METAL MIXTURE TOXICITY TO *Hyalella azteca*: RELATIONSHIPS TO BODY CONCENTRATIONS

by

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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.

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GENERAL ABSTRACT

A literature review of metal mixture interaction analyses identified that there was not a consistent method to determine the impact of metal mixtures on an aquatic organism. The review also revealed that a majority of the research on mixtures made use of water concentrations only. Therefore research was conducted to determine the relationship between exposure, bioaccumulation and chronic effects of the four elements As, Co, Cr and Mn individually. Mechanistically based saturation models of bioaccumulation and toxicity were determined for the benthic invertebrate *Hyalella azteca,* from which lethal water concentrations and body concentrations were also determined. These models were then combined with those previously done for the metals Cd, Cu, Ni, Pb, Tl and Zn to model the impact of 10 metal mixtures on bioaccumulation in short term (1-week) exposures and on bioaccumulation and toxicity in chronic (4-week) exposures at "equi-toxic" concentrations. Interactions between the metals were identified in which; Cd, Co and Ni bioaccumulations were significantly inhibited, Tl and Zn bioaccumulations were marginally inhibited, there was no impact on Cr, Cu or Mn bioaccumulation, and both As and Pb bioaccumulation were enhanced by some mixtures of metals. It was determined that strict competitive inhibition may be a plausible mechanism of interaction affecting Co, Cd and Ni bioaccumulation but not for any of the other metals. However, it is possible that other interactions such as non-competitive or anti-competitive inhibition may have been responsible.

A metal effects addition model (MEAM) was developed for *Hyalella azteca* based on both the bioaccumulation (body concentrations) to effects and the exposure (water concentration) to effects relationships developed from the single metal only studies The MEAM was used to predict the impact of metal mixture exposures on mortality. Toxicity was under-estimated when based on measured water or body concentrations, however, its best prediction was based on body concentrations. The MEAM, when based on measured body concentrations, takes bioavailability into account, which is important since the chemical characteristics of water can greatly alter the bioavailability and therefore toxicity of metals.

The MEAM was compared to the traditional Concentration Addition Model (CAM), which calculates toxic units based on water concentrations and LC50s or body concentrations and LBC50s. The CAM overestimated toxicity, but had its best prediction when based on water concentrations. Over all, the best fit to observed mortality was the prediction by the MEAM, based on body concentrations. The measurement of bioaccumulated metals and the use of the MEAM could be important in field site assessments since it takes into account changes in bioavailability due to different site water chemistries whereas the traditional CAM based on water concentration does not.

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DEDICATION

Eight years and three months is a time frame that can pass before you know it. However, in that same time, many things can be accomplished, some things can seem to take forever and many things can pass. A few of those who have passed during my time doing this PhD were relatives and friends who inspired me, supported me or were just someone whom I whish I could spend more time with. They are missed.

Beverly Louise Steele, (Mother-in-law). 1925 to 2001
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CHAPTER 1

General Introduction

Metals occur naturally in the earth's crust and are neither created nor destroyed by anthropogenic or biological process. However, their redistribution by the minerals and metals industry (mining and smelting), power generation, fossil fuel combustion, and many other industrial processes may be of concern since an increase in metal concentrations in our environment could pose a threat to human and ecosystem health (Nriagu, 1991). For example, Noranda Inc., released 790 tonnes of metals to the environment world wide in 1997 (Noranda Inc., 1997). The metals and minerals industry provides about 350,000 jobs in Canada and contributed up to 10% of Canada's Gross Domestic Product in 1996. Governments, like that of Canada, are concerned since they have a responsibility to ensure a safe and healthy environment for their people as well as foster economic development for secure employment. Consequently, continued research on the natural and anthropogenic sources of metals, transport and fate of metals, and impact of metals on biological systems is required, particularly in regards to providing data and methods for ecological risk assessment.

The study of metal toxicity in aquatic systems underwent significant changes during the 1900's. Simple toxicity tests early in the last century gave way to much more complex, sophisticated and sensitive tests. Environmental laws, guidelines and protocols, that were virtually nonexistent in the early 1900's, are also complex and incorporate new toxicity test procedures. For example, the Canadian Environmental Quality Guidelines 1999 are based on scientifically defensible toxicological data and incorporate the toxicological results of the most sensitive organism to establish the limit (Canadian Council of Ministers of the Environment (CCME), 1999). As well, a number of "Recommended Methods" for measuring and assessing the aquatic biological effects of toxic substances have been produced, such as the *Hyalella azteca* test (Environment Protection Service, 1997).

1.1 Non-Biological Factors Which Affect Metal Toxicity

In 1983, Francois Morel produced a book "Principles of Aquatic Chemistry" (Morel, 1983), from which the free-ion-activity model (F.I.A.M.) was formulated. Essentially, the model indicates that the free-metal ion activity reflects the reactivity of the metal and it is this activity that leads to the metal's bioavailability and toxicity. Any complexation of the metal by inorganic or organic ligands could render the complex non-toxic.

Two reviews found that a majority of the literature support the F.I.A.M. (Borgmann, 1983; Campbell, 1995). However, both authors indicate that not all complexing agents will reduce metal uptake and toxicity. For example, ionophores, which bind metals, are readily absorbed by animals cells and therefore can increase the uptake of the metal (Levinson et al., 1979). As well, both pH and water hardness can affect the toxicity of the free-metal-ion and hence all three factors (complexation, pH, and

hardness) must be accounted for in order to relate free metal concentration to toxicity (Borgmann, 1983; Campbell, 1995). However, both Borgmann and Campbell indicate that a majority of the literature they reviewed only dealt with copper under laboratory conditions and did not make use of natural dissolved organic material (D.O.M.). Therefore, caution is recommended if the F.I.A.M is utilized to evaluate other metals.

Most metals end up in the bottom sediments of lakes, rivers and oceans. Through a series of complex physical, chemical and biological processes, metals can: precipitate, form complexes with inorganic or organic ligands on particulates, or become incorporated into living organisms and eventually become associated with the bottom sediments (Tessier and Campbell, 1987). The metals associated with these sediments can be distributed among a variety of physico-chemical compartments which exhibit a wide range of chemical reactivity and bioavailability.

It is apparent that many interactions that affect metal bioavailability and toxicity can occur simultaneously. Dissolved organic material and a number of major and minor ions that are found in natural water can interact with metal species. Ions such as Ca^{2+} and Mg^{2+} can interfere with the binding of metals on an organic ligand (Morel, 1983). However, calcium and magnesium complexes are much less concentrated in freshwater and hence the water hardness and pH are important factors in metal availability in freshwater systems (Morel, 1983). Another literature review indicated that organic substances, pH, temperature, alkalinity and hardness, inorganic ligands, interactions and sediments can all substantially alter metal toxicity (Wang, 1987).

1.2 Biological Factors Which Affect Metal toxicity

The reactions and interactions described above take place in the “Bulk Solution” compartment (Fig. 1.1). However, these types of reactions may also occur once the metal comes in contact with an organism. The organism itself is a complex ligand that can have many interactions with the metal species. The “Bulk Solution” is anything external of the organism, such as the surrounding aqueous solution, sediment and pore water, or the gut of the organism.

Metal toxicity is the adverse effect that the uptake of the metal has on an organism (Mason and Jenkins, 1995). The bioavailability of a metal refers to the portion of the external, aquatic environmental concentration of the metal that is biologically available, to be adsorbed or absorbed by the organism (Campbell, 1995). There are two basic routes from the environment that the metal can take in order to interact with the organism, direct contact via the aqueous compartment or through ingestion of metal contaminated food (Langston and Spence, 1995). However, in both cases, once the metal has made contact with the organism it will encounter the cell membrane of the gut, the surface

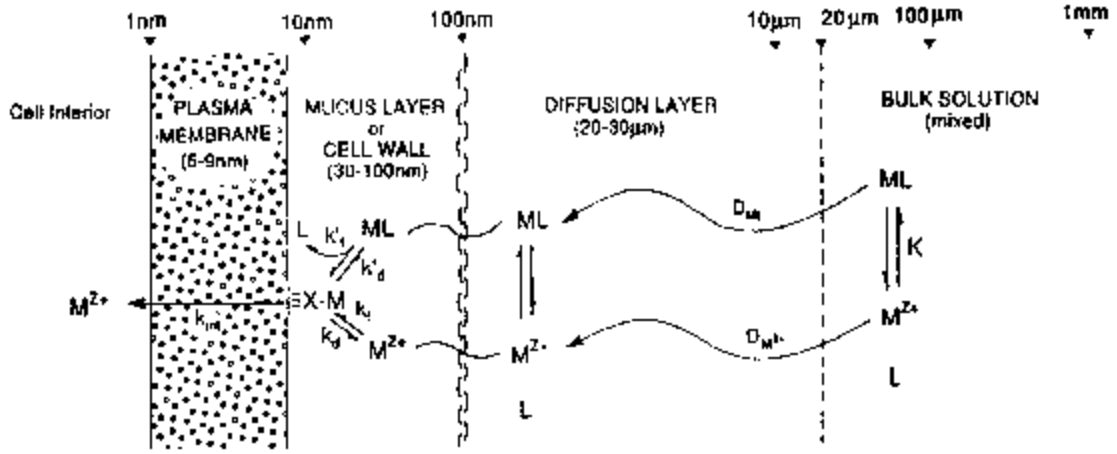

Figure 1.1 Conceptual model of metal-organism interactions. M^{Z+} = free-metal ion; ML = metal complex in solution; M-X-membrane = surface metal complex; k_f, k'_f – rate constants for formation of the surface complex; k_d, k'_d = rate constants for dissociation of the surface complex; k_{int} = rate constant for 'internalization' or transport of the metal across the biological membrane. Charges on ligand not shown for simplicity. [From Campbell (1995)]

skin or carapace, or the gill of the organism.

The cell membrane and the outer mucus layer or cell wall (Fig. 1.1) are complex formations of lipids and proteins. The lipids act as the structure of the membrane and are composed of charged or neutral, hydrophobic or hydrophilic, glycolipids and phospholipids such as esters, non-esters, sugars, cholesterol, etc. (Simkiss and Taylor, 1995). Proteins within the membrane can be involved in the transfer of essential elements into the cell by carrying or forming pores. Carriers, such as ionophores, are capable of surrounding ions and transporting them across the membrane into the cell interior. Siderophores act as a receptor sites to iron and transport the iron to the inside of the membrane for release into the cell interior. Intrinsic proteins can form pores that penetrate the entire cell membrane forming ion selective channels through the cell wall or mucus layer. The pore size and charge on the channel can limit the size and type of ions that can diffuse through. These channels act as pumps regulating ions such as K^{+}, P^{3+}, Na+, and Ca^{+}, or act as funnels and filters, or even open and close in response to specific cell-surface receptors (Simkiss and Taylor, 1995). The metal could bind to and collect on "physiologically inert sites" with no biological effect, or "physiologically active sites" (external or internal) where biological function can be impaired (Campbell, 1995). Therefore the cell membrane can act as an effective barrier or enhancement to metal uptake.

A number of metal species may permeate the membrane, such as free-metal-ions (M^{2+}), hydrated ions ($M(H_2O)_6^{2+}$), charged metal complexes ($MCl(H2O)^{5+}$), inorganic complexes (MCL_2^{0}), and organo-metallic complexes (CH_3M^{n+}) (Simkiss and Taylor, 1995). These species may enter the cell as described above. However, a number of factors can affect the permeation or transport of a metal species and hence its bioavailability and toxicity. Membrane fluidity affects the membrane proteins and ion transport. This fluidity can be affected by dietary changes (Simkiss and Taylor, 1995), as well, organism tolerance, size, life stage and nutrition can all substantially alter metal toxicity (Wang, 1987). Metal competition for binding sites (transfer proteins) or even for channels in which the pores can become blocked would also affect metal availability. Changes in environmental pH affect metal speciation as well as the biological surface. For example, membrane channel conductance reduction would change the availability and transport of a metal. It is apparent the interactions at the membrane surface or through the membrane plasma can have effects on the cell, whether the effect is the transport of a potentially toxic metal into the cell interior or the binding/blocking of essential cell membrane functions.

Once the metal has adsorbed on or been absorbed by an organism, it would be classified as available. The metal may cause a physiological effect unless the organism has some sort of protective mechanism. Some metals are essential, such as copper and zinc, and most organisms have mechanisms for handling them. *Hyalella azteca* can regulate copper concentrations in its body (Borgmann and

Norwood, 1994) and it appears that an internal ligand is the control factor, not uptake or depuration (Borgmann, 1998). An organism may control a bioavailable metal during the uptake, during internalization or through elimination of the metal. Metal-containing granules have been detected in invertebrate tissues, which effectively bind up the metals in inert forms (Brown, 1982). Other organisms produce metallothioneins which are cysteine-rich, metal-binding polypeptides and have been identified in over 80 species of fish and invertebrates (Roesijadi, 1992). These metal ligands bind and detoxify the metal, which can then be stored and/or excreted via urine or fecal matter. These mechanisms enable the organism to regulate the internal metals, by either making the metal inactive or elimination of the metal from its body.

1.3 Body Concentrations and Toxicity

Toxicology is based on the effect that a toxicant produces at a target site within an organism. Therefore, establishing the relationship between the concentration of the contaminant at the target site and the subsequent toxic effect would provide a tool for predicting toxicity (Landrum et al., 1992). This is the primary toxicological principle generally referred to as "dose-response" or "concentration-response" in which the dose or concentration of the contaminant at the target site is the concern (Connolly, 1985; McCarty, 1991). However, the target site of concern in many cases is not known or measurement of contaminant concentration at the site is not possible. Instead, surrogate measures of the target concentration have been used, such a water and sediment concentrations. For example, water and sediment quality criteria have been set for the protection of aquatic life based on laboratory bioassays or field sediment-effect correlations (Canadian Council of Ministers of the Environment (CCME), 1999; Connolly, 1985).

A number of researchers have determined that the concentration of a chemical in the organism (expressed as Body Concentration, Critical Internal Concentration, tissue residue, tissue concentration or body burden) is better for predicting effects than other measures such as water concentration, sediment concentration, QSAR's, or Equilibrium Partitioning (Connell, 1995; Driscoll and Landrum, 1997; Niimi and Kissoon, 1994). The use of body concentration as a measure of bioavailability may negate complications that can arise from uncertainties due to; interactions, multiple compartments of exposure, multiple sources and pulsed exposure (Hickie et al., 1995; Landrum et al., 1992). Body concentrations of single metals have been shown to be useful indicators of toxic effects in aquatic invertebrates especially in the presence of various complexing agents (Biesinger et al., 1982; Borgmann et al., 1991; Borgmann and Norwood, 1997; Borgmann and Norwood, 1999; McCarty, 1991; Meador et al., 1993; McCarty, 1991).

1.4 Metal Mixtures, Availability and Toxicity

It has been 23 years since Wong et al. (Wong et al., 1978) determined that 10 metals when present at levels equivalent to the objectives set by the Water Quality Subcommittee of the International Joint Commission, were not toxic to algae if present individually, but strongly inhibited primary production when present together. Yet, the most recent version of the Canadian Environmental Quality Guidelines (C.E.Q.G., 1999) does not incorporate any guidance on the effects of mixtures. It is still possible that the metal guideline concentrations which demonstrate no chronic effects individually could be chronically toxic in mixtures. However, Environment Canada has released a guidance document (Environment Canada, 1999) which recommends the Toxic Units Concept (Concentration Addition) to evaluate the effect of mixtures. However, these calculations are based on measured water concentrations and do not take into account different bioavailability due to varying water chemistries at an field sites. As well, the concentration addition method should only be applied to chemicals with similar modes of action. However, there are many chemicals that may have independent modes of action and should not be assess by the concentration addition method. Borgmann (1980) outlines an effects addition method in which the predicted impact of each toxicant (i.e. mortality) is summed to predict the mortality in mixtures.

An understanding of metal interactions in mixtures and the impact on organisms is significant since several metals are often present together at elevated concentrations in contaminated environments. Experimentation with metal mixtures, in which all other interfering or complexing agents are eliminated, kept to a minimum or held constant, should enable the use of body concentrations to help explain or determine interactions in mixtures, an area of essential research (Landrum et al., 1992; McCarty and MacKay, 1993). For example, if one metal enhances the uptake of another metal, the final body concentration of the impacted metal would be greater than expected in comparison to the uptake of that metal in a single metal exposure at the same concentrations. Therefore, this project will focus on the value of body concentrations to; predict toxicity of aqueous metal mixtures and to help resolve metal interactions.

1.5 Hypotheses

1. Bioaccumulation of the elements As, Co, Cr and Mn individually by *Hyalella azteca* is correlated to their concentration in water and can be used to predict chronic toxicity as previously determined for Cd, Cu, Ni, Pb, Tl and Zn (Borgmann et al 2004).
2. The toxicity of mixtures of metals can be predicted from *Hyalella* body concentrations.

1.6 Objectives

In order to test the above hypotheses a number of objectives had to be met as outlined below:

1. Determine the bioaccumulation and toxicity of four elements (As, Co, Cr & Mn) from individual chronic bioassays of each metal.
2. Determine the relationship between exposure concentration and the resulting body concentration (i.e. can body concentration be predicted from exposure concentration in controlled bioassays?).
3. Determine the relationship between body concentration and the resulting effect (mortality) (i.e. can mortality be predicted from body concentrations or water concentrations?).
4. Produce a 10-metal mixture (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl & Zn) based on Toxic Units (TU) such that 1 TU equals the LC25 for each metal.
5. Determine the bioaccumulation and toxicity of the ten-metal mixtures. Compare bioaccumulation from the mixture to bioaccumulation from individual metal bioassays.
6. Determine if the metal mixture toxicity is concentration additive based on water or body concentration or effect additive based on a mortality rate models.

In order to meet the above objectives, a literature review was conducted to determine the current methods available to quantify or predict the effects of metal mixtures (Chapter 2). This review determined that body concentrations had not been used to predict or evaluate the impacts of mixture exposures to aquatic organisms. This lead to the formation of a plan to integrate the individual bioaccumulation to toxicity relationships (models) for the ten elements; As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Z, commonly found in contaminated sites. However, the individual models were only known for six of the elements; Cd, Cu, Ni, Pb, Tl and Zn (Borgmann et al 2004). Therefore, the individual bioaccumulation patterns for As, Co, Cr and Mn were determined (Chapter 3) and then related to the chronic toxic effects (Chapter 4) in the formation of bioaccumulation to toxicity models for the individual elements.

Once accumulation and toxicity of all ten metals was determined, mixtures of the ten elements could then be tested in order to determine interactive effects on the bioaccumulation of each element (Chapter 5). Different types of mechanisms involved in the interactions were described but only one type, competitive inhibition, could be tested in Chapter 6. The understanding of the bioaccumulation patterns of the individual elements as well as the interactions between them is useful before evaluation of the toxic impact of mixtures. The chronic toxicity of mixtures could then be determined and evaluated using the traditional concentration addition model (Chapter 7). Moreover, an effects addition model could then be developed and tested, based on the individual bioaccumulation to toxicity models for each element (Chapter 7).

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CHAPTER 2

Effects of Metal Mixtures on Aquatic Biota: A Review of Observations and Methods

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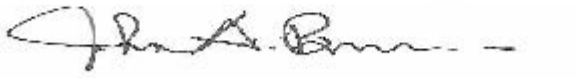

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ABSTRACT

A brief review of the historical development of metal mixture interaction analyses is presented. The two major classifications of mixture models outlined are, the “Concentration Addition” and the “Response Addition” approaches. Within these two categories, a number of graphical, mathematical and statistical methods have been used, such as the toxic unit approach, relative potencies, toxicity equivalence factors, and dose-response relationships that have been described using several methods such as probit, logit, and regression analyses. A database was generated to evaluate the frequency of occurrence of less than additive, strictly additive, and more than additive responses to metal mixture effects reported in the literature. The three responses occurred at 43, 27 and 29% respectively. The database is available electronically from the lead author. The research required to determine the most appropriate methods to quantify the effects of metal mixtures in an ecological risk assessment (ERA) framework is discussed. Until this research is completed, ERAs should use existing models such as the toxic unit or the effects addition approach. Bioaccumulation measurements by organisms for which the accumulation to response relationship is known would also be a useful complement.

2.1 INTRODUCTION

The study of metal toxicity in aquatic systems underwent significant changes during the last century. Simple acute toxicity tests on single metals first developed in the early 1900s have been replaced by more complex, sophisticated and sensitive chronic tests. Environmental laws, guidelines and protocols, virtually nonexistent until the 1970s, are now complex and incorporate new toxicity test procedures. For example, the 1999 Canadian Environmental Quality Guidelines are based on scientifically defensible toxicological data (Canadian Council of Ministers of the Environment (CCME) 1999), and methods for measuring and assessing the aquatic biological effects of toxic substances have been standardized (Environment Protection Service 1997). The US EPA is in the process of developing an integrated approach to metals assessment in surface waters and sediments based on equilibrium partitioning theory and would like to apply the Biotic Ligand Model (BLM - water column and sediment pore water metal bioavailability) and possibly tissue residue-based criteria, in the development of an integrated metals methodology that would provide a set of metals criteria for both sediments and overlying waters (Science Advisory Board 2000).

However, little progress has been made in setting water or sediment quality criteria to evaluate the impacts of metals when they occur in the environment in mixtures. For example, the Canadian Environmental Quality Guidelines do not include criteria for mixtures of metals (Canadian Council of Ministers of the Environment (CCME) 1999). As well, the European Inland Fisheries Advisory Commission (1987) has indicated that “…the tentative water quality criteria would be applicable to

situations where more than one of the toxicants considered were present. Therefore, there would appear to be little justification to set more stringent standards…" and hence there are no mixture criteria. However, they also indicate that "…it would be prudent to assume that partial addition exists, pending further research…" and thus recommend the use of additive joint action evaluations. Australia and New Zealand, on the other hand, have established a criterion (formula) for simple mixtures (<6 components) if the mixture exceeds their water quality guidelines (ANZECC and ARMCANZ 2000) as follows:

$$TTM = \sum(C_i/WQG_i)$$

"where TTM is the predicted total toxicity of the mixture, C_i is the concentration of the component and the WQG_i is the guideline for that component. If TTM exceeds 1, then the mixture has exceeded the water quality guideline" (ANZECC and ARMCANZ 2000). This is a concentration addition approach using guideline concentrations to as toxic units (TUs), which are discussed later in this document. A better understanding of metal interactions in mixtures and the impact on organisms is required to determine if changes in environmental guidelines are needed, since several metals are often present together at elevated concentrations in contaminated environments (Borgmann *et al* 2000) and whether changes are required in environmental guidelines. The questions in these situations are: To what extent does each metal contribute to any observed effect, and are the effects significantly (enough to require changes to guidelines) greater or lesser than the sum of the individual component effects?

Unfortunately, predicting the response of aquatic organisms exposed simultaneously to more than one potentially toxic chemical is one of the most difficult tasks in environmental toxicology and risk assessment. There are a number of graphical and statistical methods that have been proposed to predict the impact of metals mixtures and metal interactions. All of these methods utilize water concentrations of the contaminants of interest to generate dose-response curves for each individual contaminant, which are then used to generate specific critical concentrations for mixture models. These mixture models can be broadly classified into two basic types: Concentration Addition Models and Response (Effects) Addition Models and are described below. This is followed with the results of a literature search in an attempt to determine patterns in the toxicity of metal mixtures. Recommendations for future research and evaluation of metal mixtures in ecological risk assessment (ERA) are also discussed.

2.2 METHODS

2.2.1 Mixture Models

The concentration addition method is a mixture model that has been commonly used. The concentrations of all the toxic constituents of a mixture are added together to predict toxicity. However, each contaminant may have a different potency. Therefore, a number of different methods for

combining chemical concentrations by converting them to an equitoxic dose have been produced. Examples include the Toxic Unit approach (Sprague 1970), relative potency approach (Finney 1964; (Hewlett and Plackett 1979), or toxicity equivalence factors (TEFs) such as those summarized for dioxin-like PCBs (Ahlborg *et al.* 1994). The response-addition approach is the other main mixture model. For this model, the differing potencies of each of the mixture constituents is not important since the effect of each toxic constituent's concentration in the mixture is combined in the prediction of the mixture toxicity. In either concentration addition or response addition, mixture interactions can be detected when the observed impact of the mixture is greater than predicted (more than additive), the same as predicted (strictly additive) or less than predicted (less than additive). The terms "synergistic" and "antagonistic" are also frequently used to describe "more" or "less" than additive effects respectively, but their precise meaning and use varies, depending on the models and authors using them (Table 2.1). For example, if the toxicity of two metals present together is always equal to, and not greater than, the toxicity of the more toxic metal singly, then their effects are clearly less than additive. However, true antagonism could be argued to occur only if one metal reduces the toxicity of another, such that the combined toxicity of the metals is actually less than the toxicity of the more toxic metal present singly. There are many different models within each category that can be utilized to help distinguish these interactions as outlined below.

2.2.2 Concentration Addition

The most commonly used Concentration Addition model is the Toxic Unit approach (Sprague 1970). In this approach, the concentration of each metal in the mixture is divided by the toxic concentration for that metal and organism when present singly (*e.g.*, the lethal concentration killing 50% of the organisms, the LC50), to convert the concentration into a Toxic Unit (TU) scale for each metal. The TUs for all the metals in the test mixture are then summed. If the sum is less than 1, the mixture is predicted to affect less than half of the organisms. If the sum is greater than one, then the mixture is predicted to affect more than half of the organisms (Sprague 1970). Other values can be used instead of the LC50, such as the Lowest Observed Effect Concentration (LOEC), depending on the level of toxicity that is to be tested. If the toxic effect (*e.g.*, 50% mortality if TUs are based on LC50s) is observed when the sum of the TUs is equal to 1, then the mixture toxicity is classified as strictly additive. If the effect is observed when the sum of TUs is greater than one, the toxicity is less than additive, and if it is observed when the sum of TUs is less than one, toxicity is greater than additive. The Toxic Unit approach is usually applied only to a single effect level (*e.g.*, 50% mortality), and not to the prediction of other partial responses.

The above concept can be extended to predict toxicity at all effect levels, as long as the toxicity curves are parallel. For example, when the individual regression lines of mortality, expressed as probits, versus log doses are parallel for constituents of a mixture, then it is suspected that the modes of action in the test organism are "similar" (Finney 1964). In this case the relative potency of each toxicant is converted to doses of one toxicant and then a total dose is the sum of all the converted doses. This dose is then used in the dose-response relationship to estimate the mixture mortality, *i.e.*:

$$z = z_1 + pz_2 \quad (1)$$

where z_1 and z_2 are doses of the two toxicants and p is the corresponding relative potency ratio (Finney 1971). Chi-squared values are calculated between expected and observed responses (Finney 1971) to test for significant differences. This same method was adopted in the analysis of the "simple similar" model (Hewlett and Plackett 1979). Finney (1964) also outlines a "general test for similar action" that is the same as the Toxic Unit method outlined above, but based on probit analyses to calculate a median effective dose (LD50).

Hewlett and Plackett (Hewlett and Plackett 1979) describe isoboles that are used to examine experimental data to determine if the response of a mixture falls into a specific graphical pattern representing additive, synergism, antagonism, potentiation, subadditive and coalitive action. Generally, isobolograms have been utilized for drug and insecticide mixture analyses to determine interactions so that, for example, a formulation can be optimized so that the mixture potency can be increased, yet the doses or concentrations of the individual components can be decreased. This is a good example of a synergist drug (that is not active singly) in its ability to activate or increase potency of another (Hewlett and Plackett 1979). The isobole is simply a curve on a two-axes plot, where each axis represents the dose or concentration of each drug or insecticide in a two-component mixture. The curve at any point represents the paired concentrations that produce a fixed level of response.

Marking (Marking 1977) created an additive toxicity index to determine the additive toxicity of mixtures of chemicals. This index is based on the toxic unit concept but converts the units to a linear scale in which zero represents strict addition, greater than zero indicates "more than additive (synergistic), and less than zero indicates "less than additive" (antagonistic) effect. As well, the index includes a calculation to determine significance of deviation from zero based on 95% confidence intervals of the individual and mixture LC50s.

2.3 Response Addition (Effects Addition)

A number of studies have predicted the toxicity of metal mixtures assuming response (effects) addition. If toxic effects are strictly additive, then the control-corrected survival in the mixture ($S"_{mix}$ =

S_{mix}/S' where S' is control survival) can be predicted from the product of the survival observed when each metal is present singly.

$$S''_{mix} = S''_1 \times S''_2 \times S''_3 \ldots \quad (2)$$

where S''_i is the control-corrected survival observed when metal i is present singly at the same concentration as in the mixture. For example, if exposure to one metal results in a survival of 60%, and exposure to another metal results in a survival of 50%, then survival in a mixture of both metals at the same concentrations would be expected to be 0.6 × 0.5 = 30%. Equation 2 can be used to predict toxicity regardless of the mathematical form of the equation used to relate survival to metal concentration.

The "additivity" of equation 2 can be demonstrated clearly if toxicity is expressed as a mortality rate ($m = \ln(N/N_o)/t$, $m = m' + m''$, where N and N_o are the final and initial number of animals, m' is the mortality rate in the controls and m" is the toxicant induced mortality rate). Equation 1 then becomes

$$m''_{mix} = m''_1 + m''_2 + m''_3 \ldots \quad (3)$$

where m''_i is the mortality observed when metal i is present singly at the same concentration as in the mixture. Borgmann (Borgmann 1980) used equation 3 and an allometric relationship between m_i and metal concentration to study toxic effects of mixtures of five metals. The same approach was applied to growth, by computing a growth rate and taking its inverse (because toxicity results in a decrease in growth rate as compared to an increase in mortality rate). Equation 3 predicts the total mortality rate of the mixture, which can be directly compared to the observed mortality rate of the experiment. If the observed mortality rate is greater than the predicted, then a "more than additive" effect is occurring. If observed mortality is less than predicted, then a "less than additive" effect is occurring and finally, strict effect addition occurs when observed matched predicted. One of the advantages of using rates, such as mortality rate, instead of percent mortality, is that it provides partial effect data over a wider concentration range by allowing data from longer exposures at low concentrations to be combined with shorter exposures at higher concentrations, if the mortality rate is relatively independent of time (*i.e.*, plots of log(survival) against time must be relatively linear).

Table 2.1 Categories of mixture effects and categories of how the effect occurs. Definitions from Hewlett and Plackett (1979)

Observed Effect of a Mixture Relative to Predicted

Less Than Additive	Additive	More Than Additive
Antagonistic - toxicity of mixture is less than the toxicity of the most toxic metal when present singly at the same concentration	**Strictly Additive** - mixture toxicity matches expected toxicity based on concentration or effects addition model	**Synergistic** - a synergist is a substance that is nontoxic singly but increases the toxicity of other toxicants
Subadditive – mixture toxicity is greater than that of any single metal, but less than expected based on model prediction		**Potentiation** - both contaminants are toxic separately and greater than expected when mixed.
		Coalitive - occurs if neither contaminant is toxic separately yet together are toxic.

Joint Action of "Poisons" in a Mixture to Elicit an Effect

	Similar (same site of action) (same mode of action)	**Dissimilar** (different site of action) (different mode of action)
Non-interaction (no interaction between)	simple similar	independent dissimilar
Interactive (interaction between poisons)	complex similar	dependent dissimilar

Instead of computing mortality rates, but still applying the concept of equation 2, (Hewlett and Plackett 1952) used probit and logit transformations of quantal response data in a series of toxicity models, which were then refined and modified into biological classification of types of joint action of mixtures of "poisons" (Hewlett and Plackett 1961;Hewlett and Plackett 1964;Plackett and Hewlett 1967). Non-interactive (independent dissimilar or simple similar) and interactive (complex similar or dependent dissimilar) models were developed (Table 2.1). Similar and dissimilar refers to whether or not the "poison" acts on the same or different site of action within the organism respectively; interactive and non-interactive refers to whether one "poison" does or does not influence the biological action of the other "poison", respectively. The basic response addition model follows the following calculation:

$$P = 1-(1-P_1)(1-P_2)\ldots(1-P_n) \quad (4)$$

where P = the proportion of individuals predicted to respond to the mixture of poisons; P_1, $P_2 \ldots P_n$ = the proportion of individuals responding to each poison, based on data from individual dose-response curves (converted percent data or other quantal responses data to probit or logit data) plotted versus measured log concentration from the test solution. This is the same model proposed by Finney (1964) based on probit analyses. Note that $(1-P_i)$ in equation 4 is the same as $S"_i$ in equation 2 above. To test if a mixture falls into one of the four models of interaction, then the doses of the individual poison must be combined with each of the others in the mixture according to models for: 1) Simple similar; 2) Complex similar; 3) Independent; and 4) Dependent (Plackett and Hewlett 1967). Isoboles are used to determine synergism, potentiation, strict additivity and antagonism (Hewlett and Plackett 1979). The "simple similar" model is actually the "similar" model (Finney 1971) outlined in the Concentration Addition section above.

2.4 Mixture Interaction

A review of the literature on metal mixtures and their impacts was conducted. This review attempts to determine the "state-of-the-art" for mixture impact evaluation as well as to identify research required to produce a fully integrated metal mixture assessment technique.

2.4.1 Literature Search

The following questions were asked:

1. What species were tested?
2. How many metals were tested in the mixtures (binary pairs, three, four…., *etc*.)?
3. Were interactions between the metals identified?
4. Were there any trends in the interactions observed?
5. What methods/models were used to determine interactions?

All interactions identified in the literature were verified or recalculated using the concentration addition model utilizing toxic units (Sprague 1970) as described above or the effects addition model as described in the previous sections, depending on the type of data available in each publication and was considered a match if the predicted result fell within 10% of the observed (A cutoff of 10% was subjectively chosen as a surrogate for natural variation). This recalculation of interaction was performed in order to produce results based on the same methodology since there were many different methods utilized in the publications. As well, many indications of interaction reported in the literature were based on observations, with no formal interaction test conducted, or incorrect use of interaction models. Also, 43% of the interaction tests were based on measured concentrations whereas 57% were based on nominal concentrations and hence the recalculation of interactions was also based on this same combination. This information was included in the database.

2.3 RESULTS

A literature review of more than 68 publications dealing with metal mixtures was conducted. The database with a list of all the publications is available on request from the lead author.

2.3.1 Species Tested

More than 77 different species were tested covering a large array of phyla, family and genus and included groups such as algae, bacteria, planktonic crustaceans, benthic crustaceans, aquatic insects (benthic and pelagic), invertebrates, fish, protozoa, and aquatic macrophytes. Not only were many species utilized but also many life stages such as egg, embryo, larval, juvenile, fry and adult. To further complicate the analysis of interactions, many different endpoints were utilized such as mortality, growth, phosphorescence, enzyme production, metalothionein production, feeding rates, cough response, bioaccumulation, *etc*.

2.3.2 Multiple Metal Mixtures

Up to 11 metals in one mixture have been tested, however a majority of the literature contained results for binary mixtures (Table 2.2). Of the 191 mixture tests evaluated, 156 were binary, 18 contained three metals and all other combinations of four or more metals accounted for a total of 17 tests (Table 2.2).

Table 2.2 Metal Mixture Interactions based on reinterpretation of published data and comparison to original interpretation by the author.

In a field setting	No. of Metals in Mixture	Less Than Additive	Strictly Additive	More Than Additive	Total Tests	Could Not Test
	2	69	42	45	156	14
	3	7	6	5	18	4
	4	1	0	0	1	2
	5	3	0	3	6	2
	6	1	3	2	6	1
	7	0	0	0	0	1
	8	1	1	0	2	0
	10	0	0	1	1	1
	11	1	0	0	1	0
This Analysis	Total	83	52	56	191	25
	Percent	43	27	29	100	13
Author Interpretation	Total	89	58	63	210	12
	Percent	42	27.6	30	100	6

Table 2.3 Interactions of metals in binary mixtures based on reinterpretation of published data and comparison to original interpretation by the authors.

		Less Than Additive	Strictly Additive	More Than Additive	Total Tests
	Zn	27	8	17	52
	Cu(II)	21	8	21	50
	Cd	24	14	15	53
	Hg	11	10	11	32
	Ni	13	2	9	24
	Pb	6	6	3	15
	Al	3	1	0	4
	Mn	6	0	2	8
	Se	7	0	2	9
	V	6	0	0	6
	Cu(I)	4	0	2	6
	As	0	3	2	5
	Mo	5	0	0	5
	Mg	1	0	0	1
This Analysis	Total	134	52	84	270
	Percent	50	19	31	100
Author Interpretation	Total	145	114	100	359
	Percent	40	32	28	100

2.3.3 Interactions Between Metals

Not all of the published results identified interactions. Of the 210 tests, 12 did not indicate interaction (Table 2.2). In many of these cases, the focus of the research was not metal interactions, but rather the total impact on the organism. Other studies were based on field sediment exposures or exposure to field collected sediments that were contaminated with multiple metals. It was impossible to recalculate interactions in 25 tests because the raw data required could not be obtained from those publications.

There were 191 cases where interactions were clearly determined. These were divided into three categories: 1. Less Than Additive; 2. Strictly Additive (no interaction); and, 3. More Than Additive. Overall, the observed interactions fell into the three categories at 43, 27 and 30 percent respectively (Table 2.2) based on both the recalculated results and the author interpretations. These results indicate that there is a tendency toward "less than additive" effects. Also, the summary of interactions reported by the authors was no different than the recalculated effects.

A complete analysis of interactions of individual metals can be made based on the 156 tests of binary mixtures (Table 2.2). Fourteen metals were identified in binary mixtures. Table 2.3 lists these metals from the most to the least frequently tested and the number of times each metal was implicated in a binary mixture to be less than additive, strictly additive or more than additive. As in the overall trend for multi-metal mixtures, interaction was split between less than additive, strictly additive and more than additive at 50, 19, and 31 percent respectively (Table 2.3), again with less than additivity marginally dominating the interactions. However, there are a few metals that stand out from this trend. Aluminum, V, Mo and Mg were never reported to be "More Than Additive" in a binary mixture. Arsenic was never reported to be "Less Than Additive" in the five tests evaluated (Table 2.3). However, caution must be used in the evaluation of these two metals given the small sample size.

Binary pairs that had at least four tests in one category of interaction (more, less or strict additivity) for either the recalculated data or in the original data set of the author interpretations, are summarized in Table 2.4. It is interesting that in the 6 tests with the binary pair of Hg-Se, "Strict Additivity" was not observed and the pairing of Cu or Hg with Cd dominated the strictly and more than additive interactions.

2.3.4 Methods Used to Determine Interactions

As outlined previously, there are two basic approaches that can be used to assess the toxicity of mixtures: concentration addition and effects addition. In addition, direct comparison of the change in accumulation of metals by organisms exposed to the same concentration of a metal singly and in a

Table 2.4 Interactions in major binary pairs tested based on reinterpretation of published data and comparison to original interpretation by the authors.

		Less Than Additive	Strictly Additive	More Than Additive	Total Tests
	Cu-Zn	11	1	9	21
	Cd-Zn	9	5	5	19
	Cd-Cu	1	3	4	8
	Cd-Hg	1	4	4	9
	Cu-Ni	2	1	6	9
	Pb-Zn	2	1	2	5
	Hg-Se	5	0	1	6
	Hg-Ni	2	1	2	5
	Hg-Zn	2	0	2	4
	Al-Zn	1	1	0	2
	All Others	27	9	10	46
This Analysis	Total	63	26	45	134
	Percent	47	19	34	100
Author Interpretation	Total	72	60	49	181
	Percent	40	33	27	100

Table 2.5 Analyses used.

Methods Used to Re-evaluate Interactions	
Concentration Addition	37
Effects Addition	65
Effects & Concentration Addition	7
Means Comparison	30
Could not test	89

Calculations Used in the Publications
Berenbaum's Isobole, best fit
Effects Addition (2-factor interaction regression analysis)
Effects Addition (3-factor interaction regression analysis)
Isobole and Synergistic Ratios
Multiply regressions
Quantal effects modelling
Regressed Database of field effects against total TU
Regression comparison, linear & quadratic models of accumulation
Toxic Units based on Interstitial water, Probit analysis
Toxic Units based on sediment concentrations
Toxic Units based on water concentrations
Toxic Units from Probits

mixture are possible. There are a few other examples where direct comparison is a valid test to evaluate the impact of a mixture versus that of the individual metals, such as any decrease in a toxic effect upon exposure to two toxic metals (thus less than additive). Table 2.5 outlines the number of tests that were re-evaluated based on concentration addition, effect addition and direct comparison. However, at least nine different methods have been identified for calculating and evaluating (Table 2.5). The most prominent method of analysis was the “Concentration Addition with Toxic Units” method.

2.4 DISCUSSION

The review revealed that 30% of the cases were more than additive, 44% were less than additive and 27% were strict addition (Table 2.2). A key question is: Why are the responses to mixtures so variable, even for the same metal combinations as indicated in the binary pair interactions (Table 2.3)?

Metal interactions can be dependent on the species of organism being exposed (Braek *et al.* 1976;Wang *et al.* 1995). Since 77 different species were covered by this review, a large variation in responses would be expected. The relative concentrations of the metals in the mixture can also alter the nature of the interaction (Finlayson and Verrue 1982;Thorp and Lake 1974). Of the 68 tests identified in the database that tested and compared different ratios of metals in mixtures, 72% resulted in more than one type of interaction. It is then apparent that different effects would be expected for the entire data set dependent on the ratio used. In many cases “equi-toxic” concentrations were tested, however, any ratio could be expected in a field setting. Interactions can also be affected by the number and types of metals in a mixture. Since two to eleven metals were tested in mixtures and 21 different metals have been tested in different combinations, it would be expected that a wide range of interactions would be observed. Another impact on the results could be the type of method used to determine interactions. Approximately eight different methods were applied in either a “concentration addition” or an “effect addition” type test to determine metal interaction. However, the comparison between the results for author interpretation and the re-evaluated interactions were consistent, suggesting that it is unlikely that data analysis methods result in the wide range of observed responses summarized in Tables 2.2, 2.3 and 2.4.

There was one consistent trend throughout the database, all the tests used water concentration as the measure of exposure or dose. However, interactions can be modified by different exposure water chemistries. (Hickie *et al.* 1993) exposed larval rainbow trout to varying concentrations of a fixed-ratio mixture of Al, Mn, Fe, Ni, Zn, Cu, and Pb to determine acute toxicity over a range of pH values. Components of the mixture were deleted and the tests were rerun in order to determine which of the metals in the mixture was responsible for toxicity. Over the full pH range the toxicity of an Al, Cu and

Zn subset of the mixture was equivalent to the toxicity of the full mixture. At pH 5.8 Cu explained all toxicity. At pH 4.9, all toxicity was explained by Al. The same results were obtained when the experiments were rerun with larval fathead minnow. Bioavailability of the metal ions is also affected by other abiotic modifying factors such as alkalinity, water hardness, and the concentration of dissolved organic carbon, which can alter the nature of the chemical species of the metals (Wang 1995; Hutchinson 1987). Since the bioavailability, and hence the toxicity, of metals is dependent on the nature of the metal species present, modifying factors will alter the apparent toxicity of metals. The situation for metal mixtures is further complicated since the modifiers will influence the speciation, and hence the bioavailable fraction, of each metal differently. In addition, the metals may compete with one another for the site of uptake or action.

Considerable advances have been made on the understanding of metal toxicity when present singly, and these will ultimately impact research on metal mixtures. Morel (1983) formulated the free-ion-activity model (FIAM). Essentially, the model indicates that the free-metal ion activity reflects the reactivity of the metal and it is this activity that leads to the metal's bioavailability and toxicity. Any complexation of the metal by inorganic or organic ligands could render the complex non-toxic. Campbell (1995) has expanded and refined the FIAM, which is perhaps the best currently available surrogate for the bioavailable fraction. The FIAM does not assume that the free-metal ion activity is the only bioavailable (toxic) metal species, but rather that the biological response is proportional to the free-metal ion activity. Normally researchers do not measure the free-metal ion-activity, but rather use geo-chemical speciation models to account for the activity of the various biotic and abiotic ligands in water with any given set of water chemistry parameters. Although the FIAM does much to explain the "bioavailable fraction" of the metal, it does not fully account for metal "bioavailability" because metal bioavailability is also a function of water chemistry interactions on free metal ion uptake by the organism. In the Biotic Ligand Model (BLM), the FIAM has been expanded to incorporate stability constants for fish gill, and to treat them as just another biotic ligand in the system that competes for metal binding (Paquin *et al.* 2002). Since critical gill-metal concentrations (*i.e.*, those associated with acute toxicity) have been determined for some metals, one can literally model what metal concentration will be required to cause an impact for a given set of chemical parameters in a surface water. At present the groundwork has been laid to use this approach for a number of individual metals, most notably copper, silver and zinc (Paquin, Gorsuch, Apte, Batley, Bowles, Campbell, Delos, Di Toro, Dwyer, Galvez, Gensemer, Goss, Hogstrand, Janssen, McGeer, Naddy, Playle, Santore, Schneider, Stubblefield, Wood, and Wu 2002).

Table 2.6 Critical body concentrations of seven metals for *Hyalella azteca* in Lake Ontario water. The Lethal Body Concentrations (LBC25s) were based on chronic (4-week) water only, toxicity studies (no gut clearance).

Metal	**4-week LBC25 (nmol.g-1)**	**Source**
Cd	270	Borgmann *et al* . 1991
Cu	1900	Borgmann and Norwood 1997
Hg	350	Borgmann *et al* . 1993
Ni	197	Borgmann *et al* . 2001a
Pb	73	Borgmann *et al* . 1993
Pb	180	MacLean *et al* . 1996
Tl	290	Borgmann *et al* . 1998
Zn	1500	Borgmann and Norwood 1997

The BLM approach has significant implications for advances in the area of metal mixture risk assessment. Once gill-metal stability constants have been determined for a number of metals, it will be possible to model how the different metal ions compete with each other, and the other ligands in any given system, for binding sites on a gill surface. In theory, if two metals compete for binding to the same site of toxic action on the organism, it should be possible to model the total metal bound to that site, and hence predict metal toxicity using a mechanistic BLM approach in what would be a much more advanced type of concentration addition model. Alternatively, if two metals do not compete for the same binding site on the organism, then the BLM may provide more reliable estimates of bioavailability of the metals singly, which can be combined in more accurate effects addition models. When such models are available, we should be better able to predict the outcome of the exposure of aquatic organisms to a given mixture of metals.

