Implications of copper and nickel exposure to different members of the *Hyalella azteca* species complex

by

Jessica Leung

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Author's Declaration

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

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Abstract

Hyalella azteca, an amphipod crustacean, is frequently used in freshwater toxicity tests. Since the mid-1980s, numerous organizations have collected and established cultures of *H. azteca* originating from localities across North America. However, *H. azteca* is actually a large cryptic species complex whose members satisfy both the biological and the phylogenetic species concepts. Recently, two publications reported that members of the *H. azteca* cryptic species complex have different toxicity responses to anions and an insecticide. In this study, four members of the *H. azteca* species complex were identified with DNA barcoding. The genetic variation among the four clades was consistent with interspecific distances between species. These lineages (clades 1, 3, 6, and 8) were cultured in identical conditions and monitored on a weekly basis to determine two life history traits: adult mortality and juvenile production. The large-bodied clades had significantly better survival and juvenile production compared to the small-bodied clade 3. Clade 6 had very low juvenile production and high mortality; therefore, was not included in this study. Unique culture protocols may be required for each clade to optimize growth, survival, and juvenile production in laboratory conditions.

Genetic barcoding has identified only two clades in a survey of 17 laboratories. Therefore these two clades (1 and 8) were compared after exposure to copper and nickel 14-day toxicity tests. Clade 8 was 2.3-2.6 times more tolerant to copper exposure than clade 1 based on their LC50 and LC25. Similarly, clade 8 was more tolerant to nickel exposure than clade 1: LC50 was 1.8 times higher for the former. Nickel LBC50 and LBC25 were significantly different between clades by a factor of 2.1-2-8. Mortality (relative to copper concentrations in tissue), growth, and bioaccumulation responses were not significantly different based on overlapping confidence intervals. Although clades 1 and 8 are both large-bodied ecomorphs, these lineages had significantly different body mass (i.e., dry weight) after 14 days. The results of this study indicate that genetically characterized cultures of *H. azteca* should be used in toxicity tests.

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1. Chapter 1 – General Introduction

1.1 Overview

There are more than 30 described species of *Hyalella* (Crustacea: Amphipoda: Dogielinotidae) distributed in North and South America, which have very subtle morphological differences (Stock and Platvoet, 1991; Bousfield, 1996; Serejo, 2004). Examples of *Hyalella* species include *H. muerta* and *H. sandra* first described in Death Valley National Park, California (Baldinger *et al*., 2000); *H. chiloensis*, *H.costera* and *H. kochi*, distributed in Chili (Gonzalez and Watling, 2001); and *H. azteca*, originally described from a cistern in Vera Cruz, Mexico (Saussure,1858).

Hyalella azteca is an epibenthic detritivore that feeds primarily on algae and bacteria associated with sediment particles (<65 μm), aquatic macrophytes, as well as animal and plant detritus (Cooper, 1965; Hargrave, 1970; Wen 1993). It is abundant in benthic communities, and a major food source for larger invertebrates, fish, waterfowl, and amphibians (de March, 1981).

This amphipod occurs in permanent freshwater habitats throughout North America such as lakes, ponds, and streams (Bousfield, 1973; de March, 1978; de March, 1981; Pennak, 1989; Environment Canada, 1997, 2013). The species has been recorded from Panama to the Northwest Territories of Canada, as well as from the Atlantic to the Pacific coasts (Bousfield, 1973; de March, 1981; Pennak, 1989; Witt and Hebert, 2000).

As a result of its wide distribution, abundance, and its role as a major component of the aquatic food chain, the ecology, life history, biology, and toxicology of *H. azteca* has been frequently studied (Gonzalez and Watling, 2002). *Hyalella azteca* has many characteristics that make it useful in the laboratory, including short life cycles, easy collection and culture in captivity. Its sensitivity to contaminants, as well as the fact that it is easy to sex and age make it an ideal organism, especially for toxicity assays (Lawrence, 1981; USEPA 1994; Bousfield, 1996; Environment Canada, 1997, 2013). As a consequence, *H. azteca* has been used routinely in ecotoxicological studies of metals, acidification,

organic compounds, and sediments since the mid-1980s (USEPA 1994; Environment Canada, 1997, 2013).

Although *H. azteca* has been in culture for over 25 years in laboratories across North America, each laboratory has its own protocol for their particular stock (Environment Canada, 2013). Even though standard laboratory methods for culturing *H. azteca* have been published in Canada and the United States, laboratory personnel are given freedom of choice in several aspects of culturing, e.g., food, water sources, substrate, etc. (USEPA 1994, 2002; Environment Canada, 1997, 2013). Table 1-1 to 1-3 summarize different procedures used for culturing *H. azteca* in laboratories across Canada and the United States.

Similarly, standard protocols for sediment and reference toxicity tests using *H. azteca* are different in Canada and the United States (Environment Canada, 2013). Although standardized toxicity protocols exist, many institutions employ different test conditions. Consequently, it is difficult to compare results (e.g., LC50s) among different publications. As seen in Table 1-4, a summary of copper and nickel LC50s from various institutes compiled by Borgmann *et al.* (2005a): the LC50s for both copper and nickel ranged from 31-210 μg/L (7 fold difference) as well as 77-3620 μg/L (47 fold difference), respectively. Some of these variations in LC50s can be attributed to differences in water hardness, alkalinity, pH, and test duration. Other factors that could change the LC50s may be due to other differences in test conditions, e.g., food, feeding regime, temperature, age/size of the animal, etc. (Environment Canada, 2013).

Table 1-1: Culture vessels, test medium volume, amphipod load, water sources, hardness, method of water replacement and frequency for *Hyalella* cultures in Canada and the United States (Environment Canada, 2013): N/I = not indicated, DFO = Department of Fisheries and Oceans, ASTM = American Society for Testing and Materials, USEPA = United States Environmental Protection Agency, NWRI = National Water Research Institute.

Vessel Type	Water Volume (L)	# of Adult Amphipods/L	Water Source	Water Harness	Method of Replacement	Frequency of Replacement	Reference
2.5 L Pyrex glass jar	1	5-25	Dechl. tap	130 mg/L	Intermittent renewal	Once weekly	DFO, 1989
10 or 20 L aquarium	N/I	N/I	Well, surface, dechl. tap, or recon.	Optional	Intermittent renewal, flow-through	25-30%/week, 100 ml/min	ASTM, 1991
8 L aquarium	6	N/I	Well, surface, dechl. tap, or recon.	Optional	Intermittent renewal, flow-through	≥50%/week, 100 ml/min	USEPA, 1991a
2 L battery jar or aquarium	1	60	Surface or recon.	N/I	Intermittent renewal	Once weekly	USEPA, 1991b
30 mL cup, 1 L glass beaker, 8 L aquarium, 76 L aquarium	0.02, N/I, 6, 40	100, 80, 17-33, 13-50	Well or diluted well	100 mg/L, 200 mg/L	Intermittent renewal, flow-through	Daily	USEPA, 1991c
2.5 L Pyrex glass jar	1	5-25	Dechl. tap	130 mg/L	Intermittent renewal	Once weekly	DFO, 1992
10 L glass aquarium, 1.2 L glass jar	8, 1	20-25, 20-25	Dechl. tap	N/I	Intermittent renewal	30%, once weekly	NWRI, 1992
1-39 L aquariums	0.8-38	N/I	Well, surface, dechl. tap, or recon.	Very soft to very hard	Intermittent renewal, flow-through	N/I	USEPA, 1992
2 L glass beaker, 2.5 L glass jar, 80 L aquarium	1, 1, 5	50, 5-25, N/I	Well, surface, dechl. tap, recon., or estuarine	Optional	Intermittent renewal, flow-through	N/I	USEPA, 1994a

Table 1-2: Substrate and feeding condition among *Hyalella* laboratory cultures in Canada and the United States (Environment Canada, 2013). N/I = not indicated, DFO = Department of Fisheries and Oceans, ASTM = American Society for Testing and Materials, USEPA = United States Environmental Protection Agency, NWRI = National Water Research Institute.

Substrate	Size/Quantity	Type of Food	Quantity per Liter	Feeding Frequency	Reference
Plastic and cotton gauze	Several pieces in jar	Tetramin® fish food flakes	20 mg	1-3/week	DFO, 1989
Dried maple, alder, birch, or poplar leaves, pre-soaked several days and then rinsed	N/I	Dried maple, alder, birch, or poplar leaves, pre-soaked several days and then rinsed; rabbit pellets; ground cerel leaves; fish food pellets; brine shrimp; heat-killed Daphnia; green algae and spinach	N/I	N/I	ASTM, 1991
Shredded brown paper towel	N/I	Tetramin® fish food flakes + brine shrimp	3.3 mg	1/day	USEPA ,1991a
Medicinal gauze sponges, 10cm^2, pre-soaked in culture water for 24-28 h	1/jar	Filamentous algae and YCT	10 mL YCT	3/week	USEPA, 1991b
		Diatoms (*Synedra)*	Algae pinch	1/week	
Single layer of unbleached brown paper towel	N/I	Ground fish food flakes plus dried algae (*Spirulina*)	50-167 mg	2/day	USEPA, 1991c
Sterile 5x10 cm bandage gauze, or 210 µm Nitex nylon mesh	1/jar	Tetramin® fish food flakes	10 mg	1-3/week	DFO, 1992
2.5 cm^2 strips of 500 µm Nitex nylon mesh, pre-soaked in culture water for 24 h	8/aquarium 1/jar	Nutrafin® fish food flakes	2-4 drops	2/week	NWRI, 1992
Cotton gauze, leaves, paper towels, plastic mesh, Nitex, sand, sediment, none	1/jar	Various	Varied	Varied	USEPA, 1992
Cotton gauze, maple leaves, artificial coiled-web materials	N/I	Various	Varied	Varied	USEPA, 1994a

Table 1-3: Water temperature, aeration conditions, lighting, and source of brood stock for *Hyalella* laboratory cultures in Canada and the United States (Environment Canada, 2013): N/I = not indicated, DFO = Department of Fisheries and Oceans, ASTM = American Society for Testing and Materials, USEPA = United States Environmental Protection Agency, NWRI = National Water Research Institute.

