Chronic Nickel and Copper Toxicity in the *Hyalella azteca* Cryptic Species
Complex

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including

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Abstract

The amphipod crustacean *Hyalella azteca* was thought to be a single species, but recent molecular studies have indicated that it is a large cryptic species complex. Previous work has indicated that the *H. azteca* complex contains up to 85 genetically divergent lineages. *Hyalella azteca* has been frequently used in toxicity tests due to its sensitivity to contaminants, but past studies have examined the effects of toxicants with the assumption that *H. azteca* is a single species rather than a large cryptic species complex. Recently, a research group determined that two genetically characterized laboratory lineages (Clade 1 and 8) display different nickel and copper sensitivity in acute toxicity tests. In this study, *Hyalella* Clades 1 and 8 were subject to 28 day nickel and copper toxicity tests, and their survival and growth responses were compared. The estimated nickel and copper LC50s for Clade 8 were 2.65 and 1.47 times greater than that of Clade 1, respectively. Clade 8 amphipods also had a higher estimated LBC50, IBC25, and maximum tissue concentration for copper than that for Clade 1. However, the estimated nickel LBC50s and IBC25s were similar between the two clades, but bioaccumulation responses differed significantly. Although both clades are large-bodied ecomorphs, Clade 8 amphipods in the control and lower test concentrations had significantly higher dry weights after the 28 day exposures. These results complement an earlier study in that at least two lineages within the *H. azteca* cryptic species complex display different sensitivities to nickel and copper. Therefore, caution should be used when conducting toxicity tests with laboratory cultures that have not been genetically characterized as the results obtained may not be comparable.

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Table of Contents

List of Abbreviations

ANOVA	Analysis of variance
BLM	Biotic ligand model
CCIW	Canadian Centre for Inland Waters
CI	Confidence interval
CuZnSOD	Copper-zinc superoxide dismutase
COI	Cytochrome c oxidase I
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DO	Dissolved oxygen
EC	Environment Canada
EDTA	Ethylenediamine tetra-acetic acid
GFAAS	Graphite furnace atomic absorption spectrometry
IBC25	Inhibition body concentration at which a 25% reduction in growth occurs for the test population
IC25	Inhibition concentration at which a 25% reduction in growth occurs for the test population
LBE	Large-bodied ecomorph
LBC50	Lethal body concentration at which an inhibitory response occurs in 50% of the test population
LBC25	Lethal body concentration at which an inhibitory response occurs in 25% of the test population
LC25	Lethal concentration that results in mortality for 25% of the test population
LC50	Lethal concentration that results in mortality for 50% of the test population
ML	Maximum likelihood
NJ	Neighbour-joining
ND	Not determined
PGC	Pleasant Grove Creek

PCR	Polymerase chain reaction
QC	Quality check
ROS	Reactive oxygen species
SBE	Small-bodied ecomorph
SSD	Species sensitivity distribution
SST	Species-screening threshold
SAM	Standard Artificial Media
USEPA	United States Environment Protection Agency

Chapter 1 – Introduction

Hyalella azteca as a Cryptic Species Complex

Hyalella azteca is a fresh water amphipod that is widely distributed across lakes, streams, and ponds in Central and North America. Despite its vast geographic range, *H. azteca* has been thought to have poor dispersal capabilities due to the fact that females brood their young, and are unable to have dormant eggs (Duan, Guttman, Oris, & Bailer, 2000a; Othman & Pascoe, 2001; Witt & Hebert, 2000). However, it has been noted that *Hyalella* are able to disperse passively on waterfowl (Witt et al., 2008). Duan et al. (2000) state that there is high genetic differentiation among *Hyalella* populations that are in close proximity to each other, which would suggest that there is minimal gene flow between the populations.

Witt and Hebert (2000) build on this notion when they state that *H. azteca* has high potential for diversification since it has poor dispersal abilities, is restricted to permanent habitats due to a lack of a resting stage that is drought-resistant, and is affected by habitat selection pressures. Their claim was supported by the discovery of 7 different monophyletic clades that exhibit between 9 and 28% sequence divergence at the mitochondrial cytochrome *c* oxidase I gene (COI). These results fit the criteria for the Phylogenetic Species Concept, which defines a species as a monophyletic group that can be diagnosed "by a unique combination of character states in comparable individuals" (Baker & Bradley, 2006; Nixon & Wheeler, 1990).

Many of the lineages discovered by Witt and Hebert (2000) co-occurred in the same habitats, but displayed significant Hardy-Weinberg disequilibrium at several loci due to heterozygote deficits. These fixed allozyme differences in conjunction with significant multilocus genotypic associations indicated that the populations were reproductively isolated.

This criteria fits the Biological Species Concept, which defines a species as "groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr, 1963).

Despite the considerable allozyme differences and nucleotide sequence divergence between lineages at COI, the amphipods collected by Witt and Hebert (2000) displayed little morphological divergence. The Morphological Species Concept defines a species on the basis of morphological similarities (Wheeler & Meier, 2012), as such *H. azteca* would be considered a single species. However, this is contradictory to the results obtained by the researchers, which determined that the populations belonged to different lineages. Thus, Witt and Hebert (2000) concluded that *H. azteca* is a morphological cryptic species complex with at least 7 lineages. This resonates with previous studies that hinted at *H. azteca* being a cryptic species complex, but were unable to clearly identify lineages on a genetic level. For example the studies conducted by Duan et al. (1997, 2000b) examined genetic differences between laboratory *Hyalella* and wild populations using allozyme analyses. They determined that there were large genetic differences among populations, but they did not conclude that it was indicative of a cryptic species complex. Duan et al. (2000a) also mentioned that further studies would be required, especially to improve the validity of toxicity tests when using *Hyalella* populations from different localities

Witt et al. (2006) further assessed the *H. azteca* cryptic species complex by employing DNA barcoding in the southern Great Basin region of California and Nevada. This method uses a 648-bp segment of the COI gene and a "species-screening threshold (SST)." Based on a similar threshold set by Hebert et al. (2004) to recognize provisional species in birds, the authors utilize an SST that is ten times the average intrapopulation COI haplotype divergence. Witt et al. (2006) calculated the SST to be 3.75% after conducting pairwise haplotype comparisons among 39 *H.*

azteca populations. Using this threshold, they delineated 33 provisional species that exhibit substantial COI nucleotide divergences (4.4% - 29.9%), and these are all currently assigned to "*H. azteca*".

Additional lineages were discovered in the Great Basin in a more recent study by Witt et al. (2008). The authors examined the rates of molecular evolution and local population divergence in regions of the southern Great Basin and the Bonneville Basin in the northern Great Basin. They sampled 67 habitats, obtained a total of 362 COI sequences, and then conducted phylogenetic analyses [neighbour-joining (NJ), and maximum likelihood (ML)]. The results of the NJ analysis determined that there were 150 haplotypes, and these had an average pair-wise nucleotide sequence divergence of 21.6%.

Witt et al. (2008) discovered that the southern Great Basin as a whole displays high genetic diversity and endemism, but elaborate on the maximum likelihood results for Ash Meadows in particular – a region in the Amargosa Valley of the southern Great Basin. They discovered that six lineages resided in 11 springs in Ash Meadows, and only two of those habitats shared haplotypes while the others did not. Ash Meadows is currently isolated, but was more integrated with the White River Valley during the Pleistocene era, which indicate that barriers (vicariance) play a role in shaping genetic variation. In comparison, Witt et al. (2008) discovered that the Bonneville Basin only had four lineages, and the diversity was low. Another important result of their study was that a single lineage in one of the southern Great Basin habitats was detected as being widespread in California. The authors conclude that both dispersal and vicariance are key factors in shaping the genetic diversity in the Great Basin.

As of March 2016, Witt and Wellborn (in preparation) have proposed that the *H. azteca* complex contains up to 85 genetically divergent lineages.

Morphology and Life History

In the past, *Hyalella azteca* has been thought to be a single species, but recent molecular studies have indicated that this is not the case (Wellborn & Broughton, 2008; Wellborn & Cothran, 2004; Witt & Hebert, 2000; Witt et al., 2006). Although the various molecular studies conducted have determined that many cryptic species exist in the *H. azteca* species complex, all studies have documented that the amphipods do not differ significantly in phenotype. Wellborn (1994, 1995a, 1995b) observed that *H. azteca* collected from habitats in Southeast Michigan belonged to one of two categories: "small-bodied morphotype" and a "large-bodied morphotype". Wellborn and Broughton (2008) noted that three geographical regions in the USA (Michigan, Oklahoma, and Oregon) had *Hyalella* amphipods belonging to one of the two phenotypic groups. They deemed these two phenotypic groups as "ecomorphs" since earlier studies by Wellborn have established that the environment affects the body size and life history of the amphipods. The small-bodied ecomorph will be abbreviated as SBE, and the large-bodied ecomorph will be abbreviated as LBE. Wellborn and Broughton (2008) conclude that the evolution of small or large body size is a result of functional or ecological constraints such as predation. Earlier studies by Wellborn (1994, 1995a, 1995b) address these constraints that affect *Hyalella* body size.

Wellborn (1994) first noted that the two ecomorphs collected from Duck Lake and Duck Marsh were possibly two separate species. He observed that Duck Lake had fish such as bluegill, pumpkinseed sunfish, yellow perch, and largemouth bass inhabiting the lake. In contrast, Duck Marsh had no fish in it despite being beside the lake, but had an abundance of predatory

invertebrates such as dragonflies and damselflies. Wellborn (1994) observed that bluegill sunfish were major consumers of *Hyalella* in Duck Lake, and that mostly the larger ones were retrieved from their stomachs upon analysis. The larvae of dragonflies and damselflies were the most common invertebrate predators in both habitats, but the former were most abundant. Large and small invertebrate predators preferred *Hyalella* that were the same size, respectively. Intermediate sized invertebrate predators had no preference on *Hyalella* body length. However, there was a threshold at which predators could no longer prey on adult *Hyalella*, and this was at 3 to 4mm head widths. It was concluded that predation affected the body size of the *Hyalella* since the SBE was determined to be in areas inhabited by centrarchid fish, whereas the LBE was observed to be in areas that were fishless or had sticklebacks as the dominant fish predator (Wellborn, 1994).

Studies on reproductive success and predator community composition were also conducted by Wellborn (1995a, 1995b). He determined that reproductive success increased with body size for both males and females in the two habitats. However, the small ecomorphs showed the greatest pairing success when the male had an intermediate body size. He also noted that gnathopod size mattered in pairing success in the large ecomorphs, but not for the small ones. In contrast, female body size did not have an effect on pairing success since all were capable of finding mates. Wellborn (1995a, 1995b) concluded that since all SBE inhabit areas with well-developed centrarchid fish communities, and all LBE reside in areas that are fishless or contain sticklebacks, male body size is constrained by size-biased predation for the Lake ecomorph.

Later on, McPeek and Wellborn (1998) conducted allozyme analyses on both large and small ecotype populations of *Hyalella*, as well as infertility trials. The researchers noticed that the amount of differentiation between ecotypes was lower by almost two fold in comparison to

the differentiation among populations within each ecotype (F statistic of 0.112 vs. 0.224). They also noted that a large number of the small ecotype populations had significant heterozygote deficiencies at 4 loci. This was not the case for the large ecotype populations as there were no genotype frequencies that deviated from Hardy-Weinberg expectations at all examined loci.

A more recent study by Wellborn (2002) examined the competitive ability between the two ecomorphs in the presence and absence of *Physella virgata* – a pulmonate snail. It was noted that the presence of the snail reduced the abundance of both ecomorphs by 22%, the larger ecomorph reduced snail density by 46%, and the larger ecomorph affected the small species by depressing their performance, but the smaller ecomorph did not affect the larger ecormorph. The author indicates that activity level and resource consumption increases with body size, and that his study also confirms that this is the case with the two *Hyalella* ecomorphs.

Wellborn and Cothran (2004, 2007) discovered that three small ecomorph species co-occur in small lakes throughout the upper Midwest of North America. Species B is the largest of the three, predominantly located at the edge of the shore, and has smaller eggs than A and C. Species A is the smallest of the three and dominates a habitat farthest from the shore. Species A and C can be observed occurring at an intermediate distance from the shore and have similar egg size. The authors observed predation in Duck and South Lakes and determined that Species B was targeted the most since they had a low occurrence in the habitat, but are present at a high frequency in fish stomachs. Species A had the lowest predation risk as it had a high frequency in the habitat, and a low frequency in fish stomachs. Species C was intermediate with respect to the other two species with regard to predation. Wellborn and Cothran (2007) concluded that the increased predation on Species B is likely due to a trade-off that comes with an increased body size and competitive ability (Wellborn, 2002).

6

Hyalella azteca and Toxicity Tests

Hyalella azteca has been used extensively in toxicity tests as a biomonitor to assess contaminated sediments because it is easily cultured, widely distributed, sensitive to many pollutants, broods its young, can easily be sexed and aged, and has a short generation time (Duan et al., 1997; Ingersoll et al., 1998; Othman & Pascoe, 2001; Strong, 1972). Numerous studies employ *H. azteca* in chronic and acute whole sediment or water-only tests. Common contaminants that are frequently used in these toxicity tests include pesticides and heavy metals such as cadmium, manganese, zinc, copper, and nickel (Couillard et al., 2008; Doig & Liber, 2006; Giusto, Salibián, & Ferrari, 2014; Hall & Anderson, 2014; Ingersoll et al., 1998; Weston et al., 2013).

Although *H. azteca* is frequently used in toxicity tests, Major et al. (2013) suggest that caution should be used when conducting toxicity tests with laboratory lineages of *H. azteca*. The authors have identified six lineages that can be classified as separate provisional species due to an observed percent COI nucleotide sequence divergence of 6.98% to 25.60% between any two of the lineages. Their study involved *Hyalella* samples from 15 laboratories and 22 field sites. Among the 15 laboratory populations, two provisional species were delineated – a US laboratory lineage and a Burlington lineage. The authors also discovered that all US laboratories and four of five Canadian laboratories use populations of *Hyalella* that belong to the US laboratory lineage whereas the Canadian Centre for Inland Waters (CCIW) is the only laboratory that uses populations of *Hyalella* that belong to the Burlington lineage (Major et al., 2013). These lineages exhibit a COI nucleotide sequence divergence of 24%, and the authors suggest that this high divergence can influence toxicity test data comparisons. Also, Major et al. (2013) state that the use of the two laboratory lineages in toxicity tests may not be reliable for predicting wild

population responses since the laboratory lineages are only represented in limited locations across Eastern Canada and the US (Major et al., 2013).

Interestingly, the use of the two different lineages can also account for cross laboratory variability in toxicity test data. Even prior to this recent discovery, Duan et al. (1997) had been using what was referred to as the "Nebeker strain of *H. azteca*," which was originally collected in Oregon, USA by A. Nebeker in 1982. Duan et al. (2000b) observed that there was variability even within the same strain when employed in toxicity tests. The authors state that there are very few studies with *Hyalella* that explore the connection between toxic effects and certain genotypes. They proposed that specific allozyme genotypes would exhibit mortality while others may not when exposed to different contaminants at an acute level. The researchers tested this hypothesis by exposing the Nebeker strain of *H. azteca* to low pH, cadmium, copper, lead, and zinc. Duan et al. (2000b) discovered that there was a significant difference in mortality among the genotypes when exposed to any of the metals or low pH condition. They noted that some genotypes had opposite effects when assessing their sensitivity to the metals or pH. The example they provide are the metal-tolerant genotypes, which they determined were the most sensitive to low pH. Duan et al. (2000b) explain that this is due to the fact that lower pH conditions normally allow for higher free metal ion concentrations, and as a result increases metal toxicity.

There are standardized protocols developed by the United States Environment Protection Agency (USEPA) and Environment Canada (Environment Canada (EC), 2013; United States Environmental Protection Agency (USEPA), 2000). However, each laboratory may use slightly different protocols, such as specific food type or water hardness. Leung (2014) effectively demonstrates the aforementioned variability in protocols when evaluating the compiled data from previous studies within her work. This compilation acts as a segue into the more pressing

question of whether the discrepancies in toxicity test results could potentially be due to the use of two different laboratory lineages as mentioned by Major et al. (2013). Also, since the recent emergence of genetically characterizing *Hyalella* cultures, the standardized methods and protocols across many laboratories in Canada and the US do not account for verifying and identifying the lineage of the amphipods being used in experiments.

Hyalella and Copper

Copper is an essential metal that occurs naturally in the environment, but is present in lakes and streams due to anthropogenic causes such as mining and algicide use (Elder & Horne, 1978; Kosalwat & Knight, 1987). Although copper is required for biological processes such as complexing with oxidizing enzymes (Lepp, 1981), high concentrations of copper can be toxic to most organisms. Despite this, there are many animals that are capable of regulating this metal, and Borgmann and Norwood (1995b) determined that *H. azteca* has this ability since they observed a significant accumulation of copper that gradually decreased to control levels after a 4-6 week exposure. However, it was determined that copper regulation takes weeks to achieve, and thus another experiment was conducted with 1 week exposures to assess short-term toxicity. They concluded that toxicity is evident after 1 week exposures when the concentration is increased by an additional 1.8 µmol/g. Norwood et al. (2007) support this copper regulation discovery as they indicate that *H. azteca* are more tolerant to high levels of essential elements such as copper and zinc.

Numerous studies have been conducted to determine the effects of copper on *H. azteca* using both sediment and water-only toxicity tests. Morris et al. (2003) conducted a chronic copper experiment to assess whether the exposure would indirectly affect the nutritional composition of the amphipods by changing their metabolism. The researchers measured various

9

parameters such as fatty acid, protein, and total lipid content, and concluded that there was no evidence to support their hypothesis that the nutritional composition of *H. azteca* is affected by chronic copper exposure whether through water or food.

In contrast, Othman and Pascoe (2002) exposed *H. azteca* to chronic levels of copper to identify its direct effect on survival and reproductive aspects. Their tests were conducted with juveniles that were less than 7 days old, and over a 35 day period to accommodate a peak precopulatory time of 28-30 days of age, which they established in their previous work (Othman & Pascoe, 2001). Using measured concentrations of 13, 32, 55, and 213 µg/L, Othman and Pascoe (2002) established that after the 35 days, the control and 13 µg/L copper concentration populations had increased numbers of animals whereas there was a significant decrease in animal number at the 55 and 213 µg/L copper concentrations when compared to the control. They concluded that as the copper concentration increased, the population would be composed of the adults that they originally started with, little to no juveniles, no neonates, and smaller body size than the control adults.

Many factors can affect the toxicity of copper such as the pH and alkalinity of the water, the amount of organic matter present, and the water hardness. Although Naddy et al. (2002) do not use *H. azteca* in their work, it is still important since *Ceriodaphnia dubia*, *Daphnia magna* and *Gammarus* sp. were employed as the invertebrate test organisms. In addition to the aforementioned organisms, rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimphales promelas*) were tested to determine whether varying ratios of calcium and magnesium had an effect on the acute toxicity of copper to five different aquatic organisms since the ratios used in the laboratory are not representative of natural bodies of water. Their results for the invertebrates indicated that sensitivity to copper was greatest at the 4:0 Ca:Mg ratio except

10

for *Gammarus* sp., which the researchers concluded was equally sensitive to the metal at all of their experimental treatments. Naddy et al. (2002) conclude that the Ca:Mg ratio should be considered when testing other metals since it can also affect nickel toxicity, and that only some organisms are affected by it.

Sediments are commonly used in toxicological studies, and the sediment itself can be a factor in affecting copper toxicity. Besser et al. (2003) sought to evaluate the significance of organic matter on the bioavailability and acute toxicity of cadmium and copper to *H. azteca* in two different spiked sediments – one with purified cellulose and the other with natural humus. Their results for the cellulose sediment showed that there is a minimal effect on the bioavailability when spiked with cadmium or copper, and they expected this to be the case since previous literature states that cellulose does not have the chemical structure to bind metals like other substances such as carboxylic groups (Chapman et al., 1998). Humus on the other hand, resulted in significant reductions in toxicity of both Cd and Cu in their experiments (Besser et al., 2003). The researchers noticed that sediments with high levels of humus increased amphipod survival between 35-90% at two of their spike concentrations for both metals. Humus treated sediments had lower concentrations of Cd and Cu in comparison to the control and cellulose sediments when examining pore and overlying water. Also, Cd bioavailability was reduced by both low and high amounts of humus up to a spike level of 4 mg/L, whereby amphipod survival was impacted significantly when this concentration was exceeded in a supplementary test using a low humus sediment. Besser et al. (2003) observed different results for Cu as the low and high humus had different binding capacities. They noticed that the amphipod survival in low humus sediments was significantly reduced when exposed to Cu spikes of 100 mg Cu/L and upwards.

However, Besser et al. (2003) did not observe any significant reductions in survival when assessing the high-humus sediments at spikes of 200 mg Cu/L.

The aforementioned work is relevant because Borgmann et al. (2005) state that although water chemistry is important in affecting the toxicity of metals, using bioaccumulation methods to establish toxicity is a more reliable technique than assessing the concentration of metal in the water or sediment. However, this is not an effective methodology for copper since it is a regulated metal in *H. azteca*. Thus Borgmann et al. (2005) iterate that describing copper toxicity to this organism should be based on water concentrations, which means that water chemistry factors and their effects need to be evaluated. Their study explores the effects of pH and major ions on copper toxicity using a single-site biotic ligand model and a multi-binding site model.

Hyalella and Nickel

Nickel has not been as extensively studied as copper, and questions still remain on its toxicity and bioavailability in *H. azteca* (Keithly et al., 2004). Recent studies have determined that water hardness along with other factors, is important to the bioavailability and toxicity of nickel (Besser et al., 2013; Borgmann et al., 2004; Keithly et al., 2004). Keithly et al. (2004) suggest that more data is required to better assess the relationship between nickel toxicity and water hardness. This was partially addressed in a study by Doig and Liber (2006), which indicates that dissolved organic matter reduced the bioavailability of nickel when concentrations were at lower, sublethal levels, but did not have a significant effect in decreasing acute nickel toxicity in *H. azteca* at higher, lethal levels.

Similarly to the copper study by Besser et al. (2003), Borgmann et al. (2001) conducted an experiment to test whether different sediments affect the toxicity of nickel. The authors state

that the use of body concentrations is better at predicting toxicity than using sediment water or sediment concentrations and reference their previous work (Borgmann et al., 1998; Borgmann et al., 1991; Borgmann & Norwood, 1997b). As a result, they hypothesized that bioaccumulation should be constant if it is a reliable predictor of metal toxicity and that the type of sediment should not affect the results. They tested their hypothesis with chronic nickel exposures using sediments that had different compositions and determined that nickel toxicity and bioavailability were significantly different for each of them. The researchers also noted that the amount of bioavailable nickel was proportional to the metal toxicity, and that the LBC50s from the cone experiments were always significantly higher than the LBC50s of the beaker experiments. Borgmann et al. (2001) indicate that this is due to the fact that the cones provide better overlying water quality in comparison to the beakers, which reduces extraneous stress on the animals.

Interestingly, the authors proposed that nickel bioavailability can be measured using the overlying water concentrations if the sediment toxicity tests are conducted in cones. This is because their results showed that the LC50s and LC25s obtained from their overlying water were similar to values of their 4-week water-only tests. Borgmann et al. (2001) conclude that bioaccumulation is a better quantifier of toxicity than using the concentration of metal in the sediment. Also, overlying water concentrations can be used to determine the metal toxicity if the water quality is unaffected by the sediment and kept constant. This is important because certain metals like copper are regulated in tissues, and therefore the use of body concentrations to determine toxicity can be difficult (Borgmann et al., 2001).

Unlike copper, nickel is not regulated in *H. azteca* as multiple studies have shown that tissue concentrations of the metal elevate with increasing exposure (Doig & Liber, 2006; Keithly et al., 2004; Norwood et al., 2006; Norwood et al., 2007). Norwood et al. (2006) apply a

saturation model for nickel and state that for the model to be used, the bioaccumulation of the element of interest must increase with increased exposure concentrations.

Copper and nickel are important metals that are mined in the regions of Sudbury and Quebec (Couillard et al., 2008; Shuhaimi-Othman, Pascoe, Borgmann, & Norwood, 2006). As a result of these operations, deposits of copper and nickel can be observed in lakes surrounding the mines. Several studies have assessed the contamination levels within these lakes and the bioaccumulation in *H. azteca* as a result of the deposits. Shuhaimi-Othman et al. (2006) determined that nickel concentrations in *H. azteca* were highest in the Sudbury region, and Couillard et al. (2008) assessed the bioaccumulation of various metals in *H. azteca* around contaminated lakes in Northwestern Quebec. In Quebec, laboratory cultures of *H. azteca* were put into cages and then deployed into field sites for 17 days. A bioaccumulation factor was assessed and then linked to the potential chronic lethality. The authors concluded that 12 of 27 metals – two of these being copper and nickel – bioaccumulated in a dose-dependent manner in their cage-deployed amphipods. They also suggest that metals released in mining effluents are readily available for uptake (Couillard et al., 2008).