Unfortunately the BLM approach is not yet available to assess metal mixtures. So, what is the best approach currently available for a site-specific risk assessment of metal mixtures? The best approach is probably to do an assessment of the toxicity of the mixture as present *in situ*, including a combination of in situ community composition and laboratory toxicity testing of field collected water and sediment samples. This can be combined with bioaccumulation measurements, at least in those organisms in which metal bioaccumulation has been shown to correlate with toxic effects. Critical body concentrations have been determined for the amphipod *Hyalella azteca* (Table 2.6) based on detailed regression analysis of mortality against metal accumulated. This expresses toxicity as a function of the amount of metal actually accumulated by the organism and thus automatically takes bioavailability into account. It has been shown that metal bioaccumulated (Ni body concentration) provides a much more reliable prediction of toxicity than do concentrations in the sediment (Borgmann *et al.* 2001a). In this example the total range in LC50s determined for a variety of sediment types was over 20 fold based on sediment concentration, compared to a less than 3 fold variation based on body concentration. Borgmann *et al.* (2001b) demonstrated how critical body concentrations can be used to interpret the biological significance of environmental metal contamination. They were able to demonstrate that nickel was the primary cause of sediment toxicity since it exceeded its critical body concentration whereas other metals, though clearly elevated in the tissues of the test organism, did not approach critical levels.

Existing mixture models (*e.g.*, the Toxic Unit approach) could also be used to predict the impact of mixtures from chemical data alone, but as indicated above, the large variability in metal bioavailability from water or sediment would make it difficult to determine significant interactions and impacts.

2.5 REQUIRED RESEARCH

Research is still needed to determine the most appropriate methods of quantifying the effects of metal mixtures, particularly for environmental risk assessment (ERA). The impact of metal contamination on an aquatic organism is usually a function of bioaccumulation of that metal. However, bioaccumulation is dependent on bioavailability. Tissue concentrations of the metal should be a better predictor of biological effects than measured concentrations in water or sediment. The relationship between metal bioaccumulation and effect (toxicity) and the impact of metal mixture interaction on that relationship, must be determined in controlled laboratory experiments. The relationship between metal accumulation and environmental concentrations can be studied both in the laboratory and in situ. The relationship between metal accumulation (including the effects of metal-metal interactions) and concentrations in the environment is part of the exposure assessment in a risk assessment framework, and the relationship between accumulated metal and toxic effects is part of the effects assessment. This is a more sophisticated approach than attempting to perform risk assessments from the relationships between toxicity and environmental concentrations directly. To date, metal mixture toxicity models have been based almost exclusively on metal concentrations in the environment, rather than metals accumulated in the organism. However, research is being conducted by the Metals in The Environment Research Network (MITE-RN) to integrate the effect:accumulation functions of each metal into a metal mixture model in which an effects addition formula will be compared to a concentration addition formula, both based on body concentration.

The BLM approach explicitly recognizes the link between bioaccumulation and effects. Consequently, application of the BLM has the potential for improving our understanding of metal-metal interactions, especially if one metal affects the accumulation of another. However, the BLM has been applied primarily to acute toxicity. Broad applicability to chronic toxicity needs to be verified. Acute toxicity of metals to fish has generally been ascribed to metal-gill interactions (Paquin, Gorsuch, Apte, Batley, Bowles, Campbell, Delos, Di Toro, Dwyer, Galvez, Gensemer, Goss, Hogstrand, Janssen, McGeer, Naddy, Playle, Santore, Schneider, Stubblefield, Wood, and Wu 2002) but chronic effects for some metals could result from metal effects at internal sites deeper within the organism as well as uptake from the gut. Metal uptake then becomes a function of not just binding to the gill, but also uptake and transport processes within the body. This could make chronic mixture models more complex than acute mixture toxicity models. Nevertheless, the approach is worth pursuing.

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CHAPTER 3

Saturation models of Arsenic, Cobalt, Chromium and Manganese bioaccumulation by *Hyalella azteca*

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ABSTRACT

Bioaccumulation of As, Co, Cr and Mn by the benthic amphipod *Hyalella azteca* in Burlington City tap (Lake Ontario) water was measured in four week tests. Bioaccumulation increased with exposure concentration and demonstrated an excellent fit to a saturation model (r^2: 0.819, 0.838, 0.895 and 0.964 for As, Co, Cr and Mn respectively). The proportion of total body Mn eliminated during a 24 h depuration period decreased as Mn body concentration increased, apparently due to a saturation of the elimination rate. The high maximum body concentration of 116000 nmol•g^{-1} appears to result from the saturation of the Mn excretion which is slightly greater than the maximum Mn uptake rate. Elimination rates for As, Co and Cr were not dependent on body concentration. The four elements were not physiologically regulated in *Hyalella*. Their body concentrations should be good indicators of bioavailability and useful for environmental assessment.

3.1 INTRODUCTION

A number of researchers have determined that the concentration of a chemical in the organism (expressed as body concentration, critical internal concentration, tissue residue, tissue concentration or body burden) is better for predicting effects than environmental measures such as water concentration, sediment concentration, QSAR's, or equilibrium partitioning (Connell, 1995; Driscoll and Landrum, 1997; Niimi and Kissoon, 1994). The use of body concentration as a measure of bioavailability negates the impact of binding factors, ligands, and interactions that may occur in the exposure media, as well as integrate accumulation from exposures that may be pulsed, from multiple compartments and multiple sources (Hickie et al., 1995; Landrum et al., 1992).

In order for body concentrations to be a useful indicator of toxic effects, however, bioaccumulation of the element must increase with exposure concentration. The element cannot be regulated, such as Cu in *Hyalella* (Borgmann et al., 1993; Borgmann and Norwood, 1995) or Zn in the marine amphipod *Allorchestes compressa* (Ahsanullah and Williams, 1991) or Cr in the marine sponge *Halichondria panacea* (Hansen et al., 1995). The accumulated element must not be sequestered and stored in a non-toxic form such that there is no relationship between increasing body concentrations and increased adverse effects such as in barnacles (Rainbow and White, 1989) and *Mytilus edulis*, which can trap chromium into insoluble forms with phosphorus and sulphur (Chassard-Bouchaud et al., 1989). When physiological regulation or internal sequestration are not interfering factors, body concentrations of single metals have been shown to be useful indicators of toxic effects in aquatic invertebrates even in the presence of various complexing agents (Biesinger et al., 1982; Borgmann et al., 1991; Borgmann and Norwood, 1997; Borgmann and Norwood, 1999; McCarty, 1991; Meador et al., 1993). Therefore, in order for body concentration to be a meaningful indicator of a bioavailable element and its possible

effects, a clear understanding of the bioaccumulation pattern and relationship to exposure concentrations is required.

The Saturation Model has been recently shown to provide a better or equally good fit as the standard allometric model for describing the relationship between exposure and bioaccumulation of the metals Cd, Cu, Hg, Ni, Pb, Tl and Zn as well as the organo-metal, tributyltin (Borgmann et al., 2004). The Saturation Model can provide more insight into accumulation and acclimation since it is based on the assumption that ions bind to a finite number of sites, internally, externally or at transport sites. The saturation model is consistent with the biotic ligand model (BLM, (Paquin et al., 2002) in which toxicity is assumed to be a function of the amount of metal bound to a specific ligand, which can become saturated.

The exposure to bioaccumulation relationships of the metalloid As and the metals Co, Cr and Mn by *Hyalella azteca* were, therefore, studied using the saturation model. The objectives of the study were to determine:

1. If the bioaccumulation of each metal or metalloid demonstrates a clear dose-response relationship
2. If this relationship can be described by the Saturation Model
3. If the Saturation Model can be applied to bioaccumulation before and after a 24 h depuration period.

3.2 THEORY

3.2.1 Body Concentration at Steady State

A number of bioaccumulation models have been used for describing metal uptake in aquatic organisms with the simplest being the allometric model (McGeer et al., 2003). However, Borgmann et al., (2004) demonstrated that a more mechanistically based saturation model can describe bioaccumulation equally well or better than the allometric model for seven metals in *Hyalella*. It is described by

$$C_{TB} = \max \bullet C_w \bullet (K + C_w)^{-1} + C_{Bk} \quad (1)$$

Where C_{TB} is the total body concentration of the metal, max is the maximum above-background accumulation of the metal, C_w is the metal concentration in water, K is the half saturation constant (the concentration of C_w at which C_{TB} is halfway between the maximum accumulation and the background), and C_{Bk} is the background body concentration obtained from control animals (ie. absence of any added metals in the water media).

As explained by Borgmann et al., (2004), the biotic ligand model (BLM, (Di-Toro et al., 2001; Paquin et al. 2002) can be described by equation (1) such that max is the number of metal binding sites

(typically on a fish gill) and K is the inverse of the metal binding strength to that site. Alternatively, C_{TB} could be the ratio of the metal uptake rate divided by metal excretion rate (Borgmann et al., 2004). In this case, the metal accumulated at steady-state would be given by

$$C_{TB} = k_u \bullet C_w \bullet k_e^{-1} + C_{Bk} \quad (2)$$

where k_u is the uptake rate constant and k_e is the excretion rate constant. If the uptake rate saturates, then k_u can be replaced with

$$k_u = V_{umax} \bullet (K_{ux} + C_w)^{-1} \quad (3)$$

where V_{umax} is the maximum uptake rate and K_{ux} is the metal concentration in water at which metal uptake rate is half of maximum (V_{umax}). Combining equations (2) and (3) produces equation (1), with max = $V_{umax} \bullet k_e^{-1}$ and $K = K_{ux}$.

3.2.2 Gut Clearance and Elimination Rates

Gut clearance is necessary for determination of contaminants accumulated in *Hyalella* exposed to sediment (Neumann et al., 1999), otherwise the resulting concentration could be due to the contaminant absorbed to sediment particles in the gut plus contaminant accumulated in the tissues. However, during a 24 h depuration period, during which the gut is purged, some contaminants may also be excreted from the body tissues. Although gut clearance is usually not required in water-only metal exposures, where metal in the gut is generally minimal, it is useful to estimate metal excretion from the body after a 24 h depuration in order to make direct comparison with sediment tests. The body concentration can be described by

$$(C_{TB} - C_{Bk}) = (C_{TB0} - C_{Bk}) \bullet e^{-ke \bullet t} \quad (4)$$

where C_{TB0} is the total body concentration at time 0. If t = 24 h and k_e is independent of internal metal concentrations, then the ratio $(C_{TB} - C_{Bk}) \bullet (C_{TB0} - C_{Bk})^{-1}$ is constant at 24 h. If C_{TB0} is given by equation (1), then the bioaccumulation data after 0 and 24 h depuration can be combined and described by the equation

$$C_{TB} = \text{max} \bullet C_w \bullet (K + C_w)^{-1} \bullet (1 - \text{loss} \bullet \text{dep}) + C_{Bk} \quad (5)$$

where dep is a dummy variable set to 1 for 24 h depurated animals and 0 for animals that are not depurated, and loss represents the proportion of metal excreted in 24 h.

3.2.3 Saturation of Elimination Rate

Elimination rates could reach a maximum, analogous to metal uptake rates (Eq. 3). If this occurs, then the constant k_e in equations (2) and (4) may need to be replaced with

$$k_e = V_{emax} \bullet (K_{ex} + C_{TB} - C_{Bk})^{-1} \quad (6)$$

where V_{emax} is the maximum elimination rate and K_{ex} is the above-background metal body concentration at which metal elimination rate is half of the maximum. Combining equations (2), (3) and (6) also produces a formula analogous to equation (1), but with;

$$\text{max} = V_{umax} \bullet K_{ex} \bullet (V_{emax} - V_{umax})^{-1} \quad (6b)$$

$$K = K_{ux} \bullet V_{emax} \bullet (V_{emax} - V_{umax})^{-1} \quad (6c)$$

3.3 METHODS

Twenty, 0 - 1 week old *Hyalella* were added to 250 mL of test medium with a single piece of 5 by 5 cm cotton gauze in 500 mL Erlenmeyer flasks with inverted polyethylene sample cups as covers (Borgmann et al., 1989, 1991, 1993). Experiments were conducted in an incubator at 25°C with a 16 h light:8 h dark photoperiod. Weekly, static renewal of media and contaminants were carried out during the 4-week (chronic) test. Food additions (TetraMin fish food flakes ground to 500 *u*m mesh size), consisted of two, 2.5 mg feedings during week-1 and 2; three, 2.5 mg feedings in week-3 and two 5.0 mg feedings in week-4. The increase in food per week was incorporated to allow for animal growth throughout the experiment. Test media consisted of de-chlorinated Burlington city tap water originating from Lake Ontario (mean±95% Confidence Interval (C.I.): dissolved organic carbon 1.1±0.36 mg•L^{-1}, dissolved inorganic carbon 20±0.32 mg•L^{-1}, Alk 85±1.06 mg•L^{-1}, Cl 674±0.53 µmol•L^{-1}, SO_4 314±0.98 µmol•L^{-1}, SiO_2 19±0.10 µmol•L^{-1}, Ca 870±0.41 µmol•L^{-1}, Mg 351±0.09 µmol•L^{-1}, Na 561±0.31 µmol•L^{-1}, K 40±0.02 µmol•L^{-1}, pH 8.2±0.06 and conductivity 315±6.5 µs•cm^{-1}; analyses were conducted by the National Laboratory for Environmental Testing, Environment Canada). Two replicates were run of a concentration series for each metalloid or metal per test, controls were usually run in triplicate and each test was repeated at least once. Stock solutions of each metalloid or metal were prepared with the analytical grade salts of sodium arsenate ($Na•2H•AsO_4•7H_2O$), cobalt chloride ($CoCl_2•6H_2O$), sodium chromate ($Na•2CrO_4$), and manganous chloride ($MnCl_2•4H_2O$), dissolved in de-ionized water (Milli-Q).

Ammonia, pH, conductivity and oxygen concentrations were measured at the beginning (prior to animal additions) and end of each renewal period (mean±95% C.I.: ammonia 0.03±0.005 mmol•L^{-1}, pH 8.3±0.04, conductivity 313±4.3 µs•cm^{-1}, oxygen 8.2±0.19 mg•L^{-1}). Also, at the beginning and end of each renewal period, 1 mL filtered (0.45 *u*m Millipore membrane filter) and unfiltered water samples were collected and preserved with 10 µL nitric acid (Omni-pure) for metalloid or metal analyses. Survival was recorded at each renewal period. Final survival was recorded at the end of the 28 day exposure. One half of the survivors (or all survivors if less than 5 animals survived) were rinsed with

50 μM ethylene-diamine-tetra-acetic acid (EDTA) in de-chlorinated Burlington city tap water to remove any loosely adsorbed metal (Borgmann and Norwood, 1995; Neumann et al., 1999), weighed wet, and then placed in a pre-cleaned cryovial and dried at 60°C for 72 h before determination of dry weight. The remaining animals were also rinsed with, and then placed in, 60 mL of the same EDTA media along with a small piece of cotton gauze and fresh food for 24 h. This was analogous to the procedure used to purge the guts of amphipods in sediment tests (Neumann et al., 1999). EDTA was added to the solution to bind any contaminant released from the animal during the depuration so that the animal could not reabsorb the contaminant. Wet weight was determined after 24 h, and then the animals were placed in a pre-cleaned cryovial and dried at 60°C for 72 h before determination of dry weight.

3.3.1 Metalloid and Metal Analyses

Digestion of tissue samples were based on the methods of Borgmann et al (1991) and Stephenson and Mackie (Stephenson and Mackie, 1988). Four dried amphipods per cryovial sample were weighed and digested with 70% ultra-pure nitric acid, at room temperature for 6 days, followed by an addition of 30% hydrogen peroxide for 24 hs. Each sample was then made up to final volume with de-ionized water (Milli-Q) such that the final sample matrix consisted of 25 μL HNO_3, 20 μL H_2O_2, and approximately 1.2 mg dried *Hyalella* per mL. Four digestion volumes were used based on dry weight ranges of 0-0.749 mg, 0.750-1.499 mg, 1.500-2.249 mg and >2.249 mg in final volumes of 0.5, 1.0, 1.5, and 2.0 mL respectively.

The four elements (As, Co, Cr & Mn) in water and tissue samples were analyzed on a Varian SpectraAA 400 graphite furnace, atomic absorption spectrophotometer with Zeeman background correction. All analyses were performed in partition tubes. Analyses of arsenic in water were done with a nickel modifier and in tissues samples with a palladium/ascorbic acid modifier. Manganese analyses were also done with a palladium/ascorbic acid modifier, whereas both cobalt and chromium analyses did not require a modifier. The certified reference material CRM-DW from High-Purity Standards, Charleston, South Carolina was analyzed with every batch of samples with mean ± 95% C.I. percent recoveries of 97.8±5.0, 102.0±1.5, 89.0±5.3 and 102.1±4.5 for As, Co, Cr and Mn respectively. As well, method blanks were run with every batch of samples. Quality control blanks and standards were run every 10^{th} sample to correct for background contamination and drift.

3.3.2 Data Analyses

Comparisons were made between filtered and unfiltered water concentrations, initial and final day concentrations of each renewal period and water concentrations of repeat experiments. Bioaccumulation relationships were calculated with day 28 data. These relationships were determined

by non-linear regression of body concentration against total dissolved, water concentration. Free ion concentration was not calculated since all experiments were conducted in the same media in which the predominant complexation agents were inorganic anions present in great excess over the metalloid or metals, thus the free ion concentration would be proportional to the total dissolved metalloid or metal concentration. As well, results in relationship to total dissolved water concentration are directly comparable to previous work with other contaminants (Borgmann et al., 2004).The relationship between body concentration, expressed as dry weight, and concentration in the water for As, Co and Cr was fit to equation (5) with Systat version 10 for Windows; C_{Bk} was the background body concentration obtained from control animals (i.e. absence of any added metalloid or metals) and dep was set to 1 for 24 h depurated animals and 0 for animals that were not depurated in order to calculate the loss constant.

The loss of Mn from *Hyalella* after 24 h was dependent of the concentration of Mn in water and *Hyalella*. Therefore, the Mn accumulation data prior to gut clearance (C_{TB0}) were first fit to equation (1), and then the Mn accumulation data after the 24 h gut clearance (C_{TB24}) were fit to

$$(C_{TB24} - C_{Bk}) = (C_{TB0} - C_{Bk}) \bullet e^{-(Vemax / (Kex + CTB0 - CBk)) \bullet t} \quad (7)$$

Equation (7) was obtained by combining equation (4) and (6), but with the assumption that C_{TB} (Eq. 6) was approximately equal to C_{TB0} within the first 24 h of excretion so that the equation could be solved.

Bioaccumulation data were log transformed prior to statistical analyses to normalize the data and equalize variances. The 95% confidence limits for the log values of max, K and max$\bullet K^{-1}$ were obtained with the "funpar" (function parameter) command of Systat 10. These were then back-transformed to original values for display in Table 3.1. A dry weight to wet weight ratio ($D \bullet W^{-1}$) was determined for each experiment. The wet-weight Bio-Concentration Factor (BCF) was calculated by

$$BCF = (max) \bullet (D \bullet W^{-1}) \bullet 1000 \bullet K^{-1} \quad (8)$$

3.4 RESULTS

In order to determine the water concentration that best represented the true exposure, a number of analyses were conducted. Both filtered and non filtered samples were collected and compared. There was no significant removal of metalloid or metal due to filtering (2-way ANOVA; As P=0.561 N=78, Cr P=0.401 N=70, Mn P=0.870 N=60) except for cobalt in which filtering reduced Co concentration by

Table 3.1 Maximum metal accumulation (max) and half saturation constant (K) with 95% confidence limits (CL), r^2, number of data points (N), mean dry to wet weight ratio (D/W, S.D = 0.036-0.061, N = 54-75), bio-concentration factor (BCF = max X K^{-1} X 1000), metal lost in 24 h depuration (loss), and background metal concentration (C_{Bk}); for metal accumulation fit to a saturation bioaccumulation curve.

Metal	N	r^2	max	CL	K	CL	max/K	CL	D/W	BCF	loss	CL	C_{Bk}	CL
			(nmol•g^{-1})		(nmol•L^{-1})		(L•g^{-1})		Ratio	wet	(%•day^{-1})		(nmol•g^{-1})	
As	30	0.819	219	(117-410)	3230	(1010-10300)	0.0678	(0.0364-0.123)	0.295	20.0	33.6	(18.3-48.9)	13.9	(6.64-29.0)
Co	34	0.838	674	(395-1150)	378	(183-780)	1.79	(1.36-2.34)	0.289	515	12.6	(-8.00-33.1)	<15.5	-
Cr	30	0.895	831	(632-1090)	1090	(721-1640)	0.764	(0.631-0.925)	0.262	200	3.67	(-7.80-15.1)	<1.31	-
Mn	38	0.964	116000	(56200-238000)	146000	(63000-341000)	0.790	(0.646-0.966)	0.261	207	48.0	(39.1-56.9)	94.2	(65.6-135)

	V_{emax}	(CL)	K_{ex}	(CL)
	(nmol•g^{-1}•day^{-1})		(nmol•g^{-1})	
Mn*	20800	(5740-75300)	20300	(3950-104000)

* Saturated Elimination Rates: maximum elimination rate (V_{emax}), and K_{ex} is the body concentration when k_e (excretion rate) is 1/2 V_{emax}

N.D. Not Determined

7.5±1.7% (95% C.I.) as determined by 2-way ANOVA (P=0.028 N=25). The loss of Co suggests that a small portion of the Co had bound to particulates. To determine loss of metalloid or metals from the exposure media (i.e. precipitation, binding to container wall and absorption to the gauze substrate), water samples were collected at the beginning and end of each renewal period (7 day) and the analytical results compared. There was a slight but non-significant loss of As, Cr and Co during each renewal period (2-way ANOVA; As P=0.183, Cr P=0.065 and Co P=0.494). However, there was a significant, 49% mean loss of Mn from the water between renewal periods (2-way ANOVA, P=4.3E-10). Therefore the mean of initial and final concentration of unfiltered samples from the four renewal periods were used to determine exposure concentrations for each metalloid or metal.

Metalloid or metal bioaccumulation by *Hyalella* in the chronic 4-week tests increased with increasing water concentration for all four elements (Figs. 1 to 4). Arsenic bioaccumulation fit a saturation curve (Fig. 3.1) with an r^2 of 0.819 (Table 3.1). In order to fit the data, a background term (C_{Bk}) of 13.9 nmol•g^{-1} was required and was plotted on Fig. (1) with the 95% C.I.. A maximum body concentration of 219 nmol•g^{-1} was determined (Table 3.1). Cobalt bioaccumulation fit a saturation curve (Fig. 3.2) with a r^2 of 0.838 (Table 3.1). No background term was necessary for cobalt since levels were less than the detection limit of 7.2 nmol•L^{-1} in control water and less than the average detection limit of 15.5 nmol•g^{-1} in control tissues (Table 3.1). Hence, control data are not included on Fig. (2). A maximum body concentration of 674 nmol•g^{-1} was determined (Table 3.1). The saturation model for cobalt was confirmed with an additional short-term (1-week) bioaccumulation test with larger, adult *Hyalella*. The shorter exposure period with adult *Hyalella*, which may be more tolerant of higher doses of Co, made it possible to collect living specimens exposed to the much higher doses. These body concentrations are included in Fig. (2) (stars) and fall on the curves representing the bioaccumulation model based only on the chronic (4 wk) data.

Chromium bioaccumulation fit a saturation curve (Fig. 3.3) with an r^2 of 0.895 (Table 3.1). A background term could not be estimated by the model because Cr levels were below the detection limit of 9.3 nmol•L^{-1} in control water. However, the mean control ± 95% C.I. tissue concentration of 6.2±2.26 nmol•g^{-1} was added to Fig. (3). Manganese bioaccumulation fit a saturation curve (Fig. 3.1) with an r^2 of 0.964 (Table 3.1). In order to fit the data, a background term (C_{Bk}) of 94.2 nmol•g^{-1} was required and was plotted on Fig. (4) with the 95% C.I.. A maximum body concentration of 116000 nmol•g^{-1} was determined (Table 3.1).

A decrease in total metalloid or metal body concentration during a 24 h depuration period represents metalloid or metal lost with the waste purged from the gut as well as elimination from the

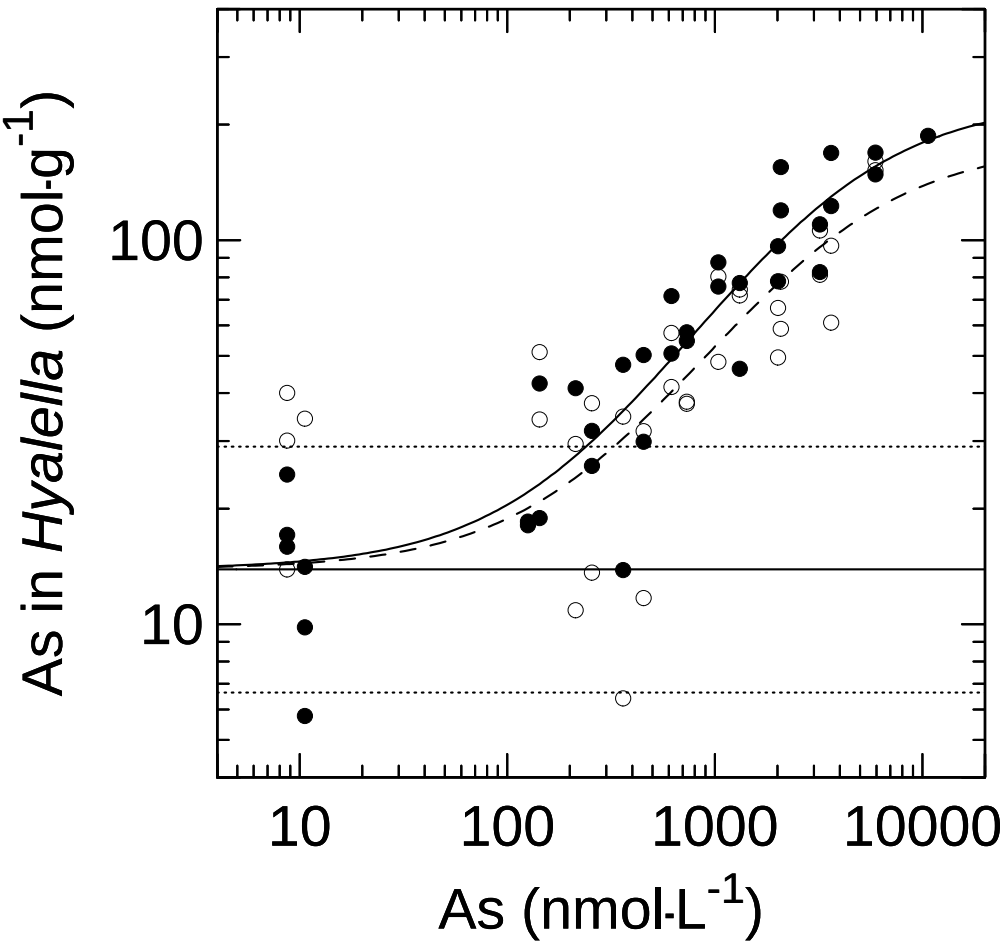

Figure 3.1 Arsenic accumulated by *Hyalella* after 4 weeks exposure to different As concentrations in water. Best fit regression to the saturation model for non-gut cleared (solid circles, solid line) and 24 hr. depurated (open circles, dashed line) total body concentrations. Solid horizontal line represents mean background body concentration (C_{Bk}) with 95% confidence intervals (doted lines).

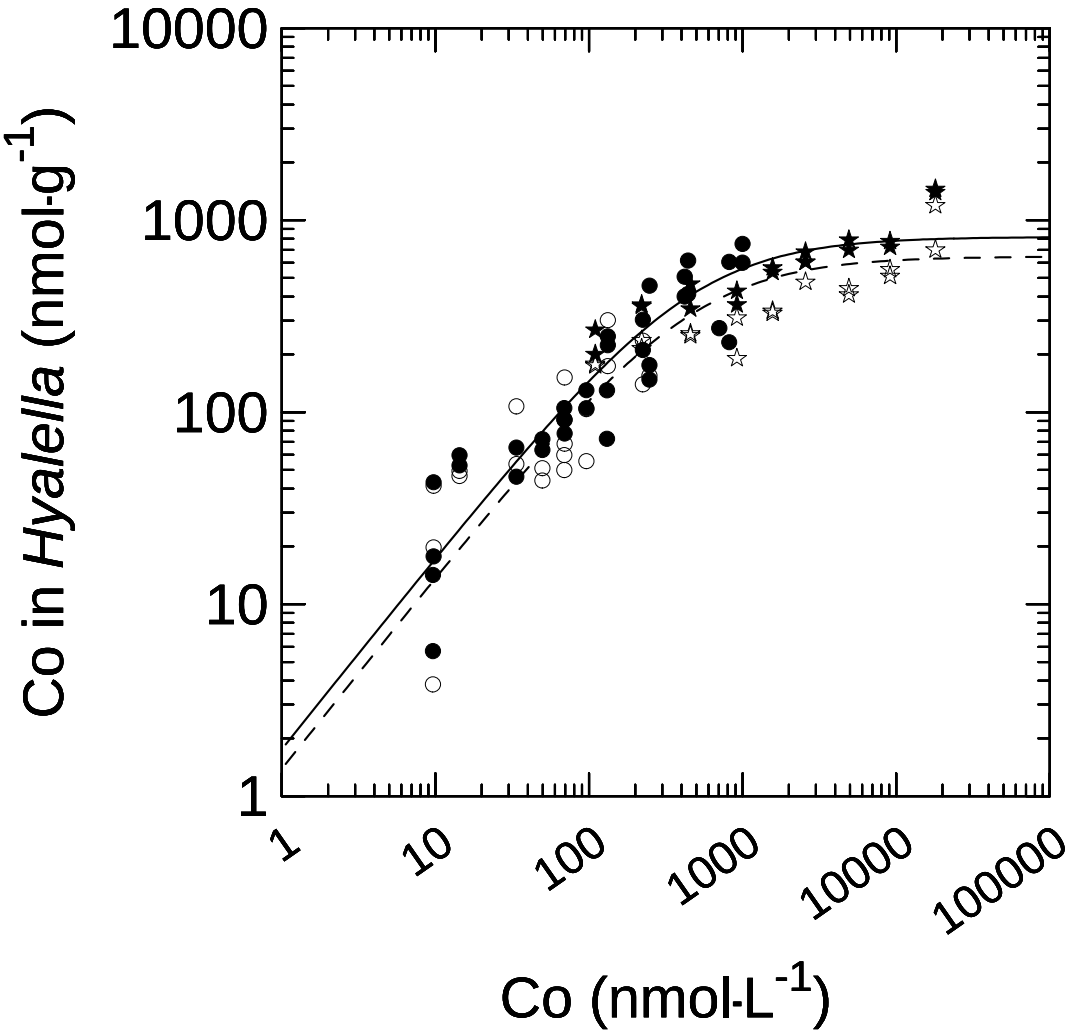

Figure 3.2 Cobalt accumulated by *Hyalella* after 4 weeks exposure to different Co concentrations in water. Format is the same as Figure 3.1. Cobalt accumulated by adult *Hyalella* after 1 week exposure to different Co concentrations in water (solid star non-gut cleared, open star 24 hr gut cleared).

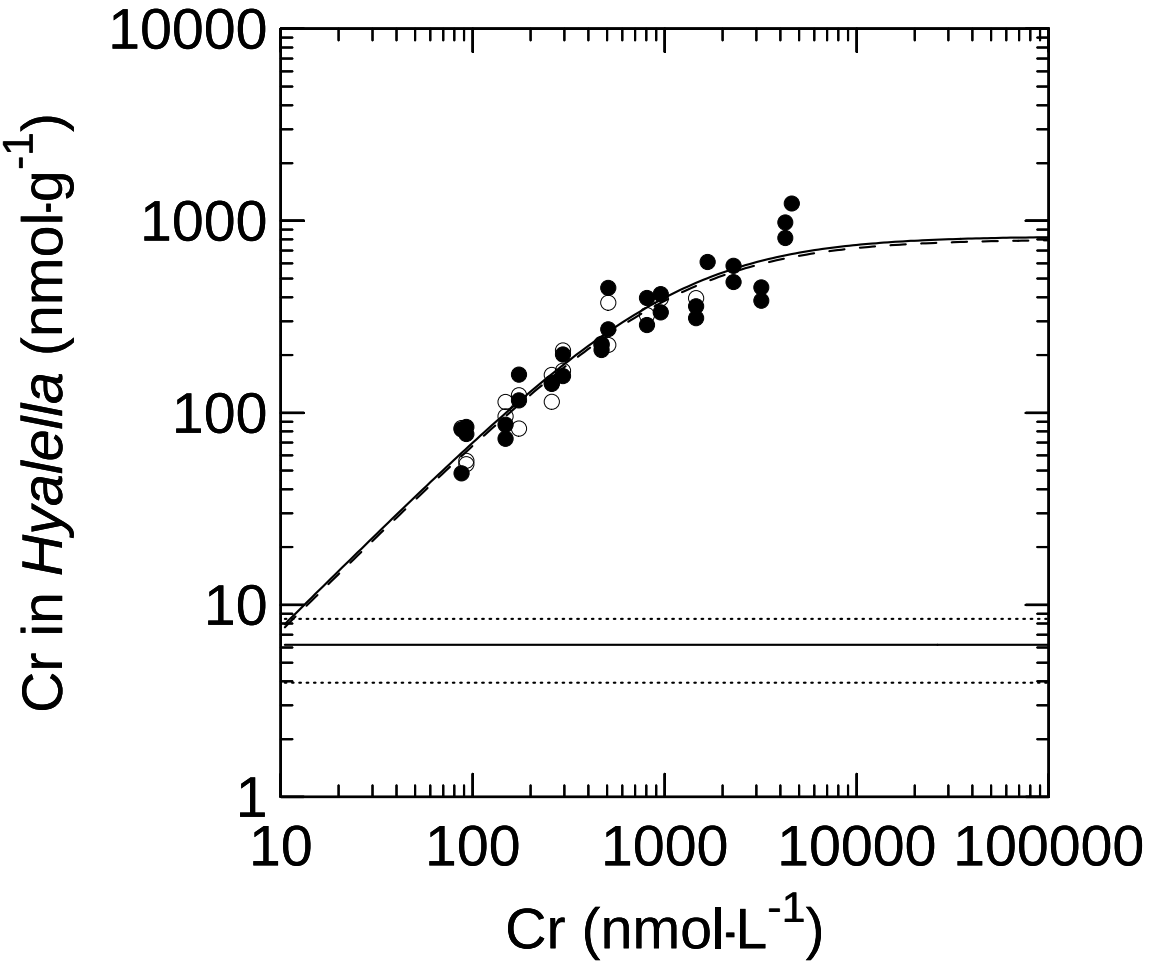

Figure 3.3 Chromium accumulated by *Hyalella* after 4 weeks exposure to different Cr concentrations in water. Format is the same as Figure 3.1.

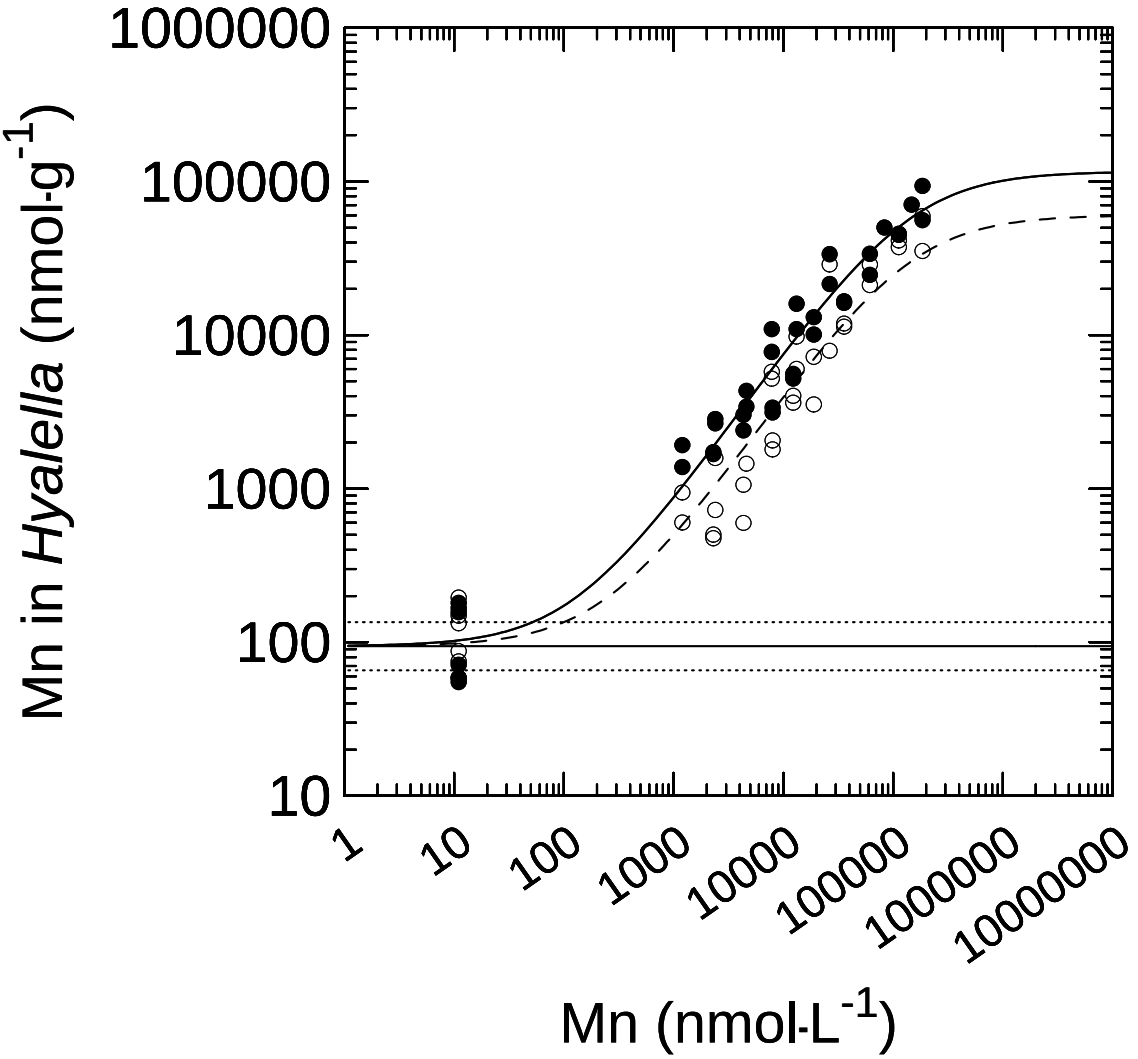

Figure 3.4 Manganese accumulated by *Hyalella* after 4 weeks exposure to different Mn concentrations in water. Format is the same as Figure 3.1.

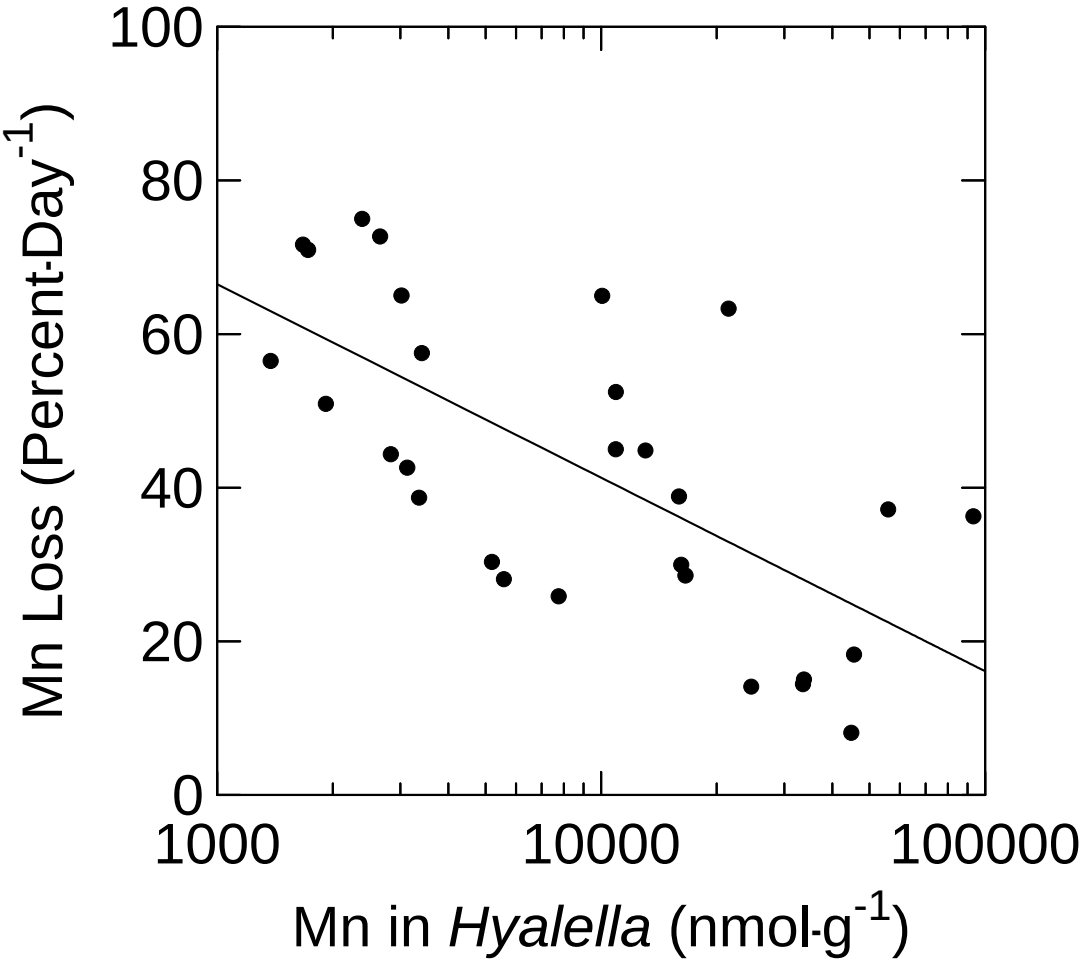

Figure 3.5 Regression of the Mn Loss (Percent per Day) versus non-depurated Mn body concentrations. (Regression $R^2 = 0.435$, Analysis of Variance P=0.00006)

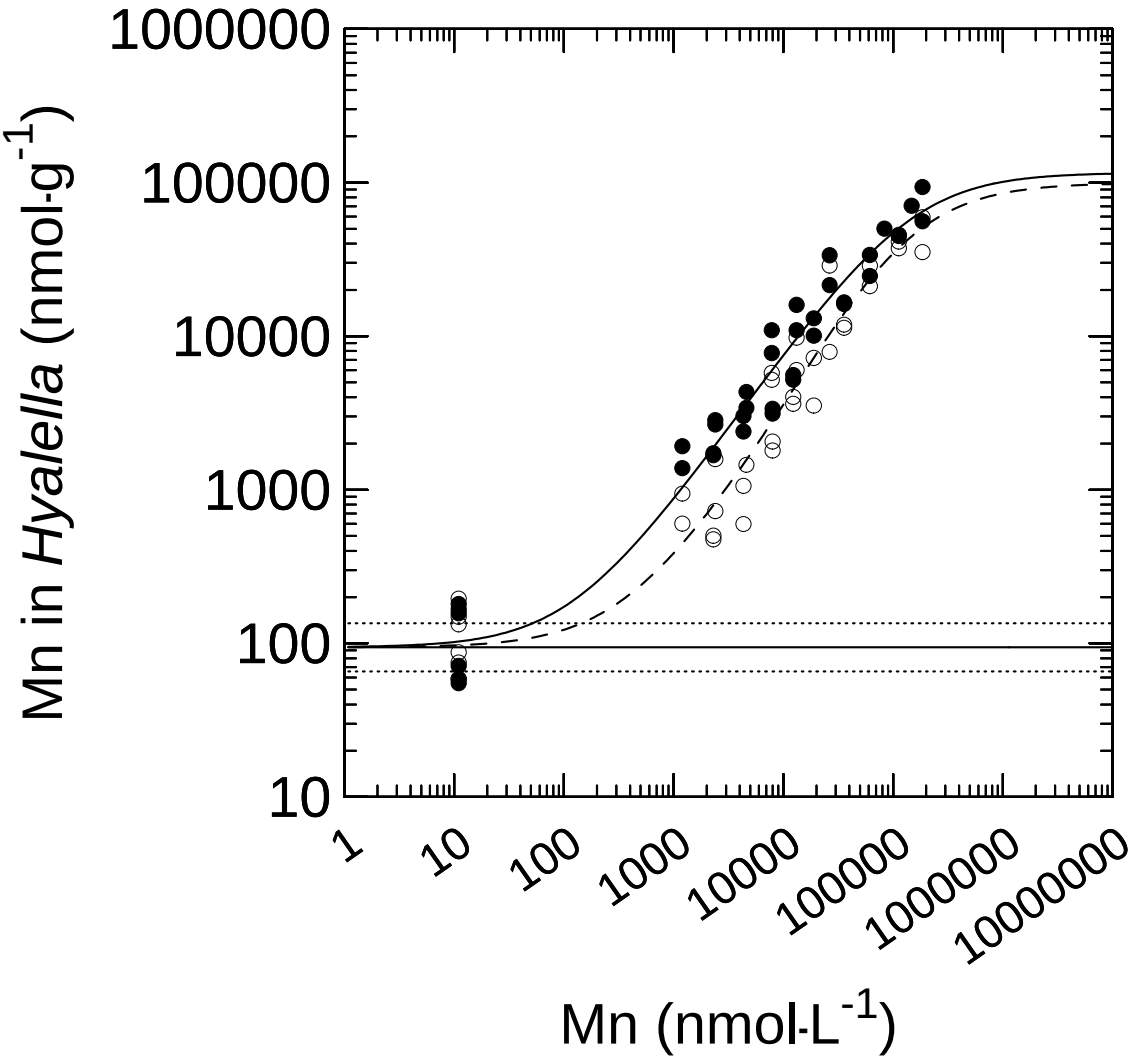

Figure 3.6 Mn accumulated by *Hyalella* after 4 weeks exposure to different concentrations in water. Best fit regressions to the saturation model for non-gut cleared (solid symbol, solid line) total body concentrations and best fit regressions to the saturation model with a variable elimination rate for the 24 hr. depurated (open circles, dashed line) total body concentrations.

tissue. The total body concentration of As decreased 33.6% (Table 3.1) and is depicted by open circles in Fig. (1). The dashed lines on the plots represent the saturation model for the 24 h depurated total body concentrations. Very little, and statistically non-significant amounts, of cobalt or chromium were lost from *Hyalella* during the 24 h depuration period (Figs. 2 and 3), equivalent to 12.6% and 3.67% loss per day respectively (Table 3.1). The loss constant calculated for Mn was 48.0% (Table 3.1) however when this term was used in the 24 h depuration model (dashed line of Fig. 4) the curve did not fit the data well. The data points within the 2,000 to 10,000 nmol•L^{-1} range fall below the curve, whereas above 20,000 nmol•L^{-1} the data points fall on or above the curve. The percentage of Mn cleared from *Hyalella* during the 24 hour depuration period decreased significantly with increased total body concentration (Fig. 3.5, Regression analysis; P=0.00006, r^2=0.435) At lower total body concentrations, *Hyalella* were able to clear greater than 66% of the accumulated Mn (gut plus tissue) but at higher total body concentrations could only clear just over 13%. This suggests that as total body concentration increased, the elimination process became saturated and *Hyalella* could only eliminate a much smaller portion of the total body burden. Therefore, the variable elimination rate equation (7) was used for the improved fit of the 24 hr depurated body concentration model for Mn (Fig. 3.6).