Water Temp. (°C)	Aeration Conditions	Lighting	Initial Source	Reference
25	none	16L:8D, fluor., 55 µE/m^2/s	Marshy shorelines of small lake near Burlington, Ontario	DFO, 1989
20±2	Gentle, if IR	16L:8D, 5382 lux	Natural freshwater source, another laboratory, or a commercial source	ASTM, 1991
25±2	Gentle, if IR	16L:8D, 5382 lux	Natural freshwater source, another laboratory, or a commercial source	USEPA, 1991a
25	Gentle (air stone)	16L:8D, 1280 lux	Best source from a Lake Superior bay, acceptable sources, other laboratories, commercial suppliers, local collection	USEPA, 1991b
23-25	IR only	16L:8D, 538-1076 lux	USEPA Newtown strain	USEPA, 1991c
25	none	16L:8D, fluor., 55 µE/m^2/s	Marshy shorelines of small lake near Burlington, Ontario	DFO, 1992
23±1	gentle	16L:8D, 51 µE/m^2/s	CCIW Burlington laboratory (W. Norwood/U. Borgmann)	NWRI, 1992
15-25	N/I	N/I	St. Louis River, lake near Burlington, Michigan State pond, Nebeker strain, USEPA Newtown	USEPA, 1992
23	Yes, if static or IR	16L:8D, 500-1000 lux	Preferably from a laboratory source unless wild populations are able to cross breed with existing laboratory populations	USEPA, 1994a

Table 1-4: Copper and nickel lethal concentrations resulting in 50% mortality (LC50) for *H. azteca* in different laboratories. Each laboratory exposed *H. azteca* to different concentrations of copper or nickel, as well as test conditions: test duration, water hardness (mg/L), alkalinity (mg/L), and pH. Both the nominal and measured concentrations were included. This table was modified from Borgmann *et al*. (2005).

Test Duration (Days)	Hardness (mg/L)	Alkalinity (mg/L)	pH	Cu LC50 (µg/L)		Ni LC50 (µg/L)		Reference
				Nominal	Measured	Nominal	Measured	
4	6-10	9-21	6.9-8.0	66	-	-	-	Suedel and Deaver (1996)
4	90	-	7.4-8.1	34-53	-	-	-	Collyard *et al.* (1994)
4	98	64	7.7-8.0	-	-	3045	-	Keithly *et al.* (2004)
4	120-140	75-100	7.5-8.5	210	-	3620	-	Milani *et al.* (2003)
4	280-300	225-245	6-6.5	17	-	2000	-	Schubauer-Berigan *et al.* (1993)
4	280-300	225-245	7-7.5	24	-	1900	-	Schubauer-Berigan *et al.* (1993)
4	280-300	225-245	8-8.5	87	-	890	-	Schubauer-Berigan *et al.* (1993)
7	6-10	9-21	6.9-8.0	53	-	-	-	Suedel *et al.* (1996)
7	18	14	7.4	56	36	77	75	Borgmann *et al*. (2005)
7	124	84	8.3	121	90	147	133	Borgmann *et al*. (2005)
10	6-10	9-21	6.9-8.0	67	-	-	-	Suedel and Deaver (1996)
10	<10	<10	6.9-7.0	42	-	-	-	Deaver and Rodgers (1996)
10	22-64	22-63	7.4-8.2	92-143	-	-	-	Deaver and Rodgers (1996)
10	44	45	7.3	31	-	-	-	West *et al.* (1993)
10	44-47	45-46	6.7-7.4	-	-	780	-	Ankley *et al.* (1991)
10	6-10	9-21	6.9-8.0	44	-	-	-	Suedel and Deaver (1996)
14	98	64	7.7-8.0	-	-	>120	-	Keithly *et al.* (2004)

Even when water hardness was normalized to compare results from different laboratories, Keithly *et al.* (2004) determined that their four-day nickel LC50 (the lethal concentration resulting in 50% mortality) for *H. azteca* (pH=7.5, 1723 μg/L) was four times greater than that reported by Schubauer-Berigan *et al.* (2003) (pH=7.9, 430 μg/L). Keithly *et al.* (2004) noted that the variation in pH (0.4 pH units) was insufficient for the four-fold difference in LC50s, and speculated that the variability could be due to differences in test protocols as well as the genetic differences in test organisms themselves.

Duan *et al*. (1997) were similarly concerned with the quality of inter-laboratory toxicity data using *H. azteca.* Since these test organisms are relatively isolated and were collected from different areas in North America, they analyzed the genetic variations among laboratory populations of *H. azteca* assaying 16 enzymatic loci in six laboratory stocks. They reported three genetically divergent groups: two groups had high levels of genetic differentiation that suggested they were distinct species of *Hyalella.*

Ecological and molecular studies have also raised the question of the taxonomic status of *H. azteca* in natural populations (Wellborn, 1994a and b, 1995a, 2002; Hogg *et al*., 1998; McPeek and Wellborn, 1998; Gonzalez and Watling, 2002; Wellborn and Broughton, 2008; Wellborn *et al*., 2005; Witt and Hebert, 2000; Witt *et al*., 2006). These studies suggest that *H. azteca* is a complex composed of many species that have been erroneously grouped together due to morphological similarities, but are genetically divergent enough to be distinct species (Witt and Hebert, 2000).

Species are defined as evolutionary independent units that are isolated by a lack of gene flow to and from other populations. The identification of a species can be a complicated task because testing genetic independence is often difficult. However, there are several species concepts or criteria for defining a species; each has their theoretical and practical problems (Agrawal and Gopal, 2013). Only the three major species concepts are presented here and applied to the *H. azteca* species complex: the biological, phylogenetic, and morphological species concepts.

The biological species concept identifies evolutionary independence by reproductive isolation. If individuals cannot mate and produce viable and fertile offspring, then they are reproductively isolated from each other, which confirm a lack of gene flow. In other words, species are populations of interbreeding individuals (Agrawal and Gopal, 2013). The phylogenetic species concept defines a species as a monophyletic group or clade, which consists of a single common ancestor and all its descendants (Agrawal and Gopal, 2013). The morphological species concept considers individuals to belong to the same species if they are phenotypically similar to a designated type specimen (Agrawal and Gopal, 2013).

In the case of *H. azteca*, two phenotypic classes were observed separately as well as sympatrically: small- and large-bodied ecomorphs (Strong, 1972; Wellborn, 1995a; McPeek and Wellborn, 1998; Wellborn *et al*., 2005). The large-bodied ecomorphs occur in habitats with little or no fish predation, are less vulnerable to predation from invertebrates, have an enhanced competitive advantage for mating success, and can outcompete small-bodied ecomorphs for resources (Wellborn, 2002). Despite being out-competed by the large-bodied ecomorphs, the small-bodied ecomorphs have a selective advantage in habitats with predatory fish (Wellborn, 1994a, 1995b, 2002). This is a result of size-biased predation since large-bodied ecomorphs are easier to identify by visual predators such as fish (Wellborn, 1994a; Wellborn *et al*., 2005).

Little morphological variations exist between ecomorphs, but each possesses different life history and ecological traits (Strong, 1972; Wellborn, 1994b, 1995a; Wellborn and Broughton, 2008). Wellborn (1994b, 1995a) reported that small-bodied adults are smaller at maturity, have smaller eggs, and have higher size-specific fecundity as well as reproductive investment than the large-bodied lineages. However, a later study by Wellborn and Cothran (2004) reported that large-bodied ecomorphs had significantly smaller and more eggs than the small-bodied ecomorphs. These life history studies of *Hyalella* suggest that survival and reproduction are likely genetically inherent and not a result of plasticity.

Furthermore, McPeek and Wellborn (1998) collected four small- and three large-bodied ecomorphs from habitats across southeastern Michigan. They conducted breeding tests using a combination of these seven populations to assess their ability to interbreed. Individuals from the same ecomorphs had an 84.6% breeding success rate. In addition, different populations with the same body size had a success rate of 64.3%. Yet when paired with a different phenotypic class (e.g., large- and small-bodied *Hyalella*), none of the replicates (n=19) resulted in reproduction. Wellborn *et al.* (2005) conducted an analogous study and confirmed the observations reported by McPeek and Wellborn (1998). They reported that crosses between small-bodied ecomorphs of *Hyalella* resulted in successful precopulatory pairings from either the same (61.5%) or different (47.8%) populations, which brought about successful fertilization and embryo development. Similarly, large-bodied morphs from either the same (50%) or different (40.4%) populations were able to pair, mate, and produce viable offspring. Consistent with McPeek and Wellborn's (1998) observations, precopulatory pairs were not frequently observed between small- and large-bodied *Hyalella* (3.3%), and any successful pairing produced minimal numbers of developing embryos (one in 90) (Wellborn *et al*., 2005). These studies suggest that the large and small-bodied ecomorphs are distinct biological species due to their inability to interbreed.

Although several other researchers reported divergent groups within *H. azteca* by analyzing allozyme and interbreeding trials (Duan *et al*., 1997; Hogg *et al*., 1998; McPeek and Wellborn, 1998; Wellborn *et al*., 2005), only Witt and Hebert (2000) delineated species boundaries. Witt and Hebert (2000) reported that the wild populations of *H. azteca* collected in Ontario, Wisconsin, New Brunswick, and the Yukon were composed of at least seven species that satisfy both the biological and phylogenetic species concepts. By observing fixed allozyme differences, these wild populations were in Hardy-Weinberg disequilibrium. This fact indicated that there were no allelic exchanges and these groups of *Hyalella* were noninterbreeding; thus, fulfilling the requirement of the biological species concept. The noninterbreeding groups were also separable into seven distinct monophyletic groups using the mitochondrial cytochrome c oxidase I (COI) gene, satisfying the phylogenetic species concept. Using

mitochondrial and nuclear markers, Witt and Hebert (2000) identified that 15 of the 24 habitats they surveyed had two or more co-occurring species in the same body of water. On the basis of the COI gene, DNA barcoding studies further revealed that there are over 30 provisional species or clades within the *H. azteca* complex in the southern Great Basin region of California and Nevada alone (Witt *et al*., 2006). To date, 85 provisional species have been identified by DNA barcoding surveys from wild populations throughout North America (Witt and Wellborn, in preparation).