Objectives

Currently, Weston et al. (2013) are the only researchers that have compared *H. azteca* lineages that inhabit contaminated lakes with laboratory lineages. The authors sampled 7 field sites in California that differed in pyrethroid insecticide sediment concentration and used 3 different laboratory cultures as a comparison group. They determined that *H. azteca* populations from field sites fell into three clades (A, B, or D), and the laboratory cultures were all grouped into another clade (C). It was then concluded that sensitivity to the contaminant (cyfluthrin) was

similar among the populations in clade C and two wild populations that did not have high concentrations of pyrethroid exposure (clade A and B).

These three clades had LC50s ranging from 1.1 ng/L to 4.8 ng/L, which is significantly lower than the LC50s of the amphipods collected from sites with high pyrethroid concentrations. All amphipods collected from sites with high pyrethroid exposure were grouped into clade D, and four of these populations showed great resistance to the contaminant with LC50s that ranged from 92 – 535 ng/L. Weston et al. (2013) cultured the resistant animals in the laboratory and discovered that resistance was not lost in the F1 generation even after 3 months of rearing. The authors then sequenced the highly conserved voltage-gated sodium channel gene and discovered that populations with decreased sensitivity to pyrethroids had mutations in specific domains in the gene.

A notable exception was that one population in clade B (Pleasant Grove Creek - PGC) also showed high resistance. This population is important as it inhabits a site in which a clade D population is also present, but the clade D population was not resistant to pyrethroid. The authors discovered that the clade B population from PGC did not have the resistant mutation in 2010, but the allele occurred at a frequency of 38% when they resampled the same site in 2013. After their toxicity tests, all individuals that survived had the resistant gene. This was not the case for the clade D population from PGC as the resistant gene was not present in any individuals in 2010 and 2013. According to Weston et al. (2013), *Hyalella* have the potential to adapt to a contaminated habitat, and the selection for the adaptation is rapid. However, the authors demonstrate that different clades in the same environment, and even lineages within the same clade, may not acquire the resistant gene.

15

Soucek et al. (2013) also conducted toxicity tests with genetically characterized lineages, but only used laboratory cultures. The amphipods employed in their study came from three separate source populations and were delineated by Major et al. (2013) as the US laboratory clade, Burlington clade, and Clear Pond clade. Prior to conducting 96 hour acute exposures using nitrate and chloride, Soucek et al. (2013) reduced the possibility of acclimation having an effect on the sensitivity observed for each lineage by culturing the animals from all three clades under the same laboratory conditions for two years. The researchers determined that during their nitrate tests where the animals were unfed, the US laboratory clade had an LC50 that was two times higher than the Clear Pond clade, while the Burlington clade had an LC50 that was intermediate between the two. This was not the case for the nitrate tests in which the animals were fed, since the US laboratory clade had a significantly lower LC50 than the other two lineages.

In contrast, Soucek et al. (2013) observed that the US laboratory clade had significantly higher LC50s than the Burlington and Clear Pond clades in chloride tests where animals were both fed and unfed, as well at 48 hour and 96 hour time points. They noted that the Burlington clade had a significantly higher LC50 than the Clear Pond clade in tests where animals were unfed, but not when the amphipods were fed. Interestingly, the researchers mention that the US laboratory and Burlington clades fit Wellborn's (1995) description of a large ecomporh, whereas the Clear Pond clade would be deemed a small ecomorph. Soucek et al. (2013) state that although the presence or absence of food affected the relative sensitivity of nitrate and chloride for the three clades, other physiological factors are also in effect. This is interesting because even though the Clear Pond clade is smaller in body size, the researchers noted that the amphipods in that lineage were the least sensitive to nitrate and more tolerant to chloride than the Burlington clade when fed.

Soucek et al. (2013) state that the geographic ranges of genetically identified *Hyalella* surveyed thus far is lacking, and according to Major et al. (2013) most laboratories are using the US laboratory clade, which is restricted to the south eastern US. These two studies imply that the range of delineated *Hyalella* needs to be expanded upon so that researchers may use a provisional species that is more reflective of its natural habitat, and precaution should be taken when comparing toxicity test data since each laboratory does not employ the same lineage. As of March 2016, the only study involving genetically characterized lineages of *Hyalella* exposed to acute exposures of copper and nickel has been completed by Leung (2014). She addresses the aforementioned implications by employing both the US laboratory and Burlington clade in her 14-day toxicity tests, and assessed if there were any significant differences in their growth and mortality responses when exposed to the two metals.

Based on the work done by Major et al. (2013), Leung (2014) established that the Burlington lineage will be deemed as Clade 1 and the US laboratory lineage as Clade 8. In her experiments, she observed that lineage 1 had a significantly lower lethal nickel concentration that resulted in 50% mortality (LC50) in comparison to lineage 8. However, she noted that there was no significant difference between the two lineages when comparing the lethal nickel concentration at which mortality occurs for 25% of the test population. In contrast, Leung (2014) determined that Clade 8 had a copper LC50 and LC25 that was 2.6 and 2.3 times higher than that of Clade 1. However, she states that despite this difference there may not be any significance as the two clades had overlapping data. In addition to her mortality data, the author observed that there was no significant difference between the two lineages when comparing lethal nickel and copper body concentrations for 50% and 25% of the test animals (LBC50 and LBC25), as well as the effects on growth. However, Leung (2014) mentions that Clade 1 was significantly larger

17

than Clade 8 even though both clades exhibited similar growth inhibition trends, and are both deemed as large-bodied ecomorphs (Wellborn, 1995).

In this current study, the effects of copper and nickel toxicity on Clade 1 and 8 were evaluated at the chronic level by employing the amphipods in 28-day tests. This study is meant to be an expansion on the previous foundation established by Leung (2014), and thus the same endpoints have been examined (mortality, growth, and bioaccumulation). However, since the test durations differ between this study and the work done by Leung (2014), there are certain modifications in the methodology and statistical analyses of the results. The following chapter will describe the experimental design in detail.

Chapter 2 – Materials and Methods

Culture Procedures

Animals belonging to Clade 1 were donated by Dr. Warren Norwood from Environment Canada, while amphipods that are classified as Clade 8 were received from Dr. Bruce Greenberg at the University of Waterloo. Amphipods were then placed in 34L aquarium tanks which contain Standard Artificial Media (SAM) developed by Borgmann (1996), a SeaPora ™ breeder sponge filter, and a net half-filled with small charcoal beads. All aquarium equipment and food were purchased from Big Al's Canada in Kitchener, Ontario. The charcoal beads were used to filter out any dissolved organic compounds (DOC) that could not be trapped by the sponge filter.

In preparation for chronic toxicity tests, culturing methods were adopted from the procedures done by Borgmann et al. (1989). Clade 8 amphipods were randomly selected from the aquarium tanks and transferred into plastic containers. These containers contained 1L of

SAM and a square piece of cotton gauze (5 x 5). The gauze served as a substrate for the animals to cling onto, which helped to reduce stress and improve juvenile survivability. The animals were left at room temperature with fluorescent lights programmed to stay on for 16 hours and turned off for 8 hours. Adult amphipods were separated from the juveniles each week on Mondays – both were counted and placed into new containers with fresh SAM. Adults and juveniles were fed with Tetra-min ® three times a week on Monday, Wednesday, and Friday. The amount of food given to adults was ~5.0 mg of food while juveniles received ~2.5 mg. Clade 1 follows the same procedures with the exception of randomly selecting from a 34L tank since the animals were reared in plastic containers upon arrival.

Lineage Verification

During preliminary chronic toxicity tests, the two lineages demonstrated identical survival and mortality results. A cross-contamination between the two clades was suspect, and lineage verification was required. The lineages within the laboratory were sequenced at the mitochondrial cytochrome c oxidase subunit I (COI) gene to confirm that the animals used in each experiment were in fact Clade 1 and 8. The procedures for DNA extraction and sequencing established by Schwenk (1996) and Witt et al. (2006), are outlined below.

DNA Extraction

A random sample of 10 amphipods were collected from each respective clade's tank and placed into separate plastic cups. The animals were then individually viewed under a dissection microscope so that two to three legs could be removed. In the event that a leg could not be obtained, another appendage such as the antennae or gnathopods was used. The dismembered body parts were ground into fine particles in a microcentrifuge tube containing 50 µL of proteinase-K extraction buffer, and the process was repeated with a new tube for each amphipod.

Once the dissection process was complete, amphipods were placed into a vial containing 95%
ethanol and stored at -20 °C (one vial per clade). The microcentrifuge tubes were then incubated
at 55 °C for ~24h, placed into a heat block and incubated at 97 °C for 12 minutes, and then
stored at -20 °C. Proteins were degraded by the extraction buffer during the first incubation at 55
°C, and thus DNA was released from the ground appendages (Schwenk, 1996).

Polymerase Chain Reaction and Sequencing

The mitochondrial COI gene (680bp) was amplified by using polymerase chain reactions
(PCR) with the primers FolA and FolB (Folmer et al., 1994) for Clade 1, and CO1 Crust DF1
and Crust DR2 for Clade 8 (Leung, 2014). Each PCR reaction had a total volume of 50 µL and
the following components: 2.5 µL of DNA template, 0.2 µM of dNTP mix, 5.0 µL Thermopol
buffer, 0.2 µM of each primer, and 1 unit of Taq DNA polymerase. The PCR conditions were
60s at 94 °C for 6 cycles, 90s at 45 °C, 60s at 72 °C; then 60s at 94 °C for 35 cycles, 90s at
51 °C, 60s at 72 °C; and finally 5 mins at 72 °C. The PCR products were then stained with
ethidium bromide, electrophoresed through a 1% agarose gel, and viewed under a UV light to
determine if there was successful amplification. All detectable PCR products were then
electrophoresed a second time so that the DNA fragments could be excised from the gel upon
reaching the UV visualization step. A Qiaex kit (QIAGEN Inc) was then used to purify the PCR
products in preparation for sequencing (Witt et al., 2006).

An ABI™ 3730 automated sequencer (Applied Biosystems) was used to sequence the
purified PCR products with their respective primers in one direction. The final sequence data
was personally analyzed, aligned, and trimmed with the aid of MEGA6 and previously
characterized sequences from Witt & Hebert (2000), Hyrcyshyn (PhD thesis in preparation), and
Leung (2014). A neighbour-joining (NJ) phylogenetic tree was then constructed in MEGA6

using the Tamura-Nei method of estimating nucleotide substitutions between sequences (Tamura & Nei, 1993), and a bootstrapping with 1000 replicates.

All 10 amphipods retrieved from the Clade 1 tank did not amplify with the FolA and FolB primers. However, 5 of the samples amplified using the CO1 Crust DF1 and Crust DR2 primers. These 5 samples and one sample from Lac Berthemet were sequenced, and are depicted in Figure 2-1. Due to the confirmation of cross-contamination, all tanks and culture containers within the lab were purged. Clade 8 cultures were restarted with amphipods kept in a separate laboratory. These cultures were maintained in the main laboratory where chronic toxicity tests were conducted, but kept in a separate area that would not allow for cross-contamination. Clade 1 tanks and cultures were restarted by receiving batches of amphipods from Dr. Warren Norwood. These amphipods have been kept in isolation and have never been in contact with Clade 8 animals (Warren Norwood personal communication). Therefore, these new animals were not verified as sequencing has already been conducted by Leung (2014) and Major et al. (2013).
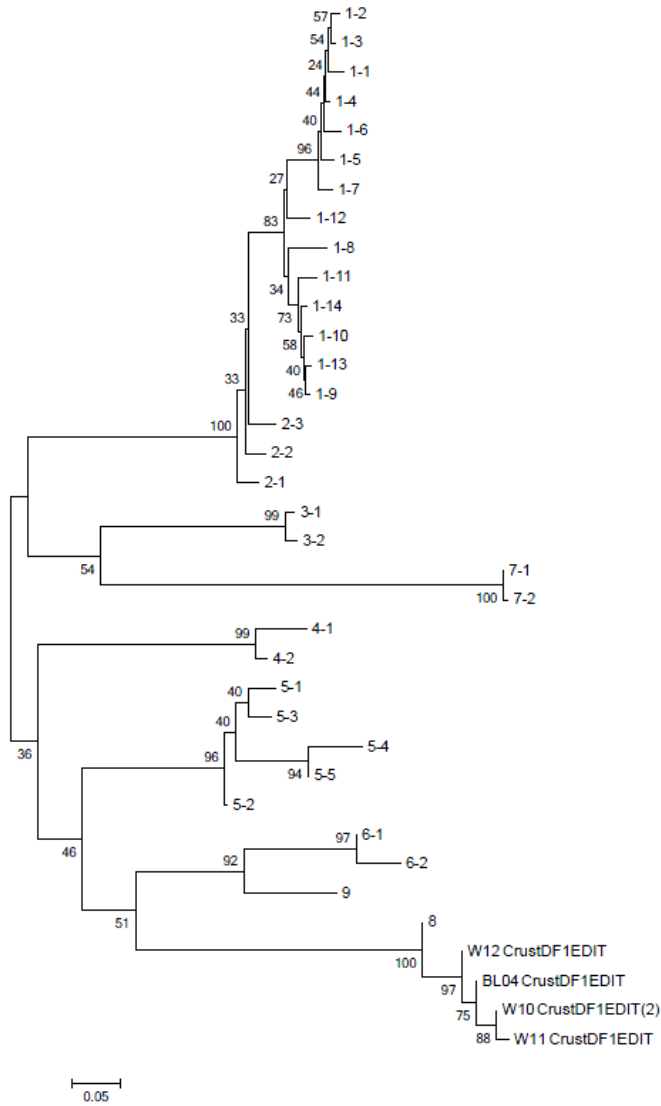
Figure 2-1: Neighbour-joining tree constructed with MEGA6 depicting the estimated phylogeny for *Hyalella* samples. Clades are indicated by the first number at the end of a branch (e.g. 1-2 is a sample belonging to Clade 1), and samples from the Canadian Centre for Inland Waters (W), and Lac Berthemet (BL) have been grouped under Clade 8. The bootstrap method was run with 1000 replicates, and values are indicated at the nodes.

Chronic Toxicity Tests

The chronic toxicity tests closely followed the procedure outlined by Norwood et al. (2006), but with some modifications. The tests were conducted over a 4 week period and consisted of weekly renewals of media and toxicants. The SAM used for culturing was prepared in a 200 litre vat and used for the entirety of the tests. This was done so that all tests would be conducted in the same water. However, due to preliminary tests to determine a suitable concentration series and a few tests that failed to meet control standards, a second batch of 200 litres of SAM was made. A concentration series was generated for both nickel and copper, and each had 8 exponentially increasing concentrations (Table 2-1). The copper and nickel concentration series used in this study were generated by taking the values from Borgmann et al. (1993) and Norwood et al. (2013), and computing a growth equation in Microsoft Excel 2013.

Table 2-1: Nominal metal concentrations and the number of replicates used in the chronic copper and nickel toxicity tests.

Metal Concentration	Number of Replicates	Nominal Concentrations (nmol/L)	
		Copper	Nickel
Control	3	0	0
Concentration 1	2	88.1	201.6
Concentration 2	2	157	351.4
Concentration 3	2	283	576
Concentration 4	2	504	887
Concentration 5	2	881	1324.8
Concentration 6	2	1574	2044.8
Concentration 7	2	2808	3168
Concentration 8	2	4978	4896

Clades 1 and 8 were tested three times with each metal for a total of 12 experiments. Each test consisted of three replicates for the control and two replicates for each metal concentration. However, there was one exception where a Clade 8 nickel test only had two replicates for the control. This test was conducted early on before a third control was added to subsequent experiments for robustness. Nineteen plastic cups were filled with 250 mL of SAM

and spiked with increasing copper or nickel concentrations from their respective stock solutions (Table 2-1). Nickel and copper chloride salts were dissolved in de-ionized water to generate these stock solutions. The metals were allowed a minimum of 3 hours to equilibrate in the containers, but were consistently prepared the night before a test.

Once the containers had equilibrated, ammonia, pH, conductivity, and dissolved oxygen (DO) concentrations were measured. The mean and 95% confidence intervals (95% CI) for these measurements were 0.31 mg/L (95% CI 0.27 – 0.35), 7.08 (95% CI 7.01 – 7.16), 365 µs/cm (95% CI 356 – 375), and 13.66 mg/L (95% CI 13.29 – 14.02), respectively. The toxic effects of ammonia, pH, and DO have been studied in the past, and thus required routine measurements to ensure mortality was due to the metals (Ankley et al., 1995; Borgmann & Borgmann, 1996; Borgmann, 1994; Irving et al., 2004). In addition to these measurements, 1.5 mL water samples were taken and preserved with 10 µL of nitric acid for metal analyses. Two samples were taken – one was filtered with a 0.45 µm Millipore membrane filter, and the other was unfiltered.

Water quality measurements and samples were taken at the beginning and end of each renewal period, and only in the first replicate of each metal concentration. However, DO and ammonia were only measured in the first replicate of the control during the start of a test and for refreshed containers on renewal days. This was because the refreshed containers did not have the amphipods transferred into them yet. Since the DO and ammonia measurements are taken before the amphipods are transferred, all containers should have the same readings, and thus, only the first replicate of the control is measured.

Since culturing was conducted on Mondays, a toxicity test could be run on Tuesday or Wednesday with the juveniles being 1 – 9 days old. Two tests were conducted simultaneously,

but were initiated a week apart to ensure that each test used a different cohort of juveniles. Therefore, a random sample of eighteen 1 – 8 day-old *Hyalella* and a 2.5 x 2.5 cm piece of gauze were added to each test container on a Tuesday to initiate the first test. The same procedure was done on Wednesday of the subsequent week for the second test with the exception that the juveniles were then 2 – 9 days old. Certain weeks had higher juvenile production, and thus some tests had a random sample of twenty juveniles added to each container to increase statistical sensitivity. However, one test failed to meet the juvenile requirement and was carried out with a random sample of 16 juveniles per container. The *Hyalella* were incubated at a temperature of ~25 °C and had a photoperiod that consisted of 16 hours of light and 8 hours of no light. Amphipods were fed twice for weeks 1, 2, and 4, and three times during week 3. The amount of TetraMin ® is ~2.5 mg for weeks 1, 2, and 3 and ~5.0 mg for week 4. Tests that were conducted on Tuesdays would be fed on Tuesday and Friday three times, and fed on Tuesday, Friday, and Sunday for the third week. The same spacing between feeding days was used for the Wednesday tests and thus feeding occurred on Wednesday and Saturday three times, and Wednesday, Saturday, and Monday for the third week of the experiment.

On the sixth day of the test, nineteen experimental containers were spiked with the appropriate metal concentrations and left to equilibrate overnight – these are the renewal containers. The next day, approximately 27 mL of solution from the first replicate of each experimental concentration for both the old and new containers, was decanted into a small polystyrene cup for water quality measurements and water samples. This step required extreme caution when working with the containers from the previous week to ensure that no juveniles were accidentally poured into the cups. Starting with the controls and working up the concentration series, the remaining contents of each container from the previous week were

transferred into a clean glass bowl. The old container was rinsed with deionized water to ensure any surviving juveniles do not accidentally get thrown out. These individuals were counted with an eyeglass dropper, placed into the new test containers with a fresh piece of gauze, and then returned to the incubator for another 7 days. The old containers are then rinsed with deionized water four more times to remove any waste matter that is stuck to the sides and hung to dry for the next renewal period.

Preliminary tests used a protocol where the old test containers were washed and a brand new set of test containers were used for each renewal. Some weeks did not have proper dish washing due to multiple technicians, and thus resulted in a few failed tests that had lower than 80% control survival. Consequently, all test containers were thoroughly bleached, acid washed, and the new protocol involving the reuse of experimental vessels was tested. After completing a successful toxicity test by rinsing each container four times with deionized water and reusing them for each renewal period, subsequent experiments employed this new protocol and improved consistency among all exposure concentrations and control survival were observed.

On the 28th day of exposure, final survival was recorded and then all of the live amphipods were taken from the container, rinsed and placed into a small plastic cup with ~60 mL of 50 µM ethylenediamine tetra-acetic acid (EDTA) in deionized City of Waterloo tap water, fresh food, and a small piece of cotton gauze. The animals were left in this media for 24 hours to allow for gut clearance (Neumann et al., 1999) before their wet weight was taken. As described by Norwood et al. (2006), the EDTA served to bind any metals that are loosely adsorbed to the amphipods, and also prevent any metals from being reabsorbed that have been released from the animal's gut. The procedure for measuring wet weights involved removing the amphipods from the EDTA containers and setting them onto folded sheets of Kimwipe® to remove any excess

water. The animals were then measured on a Mettler Toledo microbalance that is accurate to 0.001 mg, and placed into a labelled cryovial with the corresponding concentration and replicate number. The amphipods were then dried at 60 °C for 72 hours and its dry weight was then assessed.

Graphite Furnace Atomic Absorption Spectrometry

Water samples were measured at the Canadian Centre for Inlet Waters in Burlington, Ontario, using a Thermo Scientific iCE 3000 Series Atomic Absorption Spectrometer and SOLAAR Data Station V11.03. A nickel and copper method was developed and optimized prior to analyzing samples. The nickel program used Zeeman background correction and a working volume of 20 µg/L. No modifier was necessary for the analyses. Four hundred µL of each sample were placed into 500 µL plastic cups (500 µL of sample if analyzing overnight). Two standards and a blank were used to generate a calibration curve (0, 25 and 50 µg/L). A high purity nickel standard (Lot # 921108) of 1000 µg/mL was diluted to 100 µg/L, which was then modified to a working standard of 50 µg/L. Concentrations 7 and 8 were diluted by a factor of 5 and 10 respectively, whereas all other concentrations were altered by the furnace's "intelligent dilution" system. While analyzing in the morning, samples were loaded in batches of 10-15 to minimize errors due to evaporation. All overnight analyses had the samples loaded all at once. Blanks were 1% HNO_3 and 99% nanopure water.

The same parameters and procedures for the copper program were similar – Zeeman background correction and no modifier was used. However, the working volume was set to 10 µg/L and a 30 µg/L (high purity copper standard, 1000 µg/mL, Lot # 919635) working standard was made. Since the working standard was much lower than the nickel program, concentrations

28

4 and 5 were diluted by a factor of 2, concentrations 6 and 7 were diluted by a factor of 5, and concentration 8 was diluted by a factor of 10.

Quality checks and machine blanks were run every 5 samples. Machine blanks were run to ensure that no carryover occurred while analyzing each sample, and quality checks were used to correct for drift. The measured metal concentrations by the GFAAS were first corrected for any manual dilutions, and then drift was accounted for by using the appropriate quality check (QC) standard concentration (QC 25 for nickel = 25 µg/L; QC 15 for copper = 15 µg/L). Each run had a set of quality checks that occurred at specific times. Therefore, recovery rates for each run (measured amount/ QC concentration) were plotted against time and a trend line was applied. The equation of this line was then taken and applied to the dilution corrected data to give a measured metal concentration that had both dilution and drift correction.

All experiments had eight sets of filtered and unfiltered water samples collected that were labeled accordingly, and each of these sets had two respective sample blanks labelled Blank1 and Blank2. Sample names started with a "P" followed by the clade number, metal, experiment letter, fraction out of 8, and the letter F or UF to denote filtered and unfiltered (e.g. P1-Ni-A 1/8F – P1-Ni-A-8/8F, and P1-Ni-A-1/8UF – P1-Ni-A-8/8UF). The eight sets account for the water samples taken at the beginning and end of each week, where the odd numbers represent the water samples taken before amphipods are put into the test containers, and the even numbers represent the water samples taken at the end of each week. Therefore, P1-Ni-A-1/8F would be the filtered samples taken at the beginning of week 1 for Clade 1 Experiment A, and has two filtered blank samples that are separate from P1-Ni-A-2/8F. All filtered and unfiltered blanks from each set were pooled together to determine the mean filtered and unfiltered blank values. The filtered blank mean was subtracted from each filtered sample, and the same was done to the unfiltered samples

with the unfiltered blank mean. These subtracted values are the final measured concentration with dilution, drift, and blank correction taken into account. The aforementioned procedures were conducted for both copper and nickel concentrations obtained from the GFAAS. However, certain weeks had filtered and unfiltered control concentrations that were abnormally high for copper (e.g. 2-5 µg/L). Since both the filtered and unfiltered blanks were also approximately the same concentration, it is likely due to a contamination in the vials, and thus the mean of all filtered or unfiltered blanks could not be used to correct those samples. Instead, the respective blanks for that set of samples was used for correction.