The saturation model described the bioaccumulation relationship for all four elements well (r^2 > 0.8 in all cases, Table 3.1). A background value was required for the best fit of the model for As and Mn data but not for Co and Cr (Table 3.1). Values for max ranged from 219 to 116000 nmol•g^{-1} and *K* values from 378 to 146000 nmol•L^{-1} (Table 3.1). The confidence intervals for max•K^{-1} were narrower than those for either max or *K*. Since these exposures were chronic toxicity tests, there were few survivors at high concentrations. Therefore, at lower concentrations, the slope (max•K^{-1}) of the curve can be described with much greater accuracy than either max or *K*. Even the confidence interval of max•K^{-1} for the Co bioaccumulation curve, which was well described through the high concentration range, was still narrower than those for max or *K* alone (Table 3.1).

3.5 DISCUSSION

The exposure concentration ranges tested for As, Co, Cr and Mn are within the range observed at heavily contaminated sites. For example, arsenic concentrations as high as 1090 nmol•L^{-1} were detected in Fox Lake, Saskatchewan, Canada (Pyle et al., 2002) which is 100 times higher than the background levels in our experiments and 10 times higher then our lowest test concentration. Cobalt concentrations as high as 1100 and 458 nmol•L^{-1} were detected in artificial lakes and Lake Gilow respectively from near Legnica, Poland (Samecka-Cymerman and Kempers, 2004). This level is 100 times higher than our lowest test concentration and equivalent to our highest, 4 week test concentration. Chromium levels reaching as high as 4115 nmol•L^{-1} were measured at a site in the Fez River, Morocco

(Koukal et al., 2004) which was similar to our maximum exposure at 5000 nmol•L^{-1}. Manganese levels as high as 27700 nmol•L^{-1} have been measured in overlay water from toxicity tests of sediments collected in the Sudbury area of Ontario, Canada (Borgmann et al., 2001) and as high as 9360 nmol•L^{-1} from the River Erh-Jen, Taiwan (Tien, 2004) which are approximately 3000 and 1000 times higher respectively than the background levels in our experiments and well within our test range (Fig. 3.4).

The bioaccumulation of the metalloid As, and each metal, Co, Cr and Mn, increased with increasing water concentration. Since these experiments were long term (4-week) exposures initiated with young animals which produced most of their biomass within the exposure period, these measurements should represent true steady-state body concentrations. The mechanistically based saturation model had an excellent fit for all four elements (r^2 ranged from 0.819 – 0.964, Table 3.1). These relationships fell within the range of r^2's for Cd, Hg, Ni, Pb TBT, Tl, Cu and Zn determined by Borgmann et al., (2004). Accumulation patterns varied between the four elements in a way that could be accounted for in the model. Both As and Mn had background tissue concentrations that had to be included in the model, however these levels were low and only represented 6 and <0.1 percent of the maximum body concentrations respectively. These background levels are similar to the <1% of highest Ni accumulation and 1.4% of maximum for Pb determined by Borgmann et al., (2004) and were very low in comparison to the essential elements Cu and Zn which displayed backgrounds of 33 and 28 percent of maximum respectively (Borgmann et al., 2004). Both Co and Cr did not require a background term in the saturation model.

TetraMin fish food was also digested and analyzed prior to use in the bioaccumulation experiments using the same methods as described for tissue analyses. The TetraMin contained 7, <0.07, 80 and 759 nmol•g^{-1} of Co, Cr, As and Mn, respectively. As well, the concentrations of Cu, Ni, Pb and Zn in TetraMin were 168, 25, 2 and 1150 nmol•g^{-1}, respectively. The food was, therefore, probably the source leading to the background body concentrations of As and Mn found in this study and the low concentrations of Ni and Pb and higher concentrations of Cu and Zn in Borgmann et al. (2004). Even though Co was detected in the TetraMin, it was at a much lower concentration than both As and Mn, and did not lead to detectable Co in the tissue. Chromium, on the other hand, was not detectable in the TetraMin nor in the control water, yet control animals did contain 6.2 ± 2.3 (95%C.I.) nmol Cr•g^{-1} (Fig. 3.3). Nevertheless, a background term was not required. The probable source of this Cr was the water at levels below the detection limit of 9.3 nmol Cr•L^{-1}.

Maximum body concentrations of 219, 674 and 831 nmol •g^{-1} for As, Co and Cr respectively (Table 3.1) were similar to the max concentrations of 512, 1760 and 314 nmol•g^{-1} for Cd, Hg and Pb respectively (Borgmann et al., 2004) since the 95% confidence intervals all overlap. *Hyalella* could, however, accumulate and tolerate much higher body concentrations of Mn with a max of 116000

nmol•g^{-1} (Table 3.1). This was significantly higher then the max levels of 3600 and 3550 nmol •g^{-1} for the essential elements Cu and Zn respectively (Borgmann et al., 2004).

For As, Co and Cr, loss rates calculated using the saturation model with 0 and 24 h depurated body concentrations (Eq. 5) were proportional (K_e, the elimination rate was constant) across all body concentrations. However, the total body elimination rate for Mn approached a maximum as total body concentration increased. Therefore, a variable elimination model was required (Eq. 7). The total body concentration of an element may be controlled not just by the number of external and internal binding sites, but also by the ratio between elimination and uptake rates. This can be demonstrated most clearly for Mn when uptake rate was 0 (i.e., during depuration in clean water). The proportion of Mn eliminated decreased with increasing total body concentrations. This would not be expected to occur if the maximum in Mn body concentration resulted from a saturation of external or internal binding sites. It is much more likely that the elimination rate was saturated and that the maximum body concentration was, therefore, a function of the relative rates of uptake and excretion. Therefore, not only can a specific binding site (ligand) on the organism be "saturated" but possibly also the elimination and uptake rates.

Although the above explanation satisfactorily explains the observed Mn bioaccumulation (Fig. 3.6) it is possible that the variable elimination rate was an artifact caused by a saturation of the food, and therefore gut contents, at a much lower water concentration than that leading to the saturation of uptake by *Hyalella*. If this were true, the half saturation constant for food should be much lower than that for Hyalella (K = 146000 nmol•L^{-1}, Table 3.1). To test if this was plausible, Mn binding to TetraMin fish food flakes was measured in a follow up study conducted under the same experimental conditions but without *Hyalella* present. This provided an estimate of the half saturation constant K_{food} = 327000 nmol•L^{-1}, which was more than 2 times higher than that for *Hyalella* (Table 3.1) suggesting saturation of food did not contribute significantly to the variable elimination rate observed.

To further investigate the potential for Mn incorporation through the diet, the theoretical maximum amount of Mn eliminated per day by excretion and gut clearance assuming Mn uptake from food alone was calculated. Under steady-state conditions, this would equal the Mn ingestion rate divided by the final body concentration. If all the TetraMin food offered had been ingested, Mn elimination would have been approximately 16% per day. This represents the maximum possible Mn elimination per day if food was the only source of Mn. Since the actual Mn depurated was much greater than this (Fig. 3.5), and since all the food was not eaten, most of the Mn was probably accumulated from water rather than from food.

No evidence of a variable excretion rate was obtained for the other three metals, but that does not exclude the possibility that some metal may have been accumulated through ingestion of metals

adsorbed to food. Previous experiments indicated that *Hyalella* accumulation of As and Co was increased by 7 and 10% respectively, and Cr was decreased by 15% in the presence of food compared to non fed animals (unpublished data). However, these data are difficult to interpret because the effect of starvation on metabolism and metal accumulation rates is not known.

If equations (3) and (6) correctly describe the mechanism of Mn uptake and elimination, and since the terms for elimination (V_{emax} and K_{ex}, Eq. 6) have already been estimated, then it is also possible to estimate the uptake rate coefficients V_{umax} and K_{ux} (Eq. 3) from max and K (Eq. 1) by rearranging equations (6b) and (6c). This gives V_{umax} = 17700 nmol Mn•g^{-1}•day^{-1}, which is slightly smaller than V_{emax} (20800 nmol Mn•g^{-1}•day^{-1}, Table 3.1) and K_{ux} = 21800 nmol Mn•L^{-1}. Based on these calculations, it appears that the Mn concentration in water at which the uptake rate reaches half of maximum (i.e., K_{ux} = 21800 nmol•L^{-1}) is considerably lower than the Mn concentration at which the body concentration reaches half of maximum (i.e., K = 146000 nmol•L^{-1}, Table 3.1). This occurs because K is equal to K_{ux} multiplied by V_{emax}•(V_{emax} - V_{umax})$^{-1}$ (Eq. 6c) and the latter quantity is large because V_{emax} is only slightly larger than V_{umax}. Hence, the very high maximum accumulation of Mn, as well as the high Mn concentration required to reach half maximum accumulation, appear to result from the saturation of the Mn elimination rate and V_{emax} is only slightly larger than V_{umax}. These calculations provide some insight into the uptake and elimination kinetics of manganese in *Hyalella*. However, this interpretation should be verified using full time-series uptake and depuration data.

Hyalella readily accumulated arsenate at exposure concentrations up to 10 µmol As•L^{-1} (Fig. 3.1 and Table 3.1) as did the shrimp *Lysmata seticaudata*, at concentrations up to 1.33 µmol As•L^{-1} (Fowler and Ünlü, 1978). However, the mean BCF for the shrimp was 2.6 compared to 20 for *Hyalella* (Table 3.1). In contrast, a number of marine species of shrimp, copepods and barnacles did not accumulate arsenate, at exposure concentrations ranging from 0.33 to 1.468 µmol As•L^{-1} in sea water for 22 to 28 days (Hunter et al., 1998; Lindsay and Sanders, 1990; Sanders et al., 1989). The mayfly (*Heptagenia sulphurea*) and the snail (*Physa fontinalis*) both freshwater species, did not bioaccumulate As in 10 day exposures with 1.33 µmol As•L^{-1} yet four other freshwater species (two amphipods, *Gammarus fossarum* and *Niphargus rhenorhodanensis*, one isopod, *Asellus aquaticus*, and one Trichoptera insect, *Hydropsiche pellucidula*) accumulated 270, 302, 354 and 1431 nmol As•g^{-1}(dry wt) respectively in identical exposures (Canivet et al., 2001). It is evident by the above examples that different species demonstrate a wide range of arsenic bioaccumulation patterns and therefore the specific pattern must be known if the bioaccumulation data are to be used as a predictor of impact. This applies to any contaminant under study, as demonstrated below by the other three metals in this paper.

Hyalella demonstrated increased accumulation of cobalt with increasing exposures up to 20 µmol Co•L^{-1} for 28 days (Fig. 3.2 and Table 3.1) very much like the marine amphipod

Echinogammarus pirloti with exposures up to 17 µmol Co•L^{-1} for 21 d (Rainbow and White, 1990). Rainbow and White (1990) were able to demonstrate similar bioaccumulation trends for the decapod *Palaemon elegans* as well as the barnacle *Elminius modestus*. However, bioaccumulation in these three species did not appear to reach a maximum as it did in *Hyalella*. As well, Rainbow and White (1990) indicated that there was "no evidence for significant excretion of accumulated cobalt, at least during the time periods" studied. This was similar to the non-significant loss rate of 13% per day of cobalt from *Hyalella* (Table 3.1).

Hyalella demonstrated increased accumulation of chromium (VI) with increasing exposures up to 5 µmol Cr•L^{-1} for 28 days (Fig. 3.3 and Table 3.1). Rainbow trout (*Salmo gairdneri*) accumulated increasing amounts of Cr (VI) in gill, liver, kidney and digestive tract with increased exposure as well (Van der Putte et al., 1981). However, they required a much higher exposure concentration of 769 µmol Cr•L^{-1} for 4 days. Chromium elimination was slow under clean conditions and resulted in only 25% and 12% reductions in whole body and gill concentrations after 3 days respectively (Van der Putte et al., 1981). This gill loss rate was similar to the non-significant 3.7% loss in 1 day exhibited by *Hyalella* (Table 3.1). Loss rate of Cr (VI) was also fairly slow in the marine mussel *Mytilus galloprovincialis* with only 13% loss in 1 day and only 35% loss in 11 days (Parlak et al., 1999). However, the mussels were able to excrete 48% and 62% of Cr (III) in 1 and 11 days respectively. Chromate, Cr(VI), exposures as high as 4.8 µmol Cr•L^{-1} for 28 days resulted in accumulation as high as 885 nmol Cr•g^{-1} in the marine amphipod *Allorchestes compressa* (Ahsanullah and Williams, 1991), which is very similar to the max of 831 nmol Cr•g^{-1} determined for *Hyalella* (Table 3.1). The authors suggest that *A. compressa* can regulate Cr in sea water up to 1.92 µmol Cr•L^{-1} above which the amphipod begins to accumulate Cr. Their control animals contain up to 596 nmol Cr•g^{-1} whereas control *Hyalella* body concentrations were 6.2 nmol Cr•g^{-1} dry wt. The marine sponge *Halichondria panacea* also appears to regulate Cr and maintained a constant body concentration of approximately 192 nmol Cr•g^{-1} with exposures as high as 19.2 µmol Cr•L^{-1} (Hansen et al., 1995).

Hyalella do not regulate Cr since there was an increased body concentration with increased exposure concentration (Fig. 3.3). *Daphnia magna* also demonstrated increased Cr accumulation with increased exposure (Kungolos and Aoyama, 1993). The marine polychaete *Neanthes arenaceodentata* as well, demonstrated increased Cr accumulation with increased exposure to waterborne chromate (Oshida and Word, 1982) and a calculated mean BCF of 200 for all data combined. The accumulation pattern and BCF for this polychaete was similar to *Hyalella* (Table 3.1) except the accumulation does not appear to saturate. The freshwater crayfish, *Procambarus clarkii*, also demonstrated increased accumulation with increased exposure but required exposure concentrations 1,000 times higher to achieve similar body concentrations as *Hyalella* (Bollinger et al., 1997). Very little Cr was depurated

from these crayfish in 1 week clearance tests, with 55% to 100% of the Cr being retained, depending on the tissue (the carapace lost the most, all other tissues lost very little).

Accumulation and elimination kinetics studies with the Norway lobster *Nephrops norvegicus* (L.) indicated that Mn tissues concentrations would give a good indication of prevailing environmental conditions (Baden et al., 1999). They demonstrated rapid uptake and elimination half-lives that ranged from 0.8 to 4.2 days and 0.8 to 4.6 days respectively. They also determined a mean bioconcentration factor (BCF) of 2.4±0.82 (±SD) which is considerably lower than the BCF of 206 determined for *Hyalella* (Table 3.1). This could be caused by the salt water in the lobster exposures compared to freshwater for *Hyalella*. Hansen and Bjerregaard (1995) observed a linear increase in whole body BCF to as high as 12 after 23 d for the Sea Star *Asterias rubens* (L.) in a constant exposure. They determined an elimination half life of 36 d, which was considerably slower than the 48% loss per day we observed (Table 3.1) and rapid elimination observed by Baden et al., (1999). These data indicate that both *Hyalella* and the lobster tissue concentrations are good indicators of recent exposures to Mn whereas the sea star is not, at least in the time frames monitored.

Bioaccumulation of a metalloid or metal varies from one species to another. The rates of uptake and excretion, maximum accumulation (i.e. saturation), time to equilibrium, background concentrations and BCFs can be different for each metalloid or metal as well as for each organism. Therefore, to allow meaningful interpretation of bioaccumulation data, a good understanding of the organism and the way in which it handles a specific metalloid or metal is required. Toxic effects were observed with increasing exposure and bioaccumulation of all four contaminants. The relationship between contaminant bioaccumulation and effects (mortality and reduced growth) will be detailed in a follow-up paper.

3.6 SUMMARY

The bioaccumulation of As, Co, Cr and Mn by *Hyalella* demonstrated a clear dose-response relationship which could be described by the Saturation Model. The Saturation Model could be applied to bioaccumulation both before and after a 24 h depuration period. For Mn, however, accumulation after a 24 h clearance period was best described using a saturation model for initial accumulation combined with a saturation model for Mn clearance rates. This implies that, at least for Mn, maximum accumulation is a function of the relative rates of uptake and excretion, and not just a function of the number of binding sites inside or outside the animal. Based on comparisons to other organisms and habitats (i.e. marine vs. freshwater), it is apparent that bioaccumulation patterns vary considerably among different species, and that bioaccumulation data are only useful as an environmental assessment tool if the specific pattern of accumulation for each organism and metalloid or metal is understood.

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CHAPTER 4

Chronic toxicity of Arsenic, Cobalt, Chromium and Manganese to *Hyalella azteca* in relation to exposure and bioaccumulation.

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ABSTRACT

Chronic toxicity of As, Co, Cr and Mn to *Hyalella azteca* can be described using a saturation-based mortality model relative to total-body or water metal concentration. LBC25s (total-body metal concentrations resulting in 25% mortality in four weeks) were 125, 103, 152 and 57900 nmol g^{-1} dry weight for As, Co, Cr and Mn respectively. LC50s (metal concentrations in water resulting in 50% mortality in four weeks) were 5600, 183, 731, and 197000 nmol L^{-1} respectively. A hormesis growth response to As exposure was observed. Growth was a more variable endpoint than mortality for all four toxicants, however, confidence limits based on growth and mortality all overlapped, except Cr which had no effect on growth. Mn toxicity was greater in glass test containers compared to plastic. Bioaccumulation of As, Co, Cr, and Mn was strongly correlated with, and is useful for predicting, chronic mortality.

4.1 INTRODUCTION

Since toxicity is based on the effect that a toxicant produces at a target site within an organism, establishing the relationship between the concentration of the substance at the target site and the subsequent toxic effect can provide a tool for predicting toxicity (Landrum et al., 1992). This is the primary toxicological principle generally referred to as “dose-response” or “concentration-response” in which the response of an organism is proportional to the dose or concentration of the substance at the target site (Connolly, 1985;McCarty, 1991). In many cases the target site is unknown, or measurement of the substance at the site is not possible. Instead, surrogate measures of the target site concentration have been used. A number of researchers have determined that the concentration of a substance in the organism (expressed as body concentration, critical internal concentration, tissue residue, tissue concentration or body burden) was a better predictor of effect than water concentration, sediment concentration, or equilibrium partitioning (Connell, 1995;Driscoll and Landrum, 1997;Niimi and Kissoon, 1994). The use of metal and metalloid body concentrations as a measure of bioavailability may negate complications that can arise from uncertainties such as, interactions with other ions or molecules that may hinder or enhance bioaccumulation, multiple compartments of exposure, multiple sources and pulsed exposures (Hickie et al., 1995;Landrum et al., 1992). Body concentrations of single elements have been shown to be useful indicators of toxic effects in aquatic invertebrates, even in the presence of various complexing agents (Borgmann et al., 1991) and can help identify the cause of biological effects in sediment assessments (Borgmann et al., 2001a;Borgmann and Norwood, 1997;Borgmann and Norwood, 1999).

This research was undertaken to determine the toxicity of As, Co, Cr and Mn to the freshwater amphipod, *Hyalella azteca*. These elements are commonly found at metal contaminated sites and they are accumulated by *Hyalella*. However, unlike several other metals, their relative contribution to toxicity could not be assessed in previous sediment assessment studies because the relationship between

bioaccumulation and toxicity was not known (Borgmann et al. 2001a). Norwood et al. (2006) recently demonstrated that the metalloid As and the metals Co, Cr and Mn demonstrate a clear relationship between exposure concentration and bioaccumulation. The present paper examines the relationship between chronic toxicity (mortality and growth effects) and the exposure and the bioaccumulation data from Norwood et al. (2006).

4.2 THEORY

4.2.1 Metal Toxicity

The simplest metal-toxicity paradigm is the allometric model. It has been used to describe the relationship between mortality rate and metal concentration, in water or tissue (Borgmann et al., 2004;Borgmann and Norwood, 1995) in which overall mortality rate *m*, is expressed as:

$$m = m' + aC^{n} \quad (1)$$

where *m*' is the control mortality rate, *C* is the water or tissue metal concentration and a and n are constants. If applied to both water and tissue concentrations, this model can only be mathematically correct if the toxicant bioaccumulation also follows an allometric relationship. The model cannot be mathematically correct when applied to both water and body concentrations if the relationship between water and body concentrations follows a saturation curve. However, saturation curves are mechanistically based and are often more useful than allometric models for describing metal bioaccumulation (Borgmann et al. 2004). A more appropriate mortality saturation model has been described (Borgmann et al., 2004) in which the allometric relationship $a^{(1/n)}C$ in equation 1 is replaced with the saturation relationship; max"C (K" + C)$^{-1}$ such that

$$m = m' + [\max_{W}" \, C_{W} \, (K_{W}" + C_{W})^{-1}]^{nw} \quad (2a)$$

and

$$m = m' + [\max_{TBX}" \, C_{TBX} \, (K_{TBX}" + C_{TBX})^{-1}]^{nb} \quad (2b)$$

where $\max_{W}$" and $\max_{TBX}$" are the water and body metal concentrations when metal-induced mortality has reached a maximum, K_{W}" and K_{TBX}" are the water and total body metal concentrations respectively when metal-induced mortality is half of the maximum, C_{W} is the measured metal water concentration, and C_{TBX} is the background-corrected metal body concentration. The max" terms in Eqs. (2a) and (2b) can be replaced with LC50 (water concentration resulting in 50% mortality) or $LBC50_{X}$ (background-corrected body concentration resulting in 50% mortality), which are of greater toxicological interest, giving:

$$m = m' + (\ln(2)/t) \, [C_{W}(LC50^{-1} + K_{W}"^{-1}) \, (1 + C_{W} \, K_{W}"^{-1})^{-1}]^{nw} \quad (3a)$$

and

$$m = m' + (\ln(2)/t)\,[(C_{TBX}(LBC50_X^{-1} + K_{TBX}"^{-1})\,(1 + C_{TBX}\,K_{TBX}"^{-1})^{-1}]^{nb} \quad (3b)$$

where t is the exposure time corresponding to the LC50 and $LBC50_X$. These equations are consistent with the saturation uptake models for As, Co, Cr, and Mn (Norwood et al., 2006).

4.2.2 Growth Effects

The impact of the metals and metalloid on growth, expressed as final body size W (final wet weight after 4 weeks) was evaluated with a general growth model

$$W = W'\,(1 + aC^n)^{-1} \quad (4)$$

where W' is the control wet weight, C is the water metal concentration or background-corrected tissue metal concentration and a and n are constants (Borgmann et al., 1998). Since bioaccumulation was expressed as a saturation model in relation to water concentration, growth should also be expressed as a saturation model in relation to water or body concentrations to be mathematically consistent. However, saturation models, analogous to equations 2a and 2b for mortality, could not be satisfactorily fit to the final body size data for any of the four toxicants based on either water or body concentration. Therefore, the relationships of growth to water or body concentration were expressed with allometric models only. Due to this inconsistency, the IC25*s* (metal concentrations in water resulting in a 25% reduction in final body size) cannot be directly converted to $IBC25_X$*s* (total-body metal concentrations resulting in a 25% reduction in final body size) with the bioaccumulation model for each toxicant.

In some cases growth was stimulated at low toxicant concentrations (hormesis) and the exposure-response relationship could be described using

$$W = W'\,(1 + bC^m)(1 + aC^n)^{-1} \quad (5)$$

in which the term $(1 + bC^m)$ describes low-exposure concentration stimulation of growth and the $(1 + aC^n)^{-1}$ term over-rides the low-exposure term at higher concentrations.

4.3 METHODS

The chronic (4 week) toxicity test methods are described in (Norwood et al., 2006). All tests were conducted in 500 ml glass Erlenmeyer flasks. However, manganese exposure was repeated using 500 ml High Density Polyethylene (HDPE) wide mouthed containers for comparison to the results from the glass flasks since the laboratory was converting over to these significantly less expensive, shatterproof HDPE containers. Stock solutions consisted of sodium arsenate ($Na_2HAsO_4 \bullet 7H_2O$), cobalt chloride ($CoCl_2 \bullet 6H_2O$), sodium chromate anhydrous (Na_2CrO_4), and manganous chloride ($MnCl_2 \bullet 4H_2O$), dissolved in de-ionized water (Milli-Q). Therefore, each element was in the form As(V), Co(II), Cr(III) and Mn(II).

Survival was recorded weekly and at the end of the 28-day exposure. Wet weight, dry weight and body concentrations were determined for 0 and 24 hr gut purged survivors and water samples were analyzed as described by Norwood et al. (2006).

4.3.1 Bioaccumulation

Total body As, Co and Cr concentrations (C_{TB}, nmol g^{-1} dry weight) were calculated from metal concentration in water with the bioaccumulation model of Norwood et al. (2006) as follows;

$$C_{TB} = \max C_w (K + C_w)^{-1} (1 - \text{loss dep}) + C_{Bk} \quad (6a)$$

where max was the maximum above-background accumulation of the metal, C_w was the metal concentration in water, K was the half saturation constant (the concentration at which C_{TB} was halfway between the maximum accumulation and the background), C_{Bk} was the background body concentration obtained from control animals (i.e. no added metals) and dep was set to 1 for 24 h gut cleared animals, and to 0 for animals that were not gut cleared, in order to calculate the loss constant.

Total body Mn concentrations were calculated from water metal concentrations using equation (6a) for non-gut cleared animals. However, the following bioaccumulation model which accounted for saturation of the elimination rate (Norwood et al., 2006) was used to calculate the Mn body concentration of gut-cleared animals

$$(C_{TB24} - C_{Bk}) = (C_{TB0} - C_{Bk})\, e^{-(Vemax / (Kex + CTB0 - CBk))\, t} \quad (6b)$$

where C_{TB0} was the total body concentration at time (t) = 0, C_{TB24} was the total body concentration at t =1 (24 h), V_{emax} was the maximum elimination rate and K_{ex} was the above-background metal body concentration for which the metal elimination rate was half of the maximum.

Even though the body concentrations were measured (Norwood et al. 2006), the calculation of total body concentration was applied to all exposures. This was done in order to include partial effect treatments in which there were no surviving animals at the end of 4 weeks, yet the mortality rates could be determined based on the of surviving animals from weeks 1, 2, and 3.

4.3.2 Mortality

Mortality rates were determined from the regression of the natural logarithm of survival against time up to week 4. Mortality rate data were 4^{th} root transformed prior to statistical analyses to normalize the data and then fit to the saturation equations (3a) and (3b) to relate mortality to exposure concentration or tissue concentration. The 4^{th} root transformation produced more uniform variance than log or square root transformations. All models were fit using non-linear regressions in Systat 10 which provides estimates and 95% confidence limits for all parameters (constants, coefficients and exponents). In some cases, the estimated value of nw or nb in equations 3a and 3b were extremely large and could not be

determined accurately. If estimates of nw or nb were >100, they were set to 100. LC25 and $LBC25_X$ were determined as:

$$LC25 = [(LC50^{-1} + K_W"^{-1}) (\ln(4/3) \ln(2)^{-1})^{1/nw} - K_W"^{-1}]^{-1} \quad (7a)$$

and

$$LBC25_X = [(LBC50_X^{-1} + K_{TBX}"^{-1}) (\ln(4/3) \ln(2)^{-1})^{1/nb} - K_{TBX}"^{-1}]^{-1} \quad (7b)$$

where K_W" and K_{TBX}" were the concentrations when metal-induced mortality was half of the maximum, LC25 and LC50 were the lethal water concentrations at 75% and 50% survival respectively, and $LBC25_X$ and $LBC50_X$ were the background-corrected (X) lethal body concentrations at 75% and 50% survival respectively.

4.3.3 Growth

Growth data were square root transformed prior to statistical analyses to normalize the data and equalize variances. This produced more uniform variances than log and 4th root transformation of the data. Growth (4-wk. wet weight) was fit to equation (4) using Systat 10, non-linear regression. IC25 and $IBC25_X$ (water or background corrected body concentration resulting in 25% inhibition of growth) were determined as:

$$IC25 = (3a)^{-(1/n)} \quad (8)$$

where a and n were determined from equation (4) (Borgmann et al., 1998) based on ($W\ W'^{-1} = 0.75 = (1 + aC^n)^{-1}$.

For hormetic growth patterns, growth data were fit to equation (5) to determine W' (control growth), the coefficients b and a, and the exponents n and m. If n and m were approximately 2 and 1 respectively, then the IC25 and IB25 could be determined by setting n = 2 and m =1 thus converting equation 5 into a quadratic equation. Growth (W) was set to $0.75W'$ (25% reduction in control growth, W') and the equation was then resolved as the quadratic equation:

$$IC25 = (b \pm (b^2 + 0.75\ a)^{0.5}) (1.5\ a)^{-1} \quad (9)$$

The hormesis model is useful for determining if there is a hormetic effect and how significant the effect is. However, in order to estimate an IC or IBC that is not based on an over-estimated control due to the hormesis effect, leading to a lowering of the IC or IBC value, (Environment Canada, 2005) recommends a modification in the data analysis. Growth (wet weight) data greater than the highest control growth were omitted from the statistical analyses in the fit to the general growth model (4) as a means of dealing with hormesis and estimating IC25 and $IBC25_X$ values (Tables 3 and 4).

Since a log scale was used for the concentration-toxicity and concentration-growth plots, the "funpar" command in Systat 10 was used to determine the log values and 95% confidence limits for the LC50, LC25, $LBC50_X$, $LBC25_X$, IC25 and $IBC25_X$ values. These estimates and confidence limits were back-transformed for display in the tables.

4.4 RESULTS

4.4.1 Mortality (related to exposure)

Mortality rate generally increased with exposure concentration for all of the toxicants tested (Fig. 4.1). However, mortality rate with As exposure did not start to increase until the concentration exceeded 4000 nmol L^{-1} at which point there was a sharp increase in mortality. The exponents nw and nb were extremely large (Systat estimates >100) and could not be determined accurately; these were set to 100 (Tables 1 and 2). Control data (no added As) could not be included in the model since measured As water concentration was below the detection limit. However, the control mortality was plotted at the detection limit of 21.4 nmol L^{-1} which falls very close to the model line (Fig. 4.1).The mortality model fit the data with an r^2 of 0.76 and resulted in an estimated LC50 of 5600 nmol L^{-1} (Table 4.1).

Unlike the situation with As, mortality gradually increased with increasing Co exposure (Fig. 4.1). Similar to As, the control data for the Co experiments could not be included in the model since measured Co water concentration was below detection limit. However, the control mortality was plotted at the detection limit of 7.2 nmol L^{-1} which fall very close to the model line (Fig. 4.1). The Co mortality model had an r^2 of 0.86 and resulted in a LC50 of 183 nmol L^{-1} (Table 4.1). Chromium demonstrated a similar mortality curve. As for As and Co, control data (no added Cr) could not be included in the model since measured Cr water concentration was below detection limit. However, the control mortality was plotted at the detection limit of 9.3 nmol L^{-1} which also fell very close to the model line (Fig. 4.1). The Cr mortality model fit with an r^2 of 0.81 and a LC50 of 731 nmol L^{-1} (Table 4.1).

Hyalella tolerated much higher concentrations of Mn relative to the other three toxicants. Mortality increased gradually with exposures in glass containers, but exhibited a sharp increase when the exposure concentrations exceeded 100,000 nmol L^{-1} in the HDPE containers (Fig. 4.1). This resulted in significantly different LC50*s* and LC25*s* for the different container types. *Hyalella* exposed to Mn in HDPE containers tolerated significantly higher concentrations with an LC50 of 197,000 nmol L^{-1}, almost 7 times higher than the LC50 for exposure in glass (Table 4.1). Unlike As, Co and Cr, control mortality data was incorporated in the model since the measured water concentrations were well above the detection limit of 2.7 nmol L^{-1}. In both cases the models fit the data well with r^2s of 0.90 and 0.86 for exposures in glass and HDPE respectively.

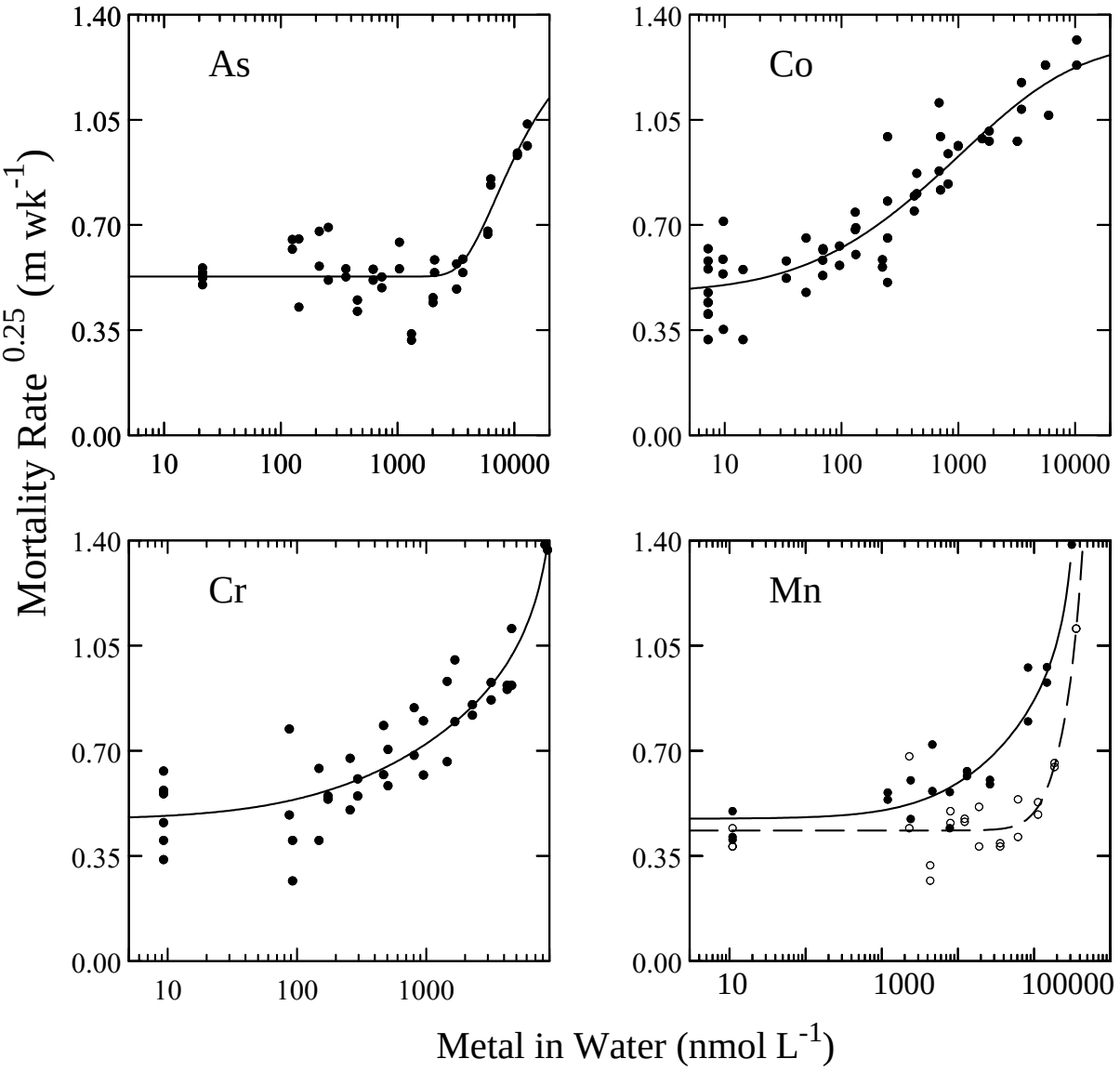

Figure 4.1 Mortality rate versus mean measured As, Co, Cr and Mn water concentrations after 4-week exposures in glass (solid circles) or HDPE (open circles) containers. Arsenic, Co and Cr control water concentrations were less than detection limits and hence mortality rates were plotted at the detection limits of 21.4, 7.2 and 9.3 nmol L^{-1}, respectively. Non-linear regressions of the mortality saturation model for exposure in glass (solid lines) and exposure in the HDPE (dashed line) containers.

Table 4.1 Control mortality (m'), half saturation constant (K_W''), exponent (nw), LC50 and LC25 (with 95% CL) and model fit (r^2) for *Hyalella* mortality as a function of water concentration.

		Saturation Mortality Model							
					(nmol/L)				
Metal	Container	m'	K_W''	nw	LC50	CL	LC25	CL	r^2
As	glass	0.078	172	100	5600	4570-6870	4320	3370-5550	0.756
Co	glass	0.050	3900	0.913	183	120-279	68	33-140	0.858
Cr	glass	0.049	-11000	0.767	731	413-1290	243	91-650	0.814
Mn	glass	0.050	-380000	0.756	29400	14600-59000	9680	2900-32400	0.904
Mn	HDPE	0.036	-808000	2.364	197000	144000-271000	147000	103000-210000	0.858

4.42 Mortality (related to bioaccumulation)

The relationship between bioaccumulation of each toxicant and mortality followed similar patterns to that of the water exposure relationships (Fig. 4.2). Mortality rate in the As exposures did not increase significantly until body concentration exceeded 100 nmol g^{-1} dry weight (dw), even though As bioaccumulation had increased by approximately a factor of 10 and exposure concentration had increased by a factor of approximately 400 (Fig. 4.2). Again, control data could not be included in the model since calculated body concentrations could not be determined from the water concentration, which was below the detection limit. However, measured control body concentrations were above the detection limit and these are included in Figure 4.2. The mortality model fit the data well with an r^2 of 0.76 and resulted in an estimated lethal body concentration causing 50% mortality ($LBC50_X$) of 139 nmol g^{-1} dw (Table 4.2).

Cobalt mortality increased gradually with increasing bioaccumulation (Fig. 4.2) with an $LBC50_X$ estimate of 220 nmol g^{-1} dw (r^2 = 0.86, Table 4.2). The control body concentrations for Co could not be included in Figure 4.2 because they were below the detection limit. However, the upper limit for the control body concentrations were estimated from the measured dry weights and the digestion sample detection limits and included as stars in Figure 4.2. The Cr mortality curve was similar to that of Co (Fig. 4.2) with an r^2 of 0.81 and an estimated $LBC50_X$ of 334 nmol g^{-1} dw (Table 4.2). As for the As plot, measured control body concentrations were included in Figure 4.2. Mortality rate gradually increased with exposure to Mn in glass (Fig 2) however, *Hyalella* tolerated up to 50,000 nmol Mn g^{-1} dw before there was a sharp increase in mortality with exposure in HDPE containers. This resulted in significantly different $LBC50_Xs$ and $LBC25_Xs$ such that the *Hyalella* exposed to Mn in HDPE could tolerate a much higher tissue load with a $LBC25_X$ six times higher and a $LBC50_X$ three times higher than for the glass exposure (Table 4.2). Control Mn body concentrations were included in the mortality model. The mortality models fit the data well with an r^2 of 0.90 for exposure in glass and an r^2 of 0.86 for exposure in HDPE (Table 4.2).

The mortality to total body concentration models reported above were based on non gut cleared body concentrations. Gut clearance was conducted on approximately 50% of the animals so that depuration of each toxicant could be determined over a 24-h period and the final body concentrations included in the mortality model with a "loss" term (Eq. 6a). The loss term was used to correct the critical body concentration to generate the 24 hr critical body concentrations $LBC50_{X24hr}$, $LBC25_{X24hr}$, $IBC50_{X24hr}$ and $IBC25_{X24hr}$ (Tables 2 and 4). The variable elimination calculation (eq. 6b) was used to determine the 24 hr critical body concentrations for Mn. These values are necessary for assessing bioaccumulation in *Hyalella* exposed to sediments since these animals must undergo gut purging to ensure that contaminated sediment particles are eliminated from the gut prior to body analysis (Neumann et al., 1999). Manganese

Table 4.2 Control mortality (m'), half saturation constant (K_{TB}''), exponent (nb), $LBC50_X$ and $LBC25_X$ (with 95% CL), loss rate, the 24hour gut cleared critical concentrations $LBC50_{X24hr}$ and $LBC25_{X24hr}$ and model fit (r^2) for *Hyalella* mortality as a function of total body concentration.

		Mortality Saturation Model										
					(nmol g^{-1} dry wt.)				loss	(nmol g^{-1} dry wt.)		
Metal	Container	m'	K_{TB}''	nb	$LBC50_X$	CL	$LBC25_X$	CL	(% day^{-1})	$LBC50_{X24hr}$	$LBC25_{X24hr}$	r^2
As	glass	0.078	12.3	100	139	129-150	125	113-140	33.6	92	83	0.756
Co	glass	0.050	-747	0.913	220	166-292	103	55-190	12.6	192	90	0.858
Cr	glass	0.049	-756	0.767	334	237-470	152	68-339	3.67	322	146	0.814
Mn	glass	0.050	-97400	0.752	25700	17400-37900	9750	4610-20600	*	16400	4880	0.904
Mn	HDPE	0.036	-142000	2.364	71000	60700-83000	57900	46300-72400	*	56500	44400	0.858

* Variable elimination calculation: $(BC_{24hr}) = (BC_{0hr}) \bullet e^{-(20800 / (20300 + BC0hr))}$

Percent Loss = $100(BC_{0hr} - BC_{24hr})(BC_{0hr})^{-1}$

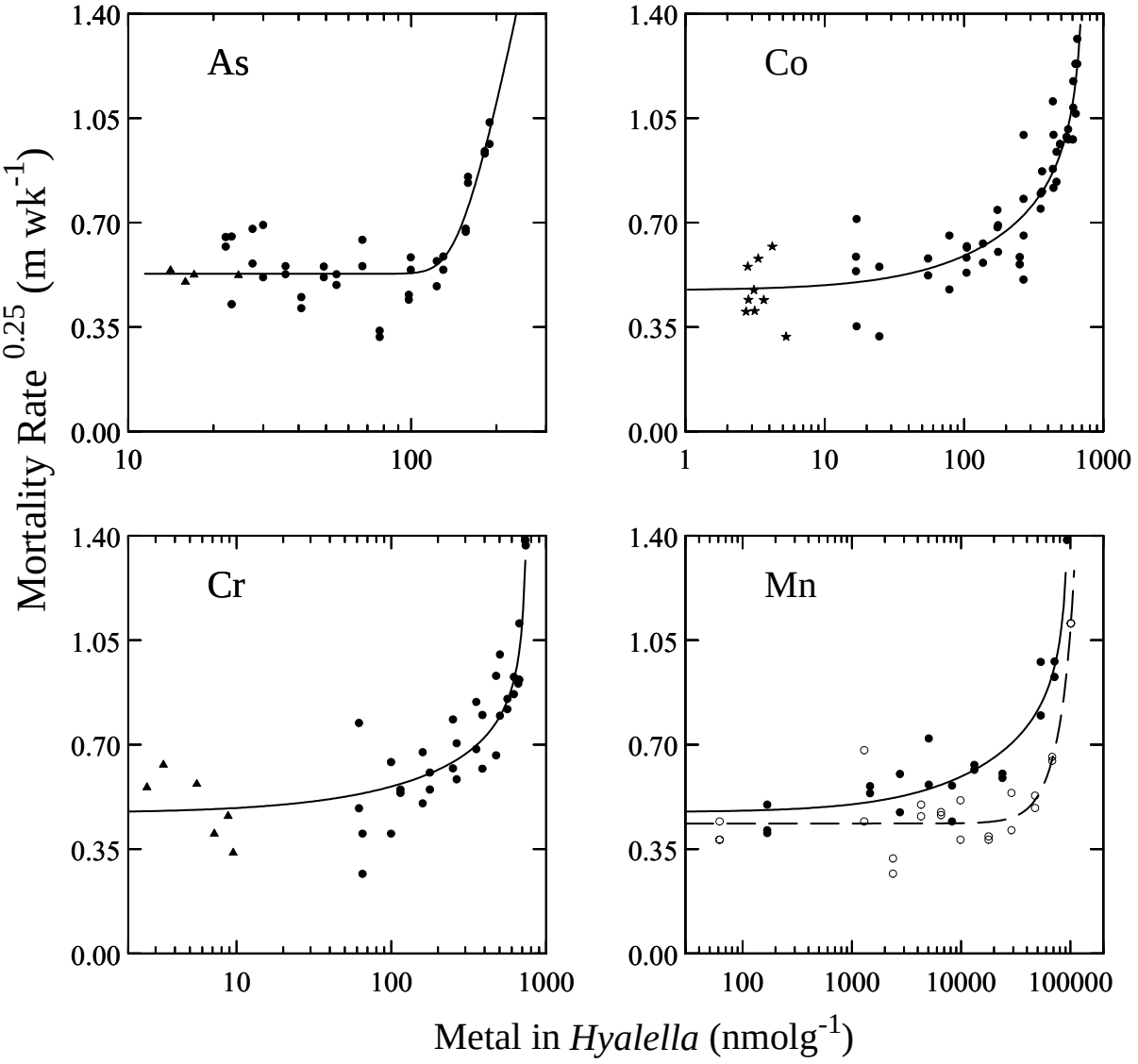

Figure 4.2 Mortality rate versus calculated As, Co, Cr and Mn body concentrations after 4-week exposures in glass (solid circles) or HDPE (open circles) containers. Format same as Figure 4.1 except solid triangles represent control mortality at measured, rather than calculated, As and Cr in *Hyalella* since body concentration could not be calculated from water concentrations below detection limits. Stars represent control mortality at maximum Co body concentrations estimated from the digestion detection limit and the digest dry weight since control water and body concentrations were both below detection limits.

had a high mean loss rate of 48% (Norwood et al. 2006), followed by As at 33.6%,Co at 12.6% and Cr at 3.7% per day (Table 4.2).