Traditional analyses, using the morphological species concept, had previously identified most North American *Hyalella* populations as *H. azteca*. The lack of obvious morphological differences had led previous investigators to believe that this amphipod was one widely distributed species present throughout North America until the application of the biological and phylogenetic species concept (Gonzalez and Watling, 2002).

The evolution of multiple species within *H. azteca* could have been the consequence of its short generation time, an absence of a dispersal stage, exposure to different environments and to geographic isolation (Witt and Hebert, 2000). In addition, these species can co-exist, likely due to behavioural differences (e.g., predator avoidance) that may have resulted in the exploitation of different ecological niches (Wellborn and Cothran, 2004; Witt *et al.*, 2006; Wellborn and Cothran, 2007).

There is a great deal of genetic diversity among *H. azteca* in natural populations, but *Hyalella* in laboratories have very little genetic variation. Major *et al.* (2013) and Weston *et al.* (2013) had sequenced *H. azteca* from 17 stocks collected from different institutes in North America. Sixteen of the 17 laboratories culture clade 8 and only one institution uses clade 1. These publications indicate that not all North American laboratories surveyed are using the same clade for toxicity tests. Major *et al*. (2013) noted that the use of two laboratory lineages may not accurately predict the responses in wild populations and expressed concern regarding the widespread use of clade 8 in North American research laboratories. This is especially true considering that clade 8 has been only been reported in the southeastern region of

the United States, e.g., Oklahoma, Nebraska, Kansas, Arkansas, Mississippi, Tennessee, Alabama, Georgia, and Florida (Wellborn and Broughton, 2008; Major *et al*., 2013; M. Hrycyshyn, PhD thesis in preparation). Clade 1, however, is widely distributed from New Brunswick to Alaska and as far south as Nevada (Witt and Hebert, 2000; Witt *et al*., 2003; M. Hrycyshyn, PhD thesis in preparation).

Although Major *et al.* (2013) and Weston *et al.* (2013) had genetically characterized *H. azteca* from numerous laboratories, these studies may not necessarily represent all clades in use by other institutions. In addition, having recently sequenced laboratory cultures provide only a "snapshot" of the clades these 17 institutes currently possess. Since laboratory stocks were originally collected from the wild in various localities (Duan *et al.*, 1997), there remains a possibility that institutes may have initially had a mix of different clades since 63% of natural habitats surveyed by Witt and Hebert (2000) had two or more species of *Hyalella*. Furthermore, some laboratories may have re-stocked *Hyalella* from another laboratory that uses a different clade, which could also explain some inter-laboratory differences.

Given that *H. azteca* has been determined to be numerous distinct species, it is possible that some of the variability among cultures and toxicity protocols could be due to genetic differences between clades. In the case of culture protocols, different clades may have diverse nutritional and/or behavioural requirements as suggested by their ability to coexist in the same body of water (Wellborn, 1994b, 1995b; Witt and Hebert, 2000; Wellborn and Broughton, 2008) and the variability among them may be the result of laboratories optimizing different parameters for their particular lineage of *Hyalella*. Major (2012) assessed the life history characteristics of two laboratory and two wild clades since these parameters are the basis for chronic toxicity test endpoints. She reported that body size and reproductive rates deviated among clades and that unique laboratory culturing conditions may be necessary to optimize the health of each clade.

Genetic differences between clades may also confound toxicity tests. Indeed, Soucek *et al.* (2013) cultured three genetically characterized clades of *H. azteca* and reported that they had different

survival in the presence or absence of food when acutely exposed to chloride or nitrate toxicity tests. They also observed that the "US lab clade" (clade 8) was also substantially more tolerant than the other clades (clades 1 and 3). Weston *et al*. (2013) reported a 550 fold difference among four members of the *H. azteca* cryptic species complex when exposed to pyrethroid (an insecticide). The evidence of different sensitivities to contaminants among members of the *H. azteca* species complex has important implications for biomonitoring programs (Weston *et al*., 2013). In this study, two metals (copper and nickel) are used to assess whether different sensitivity exists between two *Hyalella* clades.

The Web of Science (December 2013) indicated that *H. azteca* has been employed in at least 100 acute and chronic toxicity tests relating to copper as well as 33 for nickel. However, no published metal toxicity tests have genetically identified the test population of *H. azteca*. Metals are elements that originate from the Earth's crust and are naturally mobilized by the erosion of rock surfaces via running water, wind, and ice. Other factors include organisms, windblown dusts, forest fires, volcanoes, as well as sea sprays (Nriagu, 1979; Siegel, 2002; Luoma and Rainbow, 2008). However, anthropogenic annual emissions of the two metals are three times greater than natural sources worldwide (Nriagu, 1979). Anthropogenic emissions of copper and nickel (56×10^6 kg and 47×10^6 kg per year, respectively as of 1975) originate from metal production (non-ferrous, iron, and steel), fossil fuel emissions, sewage, agricultural uses (fertilizer, fungicides, and algaecides), as well as waste (mining, industrial, and domestic) (MacKenthum and Cooley, 1952; Beavington, 1973, 1977; Lopez and Lee, 1977; Elder and Horne, 1978; Forstner and Wittmann, 1979; Nriagu, 1979; Barkay *et al*., 1985; Yamamoto *et al*., 1985). Consequently, these activities have resulted in significant changes to the quantity and bioavailability of metals and are a concern to human health and environmental integrity (Flemming and Trevors; 1989).

Aquatic biota are frequently more sensitive to metals than terrestrial organisms (Hodson *et al.*, 1979) because they have low surface area to volume ratios, high respiratory rates, and high flow rates over gill surfaces, which facilitates metal uptake (Hodson *et al.*, 1979). The bioaccumulation of metals in

aquatic organisms depend on the pH, redox potential, water hardness, organic content, sediment type, or combinations of these factors (Flemming and Trevors, 1989).

Copper and nickel were chosen for this study because they have been extensively tested under variable conditions using *H. azteca* (Table 1-4). They are essential and non-essential metals, respectively, and can enter different metabolic pathways. In trace amounts, copper is essential for several biochemical processes in metabolic pathways and is usually obtained from the diet; however, high levels of copper exposure has toxic effects on aquatic fauna (Nor, 1987; Flemming and Trevors, 1989; Luoma and Rainbow, 2008). In contrast, nickel is not used in enzymes or cofactors in invertebrates and is toxic to a wide range of organisms depending on its form and concentration (Nielson *et al*., 1975; Schnegg and Kirchgessner, 1975; National Research Council, 1975).

Copper toxicity in *H. azteca* is a result of the bioaccumulation of dissolved copper ions (Deaver and Rodgers, 1996; Borgmann *et al*., 2005a). Its toxicity is highest at low pH owing to an increase in copper concentration and solubility (Campbell and Stokes, 1985; Schubauer-Berigan *et al.*, 1993). Dissolved copper quickly accumulates in the body of *H. azteca* during continuous exposure, but gradually decreases to control levels due to metabolic regulation (Borgmann and Norwood, 1995; Borgmann, 1998). Othman and Pascoe (2002) observed that juvenile *H. azteca* (<7 days old) exposed to increasing concentrations of copper (nominal concentrations of 18 μg/L, 40 μg/L, 70 μg/L and 260 μg/L) for 35 days resulted in decreases in population size, juvenile recruitment, mating pairs, and body length. Moreover, concentrations above 55 μg/L (for 35 days) resulted in a statistically significant decrease in survival compared to the control juveniles. The Canadian Water Quality Guideline for the protection of aquatic life in freshwater is 2-4 μg/L of copper (CCREM, 1987).

Borgmann *et al*. (2001) characterized the impacts of nickel on *Hyalella* exposed to spiked natural sediments and determined a number of chronic (4- and 10-week) lethal nickel concentrations in total-body and exposure solution (0.09-72.68 μmol/g dry weight of spiked sediments). Furthermore, they generated

a number of toxicity effect concentrations (e.g., IC25) based on the reduction of growth, total biomass, and reproduction, with increased nickel exposures. Nickel bioaccumulation linearly increased in relation to its concentration in solution and remained at similar levels after 10 weeks, indicating that the bioaccumulation of nickel is a reliable predictor of its toxicity (Borgmann *et al*., 2001). The Canadian Water Quality Guideline for the protection of aquatic life in freshwater is 25-150 μg/L of nickel (CCREM, 1987).

1.2 Objectives

Although standardized culture and toxicity methodologies exist for *Hyalella azteca*, variability in protocols among laboratories is very high, and may partially explain differences in toxic responses. There is a lack of research that addresses whether different clades of *H. azteca* require specific culture protocols to maximize lifespan and juvenile production. In this thesis, three *Hyalella azteca* clades (1, 3, and 8) are genetically characterized. Laboratory-relevant life histories traits (background mortality rates and juvenile production) of three clades are presented in Chapter 2. Each clade is hypothesized to have different mortality rates and juvenile production under identical culture conditions.