During the third nickel test for Clade 8, filters were not available after the first week and the new batch had not arrived. Thus, all samples collected henceforth were unfiltered. This issue was compensated by plotting all filtered water samples with their accompanying unfiltered water samples and fitting a trend line to the data. The equation obtained represented the amount of sample being filtered out and was applied to all the unfiltered samples of the third nickel test to generate filtered values. A similar procedure was done to determine how much copper was being filtered out. This was done so that any outliers that were indicative of filter contamination could be corrected for. The correction involved multiplying the unfiltered sample value by the equation of how much metal was filtered out. The arithmetic mean was calculated for each concentration and these averages were used as the final measured concentrations. Each set of filtered and unfiltered values for each week were converted from µg/L to nM and plotted against the nominal concentrations. The average filtered concentrations were then compared to unfiltered ones.

Tissue Digest

The tissue digests were conducted following the procedures outlined by Norwood et al. (2006) and Borgmann et al. (1991) that were modified from the methodology originally

described by Stephenson and Mackie (1988). Dried amphipods from the toxicity tests were placed onto a small aluminum tray and weighed using a microbalance. The animals were removed from the cryovials by gently picking them up with a small paintbrush. Amphipods were added to the aluminum tray until a weight of approximately 1.200 mg was measured, or until there were no more animals available. A new cryovial was labelled and passed through a static removal device prior to adding the weighed animals. The aluminum tray containing the amphipods was also passed through the static removal device, and the transfer of the animals into the cryovial was done in close proximity to it so that the bodies would not stick to the sides of the walls. All of the animals that were transferred needed to be at the bottom of the cryovial, so gentle taps and frequent passes through the static removal device were done if necessary.

After all dry weights were obtained, cryovials that had a weight of 0.750 – 1.499 mg received 25 µL of 70% nitric acid (Fisher Scientific LOT 136079), whereas cryovials with a weight of 0.749 mg and less received 13 µL of nitric acid. The amphipod tissues were digested for 6 days, and on the 7th day 30-32% hydrogen peroxide was added (J.T.Baker CAS NO: 7722-84-1). Cryovials that received 25 µL and 13 µL of acid had 20 µL and 10 µL of hydrogen peroxide added, respectively. The peroxide was given 24 hours to neutralize the acid, and then MilliQ water was added. The cryovials were then mixed and had final volumes of either 1.0 mL (0.750 – 1.499 mg dry weight), or 0.5 mL (0 – 0.749 mg dry weight).

A standard reference material was digested in the same manner. This material is made from lobster heptatopancreas and is called TORT-2. The TORT is stored as a very fine powder, and as such makes it difficult to weigh for analysis. A procedure has been developed at the Canadian Centre for Inland Waters that will be outlined as follows. A 15 mL Falcon tube is pre-cleaned and used to weight out 40 mg of TORT. Ten mL of nano-pure water was added to the

tube to turn the powder into a slurry. If the mixture was too thick, more water was added and then the tube is vortexed. The mixture was then transferred to pre-cleaned cryovials in volumes of 1 mL unless additional water was added, whereby the amount transferred would correspond to the increased volume (e.g. 1.5 mL if total volume was 15 mL). After each transfer, it is suggested to keep mixing with the vortex to ensure that the material is kept in suspension. Once all transferring was completed, the vials are loosely capped and placed into a 60 °C drying oven for 3 – 7 days. The vials now contain a chunk of TORT that can be broken off for weighing with the microbalance. Six TORT-2 cryovials were made for analysis and have a weight range from 1.024 mg to 1.359 mg.

Data Analysis

Control Mortality

Mean mortality rates for Clade 1 and 8 controls were compared using a two-sample t-Test with SAS® Studio Release 3.1. The mean values from each control replicate for both copper and nickel experiments were fourth root transformed to normalize the data and equalize variances prior to running the comparison. There was no observed significant difference when comparing control mortality between Clade 1 and 8 [t (30) = 0.80, p = 0.433]. The mean mortality rates computed after fourth root transformation for Clades 1 and 8 were 0.3998 (95% CI = 0.3476 – 0.4519) and 0.3730 (95% CI = 0.3274 – 0.4186), respectively.

Control Dry Weights

Clade 1 and 8 control dry weights were compared using a two-sample t-Test with SAS® Studio Release 3.1. The dry weights were left untransformed since the statistical program determined they were normally distributed for both clades (Kolmogorov-Smirnov test p value > 0.15). The two-sample t-Test determined that Clade 8 had significantly larger control dry

weights than Clade 1 [t (30) = -4.75, p < 0.0001] with a mean of 0.2508 (95% CI = 0.2207 – 0.2809) mg/individual, whereas that for Clade 1 was 0.1731 (95% CI = 0.1530 – 0.1933) mg/individual.

Filtered Vs. Unfiltered

Filtered and unfiltered measured metal concentrations were tested for normality using SAS® Studio Release 3.1. The measured concentrations differed significantly from a Normal distribution when assessed using the Kolmogorov-Smirnov test for Normality (D = 0.2291, p < 0.0100). Since the data did not fit a Normal distribution, the measured values were log transformed using the log transform command in Systat 10. Both nominal and measured metal concentrations were log transformed prior to conducting an ANOVA in Systat 10. The ANOVA command in Systat 10 was compared with results obtained from a two-way ANOVA computed with IBM SPSS and the results were identical. Non-log transformed metal concentrations resulted in a significant reduction in measured metal concentrations when comparing filtered and unfiltered data for copper [F (8, 72) = 3.109, p= 0.0045], but not for nickel [F (8, 90) = 0.693, p= 0.697]. However, when the data was log transformed, both copper [F (7, 64) = 1.445, p= 0.203] and nickel [F (7, 80) = 0.114, p= 0.997] did not show a significant reduction in measured metal concentrations when comparing filtered and unfiltered data.
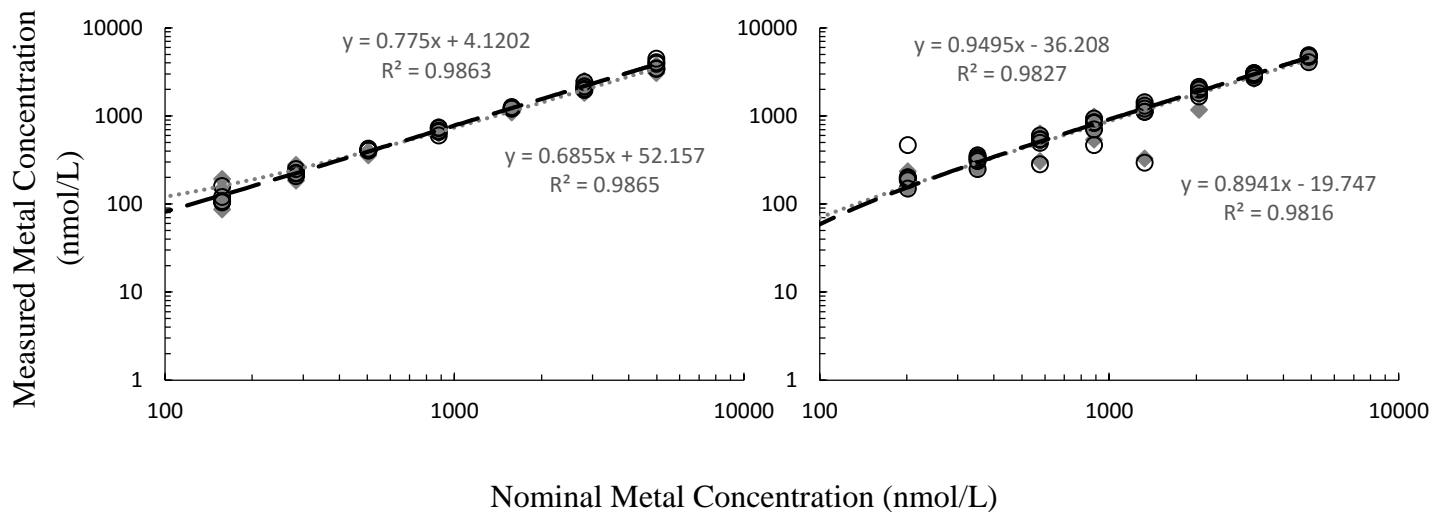
Figure 2-2: Comparison between untransformed nominal and measured metal concentrations for the 28 day copper and nickel (left to right, respectively) toxicity tests using juvenile *Hyalella*. Filtered water samples denoted by ♦ (copper: n = 45, nickel: n = 54), and unfiltered samples denoted by ○ (copper: n = 45, nickel: n = 54).

Although there was no significant reduction in metals due to the filters, the filtered concentrations were slightly lower suggesting that some of the metals could have been bound to organic matter (Norwood et al., 2006). As a consequence, the filtered metal concentrations were used to estimate parameters such as LC50, LBC50, and bioaccumulation. In addition, these water samples were used to maintain consistency between previous work, since metal bioavailability may be affected by organic matter. Thus, the filtered samples provide a more accurate representation of the bioavailable metal to the animals than the unfiltered water.

Mortality

Copper and nickel survival data were duplicated so that there is a set for modeling and another that is the raw data (Leung, 2014). Whenever a replicate reached 0 survivors, that first instance would have its value changed from 0 to 0.25 (Borgmann et al. 1998). All subsequent weeks for that replicate would then have the original 0 survivors. If a replicate had 100% survival in the final week of the test, the value was changed to having 0.5 animals die (e.g. Started with 20 amphipods and there were 20 remaining in the final week of the test, therefore number of survivors is changed to 19.5). When there are survivors present in one replicate of a particular concentration, but none in the other, the replicate with 0 animals was changed to 0.25 (Warren Norwood personal communication). This is slightly different than Leung (2014), where she assigned 0.5 to the replicate instead.

The above modifications to survival were adopted from Borgmann et al. (1998), but a change in the protocol was implemented with the 100% mortality. In the original protocol, the researchers assigned 0.5 animal surviving out of 40 and calculated a single mortality rate. However for this study, the protocol was changed to two replicates of 0.25 animal surviving out of a total of 18 or 20, and two mortality rates are computed. Generating two mortality rates was done so that both replicates are accounted for, and more statistical power is available. The survival data with and without modifications were then used to calculate mortality rates as described by Borgmann et al. (1998).

Table 3-2: Example of survival modifications used in estimating unknown parameters. An example of a concentration with no survivors in one replicate, but animals in the other is shown in concentration 5. The protocol for correcting 100% mortality is shown in concentration 7 and 8 (Week 2), and concentration 6 (Week 3).

# in Concentration Series	Rep	Survival Model				Survival Real			
		Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
5	1	18	10	6	4	18	10	6	4
	2	17	11	2	0.25	17	11	2	0
6	1	10	5	0.25	0	10	5	0	0
	2	9	4	0.25	0	9	4	0	0
7	1	3	0.25	0	0	3	0	0	0
	2	3	0.25	0	0	3	0	0	0
8	1	1	0.25	0	0	1	0	0	0
	2	1	0.25	0	0	1	0	0	0

Borgmann et al. (1998) computed mortality rates (m) by regressing survival against time. The researchers used regressions for all four weeks rather than just the final one because they state that a larger number of partial effects can be obtained. Also, they justify this action because it is more accurate in determining the LC25 since it incorporates the surviving animals that are present in weeks 1, 2, or 3. This allows for a more precise determination of the slope of the toxicity curve because some concentrations will have 100% mortality in the fourth week, and thus computation of m would not be possible if it is the only one used. Therefore, the current study also uses all four weeks when computing mortality rates. The mortality rate used to fit the model was computed using Equation 2-1,

Equation 2-1: Conversion of survival to Mortality rate

$$m = \frac{-\ln(\frac{N}{N_0})}{t}$$

where m is the mortality rate, N is the number of survivors, N_0 is the initial number of test

organisms, and t is the time in weeks.

Mortality rates in this study were fourth root transformed before fitted to the saturation

models with Systat 10. This was done instead of log or square root transformation because

Borgmann et al. (2004) and Norwood et al. (2007) have determined that using the fourth root

protocol produces more uniform variances. The data was then modeled with the measured metal

concentrations in solution using nonlinear regressions in Systat 10 to estimate the LC50 and

LC25 (lethal concentration at which 50% and 25% mortality occur, respectively), and the other

parameters (constants, coefficients, exponents) in Equation 2-2.

Equation 2-2: Mortality saturation model described by Borgmann et al. (2004) to estimate LC50.

$$m = m' + \left(\frac{\ln(2)}{t}\right) \times \left[C_W \frac{\left(LC50^{-1} + K_W''^{-1}\right)}{\left(1 + C_W K_W''^{-1}\right)}\right]^{nw}$$

where m is the overall mortality rate, m' is the control mortality rate, t is the exposure time in

weeks, C_W is the concentration of metal in the water, nw is a constant, and finally K_W'' is the

water concentration at which half of the maximum metal-induced mortality has been reached

(Borgmann et al., 2004; Norwood et al., 2007). The LC25 equation replace $\ln(2)$ with $\ln(4/3)$

respectively. The funpar command in Systat was used to generate Wald calculation 95%

confidence intervals (CI) for all of these parameters.

Total-body concentration in relation to mortality was also examined in this study, and estimates similar to those obtained for metal concentrations in solution were computed for the LBC50 and LBC25 (lethal body concentration at which 50% and 25% inhibitory responses occur, respectively), coefficients, constants, and exponents. These parameters were estimated using a similar equation given by,

Equation 2-3: Mortality saturation model described by Borgmann et al. (2004) to estimate LBC50.

$$m = m' + \left(\frac{\ln(2)}{t}\right) \times \left[C_{TBX} \frac{\left(LBC50_x{}^{-1} + K_{TBX}^{"-1}\right)}{\left(1 + C_{TBX}K_W^{"-1}\right)}\right]^{nb}$$

where all the parameters described here are the same as those in equation 2-2, except C_{TBX} is the concentration of metal within the body that has been background corrected, and K_{TBX} is the body concentration at which half of the maximum metal-induced mortality has been reached (Norwood et al., 2007). The LBC25 equation replace ln(2) with ln(4/3) respectively.

Growth

The relationship between dry weights and metal concentrations present in the test water for this study were examined by fitting the data to Equation 2-4 – the general growth model described by Borgmann et al. (1998). The dry weights for all surviving amphipods in a replicate were measured and divided by the number of animals weighed. Amphipods used for digests were weighed separately, and thus the IBC25s were estimated with those dry weights instead. Only whole bodies were used for all dry weights (total and digests) – amphipods that had split in half were only used if the break occurred while placing them on the metal tray. These dry weight per animal values were square root transformed to normalize and equalize the variances instead of

using fourth root or log transformations since Norwood et al. (2007) have determined that more uniform variances were obtained using this method. Nonlinear regressions in Systat 10 were conducted using the transformed data and Equation 2-4 described as follows,

Equation 2-4: General growth model described by Borgmann et al. (1998).

$$W = W'(1 + aC^n)^{-1}$$

where W is the final dry weight at the end of the test (in this case 4 weeks), W' is the dry weight measured for the control animals, C is the concentration of metal present in the water or background-corrected body concentration, and a and n are constants. Equation 2-4 was modified to Equation 2-5 to estimate the IC25 and IBC25, which are the water and total-body metal concentrations at which final body size is reduced by 25%, respectively (Norwood et al., 2007).

Equation 2-5: Modification to Equation 2-4 to estimate IC25 and IBC25.

$$IC25 \ or \ IBC25 = (3a)^{\frac{-1}{n}}$$

Chapter 3 – Copper

Introduction

Copper is a naturally occurring metal that has been used by mankind for over 10,000 years. Production of this metal comes from a mix of sulfide minerals ($CuFeS_2$, Cu_2S, and Cu_5FeS_4) and oxidized copper ores. The sulfide minerals constitute 90% of the total primary copper produced, and these minerals only contain approximately 0.5 to 2% elemental copper. Currently, copper is used in modern society in its purest form, or mixed with other metals to form alloys such as copper-zinc and copper-nickel compounds. Due to their strong resistance to corrosion and fatigue, great electrical and thermal conductivity, and ease of fabrication, copper and its alloys have been extensively used in commercial products. These include electrical cables, water pipes and valves, heating systems, jewellery, and even in dental products (Davis & Committee, 2001; Gaetke & Chow, 2003).

Although copper is widely produced for commercial needs, it is an essential metal that is required by many living organisms. Many biological systems use copper as a cofactor for certain proteins such as cytochrome *c* oxidase and Cu-Zn superoxide dismutase (CuZnSOD). These proteins are often involved in electron transfer and redox reactions, but are also capable of acting as chaperones and deposit sites (Cohu & Pilon, 2010; Gaetke & Chow, 2003; Mander & Liu, 2010). Since copper has strong redox capabilities, its free ion state can produce hydroxyl radicals and other reactive oxygen species (ROS) that are toxic and damaging to an organism's cells. As such, many metallo-chaperones and copper transporters have evolved in organisms to shuttle the metal to proteins and enzymes that require it as a substrate or electron receptor.

Copper proteins have been determined to be present in archaea, bacteria, and eukarya, and occur in two oxidation states – Cu(I) and Cu(II) (Cohu & Pilon, 2010; Mander & Liu, 2010).

In humans and other mammals, the liver is the main site at which copper is deposited and where chronic toxicity typically occurs (Gaetke & Chow, 2003; Grosell & Wood, 2002). Many studies have examined the oxidative effects of copper overload on animals, as well as the effects of being deficient in the metal. These studies are reviewed by Gaetke and Chow (2003), and the common result is oxidative damage. Lipid peroxidation occurs when there is a reaction between oxygen and fatty acid radicals, and is a well-known result of elevated copper levels. In a study with rats that were overloaded with copper, increased lipid peroxidation products were observed along with other factors that contributed to oxidative damage (Sokol et al., 1990). However, increased lipid peroxidation was also observed in the erythrocytes of copper deficient chickens, where the researchers determined that the animals had decreased activity in certain antioxidant enzymes (Bozkaya et al., 2001).

In contrast, Grosell and Wood (2002) state that the copper mechanisms for fish and lower invertebrate metabolism have not been as well established compared to mammal studies. They mention that like mammals, the liver is important in regulating copper levels, but the gills of fish also have a significant role in maintaining homeostasis of the metal. In their study, Grosell and Wood (2002) demonstrated that juvenile rainbow trout exposed to radioactively labelled ^{64}Cu isotopes had most of the metal uptake occur at the gills (30-70% of copper accumulation occurred at the gills in comparison to the whole-body).

Grosell and Wood (2002) also observed that increasing ambient sodium levels had the ability to reduce the amount of copper uptake at the gills substantially. This was highlighted in a

recent study by Ransberry et al. (2015), where the researchers sought to investigate whether increasing salinity would protect adult killifish (*F. heteroclitus*) from copper-induced oxidative stress. They tested this by acclimating wild saltwater killifish to a 0 ppt freshwater tank, or one with 35 ppt saltwater. The killifish were then placed into experimental tanks with one of the two aforementioned salinities, and Ransberry et al. (2015) observed that the saltwater acclimated animals had a 40% copper reduction in the liver when compared to their freshwater counterparts. They also noted that the gill copper load in the freshwater killifish was significantly higher than the saltwater condition by about 4 fold. However, the researchers conclude that the protection from oxidative stress by the lowered copper accumulation in the gills and liver is not substantial, and the only marked difference to note was the lower protein carbonyl content present in saltwater killifish intestines when compared to their freshwater counterparts.

Invertebrates have also been used to assess the toxicity of copper and two recent studies examined exposure through ingestion. Hook et al. (2014) employed the amphipod *Melita plumulosa* to investigate the effects of different copper exposure routes on gene expression. Their study involved exposing the amphipods to copper-contaminated silty sediment, silty sand, and water with added sand that could not be ingested. Hook et al. (2014) determined that there were statistically significant changes in transcript abundance for all of their copper exposures. However, they noted that the transcriptomic profile for copper exposure through ingestion was different than that of dissolved copper exposure. The researchers suggest that due to this difference, the toxic effects of copper depend on the route of exposure. For example, Hook et al. (2014) state that exposure to both dissolved and particulate copper resulted in a change in abundance of transcripts that affect the activities of hydrolase, G protein-coupled receptors, and amino acid kinases, but this was not observed when assessing only dissolved metal.

Zubrod et al. (2015) also examined the effects of diet-related copper toxicity compared to the effects of waterborne metal exposure, but used the amphipod shredder *Gammarus fossarum*. The researchers conducted a study to determine whether fungal biomass would increase on leaves due to copper exposure, and if this result would have a positive effect on the physiology of the test invertebrate. In order to test this, they had four treatments which were a control where the gammarids were not exposed to copper nor were the leaves, gammarids exposed to copper but not the leaves and vice versa, and both the leaves and gammarids exposed to copper.

In their study, Zubrod et al. (2015) discovered that copper exposed leaves did have a significantly increased fungal biomass, but it did not have a positive effect on the gammarids. Instead, they noted that although the animals preferred the leaves exposed to copper and had lower consumption rates, the test organisms also had a significant reduction in lipid content and growth. However, the researchers observed that the animals exposed to copper with clean leaves did not have any physiological impairments despite having significantly more copper content. Zubrod et al. (2015) suggest that the difference in exposure routes could affect the toxic mode of action and relate their observations to the transcript expression differences between waterborne and dietary uptake determined by Hook et al. (2014). Interestingly, they also note that the combination of exposed animals and copper treated leaves did not result in an additive body burden, suggesting that the two pathways are independent of each other.

Bioaccumulation

Borgmann and Norwood (1995a) state that using bioaccumulation data rather than water concentrations to predict toxicity should be easier, and previous literature supports this claim that body concentrations are useful when assessing metals and organic contaminants (Landrum et al., 1992; McCarty & Mackay, 1993). However, there is an issue when evaluating copper since it is

an essential metal, and therefore can be regulated and maintained at steady levels within the animal's tissues. *Hyalella* are capable of regulating copper in chronic exposures, and thus the researchers sought to determine whether or not the control concentrations of copper and zinc present in *Hyalella* is representative of the minimum physiological requirement. They tested this hypothesis by exposing their laboratory cultures to ethylenediamine tetra-acetic acid (EDTA), and also wished to determine whether this complexing agent was capable of preventing the accumulation of copper and zinc, as well as generating metal deficiencies for the animals at toxic chronic EDTA levels.

Borgmann and Norwood (1995a) determined that the control *Hyalella* used in the experiments were not contaminated with metals by treating a set of amphipods to the same conditions as the control, but exposed to 1 µM of EDTA. They observed that the EDTA exposed *Hyalella* had slightly lower concentrations of copper and zinc, but it was not statistically significant and thus concluded that the background concentrations of the two metals are close to 1 µmol/g dry weight. In their uptake prevention tests, Borgmann and Norwood (1995a) determined that 10 µM EDTA could prevent the uptake of zinc when 4.6 µM of the metal was added. However, concentrations of EDTA up to 560 µM could not completely prevent the uptake of copper, although increasing the amount of complexing agent did reduce how much metal entered the tissues. In addition to this, copper and zinc concentrations in the control amphipods were not reduced when exposed to increasing EDTA levels, and this further suggests that the control *Hyalella* used in the experiment represented true minimum physiological requirement concentrations.

Interestingly, Borgmann and Norwood (1995a) observed that the chronic toxicity induced by high EDTA concentrations were partially reversed when zinc was added to the medium.

However, they were unable to demonstrate the same capabilities for copper additions, and determined that the toxicity is due to a zinc deficiency. The researchers conclude that since excess copper has a much lower excretion rate than zinc in *Hyalella*, and that the prevention of copper uptake by EDTA was not as substantial as with zinc, it appears that a highly efficient mechanism to acquire and maintain copper has evolved in this amphipod. However, the researchers do note that *Hyalella* can maintain constant body concentrations of copper and zinc when exposed to varying concentrations of the two metals in the presence of EDTA. Also, they mention that *Hyalella* cannot prevent an increase in body concentrations when exposed to water that does not have EDTA, and has high levels of copper and zinc.

The aforementioned study is of importance because Borgmann and Norwood (1997b) observed that background concentrations of copper and zinc did not differ when comparing spiked sediment tests with waterborne exposure experiments. In their 1997 study, the researchers aimed to expand current data on toxicity and accumulation for copper and zinc. They note that body concentration and toxicity relationships could be affected by waterborne or contaminated sediment exposures, and accurate data on these interactions are required for using body concentration data to predict toxic effects.

Borgmann and Norwood (1997b) state that body concentrations of copper could not predict chronic 4-week toxicity, but could for short term 1-week exposures. This is because the LBC50 and LBC25 for the 1-week exposures represented body concentrations that were positively correlated with copper in the sediments. In contrast, the LBC50 for the 4-week exposures represented body concentrations that were not dependent on copper in the sediment. Borgmann and Norwood (1997b) noted that the 4-week LBC25 for copper in sediment was lower than the LBC25 for waterborne exposures, even though the mortalities were similar. This

was not the case for their zinc results, as the researchers observed concentrations that were higher than background levels, and thus could infer chronic toxicity.