4.4.3 Growth

Since the patterns of growth in relation to exposure and total body concentration were similar, only the growth in relation to total body concentration plots are presented (Fig. 4.3). When the hormesis model (Eq. 5) was fit to the As water concentration data, the exponents m and n were determined to be 1.16 and 2.23 respectively (Table 4.3). Since these values were approximately 1 and 2 respectively, the model was rerun with m and n set equal to 1 and 2. This was then solved as a quadratic equation (Eq. 9) in order to determine the IC25 of 4010 nmol L^{-1} (Table 4.3).

Significant reduction in wet weight relative to control occurred when As body concentration exceeded 100 nmol g^{-1} dw (Fig. 4.3). This was the same point at which mortality increased significantly (Fig. 4.2). However, stimulation of growth occurred at low concentrations of As, with a maximum stimulation at approximately 70 nmol g^{-1} dw (Fig. 4.3). Therefore, equation (5) was used to model the hormesis effect (dashed line in Fig. 4.3, As); this fit with an r^2 of 0.78 (Table 4.4). This hormesis effect was significant since the *b* coefficients, for body and water concentrations, were significantly greater than 0 (95% CL > 0, Tables 3 and 4). A coefficient of zero would indicate no stimulation of growth. An $ICB25_X$ could not be determined using this model based on body concentrations since, unlike the relationship between growth and water concentrations, the exponents m and n were not close to 1 and 2 respectively, and the equation could not be solved as a quadratic.

The general growth model (Eq. 4), was also applied to the As data, in which growth that was greater than the highest control was omitted from the analysis (solid line in Fig. 4.3, As). The IC25 and an $IBC25_X$ of 3920 nmol L^{-1} and 128 nmol g^{-1} dw with r^2*s* of 0.76 and 0.72 respectively were determined (Tables 3 and 4).

There was considerable variation in growth at the low and control concentrations of Co (Fig. 4.3). This resulted in r^2 values of 0.49 and 0.50 and a fairly wide confidence interval around the IC25 of 48.7 nmol L^{-1} and $IBC25_X$ of 121 nmol g^{-1} dw respectively (Tables 3 and 4). Chromium exposure and bioaccumulation did not significantly affect growth (Fig. 4.3), even at the point where mortality became significant. Therefore, IC25 and $IBC25_X$ could not be calculated.

Manganese growth response in the exposures with the two container types was different. Growth gradually decreased with increasing exposure and bioaccumulation in the glass containers and fit the general growth models with r^2 values of only 0.37 for both water and tissue concentrations (Fig. 4.3 solid line, Tables 3 and 4). However, good growth was maintained in the HDPE containers with bioaccumulations up to 20,000 nmol Mn g^{-1} dw before there was a significant decrease in growth

Table 4.3 Control wet weight (W'), coefficients a and b, exponents n and m, the water concentration causing a 25% inhibition of growth (IC25), 95% CL and model fit (r2) for Hyalella exposed to As, Co, Cr and Mn over 28 days.

		(mg individual^{-1})				(nmol L^{-1})				
Metal	Container	*W'*	*b*	CL	m	*a*	n	IC25	CL	r^2
As[1]	glass	1.17				1.08E-11	2.92	3920	2980-5170	0.759
As[2]	glass	1.09	3.08E-04	1.14E-04 - 0.001	1.16	6.23E-08	2.23	DNR	DNR	0.788
As[3]	glass	1.06	9.89E-04	3.59E-04 - 0.002	1.00	3.49E-07	2.00	4010	3300-4890	0.783
Co[1]	glass	0.944				1.33E-02	0.829	48.694	10.7-221	0.49
Cr	glass	1.01		No change in growth						
Mn[1]	glass	0.945				3.16E-02	0.308	2110	0.253-1.75E07	0.374
Mn[1]	HDPE	0.974				5.71E-18	3.28	128000	95700-172000	0.929

DNR - Does Not Resolve

1 general allometric growth model

2 hormesis growth model

3 hormesis quadratic growth model

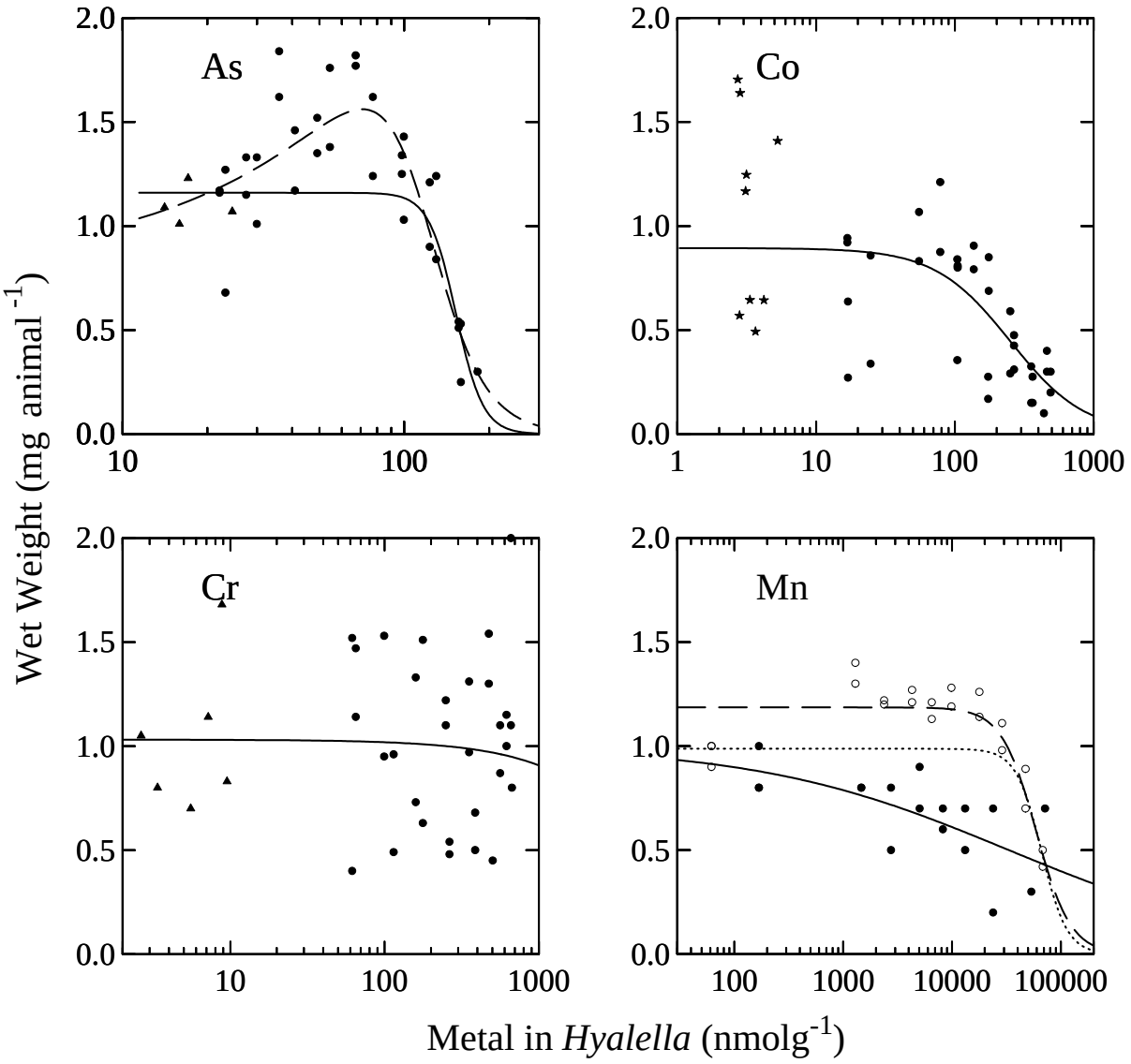

Figure 4.3 Mean wet weight versus As, Co, Cr and Mn body concentrations after 4-week exposures in glass (solid circles) or HDPE (open circles) containers. The general growth model for the glass exposures is represented by the solid lines. The hormesis model for As is represented by the dashed line. The general growth model for Mn exposures in HDPE containers using all data is represented by the dashed line, and the modified general growth model in which growth greater than control values was excluded (option 3, Environment Canada, 2005) is represented by a dotted line. Solid triangles represent control growth at measured (not calculated) As and Cr body concentration. Solid stars represent control growth at maximum Co body concentrations estimated from the digestion detection limit and the digest dry weight.

Table 4.4 Control wet weight (*W'*), coefficients *a* and *b*, exponents n and m, the body concentration causing a 25% inhibition of growth ($IBC25_X$), 95% CL and model fit (r^2) for *Hyalella* exposed to As, Co, Cr and Mn over 28 days.

		(mg individual^{-1})						(nmol g^{-1} dry wt.)			
Metal	Container	*W'*	*b*	CL	m	*a*	n	$IBC25_X$	CL	$IBC25_{X24hr}$	r^2
As[1]	glass	1.16				2.54E-20	8.98	128	116-142	85	0.724
As[2]	glass	0.79	5.70E-02	0.013 - 0.102	0.707	1.36E-10	4.76	DNR	DNR	DNR	0.782
Co[1]	glass	0.89				9.12E-05	1.71	121	56.1-262	117	0.498
Cr	glass	1.03				No change in growth					
Mn[1]	glass	1.00				2.04E-02	0.3743	1740	.027 - 1.12E08	677 *	0.374
Mn[1]	HDPE	0.99				1.43E-18	3.70	49400	43900 - 55700	36700 *	0.923

DNR - Does Not Resolve

* Variable elimination calculation: $(IBC_{X24hr}) = (IBC_X) \bullet e^{-(20800 / (20300 + IBCx))}$

1 general allometric growth model

2 hormesis growth model

(Fig. 4.3 dashed line) and fit the growth model with a r^2 of 0.92 (Table 4.4). This body concentration was lower than the body concentration at which mortality began to significantly increase (50,000 nmol Mn g^{-1} dw, Fig. 4.2). There appears to be some stimulation of growth in comparison to control at intermediate Mn exposures in the HDPE containers (open symbols Fig. 4.3). However, the hormesis model (Eq. 5) could not be fit to the data. Therefore, the general growth model (Eq. 4), in which growth that was greater than the highest control was omitted from the analysis, was applied (dotted line Fig. 4.3, Mn) resulting in the estimation of an IC25 of 128000 nmol L^{-1} (Table 4.3) and $IBC25_X$ of 49400 nmol g^{-1} (Table 4.4). If all the data were used in the estimation (dashed line in Fig. 4.3) the IC25 is reduced to 97700 (73100 – 131000 95% CL) nmol L^{-1} and the $IBC25_X$ is reduced to 39600 (34700 – 45100 95% CL) nmol g^{-1}. However, these confidence intervals overlap those of the previous general model (Tables 3 and 4).

There appears to be a greater toxic effect in the glass containers since the IC25 was 61 times lower than in the HDPE container, dropping from 128000 to 2110 nmol L^{-1} (Table 4.3). As well, the $LBC25_X$ was 28 times lower in the glass container exposure than in the HDPE container dropping from 49400 to 1740 nmol g^{-1} (Table 4.4). However, due to the high variability in the data, the confidence intervals were very large for the glass exposures and completely overlap the estimates for the exposures in HDPE (Tables 3 and 4).

4.5 DISCUSSION

4.5.1 Mortality models

The saturation-based modeling approach provided sound descriptions of the relationships between mortality and both water and body concentrations of As, Co, Cr and Mn with r^2 values ranging from 0.76 – 0.90 (Tables 1 and 2). The mortality models based on exposure-concentration and body-concentration are equivalent and interchangeable. For example, the LC50s (Table 4.1) can be converted to $LBC50_X$*s* (Table 4.2) by utilizing the bioaccumulation formula (Eq. 6a) for As, Co and Cr and (Eq. 6b) for Mn (Norwood et al., 2006). However, this is only appropriate when using water concentrations of toxicants in the same medium utilized in this research. For example, field-site water could be softer or harder, with differing levels of dissolved organic carbon and other competing or interacting ions, all of which can affect bioavailability. Therefore the water-concentration-based mortality models must be used with caution. As well, there are potentially two routes of exposure of the test elements to *Hyalella*, directly from the water and from ingestion of contaminated food. However, bioaccumulation can result from both routes of exposure and chronic toxicity of metals to *Hyalella* is more constant when expressed on a body concentration basis (Borgmann et al., 1991;Borgmann et al., 2001a;Borgmann et al., 2001b;Borgmann, 2000). Therefore, the body-concentration-based mortality models and the critical body concentrations (Table 4.2) should have a broad applicability for predicting toxic effects in amphipods exposed to field conditions.

$LBC25_X$ values were generally close to the threshold where metal induced mortality rate became greater than control mortality rate. The $LBC25_X$ was estimated rather than the lowest observed effect concentration (LOEC) because the $LBC25_X$ is an estimate of a fixed point (i.e. 25% increase in mortality rate) whereas the LOEC is an estimate of the lowest concentration in which a statistically significant increase in mortality occurs. The LOEC estimate is dependent on the variability and number of replicates in the test, whereas the $LBC25_X$ is not. The threshold point can be clearly seen for As and Mn (Fig. 4.2) in which the $LBC25_X$ occurs just at the heel of the "hockey stick". With increasing body concentrations above this point, the slope of each curve can be different leading to significantly different $LBC50_X$*s* for all four toxicants. The strength of the mortality model lies in its ability to describe the impact (mortality rate) across all body concentrations.

4.5.2 Growth effects

A saturation model for the inhibition of growth could not be resolved for any of the toxicants and the standard allometric model was used to describe the inhibition of growth, both on a water and body concentration basis. Therefore IC25*s* (Table 4.3) cannot be converted to $IBC25_X$*s* (Table 4.4) using the bioaccumulation models (6a and 6b) since the allometric model describing inhibition of growth is mathematically incompatible with the saturation model describing bioaccumulation. Although it should theoretically be possible to fit a valid saturation model to the growth data, it is difficult to fit such a model due to: the large variability in growth, no impact due to Co, and a hormesis effect of As and perhaps Mn. Generally, the modeling of growth effects to predict toxicity cannot be done in a consistent manner.

The effect of each of these toxicants on growth was different. Bioaccumulation of As produced a clear hormetic effect in which growth was stimulated at low concentrations with a maximum wet weight occurring at approximately 70 nmol g^{-1} above which there was a sharp decline in growth (Fig. 4.3). None of the other metals produced a clear hormetic affect. Arsenic compounds such as Roxarsone, have been used for decades for disease control and stimulated growth in organisms such as swine and poultry (Carpenter, 1951; Morehouse, 1949). The control of parasites (Buck et al., 1976; Morehouse and Mayfield, 1946) may allow improved growth of the animals. This might apply to *Hyalella* in this current study. However, hormesis may be an "adaptive response to environmentally induced disruptions in homeostasis" (Calabrese and Baldwin, 2001). Their findings indicate that hormetic effects can be seen across diverse biological, toxicological, and pharmacological disciplines and appears to be independent of chemical class. It is possible that a hormetic effect was not detected with Co, Cr or Mn for a couple of reasons (Calabrese, 2005). First, the dose response curves were based on a limited number of exposure concentrations, in an attempt to find and describe the toxic levels. Therefore, there may not be enough low dose concentrations to reveal the effect. Secondly, it is difficult to statistically detect hormesis due to the large variation in the growth data combined with the potentially moderate stimulatory effect.

The calculation of critical concentrations when the data undergo a hormetic pattern must be done with caution. Environment Canada (2005) has adopted the policy of determining critical concentrations in relation to the true control performance, such that the IC25 is the concentration resulting in the 25% reduction from control growth. When the growth data showing stimulation above control levels were excluded in the relation between As exposure and *Hyalella* growth, the resulting IC25 decreased from 4010 to 3920 nmol L^{-1} (Table 4.3)

Increasing Co bioaccumulation resulted in a gradual reduction in growth whereas the bioaccumulation of Cr did not have an effect on growth (Fig. 4.3). Manganese produced two different effects. Exposure in glass containers resulted in a gradual reduction in growth with increasing bioaccumulation whereas exposures in HDPE containers resulted in a sharp reduction in growth when bioaccumulation exceeded 20,000 nmol g^{-1}. As well, there may be some stimulation of growth between 1,000 and 20,000 nmol g^{-1} (Fig. 4.3). It is possible that there might also have been some differences in the impact of As, Co and Cr if the exposures were run in plastic containers; however this has not been tested. One-week reference toxicity tests with Cu have been conducted in both glass and HDPE containers in this laboratory. Slight differences have been observed (e.g., a 15% higher LC50 in glass, based on measured Cu in seven tests in both container types), but the effects of container type were much less than those observed with Mn in the present study, suggesting that the dramatic effects of container type seen here might be unique to Mn. An explanation for the differing impact of container type (glass or HDPE) on mortality and growth in the presence of Mn is not currently available and further investigation is warranted but it does indicate that container type may be important to the interpretation of toxicity test results for some metals.

Overall, the growth endpoint is more variable than the mortality endpoint when based on exposure concentrations (Tables 1 and 3). However, critical water and body concentrations based on growth or mortality were not significantly different since all the 95% confidence intervals overlap (Tables 1 to 4), except Cr exposure which did not affect growth.

4.5.3 Comparison with other metals

The $LBC25_X$*s* for As, Co and Cr were similar to those of Cd, Hg, Ni, Pb, and Tl from Borgmann et al. (2004) as demonstrated in Fig. (4.4). The geometric mean for this group was 187±81 (95% CL) nmol g^{-1} (line, Fig. 4.4), which was significantly lower than the $LBC25_X$*s* for Cu, Mn and Zn which range from 1,800 to 58,000 nmol g^{-1} (Fig. 4.4). *Hyalella* appear to tolerate much higher body concentrations of essential elements such as Cu, Mn and Zn.

The similarity in the $LBC25_X$*s* across numerous metals, excluding essential metals such as Cu, Mn and Zn, raises the question of whether the ranking of toxic elements based on chronic toxicity is predictable from metal or metalloid bioaccumulation in *Hyalella*. The toxicity of organic contaminants is

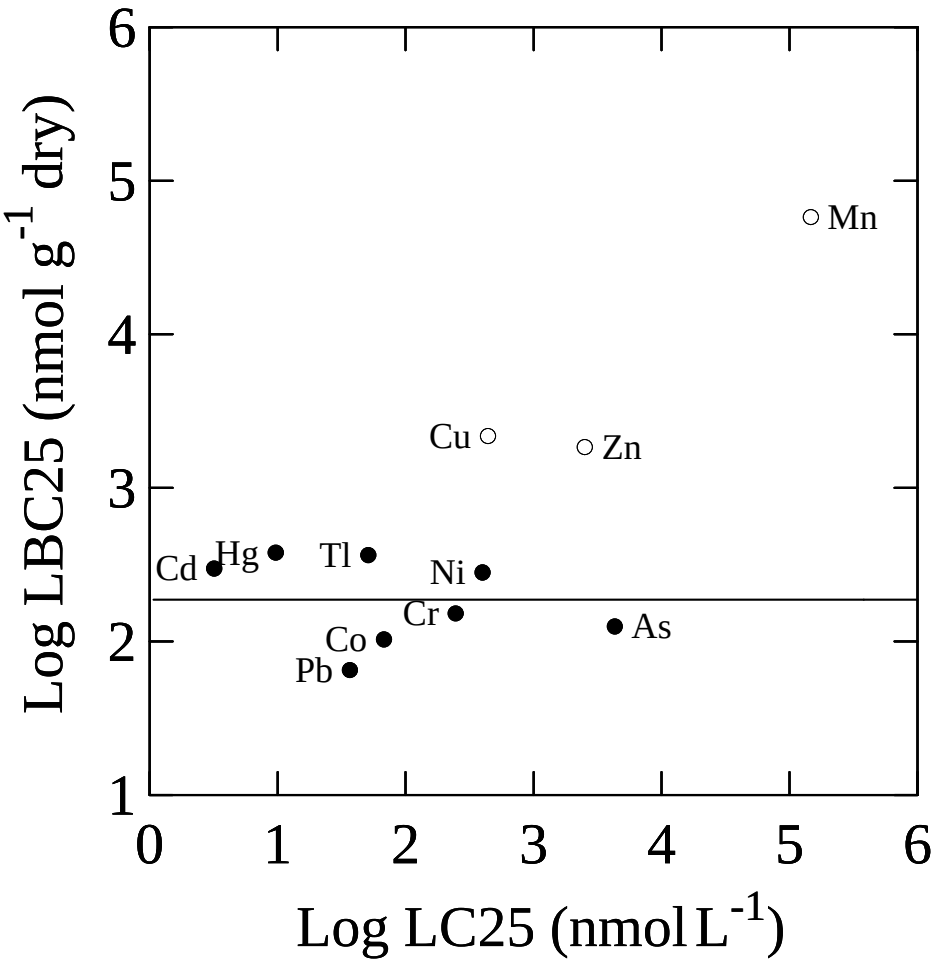

Figure 4.4 Lethal body concentration versus lethal exposure concentration, resulting in 25% mortality. Non- or sparingly essential elements (solid symbols), essential elements (open symbols) and geometric mean of 187 nmol g-1 for the non-essential elements (solid line). Values for Cd, Cu, Hg, Ni, Pb, Tl and Zn from Borgmann et al. (2004).

often predicted from bioconcentration factors (BCF*s*), especially when toxicity data are scarce or unavailable, and attempts are sometimes made to produce hazard classification schemes which treat all substances, including metals, in a consistent fashion (Environment Canada, 2000;McGeer et al., 2003). However, the BCF (as measured in the lab) or bioaccumulation factor (BAF, as measured using field data) is inversely correlated to exposure for a number of metals (McGeer et al., 2003). The reason for this is apparent after re-arrangement of equation 6a (with dep=0) giving

$$BCF = C_{TB}\, C_W^{-1} = \max\,(K + C_W)^{-1} + C_{Bk}\, C_W^{-1}$$

The BCF deceases with increasing C_W if K is small, relative to C_W and/or if there is a significant background. To avoid this concentration dependence, it is necessary to define the BCF as background-corrected accumulation at low C_W (i.e., $C_W << K$) giving

$$BCF_{LC} = (C_{TB} - C_{Bk})\, C_W^{-1} = \max K^{-1}$$

which is independent of water concentrations. When the LC25 was plotted against the BCF_{LC} for the summarized data from Borgmann et al. (2004), Norwood et al. (2006) and this current work, a strong inverse relationship between LC25 and BCF_{LC} was apparent (Fig. 4.5) in which the non-essential (or sparingly essential, e.g. Co) metals and metalloid were all within a factor of 2 of the regression line:

$$\text{Log(LC25)} = -0.897\text{Log}(\max K^{-1}) + 2.356 \qquad (r^2 = 0.95)$$

The essential elements Cu, Mn and Zn were not included in the regression, but instead lie significantly above the line. The slope (-0.859) was significantly different than 0 (p=0.000006). Therefore, there appears to be a very strong negative relationship between chronic toxicity (LC25) and BCF_{LC} (max K^{-1}) to *Hyalella* for the non- or sparingly-essential metals and metalloid studied so far in our laboratory.

The strong relationship between BCF_{LC} and chronic toxicity to *Hyalella* for the non- or sparingly-essential elements results primarily because of the similarity in the $LBC25_X$*s* for these metals and metalloid (Fig. 4.4), and is probably not applicable to all metals in the periodic table. Chemical analyses of *Hyalella* in our culture has revealed some elements with background body concentrations similar to, or higher than, the $LBC25_X$ geometric mean of 187 nmol g^{-1} from Fig.(4). These include the alkaline earth elements Ba and Sr at 224 and 3223 nmol g^{-1} respectively, and the alkali metal Rb with a background level of 130 nmol g^{-1}. Therefore, the toxicity bioaccumulation relationship seen in Fig. 4.5 probably does not apply to the first two columns of the periodic table, which also includes the essential elements Na, K, Mg and Ca. Another transition metal, Fe, is probably an essential metal like Cu, Zn and Mn and was found at background levels of 770 nmol g^{-1}. One other element that was found at a high yet non-toxic, background concentration of 328 nmol g^{-1} was Al, a light element near the top of the periodic table. It is likely that these elements would have critical body concentrations that are even higher than

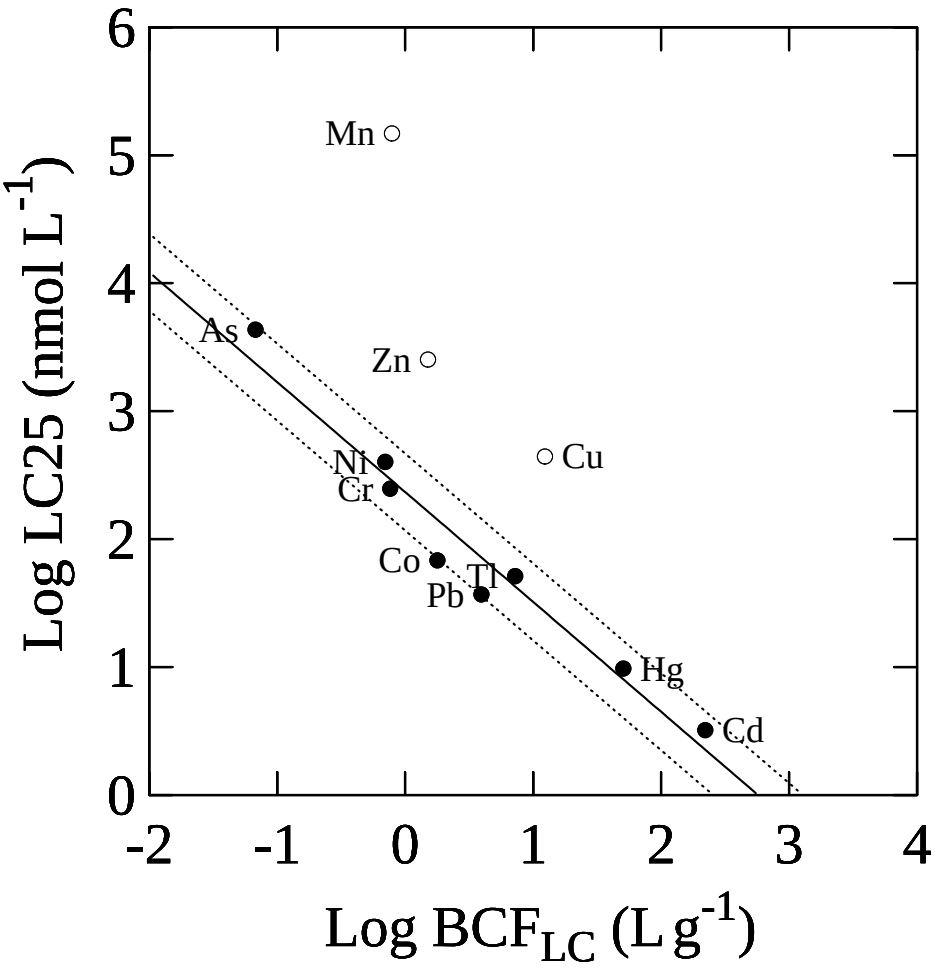

Figure 4.5 Relationship between toxicity (LC25) and BCF_{LC} as calculated from (max K^{-1}) for metals and a metalloid in *H. azteca*. Data from; Borgmann et al. (2004) for Cd, Cu, Hg, Ni, Pb, Tl and Zn; Norwood et al. (2006) and this study for As, Co, Cr and Mn. Symbols as in Figure 4.4. The solid line represents the linear regression for the non-essential elements and the dotted lines represent ±2 times the regression line.

their background levels. *Hyalella* were tolerant of these background levels and may have mechanisms to deal with them, or these elements may bind to sites that do not have an impact on *Hyalella*.

4.5.4 Relevance to metal risk assessment

This study augments data already available on bioaccumulation-toxicity relationships for Cd, Cu, Hg, Ni, Pb, Tl and Zn (Borgmann et al. 2004), and increases the suite of commonly present metals for which bioaccumulation data from environmental assessments can be compared to critical-body-concentrations. This improves our ability to identify causative agents at metal contaminated sites. These relationships have already been used to identify the cause of sediment toxicity due to atmospheric deposition of metals from Sudbury area smelters on lake ecosystems (Borgmann et al., 2001a). The study made use of one-week bioaccumulation tests with *Hyalella* to determine bioavailability of metals from sediments collected from the area, coupled with *Hyalella* toxicity tests and benthic survey of the area. The bioaccumulation data were then compared to the critical body concentrations (LCB25) to determine which metal(s) were sufficiently accumulated to levels that could be toxic. The authors concluded that Ni was the primary cause of sediment toxicity since the bioaccumulated levels exceeded the LBC25 at the most impacted sites. They could rule out Cd, Cu, Pb, Tl and Zn as causative agents since their bioaccumulations were far below the LBC25s. As, Co, Cr and Mn bioaccumulation were also determined at that time, but the LBC25s were not available for comparison. Re-examination of the Borgmann et al. (2001a) data indicates that the highest As bioaccumulation of 20 nmol g^{-1} was well below the $LBC25_{X24hr}$ of 83 nmol g^{-1} and the highest Cr bioaccumulation of 31 nmol g^{-1} was well below the $LBC25_{X24hr}$ of 146 nmol g^{-1} (Table 4.2). Bioaccumulation of Co was as high as 54 nmol g^{-1} which was well above background level and approximately 60% of the $LBC25_{X24hr}$ (90 nmol g^{-1}, Table 4.2). Manganese bioaccumulation was as high as of 29600 nmol g^{-1} which exceeded the $LBC25_{X24hr}$ of 4880 nmol g^{-1} in glass and was approximately 67% of the $LBC25_{X24hr}$ of 44400 nmol g^{-1} in HDPE (Table 4.2). Therefore both Co and Mn could be contributing to the toxicity observed at some of these sites.

The mortality models can be used to predict chronic (4 wk) toxicity by using the $LBC25_{X24hr}$ values and other coefficients from Table 4.2, along with the measured bioaccumulation from the test site exposures, in equation 3b. For example, for Co after 24 h gut clearance

$$m - m' = (\ln(2)/4)\ [(54)\ (192^{-1} - 747^{-1})\ (1 - (54)\ 747^{-1})^{-1}]^{0.913} = 0.04436$$

This is the control-corrected mortality rate, which can be converted to control-corrected survival with $S = e^{-(m-m')\bullet t}$ where S is survival and *t* is time (4 wks), giving 83.7%. Therefore Co is predicted to reduce survival to 83.7% of the control level. This type of calculation can be done for each metal to determine its contribution to mortality. Nickel bioaccumulation of 757 nmol g^{-1} was well above the LBC25 of 169 nmol g^{-1} (after gut clearance) and is predicted to reduce survival to 0.018% of control

levels (equation 3b with parameters for Ni from Borgmann et al. 2004). This would indicate that Ni was the major contributor to toxicity at that site. Repeating this procedure for all metals makes it possible to rank the relative contribution of each metal to mortality at any given site.

4.6 CONCLUSIONS

1. Mortality due to exposure and bioaccumulation of As, Co, Cr and Mn can be described satisfactorily using saturation mortality models (Eqs. 3a and 3b) from which LC50 and $LBC50_X$ can be computed (Tables 1 and 2). These models are consistent and hence compatible with the mechanistically-based saturation models of bioaccumulation described in Norwood et al. (2006).
2. Growth could not be successfully fit to a saturation model but instead was fit to a general allometric model. This model is not consistent with the saturation models for bioaccumulation and mortality. However it does describe the impact of As, Co and Mn on growth and produces IC50 and $IBC50_X$ estimates (Tables 3 and 4). Chromium had no impact on growth.
3. Growth enhancement and inhibition in *Hyalella* in response to As exposure was described using a hormesis model. This model describes growth enhancement at low concentrations as well as inhibition at higher concentrations. This hormesis model could be applied to estimate the IC25 using a quadratic equation, but not the $IBC25_X$. The IC25 estimated using the general growth model with data above control values omitted (option 3, Environment Canada 2005) and the IC25 estimated using the hormesis model were not significantly different (Table 4.3).
4. There is a significant trend of increased toxicity on a water concentration basis with increased BCF_{LC} (background-corrected accumulation at low water concentrations) for As, Cd, Co, Cr, Pb, Ni, Tl and Hg but not Cu, Mn and Zn.
5. Critical body concentrations coupled with mortality models and bioaccumulation measurements are useful tools for identifying which element(s) have the potential for adverse effects, and for estimating the magnitude of their impact, at contaminated sites.

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CHAPTER 5

Interactive effects of metals in mixtures on bioaccumulation in the amphipod *Hyalella azteca*.

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ABSTRACT

Mixtures were produced of "equi-toxic" concentrations of 10 elements at the four-week LC25 for *Hyalella azteca*. Bioaccumulation was determined in one-week exposures. The first mixtures tested included seven elements; As, Cd, Co, Cr, Ni, Pb and Tl. Copper, Mn and Zn were not included in the initial tests due to potential confounding effects, such as regulation of Cu and Zn by *H. azteca* and the high concentrations of Mn required to be "equi-toxic", which might cause adsorption of metals to Mn hydroxides if these were formed. The second set of tests included the seven element mixture in combination with; Cu, Mn and Zn individually; the binary pairs, Cu-Mn, Cu-Zn and Mn-Zn; and the tertiary group Cu, Mn and Zn. Interaction factors (*IF*) were computed which quantified each element's impact on the bioaccumulation of the other nine. Cobalt, Cd and Ni bioaccumulation was significantly inhibited with increasing number of metals in the mixture. Arsenic bioaccumulation was enhanced with increasing number of metals in the mixture exposure. Lead bioaccumulation was enhanced by some mixture combinations. Bioaccumulation of Cr, Cu, Mn, Tl and Zn were not significantly affected by exposure to other metals.

5.1 INTROLDUCTION

Body concentrations of single toxicants have been shown to be useful indicators of toxic effects in aquatic organisms even in the presence of various complexing agents and can help identify the cause of biological effects in sediment assessments (Biesinger et al., 1982; Borgmann et al., 1991; McCarty, 1991; Meador et al., 1993; Borgmann and Norwood, 1997; Borgmann and Norwood, 1999). The use of body concentration as a measure of bioavailability may negate complications that can arise from uncertainties due to interactions with other ions or molecules that may hinder or enhance availability, multiple compartments of exposure, multiple sources and pulsed exposures (Hickie et al., 1995; Landrum et al., 1992). The relationship between metal accumulation and toxic effects in *Hyalella azteca* have been established for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn (Borgmann et al., 2004; Norwood et al., 2007). It is therefore possible that body concentrations of metals bioaccumulated from mixture exposures may also be useful indicators of effects. However, interaction between the metals may affect their bioaccumulation and toxicity, therefore the determination of any interactive effect on bioaccumulation is necessary since several metals are often present together at elevated concentrations in contaminated environments. A review of the effects of metal mixtures on aquatic biota (Norwood et al., 2003) revealed that there is no consistent method of quantifying the effects of metals mixtures. The most recent version of the Canadian Environmental Quality Guidelines (Canadian Council of Ministers of the Environment, 1999) does not incorporate any guidance on the effects of mixtures. Europe does not have a mixture criterion either, but they recommend the use of additive joint action evaluation (European Inland Fisheries Advisory Commission, 1987). Australia and New Zealand have established

a water quality guideline criterion for simple mixtures of less than 6 components (ANZECC and ARMCANZ, 2000). The United States of America does not have mixture guidelines. Therefore it is evident that research is required to quantify the effects of metal mixtures and determine appropriate methods that can have practical application to the protection of aquatic life.

It has been assumed that competition of a metal with other cations for binding sites on the biotic ligand can inhibit the binding of the metal (Di Toro et al., 2001; Paquin et al., 2002; Playle, 1998). In theory, a competitive inhibition model could also be applied to metal-metal interactions. For example, Playle (2004) postulated that "competition increases as the metal concentrations increase" such that with an increased number of metals in a mixture there is a decrease in the number of binding sites occupied by each metal. Therefore, the objective of this research was to determine if there are any interactions affecting bioaccumulation of arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), thallium (Tl) and zinc (Zn) from various mixture combinations. For simplicity, these metals and the metalloid will be referred to as "metals" for the remainder of this paper.

5.2 THEORY

5.2.1 Body Concentration With Interaction Coefficients

Bioaccumulation of an individual metal from water has been described using a mechanistically based saturation model (Borgmann et al., 2004) as follows:

$$C_{\mathrm{TB}} = C_{Bk} + \frac{(\max \times C_{\mathrm{W}})}{(K_{0.5} + C_{\mathrm{W}})} \times (1 - loss \times dep) \qquad (1)$$

Where C_{TB} is the total body concentration of the metal of interest, max is the maximum above-background accumulation of the metal, C_{W} is the metal concentration in water, $K_{0.5}$ is the half saturation constant (the concentration of C_{W} at which C_{TB} is halfway between the maximum accumulation and the background), C_{Bk} is the background body concentration obtained from control animals (ie. in the absence of any added metals in the medium), loss is the coefficient of depuration which can be converted to percent loss per day by multiplication by 100 and dep is either 0 (for no depuration) or 1 (for 24 h depuration).

The modelling of accumulation follows the classical modelling of chemical kinetics of enzyme actions (Laidler and Bunting, 1973), but instead of determining the rate of enzyme action, the maximum and binding constants for accumulation of a metal are modeled. The saturation models of bioaccumulation as well as the accumulation to toxicity relationships have been determined for the metals As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn individually in *H. azteca* (Borgmann et al., 2004;

Norwood et al., 2006; Norwood et al., 2007). However, the impact of mixtures of metals on individual metal accumulation has not been investigated. The following interaction model can be used:

$$C_{\mathrm{TB}} = C_{\mathrm{Bk}} + \frac{(\max \times C_W) \times \left(1 + \sum_{n=2}^{10} \left(a'_n \times C_n\right)\right)}{\left(K_{0.5} + C_{\mathrm{W}}\right) \times \left(1 + \sum_{n=2}^{10} \left(a_n \times C_n\right)\right)} \times \left(1 - \mathrm{loss} \times \mathrm{dep}\right) \quad (2)$$

in which a_{n} is the interaction coefficient if a reduction of accumulation (decreased C_{TB}) occurs and a'_n is the interaction coefficient if enhancement of accumulation occurs. C_{n} is the water concentration of the metals influencing the accumulation of C_{W} in the mixture.

Equation (2) can also be expressed as, and is equivalent to:

$$C_{TB} = C_{Bk} + \frac{(\max \times C_W) \times \left(1 + \sum_{n=2}^{10} \left(a'_n \times C_n\right)\right)}{\left(K_{0.5} + C_W + \sum_{n=2}^{10} \left(b_n \times C_n\right)\right)} \times \left(1 - \mathrm{loss} \times \mathrm{dep}\right) \quad (3)$$

Where $b_{\mathrm{n}} = a_{\mathrm{n}} \times \left(K_{0.5} + C_W\right)$

If a_{n} is constant when C_{W} is varied, then the denominator of equation (2) is consistent with non-competitive inhibition. If b_n is constant when C_{W} is varied, then the denominator of equation (3) is consistent with competitive inhibition. If C_{W} is not varied, it cannot be determined if either a_{n} or b_n are constant and hence equations (2) and (3) are indistinguishable. Therefore, the denominator term is consistent with either competitive or non-competitive inhibition of accumulation of the metal of interest.

5.2.2 Interaction Factors

In order to compare the interactions of all the metals, their coefficients were standardized based on the exposure concentrations of each metal, producing an Interaction Factor (*IF*) as follows:

Numerator coefficients: $IF_{\mathrm{n}} = 1 + a'_{\mathrm{n}} \times C_{\mathrm{n}}$ (4)

Denominator coefficients: $IF_{\mathrm{n}} = 1/(1 + a_{\mathrm{n}} \times C_{\mathrm{n}}^{-1})$ (5a)

$= 1/(1+(b_{\mathrm{n}} \times C_{\mathrm{n}})/(K_{0.5}+ C_{\mathrm{W}}))$ (5b)

If no interaction occurs due to a metal (n) then $IF_{\mathrm{n}} = 1$. If the metal causes a stimulatory effect then $IF_{\mathrm{n}} > 1$ and if the metal causes an inhibitory interaction then $IF_{\mathrm{n}} < 1$. The values of IF_{n} for denominator coefficients are the same regardless of whether equation (2), representing non-competitive

inhibition, or equation (3), representing competitive inhibition, is used to model inhibition of metal accumulation.

5.3. METHODS

5.3.1 Bioaccumulation Tests

Twenty, 6 - 10 week old *H. azteca* were added to 400 mL of test medium with a single piece of 5 by 10 cm cotton gauze in 500 mL HDPE containers (Borgmann et al., 1991; Borgmann et al., 1993). Exposures were conducted in an incubator at 25^{o}C with a 16 h light:8 h dark photoperiod. Test media were renewed twice during the 1-week accumulation exposures (hence renewal every 2 to 3 days). The 1-week exposure period was selected since we have found a number of the metals achieve equilibrium in *H. azteca* in three days (Borgmann and Norwood, 1995; MacLean et al., 1996). The 1 week test was chosen with older animals (2-10 week olds) which are less sensitive in order to keep survival high and to produce large body size for the tissue analyses. The increased renewal rate, as opposed to a 1-week renewal, was carried out in response to known losses of Pb and Mn from test media (MacLean et al., 1996; Norwood et al., 2006). Food additions (TetraMin© fish food flakes ground to 500 *u*m mesh size) consisted of a 5 mg addition at the beginning of each exposure or renewal period. The exposure may, therefore, have been partially via food. The test medium consisted of de-chlorinated Burlington city tap water originating from Lake Ontario (mean±95% confidence interval: dissolved organic carbon 1.86±0.36 mg L^{-1}, dissolved inorganic carbon 20±0.36 mg L^{-1}, Alk 87±0.95 mg L^{-1}, Cl 670±13 µmol L^{-1}, SO_4 319±12 µmol L^{-1}, SiO_2 15±1.5 µmol L^{-1}, Ca 863±22 µmol L^{-1}, Mg 359±3.7 µmol L^{-1}, Na 528±3.7 µmol L^{-1}, and K 42±13 µmol L^{-1}; analyses were conducted by the National Laboratory for Environmental Testing, Environment Canada, Burlington, Ontario, Canada) with metal additions. Two replicates were run of each mixture and each test was repeated. Stock solutions of each metal were prepared with the analytical grade salts of sodium arsenate ($Na_2H•AsO_4•7H_2O$), cadmium chloride anhydrous ($CdCl_2$), cobalt chloride 6-hydrate ($CoCl_2•6H_2O$), sodium chromate anhydrous (Na_2CrO_4), cupric chloride ($CuCl_2•2H2O$), manganous chloride 4-hydrate ($MnCl_2•4H_2O$), nickel (II) chloride hexahydrate ($NiCl_2•6H_2O$), lead chloride ($PbCl_2$), thallous nitrate ($TlNO_3$) and zinc chloride ($ZnCl_2$) dissolved in de-ionized water (Milli-Q) acidified to 0.07% nitric acid (Omni-pure).

Each metal was spiked into the medium to achieve a final concentration equivalent to the chronic (4 wk) LC25 (Table 5.1). This concentration was selected in order to keep mortality rates low since the LC25 was determined from chronic exposures with juvenile *H. azteca*, yet the accumulation tests were only 1 week exposures with less sensitive adults (4 to 6 wk). These larger animals were also used in order to provide adequate amounts of tissue for analyses. As well, the LC25s are generally environmentally relevant since many have been exceeded in contaminated site water or in overlay waters from sediment assessment tests (Borgmann and Norwood, 1997; Borgmann et al., 2000;

Table 5.1 Geometric mean measured total dissolved exposure concentrations with 95% confidence limits (CL) and sample size (N), for spiked and non-spiked (background) treatments. Free ion forms with percent contribution to total dissolved concentration. LC25 values based on saturation mortality models. Exposure ratio is the spike concentration as a proportion of the LC25.

