data point and min is the lowest value in the set) prior to analysis. This method for transformation was selected to ensure that no data (negative values) were undefined following logarithmic transformation. The transformed data did not significantly deviate from a normal distribution (Shapiro-Wilk; $p > 0.05$), but the assumption of homogeneity of variances was violated (Levene's test for equal variances; $p = 0.036$). The two-way ANOVA did not detect a statistically significant interaction between filtering and nominal concentration level on the measured cadmium concentration ($F = 4.301$, $p = 0.912$, d.f. = 7, 79). However, many of these water samples were likely contaminated, which yielded greater-than-nominal recoveries. The contamination may have been due to sample vial contamination or use of yellow pipette tips. Therefore, to model data with dissolved (measured, filtered) cadmium concentrations, samples above the 1:1 nominal to measured ratio were excluded. The recovery of the aqueous CRM was 85% (95% CI: 80-

90), making the sub-100% recovery a conservative assumption. A linear regression was used to estimate the cadmium content of the lowest exposure concentration because no measured concentrations were less than the level of nominal recovery.

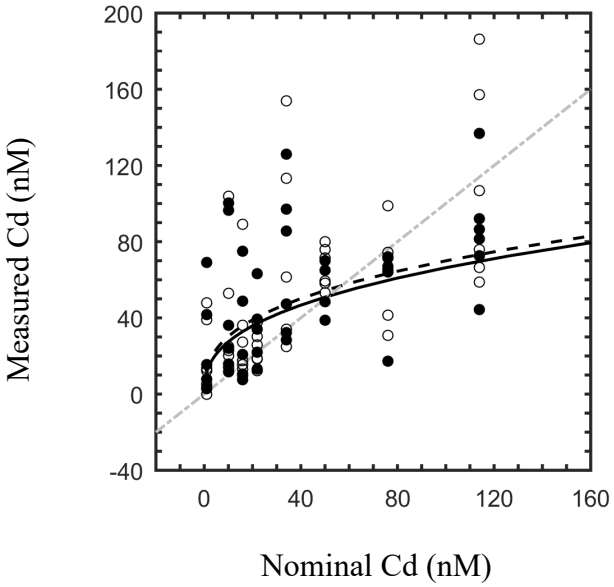

Figure 3.1: GFAAS-measured dissolved and total cadmium concentrations in short-term test media as functions of nominal metal concentration

Total (○, dashed black line) and dissolved (●, solid black line) measured cadmium concentrations (n = 47, 48). Data from all short-term experiments and both clades are included. Measurements were drift, background, and blank corrected prior to plotting. The light grey line (intermittent dotted and dashed line) is displayed for reference of a 1:1 measured-to-nominal ratio. Logarithmically transformed (log(a + min + 1)) dissolved and total cadmium data were linearly regressed, yielding R-square values of 0.34 and 0.38, respectively.

3.1.1.2 Bioaccumulation in short-term experiments

GFAAS analysis measured the cadmium content of clade 1 and 8 digested tissue (nmol/g dry weight). A two-way ANOVA was conducted to examine the effects of experiments run at different times on clade background tissue metal. All cells of the design were normally

distributed (Shapiro-Wilk; $p > 0.05$), and the data met the assumption of homogeneity of variances (Levene's test; $p = 0.588$). There was not a significant interaction effect between temporal replicates and clade on the mean measured background concentration (2-way ANOVA; F = 0.389, $p = 0.687$, d.f. = 2, 16). An analysis of main effects at the clade level also did not detect a significant difference (F = 0.026, $p = 0.875$, d.f. = 1, 16). The unweighted marginal mean of clade 1 was 30.2 (95% CI: 19.5 – 40.8) and that of clade 8 was 29.0 (95% CI: 17.5 – 40.5). Figure 3.2 is a boxplot that displays the background tissue metal (nmol/g dry weight) of both clades.

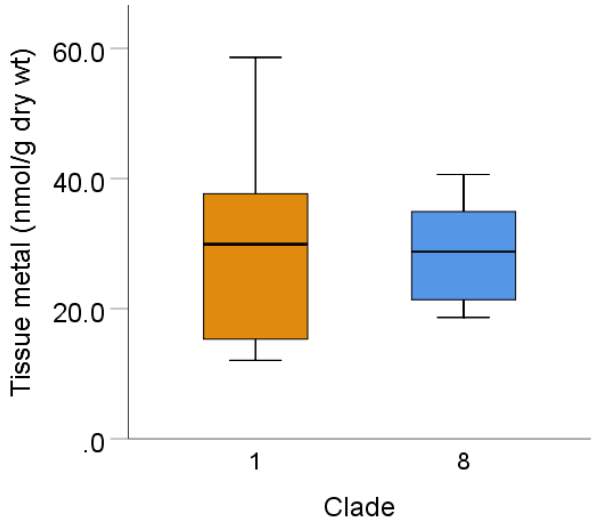

Figure 3.2: Boxplot of background cadmium measured in tissues (nmol/g dry weight) of clades 1 and 8 measured at the end of seven-day short-term toxicity experiments

The mean tissue metal (nmol/g dry wt) of clade 1 was 30.2 (95% CI: 19.5 – 40.8) and that of clade 8 was 29.0 (95% CI: 17.5 – 40.5). An analysis of main effects determined that the clades' background tissue metal was not significantly different (F = 0.026, $p = 0.875$, d.f. = 1, 16).

To model metal accumulated in the tissues over the 7-day exposure period, data were logarithmically transformed and fitted to the bioaccumulation equation of the saturation

model. The model was fitted on the basis of nominal cadmium and dissolved cadmium, independently. Data were modeled in SYSTAT 13 and the "Funpar" command was used to generate 95% confidence intervals for each parameter (*max*, *K*, and C_{BK}), all of which are displayed in Table 3.1. The background term C_{BK} was estimated by the model. The 95% confidence intervals of the estimated C_{BK} of each clade overlapped with those of the calculated background tissue concentrations. The tissue metal concentration increased with increasing metal water concentration. The Michaelis-Menten curve fit the clade 1 data with R-square values of 0.95 and 0.93, for the nominal and dissolved metal, respectively; the R-squared values for the clade 8 data fit were 0.95 and 0.94, repesctively. Based on overlapping confidence intervals, the modeled maximum body concentration, half-saturation constant, and background tissue concentration of both clades were not significantly different, whether the measurement was based on dissolved metal concentration or nominal concentration.

Table 3.1: Fitted parameters for modeled bioaccumulation of cadmium by clades 1 and 8 in seven-day short-term toxicity experiments

Cadmium estimate	Clade	*max* (nmol/g dry wt)	95% CI	*K* (nM)	95% CI	C_{BK} (nmol/g dry wt)	95% CI	R-square	RMSE
Nominal Cd	1	35600	(3.48, 3.64+E08)	11500	(0.0895, 1.49E+09)	26.6	(19.4, 36.4)	0.95	0.1126
Dissolved Cd	1	137	(29.4, 638)	8.47	(1.60, 44.9)	26.8	(18.7, 38.4)	0.93	0.1291
Nominal Cd	8	1.58E+07	(0.0397, 6.31E+15)	4.20E+06	(2.90E+04, 6.03E+16)	26.3	(18.5, 37.3)	0.95	0.1263
Dissolved Cd	8	885	(66.8, 11700)	33.3	(2.14, 516)	26.9	(18.4, 39.4)	0.94	0.1381

Logarithmically-transformed data were fitted to the bioaccumulation model. The table displays the back-transformed parameters and 95% CI for maximum tissue metal accumulated (*max)*, half saturation constant (*K*) and background tissue metal concentration (C_{BK}). Measures of model fit (R-square and RMSE) are also included in the table.

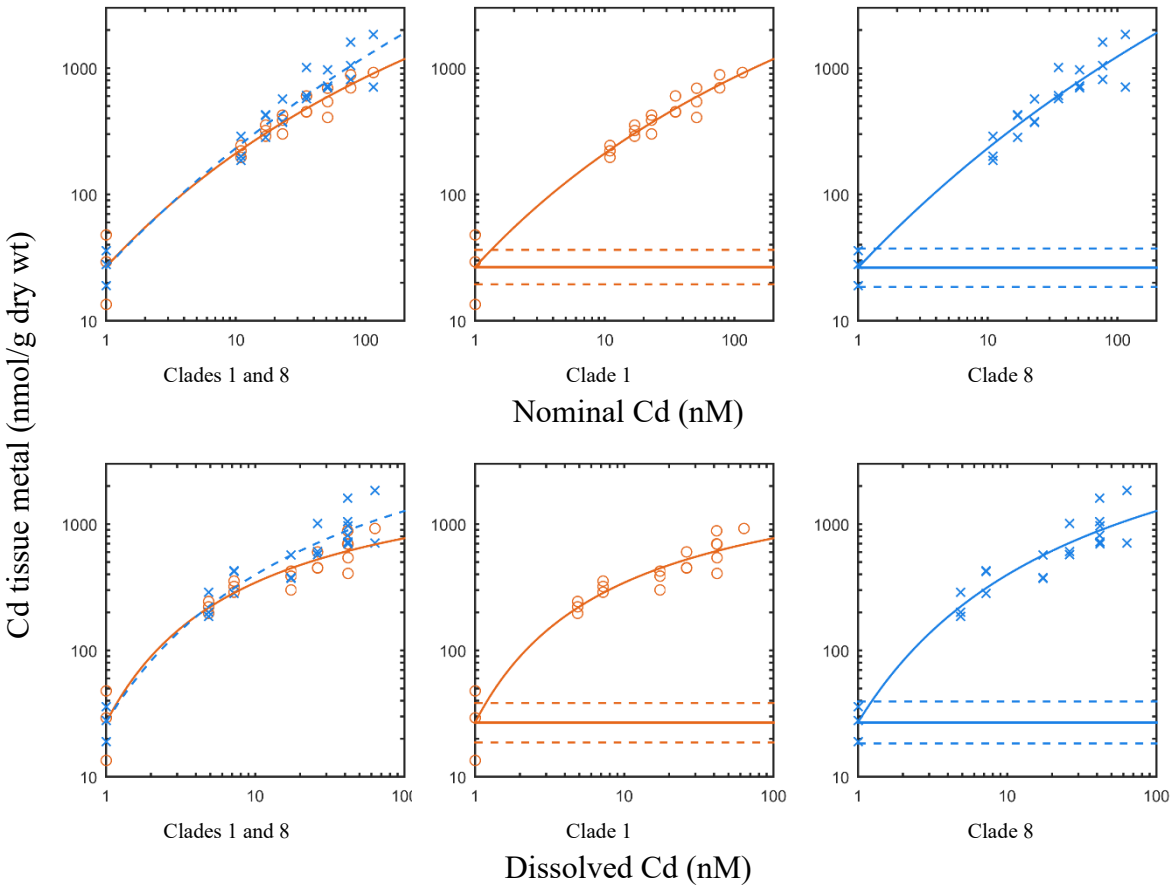

Figure 3.3: The relationship between tissue metal and nominal or dissolved cadmium

Untransformed data and the fitted saturation model for bioaccumulation for clades 1 (○, orange) and 8 (x, dashed line) are displayed in the left panels. The center panels contain clade 1 data with the addition of the model-estimated C_{BK} plotted as a line (slope = 0) with its 95% CI (dashed lines). The right panels display clade 8 bioaccumulation model and data with model-estimated C_{BK} and its 95% CI.

3.1.1.3 Mortality

The mean control survival of clade 1 across all short-term experiments was 0.963 (95% CI: 0.944 – 0.982); the mean control survival for clade 8 was 0.926 (95% CI: 0.873 – 0.9787). To determine whether the mean control survivals of clades 1 and 8 were significantly different, an independent-samples *t*-test was performed. The assumption of normality was satisfied for both species (Shapiro-Wilk; test statistic, $p > 0.05$). However, equal variances could not be assumed (Levene's test; $p = 0.045$). The independent-samples *t*-test did not detect a significant difference between the mean control survivals of clades 1 and 8 in the short-term exposure experiments (t = 1.705, $p = 0.137$, d.f. = 6.293). Figure 3.4 displays the short-term mean control survivals for both clades.

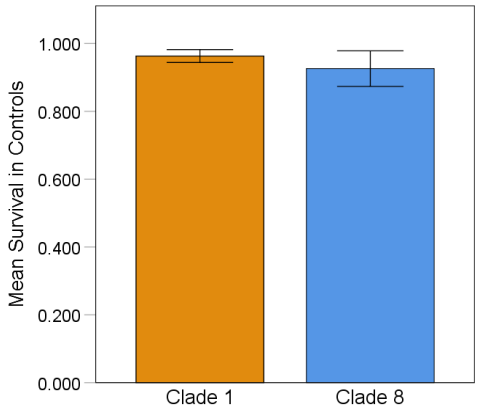

Figure 3.4: Mean survival proportions of clades 1 and 8 controls in short-term toxicity experiments

The controls of all short-term experiments (cadmium and potassium permanganate) were compared for survival. The error bars represent the 95% CI of the mean control survivals. The clade 1 controls exhibited a mean survival of 0.962 (95% CI: 0.944 – 0.982) and the survival rate of clade 8 was 0.926 (95% CI: 0.873 – 0.9787). A significant difference between the control survivals was not detected (independent samples *t*-test; t = 1.705, $p = 0.137$, d.f. = 6.293).