In addition, few studies have been conducted to assess the relationship between toxicity responses in members of the *H. azteca* species complex. Although the effects of metals, acidification, organic compounds, and sediments were well documented using *H. azteca*, the responses among laboratories were reported to have large variations due to inconsistent test protocols. The use of different clades may contribute to this variability. In Chapter 2, two groups of *H. azteca* were genetically identified by DNA barcoding to be clades 1 and 8, which are lineages that are commonly used in laboratories. These two clades were compared to observe whether mortality and growth were significantly different during metal exposures (Chapter 3). The bioaccumulation of copper and nickel was also evaluated between clades 1 and 8 (Chapter 4). Clades 1 and 8 were hypothesized to have different mortality (LC50, LC25, LBC50, LBC25), growth (IC25, IBC25), and bioaccumulation patterns upon exposure to identical copper or nickel toxicity conditions.

2. Chapter 2 – Collection and Identification of Lineages within the *Hyalella azteca* Cryptic Species Complex

2.1 Introduction

When conducting an aquatic toxicity test, the use of a well characterized organism (e.g., genetics, behaviour, etc.) is essential to extrapolate meaningful and ecologically relevant results (Shuhaimi-Othman and Pascoe, 2001). Rand and Petrocelli (1985) outlined several criteria to consider when selecting an organism for toxicity testing. The organism should be widely available, representative of the impacted ecosystem, ecologically important, and be amendable to laboratory conditions on a long term basis.

The amphipod crustacean, *Hyalella azteca* (Saussure, 1858) has many characteristics that make it an ideal organism for laboratory work. Due to its wide availability across North America, sensitivity to contaminants, relevance to the aquatic food chain, short life cycles, easy collection and culture in captivity, it has been used in numerous toxicity laboratories since the mid-1980s (Lawrence, 1981; USEPA 1994; Bousfield, 1996; Environment Canada, 1997, 2013). Several institutes across North America have collected wild populations of *H. azteca* (Duan *et al.*, 1997) and varied their culture protocols to optimize growth, reproduction, as well as survival. The optimizations of these parameters are necessary for establishing cultures on a long term basis and can increase the cost effectiveness of maintaining them. Tables 1-1 to 1-3 summarize different *H. azteca* culturing protocols utilized by various laboratories across Canada and the United States. Notably, food type and regime, water hardness, as well as substrate (which are likely the most important variables for survival, growth, and reproduction) are often left to the discretion of the laboratory personnel (Environment Canada, 2013).

Recent molecular work has indicated that *H. azteca* is actually a cryptic species complex composed of 85 morphologically similar, yet genetically distinct provisional species (Witt and Hebert, 2000; Witt et al., 2006; 2008; Witt and Wellborn, in preparation). Even before *H. azteca* was determined to be a cryptic species complex, several authors had documented that certain populations of *Hyalella* have different life history traits (Strong, 1972; Wellborn, 1994a, 1995b). Furthermore, several research groups

recognized two phenotypic classes based on size among populations of *Hyalella*, and each is associated with its own life history characteristics (Strong, 1972; Wellborn 1994a, 1995b).

The variability in culturing protocols may be a result of laboratories optimizing these parameters for their particular lineage of *Hyalella* (Environment Canada, 2013), which may be due to different nutritional/behaviour requirements for each clade. Although a standardized culture methodology exists for *H. azteca*, a great deal of variability remains in these protocols among laboratories. These variations in life history traits (such as survival and juvenile production) may also partially explain differences in toxicity responses. Currently, there is a lack of research that addresses whether specific culture protocols are needed for certain members within the complex. In this chapter, genetically identified clades of *H. azteca* are exposed to the same culture conditions to determine whether these lineages vary in two laboratory-relevant life histories traits: juvenile production and mortality. Each clade is hypothesized to have different mortality rates and juvenile production under identical culture conditions.

2.2 Materials and Methods

2.2.1 Collection, Identification, and Culture Procedures of *Hyalella azteca*

Cultures of *Hyalella "azteca"* were collected from one university and two government laboratories. Originally collected from the Valens Conservation Area (Cambridge, Ontario), one lineage has been cultured at Environment Canada in the Canadian Centre for Inland Waters (CCIW; Burlington, Ontario) since 1986. The Ontario Ministry of the Environment (MOE; Etobicoke, Ontario) has cultured its *Hyalella* for at least 10 years, and were obtained from AquaTox (Guelph, Ontario); however, the original collection site is unknown. Another group of animals was obtained from Dr. Bruce Greenberg's laboratory at the University of Waterloo (UW; Waterloo, Ontario); the source and age is unknown.

Populations of wild *H. azteca* were also collected from the field. Sampling campaigns were conducted at various localities in southern Ontario: a small fishless pond south of Guelph (Ontario), Guelph Lake (Guelph, Ontario), and Blue Springs Creek (Eden Mills, Ontario) between fall 2011 and

September 2012. Animals were collected with a dip net in littoral sites using the kick-sweep method. Once in the laboratory, *H. azteca* were separated from other invertebrates and placed into a 34 L aerated aquarium filled with a five-salt standard artificial medium (SAM-5S) consisting of 1 mM $CaCl_2 \cdot H_2O$, 1 mM $NaHCO_3$, 0.01 mM NaBr, 0.05 mM KCl, and 0.25 mM $MgSO_4 \cdot 7H_2O$ (Borgmann, 1996). This medium was chosen since it could be easily duplicated by other laboratories and its major ions (similar to Lake Ontario water) are essential to amphipod survival and growth (Borgmann, 1996).

Since the possibility existed that the amphipods collected from Guelph Lake represented several species, they were initially differentiated on the basis of colour (green or brown). Subsequently, gravid females were separated into individual containers with 2.5mg of ground TetraMin® fish food, a 2.5 cm^2 piece of cotton gauze, and 1 L of SAM-5S. These females were allowed to release their juveniles before they were preserved for DNA sequencing. Once the females were genetically identified, their juveniles were combined with others of the same clade to form a homogeneous culture.

The analysis of the cytochrome c oxidase subunit I (COI) mitochondrial gene was conducted to determine which clades individuals (within the cultures) belonged. Total DNA was extracted from 10 to 63 randomly selected amphipods from each locality (Table 2-1) according to the methods of Schwenk (1996). Two or three appendages (e.g., legs, antennae, etc.) were ground in 50µL of proteinase-K extraction buffer, which released DNA by degrading proteins. The extraction mixture was incubated at 55ºC for 18 to 24 h followed by 97ºC for 12 mins before being stored at -20ºC.

The polymerase chain reaction (PCR) amplified a 637 base pair fragment of the COI gene by using the appropriate primer combinations (Table 2-1). Each PCR reaction contained 2.5 µL of DNA template, 5.0 µL 10× Thermopol buffer, 0.2 µM of forward primer, 0.2 µM of reverse primer, 0.2 µM of dNTP mix, and 1 unit (3 µL) of *Taq* DNA polymerase, for a total volume of 50 µL. The PCR was conducted using the following: 1 min at 94°C; five cycles of 1 min at 94°C, 1.5 min at 51°C, 1 min at

72°C; followed by 35 cycles of 1 min at 94°C , 1.5 min at 51°C, 1 min at 72°C; and finally, 5 mins at 72°C (Witt *et al*., 2006).

Table 2-1: Culture origins, COI primer combinations, and sample sizes for six *H. azteca* populations collected from laboratories and field locations.

Culture origin	Primer combinations (forward, reverse)	Sample size
Canadian Centre for Inland Waters (CCIW), Burlington	Fol A, Fol B	36
University of Waterloo (UW), Waterloo	CO1 Crust DF1, Crust DR2	35
Guelph Lake (GL), Guelph	Fol A, Fol B	63
Blue Springs Creek (BSC), Guelph	Fol A, Fol B	30
Fishless pond, Guelph	Fol A, Fol B	10
Ministry of Environment (MOE), Etobicoke	CO1 Crust DF1, Crust DR2	12

In order to verify the amplification of DNA, 5 μL of the PCR products were electrophoresed in a 1% agarose gel, stained in ethidium bromide, and visualized using UV light. The products were subjected to another round of electrophoreses and ethidium bromide stain, imaged with UV light to excise the desired fragment, as well as purified using Qiaex kit (QIAGEN Inc.) (Witt *et al*., 2006).

Using the appropriate primers (Table 2-1), samples were sequenced in one direction on an ABI™ 3730 automated sequencer (Applied Biosystems) at the Genomics Facility (University of Guelph). Sequences were inspected, aligned, and trimmed using MEGA5 (Tamura *et al*., 2011) as well as compared to those collected by M. Hyrcyshyn (PhD thesis in preparation). The unweighted pair group method with arithmetic averages (UPGMA) was used to construct a phenogram (or a distance tree) to identify the clade to which individuals belonged, the number of COI haplotypes, and their frequency in each population (Sokal and Michner, 1958).

A phylogenetic tree employing the neighbour-joining (NJ) method with the Tamura-Nei model of sequence evolution was constructed to compare the COI haplotypes obtained in this study to previously characterized sequences (Witt and Hebert, 2000; M. Hrycyshyn, PhD thesis in preparation). The NJ

method was chosen to reconstruct the phylogenetic tree since it can quickly provide branch lengths and topology (Saitou and Nei, 1987). Transitional bias, unequal nucleotide frequencies, and different substitution rates were taken into account using the Tamura-Nei model of nucleotide substitution (Tamura and Nei, 1993). Finally, the bootstrap method with 1000 replicates was used to place confidence limits (i.e., the statistical reliability) on each internal node of the tree (Felsenstein, 1985).

A pairwise comparison of sequence divergence within- and between-populations was conducted using the Tamura-Nei model of nucleotide substitution (Tamura and Nei, 1993). Similarly, the pairwise amino acid sequence divergence (invertebrate mitochondrial code) between-populations was calculated using the p-distance or the proportion of differences between the sequences (Nei and Kumar, 2000). A standard error, estimated by the bootstrap procedure (1000 replicates), was obtained for each mean sequence divergence within and between groups.

Once populations of *Hyalella* were identified and established in aquaria, they were maintained at room temperature (23-25°C), aerated continuously, and exposed to a photoperiod of 16 h of light (intensity of 22 $\mu E/m^2/s$) and 8 h of darkness. Cotton balls were introduced as a substrate for the animals and ground Tetramin® was added to the tank *ad libitum*.