	Spiked			Non-Spiked			Free Ion		LC25	Exposure Ratio
	($nmol\ L^{-1}$)	CL	N	($nmol\ L^{-1}$)	CL	N	Form	(%)	($nmol\ L^{-1}$)	($Spike\ LC25^{-1}$)
As	5520	5480 - 5560	68	10.8	9.64 - 11.9	56	[a] AsO_4^{3-}	0.06	4320	1.28
Cd	2.66	2.63 - 2.69	72	0.136	0.127 - 0.144	52	Cd^{2+}	88	3.21	0.83
Co	58.4	57.8 - 59.1	68	0.565	0.494 - 0.637	56	Co^{2+}	90	68	0.86
Cr	284	280 - 287	68	5.18	5.05 - 5.30	56	CrO_4^{2-}	98	243	1.17
Ni	435	430 - 439	68	11.8	11.3 - 12.3	56	Ni^{2+}	87	400	1.09
Pb	41.8	41.3 - 42.3	68	0.274	0.246 - 0.301	56	[b] Pb^{2+}	10	36.8	1.14
Tl	42.9	42.7 - 43.1	68	0.0428	0.0364 - 0.0491	56	Tl^{1+}	99	51.2	0.84
Cu	508	499 - 515	20	36.8	34.3 - 39.4	104	[c] Cu^{2+}	6	441	1.15
Mn	78300	77000 - 79600	20	8.74	5.16 - 12.3	102	Mn^{2+}	96	147000	0.53
Zn	2360	2290 - 2420	20	21.4	20.0 - 22.8	104	Zn^{2+}	77	2520	0.94

a $HAsO_4^{2-} = 95\%$

b $PbCO_3$ (aq) = 51% and $PbOH^+ = 32\%$

c CuCO3 (aq) = 61% and $CuOH^+ = 25\%$

LC25 data: As, Co, Cr and Mn (Norwood et al. 2007), all other metals (Borgmann et al. 2004)

Borgmann et al., 2001; Pyle et al., 2002; Koukal et al., 2004; Samecha-Cymerman and Kempers, 2004).

To keep the treatments to a reasonable number, mixture groupings were devised that would incorporate combinations that would allow for the determination of interactions between each metal without doing every one of the 3,628,800 possible combinations. Copper, Mn and Zn were not included initially due to potential confounding effects such as; regulation of Cu, the small maximum elevation (2 fold) in body Zn in *H. azteca* (Borgmann et al., 1993)and the high concentrations of Mn required to be "equi-toxic" which might cause precipitation of Mn (although visible precipitation was never observed) and adsorption of metals to Mn hydroxides. Therefore, each metal was tested alone and in a 7 metal mix (7MIX) of As, Cd, Co, Cr, Ni, Pb and Tl, as well as in 7 treatments in which one of the seven metals was dropped from the 7MIX (i.e. 7MIX-As, 7MIX-Cd, 7MIX-Co, 7MIX-Cr, 7MIX-Ni, 7MIX-Pb and 7MIX-Tl). The second group of experiments contained the following mixture groups: Cu, Mn and Zn alone, 7MIX+Cu, 7MIX+Mn, 7MIX+Zn, 7MIX+Cu+Mn, 7MIX+Cu+Zn, 7MIX+Mn+Zn and all ten metals together (10MIX).

Ammonia, pH, conductivity and oxygen concentrations were measured at the beginning (prior to animal additions) and end of each renewal period (mean±95% C.I.: total ammonia 0.03±0.001 mmol L^{-1}, pH 8.2±0.03, conductivity 290±0.9 μs cm^{-1}, oxygen 8.0±0.07 mg L^{-1}). At the beginning and end of each renewal period, 10 mL non-filtered and filtered water samples were collected and preserved with 10 μL nitric acid (Omni-pure) for metalloid or metal analyses. Survival was recorded at each renewal period and at the end of the 7 day exposure, even though very little mortality was expected. One half of the survivors (or all survivors if less than 5 animals survived) were rinsed with 50 μM ethylene-diamine-tetra-acetic acid (EDTA) in de-chlorinated Burlington city tap water to remove any loosely adsorbed metal, weighed wet and then placed in a pre-cleaned cryovial and dried at 60°C for 72 h before determination of dry weight. The remaining animals were also rinsed with, and then placed in 60 mL of the same EDTA medium along with a small piece of cotton gauze and 2.5 mg fresh food for a 24 depuration period. This was analogous to the procedure used to purge the guts of amphipods in sediment tests (Neumann et al., 1999). EDTA was added to the solution to bind any metal released from the animal during the depuration so that the animal could not reabsorb the metal. Wet weight was determined after 24 h and then the animals were placed in pre-cleaned cryovials and dried at 60°C for 72 h before determination of dry weight.

5.3.2 Metalloid and Metal Analyses

Digestion of tissue samples were based on the methods of Borgmann et al. (1991) and Stephenson and Mackie (1988). Six amphipods, approximately 1.5 mg dry weight, were digested with 160 μL of70% Omni-pure nitric acid at room temperature for 6 days, followed by an addition of 120 μL

Table 5.2 Maximum metal accumulation (max) and half saturation constant (K), with 95% confidence limits (CL) and 24 h depuration loss rate (loss); based on bioaccumulation saturation model of chronic (4 wk) exposures.

Metal	Conditions	Exposure (weeks)	max (nmol g^{-1})	CL	$K_{0.5}$ (nmol L^{-1})	Loss (% day^{-1})	CL	Data Source
As	weekly renewal	4	219	(117-410)	3230	33.6	(18.3-48.9)	Norwood et al. (2006)
Cd	SAM weekly renewal[a]	1	1830	(1020-3260)	91	NA	NA	Borgmann (unpublished)
Co	weekly renewal	4	674	(395-1150)	378	12.6	(-8-33.1)	Norwood et al. (2006)
Cr	weekly renewal	4	831	(632-1090)	1090	3.67	(-7.8-15.1)	Norwood et al. (2006)
Ni	no renewal[b]	4	0.7 (max/K)	(0.59-0.84)	NA	40.0	NA	Borgmann et al. (2004)
Pb	2-day renewal	4	6520	(2120-10900)	1650	41	NA	Borgmann et al. (2004)
Tl	weekly renewal	4	18310	(6140-54600)	2525	NA	NA	Borgmann et al. (2004)
Cu	weekly renewal	4	3600	(3240-3970)	291	15	NA	Borgmann et al. (2004)
Mn	weekly renewal	4	116000	(56200-238000)	146000	48	(39.1-56.9)	Norwood et al. (2006)
Zn	weekly renewal	4	3550	(2980-4110)	2345	49.0	NA	Borgmann et al. (2004)

- [a] 1 week expsoure
- [b] data from overlay water of a Ni-spiked sediment test
- SAM - Standard Artificial Medium

30% hydrogen peroxide for 24 h in a 14 ml polypropylene test tube with snap cap. Each sample was then made up to a 6.0 ml volume with de-ionized water (Milli-Q).

All ten metals in water and tissue samples were analyzed by Inductively Coupled Plasma, Mass Spectroscopy (ICP-Mass Spec) by the National Laboratory for Environmental Testing, Environment Canada. Method blanks were run with every batch of samples to correct for background contamination and to calculate detection limits. The detection limit for each metal in water was 0.458, 0.0292, 0.0627, 0.321, 8.51, 1.54, 2.18, .0759, 0.00644 and 5.86 nmol L^{-1} for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn, respectively. The detection limits for tissue, based on the average digestion of 1.46 mg dry wt was 0.474, 0.0238, 0.367, 0.884, 3.48, 21.0, 3.68, 0.129, 0.0193 and 3.33 nmol g^{-1} respectively.

5.3.3 Data Analyses

5.3.3.1 Free Ion

Percent contribution of the free ion to the total dissolved concentration of each metal was calculated with MINTEQA2 v4.03 (U.S. EPA, 2006) which incorporated the Gaussian Model for dissolved organic matter. The majority of the metalloid arsenic was present in the species $HAsO_4^{2-}$ and $H_2AsO_4^-$. The majority of chromium was present in the species CrO_4^{2-} and $HCrO_4^-$.

5.3.3.2 ANOVA

All data were log transformed prior to statistical treatment in order to normalize the variance. In order to detect significant changes in the above background bioaccumulation of a test metal across all treatments, it was desirable to utilize both the 0 and 24 hour gut cleared data in an analysis of covariance. However, before analyzing the data with a covariance model, a general linear model (GLM) was used to test for interaction between treatments and depuration (i.e. to determine if the data was parallel with homogeneous slopes). If there was no interaction then an analysis of covariance with a Tukey pair wise comparison post hoc test was done in order to determine significantly different treatments. In addition to the Tukey test, a one-way ANOVA was performed using a GLM with a Dunnett pair wise comparison post hoc test on the 0 and 24 hr depurated tissue concentrations separately, for any data set that showed interaction between treatments and depuration. This was done in order to determine which mixture treatments significantly affected the accumulation of the test metal compared to accumulation in the single metal exposure alone.

5.3.3.3 Interaction Factors

All data were log transformed prior to modelling in order to normalize the variance. Interaction coefficients (a_n' and a_n or b_n), the max term and the loss term were estimated with equation (3) with SYSTAT 10 using the mean measured total body concentrations (C_{TB}), the mean measured unfiltered

exposure concentrations and previous $K_{0.5}$ values (Table 5.2). Initially, all the coefficients (a_n') in the numerator of equation (3) were set to zero and the model was run in order to fit the denominator coefficients (a_n or b_n). If a denominator coefficient was found to be negative, it was set to zero and the corresponding numerator coefficient was set as a variable and the model was run again. If other coefficients in the denominator became negative, they were also set to zero and the corresponding numerator coefficients were set as variables and the model was run again. As well, if any of the numerator coefficients (a_n') became negative, indicating that metal was not having an interactive effect (neither inhibition nor enhancement), it was set to zero. This process was continued until all coefficients were either positive or set to zero.

Interaction Factors (IF_n) were determined with Eq. (4) or (5a) and (5b) for each metal in relation to the metal of interest (M) for all treatments in which the metal of interest was spiked, using the non-linear regression model in SYSTAT10. Interaction factors were calculated with 0 and 24 hours depuration data separately for any metal with an interaction between treatments and depuration, in which case the (1-loss × dep) term was excluded from equation (3a). Nine interaction factors, one for each of the nine other metals (M_n where n = 2 to 10), were calculated for each metal of interest (M). IF_n is equivalent to the ratio of the accumulation of a metal in a binary mixture at concentrations equal to those in the multi-metal mixtures, divided by accumulation of the metal when present singly, as predicted from the computed coefficients. This represents the predicted effect of the second metal alone on accumulation of the first in the complex mixtures and provides a direct comparison of the predicted metal-metal interactions for each metal pair under the experimental conditions of this study.

The experiments that examined the interaction of Cu, Mn and Zn, not only investigated the interactions between these three metals but also the interaction with the 7MIX group of metals. Since these seven metals were always spiked together in the experiments investigating Cu, Mn and Zn interactions, the 7MIX was treated as if it was one metal in order to determine coefficients of interaction of each of the 7 metals on Cu, Mn and Zn bioaccumulation. This was done by using the concentration of each of the 7 metals individually as a surrogate for the 7MIX group, whereas the other 6 metal coefficients in equation (3) were set to zero. For example, to estimate the IF_ns for each of the 7MIX metals on Cu bioaccumulation, first the model was run with Eq (3) estimating the coefficient for As while the coefficients for Cd, Co, Cr, Ni, Pb and Tl were set to zero. Then this was repeated but estimating the coefficient for Cd instead of As while As, Co, Cr, Ni, Pb and Tl were set to zero. This process was repeated until coefficients for the 7 metals of the 7MIX were estimated. The IF_ns were then calculated for each metal. During this process, seven coefficients of influence were generated for each of Mn and Zn. The geometric mean of these seven coefficients was used to calculate the interaction

factors of Mn and Zn on Cu bioaccumulation. This entire process was repeated, treating Mn and then Zn as the metal of interest.

The overall response or change in the bioaccumulation of a metal of interest in the presence of other metals in solution was calculated as the sum of the absolute deviation from 1 of all the IF_Ms for that metal. As well, the overall influence of a metal in solution on the bioaccumulation of all the other metals was calculated as the sum of the absolute deviation from 1 of each IF_n where n = 2 to 10.

5.4 RESULTS

5.4.1 Water Analyses and Hyalella Survival

Dissolved and total spiked metal concentrations were equivalent throughout the test exposures for all metals since the mean dissolved (filtered) concentration was 101, 109, 101, 102, 108, 103, 107, 94, 102 and 104 percent of the total metal (non-filtered) concentrations of As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn in water respectively. As well, there was little loss from solution of the spiked metals during each renewal period (2 or 3 days), such that there was 102, 87, 100, 99, 89, 107, 98, 99 and 100 percent mean recovery of As, Cd, Co, Cr, Cu, Mn, Ni, Tl and Zn. However, there was some loss of lead which only had a recovery at 58%. Therefore, to best represent the overall exposure concentration of each metal, the mean of the initial and final measured non-filtered metal concentration of each renewal period was used to calculate the mean exposure concentrations of each metal (Table 5.1). These final exposure concentrations were similar to the desired LC25 for each metal, however the exposure ratio were not exactly equal to one (Table 5.1). This was due to variability in measurement (weighing of the stock solution salts and analyses), but also because the final published LC25 values (Borgmann et al., 2004; Norwood et al., 2007) were slightly different than the initial LC25 estimates available at the time the mixture experiments were conducted in 2002. However, fairly "equi-toxic" concentrations were achieved and very little mortality occurred (mean 1 wk survival ± 95%C.I. for all treatments was 92±1.1%).

5.4.2 Free Ion

Metal mixtures had very little effect on the free ion concentrations of all the metals. There was no change in the percent free ion concentration between non-spiked (background), individual metal treatments and the 10 metal, spiked treatments. The free ion species percent contributions for all the metals as well as the dominant species for As, Cu and Pb are presented in Table 5.1. Only Pb formed a DOM species (Pb-DOM) representing 1.8% of the total Pb. This percentage did not change across all treatments according to MINTEQA2.

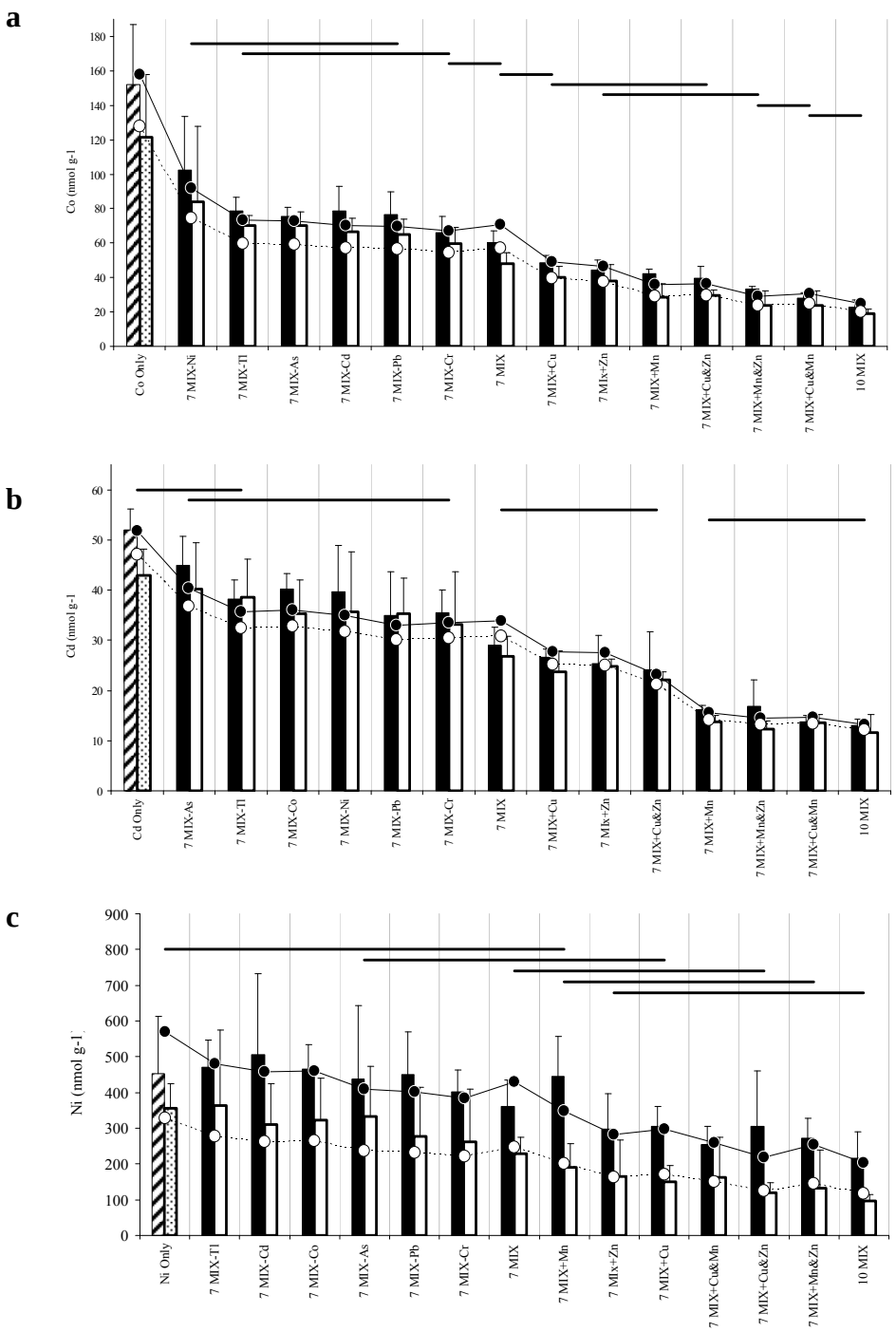

Figure 5.1 Bioaccumulation of Co (a), Cd (b) and Ni (c) by *H. azteca* in the presence of various mixtures during 1-week exposures. Solid bars represent mean 0 hr depurated total body concentrations, open bars represent 24 hr depurated, the diagonal striped bar represents the single element exposure with 0 hr depuration and the dotted bar with 24 hr depuration. Error bars represent 95% confidence limits. The horizontal lines represent treatments that are similar ($p>0.05$, ANCOVA Tukey Multiple Comparisons). The solid and open circles represent the predicted 0 and 24 hr depurated body concentrations respectively based on equation (3).

5.4.3 ANOVA: Impact of Mixtures on Body Concentrations

The analysis of variance using a general linear model (GLM) indicated that Pb was the only metal for which a significant interaction between treatment (the various mixtures) and the covariate (24 hr depuration) occurred (p=0.004), indicating that the Pb loss rate varied significantly with exposures to different mixtures. Therefore an analysis of covariance could not be performed on the Pb accumulation data. Instead, a one-way ANOVA was conducted on the 0 and 24 h data separately.

The GLM also indicated that treatment (mixtures) had a significant effect on As, Cd, Co, Cu, Ni, Pb, Tl and Zn above background, total body concentrations (p= 0.001, <0.001, <0.001, 0.003, <0.001, <0.001, <0.001 and 0.009 respectively). However, treatment did not have a significant effect on Cr and Mn above background body concentrations (p=0.259 and 0.901 respectively) indicating that Cr and Mn total body concentrations did not change across any of the mixture exposures. Depuration had a significant impact on the above background body concentrations of all the test metals (p<0.001 in all cases) except for Mn (p=0.066) indicating that there was no significant loss of Mn during the 24 h depuration period.

The analysis of covariance with a pairwise comparison using the post hoc Tukey test indicated that the impact on bioaccumulation fell into three categories: inhibition, no effect and enhancement.

5.4.3.1 Inhibition

Cobalt accumulation was significantly inhibited by every mixture combination compared to the Co only exposure (Fig. 5.1a) with the greatest impact resulting in 85 and 84% reduction in accumulation for the 0 and 24 h depurated organisms respectively when exposed to the 10-metal mixture. Cobalt accumulation was significantly reduced with increasing number of metals spiked in the exposure treatment (Fig. 5.2a, regression with the covariate depuration R^2 = 0.808, slope = -0.098±0.00841, p(2-tail) = <0.001).

Similar results were observed for cadmium accumulation with the greatest impact resulting in 75 and 73% reduction in accumulation compared to the Cd only exposure for both the 0 and 24 h depurated organisms respectively when exposed to the 10-metal mixture (Fig. 5.1b). Cadmium accumulation was also significantly reduced with an increase in the number of metals in the exposure medium (regression with the covariate depuration R^2 = 0.622, slope = -0.0636±0.00835, p(2-tail) = <0.001). Nickel was the only other metal for which accumulation was significantly inhibited by mixtures compared to the single metal only exposure, with the greatest impact resulting in a 48 and 27% reduction in accumulation by the 0 and 24 h depurated organisms, respectively, when exposed to the 10-metal mixture (Fig. 5.1c). Again, like Co and Cd, a significant decrease in Ni accumulation occurred with an increase in the number of metals in the exposure medium (regression with the

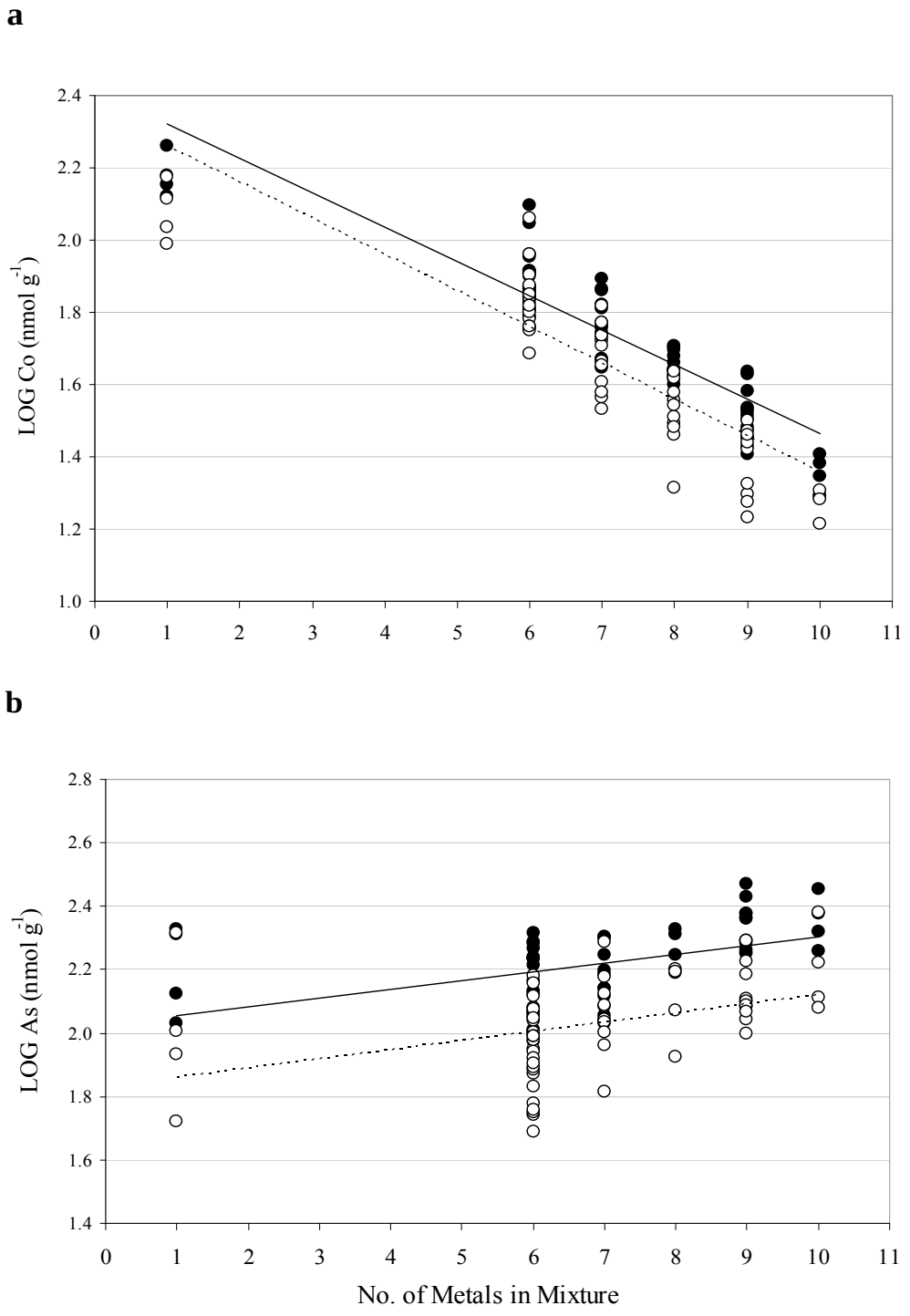

Figure 5.2 Bioaccumulation of Co (a) and As (b), versus the number of elements in the exposure mixtures. Solid symbols represent mean 0 hr depurated, open symbols represent 24 hr depurated. The solid line represents the linear regression of the 0 hr depurated body concentrations only and the dashed line represents the linear regression for the 24 hr depurated.

covariate depuration; R^2 = 0.593, slope = -0.0562±0.0119, p(2-tail) = <0.001). The Ni results had the greatest variability and the reductions were not as great as for Co and Cd, such that significant reductions only occurred when there were more than 7 metals in the mixture; the only mixture with more than seven metals that was not significantly lower than Ni alone was 7Mix+Mn.

All mixture combinations reduced thallium bioaccumulation by an average of 28 and 26% for the 0 and 24 h depurated animals respectively (Fig. 5.3a). All 14 of these mixtures were statistically the same, however, only 11 were statistically different than the Tl only treatment in individual comparisons. There was no trend of reduction with increasing number of metals in the treatment. Zinc bioaccumulation was also significantly (p=0.007) reduced by 28 and 26% for the 0 and 24 h depurated animals respectively, but only when exposed to the 10-metal mixture in comparison to the Zn only exposure (Fig. 5.3b).

5.4.3.2 No Effect

There was no significant change in Cr, Cu or Mn accumulation with exposure to any treatment in comparison to the metal only exposure (Fig. 5.4). The only significant differences in this group of metals were copper accumulation in the 10-Mix and the 7Mix+Cu+Mn, which were significantly lower than Cu accumulation in the 7Mix+Mn and the 7Mix+Cu+Mn treatments (Fig. 5.4b).

5.4.3.3 Enhancement

There was some enhancement of both As and Pb bioaccumulation by various mixtures. However, there were no treatments that had significantly different As body concentrations than the As only exposure (Fig. 5.5a) based on the paired comparisons. Nevertheless, the regression of As accumulation against the number of metals in the exposure mixtures indicated a significant increase in As accumulation with increasing number of metals spiked in the exposure medium (Fig. 5.2b, regression with the covariate depuration R^2 = 0.460, slope = 0.029±0.0103, p(2-tail) = <0.001). The results of the ANOVA using the GLM with an interaction term for depuration indicated that there was interaction between depuration and treatment on the bioaccumulation of Pb (i.e. the 0 and 24 hr data were not parallel). Therefore an analysis of covariance with a Tukey post hoc test could not be run. Instead, one-way ANOVAs with Dunnett's pairwise post hoc test was run on the 0 and 24 hr depuration data separately in order to determined any significant change in Pb bioaccumulation by any treatment compared to the Pb only exposure. There were three mixtures exposures that resulted in significantly different 0 hr depurated Pb bioaccumulation (Fig. 5.5b). The 7Mix+Cu+Mn and the 7Mix+Mn were both less than the Pb only treatment and the 7Mix-Co treatment had elevated bioaccumulation of Pb compared to the Pb only exposure. As well, there were 9 treatments with 24 hr depurated body concentrations that were significantly different than the Pb only exposure, all of which were elevated

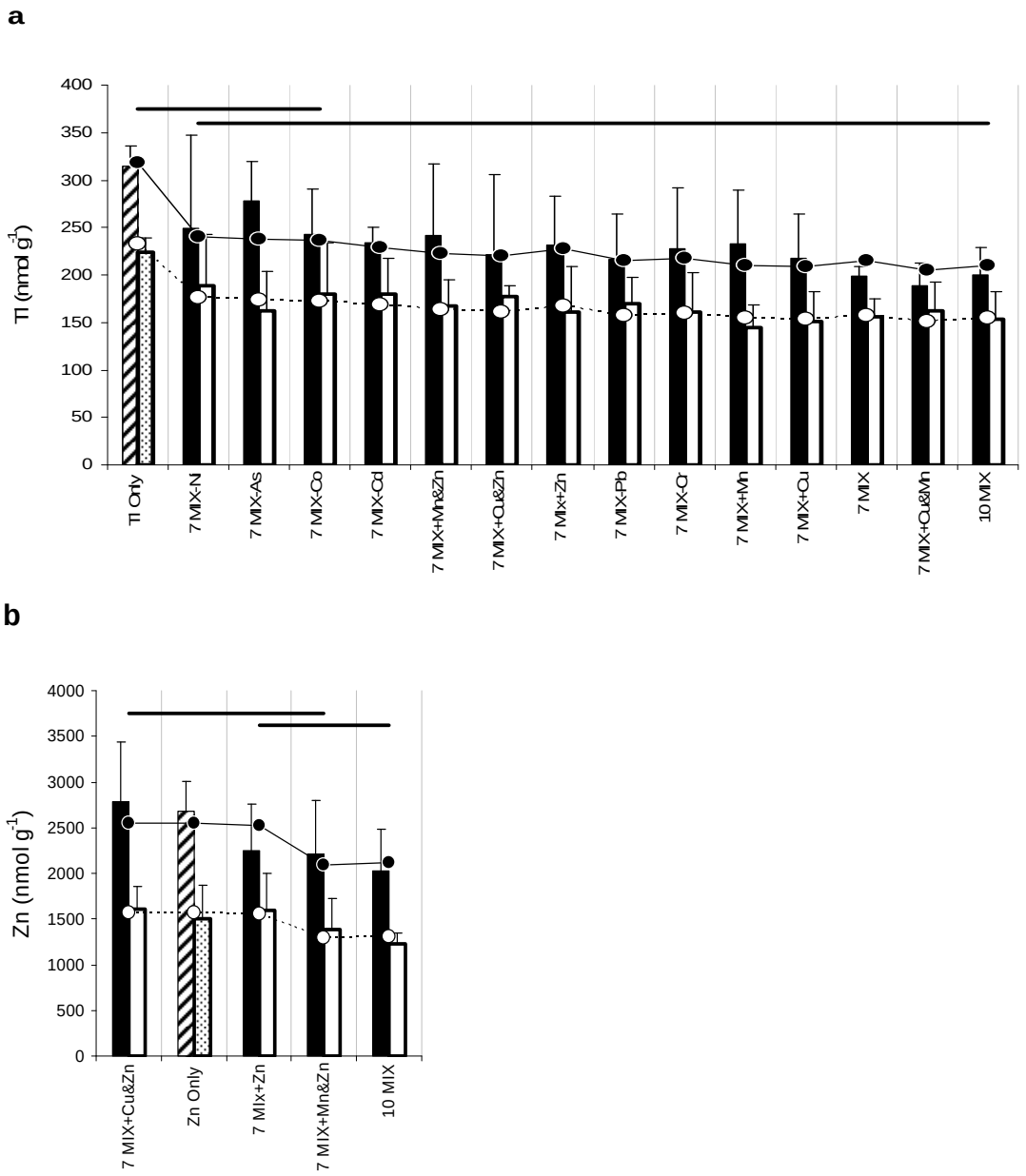

Figure 5.3 Bioaccumulation of Tl (a) and Zn (b) in the presence of various mixtures. Formats are the same as Fig. 5.1

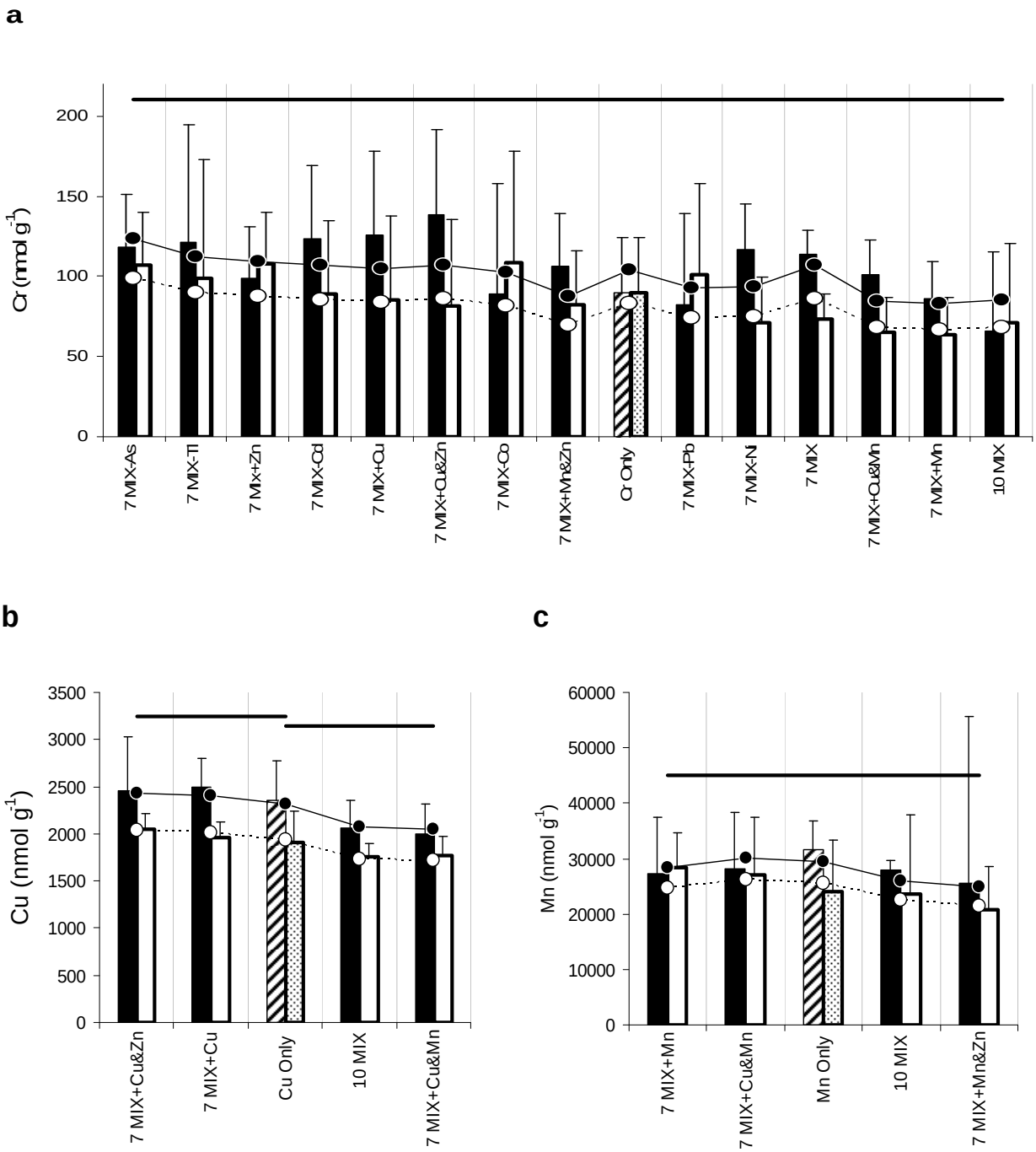

Figure 5.4 Bioaccumulation of Cr (a), Cu (b) and Mn (c) in the presence of various mixtures. Formats are the same as Fig. 5.1.

(Fig. 5.5b). However, there was no trend of increased Pb bioaccumulation with increased number of metals in the mixture (regression $R^2 = 0.409$, slope = -0.003±0.009, p(2-tail) = 0.5).

5.4.3.4 Interaction Factors

Interaction Factors (Table 5.4), representing the model predicted impact of each metal individually on each other metal in the mixture, as calculated using equation (5b), provide a more direct intuitive summary of how much the bioaccumulation of each metal was affected by every other metal at the concentrations tested in this study. The Interaction Factors indicate the factor by which the column heading metal influenced the row metal accumulation at the exposure concentrations tested. An interaction factor >1 indicates an increase of the affected metal's bioaccumulation, a factor < 1 indicates a decrease of the affected metal's bioaccumulation and an interaction factor of 1.0 indicates no change. For example, when Mn was in the exposure medium, a significant increase in As accumulation by a factor of 1.34 (Table 5.4) occurred, or in others words a 34% increase.

Cobalt bioaccumulation underwent the greatest change due to the presence of other metals in solution (Rank = 1, Table 5.4) with an overall response value of 2.97. Cobalt bioaccumulation was decreased by all other metals, except Cr, with statistically significant decreases when Ni, Cu, Mn or Zn were present (Table 5.4). The impact on As accumulation ranked second with an overall response of 2.12, of which a majority of the interactions caused enhanced bioaccumulation, except for the significant inhibition by Cr and to a lesser extent by Ni and Tl (Table 5.4). Nickel accumulation was the third most responsive, followed by Cd, Cr, Pb, Tl, Zn and Cu, with the smallest response by Mn at 0.15. Manganese had the greatest overall influence of 3.42 (Rank = 1, Table 5.4), which included significant enhancement of As accumulation and significant inhibition of Cd, Co, Cr, Cu, Ni, Pb and Zn accumulation. Zinc overall influence was ranked second at 2.22, which included significant enhancement of As accumulation and significant inhibition of Cd, Co and Ni (Table 5.4). Copper was ranked third most influential with a 1.83, which included significant enhancement of As accumulation and significant inhibitions of Cd, Co and Ni accumulation. The remainder of the metals were ranked Ni>As>Cd>Tl>Co>Cr with the least influential metal being Pb at 0.49 (Table 5.4). These included significant decreases of Cd and Tl accumulations by As, a significant decrease of As accumulation by Cr, and a significant decrease of Co accumulation by Ni (Table 5.4).

5.4.3.5 Maximum, Background and Loss

Most of the max terms (Table 5.3), computed using equation (3) with the previous *K* values (Table 5.2), were not significantly different (95% confidence limits did not overlap) from previously reported values (Table 5.2). Both the max and *K* values could not be estimated simultaneously with the current data set since only one above-background concentration was used for each metal. There were no

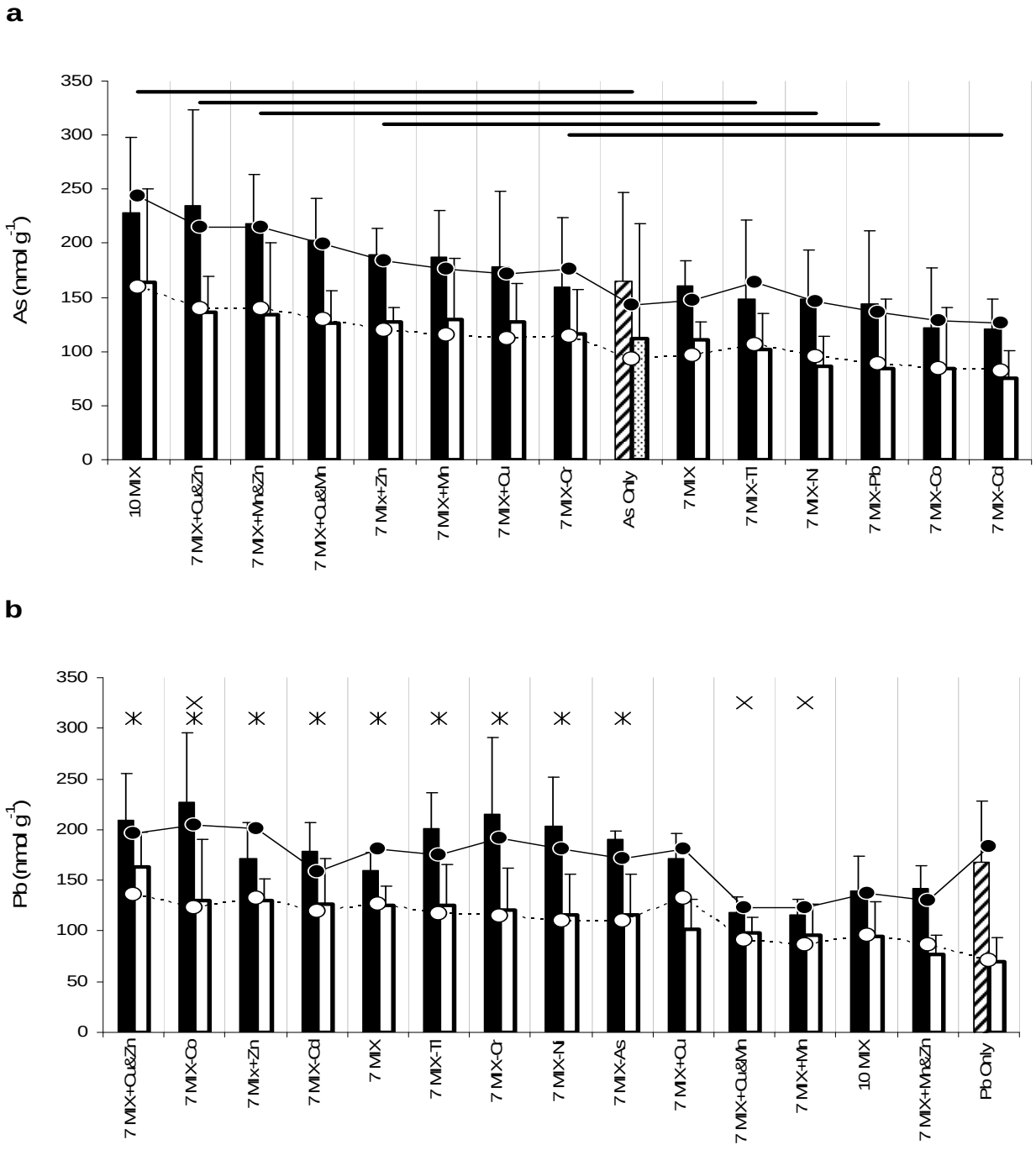

Figure 5.5 Bioaccumulation of As (a) and Pb (b) in the presence of various mixtures. Formats are the same as Fig. 5.1 except for Pb (b) in which × and ∗ indicate bioaccumulation that were significantly different than the Pb Only treatment at $p<0.05$ for the 0 and 24hr depurated total body concentrations respectively.

Table 5.3 Estimated maximum body concentration (max), 95% confidence limits (CL), background concentrations (Bkg) in which no metal was added and depuration loss rates.

	max (0 hr)	CL	Bkg	CL	Loss	CL
	(nmol g^{-1} dry wt)		(nmol g^{-1} dry wt)		(% day^{-1})	
As	195	155-235	15.0	13.2 - 16.8	34.9	29.3 - 40.5
Cd	1700	1530-1860	3.53	3.21 - 3.84	10.0	5.0 - 14.9
Co	1270	1070-1460	3.45	3.09 - 3.82	18.9	13.8 - 24.0
Cr	422	298-547	14.2	12.8 - 15.5	20.1	11.1 - 29.1
Ni	1.31 (max/*K*)	1.02-1.60	27.2	21.1 - 33.2	42.5	36.8 - 48.2
Pb (0hr)	7170	5820-8530	1.31	0.51-2.11		
Pb(24hr)	2710	2110-3310	1.00	0.60-1.40		
Tl	19300	17300-21300	0.270	0.212 - 0.328	26.7	23.1 - 30.3
Cu	1350	1090-1610	1460	1310 - 1610	16.3	11.5 - 21.1
Mn	82700	71000-94500	56.8	51.3 - 62.3	13.3	-0.2 - 26.8
Zn	3330	2740-3910	930	889 - 972	38.5	33.0 - 44.1

significant changes in the max terms for As, Cd, Co, Mn, Pb, Tl and Zn. However, it is noteworthy to point out that the Co max term increased 188% and the 95% C.L.s scarcely overlap. There were significant decreases of 49 and 63% in the max terms for Cr and Cu respectively. There was a significant increase by a factor of 2 in the max/*K* ratio for Ni (Tables 2 and 3). The observed differences in max or max/*K* might be due to differences in the methods used in this and previous studies. The two main differences between the current work and previous studies were: first, medium renewal (water, food and spiked metals) was increased to every 2 to 3 days compared to weekly for the historical work, and second, the current work utilized young adults in 1 week exposures compared to juveniles exposed for 4 weeks in the previous studies.

Background levels (no metals added) of all the metals were low (Table 5.3) in comparison to the body concentrations of the metal-only spike (Fig. 5.1, 5.3, 5.4 and 5.5) except for Cu and Zn. Background concentrations ranged from 0.1 to 15.8% of the 0 hr depurated, metal-only treatments for most metals except Cu and Zn which were 62.0 and 34.7% respectively. These percentages were also consistent when the calculations were based on the 24 hr depurated body concentrations except for Cu and Zn whose background percentages of the metal only treatment were even higher at 71.5 and 60.9% respectively.

There were no significant changes in As, Cd, Co, Cr, Cu, and Ni loss rates compared to historical values (Tables 2 and 3; for historical Cd, Borgmann et al. 2004). The Mn loss rate of 13% day^{-1} (Table 5.3) was significantly less than the 48% of previous work (Table 5.2). Both Pb and Zn loss rates of 34 and 38% day^{-1} respectively (Table 5.3) were also significantly less than the 41 and 49% day^{-1} of previous studies (Table 5.2). A comparison for Tl could not be made. The experimental conditions, including the mixtures, may have had an impact on loss rates but the exact cause is unknown.

5.5 DISCUSSION

The main objective of this work was to determine if exposure to mixtures of 10 metals results in interactions that affect their bioaccumulation. Quite clearly the answer is yes. If all the metals were competing for the same type of internal binding site, it would be expected that with increasing number of metals there would be a corresponding decrease in accumulation of each metal. There was a significant decrease in Co, Cd and Ni with increasing number of metals in the exposure medium, however this was not observed for the other seven metals. On the contrary, some enhancement of bioaccumulation occurred for As and Pb (Fig 5, Table 5.4). As well, there was very little or no effect on Tl, Zn, Cr, Cu and Mn accumulation. Therefore, not all metal interactions can be described by a competitive inhibition model such as the BLM and it is possible that not all metals bind to the same type of binding site. Both statements are plausible considering that metals, during chronic and sub-lethal exposures, could be binding to transport proteins in the membranes and metabolic sites within a

Table 5.4 Interaction Factors (IF_M, influence of the column heading element on the row element's bioaccumulation). An IF_M >1 indicates enhanced bioaccumulation and <1 indicates inhibited bioaccumulation. An IF_M = 1 indicates no interaction. Overall response of bioaccumulation of the metal in the row to the presence of other metals in solution. Overall influence of the metal in the column on the bioaccumulations of the other metals. The responses and influences are ranked.