Borgmann (1998) states that acute metal toxicity is mainly attributed with damage to the gills of fish, and mentions that inhibition of sodium or calcium uptake as one example. The researcher sought to expand on the models presented by Borgmann et al. (1993) and Borgmann and Norwood (1995a, 1995b) because the equations developed described metal uptake kinetics. However, no model had been formulated to explain their results. Thus, Borgmann (1998) developed a mechanistic equation for copper to address the aforementioned issue, and a whole animal uptake model to estimate the binding affinity of the metal for *Hyalella*. However, he mentions that the latter model only accounts for uptake of copper from the water, and accumulation happens internally rather than at the animal's surface. This is an important point because the toxicity and accumulation of copper from water exposure differs from that of contaminated sediments, since the latter potentially has higher uptake through the gut.

According to Borgmann (1998), body size does not affect copper concentrations because the binding is internal rather than at the animal's surface, and is not influenced by a surface diffusion rate. He also mentions that increasing the metal concentration in the water results in a maximum steady-state metal concentration, and this steady-state is approached at a much slower rate when considering depuration in clean water in comparison to uptake of metal from spiked water. Borgmann (1998) explains that his proposed model can provide insight into the importance of an internal ligand that binds non-essential copper, called "X". He states that in previous work, *Hyalella* is capable of maintaining constant body concentrations of copper and zinc even in the presence of a strong complexing agent like EDTA, which reduces the amount of available free metal in the surrounding water (Borgmann & Norwood, 1995a).

In Borgmann's (1998) current model, ligand X binds with a metal (M) to form a complex (MX) that will then transfer the metal to an essential macromolecule (E), which then becomes an active molecule (ME). The researcher deduced that since essential copper is maintained at a constant level, the metal-ligand complex (MX) would need to be high to saturate all essential macromolecules that require it. If the concentration of copper in the external environment is increased, his model suggests that MX would have elevated levels. However, since E has already been saturated by the high amount of MX, the amphipod has no need to continue producing ligand X. Therefore, the researcher concluded that although ligand X is physiologically important in the regulation of copper by supplying E with the metal, it has no control over the long-term toxicity involved with the internal free ions that cannot be bound, or are loosely attached, to the finite amount of ligand X. Also, he notes that from his observations there appears to be a separate ligand X for zinc, since there was no apparent competition for binding sites.

Bioaccumulation Theory

In the past, the allometric model of $Y = aX^n$ has been commonly used to describe metal uptake and bioaccumulation in aquatic organisms. This model is the simplest and has been expressed in the literature as $C_{TB} = aC_W{}^n$, where C_{TB} is the total body concentration of a particular metal, C_W is the concentration of water, and a and n are constants (Borgmann et al., 2004; McGeer et al., 2003). Although the allometric model has been successful in describing the accumulation of certain metals for *Hyalella*, Borgmann et al. (2004) developed a different model that is more mechanistically based. This new model is described by,

Equation 3-1: Mechanistic saturation bioaccumulation model developed by Borgmann et al. (2004)

$$C_{TB} = \frac{max\,C_W}{K + C_W} + C_{Bk}$$

where C_{TB} is the total body concentration of the metal, *max* is the maximum amount of metal that can be accumulated above background levels, C_W is the concentration of metal present in the water, K is the half saturation constant which signifies when the concentration of C_{TB} is halfway between the background and maximum accumulation, and C_{Bk} is the background concentration body concentration that is determined by the control animals.

When *max* and K cannot be resolved by the above model, Equation 3-1 can be simplified and the ratio given by *max*/K is determined instead. If K is very large, the addition of the C_W term to K does not alter the final ratio substantially, and therefore the equation can be simplified to

Equation 3-2: Simplified mechanistic saturation bioaccumulation model to solve for *max*/K when *max* and K cannot be resolved individually.

$$C_{TB} = \frac{max}{K} \times C_W + C_{Bk}$$

Borgmann et al. (2004) state that the biotic ligand model (Di Toro et al., 2001; Paquin et al., 2002) can be used with their aforementioned saturation model in that the *max* is defined as the total number of metal binding sites, while K is the inverse of the strength that a metal binds to that site. The researchers also propose that the ratio obtained from metal excretion rate divided by a constant metal uptake rate can be defined as K, and Norwood et al. (2006) expand on this notion by incorporating uptake and excretion factors into the original saturation model.

Results

Mortality

Clades 1 and 8 had three toxicity tests conducted for each metal (denoted A, B, C). Clade 8 copper test C did not meet control survival requirements and was not included (data not shown). Data was pooled to determine the combined LC50 and LC25, and this was done because the experiments had overlapping confidence intervals. If a single experiment did not have an overlapping confidence interval with the LC50 or LC25s of the other experiments, its data was still pooled since the overall trend was not affected (Warren Norwood personal communication). Figure 3-1 supports the notion of pooling the data as it depicts overlapping mortality points and similar trends for the individual experiments of each clade.
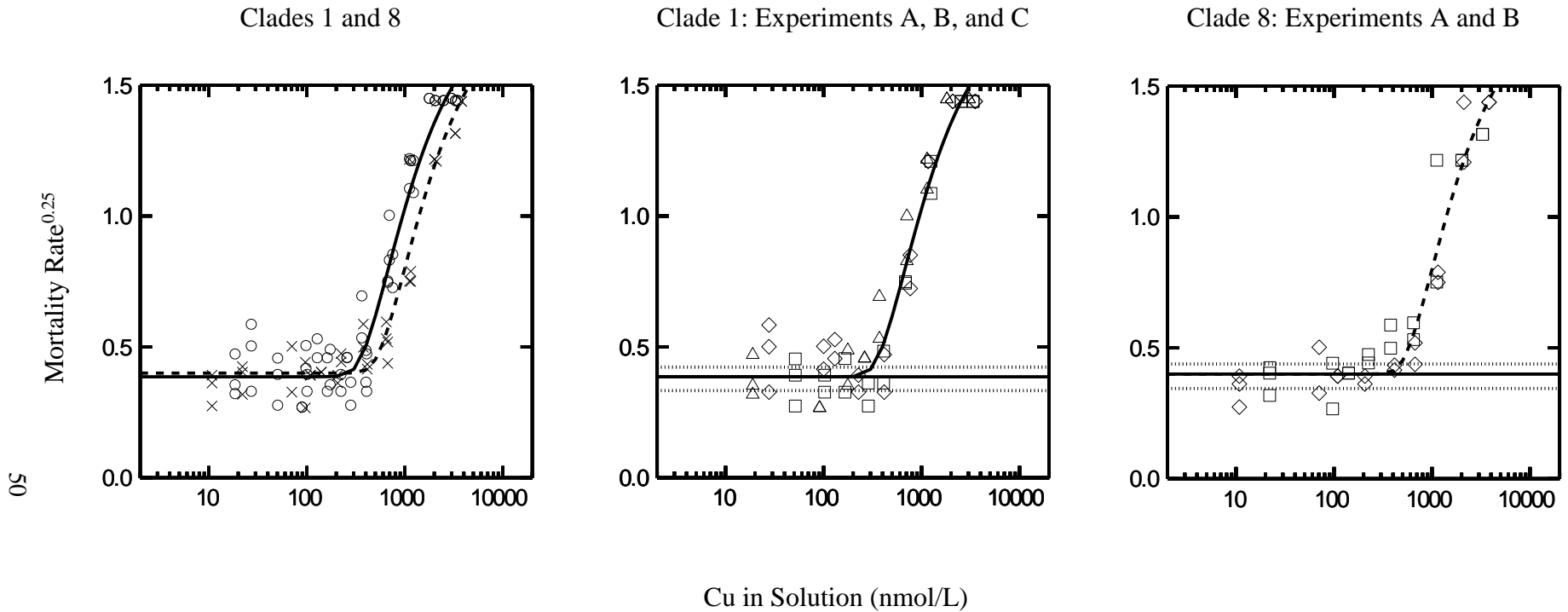
Figure 3-1: The relationship between fourth root transformed mortality rates against measured copper concentrations in experimental water from 28-day toxicity tests. Clades 1 (○) and 8 (×) were modeled with the entire data set since measured water concentrations are above detection limit. The regressions for Clades 1 and 8 were fit using a saturation-based mortality model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the estimated mean control mortality, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A (◊), B (□), C (Δ)

Measured copper concentrations in the control test solutions were above the detection

limit of 16.28 nmol/L, and therefore control data were included in the modeling. Since the

controls were included, Table 3-1 reports the estimated control mortality obtained from Systat 10

rather than the mean observed control mortality. This is also depicted in Figure 3-1, where

control data points are plotted with the estimated mean control mortality as a solid horizontal

line.

The mortality rates generally increased as the concentration of copper in solution

increased. When assessing Clade 1, it is observed from Figure 3-1 that mortality rate begins to

increase at 300 nmol/L. The estimated LC50 and LC25 computed from the regression were 544

(95% CI 483 – 605) and 446 nmol/L (95% CI 393 – 499), respectively. The model fit for these

estimates were very high as the range of r^2 was between 0.94 and 0.97 for all experiments and

the pooled data. In all cases, the estimated value for the exponent *nw* was greater than 100.

Whenever this occurred, the exponent was set to 100 instead to help the model resolve, or the

estimate for *nw* was so high that it could not be deemed accurate. The combined data for Clade 1

required *nw* to be set 100.

The combined data for Clade 8 also required *nw* to be set, and the mortality model fit was

also high – ranging from 0.92 to 0.97. The estimated copper LC50 and LC25 were 802 (95% CI

694 – 910) and 657 nmol/L (95% CI 561 – 753), respectively. When observing the nonlinear

regression in Figure 3-1, Clades 1 and 8 have a similar mortality rate at the same concentrations

until an increase in mortality for Clade 1 occurs at 300 nmol/L. However, an increase in

mortality rate for Clade 8 is not seen until 500 nmol/L. In addition, the computed LC50 and

LC25 for Clade 8 is 1.47 times higher than that of Clade 1. These differences are deemed

significant due to the LC50 and LC25s not having overlapping confidence intervals.

Table: 3-1: Estimated parameters for Clades 1 and 8 from individual (A, B, and C for Clade 1; A and B for Clade 8) and combined experiments when assessing mortality rate against measured nickel concentration in the test solution. The control mortality (m'), water exponent (nw), half saturation constant (K''_w), LC50, LC25, and model fit (r^2) are given with their respective 95% confidence intervals (CI; ±). Significant differences between Clades 1 and 8 LC50 and LC25s are indicated with bold faced text, and these inferences are based on non-overlapping CIs.

Clade	Exp	m'	±	nw	±	K''_w	±	LC50 (nmol/L)	±	LC25 (nmol/L)	±	r^2
1	Combined	0.022	0.010	100	Set	22.744	3.639	**543.965**	**60.893**	**445.842**	**53.106**	0.94
1	A	0.035	0.023	100	Set	25.215	7.386	588.459	119.138	484.342	104.570	0.95
1	B	0.012	0.012	100	Set	25.220	6.901	600.459	115.364	492.531	100.436	0.95
1	C	0.017	0.012	100	Set	16.390	3.424	415.717	62.357	337.195	53.616	0.97
8	Combined	0.026	0.011	100	Set	33.570	7.081	**801.840**	**108.299**	**657.351**	**96.147**	0.94
8	A	0.021	0.011	100	Set	41.234	9.470	907.810	132.155	754.439	118.604	0.97
8	B	0.028	0.020	100	Set	25.315	9.335	673.008	162.822	541.886	141.973	0.92

Mortality rates based on the concentration of copper in the tissues followed a similar trend to that observed for metal exposure in solution. It is to be noted that the data in Table 3-2 are the estimated values based on background corrected body concentrations of copper, and the control data are not included in the modeling. This is because the mean concentration of copper in the tissues of the control amphipods was subtracted from all other replicates during background correction. Therefore, the average measured control mortality for each clade is reported for *m'* rather than the estimated values. Also, an accurate LBC50 and LBC25 for Clade 1 experiment A could not be resolved. However, the data is still reported in Table 3-2 and combined with experiments B and C, since the tissue concentrations and number of replicates are similar to the other tests.

Table: 3-2: Estimated parameters for Clades 1 and 8 from individual (A, B, and C for Clade 1; A and B for Clade 8) and combined experiments when assessing mortality rate and measured copper concentration in the test solution. The control mortality (m'), water exponent (nb), half saturation constant (K''_w), LC50, LC25, and model fit (r^2) can be seen below with their respective 95% confidence intervals (CI; ±). Significant differences between Clades 1 and 8 LC50 and LC25s are indicated with bold faced text, and these inferences are based on non-overlapping CIs.

Clade	Exp	m'	±	nb	±	K''_{TB}	±	LBC50 (nmol/g)	±	LBC25 (nmol/g)	±	r^2
1	Combined	0.037	0.023	100	Set	142.018	104.980	**1673.969**	**225.532**	**1504.091**	**208.506**	0.30
1	A	0.063	0.059	100	Set	1000	5.44E+07	1964.056	2.0E+06	1523.301	3.7E+06	0.48
1	B	0.024	0.021	100	Set	177.269	303.208	1523.301	275.138	1404.309	310.103	0.41
1	C	0.025	0.024	100	Set	564.833	1840.702	1378.846	124.866	1338.173	77.878	0.26
8	Combined	0.019	0.008	18.574	295.283	1000	2.1E+04	**3881.737**	**665.175**	**3138.818**	**484.377**	0.48
8	A	0.015	0.010	6.567	79.925	1000	5.44E+05	3515.241	901.776	2944.964	743.174	0.37
8	B	0.023	0.013	27.777	901.491	694.425	2.71E+04	4070.792	793.967	3334.742	673.860	0.70

Clade 1 mortality rate begins to increase at approximately 1400 nmol/g and plateaus at 1000 nmol/L (Figure 4-2). The estimated LBC50 and LBC25 for Clade 1 was 1674 (95% CI 1448 – 1900) and 1504 nmol/g (95% CI 1296 – 1713), respectively. The r^2 ranges for Clade 1 were between 0.26 and 0.48 for all experiments and the combined data. These values were lower than those observed for the relationship between mortality rates and copper present in solution. All of the experiments for Clades 1 required *nb* to be set to 100.

In contrast, the data for Clade 8 fit the model better since r^2 values ranged between 0.37 and 0.70, and *nb* was not set. Also, the LBC50 and LBC25s were significantly higher at 3882 (95% CI 3217 – 4547) and 3139 nmol/g (95% CI 2654 – 3623), respectively. In addition, Clade 8 has lower mortality rates at the same concentrations as Clade 1, and the point at which mortality rate increases is around 2000 nmol/g. The two clades do not have overlapping confidence intervals when assessing their copper LBC50 and LBC25s, and thus there is a significant difference between them.
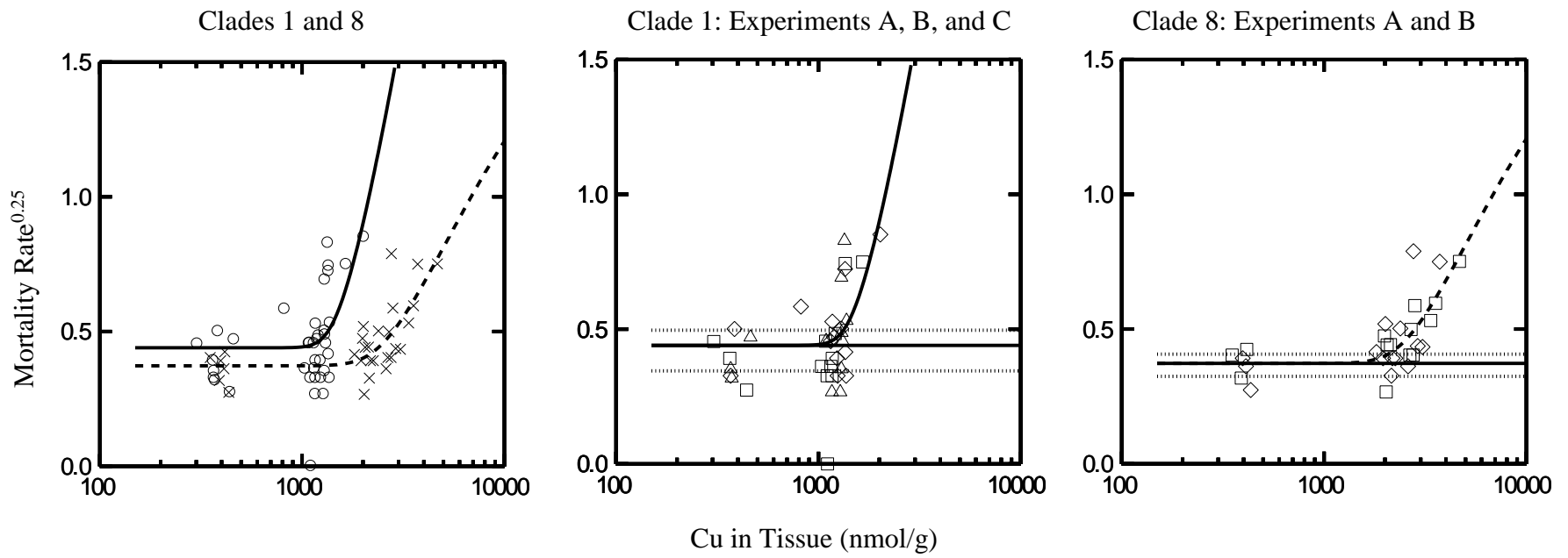
Figure 3-2: The relationship between fourth root transformed mortality rates against measured copper concentrations in amphipod tissues digested after 28 days. Clades 1 (○) and 8 (×) were modeled with the average control mortality for each clade set in the equation. Therefore, measured control tissue concentrations were plotted instead of estimates. The regressions for Clades 1 and 8 were fit using a saturation-based mortality model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control mortality, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A (◊), B (□), C (Δ)

Bioaccumulation

Experimental data for each individual test was pooled together for each clade. The models were estimated by setting the background term (C_{BK}) to the mean measured tissue concentration in the controls for each clade, and this was done to properly estimate the *max* and *K* terms. Allowing the model to estimate C_{BK} resulted in extremely high values for *max, K,* and their respective 95% confidence intervals. In addition, a second model was run where the term *max*/*K* was computed in Systat 10 with the "funpar" command, and Equation 3-2 was used instead of Equation 3-1. The control data were not included for both models because the measured tissue concentration present in the controls was used as a background correction. Therefore, the mean measured control tissue concentration is reported as C_{BK} in Table 3-3, and depicted as a solid horizontal line in Figure 3-3.

Table 3-3: Mean measured background copper concentration (C_{BK}), the estimated maximum copper accumulated (*max*), half saturation constant (*K*), model fit (r^2), and their respective 95% confidence intervals. The estimates were computed using a saturation bioaccumulation curve. The *max/K* term and its confidence interval were estimated with a model where *max* and *K* could not be estimated (See Equation 3-2).

Clade	Exp	C_{BK} (nmol/g)	±	*max* (nmol/g)	±	*K* (nmol/L)	±	*max*/*K* (L/g)	±	r^2
1	Combined	432	100	943	140	28	32	0.46	0.263	0.13
8	Combined	399.4	23	2702	525	53	46	1.29	0.677	0.30
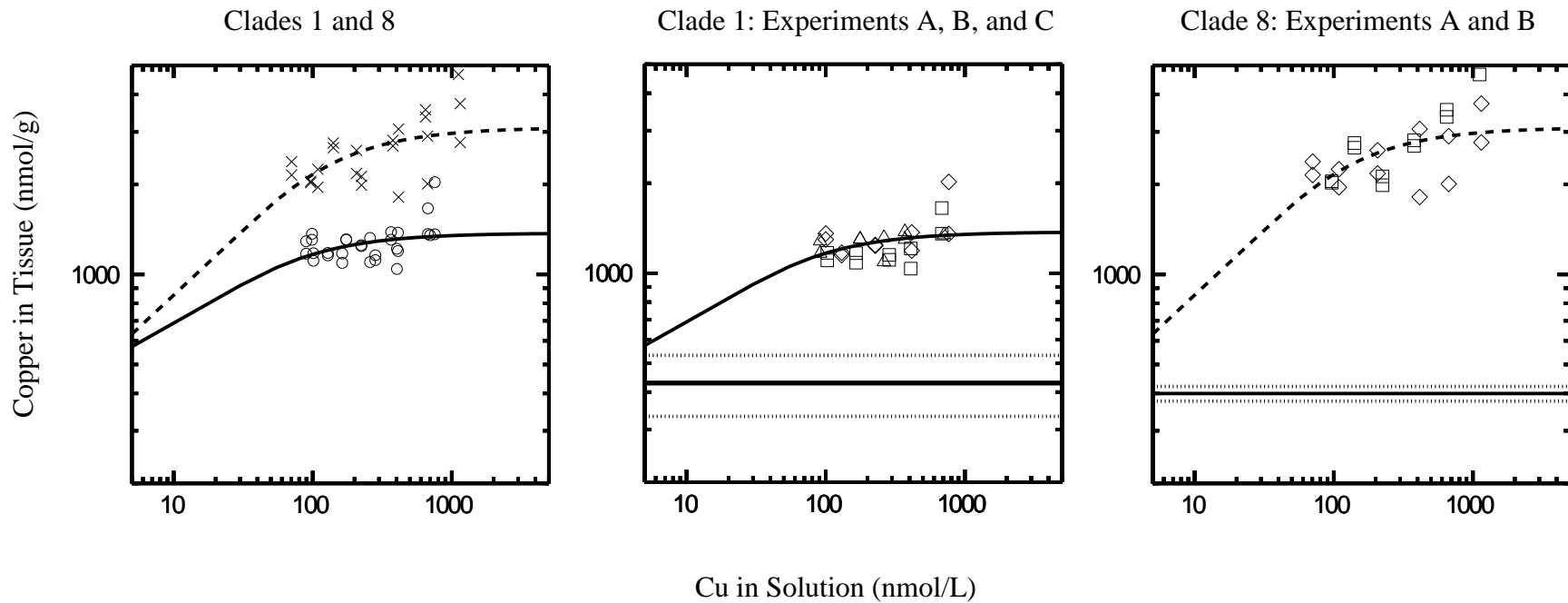
Figure 3-3: The relationship between measured copper concentrations in tissues digested from amphipods after 28 days and measured copper concentrations in the experimental water. Clades 1 (○) and 8 (×) were modeled without any control data, and thus only replicates spiked with copper are plotted. The regressions for Clades 1 and 8 were fit using a saturation bioaccumulation model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control tissue concentration, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A (◊), B (□), C (Δ)

The concentration of copper present in the tissues increased as the exposure concentrations elevated. However, the accumulation of copper was not gradual – amphipods in the replicates with low concentrations of copper had significantly more metal in their tissues than control animals, but had similar concentrations to replicates higher in the series that could be measured. A positive background concentration for copper was determined for both Clades 1 and 8 at 432 nmol/g and 399 nmol/g, respectively. There is no significant difference between the two clades for C_{BK} due to overlapping confidence intervals.

When assessing *max/K*, it is more difficult to state whether a significant difference is present or not. Clade 8 has a *max/K* term at a value of 1.29 (95% CI 0.614 – 1.968), whereas 0.461 (95% CI 0.198 – 0.725) was reported for Clade 1. However, there is a significant difference between the two clades when comparing *max* terms based on non-overlapping confidence intervals. Clade 1 is estimated to reach a maximum body concentration at 943 nmol/g (95% CI 804 – 1083), whereas Clade 8 had a value of 2702 nmol/g (95% CI 2177 – 3227). The reported mean corrected R-square from Systat 10 for Clades 1 and 8 were both low at 0.13 and 0.30, respectively.

Dry Weights

The total dry weight measured for each replicate was divided by the amount of amphipods weighed. Models were fit without the control data, and instead had *W'* set to the measured mean control dry weight. This was due to the fact that some replicates had higher dry weight than the control, and thus skewed the estimate of *W'*. However, since the control water concentrations were above the detection limit of 16.28 nmol/L, the control data is plotted in Figure 3-4.

Clade 8 had significantly larger control dry weights than Clade 1, and this is consistent with the two-sample t-Test presented in Chapter 2. Although Clade 8 amphipods are initially larger than those belonging to Clade 1, Figure 3-4 depicts that animals in both clades have a reduction in weight at a similar concentration of copper. Amphipods from both clades appear to experience weight loss at 200 nmol/L, and maintain a similar weight as the concentrations increase. Also, the data for both clades fit the model well, where Clade 1 r^2 values range between 0.68 – 0.85, and 0.91 – 0.95 for Clade 8. The estimated IC25s for Clades 1 and 8 were 299 (95% CI 219 – 380) and 225 (95% CI 173 – 278) nmol/L, respectively. Based on the overlapping confidence intervals of the combined data, the two clades may not be significantly different.

Table 3-4: Estimated parameters for Clades 1 and 8 from individual (A, B, and C for Clade 1; A and B for Clade 8) and combined experiments when assessing dry weight against measured copper concentration in solution. The control dry weight (W'), exponents (a and n_w), IC25, and model fit (r^2) are given below with their respective 95% CIs ($\pm$).