A subset of *Hyalella* from each identified clade was randomly removed from the aquaria and placed into 2 L plastic (high-density polyethylene) culture containers. The culture procedure using the 2 L containers was similar to the method outlined by Borgmann *et al.* (1989). The 2 L plastic culture container was prepared with the following: 1 L SAM-5S (dissolved organic carbon 0.3 mg/L, dissolved inorganic carbon 9.43 mg/L, hardness ($CaCO_3$) 898 µmol/L, Alk 41.53 mg/L, Cl 1892 µmol/L, SO_4 243 µmol/L, Mg 244 µmol/L, Na 965 µmol/L, and K 50 µmol/L), a single piece of 5 cm^2 pre-soaked cotton gauze (substrate), and 5 mg of Tetramin® fish food. The major ions in the SAM-5S solution were measured at the National Laboratory for Environmental Testing (Environment Canada, Burlington, Ontario, Canada).

To ensure equal numbers of males and females, 25 mating pairs (total of 50 individuals) were added to each culture container. If 25 mating pairs could not be collected, then individual amphipods were sexed: ovigerous females were selected and males were identified by their enlarged gnathopods. Adults in these containers were exposed to the same light conditions as animals in the aquariums and fed 5 mg of TetraMin® three times a week at 2-3 day intervals.

All clades were exposed to the same light schedules, feeding regimes, and water conditions using SAM-5S. To monitor culture health, juvenile production and adult survival were monitored on a weekly basis. Separating adults from the juveniles on a weekly basis also ensured that the juveniles were the similar in age (0 to 7 days). First, the cotton gauze, which served as a substrate for the *Hyalella*, was shaken in SAM-5S to remove all the amphipods that clung to the gauze. Contents in the 2 L plastic culture container were poured through two filters to separate adults and juveniles (mesh sizes 750 and 300 μm, respectively). The adults were counted and returned to their original culture container after it was gently scrubbed, filled with 1 L of fresh SAM-5S and spiked with ~2mL algae as well as 5 mg of Tetramin®. Unless completely disintegrated, the cotton gauze was also returned to the culture container; otherwise, a new piece was added. After the juveniles were counted, they were transferred to a new culture container. This culture container contained 1 L of SAM-5S and a single 5 cm^2 cotton gauze piece. They were fed at the same time as adults with 2.5 mg of ground and sifted (750 μm mesh) TetraMin® (or 5 mg if there were >100 juveniles).

2.2.2 Adult Mortality and Juvenile Production per Adult in Culture

After adult and juveniles were separated each week, they were enumerated. Adult mortality was calculated from survival using Equation 2-1, where N is the number of surviving animals, and N_o is the initial number of animals added to each exposure vessel.

Equation 2-1: Survival data converted to mortality.

$$m = -ln\left(\frac{N}{N_o}\right)$$

Mortality data did not include culture containers that were restocked with *Hyalella* over the course of four weeks. The time frame monitored for adult mortality in culture containers ranged from weeks zero to four since several chronic toxicity tests are conducted over four weeks or 28 days in length (Borgmann and Munawar, 1989; Borgmann and Norwood, 1993; Nipper and Roper, 1995; Borgmann and Norwood, 1997; Green *et al*., 1999).

The relationship between mortality and time (weeks 0 to 4) was determined by a linear regression ($y = mx+b$), where the resulting slope (m) represented the mortality rate, the variable y was the mortality, b was the y-intercept, and x was time. In order to determine longevity, the time (in weeks) for 50% of the original population of adults to survive, the variable, x, was solved from the regression by setting y to 0.693. The number 0.693 was derived from Equation 2-1 at 50% survival. In other words, if half (50%) of the population survived, then the equation was presented as –ln [50%], which resulted in a mortality of 0.693.

Juvenile production was quantified each week as the number of offspring produced per adult in each culture container. The ratio for juvenile production per adult was taken from culture containers to which new adults were not added the prior week. Utilizing culture containers with adults that were acclimatized to culture container conditions for at least a week provided consistent results and avoided skewing the number of juveniles produced per adult.

The IBM® SPSS® Statistics 22 (IBM Corporation; Armonk, NY) was used for all statistical tests. A two-way ANOVA was used to determine significance among clades and adult mortality in culture containers over time (weeks). Significance among clades when comparing the mean number of juveniles produced per adult ratio was determined using a one-way ANOVA. Both the two-way and one-way ANOVA assume that the data were normally distributed and their variances were equal (homoscedastic) (Wardlaw, 1999). To determine which clades had significantly different adult mortality or juvenile per

adult ratio, Tukey's multiple comparison of means, a post hoc test, was employed (Wardlaw, 1999). An outline of the statistical procedure is illustrated in Figure 2-1.

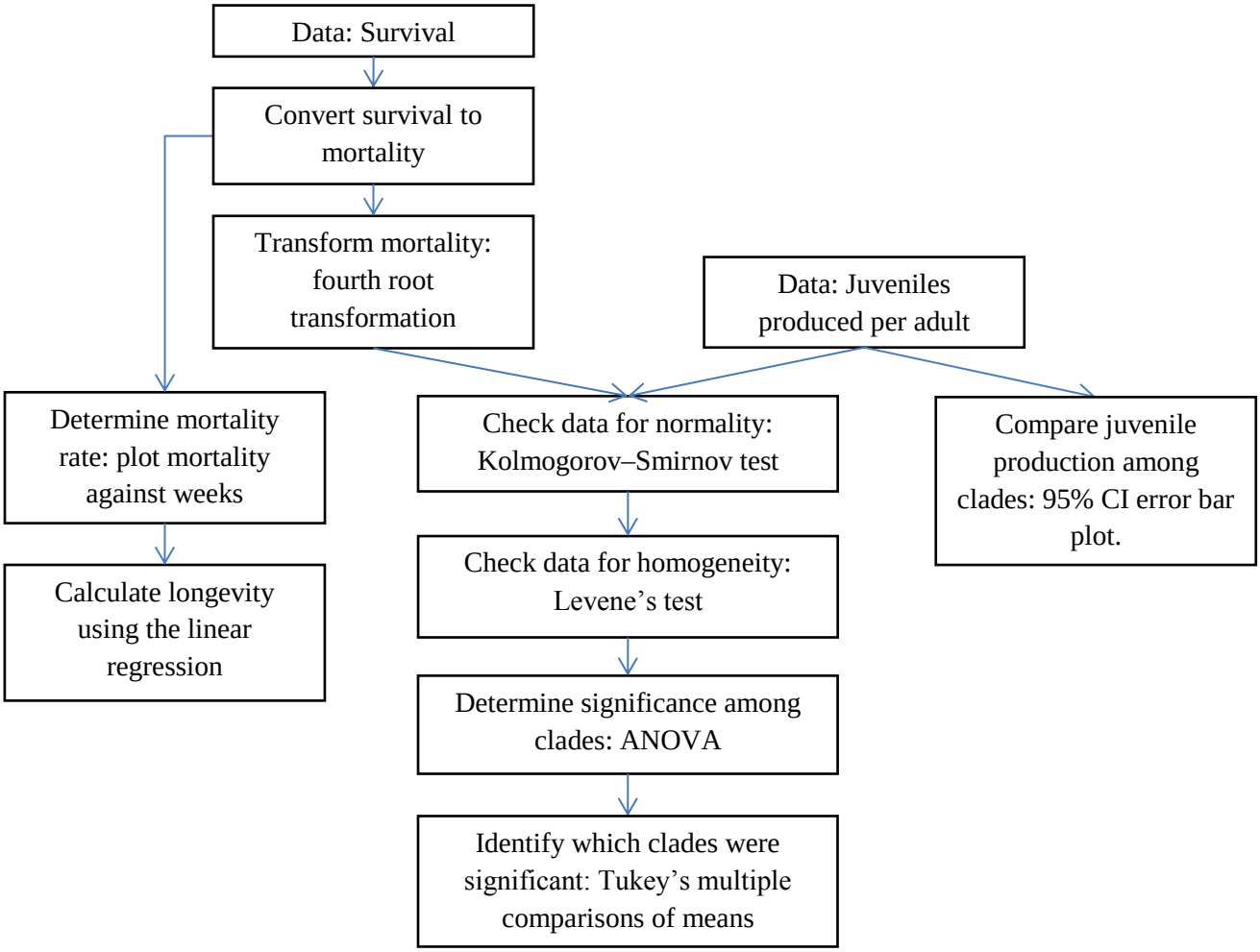

Figure 2-1: Flowchart of the statistical procedures applied to the survival data and juvenile production.

2.3 Results

2.3.1 Collection, Identification, and Culture Procedures of *Hyalella azteca*

One hundred and fifty-one *Hyalella* COI sequences were obtained from samples collected in the field and laboratories. Nucleotide sequence alignments and amino acid translations did not indicate the presence of gaps or nonsense codons. Among the populations of amphipods sequenced, a preliminary NJ analysis identified five monophyletic groups (Table 2-2) that corresponded to previously characterized clades (1, 2, 3, 6, and 8) reported by Witt and Hebert (2000) and M. Hrycyshyn (PhD thesis in preparation).

Table 2-2: Culture origins, clades, and sample size of individuals sequenced.

Origin of culture	Clade	Sample size
Canadian Centre for Inland Waters (CCIW), Burlington	1	36
University of Waterloo (UW), Waterloo	8	35
Guelph Lake (GL), Guelph	3, 6	53, 10
Blue Springs Creek (BSC), Guelph	1, 6	15, 15
Fishless pond (Guelph)	1-2 hybrid	10
Ministry of Environment (MOE), Etobicoke	8	12

The *Hyalella* received from established laboratories were observed to be much larger than the amphipods collected in the field, with the exception of those from the fishless pond outside Guelph. The COI sequences of the amphipods received from the MOE and UW corresponded to clade 8, whereas the *Hyalella* cultured at the CCIW were associated with clade 1.