Table 3.2: Clade 1 and 8 mortality rates in short-term toxicity experiments modeled as functions of nominal, dissolved, and tissue cadmium concentrations

Cadmium estimate	Clade	*m'*	95% CI	K''_W	95% CI	LC50 (nM)	95% CI	LC25 (nM)	95% CI	R-square	RMSE[1]
Nominal Cd	1	0.0555	(0.0206, 0.0903)	1.61	(1.34, 1.88)	61.0	(52.5, 69.7)	44.2	(35.8, 52.7)	0.89	0.1095
Dissolved Cd	1	0.0599	(0.0275, 0.0923)	1.79	(1.41, 2.16)	40.8	(36.9, 44.8)	32.5	(28.0, 37.0)	0.89	0.1049
Nominal Cd	8	0.0451	(0.0133, 0.0769)	1.53	(1.33, 1.73)	55.8	(48.0, 63.6)	39.7	(32.4, 47.0)	0.90	0.1049
Dissolved Cd	8	0.0509	(0.0172, 0.0846)	1.60	(1.31, 1.89)	37.9	(33.4, 42.4)	29.2	(24.3, 34.1)	0.88	0.1183
				K''_{TBX}		LBC50 (nmol/g dry wt)		LBC25 (nmol/g dry wt)			
Tissue metal	1	0.0571	(-0.0118, 0.126)	6.02	(-3.53, 15.6)	733	(588, 878)	564	(427, 702)	0.60	0.1643
Tissue metal	8	0.0347	(-0.00724, 0.0766)	3.95	(1.70, 6.20)	898	(755, 1040)	639	(508, 771)	0.80	0.1378

Logarithmically-transformed data were fitted to the mortality models. The table displays the back-transformed parameters and 95% CI fitted parameters and the 95% CI for control mortality (*m'*), half saturation constant (K''_W or K''_{TBX}), and mortality parameters (LC50 and LC25 or LBC50 and LC25). Measures of model fit (R-square and RMSE) are also included in the table. The parameters n_w and n_b were set to 100 in all cases and are not included in the table.

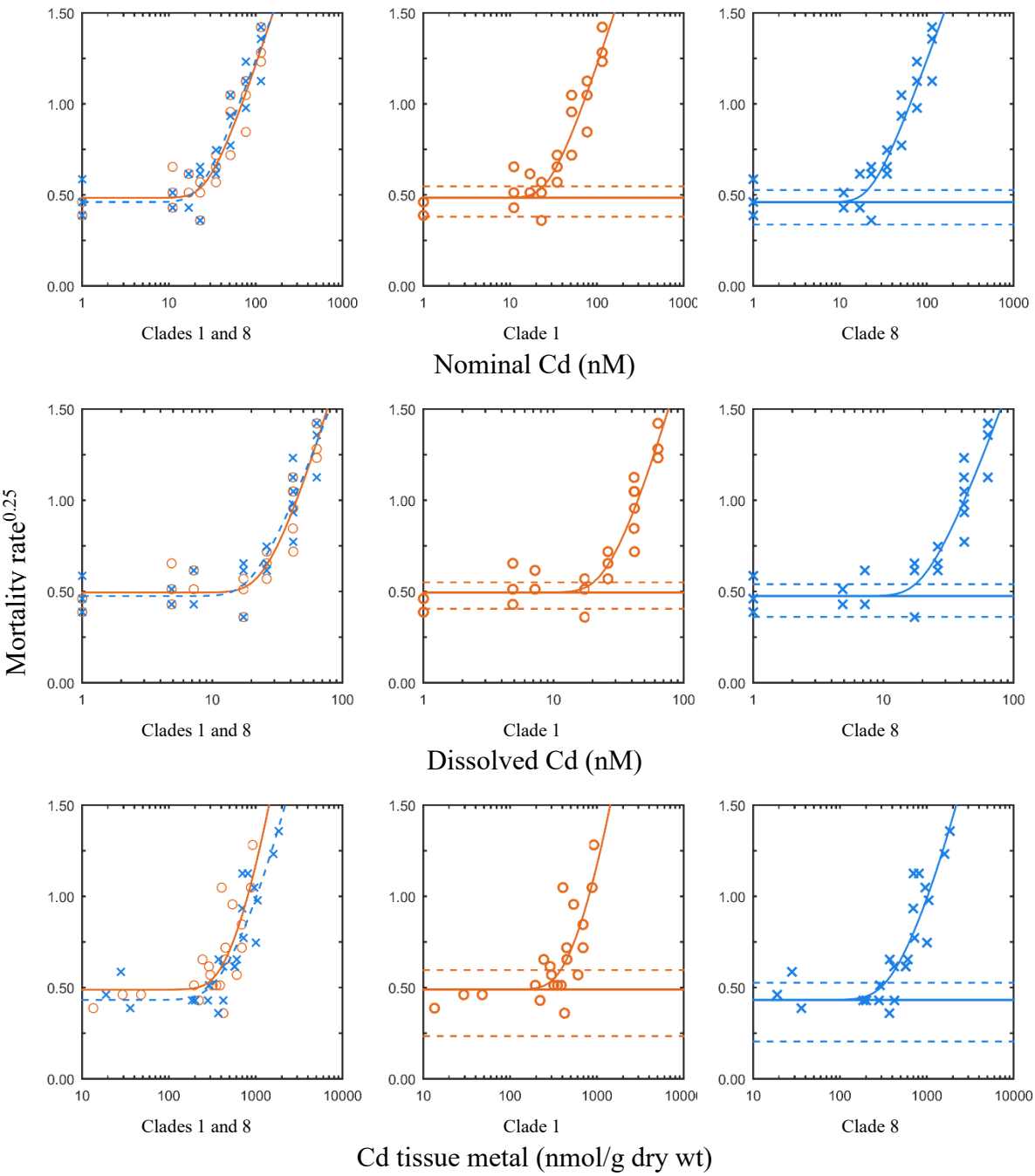

Figure 3.5: The relationship between clade mortality and nominal, dissolved, or tissue cadmium concentrations

Untransformed data and the fitted mortality model for clades 1 (○, orange, solid line) and 8 (x, blue, dashed line) are displayed in the left panels. The center panels show clade 1 data with the addition of the model-estimated control mortality rate (*m'*) plotted as a horizontal line (slope = 0) with its 95% CI (dashed lines), and the right panels displays that of clade 8. The fitted model parameters are displayed in Table 3.2.

The data from the experiments were pooled within each clade because the 95% confidence intervals of the LC50 and LC25 or LBC50 and LC25 point estimates overlapped. The parameters n_b and n_w were set to 100 in all cases. Mortality rates for both clades increased similarly with elevated cadmium concentrations, in both the water and tissue (Figure 3.5). The models fit the data well, with all water concentration-based modelling resulting in R-square values between 0.88 and 0.90 (Table 3.2). The model fits for tissue metal as the predictor variable resulted in an R-square value of 0.60 for clade 1 and 0.80 for clade 8 (Table 3.2).

The model-estimated control mortalities for clade 1 in the nominal, dissolved, and tissue models were 0.0555 (95% CI: 0.0206 – 0.0903), 0.0599 (95% CI: 0.0275 – 0.0923), and 0.0571 (95% CI: -0.0118 – 0.126), respectively. Those for clade 8 were 0.0451 (95% CI: 0.0133 – 0.0769), 0.0509 (95% CI: 0.0172 – 0.0846), and 0.0347 (95% CI: -0.00724 – 0.0766). The 95% confidence interval of all estimated parameters for clades 1 and 8 overlapped within each predictor variable (nominal, dissolved cadmium, or tissue metal). The control mortalities and the respective confidence intervals were plotted in the appropriate panels in Figure 3.5 (horizontal solid and dashed lines). The model-estimated control mortality rates were not significantly different from those that were measured: clade 1 was 0.0379 (95% CI: 0.0180 – 0.0578) and clade 8 was 0.0782 (95% CI: 0.0204 – 0.136).

Within each predictor variable no significant difference was detected among the parameters between clades (control mortality, half-saturation constant, LC50 or LBC50, and LC25 or LBC25), based on overlapping 95% confidence intervals (Table 3.2). The LC50 and LC25 values on the basis of nominal concentration were 61.0 nM (95% CI: 52.5 – 69.7) and 44.2 nM (95% CI: 35.8 – 52.7) for clade 1, and 55.8 nM (95% CI: 48.0 – 63.6) and 39.7

nM (95% CI: 34.2 – 47.0) for clade 8. The dissolved LC50 and LC25 values were 40.8 nM (95% CI: 36.9 – 44.8) and 32.5 nM (95% CI: 28.0 – 37.0) for clade 1, and 37.9 nM (95% CI: 33.4 – 42.4) and 29.2 nM (95% CI: 24.3 – 34.1) for clade 8. Finally, on the basis of tissue metal, LBC50 and LBC25 values were 733 nmol/g (95% CI: 588 – 878) and 564 nmol/g (95% CI: 427 – 702) for clade 1, and 898 nmol/g (95% CI: 755 – 1040) and 639 nmol/g (95% CI: 508 – 771) for clade 8. Above the background body concentrations of tissue metal, the model estimated that mortality began increasing even at the lowest detections of bioaccumulated cadmium.

3.1.1.4 Growth inhibition

Control data were pooled within each clade from all short-term experiments (cadmium and potassium permanganate) to examine control final body sizes. In the pooled control analysis (Figure 3.6), one outlier was removed from clade 1 control growth ($KMnO_4$, experiment D, rep 2) prior to analysis because it was determined to be an extreme outlier by analysis of a boxplot as the value was greater than 3 box lengths from the edge of the box. The mean dry weight per individual for clade 1 was 0.0341 mg (95% CI: 0.0299 – 0.0383) and that for clade 8 was 0.0310 mg (95% CI: 0.0277 – 0.0342). To determine whether clade 1 and 8 control final weights were statistically different, a pooled independent-samples *t*-test was performed. The data satisfied the assumption for normal distributions for both clade 1 and clade 8 (Shapiro-Wilk; $p < 0.05$). The assumption of equal variances was also satisfied (Levene's test; $p = 0.198$). The pooled independent-samples *t*-test determined that there was no significant difference between the two clades ($t = 1.269$, $p = 0.213$, d.f. = 1, 33).

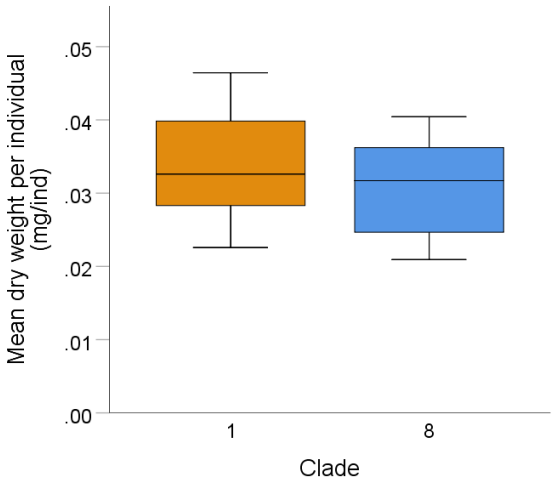

Figure 3.6: Mean dry weights per individual of clade 1 and 8 controls in short-term experiments

The mean dry weights per individual of clade 1 and 8 controls in short-term are displayed. An outlier was removed prior to plotting (Clade 1, $KMnO_4$, experiment D, rep 2, value = 0.0787). A significant difference between the control dry weights was not detected (pooled independent samples *t*-test; t = 1.269, p = 0.213, d.f. = 1, 33).

Final dry weight data were fitted to the growth inhibition model on the basis of nominal, dissolved, and tissue metal concentrations. All water concentration-based model fits resulted in R-square values between 0.27 and 0.31. Tissue metal-based R-squared values were 0.19 for clade 1 data and 0.46 for clade 8 data. All fitted parameters and their 95% confidence intervals are displayed in Table 3.3. The data and models are displayed in Figure 3.7.

The estimated clade 1 control dry weight (*W'*) was 0.0318 mg/individual (95% CI: 0.0261 – 0.0376) for nominal and 0.0310 mg/individual (95% CI: 0.0267 – 0.0352) for dissolved models; the estimated dry weight of clade 8 was 0.0305 mg/individual (95% CI: 0.0234 – 0.0376) for nominal and 0.0303 (95% CI: 0.0240 – 0.0366) for dissolved models.

The tissue metal model-estimated control dry weight was 0.0366 mg/individual (95% CI: 0.00841 – 0.0649) for clade 1 and 0.0311 mg/individual (95% CI: 0.0254 – 0.0367) for clade 8. All model estimates, within and between clades, were not significantly different, based on overlapping 95% confidence intervals, nor were they significantly different from the measured mean dry weight (mg/individual), (Figure 3.6).

The IC25 and IBC25 values were estimated by the model, with an additional "Funpar" command in SYSTAT 13. Clade 1 and 8 IC25 values are not significantly different; however, only one is significantly different from zero (does not include zero in the 95% confidence interval), which was the clade 1 IC25 value of 54.5 nM (95% CI: 16.3 – 92.6) estimated from dissolved data. The IBC25 value estimated for clade 1 was 601 nmol/g (95% CI: -5180 – 6380), and that for clade 8 was 813 nmol/g (95% CI: 228 – 1400). The clade 1 tissue metal-based model regression limited by the comparatively large confidence interval of the estimated control weight (0.00814 – 0.0649). The clade point estimates for IC25 and IBC25 values are not significantly different between those of clade 1 and clade 8.

Table 3.3: Modeled clade 1 and 8 growth inhibition in short-term toxicity experiments modeled as functions of nominal, dissolved, and tissue cadmium concentrations

Cadmium estimate	Clade	*W'*	95% CI	*a*	95% CI	*n*	95% CI	IC25 (nM)	95% CI	R-square	RMSE
Nominal Cd	1	0.0318	(0.0261, 0.0376)	0.0260	(-0.0877, 0.140)	3.99	(-2.05, 10.0)	78.6	(-3.26, 160)	0.31	0.000
Dissolved Cd	1	0.0310	(0.0267, 0.0352)	0.0198	(-0.0797, 0.119)	5.12	(-3.90, 14.1)	54.5	(16.3, 92.6)	0.27	0.000
Nominal Cd	8	0.0305	(0.0234, 0.0376)	0.0481	(-0.138, 0.234)	3.39	(-1.87, 8.65)	58.8	(-25.4, 143)	0.27	0.000
Dissolved Cd	8	0.0303	(0.0240, 0.0366)	0.0807	(-0.193, 0.355)	3.09	(-2.72, 8.90)	38.2	(-10.1, 86.5)	0.29	0.000
							IBC25 (nmol/g dry weight)				
Tissue metal	1	0.0366	(0.00841, 0.0649)	0.0434	(-0.469, 0.556)	2.00	(-6.83, 10.8)	601	(-5180, 6380)	0.19	0.000
Tissue metal	8	0.0311	(0.0254, 0.0367)	1.45E-05	(-1.33E-04, 1.62E-04)	9.41	(0.579, 18.2)	813	(228, 1400)	0.46	0.000

Logarithmically-transformed data were fitted to the growth inhibition models. The table displays the back-transformed parameters and 95% CI for control dry weight (*W')*, empirical fitting parameters *a*, and *n,* and the point inhibitive concentration point estimates (IC25 and IBC25). Measures of model fit (R-square and RMSE) are also included in the table.