		Cd	Co	Cr	Ni	Pb	Tl		Mn	Zn	Overall Response	Rank
As	-	1.261	1.228	0.771*	0.973	1.082	0.849	1.333*	1.341*	1.463**	2.12	2
Cd	0.795**	-	0.898	1.016	0.935	1	0.909	0.703**	0.278***	0.636***	1.86	4
Co	0.828	0.894	-	1.017	0.560***	0.864	0.813	0.436***	0.262***	0.396***	2.97	1
Cr	0.810	0.967	1.026	-	1.209	1.185	0.928	0.974	0.690*	1.034	1.08	6
Ni	1	0.839	0.852	1.098	-	1.051	0.834	0.527**	0.724*	0.505***	1.87	3
Pb (0hr)	1.076	1.174	0.883	0.901	0.958	-	1.034	1.026	0.648***	1.083	1.00	7
Pb (24hr)	1.230	1.105	1.072	1.118	1.185	-	1.130	1.130	0.707***	1	1.26	5
Tl	0.874*	0.921	0.882	1.000	0.892	1	-	0.942	0.957	1.045	0.58	8
Cu	1.000^S	1.000^S	1.000^S	1.000^S	1.000^S	1.000^S	1.000^S	-	0.622^{G}***	1.019^G	0.40	10
Mn	1.000^S	1.000^S	1.000^S	1.000^S	1.000^S	1.000^S	1.000^S	1.000^G	-	0.848^G	0.15	11
Zn	0.980^S	0.971^S	0.973^S	0.973^S	0.969^S	0.966^S	0.976^S	1.000^G	0.708^{G}***	-	0.48	9
Overall Influence	0.87	0.81	0.74	0.50	0.99	0.49	0.77	1.83	3.42	2.22		
Rank	5	6	8	9	4	10	7	3	1	2		

Significantly different than 1: * p<0.05, ** p<0.01, *** p<0.001

^{S} Surrogates values for the 7Mix group calculated with the other 6 in the group set to 0.

^{G} Geometric mean of The 7 values determined for this metal when each surrogate value^S was calculated.

cell, including cytosolic ligands such as enzymes and metalothioneins (Amiard-Triquet and Amiard, 1998). The current BLM is based on acute accumulation and toxicity at the plasma membrane, specifically the fish gill, with the assumption that binding occurs on a single 1:1 binding site (Slaveykova and Wilkinson, 2005). Since one week exposures were conducted with *H. azteca*, in which metal accumulation was evaluated on a whole body basis, a number of mechanistic bioaccumulation models may be necessary to cover the range of possible binding interactions and internal binding sites.

Other relationships, such as non-competitive and anti-competitive interactions, may be occurring and there is the possibility that there may be more than one binding site on the organism, internal or external. These scenarios may also account for increased accumulation of arsenic and lead. Other case studies have observed synergistic or enhanced bioaccumulation of one metal in the presence of a second metal, in plants (15 cases), molluscs (6 cases), crustaceans (9 cases), and fish (19 cases) (Amiard-Triquet and Amiard, 1998). Another possibility in which enhanced bioaccumulation or no change in a metal's bioaccumulation, even under competitive inhibition, could occur if a corresponding inhibition of excretion occurs. Hence, the difference between the uptake and excretion rates may be responsible for the final body concentration. Since the concentrations of the metals were not varied in our experiments it is not possible to distinguish the difference between non-competitive and competitive inhibition.

The use of the simplistic mathematical models (equations 2 or 3) provides a means to identify the interactions that are occurring and permit the calculation of interaction factors with equations (4), (5a) and (5b) which describe the ratio of the accumulation of a metal in a binary mixture divided by accumulation of the metal when present singly. These ratios are based on concentrations in the multi-metal mixtures exposures. The interaction factors can be used to predict metal-metal interactions for each metal pair under the experimental conditions of this study and predict bioaccumulation of each metal in each mixture exposure (Fig. 5.1, 5.3, 5.4 and 5.5).

Another way to examine the interactions between the metals is to determine if the interaction between two metals is reciprocal. For example, if the interaction between two metals is competitive inhibition at a single uptake site, then the interaction factors of the two metals should be reciprocal and less than one. Arsenic and Tl followed this pattern with a significant interaction factor of 0.874 for As influence on Tl accumulation, and the interaction factor of 0.849 for Tl influence on As accumulation (Table 5.4). The opposite was observed for the As-Cd interactions. Arsenic inhibited Cd accumulation with a significant interaction factor of 0.795, where as the interaction factor of Cd on As accumulation was 1.261 (Table 5.4). Of the 24 metal pairs that can be evaluated for reciprocity, which represents 48 interaction factors, a change in the interaction factor of 5% or greater was considered. Nine pairs demonstrated reciprocal inhibition, three pairs demonstrated reciprocal enhancement, two pairs showed

reciprocal inhibition to enhancement, four pairs showed no reciprocal effects, 3 pairs demonstrated enhancement for one metal and no effect for the other, and 3 pairs demonstrated inhibition of one metal and no effect on the other (Table 5.4). This indicates that only nine pairings out of 24 may represent competitive inhibition at a single uptake site (reciprocal inhibition). However, to accurately identify the type of interaction, the metal concentrations must be varied. For example, in a paired comparison, one metal exposure should be fixed and the second metal exposure should be varied, then the reverse should be tested.

Since the metals As, Cd, Co, Cr, Ni, Pb and Tl were always added together in tests with Cu, Mn and Zn in combination, each of the 7 metals should not be tested for reciprocal effects individually with Cu, Mn and Zn. However, the effect of the 7MIX as a group on Cu, Mn or Zn accumulation can be evaluated by taking the geometric mean of the 7 interaction coefficients. There was no impact of the 7MIX on Cu or Mn accumulation (geomean $IF_{7MIX} = 1$ for both, Table 5.4) and the 7MIX had a non-significant inhibitory effect on Zn accumulation (geomean $IF_{7MIX} = 0.973$, Table 5.4). Basically the 7MIX group of metals has very little impact on Cu, Mn or Zn bioaccumulation.

It was clear that interactions between metals did affect bioaccumulation; however the mechanisms that control these interactions cannot be determined from these experiments. These results do, however, allow for the design of more focussed investigations that can specifically target metals that were interacting. As well, it is not yet known if these changes in bioaccumulation will translate into changes in effects and thus the next step must be to determine the resulting chronic toxicity of the 10 metal mixture and the relationship to bioaccumulation.

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CHAPTER 6

Competitive Interactions of Metal Bioaccumulation in Multi-metal Exposures

6.1 Introduction

It was clearly demonstrated in Chapter 5 that the exposure of *Hyalella azteca* to mixtures of 10 metals resulted in interactions that affected the bioaccumulation of metals. If all the metals were competing for the same type of binding or uptake site, as in a strictly competitive inhibition model such as the BLM, it would be expected that with increasing number of metals in the mixture there would be a corresponding decrease in accumulation of each metal (Playle, 2004). There was a significant decrease in Co, Cd and Ni with increasing number of metals in the exposure medium, however this was not observed for the other seven metals (Chapter 5). There may have been more than one type of binding site, internally or externally, such that there was no competition between some metals, as well, other interactions may have occurred, such as non-competitive and anti-competitive (Borgmann et al., 2007)

The mathematical models in Chapter 5 provided a means to determine that interactions were occurring and to calculate interaction factors to describe the impact of a metal on the accumulation of each of the other metals in a mixture. However, these interaction factors do not identify the mechanism or the type of interaction that was occurring. The best experimental design to identify the type of interaction is one in which the concentration an individual metal is varied while all other metal concentrations in the mixtures are kept constant (Borgmann et al., 2007). Unfortunately, to evaluate the mechanisms of interaction in mixtures of 10 metals, the number of treatments required to fully evaluate the interactions becomes laboriously large. Instead, a constant-ratio experimental design was used to detect interactions in Chapter 5 and, even though this design was not well suited to identify the type of interaction, it is possible to do some testing of these data to determine if competitive inhibition is a plausible mechanism of interaction between some of the metals. Therefore the objective of this chapter is to examine the data in Chapter 5 in more detail to determine if competitive inhibition is a plausible mechanism occurring between some or all of the metals, leading to the decreased accumulations relative to single metal exposures observed in Chapter 5.

If metals compete for binding on the same ligand, the bioaccumulation of each metal can be predicted with models based on bioaccumulation in single exposures. Individual metal bioaccumulation has been described using the mechanistically based saturation model (Borgmann et al., 2004), as follows:

$$C_{TB} = \frac{(max \times C_W)}{(K_{0.5} + C_W)} + C_{Bk} \quad (1)$$

where C_{TB} is the total body concentration of the test metal, *max* is the maximum above-background accumulation of the metal, C_W is the metal concentration in water, $K_{0.5}$ is the half saturation constant

determined in the absence of other added metals, C_{Bk} is the background body concentration obtained from control animals (ie. absence of any added metals). The total body concentrations term can be replaced with C_{TBX}, which is the background-corrected body concentration (i.e. $C_{TBX} = C_{TB} - C_{Bk}$) gives

$$C_{TBX(single)} = \frac{(\max \times C_M)}{(1/K_M + C_M)} \quad (2)$$

where C_W is replaced with C_M, the water concentration of the metal of interest (M), and $K_{0.5}$ is replaced with K_M, the inverse of the half saturation constant $(K_M = 1/K_{0.5})$. K_M is equivalent to the metal binding constant in the biotic ligand model (BLM) or the conditional equilibrium stability constant as outlined by Playle et al (1993). These changes to Eq. (1) facilitate further modification of equation (2) to account for other metal interactions. When other metals are present and accumulate via the same ligand in a purely competitive manor, the bioaccumulation of the metal of interest (M) can be predicted by including the exposure concentrations and inverse of the half saturation constants of the other metals in Eq. (2) (Borgmann et al., 2007) giving

$$C_{TBX(mix)} = \frac{(\max \times C_M)}{((1 + \sum_{n=2}^{10} K_n \times C_n)/K_M + C_M)} \quad (3)$$

The predicted change (PC) in bioaccumulation of the metal of interest (M) in the presence of other metals relative to its bioaccumulation in the absence of other metals can be determined from the ratio of (Eq. 3) divided by (Eq. 2) giving

$$PC = (1 + K_M \times C_M) \div \left(1 + K_M \times C_M + \sum_{n=2}^{10} (K_n \times C_n)\right) \quad (4)$$

However, competitive inhibition is only one form of metal-metal interaction and other metal-metal interactions, such as such as non-competitive, anti-competitive and combined interactions, could occur (Borgmann et al., 2007). Unfortunately, these other two main models do not enable the prediction of changes in bioaccumulation since the max terms can be affected by metal competition and the K_M terms for interaction are not predictable from single-metal uptake studies. For example, for non-competitive interactions only the max term of the metal of interested is affected and can be decreased or increased. In anti-competitive interactions, both the $K_{0.5}$ and max terms can be affected, again the max term may be decreased or increased.

Table 6.1(a): Mixture treatment, exposure concentrations (nmol/L), total body accumulation before and after 24 hr depurations, observed bioaccumulation change factor from single metal to mixture exposures, predicted change based on all metals, prediction within 10% of observed, predicted change based on cations only, and prediction within 10% of observed.

												Observed		All Metals		All Metals		Only Cations		Only Cations	
			Water (nmol/L)			Body 0 hr (nmol/g)			Body 24 hr (nmol/g)			Change Factor		Predicted Factor		Within 10%?		Predicted Factor		Within 10%?	
Treatment	Metal	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	(0 hr)	(24 hr)	Mean	Std	(0 hr)	(24 hr)	Mean	Std	(0 hr)	(24 hr)
10 MIX	As	4	5627	8.76	2	228	43.99	4	164	54.5	4	1.42	1.54	0.41	0.0082	N	N				
10 MIX	Cd	4	2.77	0.176	2	13.0	0.769	4	11.6	2.31	4	0.20	0.22	0.17	0.0020	Y	Y	0.25	0.0042	Y	Y
10 MIX	Co	4	61.1	4.14	2	22.9	2.57	4	18.9	1.70	4	0.13	0.13	0.19	0.0019	Y	Y	0.22	0.0061	Y	Y
10 MIX	Cr	4	286	14.6	2	65.8	11.1	4	71.1	30.88	4	0.68	0.75	0.15	0.0031	N	N				
10 MIX	Cu	4	524	0.05	2	2057	191	4	1762	82	4	0.67	0.72	0.23	0.0014	N	N	0.33	0.0039	N	N
10 MIX	Mn	4	79163	3289	2	27947	1121	4	23642	8968	4	0.88	0.98	0.31	0.0062	N	N	0.44	0.0065	N	N
10 MIX	Ni	4	455	32.5	2	216	46.2	4	96.7	10.7	4	0.44	0.22	0.15	0.0030	N	Y	0.22	0.0055	N	Y
10 MIX	Pb	4	41.9	2.65	2	139	21.3	4	94.8	21.4	4	0.83	1.35	0.15	0.0031	N	N	0.22	0.0056	N	N
10 MIX	Tl	4	43.0	1.32	2	200	18.4	4	153	18.4	4	0.64	0.69	0.42	0.0087	N	N	0.60	0.0158	Y	Y
10 MIX	Zn	4	2484	197	2	2020	288	4	1223	74.1	4	0.62	0.52	0.22	0.0054	N	N	0.46	0.0063	N	Y
7 MIX+Cu&Mn	As	4	5578	12.7	2	203	24.6	4	126	18.66	4	1.25	1.15	0.50	0.0013	N	N				
7 MIX+Cu&Mn	Cd	4	2.76	0.00976	2	13.8	0.700	4	13.6	1.05	4	0.21	0.26	0.21	0.0003	Y	Y	0.33	0.0003	N	Y
7 MIX+Cu&Mn	Co	4	60.3	2.32	2	27.7	2.11	4	23.9	5.24	4	0.16	0.17	0.23	0.0003	Y	Y	0.30	0.0013	N	N
7 MIX+Cu&Mn	Cr	4	293	3.50	2	101	28.1	4	65.3	13.5	4	1.15	0.67	0.18	0.0007	N	N				
7 MIX+Cu&Mn	Cu	4	504	2.05	2	1998	196	4	1765	128	4	0.60	0.73	0.28	0.0023	N	N	0.44	0.0035	N	N
7 MIX+Cu&Mn	Mn	4	76963	2725.88	2	28126	6375	4	27060	6550	4	0.89	1.12	0.18	0.0014	N	N	0.29	0.0023	N	N
7 MIX+Cu&Mn	Ni	4	442	17.5	2	255	31.3	4	162	71.2	4	0.53	0.42	0.19	0.0005	N	N	0.29	0.0009	N	N
7 MIX+Cu&Mn	Pb	4	39.5	1.83	2	118	10.1	4	98.7	9.67	4	0.70	1.41	0.19	0.0008	N	N	0.29	0.0013	N	N
7 MIX+Cu&Mn	Tl	4	43.7	0.396	2	189	15.0	4	162	19.2	4	0.60	0.72	0.50	0.0033	N	N	0.78	0.0054	N	Y
7 MIX+Cu&Zn	As	4	5709	26.0	2	235	55.5	4	137	20.8	4	1.47	1.26	0.46	0.0120	N	N				
7 MIX+Cu&Zn	Cd	4	2.73	0.0267	2	24.1	4.71	4	22.0	1.05	4	0.42	0.48	0.19	0.0036	N	N	0.29	0.0086	N	N
7 MIX+Cu&Zn	Co	4	60.2	1.90	2	39.5	4.26	4	29.4	2.09	4	0.24	0.22	0.21	0.0028	Y	Y	0.26	0.0092	Y	Y
7 MIX+Cu&Zn	Cr	4	285	13.5	2	138	75.4	4	81.7	33.7	4	1.64	0.89	0.17	0.0039	N	N				
7 MIX+Cu&Zn	Cu	4	516	20.0	2	2452	363	4	2046	106	4	1.11	1.24	0.17	0.0039	N	N	0.25	0.0085	N	N
7 MIX+Cu&Zn	Ni	4	438	22.4	2	305	96.6	4	119	18.0	4	0.65	0.29	0.17	0.0039	N	N	0.26	0.0086	N	Y
7 MIX+Cu&Zn	Pb	4	42.4	0.652	2	210	29.0	4	163	21.0	4	1.25	2.35	0.17	0.0039	N	N	0.25	0.0086	N	N
7 MIX+Cu&Zn	Tl	4	43.6	0.147	2	221	52.9	4	177	6.93	4	0.70	0.79	0.46	0.0007	N	N	0.69	0.0064	Y	N
7 MIX+Cu&Zn	Zn	4	2355	146	2	2779	411	4	1606	156	4	1.06	1.17	0.26	0.0088	N	N	0.52	0.0021	N	N
7 MIX+Mn&Zn	As	4	5568	45.7	2	218.7	27.9	4	134	42.2	4	1.36	1.23	0.55	0.0058	N	N				
7 MIX+Mn&Zn	Cd	4	2.86	0.155	2	16.8	3.34	4	12.4	0.920	4	0.27	0.24	0.24	0.0022	Y	Y	0.40	0.0040	N	N
7 MIX+Mn&Zn	Co	4	61.4	2.78	2	33.1	1.01	4	23.6	5.53	4	0.20	0.17	0.26	0.0010	Y	Y	0.36	0.0063	N	N
7 MIX+Mn&Zn	Cr	4	293	16.4	2	106	42.1	4	82.6	21.0	4	1.21	0.91	0.20	0.0032	N	N				
7 MIX+Mn&Zn	Mn	4	79623	2276	2	25466	3357	2	20794	4838	4	0.80	0.86	0.41	0.0036	N	N	0.69	0.0056	N	N
7 MIX+Mn&Zn	Ni	4	455	21.5	2	271	35.5	4	131	68.3	4	0.57	0.33	0.21	0.0031	N	N	0.35	0.0054	N	Y
7 MIX+Mn&Zn	Pb	4	40.3	1.39	2	142	13.9	4	76.6	12.3	4	0.85	1.09	0.21	0.0032	N	N	0.35	0.0057	N	N
7 MIX+Mn&Zn	Tl	4	44.0	0.260	2	241	47.7	4	168	16.9	4	0.77	0.75	0.23	0.0091	N	N	0.38	0.0156	N	N
7 MIX+Mn&Zn	Zn	4	2421	117	2	2214	363	4	1388	215	4	0.73	0.80	0.35	0.0052	N	N	0.73	0.0053	Y	Y
7 MIX+Cu	As	4	5635	110	2	179	43.7	4	127	22.3	4	1.10	1.16	0.56	0.0105	N	N				
7 MIX+Cu	Cd	4	2.57	0.123	2	26.6	1.04	4	23.7	2.63	4	0.48	0.52	0.23	0.0047	N	N	0.40	0.0093	Y	N
7 MIX+Cu	Co	4	58.1	5.01	2	48.7	2.38	4	39.9	4.23	4	0.30	0.31	0.26	0.0043	Y	Y	0.36	0.0134	Y	Y
7 MIX+Cu	Cr	4	283	19.8	2	125	40.5	4	85.2	32.9	4	1.47	0.94	0.20	0.0064	N	N				
7 MIX+Cu	Cu	4	490	25.1	2	2489	199	4	1957	105	4	1.15	1.08	0.20	0.0063	N	N	0.34	0.0119	N	N
7 MIX+Cu	Ni	4	434	30.3	2	305	35.7	4	151	28.4	4	0.65	0.39	0.21	0.0064	N	N	0.35	0.0121	N	Y
7 MIX+Cu	Pb	4	41.7	1.10	2	171	15.4	4	102	18.3	4	1.02	1.46	0.21	0.0065	N	N	0.35	0.0121	N	N
7 MIX+Cu	Tl	4	42.9	0.272	2	218	28.9	4	151	20.1	4	0.69	0.68	0.55	0.0004	N	N	0.92	0.0026	N	N

Table 6.1(b): Continued

			Water (nmol/L)			Body 0 hr (nmol/g)			Body 24 hr (nmol/g)			Observed Change		All Metals Predicted Change		All Metals Within 10%?		Only Cations Predicted Change		Only Cations Within 10%?	
Treatment	Metal	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	(0 hr)	(24 hr)	Mean	Std	(0 hr)	(24 hr)	Mean	Std	(0 hr)	(24 hr)
7 MIX+Mn	As	4	5667	181	2	187	27.1	4	130	35.72	4	1.15	1.18	0.71	0.0030	N	N				
7 MIX+Mn	Cd	4	2.67	0.0571	2	16.1	0.541	4	13.7	0.896	4	0.26	0.27	0.30	0.0038	Y	Y	0.62	0.0029	N	N
7 MIX+Mn	Co	4	58.5	1.53	2	42.2	1.69	4	28.3	5.27	4	0.26	0.21	0.32	0.0030	Y	N	0.59	0.0007	N	N
7 MIX+Mn	Cr	4	279	9.41	2	86.4	15.7	4	63.7	14.6	4	0.95	0.65	0.26	0.0042	N	N				
7 MIX+Mn	Mn	4	75955	2830	2	27338	6430	4	28343	3926	4	0.86	1.17	0.26	0.0047	N	N	0.54	0.0004	N	N
7 MIX+Mn	Ni	4	427	17.9	2	444	70.7	4	191	42.0	4	0.98	0.50	0.26	0.0043	N	N	0.55	0.0006	N	Y
7 MIX+Mn	Pb	4	40.3	0.00829	2	116	9.85	4	95.4	19.9	4	0.69	1.36	0.26	0.0042	N	N	0.55	0.0007	N	N
7 MIX+Mn	Tl	4	42.9	0.351	2	232	35.6	4	144	15.6	4	0.74	0.64	0.29	0.0109	N	N	0.59	0.0125	N	Y
7 MIx+Zn	As	4	5482	18.8	2	189	15.55	4	128	8.06	4	1.16	1.17	0.63	0.0058	N	N				
7 MIx+Zn	Cd	4	2.76	0.0521	2	25.4	3.49	4	24.9	0.874	4	0.45	0.55	0.27	0.0010	N	N	0.50	0.0043	Y	Y
7 MIx+Zn	Co	4	59.3	1.47	2	44.2	3.77	4	38.0	6.02	4	0.27	0.29	0.29	0.0001	Y	Y	0.46	0.0060	N	N
7 MIx+Zn	Cr	4	282	9.22	2	98.5	36.49	4	108	20.3	4	1.11	1.24	0.23	0.0017	N	N				
7 MIx+Zn	Ni	4	435	14.2	2	297	61.85	4	166	63.4	4	0.63	0.43	0.24	0.0016	N	N	0.44	0.0050	N	Y
7 MIx+Zn	Pb	4	44.2	0.918	2	171	22.53	4	130	13.4	4	1.02	1.86	0.24	0.0017	N	N	0.44	0.0053	N	N
7 MIx+Zn	Tl	4	43.358	0.492	2	231	32.54	4	160	30.3	4	0.74	0.72	0.26	0.0071	N	N	0.48	0.0154	N	N
7 MIx+Zn	Zn	4	2308	109	2	2254	316	4	1593	254	4	0.76	1.15	0.44	0.0055	N	N	0.92	0.0098	N	N
7 MIX	As	12	5555	172	6	161	31.9	10	111	23.3	10	0.97	0.99	0.81	0.0078	N	N				
7 MIX	Cd	12	2.60	0.077	6	29.0	5.82	12	26.8	6.13	12	0.52	0.60	0.35	0.0034	N	N	0.84	0.0180	N	N
7 MIX	Co	12	59.5	1.31	6	60.2	11.1	12	48.1	9.69	12	0.38	0.38	0.38	0.0038	Y	Y	0.83	0.0180	N	N
7 MIX	Cr	12	284	7.16	6	114	37.7	12	73.5	24.0	12	1.31	0.78	0.30	0.0034	N	N				
7 MIX	Ni	12	437	13.9	6	361	117	12	229	70.1	12	0.78	0.62	0.31	0.0035	N	N	0.75	0.0145	Y	N
7 MIX	Pb	12	42.5	0.992	6	160	27.9	12	126	28.3	12	0.95	1.80	0.30	0.0035	N	N	0.74	0.0140	N	N
7 MIX	Tl	12	42.6	1.63	6	199	15.8	12	156	29.8	12	0.63	0.70	0.34	0.0104	N	N	0.83	0.0046	N	N
7 MIX-As	Cd	4	2.66	0.0548	2	44.9	3.69	4	40.3	5.72	4	0.85	0.94	0.69	0.0075	N	N	0.81	0.0056	Y	N
7 MIX-As	Co	4	56.5	0.361	2	75.5	3.43	4	70.0	4.94	4	0.48	0.57	0.75	0.0036	N	N	0.80	0.0070	N	N
7 MIX-As	Cr	4	275	9.41	2	118	19.5	3	107	20.9	4	1.37	1.23	0.60	0.0071	N	N				
7 MIX-As	Ni	4	422	8.64	2	437	83.0	3	334	153	2	0.96	0.93	0.61	0.0067	N	N	0.73	0.0050	N	N
7 MIX-As	Pb	4	42.3	1.51	2	191	5.19	4	116	25.4	4	1.14	1.66	0.61	0.0071	N	N	0.72	0.0056	N	N
7 MIX-As	Tl	4	42.2	0.0581	2	278	26.1	4	162	26.0	4	0.88	0.72	0.71	0.0017	N	Y	0.84	0.0013	Y	N
7 MIX-Cd	As	4	5444	80	2	121	17.3	4	74.8	16.2	4	0.71	0.62	0.81	0.0038	N	N				
7 MIX-Cd	Co	4	56.6	0.469	2	78.6	9.14	4	66.4	4.98	4	0.51	0.53	0.38	0.0030	N	N	0.82	0.0037	N	N
7 MIX-Cd	Cr	4	276	4.63	2	123	48.4	4	89.4	28.5	4	1.44	1.00	0.30	0.0014	N	N				
7 MIX-Cd	Ni	4	423	5.60	2	506	90.4	3	310	71.7	4	1.13	0.86	0.31	0.0019	N	N	0.74	0.0019	N	N
7 MIX-Cd	Pb	4	42.2	2.50	2	179	17.8	4	127	28.1	4	1.07	1.81	0.31	0.0014	N	N	0.74	0.0030	N	N
7 MIX-Cd	Tl	4	42.6	0.201	2	234	10.4	4	180	23.7	4	0.74	0.81	0.35	0.0024	N	N	0.85	0.0014	N	Y
7 MIX-Co	As	4	5428	162	2	122	34.8	4	84.6	35.2	4	0.71	0.72	0.84	0.0018	N	N				
7 MIX-Co	Cd	4	2.59	0.111	2	40.3	1.94	4	35.3	4.29	4	0.76	0.81	0.31	0.0064	N	N	0.80	0.0048	Y	Y
7 MIX-Co	Cr	4	271	8.88	2	88.8	15.9	4	109	43.6	4	0.98	1.25	0.31	0.0066	N	N				
7 MIX-Co	Ni	4	427	24.8	2	466	42.9	4	322	74.7	4	1.03	0.90	0.32	0.0063	N	N	0.82	0.0040	N	Y
7 MIX-Co	Pb	4	43.1	2.32	2	227	42.8	4	130	37.4	4	1.36	1.87	0.32	0.0066	N	N	0.81	0.0050	N	N
7 MIX-Co	Tl	4	42.3	0.366	2	243	29.6	4	180	33.9	4	0.77	0.81	0.37	0.0062	N	N	0.93	0.0022	N	N

Table 6.1(c): Continued

			Water (nmol/L)			Body 0 hr (nmol/g)			Body 24 hr (nmol/g)			Observed Change		All Metals Predicted Change		All Metals Within 10%?		Only Cations Predicted Change		Only Cations Within 10%?	
Treatment	Metal	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	(0 hr)	(24 hr)	Mean	Std	(0 hr)	(24 hr)	Mean	Std	(0 hr)	(24 hr)
7 MIX-Cr	As	4	5351	42.9	2	160	39.9	4	117	25.6	4	0.97	1.05	0.86	0.0036	N	N				
7 MIX-Cr	Cd	4	2.604	0.0539	2	35.4	2.92	4	33.2	6.56	4	0.66	0.76	0.37	0.0016	N	N	0.81	0.0031	N	Y
7 MIX-Cr	Co	4	57.3	1.49	2	66.2	5.89	4	59.7	5.82	4	0.42	0.48	0.33	0.0004	Y	N	0.80	0.0066	N	N
7 MIX-Cr	Ni	4	430	13.8	2	401	39.6	4	262	92.5	4	0.88	0.72	0.33	0.0012	N	N	0.72	0.0033	N	Y
7 MIX-Cr	Pb	4	41.1	4.59	2	216	47.2	4	121	25.9	4	1.29	1.73	0.33	0.0004	N	N	0.72	0.0050	N	N
7 MIX-Cr	Tl	4	42.8	1.02	2	228	40.0	4	161	25.7	4	0.73	0.72	0.38	0.0016	N	N	0.84	0.0034	N	N
7 MIX-Ni	As	4	5455	15.7	2	148	28.6	4	86.6	17.3	4	0.89	0.74	0.80	0.0050	Y	Y				
7 MIX-Ni	Cd	4	2.57	0.0651	2	39.7	5.78	4	35.7	7.53	4	0.75	0.82	0.34	0.0001	N	N	0.82	0.0059	Y	Y
7 MIX-Ni	Co	4	55.4	1.86	2	102	19.5	4	83.8	27.7	4	0.66	0.68	0.38	0.0005	N	N	0.81	0.0106	N	N
7 MIX-Ni	Cr	4	284	4.14	2	117	41.9	4	71.0	18.0	4	1.35	0.75	0.30	0.0013	N	N				
7 MIX-Ni	Pb	4	40.7	0.490	2	203	30.5	4	116	25.3	4	1.21	1.66	0.30	0.0013	N	N	0.73	0.0082	N	N
7 MIX-Ni	Tl	4	43.4	0.786	2	249	62.0	4	188	34.3	4	0.79	0.84	0.35	0.0015	N	N	0.84	0.0024	Y	Y
7 MIX-Pb	As	4	5256	238	2	144	42.5	4	83.7	40.9	4	0.86	0.71	0.81	0.0123	Y	Y				
7 MIX-Pb	Cd	4	2.51	0.0128	2	34.9	5.56	4	35.3	4.46	4	0.65	0.81	0.35	0.0048	N	N	0.83	0.0160	N	Y
7 MIX-Pb	Co	4	54.8	0.485	2	76.3	8.44	4	65.0	5.64	4	0.49	0.52	0.38	0.0060	N	N	0.82	0.0190	N	N
7 MIX-Pb	Cr	4	272	4.17	2	82.6	27.6	4	101	35.6	4	0.90	1.15	0.31	0.0039	N	N				
7 MIX-Pb	Ni	4	428	23.4	2	450	67.7	2	277	86.0	4	0.99	0.76	0.31	0.0039	N	N	0.72	0.0148	N	Y
7 MIX-Pb	Tl	4	41.9	0.411	2	216	29.8	4	169	17.4	4	0.69	0.76	0.36	0.0128	N	N	0.86	0.0018	N	Y
7 MIX-Tl	As	4	5485	195	2	149	46.2	4	102	20.9	4	0.89	0.89	0.81	0.0085	Y	Y				
7 MIX-Tl	Cd	4	2.59	0.177	2	38.3	2.31	4	38.5	4.75	4	0.72	0.89	0.34	0.0056	N	N	0.83	0.0041	N	Y
7 MIX-Tl	Co	4	57.3	1.91	2	78.8	4.75	4	70.1	3.85	4	0.51	0.57	0.38	0.0067	N	N	0.81	0.0086	N	N
7 MIX-Tl	Cr	4	282	8.39	2	121	37.5	4	99.1	46.3	4	1.41	1.13	0.30	0.0035	N	N				
7 MIX-Tl	Ni	4	438	15.7	2	471	47.0	4	363	133	4	1.04	1.02	0.31	0.0042	N	N	0.74	0.0056	N	N
7 MIX-Tl	Pb	4	42.0	2.97	2	201	22.4	4	125	25.9	4	1.20	1.79	0.30	0.0035	N	N	0.72	0.0067	N	N
CONTROL	As	12	15.8	7.23	6	15.0	2.77	12	15.4	1.90	12										
CONTROL	Cd	12	0.149	0.0530	6	3.53	0.496	12	3.03	0.594	12										
CONTROL	Co	12	0.562	0.252	6	3.45	0.573	12	3.22	0.497	12										
CONTROL	Cr	12	5.16	0.443	6	14.2	2.03	11	14.9	1.95	11										
CONTROL	Cu	12	43.5	16.6	6	1461	231	12	1370	259	12										
CONTROL	Mn	12	12.7	9.40	6	56.8	8.64	12	54.7	9.61	11										
CONTROL	Ni	12	12.0	1.26	6	27.2	9.03	11	22.0	7.91	10										
CONTROL	Pb	12	0.264	0.0594	6	1.77	2.14	11	0.991	0.620	11										
CONTROL	Tl	12	0.0538	0.0423	6	0.270	0.0906	12	0.375	0.353	12										
CONTROL	Zn	12	25.0	13.8	6	930	65.2	12	918	58.3	12										
As Only	As	4	5497	67.0	2	165	52.2	4 8	112	66.6	4										
Cd Only	Cd	8	2.77	0.130	4	52.0	4.90	8 8	42.8	6.37	8										
Co Only	Co	4	58.8	0.804	2	152	21.7	4 8	121	23.0	4										
Cr Only	Cr	4	291	4.69	2	89.9	14.9	3 8	89.7	21.5	4										
Cu Only	Cu	4	506	20.3	2	2356	266	4 8	1915	204	4										
Mn Only	Mn	4	80067	2622	2	31698	3245	4 8	24131	5739	4										
Ni Only	Ni	4	442	9.63	2	453	100	4 7	357	42.6	4										
Pb Only	Pb	4	42.7	0.254	2	168	37.8	4 8	70.23	14.3	4										
Tl Only	Tl	4	42.8	0.382	2	314	13.3	4 8	223	10.2	4										
Zn Only	Zn	4	2244	15.1	2	2678	204	4 #	1507	231	4										

Both non-competitive and anti-competitive interactions could lead to enhanced bioaccumulation of the metal of interest (Borgmann et al., 2007). Furthermore, in competitive inhibition the K_M for metal binding in a single metal uptake study is the same as the K_M for that metal's inhibition of uptake of another metal (i.e., binding is to only one site). However, in non-competitive inhibition a metal causing inhibition can bind to a site other that the one that transports metals into the organism (i.e., there is more than one binding site on the ligand, an uptake site and a control site). The K_M for binding to the site of uptake is not necessarily the same as the K_M for binding to the control site. The correct K_M for predicting a metals inhibitory effects on other metals is not necessarily the same at the K_M for uptake of the metal when present singly. Therefore, only the competitive inhibition model can be used to predict change in bioaccumulation of a metal of interest in the presence of other metals since the max and K_M terms are not expected to be affected.

6.2 Methods

The predicted change in the metal of interest was determined with equation (4) using the inverse of the half saturation constants for each metal (Table 5.2, Chapter 5) and the exposure concentrations of each metal (Table 6.1). The predicted change was then compared to the observed change (*OC*), as given by

$$OC = (C_{\mathrm{TB}}(\mathrm{mix}) - C_{\mathrm{TB}}(\mathrm{control})) \div (C_{\mathrm{TB}}(\mathrm{single}) - C_{\mathrm{TB}}(\mathrm{control})) \qquad (5)$$

in which C_{TB} (mix) was the measured total body concentration of the metal of interest in each mixture treatment and C_{TB}(single) was the measured total body concentration of the metal of interest metal only treatment, both corrected for background (control) concentration (Table 6.1a,b,c). This was done for both 0 and 24 hr depurated body concentrations. When the predicted change was within 10% (0.1 units of change) of the observed change it was considered a match, indicating that competitive inhibition was plausible. If the predicted change did not match the observed, it was possible no competitive interactions occurred or other types of interactions were occurring. The above calculations were performed with all ten metals (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn) included. Since both As and Cr were added as anions and the other metals as cations it was unlikely that the two groups would interact competitively. Therefore, predicted change was also calculated with As and Cr $K_{0.5}$ values and concentrations excluded from equation (4) in order to examine the interactions based on the eight cations only. Observed change (*OC*) was regressed against predicted change (*PC*) for all mixture treatments, using both the "anion & cations model" and the "cations only model". Control and single metal only treatments were excluded from the regression since there was no change. A regression line with a slope of 1 that passes through the origin signifies that observed change in bioaccumulation was

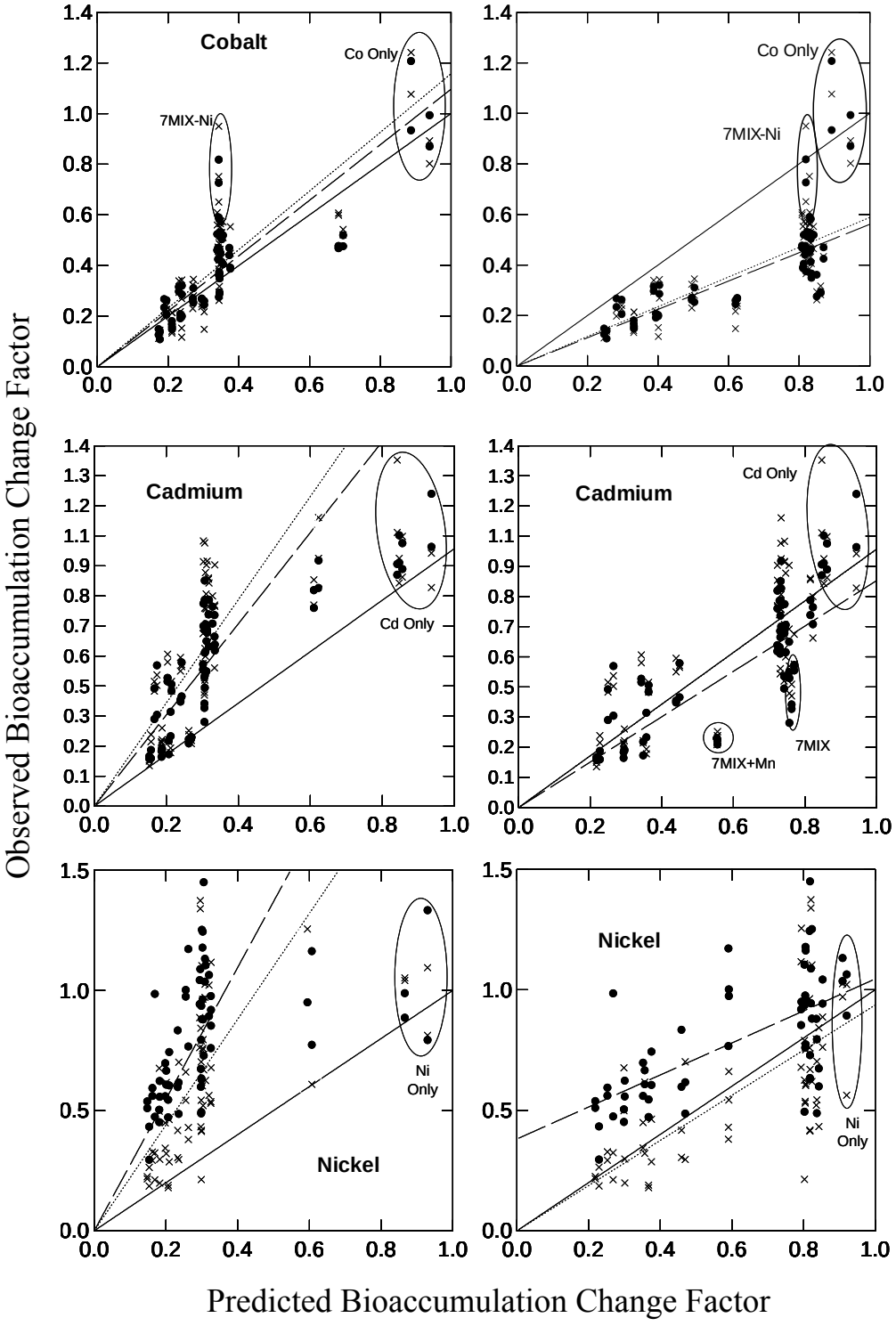

Figure 6.1 Observed bioaccumulation change (factor) for Co, Cd and Ni bioaccumulation based on 0 hr (●) and 24 hr (X) depurated body concentrations versus predicted bioaccumulation change factor based on anions & cations or cations only models. A 1:1 ratio of observed to predicted with a slope of 1(—) is plotted for comparison along with change in 0 hr depurated body concentration regressed against predicted (----) and regression of the change in 24 hr depurated body concentration versus predicted (·····).

the same as the predicted change, and indicates that competitive inhibition of the metal of interest is plausible. Data points that fall above this line, indicates that inhibition of accumulation was less than additive (less inhibition than expected by pure competitive inhibition) and data points that fall below the line indicate greater than additive effects (greater inhibition than expected by pure competitive inhibition).

6.3 Results and Discussion

6.3.1 Metals with Inhibited Bioaccumulation in Mixtures

Cobalt bioaccumulation was the most significantly inhibited in mixture exposures (Chapter 5). The change in Co bioaccumulation from the single exposure to mixture exposures was within 10% of predicted change in bioaccumulation in 22 of the 56 comparisons made, indicating a plausibility of competitive inhibition (Table 6.1a,b,c). However, 16 of these comparisons were based on prediction using the "anions & cations" model using equation (4). When the predicted change was based on the "cations only" there were only 3 treatments for which the change in Co accumulation was within 10% of the predicted values for both 0 and 24 hr depurated body concentrations. A plot of the observed change in Co accumulation versus predicted change based on the "anions & cations" models indicates that all mixtures, except the 7MIX-Ni and 7MIX-As, fell on the 1:1 line (Fig. 6.1, Cobalt: Anions & Cations Model). The 7MIX-Ni (ie. As, Cd, Co, Cr, Pb and Tl) mixture data points fall above the 1:1 line and therefore didn't appear to inhibit Co accumulation as much as predicted by the "anions & cations" model, whereas the 7MIX-As (ie. Cd, Co, Cr, Ni, Pb and Tl) mixture appeared to inhibit Co accumulation more than expected since the data points fall below the 1:1 line. When the prediction was based on the "cations only" model, the 7MIX-Ni data points were shifted to the right and fell on the 1:1 line, whereas all other treatments fell below the line resulting in a regression line with a slope±95% confidence level of 0.56±0.03 (0 hr depurated) and 0.59±0.04 (24 hr depurated) with an R^2 of 0.946. It appears then that As and Ni may be negating or balancing their respective effect on Co accumulation when both are present in the mixture. When the anions (As & Cr) were removed from the predictive model, the 7MIX-Ni treatment shifted to the right and fell on the 1:1 line and all other treatments, which include Ni, also shifted to the right and below the 1:1 line. Hence, the only metals that may be acting in a competitive inhibition interaction with Co were Cd, Co, Cr, Pb and Tl (i.e. the 7MIX-Ni treatment). The addition of Ni appears to interact with all the other metals resulting in a more than additive effect on the inhibition of Co accumulation. Therefore, it is possible that more than just competitive inhibition may be occurring.

Cadmium bioaccumulation was also significantly inhibited in mixtures exposures (Chapter 5). The change in Cd bioaccumulation from single to mixtures exposures was within 10% of the predicted

change in 20 of the 56 comparisons made, indicating the plausibility of competitive inhibition (Table 6.1 a,b,c). However, when the predictions were based on the "anions & cations" model, only 4 out of 14 treatments (for both 0 and 24 hr depurated body concentrations) inhibited Cd bioaccumulation within 10% of predicted. The observed change in Cd accumulation versus predicted change based on the "anions & cations" models indicated that most of the data points were above the 1:1 line resulting in regressions with slopes of 1.77±0.12 and 2.00±0.16 and R^2s of 0.928 and 0.908 for the 0 and 24 hr depurated respectively (Fig. 6.1, Cadmium; Anions & Cations Model) indicating that competitive inhibition was not the dominating interaction. When the prediction was based on the "cations only" model, 6 of the 14 treatments (for both 0 and 24 hr depurated body concentrations) were within 10% of predicted. This exclusion of the two anions As and Cr, shifted the data points to the right resulting in a majority of the data falling on the 1:1 line with regressions with slopes of 0.878±0.059 and 0.999±0.073 and R^2s of 0.933 and 0.933 for the 0 and 24 hr depurated groups respectively indicating plausible competitive inhibition (Fig. 6.1, Cadmium; Cations Only Model). However the 7MIX+Mn and 7MIX treatments then fell well below the 1:1 line, indicating more than additive effect on Cd inhibition (Fig. 6.2, Cd; Cations Only Model). This seems to imply that something other than competitive inhibition alone was acting on Cd accumulation in some of the treatments.

The observed change in Ni accumulation versus the predicted change based on the "anions & cations" model had a similar pattern to that of Cd, and a majority of the data point lie above the 1:1 line, resulting in slopes of 2.738±0.236 and 2.200±0.226 with R^2s of 0.901 and 0.862 for the 0 and 24 hr depurated groups (Fig. 6.1, Nickel; Anions & Cations Model). Again, this would indicate that competitive inhibition was not the dominating interaction. The "cations only" model did shift the data points closer to the 1:1 line (Fig. 6.1, Nickel; Cations Only Model). However, the regression of the 0 hr depurated data did not pass through the origin and resulted in a y-intercept of 0.381±0.137 and a slope of 0.664±0.207 (R^2=0.416) again indicating the competitive inhibition was not the dominating interaction. Depuration (24 hr) did make a difference in the observed change in bioaccumulation and the regression resulted in a line that passes through the origin with a slope of 0.937±0.086 thus indicating plausible competitive inhibition.