Clades 3 and 6 co-occurred in the population from GL. Initially, amphipods from BSC consisted of clade 6 only, but after a second sampling trip, the COI sequences were identified as clade 1. Finally, a mix of clades 1 and 2 was detected from the fishless pond outside of Guelph (Table 2-2).

The amphipods from CCIW (clade 1), GL (clades 3 and 6) and UW (clade 8) are depicted in the NJ phenogram (Figure 2-2) and were cultured. Clades from BSC and the fishless pond outside of Guelph were not included in the tree since they represented a mix of two species. In addition, the amphipods from the MOE were not sufficiently acclimatized to laboratory conditions prior to the experiments and were not included in the NJ phenogram. The four clades are well supported by the NJ analysis and form distinct monophyletic groups (Figure 2-2).

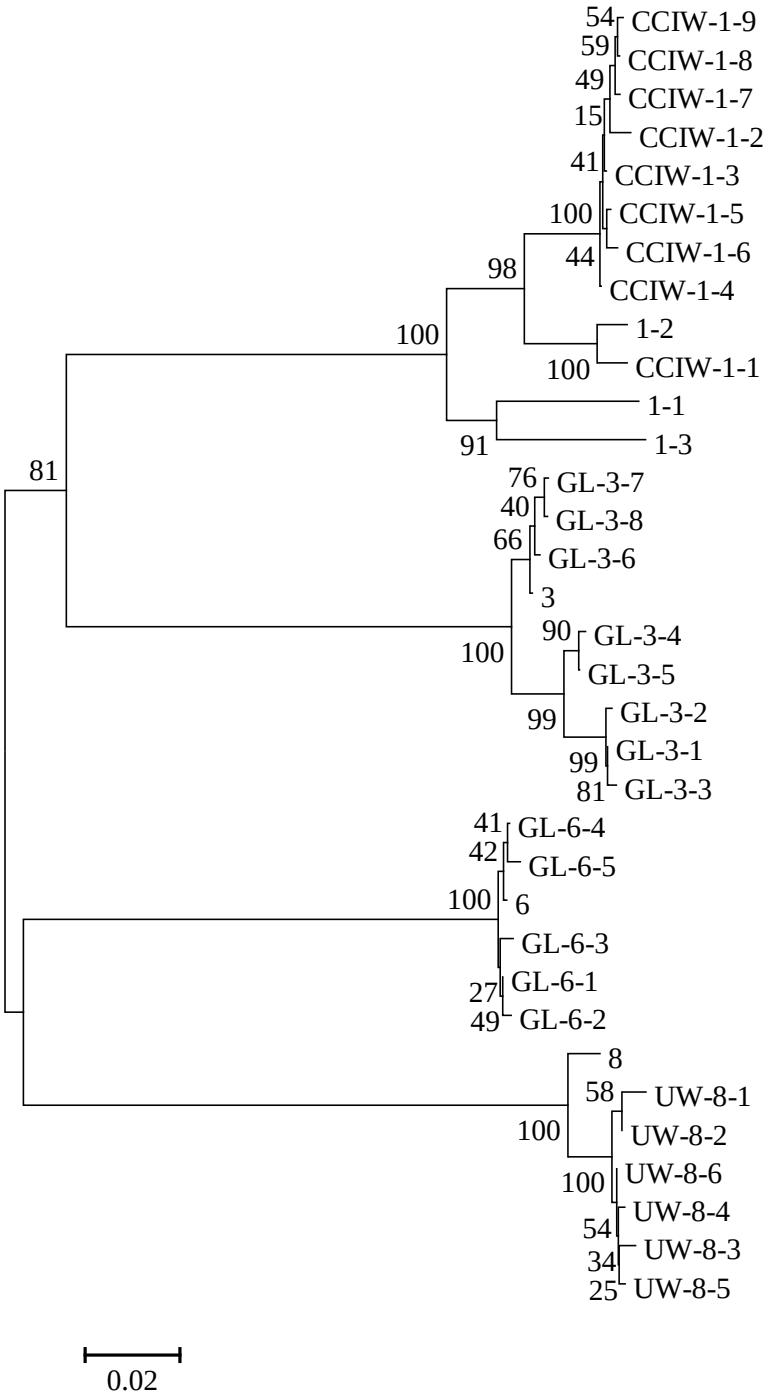

Figure 2-2: NJ phenogram 28 COI *Hyalella* haplotypes from the Canadian Centre for Inland Waters (CCIW), University of Waterloo (UW), and Guelph Lake (GL). Haplotypes from each site are indicated by their acronym, clade number, and haplotype number. The phylogeny was estimated in MEGA5 using the bootstrap method with 1000 replicates. Bootstrap values are given in the above nodes.

The analysis of the 637 bp COI sequences using UPGMA identified 28 haplotypes among the four clades (Figure 2-2 and Table 2-3). Clade 1 had the highest number of haplotypes, followed by clades 3, 8 and 6 (Table 2-3).

Table 2-3: Clade, collection location, haplotypes and their frequencies for lineages of *Hyalella* used in this study.

Clade	Location	Haplotypes	Frequency
1	CCIW	1-1	1/36
		1-2	1/36
		1-3	9/36
		1-4	1/36
		1-5	2/36
		1-6	1/36
		1-7	1/36
		1-8	19/36
		1-9	1/36
3	GL	3-1	27/53
		3-2	1/53
		3-3	1/53
		3-4	1/53
		3-5	9/53
		3-6	1/53
		3-7	1/53
		3-8	12/53
6	GL	6-1	6/10
		6-2	1/10
		6-3	1/10
		6-4	1/10
		6-5	1/10
8	UW	8-1	2/35
		8-2	8/35
		8-3	1/35
		8-4	1/35
		8-5	1/35
		8-6	22/35

Clade 3 was the most diverse clade in this study with a mean nucleotide sequence divergence among its haplotypes of 1.5% (standard error or SE 1.2-1.7%), followed by clades 1, 8, and 6 at 1.3% (SE 0.9-1.7%), 0.5% (SE 0.3-0.7%), and 0.4% (SE 0.1-0.7%), respectively (Table 2-4). The pairwise comparisons of the mean nucleotide sequence divergences between clades ranged from 22.6-26.0%

(Table 2-4). Similarly, the average amino acid divergences (invertebrate mitochondrial code) observed between clades ranged from 3.3-5.3% (Table 2-4).

Table 2-4: The mean COI nucleotide sequence divergence in decimal percentages (± standard error or SE) within populations using the Tamura-Nei model is shown on the diagonal in boldface. Below the diagonal are the mean COI nucleotide sequence divergences using the Tamura-Nei model between populations. Above the diagonal are the amino acid sequence divergences between populations, which are the p-distances.

	CCIW – 1	GL – 3	GL – 6	UW – 8
CCIW - 1	**0.013±0.004**	0.043±0.012	0.053±0.014	0.051±0.013
GL – 3	0.226±0.020	**0.015±0.003**	0.035±0.011	0.033±0.010
GL – 6	0.233±0.020	0.235±0.020	**0.004±0.003**	0.039±0.012
UW – 8	0.260±0.022	0.245±0.023	0.228±0.020	**0.005±0.002**

Although the clades were not directly categorized by size in this study, the mitochondrial COI sequences were compared to those stored in GenBank. This comparison confirmed that both clades 3 and 6 were small-bodied ecomorphs, whereas clades 1 and 8 were large-bodied ecomorphs reported by Wellborn and Broughton (2008).

2.3.2 Adult Mortality and Juvenile Production per Adult in Culture

In general, the large-bodied clades (1 and 8) were relatively more successful in survival and juvenile production in laboratory conditions than their smaller counterparts (3 and 6). The poor success demonstrated by the small-bodied clades may be a result of insufficient acclimation time in the laboratory, water conditions, incompatible food sources, etc.

Several attempts to culture individuals from clade 6 in the 2 L plastic containers were made during this study. Unfortunately, approximately half the individuals from clade 6 were lost on a weekly basis. Attempts to improve survival by using different culture mediums (e.g., SAM-5S, dechlorinated water) were not successful. Regardless of adjustments to water softness to the culture medium, mortality remained high and individuals from clade 6 were returned to the aquariums where they appear to have better survival. As a result of the lack of data from this group, clade 6 was excluded from this thesis. In

total, weekly culture records for mortality rates and juvenile production for clades 1, 3, and 8 were assessed.

Adult mortality was square root transformed to normalize data and equalize variances. A two-way ANOVA tested the adult mortality of each clade over time (weeks) and yielded an effect for clade [$F(2, 50) = 13.351$, $p < 0.001$]. The effect of time (weeks) was also significant ($p < 0.01$), indicating that the mortality increased over time (Figure 2-3). The post-hoc Tukey's test indicated that the adult mortality rate for clade 3 was significantly different than from clades 1 and 8 (both $p < 0.01$), but clades 1 and 8 were the same ($p = 0.270$). The mortality rate (*m*) was three fold greater for clade 3 than clades 1 and 8, as outlined in Table 2-5. Consequently, the slope for clade 3 (green triangles) was much steeper than for the two other lineages (Figure 2-3).

Table 2-5: Mortality rate (*m*), y-intercept (*b*), longevity (*x*), and the regression fit (r^2) for adult mortality observed in clades 1, 3, and 8 over a span of four weeks.