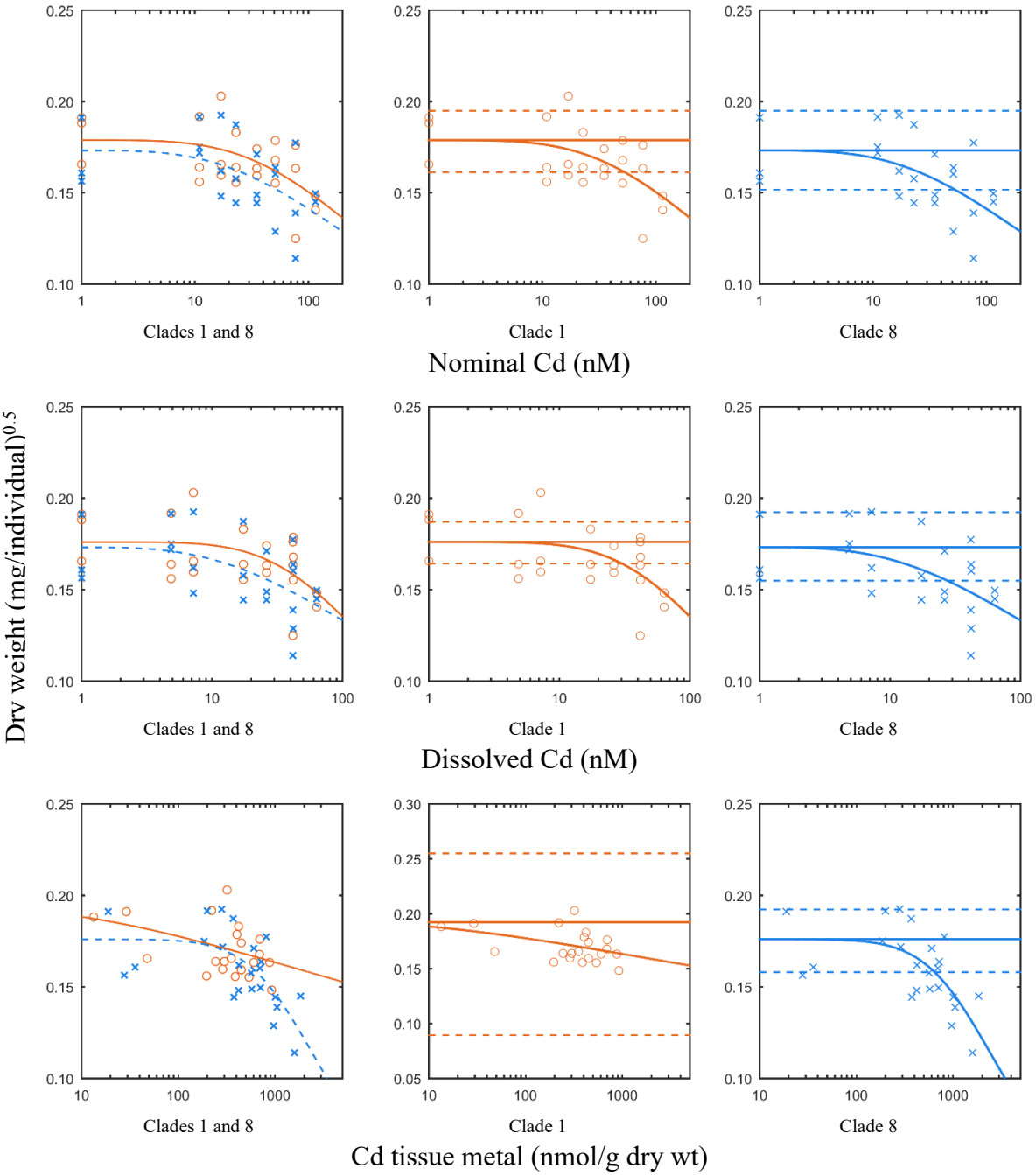

Figure 3.7: The relationship between clade 1 and 8 final mean dry weight and nominal, dissolved, and tissue cadmium concentrations

Untransformed data and the fitted growth inhibition models for clades 1 (○, orange, solid line) and 8 (x, blue, dashed line) are displayed in the left panels. The center panels show clade 1 data with the addition of the model-estimated control dry weight (*W'*) plotted as a horizontal line (slope = 0) with its 95% CI (dashed lines), and the right panels display those of clade 8. Controls in the nominal figures were plotted at x = 1.

3.1.2 Uptake and depuration experiments

All data were pooled from the two cadmium uptake and depuration experiments. A two-way ANOVA was performed on water samples to determine whether there was a significant difference between dissolved and total treatments at the beginning and end of each exposure renewal period. The measured concentrations were logarithmically transformed, but the two-way ANOVA was limited because all but one cell of data was normally distributed (Shapiro-Wilk; $p > 0.05$), nor was there homogeneity of variance (Levene's test; $p < 0.05$). There was a significant interaction between filtering and time from beginning to end of the renewal (2-way ANOVA; $F = 25.117$, $p < 0.0005$, d.f. = 1, 139). An analysis of simple main effects determined that there was a significant difference between the beginning and end of a renewal period for dissolved samples ($F = 66.186$, $p < 0.0005$, d.f. = 1, 139). However, no significant reduction in cadmium concentration was detected from the beginning to end of renewal periods in total fraction samples ($F = 0.947$, $p = 0.335$, d.f. = 1, 139) and therefore mean concentration was used. There was no significant difference between dissolved and total samples at the beginning of renewal periods ($F = 0.969$, $p = 0.329$, d.f. = 1, 139), but there was at the end ($F = 66.377$, $p < 0.0005$, d.f. = 1, 139); however, mean values of day 0 and day 7 were used. Table 3.4 displays the water sample data for control and exposure data in the uptake and depuration experiments.

Over the 14-day uptake portion of the experiment, the tissue metal concentration of both clades significantly increased. The uptake model was fitted to the data, and the exposure concentration was set to the average dissolved concentration: 14.5 nM. The uptake model fitted clade 1 and 8 both with an R-square value of 0.94. The depuration model fit clade 1 and 8 with R-square values of 0.58 and 0.70, respectively (Table 3.6).

Mean body concentration increased over the duration of the uptake experiment, but appeared to have begun to approach steady state by the 14th day in both clades (Figure 3.8, top row). The model estimated that the body concentration at steady state (C_{SS}) was 559 nmol/g (95% CI: 270 – 849) for clade 1 and 489 nmol/g (95% CI: 314 – 664) for clade 8. The estimated C_{SS} values were not significantly different between clades (overlapping 95% confidence intervals), (Table 3.6).

Growth, calculated as the slope of a linear regression of mean logarithmically-transformed dry weight over time, was not significantly different between the two clades (Table 3.4). As the growth constants, g, were much smaller than the estimates of the elimination constants, k_e, growth was not incorporated into the uptake and depuration models. For both clades, the k_e value estimated for the uptake experiments were about twice what was estimated for the depuration portion; however, none were significantly different from the others based on overlapping 95% confidence intervals.

Data at the end of the uptake portion and beginning of the depuration portion were comparable with the initial estimated depuration body concentration, C_{b0}, and were not significantly different between clades. The estimated C_{b0} for clade 1 was 435 nmol/g (95% CI: 384 – 486) and that for clade 8 was 420 nmol/g (95% CI: 379 – 461). The mean clade 1 body concentration at the beginning of the depuration experiment was 445 nmol/g, and that of clade 8 was 477 nmol/g (Table 3.6).

The half-lives ($t_{0.5}$) calculated in the depuration experiment were 13.6 days (95% CI: 9.53 – 17.7) and 12.2 days (95% CI: 9.45 – 15.0), and were not significantly different by clade (Table 3.6). By the end of the 14-day depuration experiment, both clades continued to

have elevated tissue metal concentrations. The mean measured control concentrations over all experimental, uptake and depuration, time points were 1.71 nmol/g (95% CI: 1.52 – 1.94) and 1.70 nmol/g (95% CI: 1.46 – 1.98) for clade 1 and clade 8, respectively (Table 3.6). The tissue metal concentration at the final depuration time point for clade 1 exposure was 190 nmol/g (95% CI: 150 – 241), and that of clade 8 was 182 nmol/g (95% CI: 161 – 208), (Table 3.6).

Table 3.4: Measured dissolved cadmium concentrations in water samples from the uptake and depuration experiments

	Control	95% CI	Exposure, uptake	95% CI	Exposure, depuration	95% CI
Dissolved	0.0212	(-0.677, 0.720)	14.6	(11.3, 18.0)	1.75	(-1.42, 4.92)
Total	-0.219	(-0.480, 0.0428)	24.9	(23.6, 26.3)	0.197	(-0.257, 0.651)

Total and dissolved cadmium concentrations in the uptake and depuration experiments with 95% confidence intervals. All concentrations are in nM. Control data were pooled over the entire experiment.

Table 3.5: Growth rates of clades 1 and 8 in cadmium uptake and depuration experiments

Experiment	Clade	g	95% CI	W_0	95% CI	r^2	RMSE
Uptake and Depuration, Control and Exposure	1	0.00473	(0.00172, 0.00775)	0.402	(0.360, 0.445)	0.07	0.1472
	8	0.00844	(0.00673, 0.0102)	0.255	(0.234, 0.276)	0.43	0.1082

The growth rate (mg/day) of each clade was estimated from dry weight data combined from the uptake and depuration experiments because all 95% confidence intervals of growth rate overlapped. Growth was estimated with a linear regression of log-transformed data.

Table 3.6: Model-estimated parameters of clades 1 and 8 two-week uptake and two-week depuration of manganese

Experiment	Clade	k_u	95% CI	k_e	95% CI	C_{SS}	95% CI	BCF	95% CI	R-square	RMSE
Uptake	1	4.09	(3.22, 4.96)	0.107	(0.0358, 0.177)	559	(270, 849)	38400	(18500, 58300)	0.94	0.1738
	8	5.44	(4.27, 6.62)	0.162	(0.0809, 0.243)	489	(314, 664)	33600	(21600, 45700)	0.94	0.1728
		C_{b0}	95% CI	k_e	95% CI	$t_{0.5}$	95% CI	-	-	R-square	RMSE
Depuration	1	435	(384, 486)	0.0510	(0.0357, 0.0662)	13.6	(9.53, 17.7)	-	-	0.58	0.09678
	8	420	(379, 461)	0.0570	(0.0438, 0.0696)	12.2	(9.45, 15.0)	-	-	0.70	0.08312

Body concentration data were logarithmically transformed prior to model fitting. The table displays the back-transformed parameters and the 95% CI for cadmium uptake rate constant (k_u, day^{-1}), depuration constant from the uptake and depuration experiments, separately (k_e, day^{-1}), body concentration at steady state ($C_{SS,}$ nmol/g dry weight), bioconcentration factor (BCF, calculated as k_u over k_e and multiplied by 1000), body concentration at the beginning of the depuration experiment (C_{b0}, nmol/g dry weight), and cadmium half-life in days ($t_{0.5}$, days). Measures of model fit (R-square and RMSE) are also included in the table. The model was fitted with dissolved cadmium concentration as the exposure concentration. The background concentration (C_{BK}) was set to zero

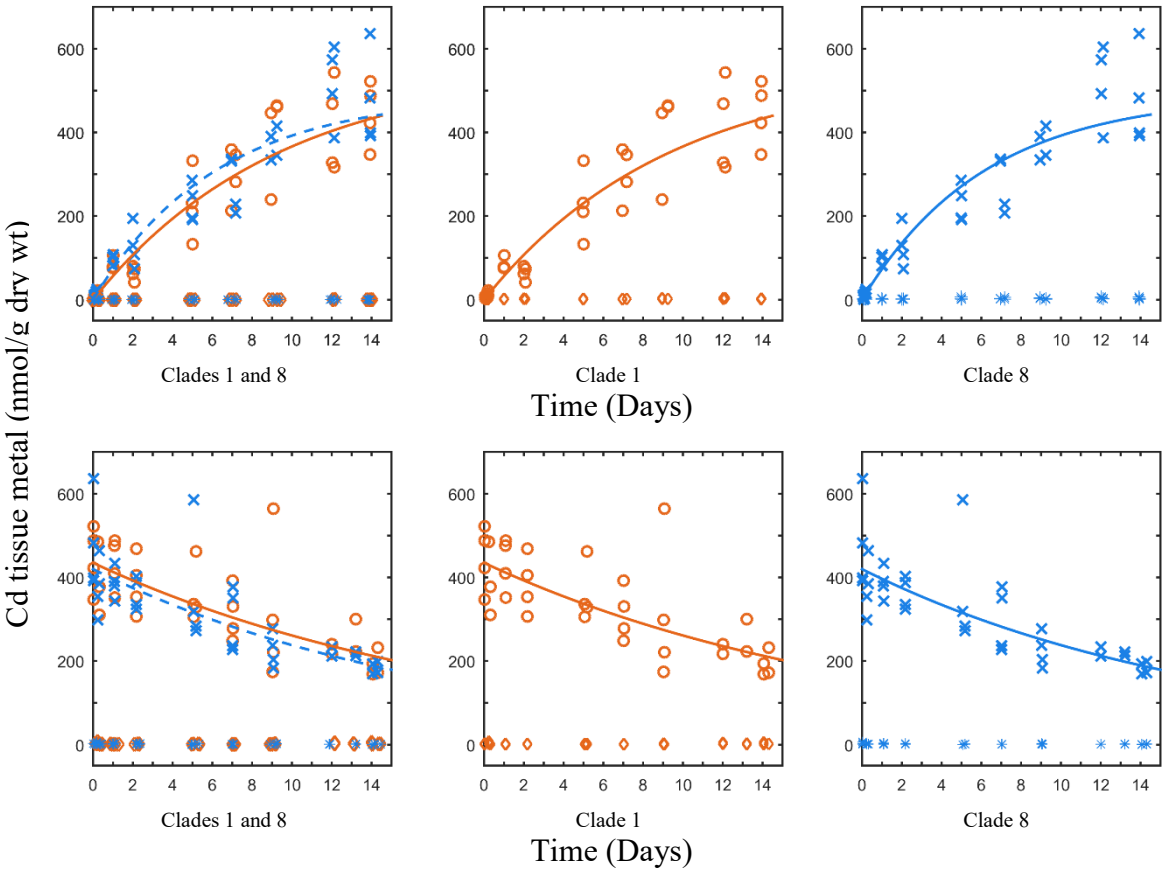

Figure 3.8: The relationship between clade 1 and 8 tissue metal bioaccumulated from a single exposure concentration (top row) and depurated (bottom row) over time

Untransformed data and the fitted uptake and depuration models for clades 1 (orange, exposure: ○; control: ◊) and 8 (orange, exposure: x; control: □) are displayed in the left panels. The centre panels show clade 1 data and the right panels display that of clade 8. The model was fitted with dissolved cadmium concentration as the exposure concentration.