6.3.2 Metals with Limited Inhibition of Bioaccumulation in Mixtures

Both thallium and zinc bioaccumulation were inhibited by some mixtures relative to metal only treatments (Chapter 5). All observed changes in Tl accumulation lie above the 1:1 line when compared to the predictions based on the "anions & cations" model except for two points for 24 hr depurated accumulation in the 7MIX-As treatment (Fig. 6.2, Thallium: Anions & Cations Model). However, the change in Tl accumulation was predicted to within 10% of observed change for 10

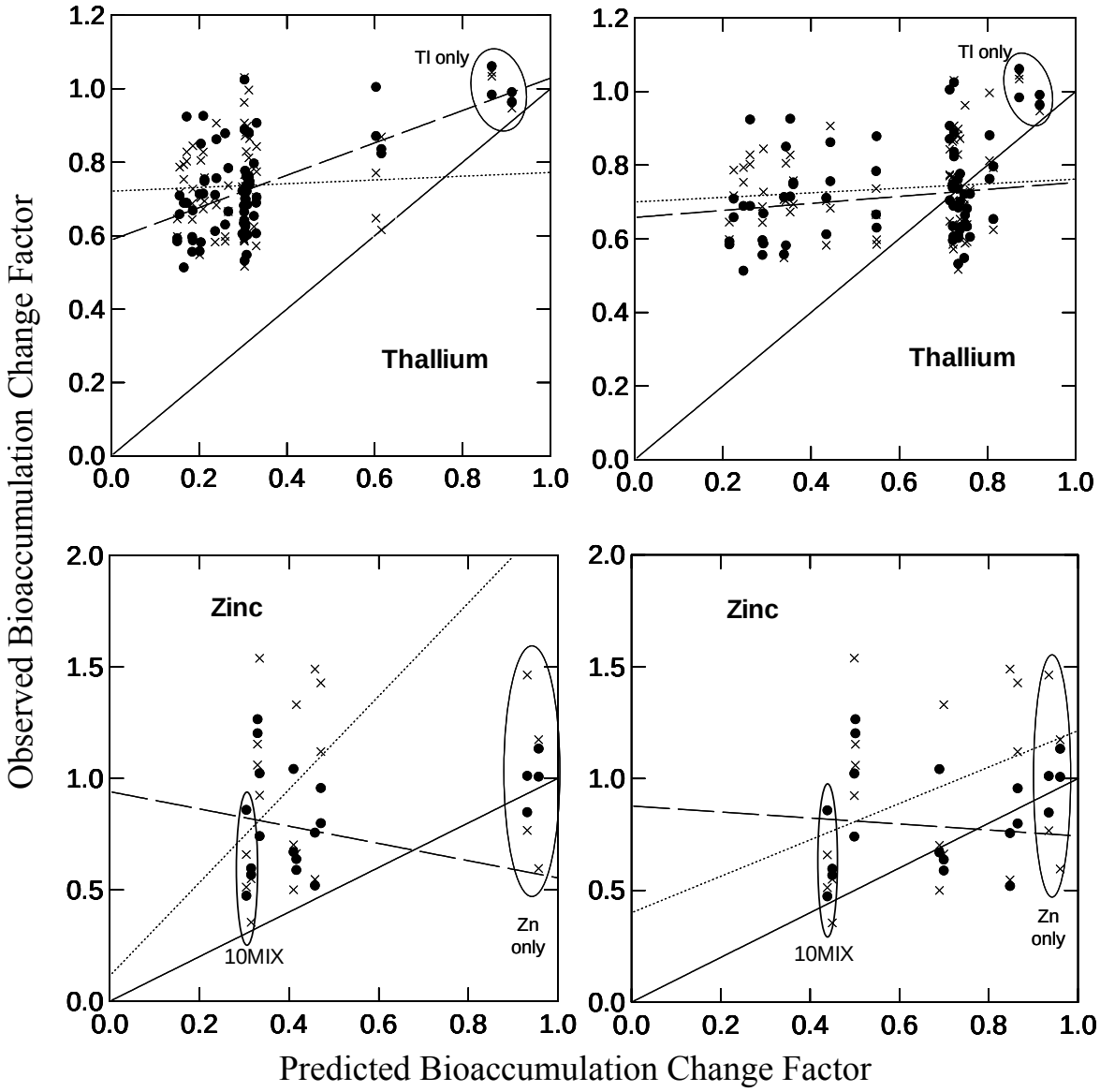

Figure 6.2 Observed change (factor) for Tl and Zn bioaccumulation. Formats the same as in Fig. 6.1

different comparisons out of 56 (Table 6.1a,b,c) when based on the "cations only" model (Table 6.1a). These data points are clustered around the 1:1 line when observed change was plotted versus predicted change based on the "cations only" model. The regressions of observed versus predicted based on the cations only model produced slopes of 0.095±0.140 and 0.062±0.137 with intercepts of 0.658 and 0.700 for the 0 and 24 hr depurated data sets respectively. These lines are horizontal indicating that there was approximately a 32% decrease in accumulation of both 0 and 24 hr depurated Tl body concentrations across all mixtures relative to the Tl only treatment (Fig. 6.2, Thallium: Cations Only Model). Zinc accumulation was significantly inhibited by the 10-metal mixture only (Chapter 5) but the observed change factor of Zn accumulation for this treatment, as well as all other treatments, fell above the line predicted by competitive inhibition based on the "Anions & Cations" model (Fig. 6.2). These data points were shifted to the right onto, or much closer to, the predicted line when based on the "Cations Only" model (Fig. 6.2) indicating the plausibility of competitive inhibition. However, there was high variability in the data and the regression analyses did not provide any additional information (The R^2 values for all regression lines were <0.12). Therefore no conclusions concerning Zn are made..

6.3.3 Metals with No Change in Bioaccumulation in Mixtures

There was no significant changes in Cr, Cu or Mn accumulations with exposure to any treatment in comparison to the metal-only exposures (Chapter 5). Therefore the regressions of observed changes versus predicted changes based on either model did not produce any useful information other than indicating high variability in change. There were a large number of data points indicating an increase in Cr accumulation (Observed change > 1) with exposures to various mixtures but there was no consistent pattern to this enhanced bioaccumulation (Fig. 6.3, Chromium). Therefore there was no indication of competitive inhibition of Cr, Cu or Mn by the other metals.

6.3.4 Metals with Enhanced Bioaccumulation in Mixtures

The was significant enhancement of both As and Pb bioaccumulation by various mixtures (Chapter 5). The regression of observed change in 0 and 24 hr depurated As accumulations versus predicted changes based on the "anions & cations" model had slopes of -1.383±0.393 and -1.323±0.512 respectively significantly (Fig. 6.4, Arsenic). These lines were completely opposite to that of the 1:1 line and provide further evidence that competitive inhibition was not occurring. A majority of al the observed bioaccumulation change factors versus both the "anions & cations" or the "cations only" models fell above the 1:1 line indicating no plausibility of competitive inhibition. Many of the points, especially the accumulation change based on 24 hr depuration were above a factor of 1, indicating increased accumulation of Pb from mixtures compared to Pb-only exposure.

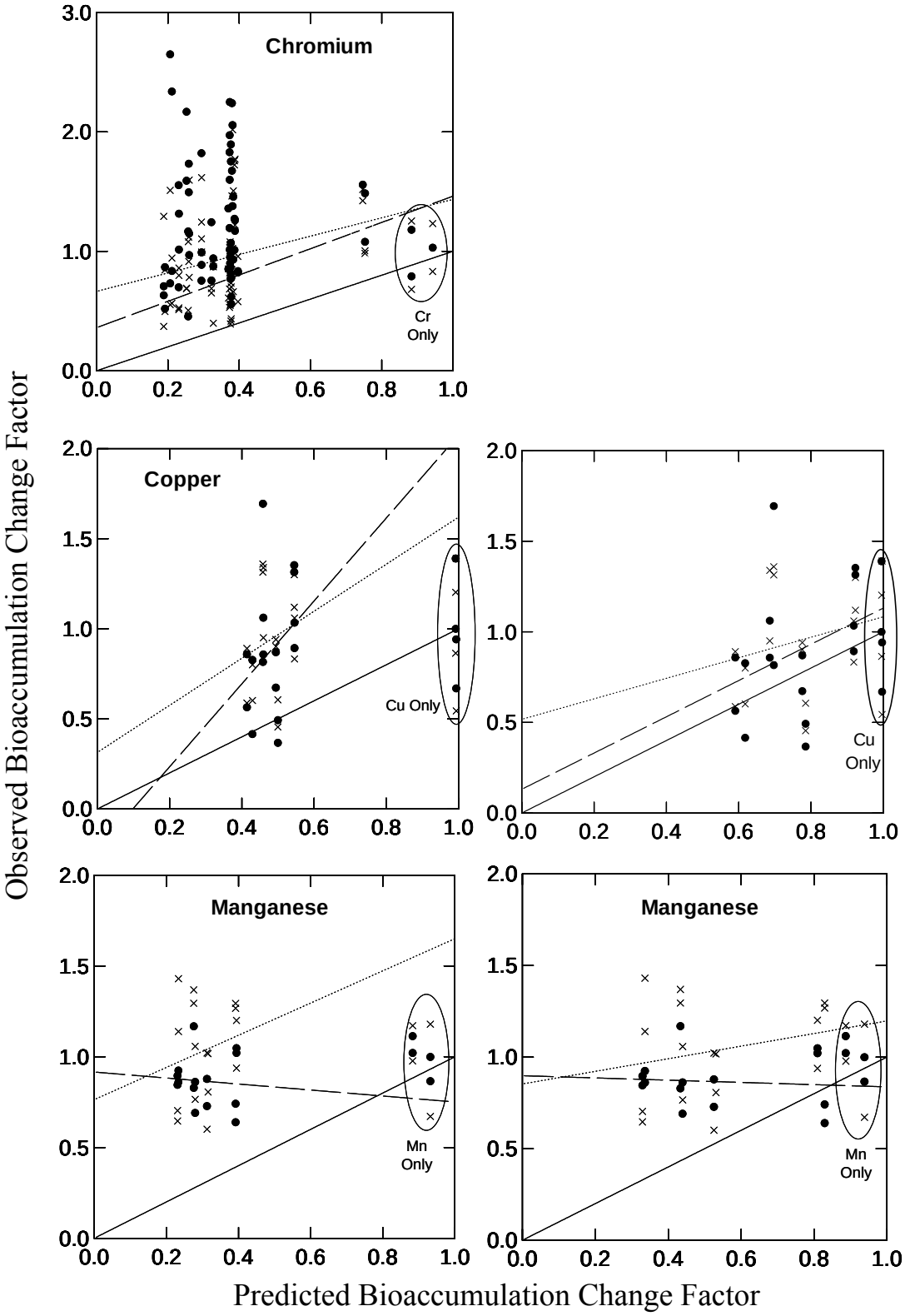

Figure 6.3 Observed change (factor) for Cr, Cu and Mn bioaccumulation. Formats the same as in Fig. 6.1

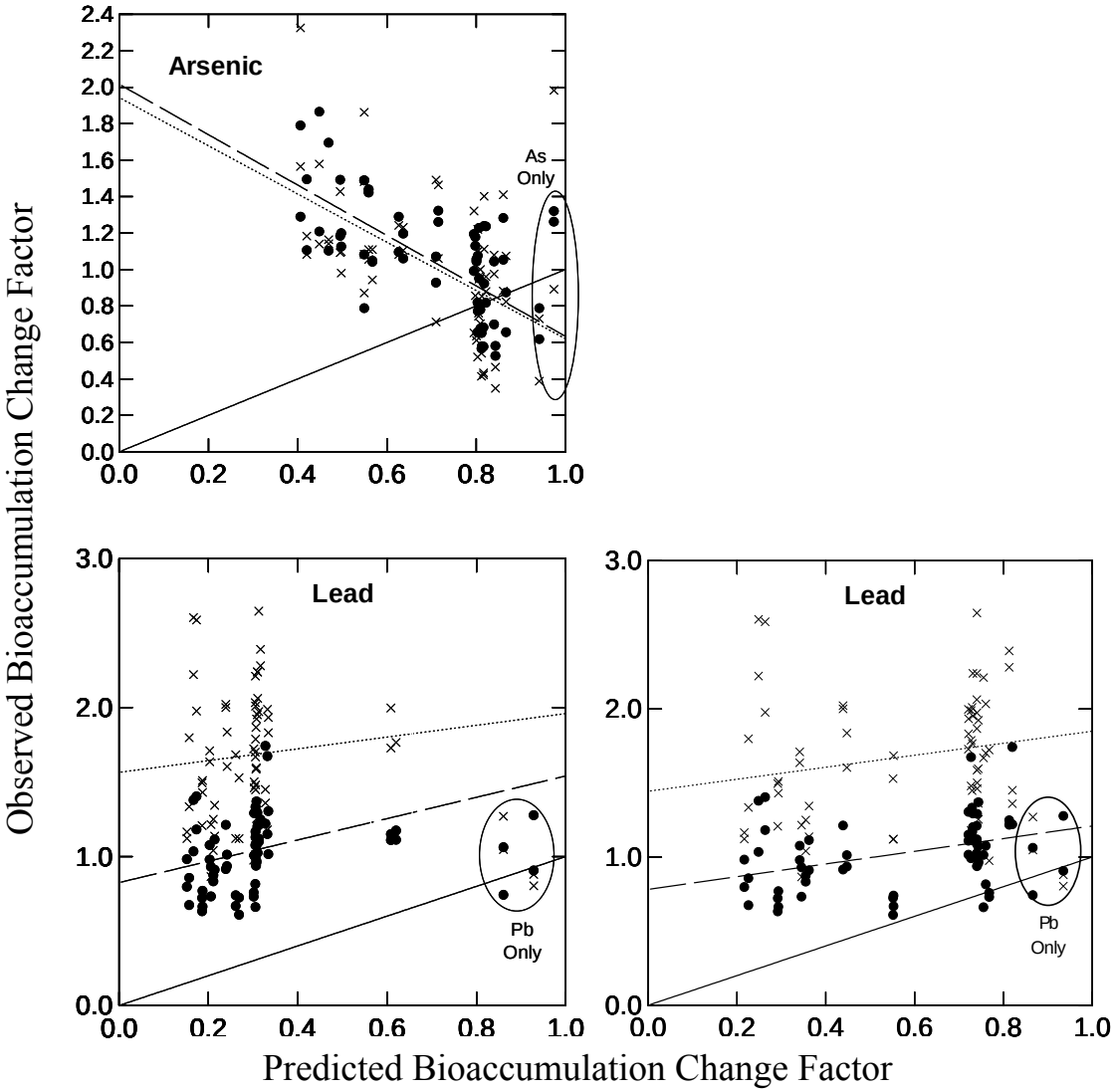

Figure 6.4 Observed change (factor) for As and Pb bioaccumulation. Formats the same as in Fig. 6.1

6.4 General Discussion

The objective of this chapter was to determine if competitive inhibition was a plausible interaction occurring between some or all of the metals, leading to the decreased accumulations relative to single metal exposures. Cobalt, Cd and Ni bioaccumulation was inhibited by some treatments and it is plausible that competitive inhibition was involved. The other 7 metals (As, Cr, Cu, Mn, Pb, Tl & Zn) did not fit a competitive inhibition model. This was not unexpected since there was little or no significant change in bioaccumulation in mixtures relative to single metal only exposures for Cu, Mn, Tl and Zn. As well, there was either no change or some increase in accumulation of As, Cr and Pb in mixtures exposures relative to single metal exposures.

For strict competitive interaction between two metal, both metal accumulations should be affected, however, if there was more than one binding site on the ligand, other types of inhibition may be involved (Borgmann et al., 2007), such as anti-competitive and non-competitive inhibition. Actually, both anti-competitive and non-competitive models can account for enhancement of metal accumulation in mixtures, such as seen in some of the cases with As, Cr and Pb. Other mechanisms may occur in which one metal is not affected by the presence of the second metal, but the second metal may be inhibited by the presence of the first. This could be occurring between Co and other metals individually or in combination, where accumulation of the other metals does not change in the presence of Co (for example Mn, Cu or Cr). The only way to identify these mechanisms or to determine the difference in inhibition between competitive, anti-competitive or non-competitive is to vary the metal exposures independently (Borgmann et al., 2007). Since body concentrations of single toxicants have been shown to be useful indicators of toxic effects in aquatic organisms, it may be possible that body concentrations of metals bioaccumulated from mixture exposures may also be useful indicators of effects. However, interactions between metals that affect accumulation may also affect the resulting toxicity. If the effect is strictly competitive inhibition of bioaccumulation, then metal toxicity should be concentration additive (predictable from water or body concentrations), however, if other types of interactions occur, concentration addition may not predict toxicity adequately. An effects addition model based on bioaccumulation may better predict toxicity, at least for certain metals. There is a possibility that neither concentration addition nor effects addition can predict the toxicity of metal mixtures but instead a combination of both may be required if a combination of the different interactive mechanisms of metal bioaccumulation is involved.

As stated in the introduction, if all the metals were competing for the same type of binding or uptake site, as in a strictly competitive inhibition model such as the Biotic Ligand Model (BLM), it would be expected that with increasing number of metals in the mixture there would be a corresponding decrease in accumulation of each metal (Playle, 2004). It is clear that not all the metal accumulations

were inhibited in *Hyalella* and that strict competitive inhibition did not occur for all the metals. The BLM, as described by Niyogi and Wood (2004) "incorporates the competition of the free metal ion with other naturally occurring cations (eg. Ca2+, Na+, Mg2+, H+)….for binding with the biotic ligand, the site of toxic action on the organism". Since the BLM is a model of the competitive binding of cations, it may be ideal for evaluating mixtures of metal cations in site specific assessments or setting of site-specific water quality criteria. However, the BLM has been developed on the basis of fish gill research in acute exposures of single metals only, relating short-term binding on the gill to acute toxicity (Niyogi and Wood, 2004). Therefore The BLM at this time is not appropriate for modelling metal mixtures. As well, it has been found that individual metals bind or block different sites. For example, metals such as Cu^{2+} and Ag^{+} which specifically block Na^{+} and Cl^{-} transport sites on the gills and Cd^{2+}, Zn^{2+} Co^{2+} and Pb^{2+} which block Ca^{2+} transport sites on the gills (Niyogi and Wood, 2004). The number of different types of critical binding sites may increase further if chronic bioaccumulation is considered since under chronic conditions the exposure concentrations are lower and the metals become internalized. It is highly probably that there may be additional critical binding sites in organs and locations other than the gill. Also, metals may be accumulated through different pathways, such as from dietary sources in which the metals are absorbed through the gut (Niyogi and Wood, 2003).

Only Cd, Co and Ni bioaccumulations in *Hyalella* were significantly reduced in the presence of metal mixtures in this current study and perhaps these metals compete for the same binding or transport sites. Cadmium has been implicated as competing or interacting with Ca^{2+} for accumulation in the crustacean *Gammarus pulex* (Wright, 1980), the insect *Chironomus sp.* (Craig et al. 1999; Gillis and Wood, 2007), the fish *Oncorhynchus mykiss* (Franklin et al 2005) and humans (Visser et al., 1993). Cobalt has also been implicated as a competitor with Ca^{2+} in the fish *Cyprinus carpio* (Comhaire 1998). Nickel is also a competitor with Ca^{2+} in the trout *Oncorhynchus mykiss* (Meyer et al., 1999) and in rat hepatocytes (Funakoshi et al., 1997). Therefore, it is plausible that these three metals were competing for the same Ca^{2+} uptake and binding sites in a strictly competitive inhibitory manner.

Of the remaining 7 metals investigated in this study, Pb, Mn and Zn have also been implicated as calcium antagonists in fish (Roger and Wood, 2004) in parathyroid cells from rats or cattle (Johansson et al., 1988) and in fish gills (Hogstrand et al., 1996) respectively. However, in this current study, there was no consistent change in bioaccumulation of each of these three metals from that of single exposures (Fig. 6.4, 6.3 and 6.2). There are a couple of possible explanations as to why very little change was seen in the accumulation of these three metals. First, it is possible that the binding strength of these metals was sufficiently high to out-compete other metals, but this doesn't seem likely since, for example, the log K values for Cd are higher than those for Pb, at least for fish gills (Niyogi and Wood, 2004). However, there is a possibility that these metals were not acting strictly competitively, but

instead may have caused non-competitive inhibition of the other calcium analogues; Cd, Co and Ni but not Pb, Mn or Zn.

The last four elements tested here have been implicated as antagonists for different uptake pathways or binding locations. Arsenic competes with phosphorus in freshwater algae (Levy et al., 2005), Cr (probably as CrO_4^{2-}) competes with sulphate in mammalian livers and kidneys (Markovich and James, 1999), Cu uptake in the gills of rainbow trout occurs through Na^+ channels (Grosell and Wood, 2002) and Tl competes with K^+ for uptake in the amphipod, *Hyalella azteca* (Borgmann et al., 1998). Since these four elements may be accumulated through different uptake mechanisms and binding sites, it would be expected that there would be no competition and hence no inhibition of bioaccumulation from mixtures compared to single exposures, as was observed (Fig. 6.2, 6.3, 6.4)

All of the above "implications" of interaction at various uptake pathways or binding sites only serve as examples of possible mechanisms in which interactions of the metals of interest could occur. However, most of these examples are from different organisms, and in many cases from completely different phyla, and therefore might not occur in *Hyalella*. Nevertheless, they do help interpret possible interactions and reveal the complexity of the interactions that may occur in the accumulation of metals from mixtures.

6.5 Summary

Competitive inhibition may be a plausible mechanism of interaction in the accumulation of Co, Cd and Ni from metal mixtures. However, it is possible that other interactions may have been responsible or occurred in combination with competitive inhibition resulting in the observed changes in bioaccumulation. Even though the fixed ratio and exposure mixture experiment conducted cannot provide the data necessary to identify the mechanism of interaction, it did provide insight into possible mechanisms and did demonstrate that interactions were occurring.

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CHAPTER 7

Chronic exposure of the amphipod *Hyalella azteca* to metal mixtures. Impact on bioaccumulation and toxicity

7.1 INTRODUCTION

Body concentrations of single toxicants have been shown to be useful indicators of toxic effects in aquatic organisms (Biesinger et al., 1982; Borgmann et al., 2004; McCarty, 1991; Chapter 4) especially in the presence of various complexing agents (Borgmann et al., 1991) and can help identify the cause of biological effects in sediment assessments (Borgmann and Norwood, 1997; Borgmann and Norwood, 1999; Lemke and Kennedy, 1997; McCarty, 1991; Meador et al., 1993). It is therefore possible that body concentrations of metals bioaccumulated from mixture exposures may also be useful indicators of effects. However, interaction between the metals can affect their bioaccumulation (Chapter 5). The review of the effects of metal mixtures in Chapter 2 revealed that there was no consistent method of quantifying the effects of metal mixtures. It is evident that research is required to quantify the effects of metal mixtures and determine appropriate methods for assessment and prediction of mixture effects that can have practical application to the protection of aquatic life.

Only three studies were reported in Chapter 2 that examined the impact of 10 or 11 metals on different end points such as acetylcholine activity, primary production, algal cell numbers, and metal accumulation (Dyer et al., 2000; Olson and Christensen, 1980; Wong et al., 1978). However, only Olson and Christensen (1980) tested a full concentration series of each metal individually in order to determine no effect concentrations which were then used in mixture testing. The mixture experiments indicated that the metals had an additive impact on the inhibition of acetylcholinesterase. Wong et al (1978) only tested algal species with metals at no effect concentrations (International Joint Commission, 1976) in mixtures and found toxic effects with up to 70% inhibition of algal primary productivity. The Dyer et al (2000) investigation was based on the bioaccumulation of metals in 43 fish species regressed against adverse effects in the field, from which toxic units were derived. Other studies with fewer metals in the mixture have also been done, however there has not been a consistent, lab controlled, study in which individual, exposure-bioaccumulation-effects relationships have been determined for a number of metals individually and then in mixtures for a single species of an aquatic organism (Chapter 2).

The two main models that have been utilized to predict or quantify the effects of metal mixtures are concentration addition and effects addition based on water concentrations only (Chapter 2). It is possible that these two models can also be used to predict effects based on metal accumulation. Exposure-bioaccumulation-effects relationships have been established for the aquatic, benthic, crustacean, *Hyalella azteca* with the following 10 elements: As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn (Chapters 3, 4, and Borgmann et al., 2004). The main objective of this chapter is to determine the chronic impact of a concentration series of the 10-element mixture. The impacts of the mixtures were assessed by the Concentration Addition Model (CAM) and the Metal Effects Addition Model (MEAM)

based on both measured water concentrations and measured body concentrations. Finally, the CAM and the MEAM were evaluated to determine the best methods for assessment and prediction of mixture effects. Even though arsenic is a metalloid and the other nine elements are metals, for simplicity these 10 elements will be referred to as "metals" for the remainder of this chapter.

7.2 METHODS

7.2.1 Toxicity Test

The chronic (4 week) toxicity test followed the basic methods outlined in Chapter 5. Twenty, <1-wk old *Hyalella azteca* were added to 400 mL of test medium with a single piece of 5 by 10 cm cotton gauze in 500 mL high density polyethylene (HDPE) plastic containers (Borgmann et al., 1991; Borgmann et al., 1993). Exposures were conducted in an incubator at 25^{o}C with a 16 h light:8 h dark photoperiod. Test media were renewed every 2 to 3 days. This increased renewal rate was carried out in response to known losses of Pb (MacLean et al., 1996) and Mn (Chapter 3) from test media. Food additions (Tetra-Min fish food flakes ground to 500 *u*m mesh size) consisted of two, 2.5 mg feedings during week-1 and 2; three, 2.5 mg feedings in week-3 and two 5.0 mg feedings in week-4. The increase in food per week allowed for animal growth throughout the experiment by providing a maximum food availability without excess that could cause fouling of the media. Test media consisted of de-chlorinated Burlington city tap water originating from Lake Ontario (mean±95% confidence interval: dissolved organic carbon 1.06±0.26 mg L^{-1}, dissolved inorganic carbon 21.5±0.89 mg L^{-1}, Alk 87±0.95 mg L^{-1}, Cl 698±34 µmol L^{-1}, SO_4 322±10 µmol L^{-1}, SiO_2 16±1.7 µmol L^{-1}, Ca 832±19 µmol L^{-1}, Mg 354±5.9 µmol L^{-1}, Na 529±12 µmol L^{-1}, and K 41±2.2 µmol L^{-1}; analyses were conducted by the National Laboratory for Environmental Testing, Environment Canada, Burlington, Ontario, Canada) with metal additions. Two replicates of each mixture combination and three control (no added metals) replicates were tested. Stock solutions of each metal were prepared with the analytical grade salts of sodium arsenate (Na_2HAsO_4•$7H_2O$), cadmium chloride anhydrous ($CdCl_2$), cobalt chloride 6-hydrate ($CoCl_2$•$6H_2O$), sodium chromate anhydrous (Na_2CrO_4), cupric chloride ($CuCl_2$•2H2O), manganous chloride 4-hydrate ($MnCl_2$•$4H_2O$), nickel (II) chloride hexahydrate ($NiCl_2$•$6H_2O$), lead chloride ($PbCl_2$), thallous nitrate ($TlNO_3$) and zinc chloride ($ZnCl_2$) dissolved in de-ionized water (Milli-Q) acidified to 0.07% nitric acid (Omni-pure).

Each metal was spiked in a dilution series. For each treatment the metals were kept equi-toxic by maintaining the ratio between metals equivalent to the ratio between the LC25 values (Table 7.1). Treatment A was the control (no metals added), B was 0.1 × LC25, C was 0.32 × LC25, D was 1.0 × LC25 and E was 5.6 × LC25 for each metal. The sum of toxic units (based on individual LC25s) for the 10-metal mixture treatment was A=0, B=1, C= 3.2, D= 10 and E=56. Testing at equi-toxic concentrations allowed for the production of a concentration series of increasing toxicity.

Table 7.1 Geometric mean measured total dissolved exposure concentrations with 95% confidence limits (CL) and sample size (N), of the initial and final concentration of each element during each renewal period. Free ion contribution from MINTQA2 (USEPA 2006), published lethal concentrations causing 25% mortality and Toxic Units based on LC25.

	A			B			C			D			E			Free Ion		LC25	Toxic Unit based on LC25			
	(nmol L^{-1})	CL	N	(nmol L^{-1})	CL	N	(nmol L^{-1})	CL	N	(nmol L^{-1})	CL	N	(nmol L^{-1})	CL	N	Form	(%)	(nmol L^{-1})	B	C	D	E
As	7.10	0.765	8	576	22.9	8	1806	47.9	8	5702	133	6	33713	NA	1	[a] AsO_4^{3-}	0.06	4320	0.1	0.4	1.3	7.8
Cd	0.141	0.020	7	0.415	0.027	8	1.07	0.0547	8	3.06	0.0886	6	18.4	NA	1	Cd^{2+}	88	3.21	0.1	0.3	1.0	5.7
Co	0.553	0.198	8	6.52	0.274	8	20.6	0.668	8	64.6	1.41	6	389	NA	1	Co^{2+}	90	68	0.1	0.3	0.9	5.7
Cr	5.88	0.829	8	34.3	1.26	8	99.5	2.82	8	302	7.09	6	1699	NA	1	CrO_4^{2-}	98	243	0.1	0.4	1.2	7.0
Cu	19.1	14.8	8	68.9	15.9	8	184	16.8	8	552	29.9	6	3247	NA	1	[c] Cu^{2+}	6	441	0.16	0.4	1.3	7.4
Mn	4.4	1.96	7	7281	368	8	25101	709	8	80062	1993	6	442376	NA	1	Mn^{2+}	96	147000	0.05	0.2	0.5	3.0
Ni	12.2	1.45	8	55.0	2.92	8	157	5.33	8	484	6.54	6	2891	NA	1	Ni^{2+}	87	400	0.1	0.4	1.2	7.2
Pb	0.216	0.0451	8	3.84	1.30	8	14.9	2.82	8	46.2	9.91	6	334	NA	1	[b] Pb^{2+}	10	36.8	0.1	0.4	1.3	9.1
Tl	0.066	0.0059	8	4.67	0.160	8	15.0	0.351	8	45.3	0.797	6	260	NA	1	Tl^{1+}	99	51.2	0.1	0.3	0.9	5.1
Zn	11.0	2.77	8	239	18.0	8	823	38.9	8	2651	78.1	6	16544	NA	1	Zn^{2+}	77	2520	0.1	0.3	1.1	6.6

a $HAsO_4^{2-}$ = 95%

b $PbCO_3$ (aq) = 51% and $PbOH^+$ = 32%

c CuCO3 (aq) = 61% and $CuOH^+$ = 25%

LC50 and LC25 data: As, Co, Cr and Mn from Chapter 3 (Norwood et al. 2007), all other metals (Borgmann et al. 2004)

Varying the concentration of each metal independently would provide much more data for interpreting interactions, however the number of treatments would be unmanageable.

Conductivity, total ammonia, oxygen, and pH concentrations were measured at the beginning (prior to animal additions or transfer) and end of each renewal period (geometric mean±95% C.I.: conductivity 307±10 μs cm^{-1}, total ammonia 0.007±0.011 mmol L^{-1}, oxygen 8.9±0.15 mg L^{-1}, pH and end of each renewal period, 10 mL unfiltered water samples were collected and preserved with 10 μL nitric acid (Omni-pure) for metalloid or metal analyses. Survival was recorded at each renewal period and at the end of the 28 day exposure. One half of the survivors (or all survivors if less than 5 animals survived) were rinsed with 50 μM ethylene-diamine-tetra-acetic acid (EDTA) in de-chlorinated Burlington city tap water to remove any loosely adsorbed metal, weighed wet and then placed in a pre-cleaned cryovial and dried at 60°C for 72 h before determination of dry weight. The remaining animals were also rinsed with, and then placed in 60 mL of the same EDTA media along with a small piece of cotton gauze and 2.5 mg fresh food for a 24 depuration period. This was analogous to the procedure used to purge the guts of amphipods in the one week bioaccumulation test in Chapter 5. EDTA was added to the solution to bind any contaminant released from the animal during the depuration so that the animal could not reabsorb the contaminant (Borgmann and Norwood, 1995: Neumann et al., 1999). Wet weight was determined after 24 h and then the animals were placed in a pre-cleaned cryovials and dried at 60°C for 72 h before determination of dry weight.

7.2.2 Metalloid and Metal Analyses

Digestion of tissue samples were based on the methods of Borgmann et al (1991) and Stephenson and Mackie (1988). Six dried amphipods were weighed (mean dry weight = 0.752 mg, maximum = 1.26 and minimum = 0.313 mg) and digested with 150 μL 70% Omni-pure nitric acid at room temperature for 6 days, followed by an addition of 120 μL 30% hydrogen peroxide for 24 h in a 14 ml polypropylene test tube with snap cap. Each sample was then made up to a 10 ml volume with de-ionized water (Milli-Q).

All ten metals in water and tissue samples were analyzed by Inductively Coupled Plasma, Mass Spectroscopy (ICP-Mass Spec) by the National Laboratory for Environmental Testing, Environment Canada. Method blanks were run with every batch of samples to correct for background contamination and to calculate detection limits. Detection limits were calculated as 3 times the standard deviation of the blank analyses. The detection limit for each metal in water was 0.14, 0.027, 0.034, 0.15, 0.19, 1.0, 0.36, 0.02, 0.0006, and 2.05 nmol L^{-1} for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn respectively. The mean detection limits for tissue, based on the average digestion of 0.752 mg dry wt was 1.39, .10, 1.36, 3.57, 13.0, 78.1, 13.5, 0.32, 0.049, 13.7 nmol g^{-1} for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl and Zn respectively.

7.2.3 Free Ion

Percent contribution of the free ion to the total dissolved concentration of each metal was calculated with MINTEQA2 v. 4.03 (US EPA, 2006) which incorporated the Gaussian model for dissolved organic matter. The majority of the metalloid arsenic was present in the species $HAsO_4^{2-}$ and $H_2AsO_4^-$ and the majority of chromium was present in the species CrO_4^{2-} and $HCrO_4^-$ (Table 7.1). Most of the other metals were generally at a high percentage in the free ion form. Percent contribution of each metal species did not change with increasing metal concentrations.

7.2.4 Bioaccumulation

All bioaccumulation data were corrected by subtraction of the background body concentration determined in controls (no added metals), thus producing above-background bioaccumulation for each metal in an exposure treatment. The above-background bioaccumulation values were graphical checked for homogeneity of variance and it was determined that the variance increased with increasing exposure concentration. A log transformation was required to normalize the variance prior to statistical treatment. Statistical analysis were performed with SYSTAT 10, SPSS Inc. (2000©). A one way analysis of variance with a Tukey post hoc comparison was used to determine differences between; 1) above background bioaccumulation of each metal from each 4-wk, 10-metal mixture exposure with young *Hyalella*; 2) above background bioaccumulation of each metal from 1-wk, 10-metal mixture exposure with adult *Hyalella* from Chapter 5, exposed to the 10-metal mixture at the same ratio; and 3) one week background corrected bioaccumulation in single metal exposure with adult *Hyalella* from Chapter 5. As well, each metal's bioaccumulation in all the above tests (4-wk accumulations from this chapter as well as the 1-wk accumulations from chapter 5) were plotted against exposure concentration along with the historical bioaccumulation models based on single metal generated from four week exposures with young *Hyalella* in Chapter 3 and Borgmann et al (2004) in order to determine if bioaccumulations were consistent.

7.2.5 Mortality

Mortality rate was determined as the slope of the regression of the negative natural logarithm of the number of survivors versus time in weeks (Chapter 4). This allowed for the computation of mortality for partial effect concentrations in which there were no survivors at the end of four weeks. Observed survival was also determined at day 28 as

$$S = N/N_0$$

Where N was the final number of survivors and N_0 was the initial number of animals at day 0.

Table 7.2 Geometric mean (GM), 95% confidence level (CL) and sample size (N) for each treatment for conductivity, total ammonia, oxygen and pH measured through-out the exposure, at the beginning and end media renewals.

	A			B			C			D			E			Overall		
	GM	CL	N	GM	CL	N	GM	CL	N	GM	CL	N	GM	CL	N	GM	CL	N
Conductivity (µs)	288	3.99	24	291	3.51	24	295	3.16	24	305	2.92	11	359	36.3	3	307	10.0	86
Ammonia (mmol/L)	0.008	0.009	12	0.008	0.011	12	0.008	0.011	12	0.003	0.014	5	0.210	NA	1	0.007*	0.011*	41*
Oxygen (mg/L)	9.0	0.18	24	8.8	0.13	12	8.9	0.22	12	8.8	0.06	5	8.8	NA	1	8.9	0.15	54
pH	8.3	0.05	24	8.3	0.06	24	8.3	0.05	24	8.2	0.06	11	7.8	0.53	3	8.2	0.15	86

* excluding treatment E

7.2.6 Metal Mixture Toxicity Evaluation

The impact of mixtures on mortality was evaluated with the Concentration Addition and Effects Addition models. Both of these models can be based on either the measured water concentration or the measured, above background body concentration of each of the ten metals as follows:

7.2.6.1 Concentration Addition Model (CAM)

The concentrations addition approach is based on Toxic Units (TUs) (Environment Canada, 1999). TUs were calculated by dividing the measured exposure concentrations (Table 7.1) by the LC50 (Table 7.3) when based on water concentration, or the measured above background body concentration for each replicate of all treatments divided by the $LBC50_X$ (Table 7.3) as in the following;

$$TU_M = C_M (LC50_M)^{-1} \quad (1a)$$

$$TUB_M = BC_M (LBC50_M)^{-1} \quad (1b)$$

The sum of the TUs for the mixture was calculated as in the following;

$$TU_{MIX} = \sum_{n=1}^{10} TU_{M(n)} \quad (2)$$

Where M_1 was the first metal, M_2 was the second metal, etc.. MIX refers to mixture, LC50 was the water concentration and $LCB50_X$ was the background corrected body concentration, at 50% mortality.

Observed survival was plotted against mixture concentrations expressed as toxic units, based on measured water concentrations or measured background corrected body concentrations. The toxicity was evaluated as strictly concentration additive when the observed survival curve intersected the 50% survival point at 1 toxic unit. If the curve fell below this intersect point, the toxicity was more than concentration additive and if the curve fell above this intersect point, the toxicity was less than concentration additive

7.2.6.2 Metal Effects Addition Model (MEAM)

Mortality was predicted based on measured water concentrations or measured body concentrations (background corrected) using a modification of the saturation model of metal toxicity (Borgmann et al., 2004)

$$m = m' + [\max'' \; C \; (K'' + C)^{-1}]^n \quad (3)$$

where m is the total mortality, m' is the control mortality and the remainder of the equation represents the metal-induced mortality in which max" is the maximum mortality, K'' is the half saturation constant (concentration at which the metal-induced mortality is half way between the control mortality and the

maximum) and C is the metal concentration in water (W) or the background corrected metal concentration in the body (TB_X).

Equation (3) was modified according to Borgmann et al. (2004) by replacing the max" term with the LC50 or $LBC50_X$ (background-corrected lethal body concentration) at time (t) in weeks, giving

$$m = m' + (\ln(2)/t)\,[C_W(1/LC50 + 1/K_W'') / (1 + C_W/K_W')]^{nw} \quad (4a)$$

$$m = m' + (\ln(2)/t)\,[C_{TBX}(1/LBC50_X + 1/K_{TBX}'') / (1 + C_{TBX}/K_{TBX}')]^{nb} \quad (4b)$$

The sum of mortality rates (product of survival) per time per metal can be used to predict mixture toxicity (Chapter 2).

$$m''_{mix} = m' + \sum_{n=1}^{10} m''_n \quad (5)$$

Where m''_{mix} is the total mortality rate of the mixture, and m''_n is the control corrected mortality rate predicted for each of the ten metals (n = 1-10) using (Eqs. (4a) and (4b)), based either on measured water or measured background-corrected body concentrations. Each mixture treatment was then evaluated to be; a) additive when observed mortality rate was the same as predicted, b) more than additive when the observed mortality rate was greater than predicted, and c) less than additive when the observed mortality rate was less than predicted.

For comparison to the CAM, MEAM predicted survival, calculated from water concentrations or background corrected body concentrations, was plotted against mixture concentrations expressed as toxic units, based on water or background corrected body concentrations respectively. As well, both types of MEAM predicted survivals were plotted together on a third graph, against mixture concentrations expressed as toxic units based on water concentrations, to compare the two types of MEAMs.

7.3 RESULTS

7.3.1 Exposure Concentrations

A logarithmic series of increasing mixture exposure concentration, treatments "A" through to "E", was produced in which the ratio between the metals remained constant across treatments, based on the LC25 (Table 7.1). Treatment "D" individual metal concentrations were approximately equivalent to the LC25 (Table 7.1). This insured a constant increase in exposure concentrations, in order to cover a large range of impact on the test organism, while maintaining equivalent contribution by each metal

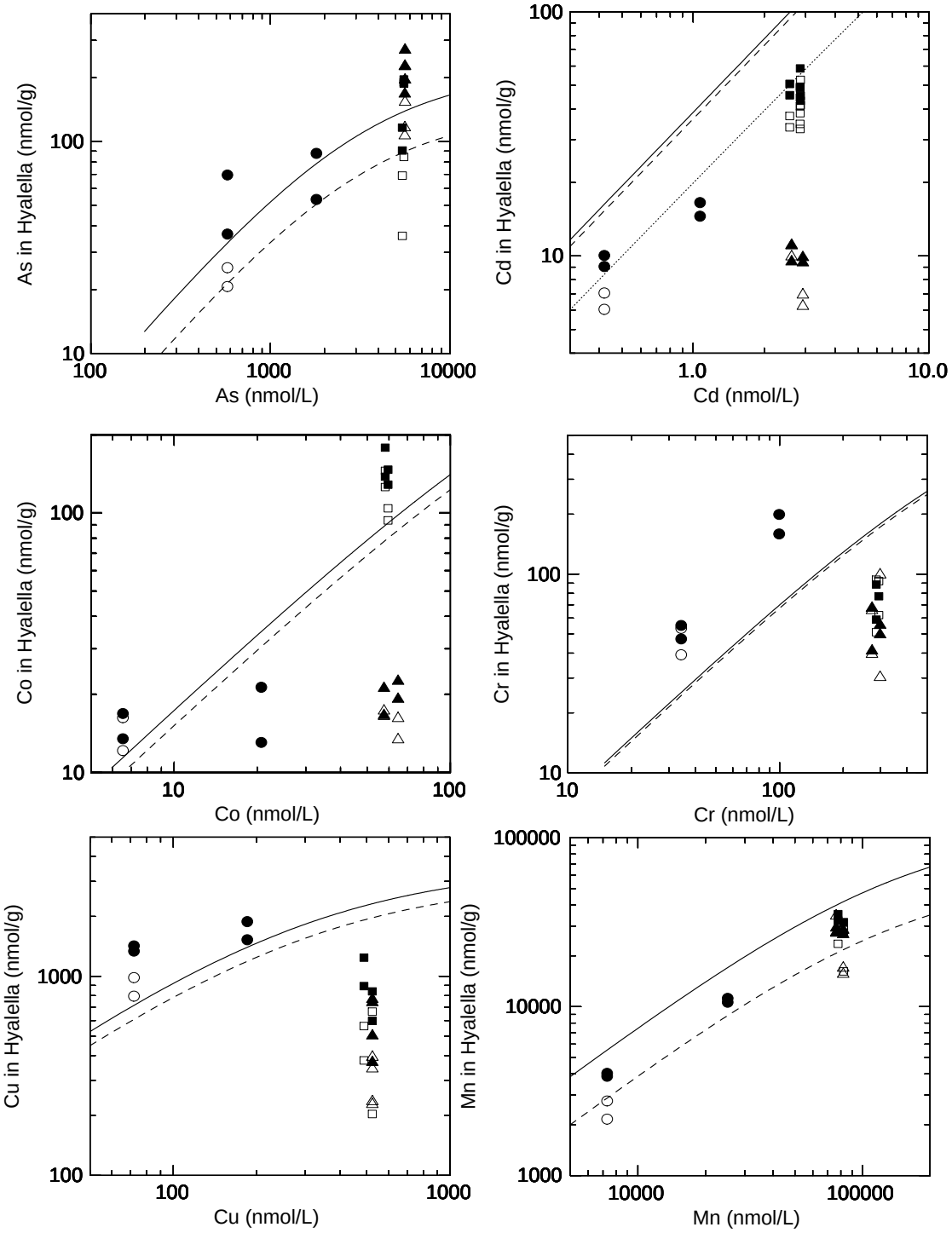

Figure 7.1 As, Cd, Co, Cr, Cu and Mn background corrected accumulation by *Hyalella* after; 4-week exposure in 10-metal mixtures (● non-depurated; ○ 24-hr depurated) from this chapter; 1-week exposure of adult *Hyalella* to 10-metal mixtures (▲ non-depurated; Δ 24-hr depurated) and 1-week exposures of adult *Hyalella* to single metals only (■ non-depurated; □ 24-hr depurated) from chapter 5. Non-depurated saturation model (—) and 24 hr depuration saturation model (----) for each metal (Borgmann et al, 2004; chapter 3). Additional non-depurated Cd 1-wk model (·····) from Borgmann (unpublished).