Linear regression parameters	Clade 1	Clade 3	Clade 8
Mortality Rate, m	0.094	0.258	0.073
Y-intercept, b	0.008	-0.050	-0.004
Longevity (weeks), x	7.308	2.882	9.588
Regression fit, r^2	0.577	0.576	0.340

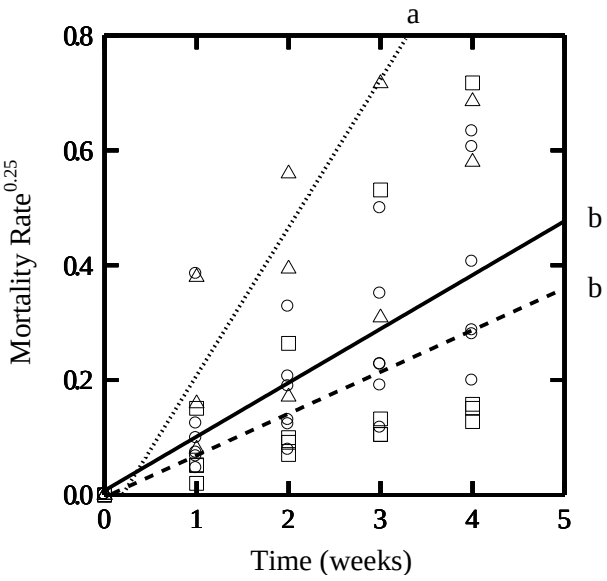

Figure 2-3: Fourth root transformed mortality as a function of time (weeks). Clades 1, 3, and 8 are indicated by a solid line/o, small dotted line/Δ, and dashed line/□, respectively. The letters (a and b) indicate significant differences between clades for mortality rates as determined by the two-way ANOVA.

A one-way ANOVA was conducted to compare the effects of the three clades (1, 3, and 8) on the ratio for juveniles produced per adult. This ratio required a square root transformation to fulfill parametric assumptions (normality and homoscedasticity) of the one-way ANOVA. There was a significant effect of clade, where $p < 0.05$ level, [$F(2, 54) = 15.172$, $p < 0.001$]. This indicates that the number of juveniles produced per adult varied depending on clade. The post hoc (Tukey's test) comparison of the mean number of juveniles produced per adult indicated that clade 3 (mean = 0.933, 95% CI 0.765-1.10) was significantly different than those for clade 1 (mean = 1.44, 95% CI 1.29-1.60) and 8 (mean = 1.66, 95% CI 1.45-1.87). However, clade 1 and 8 did not significantly differ from each other ($p = 0.166$).

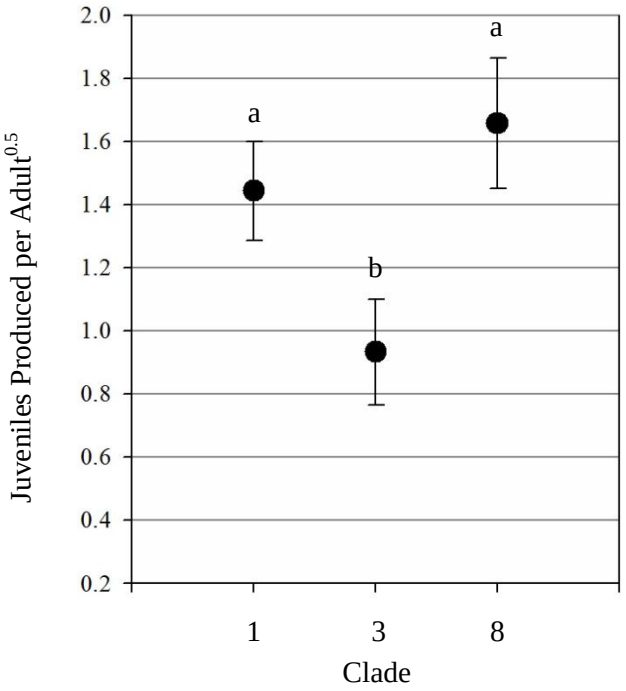

Figure 2-4: The 95% CI error bar plot for the ratio of juveniles produced per adult (square root transformed) for clades 1, 3 and 8. The letters (a and b) indicate significant differences between clades as determined by the one-way ANOVA.

These results suggested that clades had different adult mortality and juvenile to adult production ratios in culture. Specifically, clade 3 had higher rates of adult mortality and produced less juvenile per adult than clades 1 as well as 8. Clades 1 and 8 did not have any significant differences in adult mortality or the amount of juveniles produced per adult.

2.4 Discussion

The COI nucleotide sequence divergences among the three clades tested for life history traits were greater than 22.6% and were consistent with interspecific differences (Witt *et al.,* 2006). The COI nucleotide sequence divergence between clades 1 and 8 in this study was 22.6-26.0%. This is consistent with the percent divergence reported by Major *et al.* (2013), which ranged from 23.1-24.9%.

The small-bodied clades (3 and 6) also formed distinct monophyletic groups, and the magnitude of COI nucleotide sequence divergences between these two lineages was 23.5%. This result was

consistent with that reported by Witt and Hebert (2000), who also employed the 637bp COI fragment to determine the average nucleotide sequence divergence in clades 1, 3, and 6.

This study compared two laboratory relevant aspects of life history among three genetically characterized members of the *Hyalella azteca* cryptic species complex. The life history parameters (mortality and juvenile production) were hypothesized to be significantly different among clades. Despite exposure to identical culture conditions, the large-bodied clades (1 and 8) outperformed the small-bodied clades (3 and 6) by having the lowest adult mortalities and highest juvenile productions.

It may be that "what might work well for one laboratory might not work as well for another laboratory" (USEPA, 1994) could simply be due to the use of different clades, which may have resulted in the development of different laboratory protocols. Notably, food type and regime, water hardness, as well as substrate are the most important variables for survival, growth, and reproduction. These factors are often left to the discretion of the laboratory personnel (Environment Canada, 2013), which may be a result of compensating for the different nutritional and/or behaviour requirements for each clade. Although the Canadian and American culturing protocols are available for rearing *Hyalella* (Environment Canada, 1997; 2013), these methods may not produce conditions that guarantee universal success for all clades. In addition, none of these protocols were developed with a specific genetically characterized species of *Hyalella*, although the brood source was indicated. Ideally, clade specific culture protocols should be developed.

The differences in the number of juveniles produced per adult in this study was consistent with those reported by Wellborn and Cothran (2004), who determined that the large-bodied morphs had more, yet smaller eggs than their small-bodied counterparts. They also documented that small-bodied ecomorphs produced fewer juveniles as a result of smaller clutch sizes. However, the differences in mortality and juvenile production between ecomorphs may be a result of insufficient acclimatization time for clades 3 and 6, which were collected from the wild instead of a laboratory. Four clades (1, 3, 6, and 8)

were reared for several generations prior to the analyses for mortality and juvenile production, but the large-bodied clades (1 and 8) had previously been in culture for many years. In contrast, the small-bodied clades (3 and 6) had only been in culture for a few months.

In addition to potentially insufficient acclimatization times for clades 3 and 6, the poor survival of adults and low juvenile production for these small-bodied clades may be due to an unknown environmental constraint. The differences in ionic composition may have been too extreme for clades 3 and 6 to tolerate since these clades were from Guelph lake and SAM-5S mimicked lake Ontario (API, 1999). Although SAM-5S is recommended by Environment Canada (2013) because it has more universal success with respect to *Hyalella* growth and reproduction, this culture medium may either be deficient or have an excess of a particular nutrient that is required by clades 3 and 6. As a result, the water conditions may have been suboptimal for the small-bodied clades, stressing the importance of species-specific culturing conditions (Neuparth *et al*., 2002).

Regardless of the potential confounding problems caused by insufficient acclimatization and culture media, different life history traits are not uncommon among members of a cryptic species complex. For example, two cryptic lineages of the *Lessonia nigrescens* complex (kelp) have different life history strategies and temperature tolerances (Oppliger *et al*., 2012).

The different life history traits among members of a cryptic species complex may influence toxicity responses and the interpretation of toxicity results must be made cautiously. This is the case for Atrazine, a popular herbicide, which has been the subject of debate as to whether it causes testicular ovarian follicles (TOF) in *Xenopus laevis*, the African clawed frog. Du Preez *et al*. (2009) conducted a phylogenetic analysis employing DNA barcoding for *X. laevis* collected from South Africa. They concluded that the population southwest of the Cape Fold Mountains was genetically divergent in comparison to those to the northeast and beyond. In addition, they reported that *X. laevis* northeast of the Cape Fold Mountains had incidences of TOF regardless of exposure to Atrazine, but those from the

southwest region did not. Du Preez *et al*. (2009) demonstrated that the incidences of TOF may vary according to the *X. laevis* lineage due to its life history. Life history traits should be studied in cryptic species complexes since they may influence the interpretation of toxicity responses.

2.5 Conclusion

Four clades were exposed to identical holding conditions, but the large-bodied clades (1 and 8) had significantly lower mortality rates and higher juvenile recruitment per amphipod than the smalle-bodied clades, 3 and 6. This study clearly indicated that there were statistical differences among clades for adult mortality and juvenile production; although further study on additional life history traits needs to be conducted in order to better characterize the many lineages of *Hyalella* (e.g., length and weight as a representative for growth, clutch size per female, juvenile mortality rate, and time until sexual maturity, etc.).

Since the proper understanding of life history traits was necessary to study toxicity responses at the organismal level, optimal culture protocols should be established for each clade to have comparable life history information among laboratories. In addition, toxicity responses should be cautiously interpreted when life history details among lineages from a cryptic species complex are not fully characterized.

3. Chapter 3 – Bioaccumulation Responses for Two Members of the *Hyalella azteca* Cryptic Species When Exposed to Copper and Nickel

3.1 Introduction

The amphipod crustacean, *Hyalella azteca* (Saussure, 1858), occurs in a variety of permanent freshwater habitats throughout North America and has many characteristics that make it an ideal organism to study in the laboratory, including short life cycles, easy collection and culture in captivity. In addition, this amphipod is sensitive to contaminants and relevant to the aquatic food chain (Lawrence, 1981; USEPA 1994; Bousfield, 1996; Environment Canada, 1997, 2013).