3.2 Discussion

Mortality is the most sensitive endpoint in *H. azteca* toxicity testing (EC, 2013). The saturation-based mortality model determined that the nominal LC50 and LC25 are 1.09- and 1.11-fold greater for clade 1 in comparison to clade 8. The dissolved LC50 and LC25 values are, similarly, 1.08- and 1.14-fold greater for clade 1 than clade 8. The small relative difference in lethal tolerance to cadmium between clades was not statistically significant, based on overlapping 95% confidence intervals.

The free metal ion, Cd^{2+}, is the most bioavailable (CCME, 2014). The speciation of cadmium in freshwater is variable, though dissolved it may occur as a hydrated ion, salt, or be complexed with organic or inorganic ligands (CCME, 2014). Abiotic conditions, including pH, alkalinity, hardness, temperature, and organic content, determine the fate of cadmium (CCME, 2014). Bioaccumulated cadmium increased with increasing exposure concentration in both clades. Clade 8 tissue concentrations were greater than clade 1 at the higher exposure concentrations. At the highest exposure level, the mean tissue concentration was 1280 nmol/g in clade 8 and 926 nmol/g in clade 1. The *max* value was 6.50-fold greater for clade 8 than clade 1, at 885 nmol/g and 137 nmol/, respectively. Both clades did reach their respective *max* values in this experiment, and many tissue concentrations measured were greater than *max*, which is reflective of the wide 95% confidence intervals.

The confidence intervals on the estimated parameters were wide. Some of the uncertainty in the model may be attributed to the lack of data between the controls and first exposure level, which was nominally 10 nM. Four-week cadmium bioaccumulation in *H. azteca*, clade 1 (Major *et al*., 2013), was measured and fitted to the saturation model

(Borgmann *et* al., 1991; Borgmann *et al*., 2004). The *max*/*K* ratio was 222 L/g (95% CI: 169 – 292), which is proportional to uptake at low cadmium concentrations (Borgmann *et al*., 2004). However, improvements in analytical methods since the original analysis was conducted in 1991 (Borgmann *et al*., 1991) may have partially contributed to an overestimate of cadmium bioavailability at low concentrations (Borgmann *et al*., 2010). In the nominal and measured regressions respectively, the ratios of *max*/*K* for clade 1 were 16.2 and 3.10 L/g, and those for clade 8 were 26.6 and 3.76 L/g. However, the lowest nominal exposure concentration in the present study was 10 nM, in contrast to that analyzed by Borgmann *et al*. (2004), which included four nominal exposure concentrations below 10 nM (1.6, 2.8, 5.0, and 8.9 nM), (Borgmann *et al*., 1991).

The discrepancy between the nominal and dissolved *max* terms likely results from the relative distance between the greatest exposure concentrations in the two series; it cannot simply be explained by the x-axis shift. The upper portion of the slope in the dissolved regressions were stretched along the x-axis, and predicted that body concentrations were approaching the maximum. In contrast, the slope at the higher concentrations in the nominal regressions was not stretched to the same effect. The estimated four-week *max* term was 512 nmol/g (95% CI: 376 – 698) in tap water (Lake Ontario water, on which the SAM-5S media is based, (Borgmann, 1996). Bioaccumulation does not need to reach a maximum if 100% mortality occurs at tissue concentrations lower than *max*. However, using a modified biotic ligand model, Borgmann *et al.* (2010) determined a 7-day max term of 1429 nmol/g (95% CL: 884 – 1973) with cadmium activity (Cd^{2+}). Of all competitive ions, calcium has the

greatest impact on cadmium uptake and has been demonstrated to impact *H. azteca* acclimation, and therefore the *max* term (Borgmann *et al*., 2010).

In the waterborne regressions, clade 1 appeared slightly more tolerant than clade 8. In contrast, clade 8 was slightly more tolerant than clade 1 based on tissue concentrations. The clade 8 LBC50 and LBC25 values were, respectively, 1.13- and 1.23-fold greater than that of clade 1. However, tissue-based mortality of cadmium, expressed as endpoints LBC50 and LBC25 were also not significantly different between clades, based on overlapping 95% confidence intervals. Tissue cadmium concentration is more consistent for predicting survival, due primarily to negating the presence of complexing factors (Borgmann *et al*., 1991). Borgmann *et al*. (2010) measured a range of LBC50 values from 609 – 695 nmol/g for clade 1 (clade was later determined by Major *et al*., 2013), which is within the range of the 95% confidence interval of the LBC50 value for clade 1 in this study, but not that estimated for clade 8. The relative difference of clade tolerance to waterborne and tissue cadmium may be due to internal amelioration of effects through metal-binding proteins called metallothioneins (MT) which are also induced by the presence of cadmium and other metals (Wright & Welbourn, 1994). Poynton *et* al. (2018) identified MT genes in clade 8, and suspected that recent gene duplications had occurred in the genome. In the same study, 116 genes were upregulated compared to controls following exposure to cadmium. Significantly upregulated genes included heat shock proteins for general stress response, genes involved in oxidative stress response, MT genes (15-fold upregulation), and genes involved in the cuticle. Although no similar studies have been conducted with clade 1, MT genes have been well documented across the animal kingdom, including invertebrates

(Couillard *et al*., 1993, 1995a, b; Perceval *et al*., 2002). In a study with the South American *Hyalella curvispina*, Giusto *et al*. (2012) observed that significant mortality increased in a ten-day exposure at tissue concentrations greater than 1957 nmol/g, which they used as an approximate LBC50. The study used juveniles from lab cultures (3-4.5 mm in length), but the age was not specified. This LBC50 is over 2-fold greater than the LBC50 estimates in this study.

The regressions of clades 1 and 8 are very similar. The IC25 values were also similar between clades, but these values were not very precise, based on large 95% confidence intervals. In addition, the IC25 estimates are greater than the LC50 values calculated for the respective clades for both waterborne estimates. The clade 1 dissolved IC25 was 54.5 nM (95% CI: 16.3 – 92.6) and that for clade 8 was 38.2 nM (95% CI: -10.1 – 86.5). The 95% confidence intervals are respectively 140% and 250% of the estimates and the R-square values for growth inhibition fitted against waterborne cadmium indicate a lower fit than the mortality models, as values range between 0.27 and 0.31. In contrast, the 95% confidence interval ranges for dissolved LC50 values are 19% and 24% of the clade 1 and 8 parameter estimates. Growth inhibition as an endpoint in *H. azteca* is less sensitive than mortality (EC, 2013). Borgmann *et al*. (2010) observed a reduction in growth following a seven-day exposure to waterborne cadmium in a low hardness medium. The relatively lower concentration of competitive calcium ions in the low hardness media may have resulted in greater effects. In this study, the highest exposure levels have the least number of amphipods in each measurement due to mortality. Therefore, the uncertainty also increases with the increasing concentrations.

The tissue metal-based IBC25 values were not estimated at concentrations greater than the LBC50s. In clade 8, dry weight rapidly declines above tissue concentrations of 300 nmol/g. Clade 1, in contrast, does not have data points beyond 920 nmol/g and the regression does not dramatically decline in the same fashion as clade 8, though there is a decrease in dry weight similar to clade 8 up to 920 nmol/g. Therefore, the clade 1 curve cannot be considered valid beyond this point due to the nature of the relationship and the limiting (mortal) factor.

The measured background concentrations of tissue cadmium concentration were not statistically significantly different between the two clades: mean tissue cadmium of clade 1 was 30.2 nmol/g, and that of clade 8 was 29.0 nmol/g. The model-estimated background terms (C_{BK}) were also not significantly different between clades or from the measured mean background concentrations. Model fitting resulted in parameters that were not significantly different between clades, for both nominal and measured cadmium input arguments. However, the 95% confidence limits were wide, particularly for the nominal concentration-based estimates. The inclusion of a non-zero background term (C_{BK}) was reflective of background contamination in the cryovials used in the toxicity digest assay.

The uptake and depuration experiments were each two-week portions of a four-week experiment. The first two-weeks of exposure to waterborne cadmium resulted in increased tissue cadmium concentrations for both clades. The control values varied little throughout the experiment, and were significantly different from the exposure values. All estimated parameters from uptake and depuration modeling were not significantly different between clades (based on overlapping 95% confidence intervals), with the exception of the fitted

parameter for mean starting weight (W_0) in the linear regression for growth rate. The clade 1 W_0 was 0.402 mg (95% CI: 0.360 – 0.445) and that of clade 8 was 0.255 mg (95% CI: 0.234 – 0.276). Higher mortality rates in clade 1 culture containers (amphipods were maintained in culture containers for approximately four weeks prior to starting tests) may have contributed to the observation of greater starting weights, because relatively more resources would have been available per individual during the four-week growth period. The growth rate for clade 8 was greater than that of clade 1, though neither were included in the regression. Integration of the same equation from MacLean *et al*. (1996) with the growth term results in an equation that is different only by the original k_e term equal to new k_e minus g (the growth constant). MacLean *et al*. (1996) did not use this model because growth in their experiment was not linear, however in this experiment it could be estimated, though poorly due to high variability, using a constant growth coefficient.

The uptake rate constant, k_u, was 1.33-fold greater for clade 1 than clade 8. The elimination rate constant, k_e, estimated from the uptake data was greater in clade 8 (0.162 day^{-1}) than clade 1 (0.107 day^{-1}). Both clades reached their estimated steady state values within the 14-day uptake period. The one compartment elimination model described the data well, although the measures of model fit were 0.58 and 0.70 for clade 1 and 8, respectively. The 14-day depuration period was not sufficient for tissue concentrations to return to control levels. The half-life of clade 1 is over one day greater than that of clade 8, and both are within two days of the entire depuration period. The elimination rate constants estimated from the depuration data were less than half of those estimated in the uptake period. The

difference between clade 8 elimination constants from the uptake and depuration phases was statistically significant (non-overlapping 95% confidence intervals).

In the saturation bioaccumulation experiment over a range of concentrations, clade 8 was observed to reach higher tissue concentrations than clade 1 at high concentrations. The concentrations reached by each clade after seven days in the uptake experiment were slightly greater than expected based on the short-term bioaccumulation regressions.

Bioaccumulation of cadmium in *Gammarus pulex* was studied by Pellet *et al*. (2009) over seven-day uptake and 21-day depuration phases. Depuration k_e values ranged between 0.03 and 0.035 day^{-1}, but the uptake rate constant is difficult to compare because the estimate is based on labile cadmium. The *H. azteca* estimates of depuration k_e in this study are more similar with each other than with that of *G. pulex.* In five-day uptake and five-day depuration experiments, Shuhaimi-Othman and Pascoe (2007) observed accumulation of cadmium in *H. azteca* (unspecified clade) over the uptake period. However, similar to this study, the elimination period was insufficient for the amphipods to return to background concentrations.

The only significant difference detected between clade 1 and 8 in cadmium the experiments was the initial dry weight in the uptake experiment. Other than this, no significant differences were identified in terms of bioaccumulation over a range of exposure concentrations, or in uptake and elimination rate constants over time-based experiments. The toxicity of cadmium to both clades was similar, with insufficient evidence to determine a significant difference based on water or tissue concentrations. Despite no differences detected in the cadmium experiments, previous work with these clades has indicated that

differences in the tolerance of the clades likely exist, and genetically identifying species is one method for improving reporting in toxicology (Hanson *et al*., 2017).

Chapter 4
Potassium Permanganate and Manganese

4.1 Results

4.1.1 Short-term toxicity experiment

Results from a comparison of pooled short-term control data for clade 1 and 8 control mortalities and final dry weights are presented in the chapter 3 results.

4.1.1.1 Water samples and test media

Spiked test media were translucent purple which increased with increasing potassium permanganate concentration. Green algae, which is apparent in culture and stock *Hyalella* containers, was not visually detected in any test containers throughout the tests. At the end of a test, the two highest exposure levels had a reddish-brown hue.

A two-way ANOVA was conducted to determine whether there was a significant difference the manganese measured in dissolved (filtered) and total (unfiltered) spiked samples. Data were logarithmically transformed, and all data were normally distributed (Shapiro-Wilk; $p > 0.05$), with the exception of total samples at spiked level 16000 nM (Shapiro-Wilk; $p = 0.10$). The assumption for homogeneity of variances was met (Levene's test; $p = 0.077$). There was a statistically significant interaction between nominal concentration and filtering procedure (2-way ANOVA; $F = 2.551$, $p = 0.015$, d.f. = 8, 88). A simple main effects analyses was run which determined there was a statistically significant difference in measured manganese at each level of nominal concentration. Figure 4.1 displays the recovered dissolved and total fractions of manganese.