Clade	Exp	W'	$\pm$	a	$\pm$	n_w	$\pm$	IC25 (nmol/L)	$\pm$	r^2
1	Combined	0.194	0.024	3.88E-07	2.41E-06	2.397	1.002	299.409	80.512	0.70
1	A	0.206	0.036	1.00E-06	1.00E-05	2.244	1.276	264.768	118.747	0.85
1	B	0.154	0.012	1.00E-09	8.00E-09	3.417	2.424	371.044	130.570	0.80
1	C	0.176	0.024	1.00E-06	1.27E-05	2.246	2.090	287.613	154.108	0.68
8	Combined	0.278	0.040	4.00E-06	1.10E-05	2.110	0.505	225.349	52.203	0.91
8	A	0.306	0.053	5.36E-07	2.57E-06	2.440	0.763	236.667	69.986	0.93
8	B	0.251	0.047	2.00E-05	6.60E-05	1.800	0.541	222.831	63.417	0.95
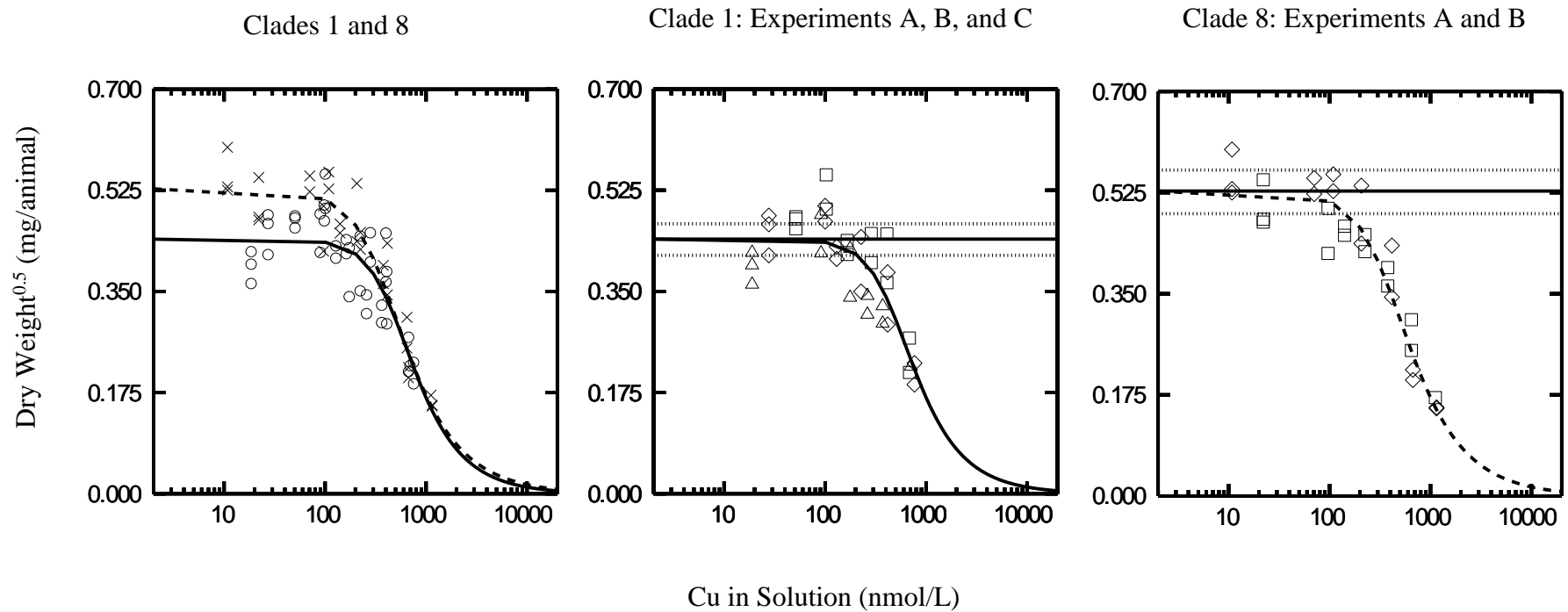
Figure 3-4: The relationship between square-root transformed total dry weight and measured copper concentrations in the experimental water after 28 days. Clades 1 (○) and 8 (×) were modeled without any control data, but all samples are plotted. The regressions for Clades 1 and 8 were fit using the general growth model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control dry weight, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A (◊), B (□), C (Δ)

The concentration of copper in the body and its effects on growth were assessed using the tissue digest dry weights rather than the total dry weight. The nonlinear regressions did not resemble the models for dry weight against copper present in solution. Although there is a similar steep downward trend indicating a reduction in dry weight, the decrease is not corresponding to an increase in copper tissue concentration. It is observed in Figure 3-5 that the dry weight decreases at a certain concentration for both clades. Also, the data for both clades had poor model fitting, as the r^2 values ranged between 0.29 – 0.56 and 0.21 – 0.80 for Clades 1 and 8, respectively.

The estimated IBC25s from the models were 1146 (95% CI 946 – 1345) and 2114 nmol/g (95% CI 1580 – 2648) for Clades 1 and 8, respectively. Based on non-overlapping confidence intervals, there is a significant difference between the two IBC25s. In addition, it is to be noted that the data for Clade 1 experiment C could not be included in Table 3-5 due to the fact that the model could not resolve it. Clade 1 experiment B had a model that resolved, but a confidence interval could not be estimated for the exponent a. Thus the data was reported and the confidence interval was deemed "not determined (ND)".

A single factor ANOVA was conducted in Microsoft Excel 2013 to determine if tissue concentrations differed significantly between amphipods obtained from replicates of the first four and five concentrations within the series for Clades 1 and 8, respectively. The ANOVA for Clade 1 indicated that there was no significant difference [$F_{(3, 23)} = 0.418$, p=0.742] for the first four concentrations, and the ANOVA for Clade 8 had a similar result for the first five concentrations within the series [$F_{(4, 19)} = 2.09$, p=0.132]

Table 3-5: Estimated parameters for Clades 1 and 8 from individual and combined (A, B, and C for Clade 1; A and B for Clade 8) experiments when assessing dry weight against measured copper tissue concentration. Clade 1 experiment C did not resolve and estimated parameters for it are not shown. The control dry weight (W'), exponents (a and nb), IBC25, and model fit (r^2) can be seen below with their respective 95% CIs ($\pm$). Bold faced text indicate significant differences based on non-overlapping CIs.

Clade	Exp	W'	$\pm$	a	$\pm$	nb	$\pm$	IBC25 (nmol/g)	$\pm$	r^2
1	Combined	0.185	0.021	7.09E-16	1.91E-14	4.796	3.736	**1145.74**	**199.45**	0.34
1	A	0.199	0.039	9.23E-12	9.91E-10	3.494	8.139	1051.78	888.42	0.29
1	B	0.203	0.015	9.62E-21	ND	6.377	7.721	1154.40	311.67	0.56
8	Combined	0.319	0.047	2.53E-16	6.68E-15	4.547	3.322	**2114.23**	**533.83**	0.37
8	A	0.337	0.083	6.51E-16	3.65E-14	4.469	7.119	1956.88	1086.09	0.21
8	B	0.300	0.053	8.50E-18	1.68E-16	4.929	2.460	2326.93	384.53	0.80

ND = Not determined (model could not estimate)
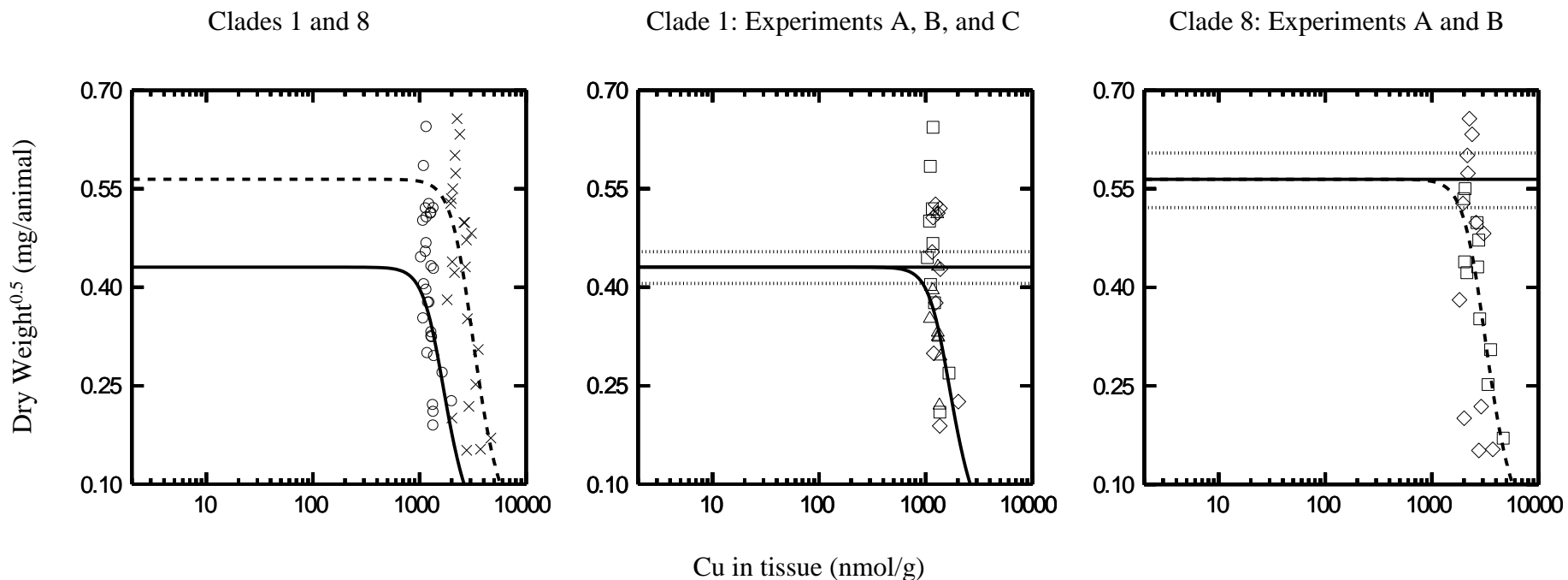
Figure 3-5: The relationship between square-root transformed total dry weight and measured copper concentrations in the experimental water after 28 days. Clades 1 ($\circ$) and 8 ($\times$) were modeled without any control data, and thus only replicates spiked with copper are plotted. The regressions for Clades 1 and 8 were fit using the general growth model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control dry weight, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A ($\diamond$), B ($\square$), C (Δ)

Discussion

To the author's knowledge, this is the first study to compare copper accumulation and toxicity in genetically characterized clades of *Hyalella* over a 4-week period. This study is a continuation of the work done by Leung (2014), but with slight changes in protocol. For instance, the nominal concentrations employed by Leung (2014) are similar to the nominal concentrations used in this study (Concentrations 5 to 7 differ by 0.24, 0.39, and 0.70 µmol/L, respectively). Although the test durations were different, the reported LC50 and LC25 in this study are comparable to the estimates in the work done by Leung (2014).

LC50 and LC25 values were estimated for Clade 1 at 491 (95% CI 423 – 559) and 383 nmol/L (95% CI 324 – 442), respectively, which have overlapping confidence intervals with the reported LC50 and LC25 for Clade 1 in this thesis. The Clade 8 LC50 in the current study is similar to that reported by Leung (2014) at 1260 nmol/L (95% CI 998 – 1522), but the confidence intervals do not overlap. However, Leung (2014) determined the LC25 for Clade 8 was 876 nmol/L (95% CI 585 – 1167), which does have overlapping confidence intervals with the LC25 presented in this thesis.

In addition to similar LC50 and LC25 values, this study also shares consistency with Leung (2014) for general trends. For example, when comparing Figure 4-2 in the work by Leung (2014) with Figure 3-1 in this thesis, it can be observed in both studies that Clade 1 increases in mortality at approximately 300 nmol/L, whereas Clade 8 increases around 400 nmol/L. Also, Clade 8 was observed to be significantly more tolerant to copper than Clade 1 by 1.47 times, which is slightly lower than the 2.3-2.6 fold difference reported by Leung (2014).

Interestingly, the LBC50 and LBC25 values reported by Leung (2014) could not be compared to the values presented in this thesis. Also, the concentration of copper in the tissues at which mortality increases could not be compared between the two studies. Only a general trend could be compared – Leung (2014) observed an LBC50 and LBC25 for Clade 8 that was 2.52 and 1.42 times higher than that of Clade 1, respectively. This thesis reports similar factors for Clade 8, where the LBC50 and LBC25 are higher by 2.32 and 2.09 times, respectively.

In addition, the Clade 1 LC50, LC25, LBC50, and LBC25 can be compared to the data reported by Borgmann et al. (1993). The results obtained in their study were re-evaluated by Borgmann et al. (2004), where they presented estimates of 718 (95% CI 545 – 946) and 441 nmol/L (261 – 743) for the LC50 and LC25, respectively. These values are higher, but also have overlapping confidence intervals with the current study. This is not the case with the LBC50 and LBC25, where Borgmann et al. (2004) estimates are 2560 (95% CI 2370 – 2770) and 2170 nmol/g (95% CI 1760 – 2670), respectively. The values are higher than the ones presented in this study and do not overlap with the confidence intervals.

A possible explanation for the discrepancy between the results obtained in this study and those reported by Borgmann et al. (1993) could be due to the methodology, since it has been established that they employ Clade 1 in the laboratory (Major et al., 2013). The researchers conducted 10 week tests and do not mention gut clearance. Although gut clearance is more important in sediment toxicity tests (Borgmann et al., 2001), the amount of copper ingested can increase the total body concentration since Norwood et al. (2006) measured 168 nmol/g of copper present in TetraMin® fish flakes.

Data from Borgmann and Norwood (1995b) were also revaluated by Borgmann et al. (2004) to fit a saturation bioaccumulation curve. The researchers estimated a background of 1170 nmol/g, and a *max* at 3600 nmol/g (95% CI 3240 – 3970). The background and *max* terms for Clade 1 in this study are significantly lower than the estimates reported by Borgmann et al. (2004), at values of 432 nmol/g and 943 nmol/g (95% CI 804 – 1083), respectively. Again, this difference is likely due to the fact that Borgmann and Norwood (1995b) did not allow the amphipods to clear their guts before assessing dry weights. It is unlikely that the use of amphipods that were 4-6 weeks old for their uptake experiments plays a role in the discrepancy since the researchers have noted that body size differences did not significantly change the amount of copper present in the tissues. The *K* and *max/K* terms reported by Borgmann (2004) were 291 nmol/L, and 12.4 L/g, respectively. These values are significantly different from the reported estimates in this study, and therefore will not be compared.

In contrast, the bioaccumulation data obtained by Leung (2014) are more similar to this study. The *max* value reported by Leung (2014) for Clade 1 is almost identical to the estimated *max* in the current study, and both terms have overlapping confidence intervals. Clade 8 *max* terms between the two studies appear significantly different, but the confidence intervals overlap with each other, suggesting that they may or may not be similar. The *K* estimates could not be compared because Leung (2014) reported confidence intervals for *K* that were very wide, and thus accommodate the significantly lower estimates presented in this thesis. Also, this study indicated that Clade 8 had a significantly higher maximum body concentration than Clade 1 by 2.87 times. Leung (2014) indicated that Clade 8 reaches a maximum that is 1.84 times higher than Clade 1, but had overlapping confidence intervals. Therefore, Leung (2014) determined that no significant difference was present.

The concentration series employed by Borgmann et al. (1993) was adopted for this study, but with the inclusion of an extra concentration. They state that no effects were observed for growth at concentrations that do not cause chronic mortality. Also, copper accumulated in *Hyalella* at all test concentrations were not significantly different from the controls. This was not the case for the current study as the dry weight per animal decreased with increasing copper concentration in the water (Figure 3-4). However, the decrease in dry weight did not follow an increase in copper present in the tissues (Figure 3-5). Also, the copper present in the controls was significantly different than the other test animals.

Figure 3-5 shows that the amounts of copper in the amphipods do not significantly differ, but the dry weights range between approximately 0.2 to 0.6 mg/animal. This was also supported by the two separate ANOVAs conducted that determined there was no evidence to suggest that the first four test concentrations differed in accumulated copper concentration for Clade 1, while Clade 8 did not differ in tissue concentration for the first five test concentrations.

Borgmann et al. (1993) indicate that copper is completely regulated by *Hyalella*. They also mention that the amphipods were capable of regulating the metal at all the concentrations that induced chronic mortality. This could likely explain why the amount of metal accumulated in the tissues remained relatively constant for the first four and five test concentrations – the animals are capable of regulating copper only to a fixed amount. This resonates with the model proposed by Borgmann (1998), where ligand X is the internal binding site at which copper accumulates until required by essential macromolecules. However, ligand X rapidly saturates and is limited in quantity. Therefore, it is plausible that the reduction in dry weight and mortality is due to the accumulated copper that cannot be bound since there are a finite number of ligands (Borgmann, 1998). The aforementioned implication is supported by test concentration 5 and 6

for Clades 1 and 8, respectively, since the replicates at those concentrations had the lowest number of amphipods that survived (1-6) and dry weight per animal. Also, these concentrations had the highest amount of copper present in their tissues relative to the other amphipods that survived.

Interestingly, Borgmann et al. (1993) report that the measured copper concentration accumulated by 4-week old *Hyalella*, and these values are significantly greater than the controls. Their results for the adult *Hyalella* are very similar to the results obtained in this study. Since the nominal concentrations in this study were adopted from Borgmann et al. (1993) (excluding the additional 8th concentration), the measured water concentrations are almost identical. Also, despite the use of 2-9 day old *Hyalella*, the mean measured tissue concentrations in this thesis are very similar to the values reported by Borgmann et al. (1993) (see Appendix).

The most intriguing observation is that the amount of copper accumulated by Clade 8 is closest to the values reported for the 4-week old *Hyalella* used by Borgmann et al. (1993), whereas Clade 1 has values that are off by approximately 45 µg/g. This discrepancy could be due to the age difference, but may not be likely since Borgmann and Norwood (1995b) observed very little change in copper body concentrations between amphipods that had different body sizes over a 100 fold range. Borgmann (1998) also supports this notion that body size does not affect copper concentrations, as he states that the binding is internal rather than at the animal's surface, and is not influenced by a surface diffusion rate. In addition, Borgmann and Norwood (1995b) state that the accumulation of copper is rapid, and the amphipod is capable of maintaining a constant internal concentration of the metal. Also, their study shows that the regulation of the metal is slow and takes weeks to reach control levels. This also helps to explain why the

amphipods at different dry weights have similar body concentrations, and the earlier implication that the amphipods maintain a constant body concentration.

Figure 2 from Borgmann and Norwood (1995b), indicates that the amphipods accumulate copper up to approximately 2800 nmol/g, and at 28 days the amount of metal in the *Hyalella* is reduced to approximately 2000 nmol/g dry weight. This is very similar to the highest measurable concentration for a single replicate in Experiment A for Clade 1 (2022 nmol/g dry weight). Since body copper is gradually decreased after the first week (Borgmann and Norwood, 1995b), it is plausible that this slow decrease is enough for the amphipods at the lower concentrations to be unaffected by the rapid accumulation.

On the other hand, at the higher concentrations it is likely that the regulation of copper is too slow and the constant exposure to excess levels of the metal affect the growth of the animals. Since Borgmann and Norwood (1995) have mentioned that body size does not significantly change the amount of copper accumulated in the body, the amphipods with reduced weight will have similar body concentrations to those in the replicates at lower water concentrations. Also, the researchers note that the reduction of copper does not mean the metal has been excreted. Borgmann and Norwood (1995b) indicate that even though the concentrations decrease, the total body burdens increase over time as the amphipods continually grow. This explains why the few survivors that remained at the highest measurable concentration had similar, or slightly higher amounts of copper in comparison to the lower concentrations within the series.

Leung (2014) determined that Clade 1 amphipods were significantly larger than Clade 8 in the control animals and in the exposure concentrations. This was not observed in the current study since Clade 8 control animals were significantly larger than Clade 1. Although they were

71

larger, Clade 8 amphipods eventually reached a similar dry weight as Clade 1 at high

concentrations (Figure 3-4). As stated earlier, the nominal concentrations used in this study and

Leung (2014) are similar. Despite this, the results for the IC25 and IBC25 cannot be compared,

and is likely due to the difference in test durations.

Leung (2014) observed similar IC25 estimates between both clades and the confidence

intervals for her IC25s were overlapping – this is consistent with the results obtained in the

current study. However, the IBC25s estimated for Clades 1 and 8 in this thesis were significantly

different based on non-overlapping confidence intervals, and this contrasts the results in the

study conducted by Leung (2014). The reported Clade 8 IBC25 in this study is 1.85 (2114

nmol/g) times higher than that of Clade 1 (1146 nmol/g). These results are consistent with the

reported *max*, as Clade 8 reaches a maximum body concentration (2702 nmol/g) that is 2.87

times higher than Clade 1 (943 nmol/g).

Figure 3-4 and Figure 3-5 indicate that copper has a prominent hormetic effect on growth

for Clade 1 – the amphipods demonstrate a higher dry weight than the controls (Environment

Canada, 2007). There does not appear to be a similar effect for Clade 8, as most of the higher dry

weights fall within the upper confidence limit of the controls. However, Clade 8 experiment A

does show two replicates that are greater than the mean measured control dry weight and its

confidence limits. Environment Canada (2007) states that hormesis is a common phenomenon,

and provide guidelines on assessing data that contain low-dose stimulation.

Norwood et al. (2007) encountered hormesis in their arsenic data and applied the general

growth model, as well as a hormesis model. Since the modeling methodology applied in this

study has been adopted from Norwood et al. (2007), the hormesis model was attempted for both

72

Clades 1 and 8. However during the analysis with the hormesis model, a 95% CI for the parameters h and m could not be estimated. In addition, the estimated IC25s were the exact same as the general growth model. Since the hormesis model could not fit the data for both clades, option 3 described by Environment Canada (2007) was used instead. Dry weights that were greater than the highest control were excluded from the analyses, and the general growth model was applied. It is to be noted that data points were only removed for the assessment of dry weight and copper present in tissue because the model would not resolve. However, the removal of data points with the analysis between dry weight and copper in solution did not change the IC25.

Conclusion

The two clades demonstrated similar control mortality rates and background concentrations of copper in their tissues. However, Clade 8 was significantly more tolerant to copper in all aspects (LC50, LBC50, *max*, IBC25) other than the IC25, where the estimated value was similar to that of Clade 1. The latter demonstrated mortality at lower water and body concentrations, and exhibited a significantly lower maximum body concentration that could be reached. Both clades had similar dry weights at the higher concentrations, but Clade 8 was larger than Clade 1 in the controls and early exposure levels. Based on the results presented in this chapter, laboratory organisms should be genetically characterized prior to conducting toxicity tests (Leung, 2014)

Chapter 4 – Nickel

Introduction

Nickel occurs in low amounts in freshwater that is uncontaminated by human activity. Boyle and Robinson (1988) report that the amount of nickel deposited in undisturbed lakes, streams, and rivers is usually less than 0.01 ppm, but can range from 0.0005 – 0.02 ppm. This metal is ranked as the fifth most abundant metal and predominantly occurs in igneous and metamorphic rocks. In water, the ionic state of nickel is predominantly Ni^{2+} when the pH is between 5 and 9, and it is usually adsorbed to organic matter, or iron and manganese oxides. However, the nickel may also form complexes with inorganic ligands at that pH range (Hertel et al., 1991).

Nickel has been thought to have no biological significance, but it has been discovered that many living systems make use of this trace metal (Boyle & Robinson, 1988). Quiroz et al., (2007) provide an overview of nickel-dependent enzymes and support the notion that nickel is essential as a cofactor in many organisms. These enzymes include urease, hydrogenase, carbon monoxide dehydrogenase, superoxide dismutase, and glyoxalase. Superoxide dismutase is of particular interest as the copper superoxide dismutase was discussed in Chapter 3. A nickel derivative of this enzyme has recently been discovered to be present in certain species of cyanobacteria and *Streptomyces* (Quiroz et al., 2007)

Küpper and Kroneck (2007) discuss how nickel is important in plants and cyanobacteria by being the active center for certain enzymes such as hydrogenase and urease. The latter enzyme plays a role in nickel deficiency, as a lack of urease leads to toxic levels of urea. They note in their review that urease is constitutively expressed if nickel is readily available to the

plant. Interestingly, Küpper & Kroneck (2007) mention plants that are capable of "hyperaccumulating" nickel. These plants are thought to hyperaccumulate the metal as a defense strategy to ward off herbivores and pathogens, but as a consequence makes them more susceptible to nickel deficiency, which may be attributed to their sequestering of the metal into epidermal vacuoles. Küpper & Kroneck (2007) also discuss how nickel toxicity can be a result of oxidative stress, whether it is induced by peroxides, or the decrease of anti-oxidative enzymes.