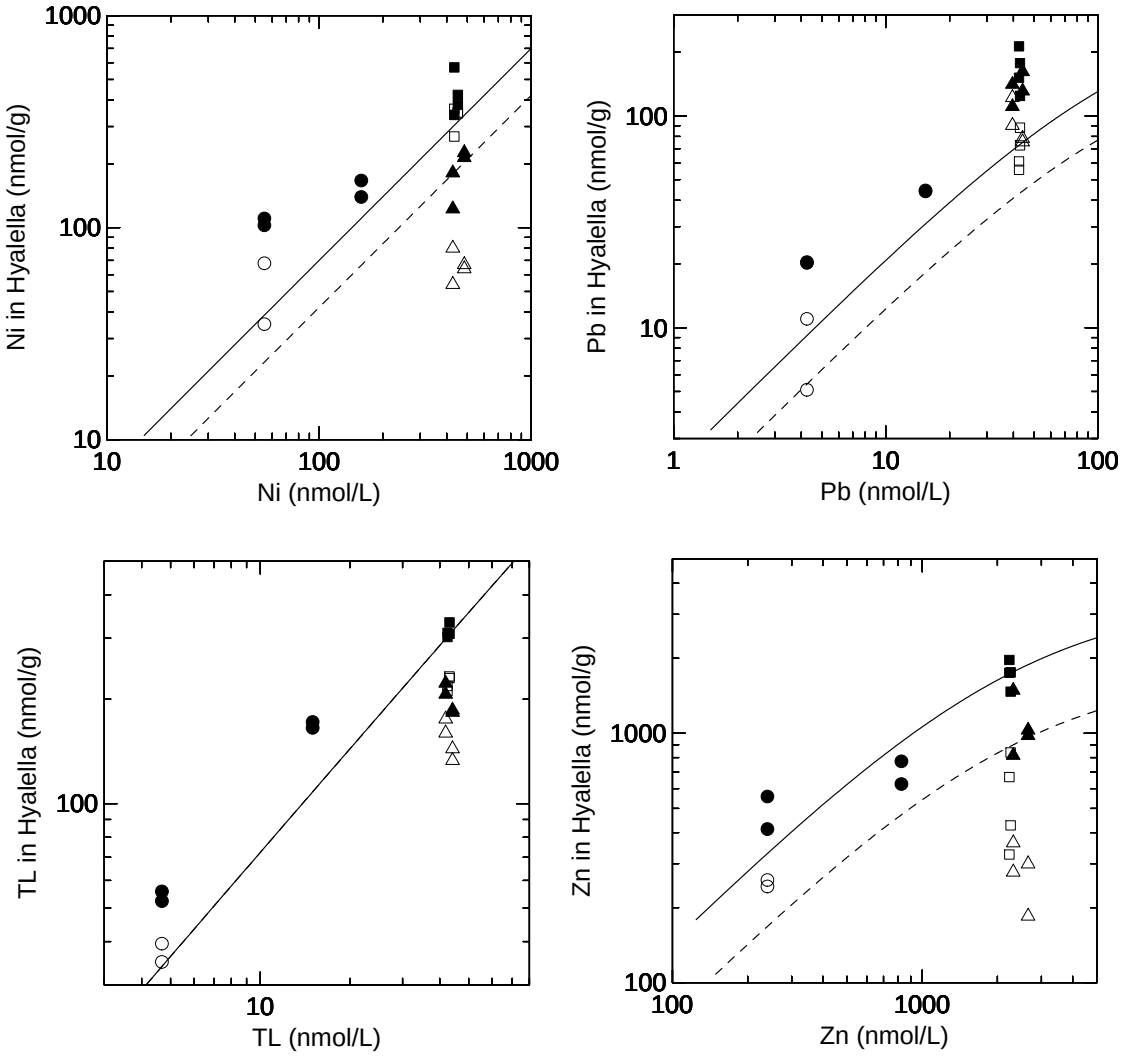

Figure 7.2 Ni, Pb, Tl and Zn accumulated by *Hyalella*. Symbols and lines as in Figure 7.1.

7.3.2 Test Conditions

Test conditions were consistent throughout the experiment (Table 7.2). Oxygen levels remained at saturation throughout the experiment (Table 7.2). Total ammonia levels were very low in all tests except treatment E, (Table 7.2) in which the ammonia level reached 0.21 mmol/L. This level of ammonia was well below the chronic (4-wk) LC50 of 0.95 mmol/L for *Hyalella* (Borgmann, 1994), using the same test media, however, 100% of the test animal were already dead after a 2 day exposure to the metals in this treatment. There was a slight, but consistent decrease in pH with increasing metal concentrations, but all within tolerance levels for *Hyalella*.

7.3.3 Bioaccumulation

There were measurable concentrations (greater than detection limit) of all metals in the bodies of the control group of *Hyalella*, treatment A (Table 7.4) to which no metals were added. These control body concentrations were significantly different (lower) than all other treatments ($p<0.05$, ANOVA Tukey post hoc). All values listed in Table 7.4 were not background corrected. The mean control was used to background correct all other treatment values prior to any further graphical or statistical analyses.

Arsenic bioaccumulation in the toxicity test as well as in the 1-wk bioaccumulation tests with adults, followed the same trend as that predicted by the saturation model (Fig. 7.1). Cadmium bioaccumulation in the toxicity test and in the single metal only treatment in the 1-wk bioaccumulation test with adults, also followed the same trend as that predicted by the saturation model, however, the 1-wk accumulation of Cd in adults in the 10-metal exposure (at approximately 3 nmol Cd/L) was significantly lower than in the single metal exposure and treatment C, 1.07 nmol/L Cd (Fig. 7.1, Table 7.4). There was an indication that accumulation in treatment C was reduced but there were insufficient data to determine if this tendency would continue at higher exposures. Cobalt accumulations in all 10-metal mixtures, from both the 4-wk toxicity test as well as the 1-wk accumulation test with adults, were the same and were significantly lower than Co accumulation by adults in the 1-wk exposure to Co singly (Fig. 7.1, Table 7.4). This accumulation in the Co singly treatment was very close to that predicted by the saturation model.

Chromium bioaccumulation in the 4-wk toxicity test increased with increasing exposure concentration as predicted by the saturation model, but at a significantly higher levels (Fig. 7.1, Table 7.4). On the other hand, Cr accumulation by adults in the 1-wk accumulation test was significantly lower than that predicted by the saturation model, in both the single metal or the 10-metal treatments. Copper accumulation follow similar trends to that of Cr in that the accumulation in the 4-wk toxicity test was elevated above the adults accumulation as well as that predicted by the saturation model (Fig. 7.1). However, the actual differences in accumulation between all treatments were not significantly

different, except for the single metal exposure treatment being significantly different that that in the 4-wk toxicity test (Table 7.4). Manganese bioaccumulation in all treatments followed the saturation model prediction quite closely and there appears to be no influence by 10-metal mixture or *Hyalella* age (Fig. 7.1, Table 7.4).

Nickel, Pb and Tl bioaccumulations in the 4-wk toxicity test followed the trend predicted by the saturation models, but the concentrations were elevated (Fig. 7.2). The Ni and Tl accumulations by the adults in the 1-wk accumulation test with single metal exposures matched the saturation model but their accumulation in the 10-metal treatment was significantly lower than the single metal exposures (Table 7.4). Lead accumulations by the adults in the 1-wk bioaccumulation continued to follow the saturation model predicted trend, but with elevated accumulations like those in the 4-wk toxicity test (Fig. 7.2).

Zinc accumulations in both the 4-wk toxicity test in mixtures and the 1-wk accumulation test with adults exposed to the single metal only, followed the saturation model but there is an indication that the accumulation levels off in 10-metal exposure such that treatment C (825 nmol Zn /L) was not significantly different than the accumulation by adults in the 1-wk test in the 10-metal mixture at 2300 – 2700 nmol Zn /L (Fig. 7.2, Table 7.4).

7.3.4 Mixture Toxicity Evaluation

The toxicity of 10-metal mixtures, kept at a constant ratio, increased significantly with increasing exposure concentration. Observed survival was reduced to zero, (i.e. significantly increased mortality rate, p=0.01, regression analyses) and there was a significant reduction in growth (p=0.007, regression analyses) (Table 7.5). The regression analyses were performed with 4^{th} root transformed mortality data , 2^{nd} root transformed growth data (mg animal^{-1}), and log-transformed exposure data (ul of stock added). Appropriate data transformation were tested in Systat 10 for each endpoint (Borgmann 2002) in which one-way ANOVAs were conducted by experiment. Plots of residuals were examined for homogeneity of variance to determine the best transformation. Observed mortality was also converted to control corrected survival (Table 7.5) for comparisons to observed survival and predicted survival based on the effects addition model: relative to exposure concentrations, background corrected body concentrations, or toxic units.

7.3.4.1 Concentration Addition Model

A simple correlation curve of control corrected observed survival versus toxic units should pass through the intercept point of 50% survival and 1.0 Toxic Unit when the concentration addition model describes the toxicity well. When the toxic units were based on measured water concentrations (Table 7.1) and LC50s (Table 7.3), the observed survival curve intersected the 50% survival point at 1.62 toxic

Table 7.3 Half saturation constants K_W" and K_{TBX}" (nmol L^{-1} and nmol g^{-1}), lethal concentrations LC50 and $LBC50_X$ (nmol L^{-1} and nmol g^{-1} with 95% confidence limits, CL) and exponent (n) for mortality as a function of water and background corrected body concentrations based on saturations models.

Metal	K_W"	LC50	CL	K_{TBX}"	$LBC50_X$	CL	$LBC50_{X24hr}$	n	Data Source
As	172	5600	4570-6870	12.3	139	129-150	92.3	100	Chapter 3 (Norwood et al. 2007)
Cd	14.1	4.61	3.61-5.88	-625	341	315-370	321	3.10	[b]Borgmann et al. (2004)
Cd	27.4	22.3	18.0-26.5	1060	856	714-1020	805[a]	5.92	Borgmann (unpublished)
Co	3900	183	120-279	-747	220	166-292	192	0.913	Chapter 3 (Norwood et al. 2007)
Cr	-11000	731	413-1290	-756	334	237-470	322	0.767	Chapter 3 (Norwood et al. 2007)
Cu	62500	718	545-946	-3330	2560	2370-2770	2176	1.82	Borgmann et al. (2004)
Mn	-808000	197000	144000-271000	-142000	71000	60700-83000	56535	2.36	Chapter 3 (Norwood et al. 2007)
Ni	1430	576	504-659	1000	405	355-463	243	3.21	Borgmann et al. (2004)
Pb	7.66	48.1	40.5-57.1	18.1	80	70-91	47	21.4	Borgmann et al. (2004)
Tl	256	78.1	65.1-93.8	2000	550	460-656	na	2.60	Borgmann et al. (2004)
Zn	2000	3100	2600-3690	10000	2020	1880-2180	1717	10.29	Borgmann et al. (2004)

[a] The 6 (% day^{-1}) Loss rate from Borgmann et al. (2004) was applied since data were not available for this data set.

[b] Borgmann (unpublished, 4-wk toxicity test with standard artificial media, 2006)

na Not available.

Table 7.4 Mean Body Concentration (nmol g^{-1}, dry weight), 95% confidence limit (CL) and sample size (N) for each treatment. Means in a row with the same superscript indicates treatments that were statistically the same after background correction ($p>0.05$, ANOVA Tukey multiple comparisons), all other means were significantly different at $p<0.05$.

		Bioaccumulation in 4 week Toxicity Test									* Bioaccumulation in 1 week Test With Adults					
		A (0 ul stock additions)			**B** (10 ul stock additions)			**C** (32 ul stock additions)			10 Metal Mixture			Single Metal		
	Depuration (hr)	(nmol g-1)	CL	N	(nmol g-1)	CL	N	(nmol g-1)	CL	N	(nmol g-1)	CL	N	(nmol g-1)	CL	N
As	0	32.0	3.7	3	85.1[a]	32.2	2	103[a,b]	33.9	2	228[c]	70.0	4	165[b,c]	83.0	4
	24	31.0	15.5	3	55.1	4.6	2	NA	-	-	164[c]	86.7	4	112[c]	106.0	4
Cd	0	1.45	0.572	3	10.8[a]	0.307	2	17.00	1.960	2	13.0[a]	1.22	4	52.0	4.10	8
	24	1.53	0.709	3	8.03[a]	0.769	2	NA	-	-	11.6[a]	3.68	4	42.8	5.32	8
Co	0	2.7	1.02	3	17.8[a]	3.30	2	19.8[a]	8.04	2	22.9[a]	4.09	4	152	34.6	4
	24	2.6	1.17	3	16.9[a]	4.03	2	NA	-	-	18.9[a]	2.70	4	121	36.6	4
Cr	0	25.3	11.3	3	76.4[a]	7.8	2	204.0	39.5	2	65.8[a]	17.6	4	89.9[a]	37.0	3
	24	31.3	6.2	3	71.5[a]	13.8	2	NA	-	-	71.1[a]	49.1	4	89.7[a]	34.2	4
Cu	0	925	370	3	2300[a]	83	2	2630[a]	349	2	2060[c]	303	4	2360[a,c]	423	4
	24	907	259	3	1810[a]	186	2	NA	-	-	1760[c]	130	4	1920[a,c]	324	4
Mn	0	86.7	23.2	3	4040	157	2	11000	569	2	28000[c]	1784	4	31700[c]	5160	4
	24	90.2	17.9	3	2550	594	2	NA	-	-	23600[c]	14271	4	24100[c]	9130	4
Ni	0	23.8	11.3	3	130[a]	7.7	2	177[a]	26.8	2	216[a]	73.6	4	453	160	4
	24	26.3	4.3	3	75.2[a]	32.1	2	NA	-	-	96.7[a]	17.1	4	357	67.7	4
Pb	0	1.34	0.483	3	21.70	0.176	2	45.60	0.421	2	139[c]	33.8	4	168[c]	60.2	4
	24	1.64	0.655	3	9.39	5.801	2	NA	-	-	94.8[c]	34.1	4	70.2[c]	22.8	4
Tl	0	0.147	0.088	3	54.2	3.39	2	169[b]	6.29	2	200[b]	29.3	4	314	21.2	4
	24	0.400	0.090	3	37.3	4.40	2	NA	-	-	153[c]	29.3	4	223[c]	16.2	4
Zn	0	698	268	3	1180[a]	142	2	1400[a,b]	143	2	2020[b]	458	4	2680	325	4
	24	752	270	3	949[a]	15	2	NA	-	-	1220[a]	118	4	1510[a]	368	4

* Norwood et al (2007b)

Table 7.5 Observed survival%, total wet weight, wet weight per animal, mortality rate per week converted to percent survival, Toxic Units (TU) based on water and body concentrations, and predicted mortality and survival based on measured water and body concentrations with the Metal Effects Addition Model (MEAM).

	Treatment								
	A			B		C		D	
Stock Addition (ul per metal)	0			10		32		100	
Rep	1	2	3	1	2	1	2	1	2
Observed Surival% (day 28)	50.0	80.0	70.0	60.0	50.0	20.0	20.0	0.0	0.0
Total Wet Weight (mg)	5.5	9.1	9.7	5.9	3.9	1.3	1.6	NA	NA
Wet Wt/animal (mg)	0.550	0.569	0.693	0.492	0.390	0.325	0.400	NA	NA
Observed Mortality Rate (m/wk)	0.190	0.057	0.094	0.146	0.171	0.448	0.475	2.083	1.388
Observed m converted to Survival%	46.7	79.5	68.7	55.7	50.5	16.6	14.9	0.02	0.39
Percent of mean Control Survival	71.9	122.3	105.7	85.7	77.6	25.6	23.0	0.04	0.60
Sum TU (based on LC50 Table 3)	0.10	0.10	0.10	0.72	0.72	2.2	2.2	6.9	6.9
Sum TU (based on $LBC50_X$ Table 3)	0.01	0.12	0.25	2.2	1.9	3.5	3.7	NA	NA
Predicted Mortality (MEAM H2O)	0.116	0.116	0.116	0.139	0.139	0.202	0.202	0.979	0.979
Predicted Survival (MEAM H2O)	62.8	62.8	62.8	57.4	57.4	44.6	44.6	2.0	2.0
Predicted Survival (MEAM H2O) Percent of Control	96.7	96.7	96.7	88.4	88.4	68.6	68.6	3.1	3.1
Predicted Mortality (MEAM BC)	0.111	0.112	0.120	0.165	0.167	0.273	0.262	NA	NA
Predicted Survival (MEAM BC)	64.1	64.0	61.8	51.7	51.3	33.6	35.0	NA	NA
Predicted Survival (MEAM BC) Percent of Control	98.8	98.5	95.2	79.5	79.0	51.8	53.9	0.0	0.0

units (Fig. 7.3) not at 1.0 toxic units as expected (asterisk, Fig. 7.3). Therefore the CAM over predicted toxicity, or in other words the observed toxicity was less than concentration additive by a factor of 1.62. When the toxic units were based on measured body concentrations (Table 7.4) and $LBC50_X$ (Table 7.3), the observed survival curve intersected the 50% survival point at 3.05 toxic units (Fig. 7.4) not at 1.0 toxic units as expected (asterisk, Fig. 7.4). Again, the CAM over predicted toxicity, or in other words the observed toxicity was less than concentration additive by a factor of 3 compared to the CAM predicted.

7.3.4.2 Metal Effects Addition Model (MEAM)

Survival predicted by the MEAM based on water or body concentrations (Table 7.5) was plotted versus the same toxic units as above. The MEAM predicted survival, calculated with water concentrations, was greater than observed, resulting in a 50% survival intercept point at 3.52 TUs (Fig. 7.3). This intercept point was 2 times higher than that for observed survival (intercept point 1.62 TUs, Fig. 7.3). This would indicate that the observed toxicity was more than effects additive when based on water concentrations compared to the MEAM predicted..

The MEAM predicted survival, calculated with body concentrations, was similar to observed, resulting in the 50% survival intercept at 3.7 toxic units, only 1.2 times greater than that for the observed survival intercept point at 3.05 (Fig. 7.4). This would indicated that the observed toxicity was just slightly more than effects additive when based on body concentrations compared to the MEAM predicted..

Survival, predicted by the MEAM, whether based on water or body concentrations, was accurate at all test mixture concentrations except at 2.2 toxic units (Fig. 7.5, Table 7.5). At this mixture concentration, the MEAM water-concentration predicted survival was 44% greater than the observed and when based ons body-concentration the predicted survival was 29% greater than observed (Fig. 7.5, Table 7.5).

7.4. DISCUSSION

7.4.1 Bioaccumulation

In general, most of the metal bioaccumulations followed the predicted saturation curves (0-hr depurated - solid lines, 24-hr depurated - dashed lines), but the 4-week accumulations of Cr, Cu, Ni, Pb and Tl (solid and open circles, Fig. 7.1 and 7.2) in the toxicity experiment were shifted to the left of the curve by 25 to 60% and Mn was shifted to the right by 40%. A potential explanation for these shifts was a change in methods from historical to current. First, all the saturation models for single metals were derived from experiments which were conducted in glass Erlenmeyer flasks

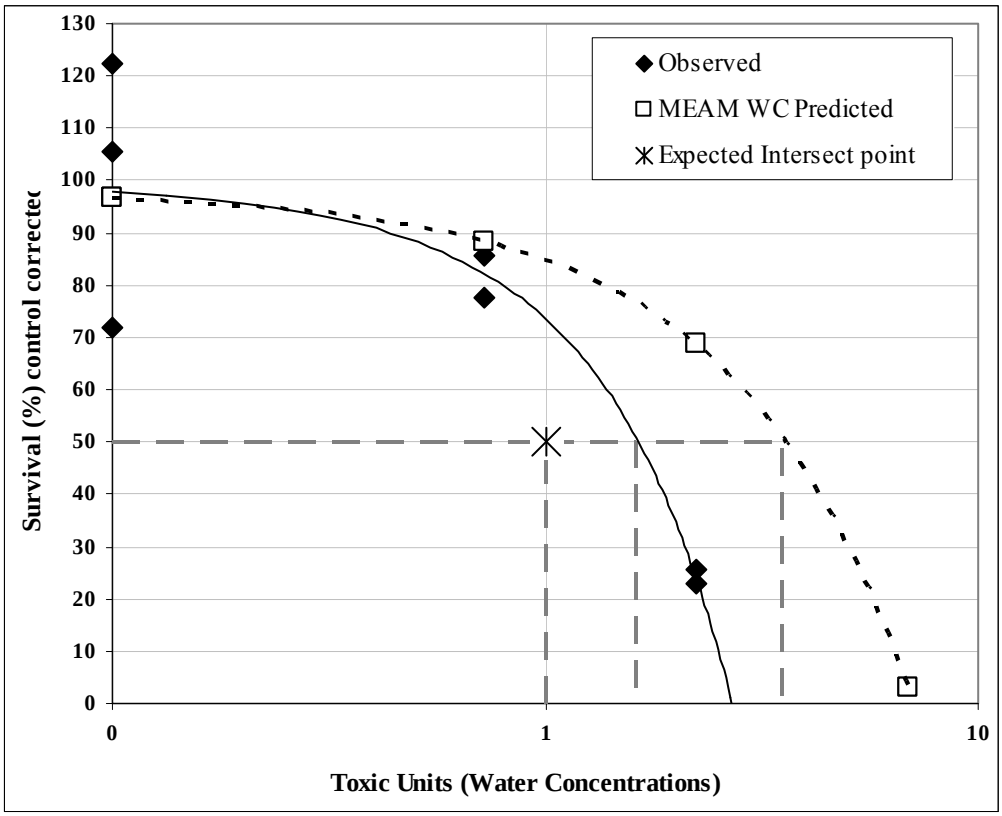

Fig. 7.3 *Hyalella* survival in relation to Toxic Units based on measured water concentrations. Observed survival (solid diamonds, solid line) and survival predicted with the Metal Effects Addition Model (MEAM WC) based on measured water concentrations (open squares, dotted line). The dashed gray lines represent the intersect points at 50% of control survival, and the expected intersect point (asterisk) of survival at a toxic unit of 1 for the Concentration Addition Model (CAM).

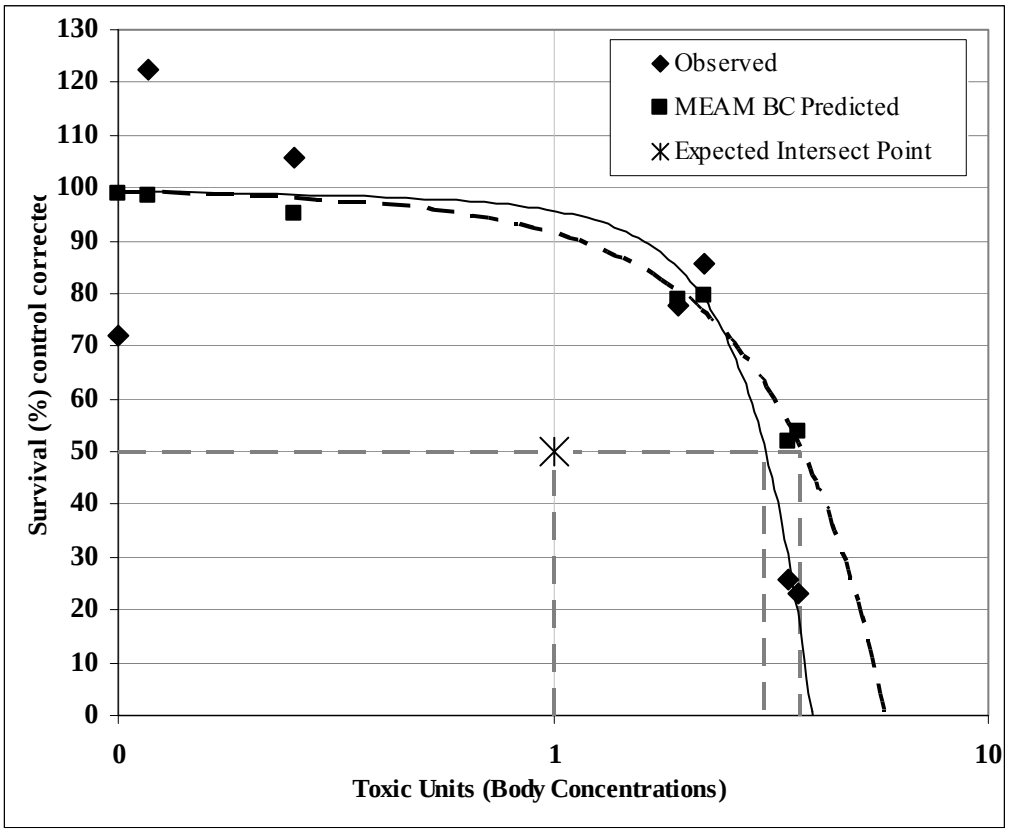

Fig. 7.4 *Hyalella* survival in relation to Toxic Units based on measured body concentrations. Observed survival (solid diamonds, solid line) and survival predicted with the Metal Effects Addition Model (MEAM BC) based on measured body concentrations (solid square, dashed line). The gray dashed lines represent the intersect points at 50% of control survival, and the expected intersect point (asterisk) of survival at a toxic unit of 1 for the Concentration Addition Model (CAM).

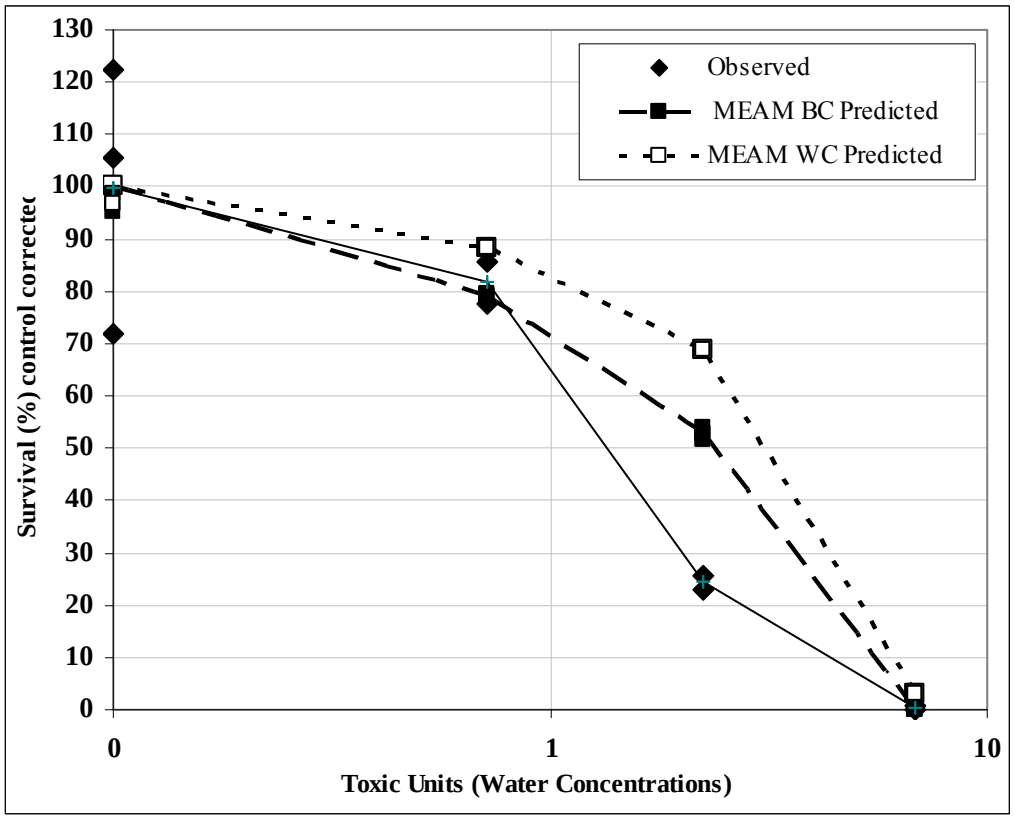

Fig. 7.5 *Hyalella* survival during chronic exposure (4 wk) to 10-metal mixtures. Observed survival (solid diamonds, solid line), predicted survival based on MEAM WC (water concentrations, open squares, dotted line) and on MEAM BC (body concentrations, solid squares, dashed line).

(Borgmann et al., 1991; Borgmann et al., 1993; Borgmann et al., 1998; Borgmann et al., 2001; Borgmann et al., 2004; Chapter 3) whereas the mixture experiments were conducted in HDPE plastic containers (Chapter 5). Different metals can adhere to the glass and plastic containers differentially over an exposure time.

Secondly, the timing of when the water samples were collected from the container was critical. Historically, Cd, Cu, Ni, Pb, Tl and Zn samples were collected at the end of each weekly turnover period, thus representing the concentration remaining in solution after any absorption of the metal to the container walls, gauze, food and detritus. Therefore, the final measured concentrations may not represent the true mean exposure. In contrast, As, Co, Cr and Mn water samples were collected at both the beginning and end of each weekly renewal period and the mean concentration was used in the models (Chapters 3 and 4). Thirdly, all the metal mixture accumulation and toxicity experiments had turnover periods of 2 and 3 days in order to reduce metal loss and produce more consistent exposures (Chapter 5). Again, the water samples were collected at the beginning and end of each of these shorter periods and mean values calculated. Finally, the analyses of Cd in water samples in Borgmann et al (1991) was done by solvent extraction and analyzed by flame AA. All other water samples and tissue samples (digested in nitric acid and peroxide) for the single metal experiments were analyzed on a Varian SpectraAA 400 graphite furnace, atomic absorption spectrophotometer with Zeeman background correction and samples from the mixtures experiments were analyzed for all 10 metal simultaneously by Inductively Coupled Plasma, Mass Spectroscopy (ICP-Mass Spec) by the National Laboratory for Environmental Testing, Environment Canada. All of these small changes could lead to shifts in the bioaccumulation curves relative to the measured water concentrations.

Some changes in bioaccumulation patterns for each metal can still be examined, keeping the above information in mind. An examination of the bioaccumulation of Co, Cd and Ni was warranted since these three metals were significantly inhibited in mixtures exposures in chapter 5, and As bioaccumulation requires some examination as well since its bioaccumulation was increased with exposure to the same metal mixtures (Chapter 5). Cobalt, above background, accumulation in the 1-wk, 10-metal exposure had the greatest inhibition at 85 and 84% in the 0 and 24 hr depurated adult organisms respectively (square symbols to triangle symbols, Co Fig. 7.1). This inhibition of accumulation was mirrored at the 6.52 and 20.6 nmol Co/L exposures in the 4-wk exposures and all Co accumulations in 10-metal exposure were statistically the same (round and triangle symbols in Fig. 7.1 Co, Table 7.4). Cadmium above background, 1-wk accumulations were second most inhibited by 81 and 79% in the 0 and 24 hr depurated adults respectively when exposed to 10-metals mixtures compared to single exposure (square symbols to triangle symbols, Cd Fig. 7.1). This inhibition was not strongly mirrored in the 4-wk exposures (round symbols, Cd Fig. 7.1), however, it was recently

Table 7.6 Geometric mean of initial and final Cd exposure concentation, measured background corrected Cd body concentrations, 95% confidense limits, sample size (N), and predicted Cd body concentration based on 4 and 1 week Cd only exposures

Treatment	Exposure (nmol Cd/L)	Observed Body (nmol Cd/g dry)	95% CL	N	[a]Predicted Body 4-wk Model (nmol Cd/g dry)	[b]Predicted Body 1-wk Model (nmol Cd/g dry)
4-wk 10-Metal Toxicity Test	0.71	9.6*	0.98	2	27.4*	NA
4-wk 10-Metal Toxicity Test	1.41	15.6*	1.96	2	54.8*	NA
1-wk Cd-Only Bioaccumulation Test	2.78	49.1	3.43	8	NA	54.2
1-wk 10-Metal Bioaccumulation Test	2.77	10.0*	0.75	4	NA	54.1*

[a] Bioaccumulation model based on final water concentrations (Borgmann et al 2004) based on Borgmann et al (1991) data.

[b] Bioaccumulation model based on mean (initial and final) water concentrations (personnel communication Borgmann 2006).

* significantly different (95% CL do not overlap)

determined that Cd bioaccumulation does not come to equilibrium within one week (J. Schroeder unpublished, 2006) as previously suggested (Borgmann et al., 1991) and the 1-wk bioaccumulation curve falls about 45% lower than the 4-wk bioaccumulation curve (Fig. 7.1, Cd). The Cd-only, above background, 1-wk bioaccumulation matches the predicted 1-wk model instead of the 4-wk model (square symbols, dotted curve, Fig. 7.1, Cd) at 49 nmol/g observed and 54.2 nmol/g predicted (Table 7.6). All other observed background corrected Cd bioaccumulations in mixtures exposures were significantly lower by an average of 73% than the bioaccumulations predicted by the appropriate 1-wk or 4-wk models (Table 7.6). Therefore the inhibition observed in Chapter 5 did occur in the 4-wk toxicity experiment.

Nickel accumulation decreases significantly by 57 and 75% in the 0 and 24 hr depurated adults respectively in the 1-wk accumulation test (square symbols to triangle symbols, Ni Fig. 7.2, Table 7.4) however, this inhibition was not strongly mirrored in the 4-wk exposures even though the accumulation at the highest Ni exposure of 157 nmol/L was not significantly different than the accumulation in the 1-wk test with 10-metals (round compared to triangle symbols in Ni Fig. 7.2, Table 7.4). Arsenic accumulations in the 4-wk toxicity test did not provide any indication of a stimulatory effect (Fig. 7.1, As, Table 7.4).

In summary, the Cd and Co accumulations were significantly reduced in the 4-wk toxicity test as predicted by the 1-wk mixture bioaccumulation test but the bioaccumulation data for As and Ni did not clearly identify the impact predicted. The identification of the impacts could potentially be improved with further study of 1-wk to 4-wk bioaccumulation, for not only the As and Ni but all the metals, in order to verify the time to steady state bioaccumulation.

7.4.2 Mixture Toxicity Evaluation

7.4.2.1 Water Concentrations

The most traditional method of assessing mixture impacts is the concentration addition model (CAM) using toxic units based on LC50s and measured water concentrations (Environment Canada, 1999; Sprague, 1970). The CAM over-estimated toxicity such that the observed 50% survival intersect point occurred at a toxic unit of 1.62 instead of 1.0 (Fig. 7.3). This would indicate that the impact of the mixture was less than additive, or in other words there was less impact observed then predicted. The Metal Effects Addition Model (MEAM) based on measured water concentrations under-estimated toxicity such that the MEAM predicted 50% survival intersect point occurred at 3.52 TUs (Fig. 7.3). Therefore, the observed toxicity of the mixture was greater than additive relative to that predicted by the MEAM based on measured water concentrations. Therefore the two different assessment techniques have opposing interpretations of the impact of the mixture, when in reality the impact lies in-between.

The difference between the two predictions lies in the assumptions of each model. A strict concentration addition models assumes that each metal acts on the same site of action, or in other words they act in a dependent manor. In this case the sum of the concentrations, converted to a common unit (TUs), of all the metals should predict the mixture toxicity. The effects addition model assumes that each metal acts on different sites of action, or in other words they act independently. In this case the sum of the effect by each metal should predict toxicity. Since the observed toxicity of the mixture was in-between the two predictions, it is possible that some of the metals act independently (act on different sites of action) and some of the metals act in a dependent manor (act on the same site of action).

7.4.2.2 Body Concentrations

Toxic units were calculated based on background corrected, measured accumulated metal concentrations and the $LBC50_X$. The CAM over-predicted toxicity since the observed 50% survival intersect point occurred at 3.05 TUs instead of 1.0 (Fig. 7.4). This would indicate that the impact of the mixture was less than additive. The MEAM predicted survival curve was similar to the observed but did extend more to the right such that the predicted 50% survival intersect point occurred at 3.7 TUs (Fig. 7.4). This indicates that the observed impact was more than additive relative to the MEAM prediction.

Again, the difference between the two predictions lies in the assumptions of each model as explained in the section 4.2.1 above. Since the observed toxicity of the mixture was in-between the two predictions, it is possible that some of the metals act independently (act on different sites of action) and some of the metals act in a dependent manor (act on the same site of action). Most of the metals appear to act independently, but there may be some competition for the same binding sites resulting in inhibition of Cobalt, Cd and Ni accumulations as seen in Chapters 5 and 6, during the 1-week bioaccumulation test. As well, Co accumulation appears inhibited in treatment C (32 ul stock addition, 20 nmol/L) indicating some competition for the same binding site (Table 7.4, Fig. 7.1). Therefore, there could be a small but noticeable influence of a concentration addition effect and hence the MEAM would under predict toxicity at this treatment.

7.5 SUMMARY

The CAM over-estimated toxicity when toxic units were based on water or body concentrations, with the best prediction based on water concentrations. The CAM has been previously found to overestimate the joint toxicity of 16 organic biocides (Faust et al., 2003) yet it was determined that the method of independent action or effects addition, accurately predicted the mixture toxicity. In this current study, the (MEAM) under-estimated toxicity based on measured water or body concentrations, with the best prediction based on body concentrations. Even though the MEAM, based

on body concentrations, underestimated toxicity at the mid-toxic range, overall it was quite close to observed.

The MEAM does have a couple of advantages. First, it can predict survival at any measured body concentration, whereas the CAM traditionally focuses on only one critical concentration, like an LC50, using the recommended procedures for calculating toxic units (Environment Canada, 1999; Sprague, 1970). The sum of toxic units can only determine that the mixture impact is expected to be less than, equal to, or greater than that of the critical value impact (such as 50% mortality for an LC50) when the sum of TUs is <1, 1 or >1 respectively. Secondly, the MEAM, when based on measured body concentrations, takes bioavailability into account. This is important since the chemical characteristics of water can greatly alter the bioavailability and therefore toxicity of metals. Toxic units when based on body concentrations would also take bioavailability into account, however this method greatly overestimated toxicity.

7.5.1 Limitations

There were some limitations to this study. First, the study was designed to examine the interaction between the test metals while maintaining a constant media. Therefore, it cannot be determined whether the MEAM or the CAM based on water concentrations, was better at predicting effects under varying chemical characteristics in the media, such as different hardness, pH and complexing agents. Secondly, this study only examined a fixed, equi-toxic ratio between the 10 metals and therefore may be inadequate for the assessment of mixtures of metals at different ratios. However, the study does provide evidence that the MEAM works when based on tissue concentrations even though it marginally underestimated toxicity at the mid toxic concentrations. The CAM when based on water concentrations overestimates toxicity. However, overestimation of toxicity does fit with the cautionary principle.

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CHAPTER 8

General Summary

Table 8.1(a) Summary of the major conclusions of metal bioaccumulations, interactions and impacts on the freshwater benthic invertebrate *Hyalella azteca* from single metal chronic exposures through to metal mixture exposures.

Major Conclusions	Page
Historically, there has been high variability in mixture responses reported. Strictly additive, less than additive and more than additive impacts for the same metals.	29
No consistent species, tests, endpoints or models have been used historically to evaluate the impact of mixtures.	29
The Biotic Ligand Model (BLM) has not been applied to mixtures.	30
Arsenic, Co, Cr & Mn follow clear dose-response relationships described by saturation models for both bioaccumulation and mortality	46, 75, 89
Elimination rates for As, Co & Cr were constant at 34, 13 & 4 percent respectively	45
Mn elimination rate saturates (reaches a maximum)	53
Bioaccumulation may be a function of the relative uptake and elimination rates	55
Hyalella do not regulate Cr	57
Arsenic, Co, Cr & Mn bioaccumulation saturate at maximum body concentrations of 219, 674, 831 and 116000 nmol g-1 respectively	46
Arsenic, Co, Cr & Mn have good bioaccumulation patterns, useful for environmental assessment	58
Arsenic, Co, Cr & Mn have Bioconcentration Factors (BCFs) of 20, 515, 200 & 207 respectively	45
Arsenic, Co, Cr & Mn have LC50s of 5600, 183, 731 & 197000 nmol/L respectively	74
Arsenic, Co, Cr & Mn have background corrected, 24 hr depurated, Lethal Body Concentrations (LBC25's) of 83, 90, 146 & 44400 nmol g^{-1} dry respectively	76
LBC25s and LBC50s were also determined for 0 and 24 hr depurated animals	76
Arsenic, Co & Mn have growth Inhibition Concentrations (IC25) of 4010, 49, 128000 nmol/L respectively	79
There was no impact of Cr on *Hyalella* growth	79
As caused a hormesis effect in *Hyalella* growth	78
Plastic (HDPE) containers are better than glass for conducting metal toxicity tests	82

Table 8.1(b) Summary of the major conclusions of metal bioaccumulations, interactions and impacts on the freshwater benthic invertebrate *Hyalella azteca* from single metal chronic exposures through to metal mixture exposures.

Major Conclusions	Page
Non-essential elements As, Cd, Co, Cr, Hg, Ni, Pb & Tl have similar lethal body concentrations	84-85
Essential elements Cu, Mn & Zn have significantly higher lethal body concentrations than the non-essential elements	84-85
There is a strong inverse relationship between LC25 and BCF for the non-essential elements	86-87
Interactions between metals in mixture exposures affect their bioaccumulation	115
Co, Cd & Ni bioaccumulation in metal mixtures was significantly inhibited up to 85, 75 and 48 percent respectively	107
Competitive Inhibition of metal accumulation is plausible for only Co, Cd and Ni	132-133
The Biotic Ligand Model (BLM) at this time is not appropriate for metal mixtures	139
The concentration addition model based on exposure or body concentrations over-estimated mixture toxicity	157
The effects addition model (MEAM) under-estimated toxicity of mixtures	164
Some metals act independently (act on different sites of action) and some act dependently (act on same site of action)	164
The Metal Effects Addition Model (MEAM), based on body concentrations, predicted toxicity the best	164

This research focused on the value of body concentrations as predictive of metal mixture toxicity. Metal mixtures were selected since several metals are often present together at elevated concentrations in contaminated environments and existing regulations and guides lines are for single metals only. The use of body concentrations was appropriate since it has been demonstrated that the concentrations of a chemical in an organism was better for predicting effects than environmental measures such as water or sediment concentrations. As well, the use of body concentrations negates the impact of interactions with other ions and ligands in the exposure media, which affect bioavailability. In order to relate body concentrations to effects under mixture exposures a number of steps were taken. First, a background literature review of all the major methods available that examine the impact of mixtures was conducted. Secondly, single metal exposures and the relationship to bioaccumulation and then to effects was determined for As, Cr, Co and Mn. This work on individual metals was combined with previous work on other metals, so that the individual bioaccumulation to chronic effects relationship for 10 elements was known. Third, interactions between metals and the impact on bioaccumulation from mixture exposures were investigated, since a significant change in a metal's bioaccumulation from mixtures compared to single exposure could have a significant impact on the contribution of that metal to the overall effect on the organism. And finally, predictive models of the impacts of mixtures on mortality were developed and compared based on the two main paradigms; concentration addition and effects addition, in which body concentrations were used as well as the traditional exposure concentration or dose. The major results and conclusions of the body of research is summarized in Table 8.1.

The main two underlying hypotheses which were tested were;

1. The bioaccumulation of the elements As, Co, Cr and Mn individually by *Hyalella azteca* was proportional to their concentration in water and can be used to predict chronic toxicity.
2. The toxicity of mixtures of metals can be predicted with *Hyalella* body concentrations.

In order to test the above hypotheses, a number of objectives were met as outlined below:

1. Determine the bioaccumulation and toxicity of four elements (As, Co, Cr & Mn) from individual chronic bioassays of each metal (Chapters 3 & 4).
2. Determine the relationship between exposure concentration and the resulting body concentration (body concentration were predicted from exposure concentration in controlled bioassays and bioaccumulation models were developed, Chapter 3).
3. The relationship between body concentration and the resulting effects, mortality and growth were determined in Chapter 4 (mortality was predicted from body and water concentrations; growth models could only be generated for As, Co and Mn; no impact of Cr on growth).

4. Up to 10-metal mixtures of As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Tl & Zn, at equi-toxic concentrations based on Toxic Units (TUs) in which 1 TU equaled the LC25 for each metal, were produced and bioaccumulation determined in 1-week exposures. Interactions affecting bioaccumulation from mixtures were determined (Chapters 5 & 6)
5. The bioaccumulation and toxicity of the metal mixtures were determined (Chapter 7).
6. Metal mixture toxicity was evaluated with the concentration additive model, based on water and body concentrations, and the effects addition model, using mortality rate models for each individual element based on water and body concentrations (Chapter 7).

The review of the historical development of metal mixture interaction analyses performed in Chapter 2 identified the two major classifications of mixture models, the "Concentration Addition" and the "Response Addition" approaches which then became the main models to be tested in Chapter 7. This review also clearly identified the potential problems of mixture testing and modeling, and indicated that there was excessive variability in the historical results. Specifically, it was evident that the testing should be done on one species, using the same method and duration for mixture and single metal exposures, using the same end points across all experiments, using the same exposure media for every test and using the same data analyses so that the results could be controlled for direct comparison in order to determine changes and interactions. As well, bioaccumulation data had not been used in the past to evaluate the impact of mixture exposure and therefore this was included since it has been shown that bioaccumulation data is better for predicting effects than other measures such as water concentration or sediment concentration.

The bioaccumulation and toxicity of As, Co, Cr and Mn in *Hyalella azteca* was determined in chronic exposures and fit saturation models of bioaccumulation and mortality (Chapters 3 & 4). Bioaccumulation of As, Co, Cr, and Mn was strongly correlated with chronic mortality. Growth was a more variable endpoint than mortality for As, Co and Mn, and Cr had no effect on growth. Therefore, it was evident that body concentrations of these four elements were good indicators of bioavailability and predictors of mortality and should be useful for environmental assessment.

With the mortality models determined for As, Co, Cr and Mn combined with historical bioaccumulation and mortality models developed for Cd, Cu, Ni, Pb, Tl and Zn, mixtures were produced of "equi-toxic" concentrations of all 10 elements in order to determine bioaccumulation and toxicity (Chapters 5, 6 & 7). Bioaccumulation was determined in one-week exposures to determine interaction between the metals on bioaccumulation. Interaction factors (*IF*) were computed which quantified each element's impact on the bioaccumulation of the other nine (Chapter 5). Cobalt, Cd and Ni bioaccumulations were significantly inhibited with increasing number of metals in the mixture and

there was some inhibition of Tl and Zn bioaccumulation with some mixtures. Arsenic and Pb bioaccumulations were enhanced by mixture exposure. Whereas, Cr, Cu, Mn, Tl and Zn bioaccumulations were not significantly affected by exposure to other metals (Chapter 5). It was determined that competitive inhibition may be a plausible mechanism of interaction in the accumulation of Co, Cd and Ni (Chapter 6). However, it is possible that other interactions may have been responsible, especially in the cases of As and Pb enhanced bioaccumulations. This is not possible in a strictly competitive interaction model. Thus a BLM for mixtures would not be appropriate since the BLM currently is a strictly competitive inhibition model.

The toxicity of metal mixtures in a concentration series of a fixed ratio of the 10 metal concentrations was determined in Chapter 7. It was determined that the concentration addition model over-estimated toxicity when toxic units were based on water or body concentrations and the metal effects addition model (MEAM) under-estimated toxicity based on measured water or body concentrations. However, the MEAM when based on body concentrations was the best predictor of observed toxicity. This was very encouraging since it has been shown that body concentrations are much better at predicting toxicity of individual metals under varying environmental conditions (i.e. different ligands such as organic mater, sediment particles and varying water chemistry). It is therefore expected that the MEAM, when based on measured body concentrations, takes bioavailability into account and should be superior at predicting effects.