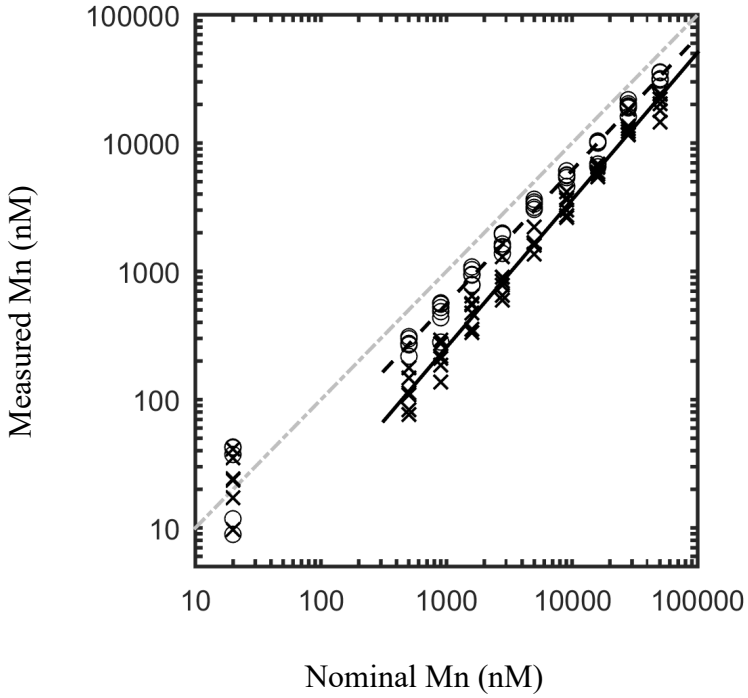

Figure 4.1: GFAAS-measured dissolved and total manganese concentrations in short-term test media as functions of nominal metal concentration in toxicity experiments

Total (○, dashed black line) and dissolved (x, solid black line) measured manganese concentrations are plotted (n = 60, 58). Measured samples from all clade 1 and 8 short-term tests are displayed. Measurements were drift, background, and blank corrected prior to plotting. The light grey line (intermittent dotted and dashed line) is displayed for reference of a 1:1 measured-to-nominal ratio. Logarithmically transformed ($\log(a + min + 1)$) total and dissolved cadmium data were linearly regressed, yielding R-square values of 0.98 and 0.99, respectively. Control values were not included in the regression, but were arbitrarily plotted at x = 20.

4.1.1.2 Bioaccumulation

GFAAS analysis measured the manganese content of clade 1 and 8 digested tissue (nmol/g dry weight). An independent samples *t*-test was run to determine whether there was a significant difference between control-level manganese content of clade 1 and 8 tissue. Data analysis was conducted with logarithmically transformed data. The assumptions of normality

(Shapiro-Wilk; $p > 0.05$) and homogeneity of variances (Levene's test; $p = 0.185$) were met. There was not a statistically significant difference between control-level tissue manganese concentrations (t = 1.413, $p = 0.180$, d.f. = 14). Figure 4.2 is a boxplot that displays the background tissue metal (nmol/g dry weight) of both clades. The mean control concentration for clade 1 was 228 (95% CI: 181 – 286) and clade 8 was 176 (95% CI: 124 – 246).

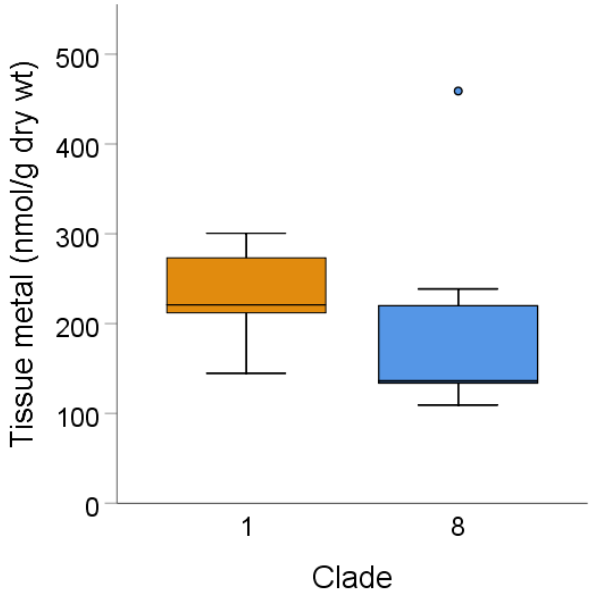

Figure 4.2: Boxplot of background tissue manganese in clades 1 and 8 measured at the end of the seven-day short-term toxicity experiments

The mean tissue metal (nmol/g dry wt) of clade 1 was 228 (95% CI: 181 – 286) and that of clade 8 was 176 (95% CI: 124 – 246). An independent samples *t*-test determined that the clades' background tissue metal was not significantly different (t = 1.413, $p = 0.180$, d.f. = 14).

The saturation bioaccumulation model could not resolve to the data, on the basis of nominal manganese or measured manganese. Instead, a simplified bioaccumulation model was used (Eq. 4.1). At concentrations of manganese far below which the uptake rate is saturated, the

bioaccumulation model predicts metal accumulation similarly to the allometric model in that uptake is linear.

$$C_{TB} = \frac{\max \cdot C_W}{K + C_W} + C_{BK}$$

$$C_{TB} = \frac{\frac{\max \cdot C_W}{K}}{1 + \frac{C_W}{K}} + C_{BK}$$

If K is relatively large, then $\frac{C_W}{K}$ becomes very small and insignificant.

$$C_{TB} = \frac{max}{K} \cdot C_W + C_{BK} \qquad \text{(Eq. 4.1)}$$

The simplified model was fit on the basis of nominal and dissolved manganese concentrations in SYSTAT 13. Parameters (*max/K* and C_{BK}) and their 95% confidence intervals were estimated. The model fitted the data well, with R-square values ranging from 0.87 to 0.92. The term C_{BK} estimated by the model was treated as a fitting parameter, rather than a true estimate of the background manganese tissue concentration. The values of C_{BK} for clade 1 were 0.879 nmol/g (95% CI: 0.297 – 2.60) and 2.90 nmol/g (95% CI: 2.90 – 4.74) for nominal and dissolved concentrations, respectively. Those of clade 8 were, respectively, 1.23 nmol/g (95% CI: 0.402 – 3.76) and 3.54 nmol/g (95% CI: 1.29 – 9.75). Based on overlapping 95% confidence intervals, the different exposure estimates of manganese resulted in statistically significant differences in C_{BK} values within both clades; however, there was not a difference between clades when estimated with nominal or measured manganese. These values were statistically different from the mean control tissue concentrations for both clades (non-overlapping 95% confidence intervals).

The *max/K* ratios for clade 1 on the basis of nominal and dissolved manganese were 8.29 L/g (95% CI: 6.08 – 11.3) and 7.80 L/g (95% CI: 5.70 – 10.7), respectively, and they are not statistically different from each other. The *max/K* ratios estimated for clade 8 were 6.48 L/g (95% CI: 4.72 – 8.88) and 6.16 L/g (95% CI: 4.42 – 8.57), respectively, which are also not statistically different from each other. In addition, these ratios were not significantly different between clades.

Table 4.1: Fitted parameters for modeled bioaccumulation of manganese by clades 1 and 8 in seven-day short-term toxicity experiments

Metal estimate	Clade	*max/k* (L/g)	95% CI	C_{BK} (nmol/g dry wt)	95% CI	Mean control (nmol/g dry wt)	95% CI	R-square	RMSE
Nominal Mn	1	8.29	(6.08, 11.3)	0.879	(0.297, 2.60)	228	(181, 287)	0.92	0.1473
Dissolved Mn	1	7.80	(5.70, 10.7)	2.90	(2.90, 4.74)			0.91	0.1531
Nominal Mn	8	6.48	(4.72, 8.88)	1.23	(0.402, 3.76)	175	(124, 246)	0.88	0.1639
Dissolved Mn	8	6.16	(4.42, 8.57)	3.54	(1.29, 9.75)			0.87	0.1748

Logarithmically-transformed data were fitted to the bioaccumulation model. The table displays the back-transformed parameters and 95% CI for the ratio of maximum tissue metal to the half saturation constant (*max/K*) and background tissue metal concentration (C_{BK}). The mean control concentrations are also included in the table, as they are likely a better estimate of background concentration; C_{BK} was treated as a parameter to achieve best model fit. Measures of model fit (R-square and RMSE) are also included in the table.

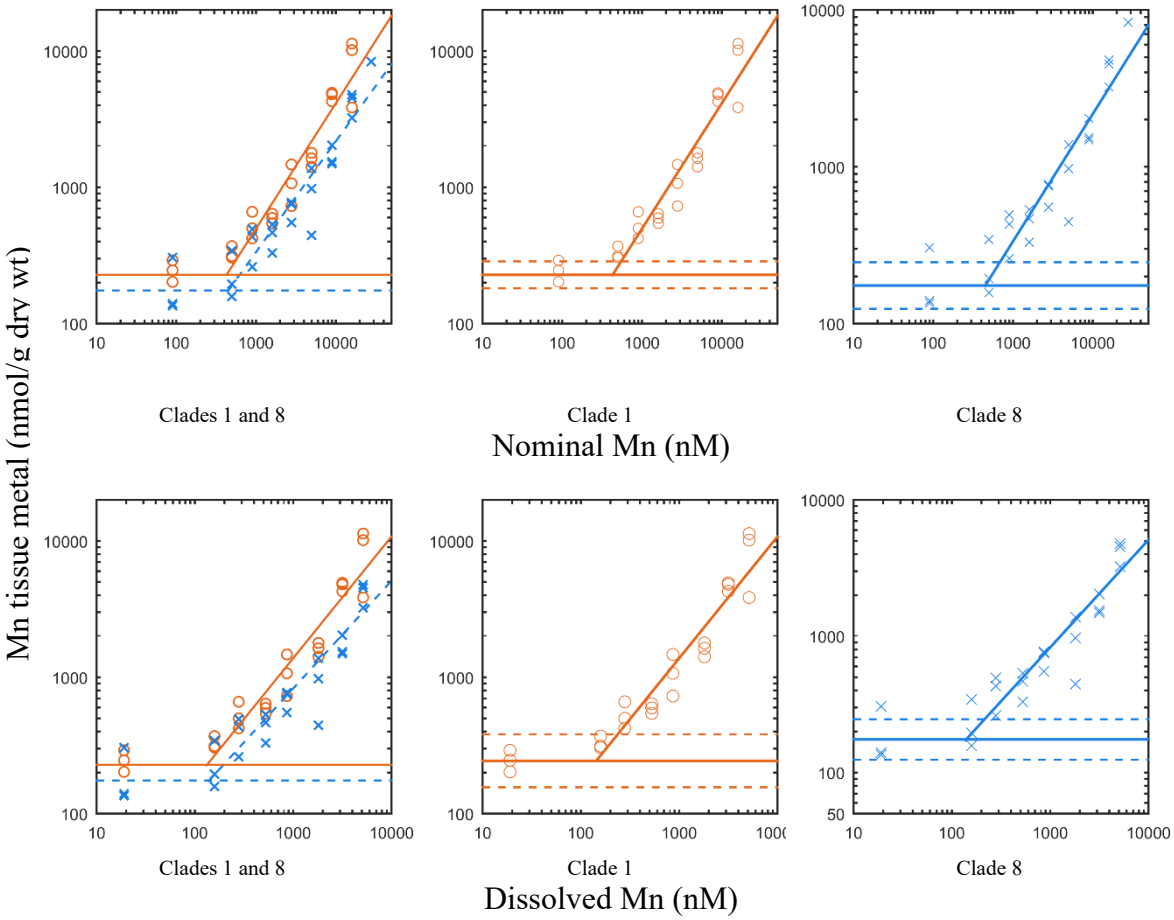

Figure 4.3: The relationship between tissue manganese (nmol/g dry weight) and nominal or dissolved manganese (nM)

Untransformed data and the fitted simplified saturation model for bioaccumulation are displayed for clades 1 (○, orange, solid line) and 8 (x, blue, dashed line) in the left panels. The center panels contain clade 1 data with the addition of the model-estimated C_{BK} plotted as a line (slope = 0) with its 95% CI (dashed lines). The right panels display clade 8 bioaccumulation model and data with model-estimated C_{BK} and its 95% CI.

4.1.1.3 Mortality

The mortality model fit the data well, with R-square values ranging between 0.88 and 0.91 for water-based toxicant estimates. R-square values for model fits to tissue metal data were 0.60 and 0.80 for clade 1 and clade 8, respectively. The exponents n_w and n_b were set to 100 in all cases to resolve the model. The mortality model estimated the background mortality of clade 1 to be 0.0336 (95% CI: 0.0159 – 0.0546), 0.0345 (95% CI: 0.0141 – 0.0549), and 0.0570 (95% CI: -0.0120 – 0.126), on the basis of, respectively, nominal potassium permanganate, dissolved Mn, and tissue metal. The estimated background mortality of clade 8 was determined to be 0.0582 (95% CI: 0.0293 – 0.0872), 0.0600 (95% CI: 0.0346 – 0.0854), and 0.0350 (95% CI: -0.00700 – 0.0770). All background mortality rates were not significantly different within or between clades, or from the control mortality rates: clade 1 was 0.0379 (95% CI: 0.0180 – 0.0578) and clade 8 was 0.0782 (95% CI: 0.0204 – 0.136).