Mechanisms of nickel toxicity are not well known for the invertebrate *Hyalella azteca*. Previous studies so far have only looked at the effects of dissolved organic matter and different spiked sediments on nickel toxicity and bioavailability (Borgmann et al., 2001; Doig & Liber, 2006, 2007; Keithly et al., 2004; Liber et al., 2011). Borgmann et al. (2001) conducted a chronic toxicity tests using *Hyalella azteca* to determine whether or not bioaccumulation would be a reliable predictor of nickel toxicity in sediments that had different compositions. Three sediments were used and the researchers determined that the LC50 and LC25s were significantly different by more than 20 fold. However, when they assessed the LBC50 and LBC25s for each sediment, the variation was less than 3 fold.

As a consequence, Borgmann et al. (2001) concluded that the use of bioaccumulation was a reliable method of predicting nickel toxicity. The researchers were also able to demonstrate that metal toxicity in sediment tests can be predicted by the overlying water if the sediment does not affect it, and if the water quality is kept constant. Keithly et al. (2004) also wished to determine the reliability of bioaccumulation to predict nickel toxicity in *Hyalella azteca*. Unlike Borgmann et al. (2001), the researchers decided to change the experimental conditions to test whether a similar lethal body burden would be observed. Therefore, Keithly et al. (2004)

conducted 14 day tests with different water chemistry, and they were able to report a lethal body burden that was similar to Borgmann et al. (2001).

Wu et al. (2003) had mentioned that toxicity data for nickel was limited in comparison to other metals that had biotic ligand models (BLM). Another point brought up in their report was that no studies systematically varying DOC had been conducted, and thus Doig et al. (2006) sought to address this issue. Doig et al. (2006) employed *Hyalella azteca* in acute lethal and sublethal tests that involved the use of three different sources of dissolved organic matter (DOM). They discovered in their 48 hour acute tests that the nickel toxicity in their amphipods was not significantly reduced regardless of the DOM used. However at sublethal concentrations, the researchers observed significant reductions in nickel tissue concentrations and free Ni^{2+} concentrations in solution, regardless of the DOM used. Doig et al. (2006) determined that the concentration of DOM was more important than the source or fraction, since approximately 130-140 mg/L DOC was capable of reducing free nickel by 91% at a concentration of 200 ug Ni/L. Therefore, they suggest that the concentrations for acute mortality are too high for metal complexion at concentrations of DOM that are environmentally relevant.

The previously discussed studies employed *Hyalella* that were not genetically characterized. The most recent study by Leung (2014) assessed the effects of nickel on growth and survival for two clades of *Hyalella* by conducting two week water-only toxicity tests. This chapter aims to expand on the results obtained by Leung (2014) by examining the effects of nickel on growth and survival after 28 day exposures on *Hyalella* clades 1 and 8.

Bioaccumulation

The bioaccumulation theory pertinent to this chapter has been described previously. See Chapter 3 – Bioaccumulation

Results

Mortality

Clades 1 and 8 had three toxicity tests conducted for nickel (denoted A, B, C). Data was pooled to determine the combined LC50 and LC25. This was done because the experiments had overlapping confidence intervals and also followed a similar trend. Therefore, even if a single experiment did not have an overlapping confidence interval with the LC50 or LC25s of the other two experiments, it was still pooled since the overall trend was unaffected (Warren Norwood personal communication). This can be seen in Figure 4-1, where the three experiments for Clades 1 and 8 are depicted in separate charts. It is to be noted that the control mortality could not be estimated from the models since the control data were not included. This was due to the fact that the measured nickel concentration in the control water was lower than the detection limit. Therefore, the average control mortality was set in the equation instead of being estimated, and the control data are plotted on Figure 4-1 at the detection limit of 19.7 nmol/L.

As nickel water concentrations increased, the mortality rates generally increased as well. When assessing Clade 1, it can be seen from Figure 4-2 that mortality rate begins to increase at 200 nmol/L. The estimated LC50 and LC25 computed from the regression were 434 (95% CI 342 – 526) and 293 nmol/L (95% CI 202 – 384), respectively. These estimates had good model fitting as the range of r^2 were between 0.78 and 0.97 for all experiments and the pooled data. When the estimated value for the exponent nw was greater than 100, it was set to 100 instead.

This was done to help the model resolve, or the estimate for *nw* was so high that it could not be deemed accurate. The combined data for Clade 1 did not require *nw* to be set.

In contrast, the combined data for Clade 8 required *nw* to be set to 100 and the mortality model fit ranged from 0.89 to 0.98. The estimated nickel LC50 and LC25 were 1151 (95% CI 932 – 1257) and 883 nmol/L (95% CI 676 – 950), respectively. The nonlinear regressions (Figure 4-1) indicate that Clade 8 has a lower mortality rate than Clade 1 at the same concentrations, and an increase in mortality is not seen until 500 nmol/L. In addition, the computed LC50 and LC25 for Clade 8 is 2.65 and 3.01 times higher than that of Clade 1, respectively. These differences are deemed significant due to the LC50 and LC25s not having overlapping confidence intervals.

Table 4-1: Estimated parameters for Clades 1 and 8 from individual (A, B, C) and combined experiments when assessing mortality rate against measured nickel concentration in the test solution. The control mortality (m'), water exponent (nw), half saturation constant (K''_w), LC50, LC25, and model fit (r^2) are given below with their respective 95% CIs ($\pm$). Significant differences between Clades 1 and 8 LC50 and LC25s have been indicated through bold faced text, and these inferences are based on non-overlapping CIs.

Clade	Exp	m'	$\pm$	nw	$\pm$	K''_w	$\pm$	LC50 (nmol/L)	$\pm$	LC25 (nmol/L)	$\pm$	r^2
1	Combined	0.034	0.020	4.128	6.806	424.505	1046.622	**433.724**	**91.834**	**293.068**	**91.287**	0.78
1	A	0.042	0.045	51.638	4915.884	10	961.334	201.656	335.958	147.892	357.977	0.90
1	B	0.032	0.026	2.855	4.902	1000	2720.126	550.566	227.341	353.089	254.298	0.97
1	C	0.029	0.046	100	Set	18.76	4.513	599.374	101.27	464.258	84.38	0.96
8	Combined	0.028	0.017	100	Set	34.462	8.059	**1150.836**	**154.239**	**882.681**	**134.248**	0.89
8	A	0.047	0.035	100	Set	25.212	7.029	853.736	152.762	652.74	128.38	0.95
8	B	0.007	0.003	8.461	34.619	493.716	2679.614	1062.29	234.341	789.687	213.613	0.93
8	C	0.037	0.027	6.16	10.114	493.716	2527.284	1479.304	179.372	1071.623	145.078	0.98
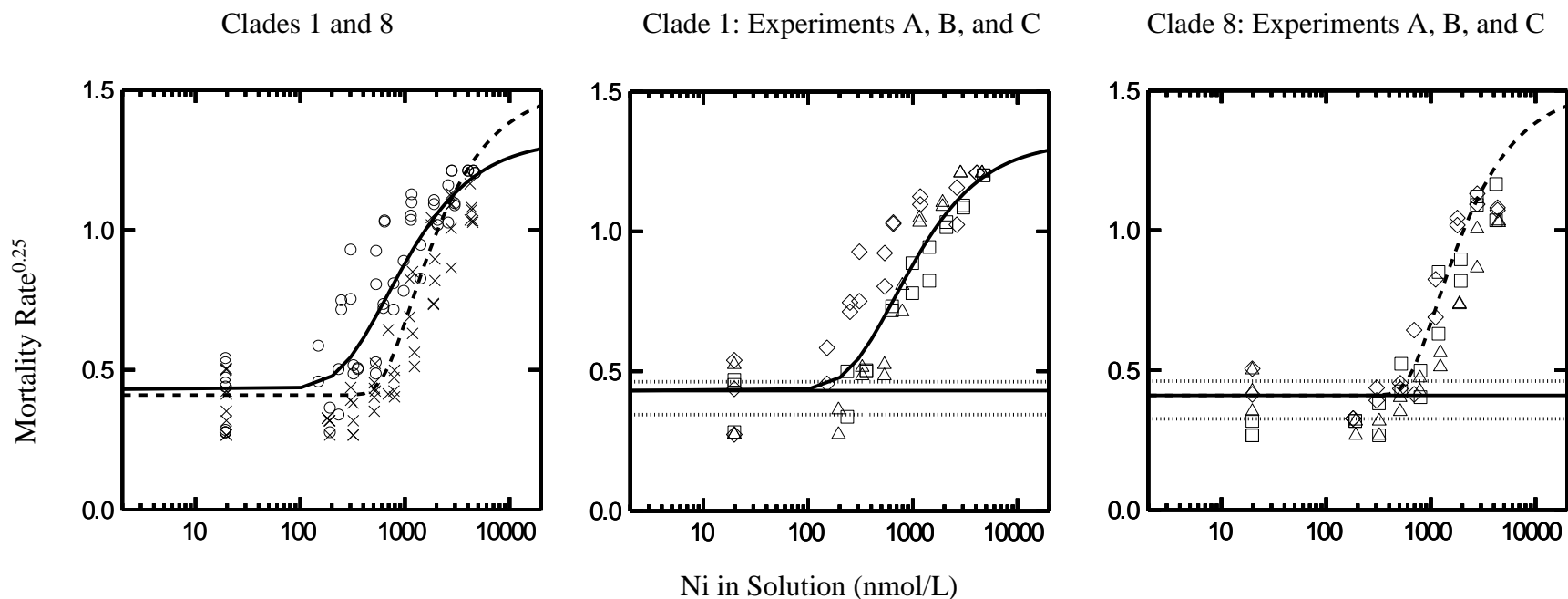
Figure 4-1: The relationship between fourth root transformed mortality rates against measured nickel concentrations in experimental water from 28-day toxicity tests. Clades 1 ($\circ$) and 8 ($\times$) were modeled with the average control mortality for each clade to fit the nonlinear regression since measured water concentrations are below detection limit. Controls are plotted on detection limit of 19.7 nmol/L. The regressions for Clades 1 and 8 were fit using a saturation-based mortality model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control mortality, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A ($\Diamond$), B ($\square$), C (Δ)

Mortality rates and nickel concentration in the tissues followed a similar trend to that observed for nickel exposure in solution. It is to be noted that the data in Table 4-2 are the estimated values based on corrected body concentrations of nickel, and the control data are not included in the modeling. This is because the background correction involved subtracting the measured nickel concentration present in the control amphipods from that present in all treatments. Therefore, the average control mortality for each clade is reported for *m'* rather than the estimated values. However, the measured control tissue concentrations were plotted in Figure 4-2 because they were all above the detection limit.

The Clade 1 mortality rate begins to increase at approximately 110 nmol/g and plateaus at 1000 nmol/g (Figure 4-2). The estimated LBC50 and LBC25 given by the nonlinear regression were 358 (95% CI 241 – 476) and 231.727 nmol/g (95% CI 136 – 328), respectively. The relationship between mortality rates and concentrations of nickel in the tissues had a lower fit than observed for metal present in solution. The r^2 ranges for Clade 1 were between 0.29 and 0.62 for all experiments and the combined data. All of the experiments for Clades 1 and 8 required *nb* to be set to 100.

Clade 8 data fit the model better slightly, with r^2 values ranging between 0.51 and 0.92. Also, the combined LBC50 and LBC25s were slightly higher at 425 (95% CI 283 – 566) and 271 nmol/g (95% CI 192 – 351), respectively. Although Clade 8 has lower mortality rates at the same concentrations as Clade 1, the point at which mortality rate increases and plateaus is very similar at around 110 nmol/g and 1000 nmol/g, respectively. Furthermore, the two clades have overlapping confidence intervals when assessing their nickel LBC50 and LBC25s, and thus there may not be a significant difference between them.

Table 4-2: Estimated nickel parameters for Clades 1 and 8 from individual (A, B, C) and combined experiments when assessing mortality rate against measured corrected body concentration. The control mortality (m'), body exponent (nb), half saturation constant (K''_{TB}), LBC50, LBC25, and model fit (r^2) can be seen below with their respective 95% CIs ($\pm$).

| Clade	Exp	m'	$\pm$	nb	$\pm$	K''_{TB}	$\pm$	LBC50 (nmol/g)	$\pm$	LBC25 (nmol/g)	$\pm$	r^2
1	Combined	0.034	0.020	100	Set	5.89	4.99	358.230	117.323	231.727	96.175	0.37
1	A	0.042	0.045	100	Set	4.913	4.452	237.021	99.798	165.179	86.783	0.61
1	B	0.032	0.026	100	Set	11.427	25.210	449.111	523.105	331.209	468.236	0.62
1	C	0.029	0.046	100	Set	1.671	16.809	470.579	2911.12	134.608	1253.98	0.29
8	Combined	0.028	0.017	100	Set	6.752	4.491	424.701	141.473	271.471	79.333	0.51
8	A	0.047	0.035	100	Set	6.758	3.756	238.414	38.452	180.56	37.267	0.92
8	B	0.007	0.003	100	Set	7.359	7.08	283.96	97.067	210.397	83.379	0.65
8	C	0.037	0.027	100	Set	12.439	10.524	565.212	155.206	400.811	124.946	0.81
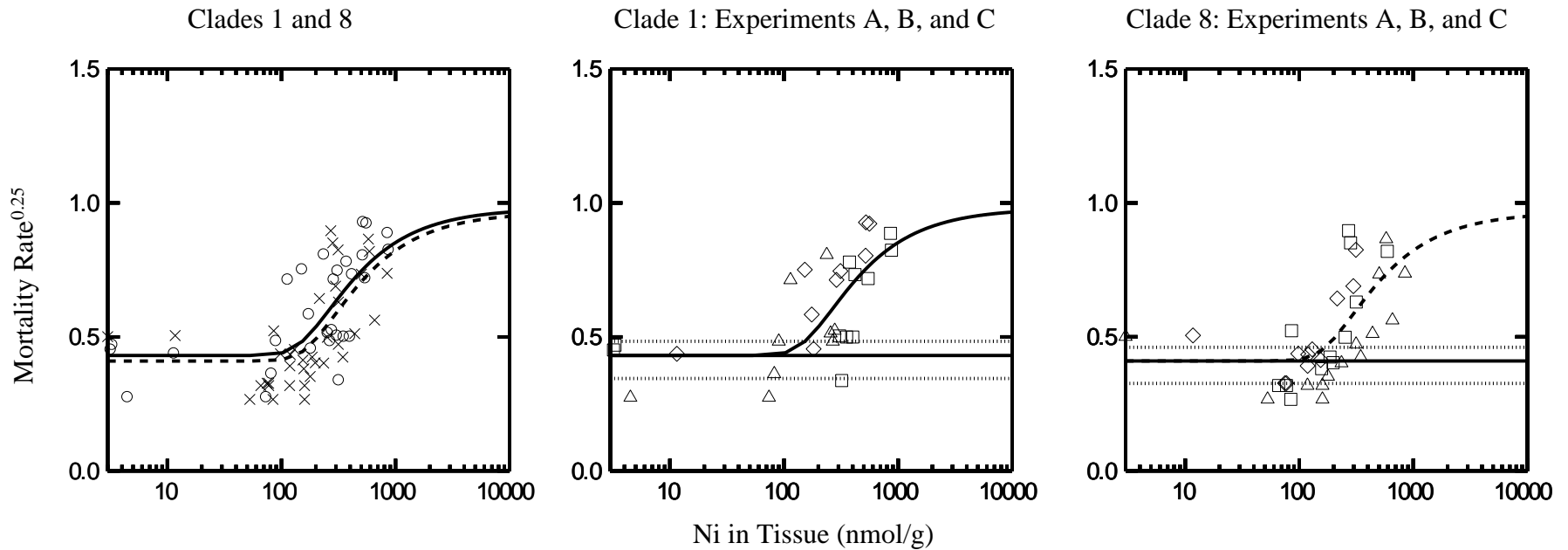
Figure 4-2: The relationship between fourth root transformed mortality rates against measured nickel concentrations in amphipod tissues digested after 28 days. Clades 1 ($\circ$) and 8 ($\times$) were modeled with the average control mortality for each clade set in the equation. Therefore, measured control tissue concentrations were plotted instead of estimates. The regressions for Clades 1 and 8 were fit using a saturation-based mortality model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control mortality, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A ($\Diamond$), B ($\square$), C (Δ)

Bioaccumulation

Similar to mortality, each clade had their respective experimental data pooled together. The *max* and *K* values could not be estimated accurately since the 95% confidence intervals were too wide. Therefore, the term *max*/*K* was computed in Systat 10 with the "funpar" command, and Equation 3-2 was used instead of Equation 3-1. In addition, a background term could not be estimated because the amount of nickel measured in control water was below the detection limit of 19.7 nmol/L. Therefore, the control data were not included in the modeling, but the average measured tissue concentration was included in Figure 4-3.

The concentration of nickel present in the tissues increased as the exposure concentration increased. A positive background concentration for nickel was determined for both Clades 1 and 8 at 2.2 nmol/g and 3.2 nmol/g, respectively. The reported mean corrected R-square from Systat 10 for Clade 1 was 0.16, and this is significantly lower than the r^2 of Clade 8, which is 0.79. There was also a significant difference observed between Clades 1 and 8 *max*/*K* values since the confidence intervals did not overlap; Clade 1 *max/K* = 0.704 (95% CI 0.518 – 0.890), whereas Clade 8 *max/K* = 0.319 (95% CI 0.282 – 0.355). However, it is likely that no significant difference between the two clades for C_{BK} is present since the confidence intervals are overlapping considerably, and the mean values are very similar.

Table 4-3: Mean measured background nickel concentration (C_{BK}), the estimated maximum nickel accumulated relative to the half saturation constant (*max/K*) , model fit (r^2), and their respective 95% confidence intervals. The estimates were computed using a saturation bioaccumulation curve. Bold faced text indicate significant differences based on non-overlapping CIs.

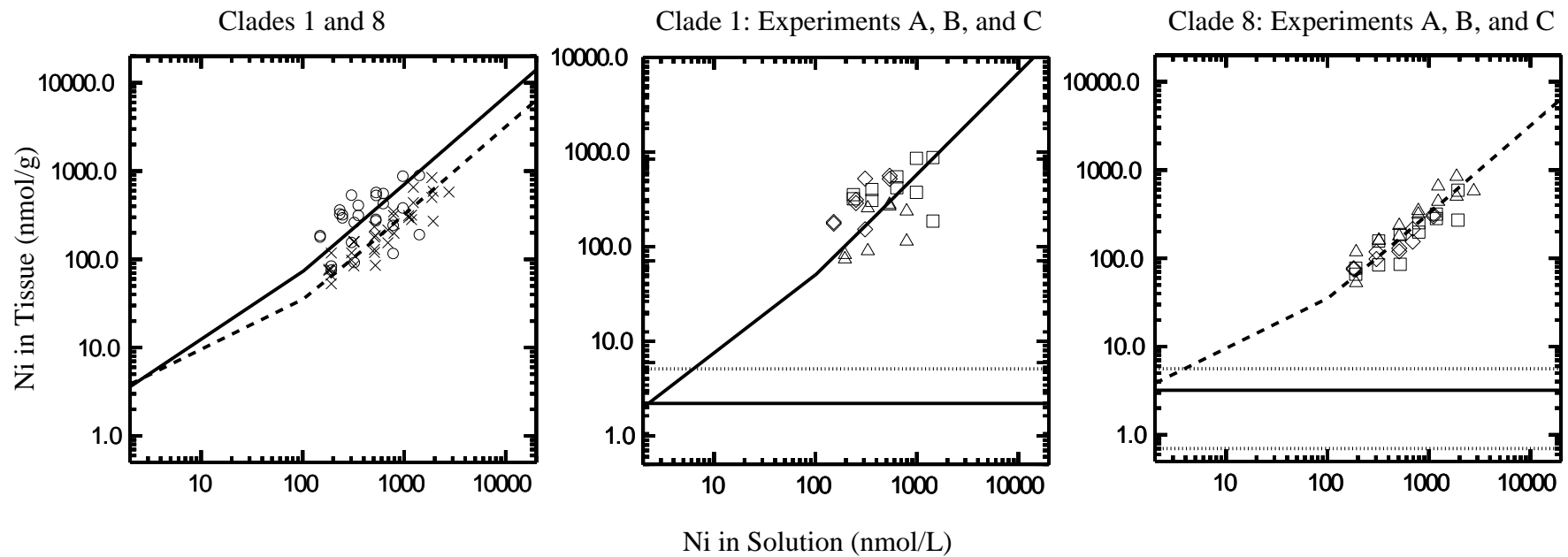| Clade	Exp	C_{BK} (nmol/g)	±	*max/K* (L/g)	±	r^2
1	Combined	2.2	2.874	**0.704**	**0.186**	0.16
8	Combined	3.2	2.466	**0.319**	**0.037**	0.79

Figure 4-3: The relationship between measured nickel concentrations in amphipod tissues digested after 28 days and measured nickel concentrations in the experimental water. Clades 1 (○) and 8 (×) were modeled without any control data, and thus only replicates spiked with nickel are plotted. The regressions for Clades 1 and 8 were fit using a saturation bioaccumulation model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control tissue concentration, while the horizontal dotted lines signifies the 95% confidence interval. The lower confidence interval of mean control tissue concentration cannot be seen for Clade 1 (-0.7 nmol/g). Experiments: A (◊), B (□), C (Δ)

Dry Weights

The total dry weight measured for each replicate was divided by the amount of amphipods weighed and modeled against the amount of nickel in solution. As with the other models, the control data could not be used since the measured nickel in the control water was below the detection limit of 19.7 nmol/L. Therefore, control data were excluded and the nonlinear regressions were modeled by setting the average control dry weight for W'.

Clade 8 had significantly larger control dry weights than Clade 1, and this was consistent with the two-sample t-Test reported in Chapter 2. Although Clade 8 animals were significantly larger at the control and low concentrations, Figure 4-4 depicts these amphipods to drop in weight at a faster rate than Clade 1. The latter appears to decrease gradually in dry weight starting at about 110 nmol/L, whereas Clade 8 has a more pronounced drop in mass at 250 nmol/L. Also, the two clades have a similar dry weight at approximately 2500 nmol/L.

The estimated IC25s for Clades 1 and 8 were 193 (95% CI 97 – 289) and 364 (95% CI 294 – 435) nmol/L, respectively. Based on the non-overlapping confidence intervals of the combined data, the two clades are significantly different. However, if individual experiments are compared to each other (e.g. Clade 1 Experiment B compared to Clade 8 Experiment B), the two clades have overlapping confidence intervals for experiments B and C. The model fit for all Clade 8 data (pooled and individual experiments) ranged from 0.84 – 0.95. In contrast, the model did not fit the data as strongly for some Clade 1 experiments since the ranges were between 0.57 and 0.86.

Table 4-4: Estimated nickel parameters for Clades 1 and 8 from individual (A, B, C) and combined experiments when assessing dry weight against measured nickel concentration in solution. The control dry weight (W'), exponents (a and n_w), IC25, and model fit (r^2) are given below with their respective 95% CIs (±). Bold faced text indicate significant differences based on non-overlapping CIs.