Half-saturation constants (K''_W or K''_{TBX}) and LC50 values were not significantly different between clades. Within each toxicant estimate, no significant difference was detected between clades for LC50 or LC25 values. The LC50 values for nominal potassium permanganate were 14300 nM (95% CI: 11600 – 17000) and 19800 nM (95% CI: 16300 – 23400), for clades 1 and 8 respectively. The LC50 values for dissolved manganese were 4700 nM (95% CI: 3970 – 5430) and 6310 nM (95% CI: 5510 - 7110), for clades 1 and 8. The LBC50 values were estimated to be 957 nM (95% CI: 672 – 1240) and 1280 nM (95% CI: 1040 – 1510).

The mortality model estimated that mortality increased above background rates when potassium permanganate concentrations increased above 2000 – 5000 nM, when dissolved

Mn concentration increased above 1000 – 3000 nM, and when tissue metal concentrations increased above 1000 – 3000 nmol/g. Table 4.2 and Figure 4.4 display the mortality data.

Table 4.2: Clade 1 and 8 mortality rates in short-term toxicity experiments modeled as functions of nominal potassium permanganate, dissolved, and tissue manganese concentrations

Toxicant estimate	Clade	*m'*	95% CI	K''_W	95% CI	LC50 (nM)	95% CI	LC25 (nM)	95% CI	R-square	RMSE
Nominal $KMnO_4$	1	0.0336	(0.0159, 0.0546)	9.40	(2.67, 16.1)	14300	(11600, 17000)	9340	(6980, 11700)	0.89	0.1225
Dissolved Mn	1	0.0345	(0.0141, 0.0549)	8.37	(2.58, 14.2)	4700	(3970, 5430)	3300	(2590, 3990)	0.90	0.1140
Nominal $KMnO_4$	8	0.0582	(0.0293, 0.0872)	23.0	(-2.97, 48.9)	19800	(16300, 23400)	14000	(10600, 17300)	0.88	0.1183
Dissolved Mn	8	0.0600	(0.0346, 0.0854)	32.5	(-9.09, 74.1)	6310	(5510, 7110)	4850	(3990, 5700)	0.91	0.1049
				K''_{TBX}		LBC50 (nmol/g dry wt)		LBC25 (nmol/g dry wt)			
Tissue metal	1	0.0570	(-0.0120, 0.126)	6.02	(-3.53, 15.6)	957	(672, 1240)	723	(580, 865)	0.60	0.1643
Tissue metal	8	0.0350	(-0.00700, 0.0770)	3.95	(1.70, 6.20)	1280	(1040, 1510)	881	(740, 1020)	0.80	0.1378

Logarithmically-transformed data were fitted to the mortality models. The table displays the back-transformed parameters and 95% CI for control mortality (*m'*), half saturation constant (K''_W or K''_{TBX}), and mortality parameters (LC50 and LC25 or LBC50 and LC25). Measures of model fit (R-square and RMSE) are also included in the table. The parameters n_w and n_b were set to 100 in all cases and are not included in the table.

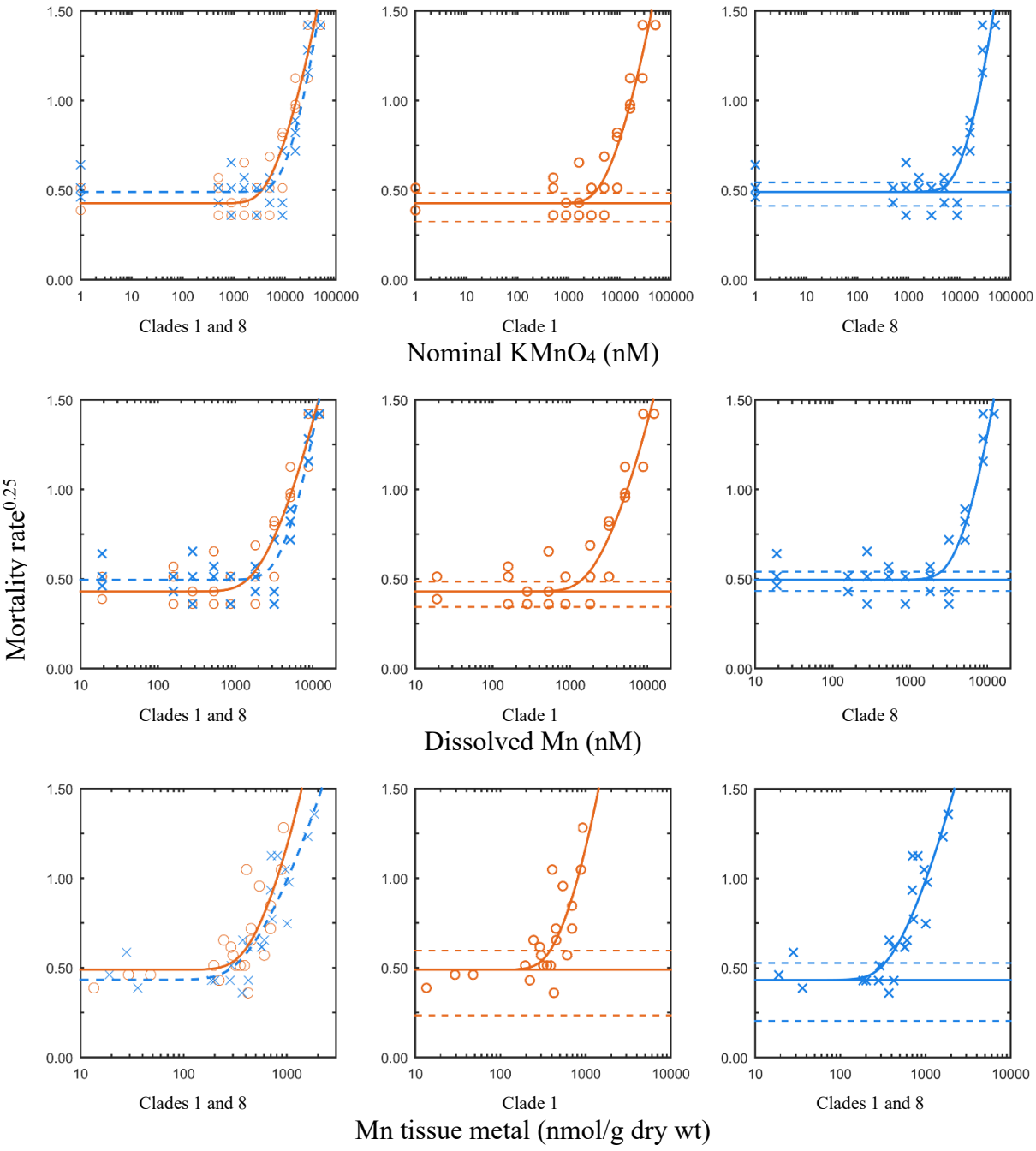

Figure 4.4: The relationship between clade 1 and 8 mortality and nominal potassium permanganate, dissolved manganese, or tissue manganese concentrations

Untransformed data and the fitted mortality model for clades 1 (○, orange, solid line) and 8 (x, blue, dashed line) are displayed in the left panels. The center panels show clade 1 data with the addition of the model-estimated control mortality rate (*m'*) plotted as a horizontal line (slope = 0) with its 95% CI (dashed lines), and the right panels displays that of clade 8. The fitted model parameters are displayed in Table 4.2.

4.1.1.4 Growth inhibition

Final dry weights were measured against toxicant concentration and fitted to the growth inhibition empirical equation. R-squared measures of fit were between 0.10 and 0.21 for water-based toxicant estimates. On the basis of tissue metal, R-square fits were 0.34 for clade 1 and 0.07 for clade 8. Table 4.3 displays the fitted parameters and the respective 95% confidence intervals. Figure 4.5 displays the plotted data and models.

All model estimates for the control dry weights of each clade were not significantly different from the measured dry weights: clade 1 measured was 0.0341 mg (95% CI: 0.0299 – 0.0383) and that for clade 8 was 0.0310 mg (95% CI: 0.0277 – 0.0342), (Figure 3.6). The estimated IC25 values for nominal potassium permanganate were 10600 nM (95% CI: -7460 – 28600) for clade 1 and 53300 nM (95% CI: -34200 – 66000) for clade 8. The clade 8 IC25 estimate is above the concentration range of potassium permanganate that was administered in this study. Similarly, the dissolved IC25 and IBC25 estimates for clade 8 are 16000 nM (95% CI: -126000 – 232000) and 12800 nmol/g (95% CI: -19600 – 44700), respectively. These estimates are model calculations, but are not reliable estimates because, like the estimate for potassium permanganate, the concentration range does not extend to the toxicant estimate for IC25 or IBC25. Values for IC10 and IBC10 were reported due to this limitation (Table 4.4). Equation 4.2 was derived from the growth inhibition model.

$$W = \frac{W'}{1 + aC^n}$$

$$9 = \frac{10}{1 + a(IC10)^n}$$

$$\frac{10}{9} = 1 + a(IC10)^n$$

$$\frac{1}{9} = a(IC10)^n$$

$$\sqrt[n]{\frac{1}{9a}} = \sqrt[n]{(IC10)^n}$$

$$IC10 \; or \; IBC10 = (9a)^{\frac{-1}{n}} \qquad \text{(Eq. 4.2)}$$

The IC10 and IBC10 values are within the range of the concentration series in this experiment, and there is not a significant difference between those estimated for clades 1 and 8, based on overlapping 95% confidence intervals. In addition, the other parameters, *a* and *n*, that were estimated by the model were not significantly different between clades or estimates of potassium permanganate or manganese exposure, also based on overlapping 95% confidence intervals.

Table 4.3: Modeled clade 1 and 8 growth inhibition in short-term toxicity experiments as functions of nominal, dissolved, and tissue manganese concentrations

Toxicant estimate	Clade	*W'*	95% CI	*a*	95% CI	*n*	95% CI	IC25 (nM)	95% CI	R-square	RMSE
Nominal $KMnO_4$	1	0.0361	(0.0276, 0.0446)	1.28E-05	(-2.38E-04, 2.64E-04)	7.30	(-6.13, 20.7)	10600	(-7460, 28600)	0.21	0.02507
Dissolved Mn	1	0.0358	(0.0280, 0.0437)	3.81E-05	(-6.53E-04, 7.29E-04)	1.14	(-6.45, 20.7)	3670	(-1950, 9290)	0.21	0.02508
Nominal $KMnO_4$	8	0.0337	(0.0297, 0.0378)	1.37E-06	(-4.43E-05, 4.70E-05)	7.99	(-14.8, 30.8)	53300[1]	(-126000, 232000)	0.10	0.01384
Dissolved Mn	8	0.0339	(0.0296, 0.0381)	2.68E-05	(-6.55E-04, 7.08E-04)	6.52	(-12.4, 25.5)	16000[1]	(-34200, 66000)	0.11	0.01377
								IBC25 (nmol/g dry weight)			
Tissue metal	1	0.0351	(0.0270, 0.0432)	3.75E-06	(-7.02E-04, 7.96E-05)	8.88	(-5.77, 23.5)	4072	(-2140, 10300)	0.34	0.02330
Tissue metal	8	0.0329	(0.0301, 0.0357)	1.60E-08	(-4.93E-07, 5.25E-07)	11.9	(-9.66, 33.5)	12800[1]	(-19600, 44700)	0.07	0.01411

Logarithmically-transformed data were fitted to the growth inhibition models. The table displays the back-transformed parameters and 95% CI for control dry weight (*W'*), empirical fitting parameters *a*, and *n,* and the point inhibitive concentration point estimates (EC25 and EBC25). Measures of model fit (R-square and RMSE) are also included in the table. [1]Denotes IC25 or IBC25 estimates that are above the concentration range set or measured in this experiment, and therefore an unreliable estimate.

Table 4.4: Estimates of clade 1 and 8 10% growth inhibition in short-term toxicity experiments modeled as functions of nominal, dissolved, and tissue manganese concentrations

Manganese estimate	Clade	IC25 (nM)	95% CI	R-square	RMSE
Nominal $KMnO_4$	1	2990	(-6180, 12800)	0.21	0.02507
Dissolved Mn	1	1140	(-2330, 4610)	0.21	0.02508
Nominal $KMnO_4$	8	13100	(-15900, 42100)	0.10	0.01384
Dissolved Mn	8	4070	(-4340, 12500)	0.11	0.01377
		IBC25 (nmol/g dry weight)			
Tissue metal	1	1570	(-2650, 5790)	0.34	0.0233
Tissue metal	8	5520	(-2960, 14000)	0.07	0.01411

Logarithmically-transformed data were fitted to the growth inhibition models. The table displays the back-transformed IC10 values and associated 95%. Measures of model fit (R-square and RMSE) are also included in the table. All other parameters of the model are in Table 4.3.

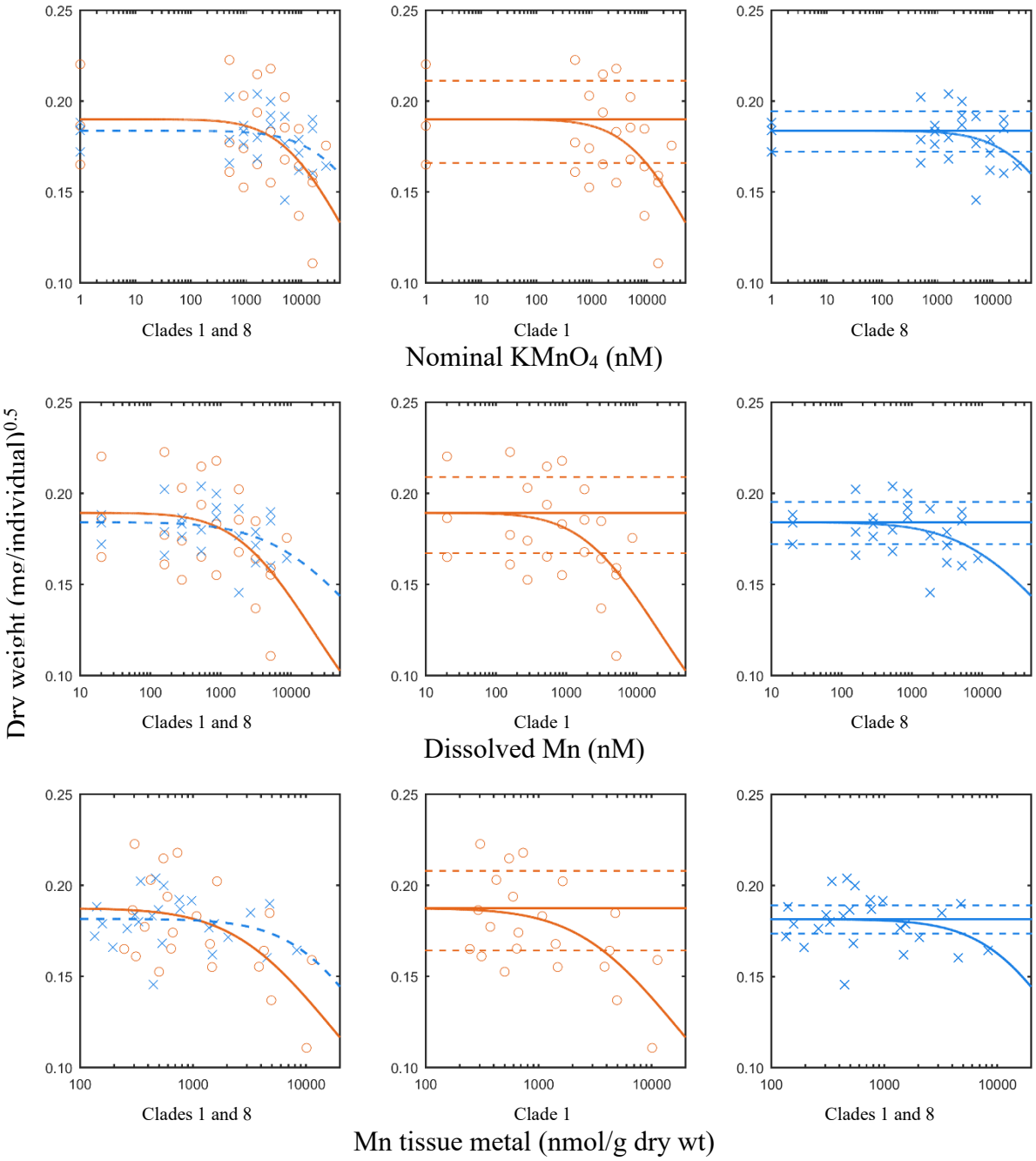

Figure 4.5: The relationship between square-root transformed clade 1 and 8 final mean dry weights and nominal potassium permanganate, dissolved manganese, or tissue manganese concentrations

Untransformed data and the fitted growth inhibition models for clades 1 (○, orange) and 8 (x, dashed line) are displayed in the left panels. The center panels show clade 1 data with the addition of the model-estimated control dry weight (*W'*) plotted as a horizontal line (slope = 0) with its 95% CI (dashed lines), and the right panels display those of clade 8. Controls in the nominal figures were plotted at x = 1.

4.1.2 Uptake and Depuration

Tissue and water concentration data were logarithmically transformed prior to analyses. A two-way ANOVA was conducted to determine whether the manganese concentration in exposure dissolved and total fraction samples was significantly different at the beginning and end of the uptake renewal periods. All cells of data were normally distributed (Shapiro-Wilk; $p > 0.05$), except the total samples at the beginning of the renewal periods ($p = 0.044$). The data fit the assumption for homogeneity of variances (Levene's test; $p = 0.168$). There was not a significant interaction effect between filtering treatment and sampling time (2-way ANOVA; F = 3.825, $p = 0.058$, d.f. = 1, 36). Therefore, a main effects analysis was conducted, and indicated that the main effect of filtering was statistically significant (F = 140.592, $p < 0.0005$, d.f. = 1, 36), but time over the renewal period was not (F = 0.005, $p = 0.942$, d.f. = 1, 36). Table 4.5 displays the mean dissolved and total concentrations for the exposure uptake and depuration experiment. The control concentrations were pooled over the four weeks, and are also presented in the table.

Table 4.6 displays the fitted parameters of the uptake and depuration experiments. The model fits of the uptake experiment yielded R-square values of 0.53 and 0.49 for clades 1 and 8, respectively. The R-square value for the depuration portion indicated better model fits: 0.85 and 0.89 for clades 1 and 8. The initial body concentrations (C_{b0}) were set to the mean measured control values because the rapid increase in body concentration to the steady state concentration (C_{SS}) at the beginning of the exposure period caused the model to overestimate C_{b0}. The mean control body concentrations were 103 nmol/g (95% CI: 95.9 – 110) for clade 1 and 101 nmol/g (95% CI: 104 – 118) for clade 8, which based on

overlapping 95% confidence intervals, are not significantly different. The estimate for C_{ss} was 2990 nmol/g (95% CI: 2550 - 3420) and 3730 nmol/g (95% CI: 2990 - 4480) for clades 1 and 8, respectively. These estimates were not significantly different and both clades reached C_{ss} within approximately 24 hours. In the depuration portion of the experiment, C_{b0} was estimated by the model and was not significantly different between clades, or in comparison to the mean control tissue concentrations.

The growth values estimated for the two clades were significantly different (non-overlapping 95% confidence intervals), but they were not included in the model because the elimination rate constants (k_e) were much larger (orders of magnitude) than the growth constants. There is not a significant difference between the clades in the estimates of k_e in the uptake portion of the experiment, and those in the depuration portion of the experiment; however, the k_e values estimated within each clade was different between the uptake and depuration experiments. The estimated k_u values were not significantly different between clades. Although there was manganese present during the depuration portion of the experiment, including an uptake rate constant for manganese uptake resulted in a negligible estimate and did not improve the fit of the model.

The half-lives of manganese in the body was estimated to be 2.92 days (95% CI: 1.45 – 4.40) and 1.72 days (95% CI: 1.23 – 2.35) for clades 1 and 8. The half-lives are not significantly different between the clades and the tissue concentration of both clades decreased to within the 95% confidence limits of the background concentrations within time of the depuration experiment.

Table 4.5: Mean measured manganese concentrations in water samples of the two-week uptake and two-week depuration experiments

	Control	95% CI	Exposure, uptake	95% CI	Exposure, depuration	95% CI
Dissolved	22.7	(20.5, 25.0)	1950	(1750, 2140)	25.3	(21.5, 29.9)
Total	16.7	(14.9, 18.7)	3840	(3560, 4150)	23.2	(18.4, 29.4)

Untransformed total and dissolved manganese concentrations in the uptake and depuration experiments with 95% confidence intervals. All concentrations are in nM. Control data were pooled over the entire experiment.

Table 4.6: Growth rates of clades 1 and 8 in manganese uptake and depuration experiments

Experiment	Clade	g	95% CI	W_0	95% CI	R-square	RMSE
Uptake and Depuration, Control and Exposure	1	0.00127	(-0.000809, 0.00334)	0.281	(0.250, 0.312)	0.02	0.1275
	8	0.00514	(0.00378, 0.00650)	0.237	(0.219, 0.255)	0.37	0.0864

The growth rate (mg/day) of each clade was estimated from dry weight data combined from the uptake and depuration experiments because the 95% confidence intervals of growth rate overlapped. Initial weight (W_0) was also estimated from the linear regression (mg). Growth was estimated with a linear regression of log-transformed data.

Table 4.7: Model-estimated parameters of clades 1 and 8 in two-week uptake and two-week depuration experiments

Experiment	Clade	k_u	95% CI	k_e	95% CI	C_{BK}	95% CI	C_{SS}	95% CI	BCF	95% CI	R-square	RMSE
Uptake	1	7.40	(4.22, 10.6)	4.83	(2.54, 7.13)	Set to 103		2990	(2550, 3420)	1530	(1310, 1750)	0.53	0.1368
	8	7.05	(3.55, 10.5)	3.68	(1.61, 5.76)	Set to 110		3730	(2990, 4480)	1910	(1530, 2290)	0.49	0.1703
		C_{b0}	95% CI	k_e	95% CI	C_{BK}	95% CI	$t_{0.5}$	95% CI	-	-	R-square	RMSE
Depuration	1	1210	(822, 1590)	0.237	(0.114, 0.360)	50.0	(-57.6, 158)	2.92	(1.45, 4.40)	-	-	0.85	0.1756
	8	1740	(1210, 2270)	0.387	(0.265, 0.509)	75.0	(32.7, 117)	1.72	(1.23, 2.35)	-	-	0.89	0.1891

Body concentration data were logarithmically transformed prior to model fitting. The table displays the back-transformed parameters and the 95% CI for manganese uptake rate constant (k_u, day^{-1}), depuration constant from the uptake and depuration experiments, separately (k_e, day^{-1}), background body concentration (C_{BK}), body concentration at steady state (C_{SS}, nmol/g dry weight), bioconcentration factor (BCF, calculated as k_u over k_e and multiplied by 1000), body concentration at the beginning of the depuration experiment (C_{b0}, nmol/g dry weight), and cadmium half-life in days ($t_{0.5}$, days). Measures of fit (R-square and RMSE) are also included in the table. The model was fitted with the dissolved manganese concentration as the exposure concentration. The background concentration (C_{BK}) was set for uptake modeling.

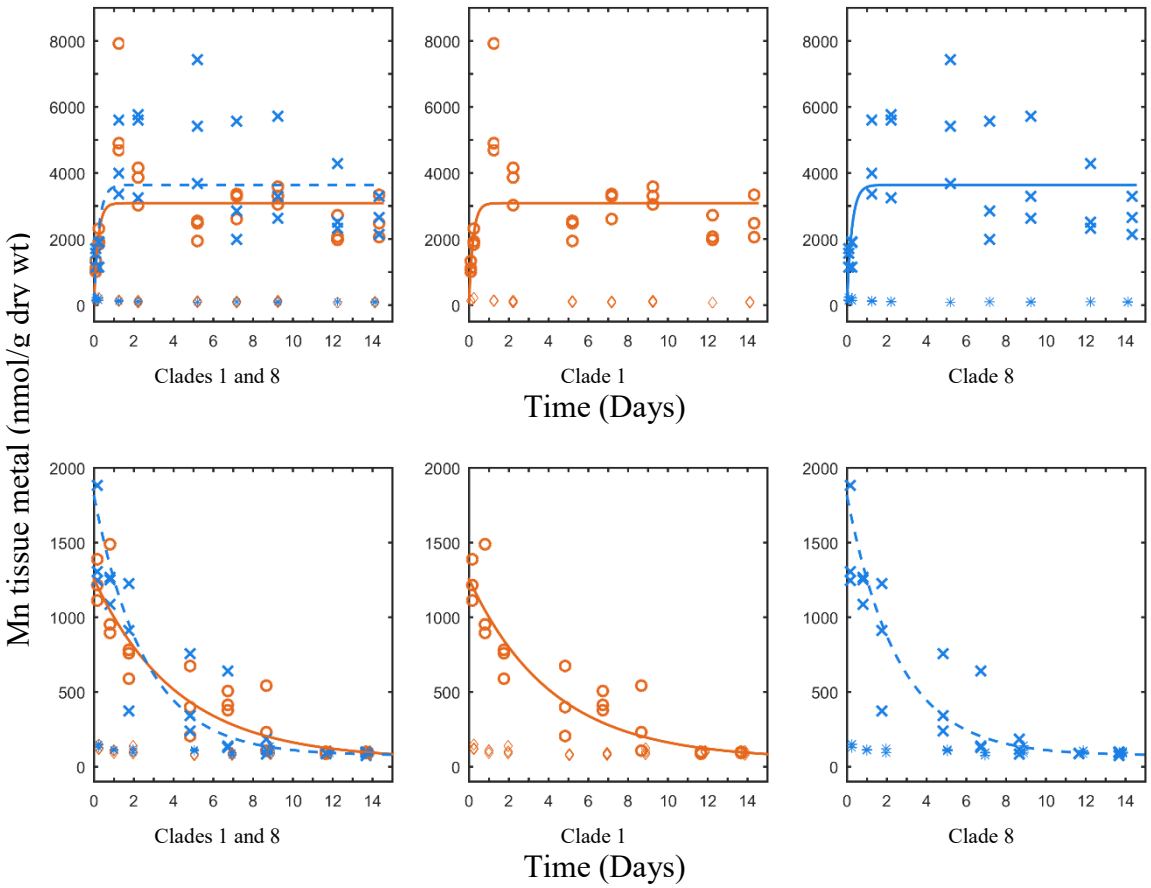

Figure 4.6: The relationship between clade 1 and 8 tissue metal bioaccumulated at a single exposure concentration (top row) and depurated (bottom row) over time

Untransformed data and the fitted uptake and depuration models for clades 1 (orange, exposure: ○; control: ◊) and 8 (orange, exposure: x; control: □) are displayed in the left panels. The center panels show clade 1 data and the right panels display that of clade 8. The model was fitted with dissolved manganese concentration as the exposure concentration.

4.2 Discussion

A clear mortality-exposure relationship was observed in both clades for nominal potassium permanganate, dissolved manganese, and tissue manganese. Mortality was correlated with waterborne or tissue concentration in both clades. The nominal potassium permanganate clade 8 LC50 value was 1.38-fold greater than that of clade 1, the dissolved LC50 value was 1.34-fold greater in clade 8, and the LBC50 value was 1.24-fold greater in clade 8. In water, potassium permanganate rapidly dissociates into a potassium (K^+) cation and a permanganate ion (MnO_4^-) that was the source of the purple colour observed in the exposures (Duncan, 1978). The MnO_4^- ion is a powerful oxidizer and the initial action by this ion was most likely the major source of above-background mortality (Griffin *et al.*, 2002; Hobbs *et al*., 2006). Hobbs *et al*. (2006) estimated 96-hour LC50s of potassium permanganate to five common aquatic test species. Of these, the *H. azteca* (unspecified clade) LC50 in synthetic moderately hard water was 28300 nM (1 SD = 13300). The toxicity endpoints reported by Hobbs *et al*. (2006) were based on nominal concentration values because the MnO_4^- ion is difficult to recover, and the toxic action is due to oxidative potential and occurs rapidly. The LC50 value, 28300 nM, is comparable to the nominal 7-day LC50 values obtained in this study: 14300 nM (95% CI: 11600 – 17000) and 19800 nM (95% CI: 16300 – 23400) for clades 1 and 8, respectively. The literature value is 2- and 1.43-fold greater than these clade 1 and 8 values.