Clade	Exp	W'	±	a	±	n_w	±	IC25 (nmol/L)	±	r^2
1	Combined	0.152	0.022	5.40E-04	1.78E-03	1.221	0.524	**193.188**	**96.201**	0.57
1	A	0.154	0.004	3.00E-06	2.80E-05	2.238	1.756	187.279	104.256	0.74
1	B	0.126	0.008	7.41E-04	3.71E-03	1.123	0.753	230.754	202.775	0.68
1	C	0.176	0.058	6.00E-05	2.84E-04	1.541	0.754	269.789	106.476	0.86
8	Combined	0.230	0.032	7.78E-06	1.80E-05	1.808	0.343	**364.322**	**70.421**	0.88
8	A	0.208	0.003	2.00E-06	1.10E-05	2.070	1.088	379.257	149.387	0.84
8	B	0.216	0.076	1.00E-06	2.00E-06	2.131	0.540	451.501	104.300	0.95
8	C	0.259	0.035	2.10E-05	7.80E-05	1.670	0.542	328.102	128.882	0.90
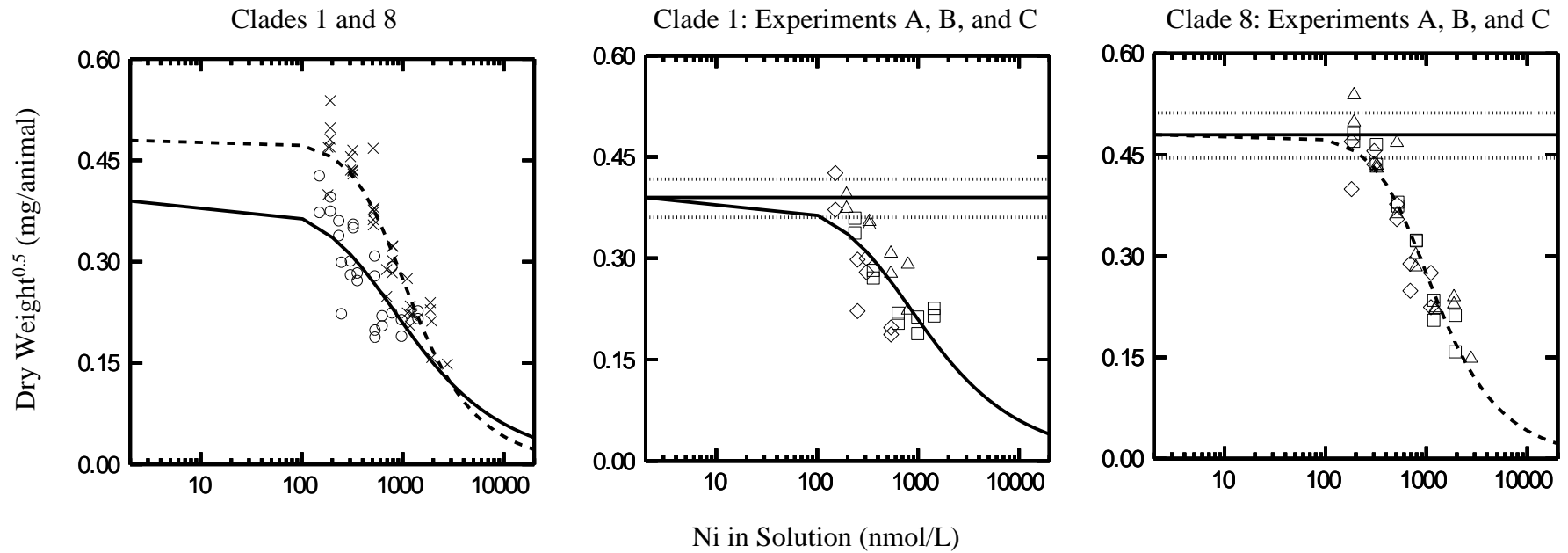
Figure 4-4: The relationship between square-root transformed total dry weight and measured nickel concentrations in the experimental water after 28 days. Clades 1 (○) and 8 (×) were modeled without any control data, and thus only replicates spiked with nickel are plotted. The regressions for Clades 1 and 8 were fit using the general growth model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control dry weight, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A (◊), B (□), C (Δ)

Similar to the effects of nickel concentration on growth, the amount of metal present in the tissues reduced the dry weight of the amphipods. As with the growth against measured nickel in solution, Clade 8 amphipods start off at a higher dry weight, which rapidly decreases as tissue concentrations increase, whereas Clade 1 animals have a gradual reduction with nickel accumulation. In Figure 4-5, it is observed that both clades have a similar dry weight at 300 nmol/g, but Clade 8 has lower dry weight at tissue concentrations beyond this point.

Again, Clade 1 data does not fit the model well with r^2 values ranging from 0.44 – 0.86, whereas Clade 8 ranges from 0.71 – 0.90 (Table 4-5). The IBC25 estimates for Clades 1 and 8 are 154 (95% CI 59 – 249) and 112 nmol/g (95% CI 78 – 146), respectively. The confidence intervals for the combined data and individual experiments for both clades are overlapping, and thus there may not be a significant difference between the two. This is apparent in Figure 4-5 as the two clades begin to decrease in dry weight when approximately 70 nmol/g of nickel is present in their tissues.

Table 4-5: Estimated parameters for Clades 1 and 8 from individual (A, B, C) and combined experiments when assessing dry weight against measured tissue concentration. The control dry weight (W'), exponents (a and nb), IBC25, and model fit (r^2) are given below with their respective 95% CIs ($\pm$).

| Clade	Exp	W'	$\pm$	a	$\pm$	nb	$\pm$	IBC25 (nmol/g)	$\pm$	r^2
1	Combined	0.146	0.023	5.36E-4	2.49E-3	1.277	0.783	153.908	95.038	0.44
1	A	0.149	0.007	9.00E-06	6.80E-05	2.308	1.366	178.222	89.541	0.78
1	B	0.123	0.015	3.78E-04	2.26E-03	1.213	0.893	267.004	236.771	0.66
1	C	0.167	0.064	1.29E-07	8.71E-07	2.462	1.577	403.335	138.422	0.86
8	Combined	0.259	0.033	5.10E-05	1.65E-04	1.862	0.585	112.100	34.114	0.71
8	A	0.282	0.076	1.82E-04	6.80E-04	1.761	0.734	71.218	27.180	0.83
8	B	0.219	0.045	3.13E-07	2.69E-06	2.252	1.041	474.194	191.560	0.88
8	C	0.284	0.040	4.00E-06	1.80E-05	1.922	0.671	370.577	142.470	0.90
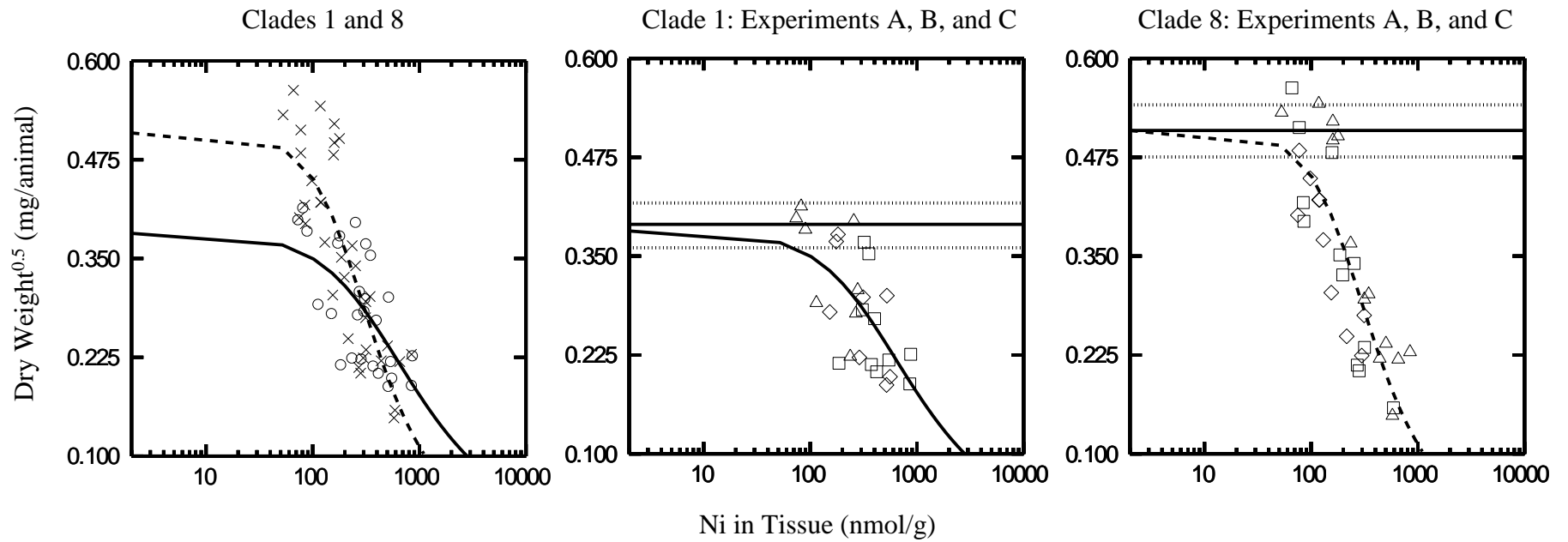
Figure 4-5: The relationship between square-root transformed digested dry weight and measured nickel tissue concentrations after 28 days. Clades 1 (○) and 8 (×) were modeled without any control data, and thus only replicates spiked with nickel are plotted. The regressions for Clades 1 and 8 were fit using the general growth model, and are depicted by a solid and dashed line, respectively. The solid horizontal line represents the measured mean control dry weight, while the horizontal dotted lines signifies the 95% confidence interval. Experiments: A (◊), B (□), C (Δ)

Discussion

Mortality was the most sensitive endpoint when comparing toxicity between Clades 1 and 8. The estimated LC50 and LC25 for the latter clade was 2.65 and 3.01 times greater than the former, respectively. However, LBC50 and LBC25 estimates between the two clades were very similar and may not be significantly different based on overlapping confidence intervals. These results suggest that Clade 8 amphipods accumulate nickel at a slower rate, but experience mortality at similar tissue concentrations as Clade 1. This is supported by the bioaccumulation data in that Clade 1 animals accumulated more nickel in the tissues in comparison to Clade 8 at the same concentrations (Figure 4-3). Despite this, the amphipods belonging to both clades had IBC25 estimates that were very similar, and had overlapping confidence intervals. This indicates that a reduction in growth occurred at similar concentrations of nickel present in the tissues. However, Clade 8 amphipods had an estimated IC25 that was greater than the value computed for Clade 1 animals, and is considered significant due to non-overlapping confidence intervals.

Borgmann et al. (2001) reported three nickel water-only LC50s at 462, 578, and 655 nmol/L, and three LC25 estimates at 255, 186, and 540 nmol/L, respectively. These estimates were based on LC50s estimated for sediment. The researchers linearly regressed the measured nickel in water against the amount of metal in sediment, and then used these estimates to convert their sediment based LC50s to values that are water concentration based. Borgmann et al. (2004) re-evaluated the data from Borgmann et al. (2001) using a saturation model, and reported an LC50 and LC25 of 576 (95% CI 504 – 659) and 400 nmol/L (95% 325 – 493), respectively.

Similarly, three LBC50 and LBC25 water-only values are reported (LBC50= 252, 375, 378; LBC25= 182, 134, 315). These estimates are for amphipods that were not gut cleared, and therefore Borgmann et al. (2001) assessed the uptake and depuration rates to estimate what the

gut cleared LBC50 and LBC25 would be. They observed that about 40% of the nickel is lost

after 24 h, and therefore the LBC50 and LBC25 would be equal to 60% of the original value.

Borgmann et al. (2004) re-evaluated the LBC50 and LBC25 by pooling the water-only and

sediment data together in a single saturation model. This yielded LBC50 and LBC25 values of

405 (95% CI 355 – 463) and 281 nmol/g (228 – 347), respectively.

It is known that Clade 1 has been used in Burlington (Major et al., 2013), and therefore

the endpoints for that lineage presented in this thesis will be compared. The LC50 and LC25 that

I determined were 434 (95% CI 342 – 526) and 293.068 nmol/L (95% CI 202 – 384),

respectively. Although the LC50 reported in this thesis is slightly lower than the value reported

by Borgmann et al. (2001), the confidence intervals of the two estimates overlap. However, the

LBC50 and LBC25 estimated in this thesis are much more consistent with Borgmann et al.

(2001), and have almost identical confidence intervals. The estimates of the LBC50 and LBC25

reported here are 358 (95% CI 241 – 476) and 231.727 nmol/L (95% CI 136 – 328), respectively.

Although this comparison is between gut cleared (this study) and non-gut-cleared amphipods, it

is still valid since Borgmann et al. (2001) state that gut clearance is a major factor when

assessing bioaccumulation between sediment toxicity tests, but is not as important when

conducting water-only experiments.

Keithly et al. (2004) report an LBC20 of 247 nmol/g wet weight when conducting a 14

day toxicity test with *Hyalella azteca.* Although it is unknown which clade was used, the results

the researchers obtained are very similar to the LBC25s of both Clades 1 and 8 estimated in this

study. Also, this consistency supports the notion that body concentrations are better predictors of

metal toxicity than using water alone, and can be compared to other experiments that differ in

methodology (Borgmann et al., 1998, 1991; Borgmann & Norwood, 1997a; Keithly et al., 2004).

Clades 1 and 8 had similar background concentrations of nickel at 2.2 and 3.2 nmol/g, respectively. These measured values are slightly lower than the estimate of 7.55 nmol/g reported by Borgmann et al. (2004), and 21.5 nmol/g reported by Leung (2014). However, the former study did not gut clear the amphipods and this slight difference could be due to the fact that nickel could have bound to food particles and remained in the gut. The discrepancy between background tissue concentrations reported here in comparison to Leung (2014) cannot be explained since experimental methods were almost identical except for duration and exposure concentrations. The most likely explanation is contamination, as the researcher states the background concentrations ranged from 2 to 41 nmol/g. Therefore, the lower range is consistent with this thesis.

The *max* and *K* could not be estimated by the model, and thus the ratio of *max/K* was determined instead. This is similar to previous literature (Borgmann et al., 2004; Leung, 2014) in which the model does not level off (Figure 4-3), and thus a maximum cannot be determined. The *max/K* determined in this study were 0.704 (95% CI 0.518 – 0.890) and 0.319 (95% CI 0.282 – 0.355) for Clades 1 and 8, respectively. Borgmann et al. (2004) reported a *max/K* ratio of 0.70 (95% CI 0.59 – 0.84), which is almost identical in value and confidence interval for the ratio determined in this thesis for Clade 1. However, the *max/K* determined by Leung (2014) is 2.6 times lower than this study and Borgmann et al. (2004). This could be due to the difference in experimental conditions since Borgmann et al. (2004) and this study are assessing endpoints after 4 weeks, in comparison to the 2 week duration conducted by Leung (2014). In addition, Clade 1 had a *max/K* ratio that was 2.21 times higher than Clade 8, which is significantly different based on non-overlapping confidence intervals. This was not observed by Leung (2014), who reported no significant difference between the two clades. Again, this is likely due to the duration of the

study and exposure concentrations – Leung (2014) exposed the amphipods to much higher concentrations for a shorter period of time.

Earlier, it was mentioned that the results presented in this thesis suggest Clades 1 and 8 accumulate nickel at different rates. This is because Clade 8 has less mortality at higher nickel water concentrations, but similar mortality at the same tissue concentrations as Clade 1. However, this implication contrasts with the work done by Leung (2014), who observed a significant difference between Clades 1 and 8 for both water and body concentration mortality. She determined that Clade 8 had an LBC50 and LBC25 that were 2.1 and 2.66 times higher than Clade 1, respectively. Leung's (2014) results suggest that Clade 8 is more tolerant to nickel than Clade 1 due to higher LBC50 and LBC25 estimates, and that the two clades accumulate nickel at the same rate.

Interestingly, the LC50, LC25, LBC50, and LBC25 reported by Leung (2014) were much greater than the estimates in this study by as much as 4.5 times. Therefore, the difference in observations could be due to the exposure concentrations and duration in which the tests were conducted. The highest nominal concentration used in this study was 4896 nmol/L, which is the second highest concentration used in the work done by Leung (2014). At two weeks there are still Clade 8 amphipods that survive at 4896 nmol/L (data not shown). Thus, if Leung's (2014) test duration was only two weeks long, there would be amphipods available for tissue assessments. This is likely the explanation as to why the reported LBC50 and LBC25 for Clade 8 by Leung (2014) are much higher than the estimated values in this study, as well as Borgmann et al. (2004).

Clade 8 control amphipods were significantly larger than Clade 1, and this also contrasts with the results reported by Leung (2014), where Clade 1 was observed to be significantly larger than Clade 8. However, the controls were weighed at the end of 4 weeks in comparison to Leung (2014), who weighed the animals after two weeks. Since the test durations were different, comparing the point at which a decrease in dry weight is observed is more plausible.

When assessing Figure 4-9 in the work conducted by Leung (2014), there are consistent trends with the results in this study. Clade 8 has a steeper drop in dry weight that appears around 250 nmol/L, while Clade 1 has a gradual decrease at around 110 nmol/L. Although Clade 8 had a steeper drop, the amphipods also started off at a higher dry weight. Based on non-overlapping confidence intervals the estimated IC25 for Clade 8 is significantly different, and is 1.89 times higher than that of Clade 1. Leung (2014) observed an IC25 for Clade 8 that was 1.6 times higher than that of Clade 1, but no significant difference may be present due to overlapping confidence intervals.

Similar trends are observed when assessing nickel concentration in tissue and dry weight. Leung (2014) observed Clade 8 amphipods to drop in weight at 70 nmol/g, while Clade 1 gradually decreases, and the same result was reported here. Interestingly, the IBC25 estimates reported by Leung (2014) for Clades 1 and 8 are 83.3 (95% CI ± 202) and 190 nmol/g (95% CI ± 226), respectively. These values are very similar to the values presented in this thesis, which are 154 (95% CI 59 – 249) and 112 nmol/g (95% CI 78 – 146), respectively. In both studies, the IBC25s between clades have overlapping confidence intervals, and thus no significant difference may be present. This once again supports that body concentrations are more consistent at predicting effects on growth than water concentrations alone (Borgmann et al., 1998, 1991; Borgmann & Norwood, 1997a).

Conclusion

Clade 8 demonstrated a higher tolerance to nickel when assessing mortality rate based on metal exposure in comparison to Clade 1 by 2.65-3.01 times. However, the two clades were observed to have no significant differences in LBC50 and LBC25s. This was also the case when assessing growth and amount of nickel present in the tissues. In contrast, there was a significant difference observed for metal exposure relative to growth, whereby the inhibitory concentration to reduce Clade 8 amphipod dry weights was higher than that for Clade 1 animals. Also, it was determined that Clade 1 accumulated nickel in tissues at a rate that was significantly higher than that of Clade 8. Therefore, the results of this chapter support the notion that laboratory organisms should be genetically characterized prior to conducting toxicity tests (Leung, 2014).

Chapter 5 – Implications, Conclusion, and Future Directions

Two large-bodied ecomorphs within the *Hyalella azteca* species complex were evaluated for their sensitivities to 4-week copper and nickel water-only toxicity tests. These clades were exposed to the same experimental conditions, but differences were observed when assessing their growth, mortality, and bioaccumulation. Clade 8 had a significantly higher dry weight than Clade 1, despite both clades being large-bodied ecomorphs (Wellborn and Broughton, 2008). Although the former was heavier, the two clades had similar sensitivity to copper spiked water when assessing growth. However, Clade 8 demonstrated a higher tolerance to copper present in the tissues before a reduction in growth occurred. This was not the case for growth effects in relation to nickel body concentrations, as both clades had similar sensitivity. However, when assessing nickel concentrations in solution, Clade 8 was more tolerant to the metal than Clade 1 when the combined data had been compared, but not when comparing two individual experiments.

Clade 1 amphipods demonstrated significantly higher mortality at lower concentrations of copper and nickel in solution than Clade 8. Also, the latter exhibited a higher tolerance to the amount of copper present in the tissues before mortality increased, which was supported by the bioaccumulation data. Clade 8 had a significantly higher estimated maximum body concentration than Clade 1, but this was not the case for nickel as both clades were observed to have increased mortality rates at similar body concentrations. However, Clade 8 had a significantly lower ratio of the maximum nickel accumulated relative to the half saturation constant (*max/K*), which supports the observation that these amphipods had lower mortalities at higher exposure concentrations. The implication derived from these results is that Clade 8 may be accumulating nickel at a slower rate, or some of the nickel could be depurated. Currently, no studies have quantified the kinetics for nickel accumulation in Clade 8 amphipods, other than Borgmann et al.

(2001). They observed Clade 1 amphipods to lose 40% of the metal in their bodies after a 24 hour gut clearance in their water-only tests. Therefore, future studies with Clades 1 and 8 should further explore the details of nickel uptake in these two lineages.

Leung (2014) determined that Clade 8 had different sensitivity to copper and nickel exposure, but similar bioaccumulation patterns to Clade 1. The researcher suggested that Clade 8 may have a better copper regulation method than Clade 1, and that metallothioneins may be involved since Geffard et al. (2010) looked at the relationship between these proteins and nickel removal in *Gammarus fossarum*. Although this study does not look at metallothioneins, the implication by Leung (2014) is expanded upon – Clade 8 appears to have a better mechanism at regulating copper. Both clades have the same background concentrations, but Clade 8 is capable of accumulating copper to a higher body concentration and maintaining it. Also, Clade 8 requires a higher body concentration before mortality starts to increase, and dry weight starts to decrease.

Although Clade 8 has a higher LC50, LBC50, and IBC25 than Clade 1 for copper, the IC25 is similar. This could indicate that the rate at which copper is regulated by Clade 8 may be the same as Clade 1. Since the latter accumulates less and requires a lower body concentration to increase mortality and reduce dry weight, the ratio of copper accumulated and excreted could be similar to Clade 8. Borgmann (1998) proposed a simple mechanistic model for copper uptake in *Hyalella*, where external metal enters the tissue and binds to a ligand (X) (See Chapter 3). This ligand is hypothesized to bind all the non-essential copper (MX) that can later be supplied to essential macromolecules (E) to form functional complexes (ME).

Borgmann (1998) proposed that the most likely reason for a reduction in MX is due to the synthesis of X being halted. This could potentially explain the difference between the two

100

lineages – Clade 8 may be capable of synthesizing more ligand X initially. More MX complexes can form if there are more ligands present to bind the free metal, and thus higher concentrations of copper would be required to reach mortality. This is because Borgmann (1998) suggests that toxicity is due to the internal copper that is loosely bound or free, rather than the concentration of MX since it is able to decline over time. This suggests that depuration tests should be conducted with these two clades to further investigate whether or not Clade 8 synthesizes more ligands, or has a different uptake and elimination rate.

Also, this implication could be applicable to small ecomorphs that have not been studied. Borgmann (1998) based his model on previous observations that copper body concentrations are independent of body size, a maximum steady-state is reached with increasing metal present in the water, and that the uptake is faster than depuration in clean water (Borgmann & Norwood, 1995b). The first two observations have been consistent with this thesis, where an increase in copper water concentrations results in a rapid accumulation of metal that is held constant (E saturates and ME remains constant), despite varying body sizes (Borgmann 1998). Therefore, even though two large-bodied ecomorphs had a similar reduced dry weight during chronic copper exposure, there was a significant difference in their copper body concentrations. This begs the question; would a small bodied ecomorph have an even greater discrepancy? Firstly, the small and large ecomorphs need to be assessed to determine whether the final dry weights at the high metal concentrations are similar. If this is observed and the body concentrations are different, it could suggest that the small ecomorphs do not synthesize as much ligand X (Borgmann 1998).

In general, the small-bodied ecomorphs have not been studied in depth. Despite this, these amphipods have been sampled and sequenced in many parts of Canada and the United

States (Matthew Hyrcyshyn, PhD thesis in preparation). However, it is to be noted that many of the small ecomorphs that have been sequenced remain on the eastern and central half of North America, with the exception of one clade that is present in Washington and Oregon. In contrast, the large-bodied ecomorphs have been observed to inhabit a wider range of North America. Interestingly, by observing the distribution maps created by Matthew Hyrcyshyn (PhD in preparation) there is considerable overlap between the two ecomorphs, yet laboratories have only employed Clades 1 and 8. What is more fascinating is that Clade 8 has only been located in Florida, Georgia, Alabama, and Oklahoma, but it is the most widely used laboratory culture (Major et al., 2013; Leung, 2014). This could likely be due to the relative ease of culturing and robustness Clade 8 animals have exhibited in the laboratory.

Recently, only one study by Soucek et al. (2013) has compared a genetically characterized small-bodied ecomorph with Clades 1 and 8 (Major et al., 2013; Wellborn & Broughton, 2008; Witt et al., 2003). However, there have not been any studies that compare heavy metal sensitivity between large-bodied ecomorphs and small-bodied ones. Since increased size affects resource consumption rate and activity level within *Hyalella* species (Wellborn & Cothran, 2004; Wellborn, 1994, 2002), it would be interesting to test whether or not the two ecomorphs have any significant difference in their sensitivity to heavy metals.

Soucek et al. (2013) had mentioned the possibility of metabolic rate and other physiological differences being present between clades. This is supported by Chapman and Reiss (1999), as they mention that body size is not the only factor to affect metabolic rate – genetic factors and life style are also important contributors. Soucek et al. (2013) discovered that their small-bodied ecomorph had a significantly higher LC50 than Clade 1 in 96h fed tests, but the reverse in unfed tests. They note that body size is not the only factor in affecting sensitivity

because in their fed 96-h nitrate tests Clade 8 had the lowest LC50, whereas the small ecomorph had the highest. These results support the notion that *Hyalella* should be genetically characterized prior to conducting toxicity tests since there is significant variability between clades.

Although the significant differences observed in the aforementioned study, Leung (2014), and this thesis are around 1.5 – 3 fold in magnitude, there is little impact when a species sensitivity distribution (SSD) is applied. Also, the variability obtained in the water-only tests may not be reflective of field data where bioavailability of the contaminant may be reduced (Shaw-Allen & Suter, 2016). Thus, the results presented in this thesis are important for site-specific tests that only involve *Hyalella*, but may not be as important from a regulatory standpoint in which multiple species are taken into consideration.

References

Ankley, G. T., Schubauer-Berigan, M. K., & Monson, P. D. (1995). Influence of pH and hardness on toxicity of ammonia to the amphipod Hyalella azteca. *Canadian Journal of Fisheries and Aquatic Sciences*, *2883*, 2878–2883.

Baker, R. J., & Bradley, R. D. (2006). Speciation in Mammals and the Genetic Species Concept. *Journal of Mammalogy*, *87*(4), 643–662.