In four-week exposures, manganese bioaccumulation (Norwood *et al*., 2006) and toxicity (Norwood *et al*., 2007) were evaluated for *H. azteca*, clade 1 (clade identified by Major *et al*., 2013). The experiments were conducted in SAM-5S spiked with manganese

chloride ($MnCl_2$). The reported LC50 was 197000 nM (95% CL: 144000 – 271000), which is 42- and 31-fold greater than the clade 1 and 8 values calculated in this study. Therefore, despite the high R-square values obtained from fitting the mortality model on the basis of dissolved manganese in this study, the relationship is likely correlative rather than causative: mortality in this study was likely due primarily to the permanganate ion in solution, rather than tissue manganese. Similarly, the LBC50 reported by Norwood *et al*. (2007) was 71000 nmol/g (95% CL: 60700 – 83000), which is 74-fold and 55-fold greater than the clade 1 and 8 values in this study, respectively.

Under anoxic conditions, Mn^{4+} is readily reduced (Stokes *et al*., 1988) to the more toxic form Mn^{2+}. However, this process was unlikely to have caused toxicity in this study because exposure media did not have lower oxygen saturation than the controls. In addition, other studies have reported values similar to those published by Norwood *et al*. (2007). Lasier *et al*. (2000) determined 96-hour LC50s for *H. azteca* (unspecified clade) and 48-hour LC50s for *Ceriodaphnia dubia*. The values correlated with water hardness and ranged between 54600 and 249000 nM in *H. azteca*, and 113000 and 277000 nM in *C. dubia*.

Tissue manganese concentrations were not observed to approach a maximum body concentration in this study. Thus, the saturation model could not be resolved to fit to the bioaccumulation data and a simplified model was instead used. At concentrations far below saturation of the uptake rate, the simplified model is analogous to the allometric model, and provides a sufficient fit for the bioaccumulation data. In contrast, the bioaccumulation of manganese in clade 1 could be modeled with the full saturation model by Norwood *et al*. (2006). The highest body concentration measured by Norwood *et al*. (2006) was over

100000 nmol/g, in contrast to the greatest body concentrations measured in this study, which were lower by a full magnitude. The authors also reported a model-estimated *max* of 116000 nmol/g and a *K* term of 146000 nM, and the *max/K* ratio was 0.790 L/g. In the present study, the *max/K* ratios were estimated without controls. On the basis of dissolved manganese, these ratios were 9.9- and 7.8-fold less than that reported by Norwood *et al*. (2006).

The control tissue concentrations calculated in the short-term study were approximately 2-fold greater than that reported by Norwood *et al*. (2006). Manganese is an essential element and is expected to occur at measurable concentrations in control tissues. In addition to manganese uptake from the water, manganese is found in TetraMin®, which was used as food in culturing procedures and the experiments in this study and those by Norwood *et al.* (2006, 2007). Norwood *et al*. (2006) digested TetraMin® and determined that the manganese content was 759 nmol/g. Although the small amount of food given to the juveniles during the gut clearance period in this study may have artificially elevated the tissue concentrations that were measured, a substantial amount of manganese was likely eliminated from the tissues over the 24-hour gut clearance period. The tissue concentrations were likely greater than were measured in this experiment, based on the evidence from the rapid depuration of manganese in the bioaccumulation kinetics experiment, and on the significant percentage loss (from 13 to 66% day^{-1}; variable due to saturation of elimination) determined by Norwood *et al*. (2006).

The measured control concentrations (103 and 100 nmol/g for clade 1 and 8, respectively) in the uptake and depuration experiments were close to the value reported by Norwood *et al.* (2006). The manganese uptake was rapid during the uptake period, and

reached steady state within the first 24 hours. Because the amphipods were not gut cleared in the uptake and depuration experiment, tissue sampling that occurred close to feeding times (day zero in both the uptake and depuration phases) may have had lower tissue concentrations than were estimated, due to the manganese content of the food. Adsorption of manganese to food would have elevated the apparent exposure tissue concentrations relative to the increase that would have occurred at the same time in the controls. Manganese uptake from food may also have contributed to an overestimated k_u value, which is the rate constant for manganese uptake from water. The variability of the tissue manganese was greater in clade 8 than it was in clade 1.

Tissue concentrations of both clades returned to background concentrations within the 14-day depuration period. The half-lives of manganese in clades 1 and 8 were 2.92 and 1.72 days, respectively. The depuration model estimated the background concentrations to be lower than the mean control values. The elimination rate constants, k_e, between clades were not significantly different, but those estimated in the uptake period were significantly different from those in the depuration period. The steady state concentrations, C_{SS}, were also significantly greater than the respective initial tissue concentrations, $C_{b0.}$ In the uptake and depuration experiment, growth rates were significantly different between the clades. In the models fitted to the data in this study, the growth and elimination rate constants are simply additive. The elimination rate constants were much greater than the growth rate constants and therefore the latter were not included in the regression.

Slightly lower dry weights were observed at the greatest potassium permanganate concentrations, but the effects in clade 8 were below a 25% growth inhibition. The clade 1

IC25 and IBC25 in this study were both smaller than those estimated by Norwood *et al*. (2006), indicating that, similar to the correlation rather than causation observed in the mortality regression, the permanganate ion was likely the predominate cause for lower dry weights at high concentrations. The data were sufficient to estimate IC10 and IBC10 values. Although the IC25 value of clade 8 was 3.6-fold greater than that of clade 1, the values were not significantly different based on non-overlapping confidence intervals.

The potassium permanganate toxicity and manganese bioaccumulation responses of clades 1 and 8 in this study were not significantly different. The only statistically significant difference detected between the clades was between the growth rate constants estimated from the uptake and depuration experiment. Proactive use of potassium permanganate in aquaculture decreases parasites and external infections, and thereby decreases application of antibiotics (Lazur and Yanong, 2013). Toxicity data for potassium permanganate action on non-target organisms is limited, though Hobbs *et al*. (2006) determined that the impact is significant at suggested disease treatment levels (2.5 times the potassium permanganate demand (PPD), which is calculated on the basis of estimated reducing content of the water (Tucker, 1989). With the desire to move away from antibiotic use in food production, potassium permanganate is an inexpensive alternative, but based on the current study and that of Hobbs *et al*. (2006) it can have detrimental effects on non-target organisms.

Chapter 5
Conclusions, Implications, and Future Directions

Hyalella azteca is a morphologically cryptic species complex of freshwater amphipod crustaceans whose member species, or clades, are distributed across North America (Hrycyshyn, 2016; Wellborn and Cothran, 2004; Wellborn *et al*., 2005; Witt and Hebert, 2000; Witt *et al.*, 2003, 2006, 2008). Phenotypic stasis persists despite marked genetic divergence among its members, except in some endemic species whose environmental constraints have been relaxed or altered (Witt *et al*., 2003). Eighty-five different provisional species have been flagged through sequencing at the mitochondrial cytochrome *c* oxidase subunit I gene (Hrycyshyn, 2016; Witt and Hebert, 2000; Witt *et al.,* 2003, 2006, 2008), but only a few have been described (*Hyalella spinicauda* and *Hyalella wellborni* (Soucek *et al.*, 2015). Some of the member species exhibit geographically wide distributions, though most are endemic to the Great Basin (Hrycyshyn, 2016; Witt *et al.*, 2006, 2008; Witt unpublished).

Members of this complex have been routinely used in toxicology since the mid-1980s because they have ideal qualities for laboratory and field studies, in that they are easy to culture, important constituents of the food web, and benthic organisms which makes them relevant to sediment and water-only tests (EC, 2013). Both Environment Canada (2013) and the USEPA (2000) have published guidelines for the use of this amphipod in toxicity tests. Despite the marked genetic divergence, test species are frequently identified as "*H. azteca*" by use of a taxonomic key (EC, 2013; Leung *et al*., 2016).

Major *et al*. (2013) detected only two of the 85 wild provisional species among samples from 17 North American laboratories. Sixteen cultures were determined to contain

only clade 8, except that maintained at the Canada Centre for Inland Waters (CCIW) in Burlington, Ontario, which was clade 1. Therefore, the present study contrasted short-term bioaccumulation and toxicity, as well as uptake and depuration kinetics of clades 1 and 8.

No significant differences could be determined, based on overlapping 95% confidence intervals, between clades 1 and 8 in this study. Elevated concentrations of cadmium and potassium permanganate resulted in significant mortality and bioaccumulation effects, but growth inhibition was a less precise effect in this study. The above-background mortality and growth inhibition endpoints in response to cadmium (LC50, LC25, LBC50, LBC25, IC25, and IBC25) were notably similar between clades, with non-significant differences ranging between factors of 1.08 and 1.43. Clade 1 was slightly more tolerant to waterborne cadmium, but clade 8 was more tolerant to tissue cadmium.

Tolerance to potassium permanganate was also not significantly different between clades. Although both clades significantly bioaccumulated manganese at elevated waterborne concentrations, there was no evidence of uptake saturation in either clade. The toxic agent was potassium permanganate due to the oxidative potential of the permanganate ion, not manganese. Therefore the LBC50 and IBC50 values may be useful to predict toxicity due to potassium permanganate, but are likely not directly informative about manganese toxicity. These conclusions were made on the basis of comparisons with manganese and potassium permanganate toxicity studies (Hobbs *et al*., 2006; Laiser *et al*., 2002; Norwood *et al*., 2007). The waterborne endpoints of mortality (LC50, LC25) due to potassium permanganate exposure were 1.38 to 1.50-fold greater for clade 8 than clade 1. The IC10 value was 4.38-fold greater for clade 8 than clade 1.

In the uptake and depuration experiments, clades 1 and 8 bioaccumulated and depurated the metals similarly. Over the uptake period, both clades reached similar steady states (C_{SS}) tissue concentrations for both metals. Within the 14-day depuration portion of the experiments, tissue manganese concentrations decreased to background concentrations similarly in both clades. Cadmium tissue concentrations did not return to background levels within the depuration period. Clades 1 and 8 were not determined to have significantly different uptake and depuration kinetics.

Two-week (Leung *et al*., 2016) and four-week (Pathammovong, 2016) nickel and copper (tested separately) bioaccumulation and toxicity studies were conducted with clades 1 and 8. The clade 8 two-week LC50 was significantly greater than that of clade 1 (Leung *et al*., 2016), by approximately 2-fold for both nickel and copper. Four-week clade 8 LC50 and LC25 values were also significantly greater than those of clade 1 for nickel and copper. In both the two- and four-week tests, the clades exhibited similar bioaccumulation over the range of exposure concentrations. Lethal endpoints on the basis of tissue concentration were also not significantly different between clades. In another study, Soucek *et al*. (2015) demonstrated that clade 8 requires greater chloride concentrations in media than does clade 1. Weston *et al*. (2013) observed up to a 550-fold difference in sensitivity between four clades exposed to a pyrethroid pesticide.

In addition to work with *H. azteca*, molecular methods have resulted in the re-classification of other morphological species as cryptic species complexes. *Gammarus forassum* is another nominal species used in toxicology that was recognized as a cryptic species complex. Feckler *et al*. (2014) observed significantly greater stress tolerance in one

of the two cryptic *G. forassum* species, and Eisenring *et al.* (2016) characterized differences in biological traits for two members of the *G. forassum* complex. Two sympatric species of the *Cletocamptus deitersi* complex (redescribed as *C. stimpsoni* and *C. fourchensis*), identifiable by sequencing the COI gene, exhibited significantly different mortality in 96-h exposures to metal (Rocha-Olivares *et al.*, 2009).

Species sensitivity distributions (SSDs) are used to derive water quality guidelines (CCME, 2007). The method requires a minimum of eight species meant to be representative of all species (in the geographic area of interest). The CCME derives national benchmarks with SSDs, but site-specific guidelines (SS-WQG-PAL) may also be determined. In addition, ecological risk assessments can inform decision making processes by assessing the likelihood of possible adverse effects (Newman, 2010). Biomonitoring is another tool to evaluate the health of biological communities and water bodies ("Science of aquatic biomonitoring," 2018).

In a large-scale SSD, like those published as national benchmarks, the relative difference between two cryptic species of the *H. azteca* complex will likely not impact the final value, even though the species are at the low end of the SSD curve. However, in SS-WQG-PAL, site-specific risk assessments, and biomonitoring, clade-level differences may have a significant impact on the (site-specific) water quality guideline, decision, or assessed quality of a habitat. The SSD method can still be improved. How to aggregate toxicity data from cryptic species, species acclimated, acclimatized, or adapted to site-specific conditions are still subjective decisions (Schneider, 2014). Hanson *et al*. (2017) recommended nine reporting requirements to improve the reliability and transparency of toxicology studies, and

thereby make them more useful to decision making. Under the recommendations for test organism reporting, genetic identification was included. This will increase comparability with current and future studies.

Hyalella azteca is a cryptic species complex that toxicology research often treats as a single species. However, toxicological studies with clades 1 and 8 of this complex have revealed statistically significantly different nickel and copper LC50s and LC25s between the two clades (Leung *et al*., 2016; Pathammovong, 2016). Clade 8 was 1.5 – 2.7 times more tolerant to copper and nickel than clade 1. In the present study, significant differences were not detected between the mortality rates in response to cadmium or potassium permanganate. Growth inhibition was greater in clade 1 in response to potassium permanganate, but greater in clade 8 in response to cadmium. Reporting test results with genetic identities of test species will improve the understanding of the role that genetic divergence plays in toxicant resistance. Future research with these clades may benefit from comparisons between acclimation capabilities and tolerance ranges in environmentally relevant manipulations of test media.

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