Besser, J. M., Brumbaugh, W. G., Ingersoll, C. G., Ivey, C. D., Kunz, J. L., Kemble, N. E., … Garman, E. R. (2013). Chronic toxicity of nickel-spiked freshwater sediments: variation in toxicity among eight invertebrate taxa and eight sediments. *Environmental Toxicology and Chemistry / SETAC*, *32*(11), 2495–506. doi:10.1002/etc.2271

Besser, J. M., Brumbaugh, W. G., May, T. W., & Ingersoll, C. G. (2003). Effects of organic amendments on the toxicity and bioavailability of cadmium and copper in spiked formulated sediments. *Environmental Toxicology and Chemistry / SETAC*, *22*(4), 805–815. doi:10.1897/1551-5028(2003)022<0805:EOOAOT>2.0.CO;2

Borgmann, U. (1994). Chronic toxicity of ammonia to the amphipod Hyalella azteca; Importance of ammonium ion and water hardness. *Environmental Pollution (Barking, Essex : 1987)*, *86*(3), 329–335. doi:10.1016/0269-7491(94)90173-2

Borgmann, U. (1996). Systematic analysis of aqueous ion requirements of Hyalella azteca: A standard artificial medium including the essential bromide ion. *Archives of Environmental Contamination and Toxicology*, *30*(3), 356–363. doi:10.1007/BF00212294

Borgmann, U. (1998). A mechanistic model of copper accumulation in Hyalella azteca. *Science of the Total Environment*, *219*(2-3), 137–145. doi:10.1016/S0048-9697(98)00229-0

Borgmann, U., & Borgmann, A. I. (1996). Control of Ammonia Toxicity to Hyalella Azteca by Soduim, Potassium, and pH. *Environmental Pollution*, *95*(3), 325–331.

Borgmann, U., Cheam, V., Norwood, W. P., & Lechner, J. (1998). Toxicity and bioaccumulation of thallium in Hyalella azteca, with comparison to other metals and prediction of environmental impact. *Environmental Pollution*, *99*, 105–114. doi:10.1016/S0269-7491(97)00181-4

Borgmann, U., Néron, R., & Norwood, W. P. (2001). Quantification of bioavailable nickel in sediments and toxic thresholds to Hyalella azteca. *Environmental Pollution*, *111*(2), 189–198. doi:10.1016/S0269-7491(00)00076-2

Borgmann, U., & Norwood, W. P. (1995a). EDTA toxicity and background concentrations of copper and zinc in Hyalella azteca. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*(4), 875–881. doi:10.1139/f95-087

Borgmann, U., & Norwood, W. P. (1995b). Kinetics of excess background copper and zinc in Hyalella azteca and their relationship to chronic toxicity. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*, 864–874.

Borgmann, U., & Norwood, W. P. (1997a). Identification of the toxic agent in metal-contaminated sediments from Manitouwadge Lake, Ontario, using toxicity-accumulation relationships in Hyalella azteca. *Can. J. Fish. Aquat. Sci*, *54*, 1055–1063.

Borgmann, U., & Norwood, W. P. (1997b). Toxicity and accumulation of zinc and copper in Hyalella azteca exposed to metal-spiked sediments. *Canadian Journal of Fisheries and Aquatic Sciences*, *54*(5), 1046–1054. doi:10.1139/cjfas-54-5-1046

Borgmann, U., Norwood, W. P., & Babirad, I. M. (1991). Relationship between Chronic Toxicity and Bioaccumulation of Cadmium in Hyalella azteca. *Canadian Journal of Fisheries and Aquatic Science*, *48*, 1055–1060.

Borgmann, U., Norwood, W. P., & Clarke, C. (1993). Accumulation, regulation and toxicity of copper, zinc, lead and mercury in Hyalella azteca. *Hydrobiologia*, *259*, 79–89.

Borgmann, U., Norwood, W. P., & Dixon, D. G. (2004). Re-evaluation of metal bioaccumulation and chronic toxicity in Hyalella azteca using saturation curves and the biotic ligand model. *Environmental Pollution*, *131*(3), 469–84. doi:10.1016/j.envpol.2004.02.010

Borgmann, U., Nowierski, M., & Dixon, D. G. (2005). Effect of major ions on the toxicity of copper to Hyalella azteca and implications for the biotic ligand model. *Aquatic Toxicology*, *73*(3), 268–287. doi:10.1016/j.aquatox.2005.03.017

Boyle, R., & Robinson, H. A. (1988). Nickel in the Natural Environment. In H. Sigel & A. Sigel (Eds.), *Metal Ions in Biological Systems: Volume 23: Nickel and its Role in Biology* (p. 496). CRC Press. Retrieved from https://books.google.ca/books?id=pmn1O78IbDIC

Bozkaya, L. A., Öztürk, R., Aydemir, T., & Tarhan, L. (2001). Effects of antioxidant vitamins A, C, E and trace elements Cu, Se on CuZnSOD, GSH-Px, CAT and LPO levels in chicken erythrocytes. *Cell Biochemistry and Function*, *18*(2), 109–115. doi:10.1002/(SICI)1099-0844(200006)18:2<109::AID-CBF861>3.0.CO;2-2

Chapman, J. L., & Reiss, M. J. (1999). *Ecology: Principles and Applications*. Cambridge University Press. Retrieved from https://books.google.ca/books?id=PoRP0g2Jh9YC

Chapman, P. M., Wang, F., Janssen, C., Persoone, G., & Allen, H. E. (1998). Ecotoxicology of metals in aquatic sediments: binding and release, bioavailability, risk assessment, and remediation. *Canadian Journal of Fisheries and Aquatic Sciences*, *55*(10), 2221–2243. doi:10.1139/f98-145

Cohu, C. M., & Pilon, M. (2010). *Cell Biology of Metals and Nutrients*. (R. Hell & R. R. Mendel, Eds.). Springer Berlin Heidelberg. Retrieved from https://books.google.ca/books?id=fxRjgmjcp_EC

Couillard, Y., Grapentine, L. C., Borgmann, U., Doyle, P., & Masson, S. (2008). The amphipod Hyalella azteca as a biomonitor in field deployment studies for metal mining. *Environmental Pollution (Barking, Essex : 1987)*, *156*(3), 1314–24. doi:10.1016/j.envpol.2008.03.001

Davis, J. R., & Committee, A. S. M. I. H. (2001). *Copper and Copper Alloys*. ASM International. Retrieved from https://books.google.ca/books?id=sxkPJzmkhnUC

Di Toro, D. M., Allen, H. E., Bergman, H. L., Meyer, J. S., Paquin, P. R., & Santore, R. C. (2001). Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environmental Toxicology and Chemistry / SETAC*, *20*(10), 2383–2396. doi:10.1897/1551-5028(2001)020<2383:blmota>2.0.co;2

Doig, L. E., & Liber, K. (2006). Influence of dissolved organic matter on nickel bioavailability and toxicity to Hyalella azteca in water-only exposures. *Aquatic Toxicology (Amsterdam,*

Netherlands), *76*(3-4), 203–16. doi:10.1016/j.aquatox.2005.05.018

Doig, L. E., & Liber, K. (2007). Nickel speciation in the presence of different sources and fractions of dissolved organic matter. *Ecotoxicology and Environmental Safety*, *66*(2), 169–177. doi:10.1016/j.ecoenv.2005.12.011

Duan, Y., Guttman, S. I., & Oris, J. T. (1997). Genetic Differentiation Among Laboratory Populations of Hyalella azteca : Implications for Toxciology. *Environmental Toxicology and Chemistry*, *16*(4), 691–695.

Duan, Y., Guttman, S. I., Oris, J. T., & Bailer, A. J. (2000a). Genetic structure and relationships among populations of Hyalella azteca and H . montezuma (Crustacea : Amphipoda). *Journal of the North American Benthological Society*, *19*(2), 308–320.

Duan, Y., Guttman, S. I., Oris, J. T., & Bailer, J. a. (2000b). Genotype and Toxicity Relationships Among Hyalella azteca: I. Acute Exposure to Metals or Low pH. *Environmental Toxicology and Chemistry*, *19*(5), 1414. doi:10.1897/1551-5028(2000)019<1414:GATRAH>2.3.CO;2

Elder, J. F., & Horne, A. J. (1978). Copper cycles and CuSO4 algicidal capacity in two California lakes. *Environmental Management*, *2*(1), 17–30. doi:10.1007/BF01866443

Environment Canada. (2007). *Guidance Document on Statistical Methods for Environmental Toxicity Tests* (EPS 1/RM/4). Ottawa, Ontario.

Environment Canada (EC). (2013). *Biological Test Method : Test for Survival and Growth in Sediment Using the Freshwater Amphipod Hyalella azteca*. *Environmental Technology* (Second). Ottawa: Method Development and Applications Unit. doi:EPS 1/RM/33

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.

Gaetke, L. M., & Chow, C. K. (2003). Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology*, *189*(1-2), 147–163. doi:10.1016/S0300-483X(03)00159-8

Geffard, A., Sartelet, H., Garric, J., Biagianti-Risbourg, S., Delahaut, L., & Geffard, O. (2010). Subcellular compartmentalization of cadmium, nickel, and lead in Gammarus fossarum: Comparison of methods. *Chemosphere*, *78*(7), 822–829. doi:10.1016/j.chemosphere.2009.11.051

Giusto, A., Salibián, A., & Ferrari, L. (2014). Biomonitoring toxicity of natural sediments using juvenile Hyalella curvispina (Amphipoda) as test species: evaluation of early effect endpoints. *Ecotoxicology (London, England)*, *23*(2), 293–303. doi:10.1007/s10646-013-1173-7

Grosell, M., & Wood, C. M. (2002). Copper uptake across rainbow trout gills: mechanisms of apical entry. *The Journal of Experimental Biology*, *205*(Pt 8), 1179–1188.

Hall, L. W., & Anderson, R. D. (2014). Spatial Analysis of Bifenthrin Sediment Concentrations in California Waterbodies from 2001 to 2010: Identification of Toxic and Non-Toxic Areas. *Human and Ecological Risk Assessment: An International Journal*, *20*(2), 497–509. doi:10.1080/10807039.2012.743434

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S., & Francis, C. M. (2004). Identification of Birds

through DNA Barcodes. *PLoS Biology*, *2*(10), 1657–1663. doi:10.1371/journal.pbio.0020312

Hertel, R. F., Maass, T., Müller, V. R., Organization., W. H., Safety., I. P. on C., Programme., U. N. E., & Organisation., I. L. (Eds.). (1991). *Nickel.* Geneva: World Health Organization.

Hook, S. E., Osborn, H. L., Golding, L. a., Spadaro, D. a., & Simpson, S. L. (2014). Dissolved and particulate copper exposure induces differing gene expression profiles and mechanisms of toxicity in the deposit feeding amphipod Melita plumulosa. *Environmental Science and Technology*, *48*(6), 3504–3512. doi:10.1021/es405322s

Ingersoll, C. G. I., Brunson, E. L., Dwyer, F. J., Hardesty, D. K., & Kemble, N. E. (1998). Use oF Sublethal Endpoints In Sediment Toxicity Tests With The Amphipod Hyalella azteca. *Environmental Toxicology and Chemistry*, *17*(8), 1508–1523.

Irving, E. C., Liber, K., & Culp, J. M. (2004). Lethal and sublethal effects of low dissolved oxygen condition on two aquatic invertebrates, *Chironomus tentans* and *Hyalella azteca.* *Environmental Toxicology and Chemistry / SETAC*, *23*(6), 1561–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15376542

Keithly, J., Brooker, J. A., DeForest, D. K., Wu, B. K., & Brix, K. V. (2004). Acute and chronic toxicity of nickel to a cladoceran (Ceriodaphnia dubia) and an amphipod (Hyalella azteca). *Environmental Toxicology and Chemistry*, *23*(3), 691–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15285363

Kosalwat, P., & Knight, a. W. (1987). Acute toxicity of aqueous and substrate-bound copper to the midge, Chironomus decorus. *Archives of Environmental Contamination and Toxicology*, *16*(3), 275–282. doi:10.1007/BF01054944

Küpper, H., & Kroneck, P. M. H. (2007). Nickel in the Environment and Its Role in the Metabolism of Plants and Cyanobacteria. In *Nickel and Its Surprising Impact in Nature* (pp. 31–62). John Wiley & Sons, Ltd. doi:10.1002/9780470028131.ch2

Landrum, P. F., Lee, H., & Lydy, M. J. (1992). Toxicokinetics in Aquatic Systems - Model Comparisons and Use in Hazard Assessment. *Environmental Toxicology and Chemistry*, *11*(12), 1709–1725. doi:10.1002/etc.5620111205

Lepp, N. (1981). *Effect of Heavy Metal Pollution on Plants*. (N. Lepp, Ed.). Liverpool: Springer Netherlands. doi:10.1007/978-94-011-7339-1_4

Leung, J. (2014). Implications of copper and nickel exposure to different members of the Hyalella azteca species complex, 92.

Liber, K., Doig, L. E., & White-Sobey, S. L. (2011). Toxicity of uranium, molybdenum, nickel, and arsenic to Hyalella azteca and Chironomus dilutus in water-only and spiked-sediment toxicity tests. *Ecotoxicology and Environmental Safety*, *74*(5), 1171–9. doi:10.1016/j.ecoenv.2011.02.014

Major, K., Soucek, D. J., Giordano, R., Wetzel, M. J., & Soto-Adames, F. (2013). The common ecotoxicology laboratory strain of Hyalella azteca is genetically distinct from most wild strains sampled in Eastern North America. *Environmental Toxicology and Chemistry*, *32*(11), 2637–2647. doi:10.1002/etc.2355

Mander, L., & Liu, H. W. (2010). *Comprehensive Natural Products II: Chemistry and Biology: 10 Volume Set*. Elsevier Science. Retrieved from

https://books.google.ca/books?id=pkzx2TeYYT8C

Mayr, E. (1963). *Populations, species, and evolution*. Cambridge, Massachusetts: Harvard University Press.

McCarty, L. S., & Mackay, D. (1993). Enhancing ecotoxicological modeling and assessment. Body Residues and Modes Of Toxic Action. *Environmental Science & Technology*, *27*(9), 1718–1728. doi:10.1021/es00046a001

McGeer, J. C., Brix, K. V, Skeaff, J. M., DeForest, D. K., Brigham, S. I., Adams, W. J., & Green, A. (2003). Inverse relationship between bioconcentration factor and exposure concentration for metals: implications for hazard assessment of metals in the aquatic environment. *Environmental Toxicology and Chemistry / SETAC*, *22*(5), 1017–1037. doi:10.1002/etc.5620220509

McPeek, M. a., & Wellborn, G. a. (1998). Genetic variation and reproductive isolation among phenotypically divergent amphipod populations. *Limnology and Oceanography*, *43*(6), 1162–1169. doi:10.4319/lo.1998.43.6.1162

Morris, J. M., Collyard, S. a., & Meyer, J. S. (2003). Effects of chronic copper exposure on the nutritional composition of Hyalella azteca. *Aquatic Toxicology*, *63*(2), 197–206. doi:10.1016/S0166-445X(02)00177-7

Naddy, R. B., Stubblefield, W. a, May, J. R., Tucker, S. a, & Hockett, J. R. (2002). The effect of calcium and magnesium ratios on the toxicity of copper to five aquatic species in freshwater. *Environmental Toxicology and Chemistry / SETAC*, *21*(2), 347–352.

Neumann, P. T. M., Borgmann, U., & Norwood, W. (1999). Effect of gut clearance on metal body concentrations in Hyalella azteca. *Environmental Toxicology and Chemistry*, *18*(5), 976–984. doi:10.1002/etc.5620180524

Nixon, K. C., & Wheeler, Q. D. (1990). an Amplification of the Phylogenetic Species Concept. *Cladistics*, *6*(3), 211–223. doi:10.1111/j.1096-0031.1990.tb00541.x

Norwood, W. P., Borgmann, U., & Dixon, D. G. (2006). Saturation models of arsenic, cobalt, chromium and manganese bioaccumulation by Hyalella azteca. *Environmental Pollution*, *143*(3), 519–528. doi:10.1016/j.envpol.2005.11.041

Norwood, W. P., Borgmann, U., & Dixon, D. G. (2007). Chronic toxicity of arsenic, cobalt, chromium and manganese to Hyalella azteca in relation to exposure and bioaccumulation. *Environmental Pollution (Barking, Essex : 1987)*, *147*(1), 262–72. doi:10.1016/j.envpol.2006.07.017

Norwood, W. P., Borgmann, U., & Dixon, D. G. (2013). An Effects Addition Model Based on Bioaccumulation of Metals from Exposure to Mixtures of Metals Can Predict Chronic Mortality in the Aquatic Invertebrate Hyalella azteca. *Environmental Toxicology and Chemistry*, *32*(7), 1672–1681. doi:10.1002/etc.2236

Othman, M. S., & Pascoe, D. (2001). Growth, development and reproduction of hyalella azteca (saussure, 1858) in laboratory culture, *74*(2), 171–181.

Othman, M. S., & Pascoe, D. (2002). Reduced recruitment in Hyalella azteca (Saussure, 1858) exposed to copper. *Ecotoxicology and Environmental Safety*, *53*(1), 59–64. doi:10.1006/eesa.2002.2174

Paquin, P. R., Gorsuch, J. W., Apte, S., Batley, G. E., Bowles, K. C., Campbell, P. G. C., … Wu, K. B. (2002). The biotic ligand model: A historical overview. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *133*(1-2), 3–35. doi:10.1016/S1532-0456(02)00112-6

Quiroz, S., Kim, J. K., Mulrooney, S. B., & Hausinger, R. P. (2007). Chaperones of Nickel Metabolism. In *Nickel and Its Surprising Impact in Nature* (pp. 519–544). John Wiley & Sons, Ltd. doi:10.1002/9780470028131.ch14

Ransberry, V. E., Morash, A. J., Blewett, T. a., Wood, C. M., & McClelland, G. B. (2015). Oxidative stress and metabolic responses to copper in freshwater- and seawater-acclimated killifish, Fundulus heteroclitus. *Aquatic Toxicology*, *161*, 242–252. doi:10.1016/j.aquatox.2015.02.013

Schwenk, K. (1996). *Evolutionary Genetics of Daphnia Species Complex: Hybridism in Syntopy*. Neuwersluis, The Netherlands: Netherlands Institute of Ecology, Centre for Limnology.

Shaw-Allen, P., & Suter, G. W. (2016). Species Sensitivity Distributions. Retrieved March 9, 2016, from http://www3.epa.gov/caddis/da_advanced_2.html

Shuhaimi-Othman, M., Pascoe, D., Borgmann, U., & Norwood, W. P. (2006). Reduced metals concentrations of water, sediment and hyalella azteca from lakes in the vicinity of the sudbury metal smelters, Ontario, Canada. *Environmental Monitoring and Assessment*, *117*(1-3), 27–44. doi:10.1007/s10661-006-7674-6

Sokol, R. J., Deverbaux, M., Mierau, G. W., Hambidge, K. M., & Shikes, R. H. (1990). Oxidant injury to hepatic mitochondrial lipids in rats with dietary copper overload. Modification by vitamin E deficiency. *Gastroenterology*, *99*(4), 1061–1071. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-0025088859&partnerID=40&md5=4c394e3d30e13ea7c3e6bba4fc4312da

Soucek, D. J., Dickinson, A., Major, K. M., & McEwen, A. R. (2013). Effect of test duration and feeding on relative sensitivity of genetically distinct clades of Hyalella azteca. *Ecotoxicology*.

Stephenson, M., & Mackie, G. L. (1988). Multivariate Analysis of Correlations Between Environmental Parameters and Cadmium Concentrations in Hyalella azteca (Crustacea: Amphipoda) from Central Ontario Lakes. *Canadian Journal of Fisheries and Aquatic Sciences*, *45*(10), 1705–1710. doi:10.1139/f88-202

Strong, D. R. J. (1972). Life History Variation Among Populations of an Amphipod (Hyalella Azteca). *Ecology*, *53*(6), 1103–1111.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, *10*(3), 512–526. doi:10.1093/molbev/msl149

United States Environmental Protection Agency (USEPA). (2000). Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates. *Environmental Protection*, (March), 192.

Wellborn, G. A. (1994). Size-Biased Predation and Prey Life Histories : A Comparative Study of Freshwater Amphipod Populations. *Ecology*, *75*(7), 2104–2117.

Wellborn, G. A. (1995). Predator Community Composition and Patterns of Variation in Life History and Morphology Among Hyalella (Amphipoda) Populations in Southeast Michigan. *American Midland Naturalist*, *133*(2), 322–332.

Wellborn, G. A. (2002). Trade-Off between Competitive Ability and Antipredator Adaptation in a Freshwater Amphipod Species Complex. *Ecology*, *83*(1), 129–136.

Wellborn, G. A., & Broughton, R. E. (2008). Diversification on an ecologically constrained adaptive landscape. *Molecular Ecology*, *17*(12), 2927–36. doi:10.1111/j.1365-294X.2008.03805.x

Wellborn, G. A., & Cothran, R. D. (2004). Phenotypic similarity and differentiation among sympatric cryptic species in a freshwater amphipod species complex. *Freshwater Biology*, *49*(1), 1–13. doi:10.1046/j.1365-2426.2003.01160.x

Weston, D. P., Poynton, H. C., Wellborn, G. A., Lydy, M. J., Blalock, B. J., Sepulveda, M. S., & Colbourne, J. K. (2013). Multiple origins of pyrethroid insecticide resistance across the species complex of a nontarget aquatic crustacean , Hyalella azteca. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(41), 16532–16537. doi:10.1073/pnas.1302023110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1302023110

Wheeler, Q., & Meier, R. (2012). *Species Concepts and Phylogenetic Theory: A Debate*. Kobo: Columbia University Press.

Witt, J. D. S., Blinn, D. W., & Hebert, P. D. N. (2003). The recent evolutionary origin of the phenotypically novel amphipod Hyalella montezuma offers an ecological explanation for morphological stasis in a closely allied species complex. *Molecular Ecology*, *12*(2), 405–13. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12535091

Witt, J. D. S., & Hebert, P. D. N. (2000). Cryptic species diversity and evolution in the amphipod genus Hyalella within central glaciated North America : a molecular phylogenetic approach. *Can. J. Fish. Aquat. Sci.*, *57*, 687–698.

Witt, J. D. S., Threloff, D. L., & Hebert, P. D. N. (2006). DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology*, *15*(10), 3073–82. doi:10.1111/j.1365-294X.2006.02999.x

Witt, J. D. S., Threloff, D. L., & Hebert, P. D. N. (2008). Genetic zoogeography of the Hyalella azteca species complex in the Great Basin: Rapid rates of molecular diversification in desert springs. *The Geological Society of America*, *2439*(05), 103–114.

Wu, K. B., Paquin, P. R., Navab, V., Mathew, R., Santore, R. C., & Di Toro, D. M. (2003). *Development of a biotic ligand model for nickel: Phase I*. Alexandria, VA, USA. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Development+of+a+Biotic+Ligand+Model+For+Nickel:+Phase+I#2

Zubrod, J. P., Englert, D., Rosenfeldt, R. R., Wolfram, J., Lüderwald, S., Wallace, D., … Bundschuh, M. (2015). The relative importance of diet-related and waterborne effects of copper for a leaf-shredding invertebrate. *Environmental Pollution*, *205*, 16–22. doi:10.1016/j.envpol.2015.05.015

Appendix

Table A-1: Bioaccumulation data comparison between results obtained from 28-day copper exposures using 2-9 day old Clade 1 *Hyalella* in the current study, and 4-week old Clade 1 *Hyalella* used by Borgmann et al. (1993).

Mean Measured Water Conc. (µg/L)	Mean Tissue Conc. (µg/L)	Measured Water Conc. (µg/L) (Borgmann 1993)	Measured Tissue Conc. (µg/g) (Borgmann 1993)	S.D (Borgmann 1993)
2.08	27.43	1.3	98	77-119
6.22	78.13	4.8	122	100-144
10.00	75.98	8	123	101-145
16.42	75.72	13.3	159	118-200
25.32	79.24	22.8	150	108-192
45.86	98.24	39.2	196	153-239

Table A-2: Bioaccumulation data comparison between results obtained from 28-day copper exposures using 2-9 day old Clade 8 *Hyalella* in the current study, and 4-week old Clade 1 *Hyalella* used by Borgmann et al. (1993).

Mean Measured Water Conc. (µg/L)	Mean Tissue Conc. (µg/L)	Measured Water Conc. (µg/L) (Borgmann 1993)	Measured Tissue Conc. (µg/g) (Borgmann 1993)	S.D (Borgmann 1993)
1.04	25.38	1.3	98	77-119
5.31	136.81	4.8	122	100-144
7.93	152.67	8	123	101-145
13.69	141.31	13.3	159	118-200
25.16	164.90	22.8	150	108-192
41.86	187.95	39.2	196	153-239
72.40	236.33	65.1	252	214-290