Since the late 1980s, this amphipod has been used in hundreds of acute and chronic aquatic toxicity investigations for metals, acidification, organic compounds, as well as sediments. Its responses to contaminants have frequently influenced water quality standards that protect aquatic life and, ultimately, affect human health (Environment Canada, 2013).

Recent ecological and genetic studies on *H. azteca* have revealed that it is actually a group of numerous distinct species that are morphologically similar (Wellborn, 1994b, 1995a, 2002; Hogg *et al*., 1998; McPeek and Wellborn, 1998; Gonzalez and Watling, 2002; Wellborn and Broughton, 2008; Wellborn *et al*., 2005; Witt and Hebert, 2000; Witt *et al*., 2006). Currently, there are 85 provisional species within the *Hyalella azteca* cryptic species complex (Witt and Wellborn, in preparation). Despite the huge genetic diversity discovered in the wild, two publications have indicated that only two clades are used in 17 laboratories throughout North America: clade 1 and 8 (Major *et al*., 2013; Weston *et al*., 2013). Further, only one of the 17 laboratories uses clade 1 while those remaining use clade 8.

The relationship between toxicity responses in members of the *H. azteca* cryptic species complex was assessed by two groups of researchers. Soucek *et al*. (2013) reported different responses among three clades of *Hyalella* when they conducted acute toxicity tests using two anions (nitrate and chloride) with fed and unfed amphipods. Weston *et al*. (2013) observed a 550 fold difference in sensitivity among four

members of the *H. azteca* complex when exposed to a pyrethroid insecticide. Currently, different sensitivities among clades using metal stressors are unknown.

In this chapter, the saturation bioaccumulation model (Norwood *et al*., 2006) is used to determine whether the two most commonly used clades in laboratories accumulate copper and nickel in the same manner. The bioaccumulation or the concentration of a chemical in the organism can be used as an indicator of toxicity. This endpoint links the amount of contaminants in the tissue of the test organism with toxic effects (McGeer *et al*., 2012). The use of bioaccumulation as a toxic response can simplify and identify the cause of biological effects in sediment assessments (Borgmann and Norwood, 1997). Complications from other endpoints (e.g. LC50) are negated when using bioaccumulation as a response variable since it integrates many factors such as metal interactions, binding factors, ligands, geochemical effects, etc. (Landrum *et al*., 1992; McGeer *et al.*, 2003).

The saturation bioaccumulation model was used in previous studies to describe the relationship between metal exposure and its accumulation in *Hyalella* (Borgmann *et al.*, 2004). In this study, the concentrations of copper and nickel accumulated within the tissues of each clade were compared. It was hypothesized that clades would have different bioaccumulation when exposed to either copper or nickel.

3.1.1 Theory for the Saturation Bioaccumulation Model

The saturation bioaccumulation model was used to mechanistically describe metal uptake in *H. azteca* (Borgmann *et al*., 2004). Using this model (Equation 3-1), Borgmann *et al*. (2004) demonstrated that copper and nickel could be described equally well or better than the allometric model developed by McGeer *et al.* (2003).

Equation 3-1: Mechanistically based saturation bioaccumulation model described 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 above background amount of metal that can be accumulated, C_W is the concentration of metal in the exposure solution, *K* is the half saturation constant or the metal concentration when C_{TB} is halfway between the background body concentration and the maximum body concentration of the metal, and C_{BK} is the background concentration of metal (control animals).

In the situation where Equation 7 can not resolve for *max* or *K*, the model is further simplified to solve for *max/K* instead (Equation 8).

Equation 3-2: The simplified mechanistically based saturation bioaccumulation model used to solve for *max/K* when the model cannot resolve *max* or *K* individually. The procedure to simplify Equation 3-1 is also included.

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

$$C_{TB} = \frac{\frac{max\ C_w}{K}}{\frac{K + C_w}{K}} + C_{Bk}$$ Divide the equation by K

$$C_{TB} = \frac{\frac{max\ C_w}{K}}{1 + \frac{C_w}{K}} + C_{Bk}$$

If K is very large, then the term $\frac{C_w}{K}$ becomes very small/insignificant.

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

3.2 Materials and Methods

3.2.1 Toxicity Procedures

The *Hyalella* collected from local ponds, lakes, and rivers in southern Ontario, as well as from established laboratories stocks, were placed into aquariums filled with SAM-5S (Borgmann, 1996), identified using DNA barcoding (Witt *et al*., 2006), and cultured for several generations before collecting juveniles for toxicity tests. As a result of poor survival and juvenile production, toxicity tests were not conducted using either of the small-bodied clades (3 and 6). Only two lineages, clades 1 (CCIW) and 8 (UW), were

successfully cultured with enough juvenile production to conduct the toxicity tests. Please refer to Chapter 2 for more details on culture protocols and results.

Two hundred liters of SAM-5S were prepared prior to the start of the toxicity tests in order to have all experiments conducted using the same water. For this reason, the major ion concentrations of SAM-5S (dissolved organic carbon 0.3 mg L^{-1}, dissolved inorganic carbon 9.43 mg/L, hardness ($CaCO_3$) 898 µmol/L, Alk 41.53 mg/L, Cl 1892 µmol/L, SO_4 243 µmol/L, Mg 244 µmol/L, Na 965 µmol/L, and K 50 µmol/L) were measured only once by the National Laboratory for Environmental Testing (Environment Canada, Burlington, Ontario) before the start of the toxicity experiments.

Each clade (1 and 8) and metal (copper and nickel) were tested three times (experiments A, B, and C) for a total of twelve toxicity experiments (3 replicates × 2 clades × 2 metals = 12 experiments) according to the methods described by Norwood *et al.* (2006), but with some modifications. Each experiment was 14 days in length with a renewal at day 7. These tests included three replicates for the control and two replicates for each of the seven metal concentrations (Table 3-1).

Table 3-1: The number of replicates for copper and nickel assays as well as their respective nominal metal concentrations.

Metal Concentration	Number of Replicates	Nominal Concentrations ($\frac{\mu mol}{L}$)	
		Copper	Nickel
Control	3	0	0
Conc. 1	2	0.012	0.28
Conc. 2	2	0.196	0.49
Conc. 3	2	0.35	0.875
Conc. 4	2	0.63	1.575
Conc. 5	2	1.12	2.8
Conc. 6	2	1.96	4.9
Conc. 7	2	3.5	8.75

Plastic (high-density polyethylene) 500 mL containers were filled with 400 mL of SAM-5S, spiked with the appropriate volume of copper or nickel stock solution (Table 3-1), and a piece of gauze (2.5 cm^2) was added. These containers served as the experimental vessels for the toxicity test. The metal concentration series for copper and nickel were determined by preliminary trials using animals belonging to clade 1 (Table 3-1). The metal stock solutions were made by dissolving the metal chloride salt ($CuCl_2 \cdot 2H_2O$ or $NiCl_2 \cdot 6H_2O$) into Milli-Q® water.

The containers were allowed to equilibrate for a minimum of 2 h. After, water quality parameters including pH, conductivity, dissolved oxygen (DO) concentrations, and ammonia were measured. The pH and conductivity were measured in the first replicate of each metal concentration. The first replicate of the control was also measured for DO and ammonia in addition to the pH and conductivity on the start and renewal days.

Twenty randomly assigned juveniles (2 to 9 days old) from genetically characterized cultures were placed into each test vessel. These juveniles were separated from adults two days prior to the experiment to ensure that there would be enough juveniles for the bioassay and to exclude the individuals that had died due to handling (Borgmann *et al*., 1989). After the addition of the 20 juveniles, 2.5 mg of ground TetraMin® was added to each experimental container. These juveniles were fed 2.5 mg of ground TetraMin® every 2-3 days. The toxicity assay was incubated at 25ºC (± 2ºC) with a photoperiod of 16 h of light (intensity of 22 μE/m2/s) and 8 h of darkness.

On the seventh day, the test containers were removed from the incubator and an aliquot from the first replicate of each experimental concentration was dispensed into a small 70 mL polystyrene cup; care was taken to not dispense any juveniles. The copper and nickel test solutions were then assayed for conductivity, pH, ammonia, and DO using these aliquots. The mean (95% confidence interval or CI) measurements of pH, conductivity, dissolved oxygen, and ammonia of test solutions were 7.69 (95% CI

7.65-7.71), 459 µs/cm (95% CI 454-463), 9.48 mg/L (95% CI 9.16-9.80), and 0.026 mmol/L (95% CI 0.026-0.029), respectively.

Surviving juveniles (days 0-7) were poured along with the contents from each plastic container into a clean glass sorting bowl, starting with the lowest concentration of metals (i.e., live *Hyalella* were removed from the glass bowl with a clean eyeglass dropper, counted, and placed into the fresh test/renewal solution). Once all of the live juveniles were accounted for, the gauze from the first toxicity test (days 0-7) was also transferred into their respective renewal container. Renewal solution at day 7 consisted of a new set of test solutions prepared as above. The measurements for conductivity, pH, ammonia, and DO as well as the collection of the subsamples of the test solutions followed the same procedures as above. The new set of test vessels were then returned to the incubator for another 7 days.

After a total of 14 days, the juveniles were decanted into a clean glass sorting bowl and counted. Juveniles from the replicates of the same metal concentration were combined and placed into a clean polystyrene container filled with 60 mL of 50 µM Ethylenediaminetetraacetic acid (EDTA) in SAM-5S solution, a fresh piece of gauze, and 2.5 mg of TetraMin®, for 24 h to allow for gut clearance (Neumann *et al.*, 1999). After 24 h, the juveniles were removed from the solution, placed on a folded Kimwipe® to remove excess moisture, counted, and their wet weights were measured using the Mettler Toledo microbalance, accurate to 0.001 mg. *Hyalella* were then placed into labelled, acid-washed cryovials, and dried in an oven set at 55°C for 72 h. Before amphipods were digested to determine the amount of metal in their tissue, their dry weights were measured. Figure 3-1 summarizes the procedure for a single toxicity test.

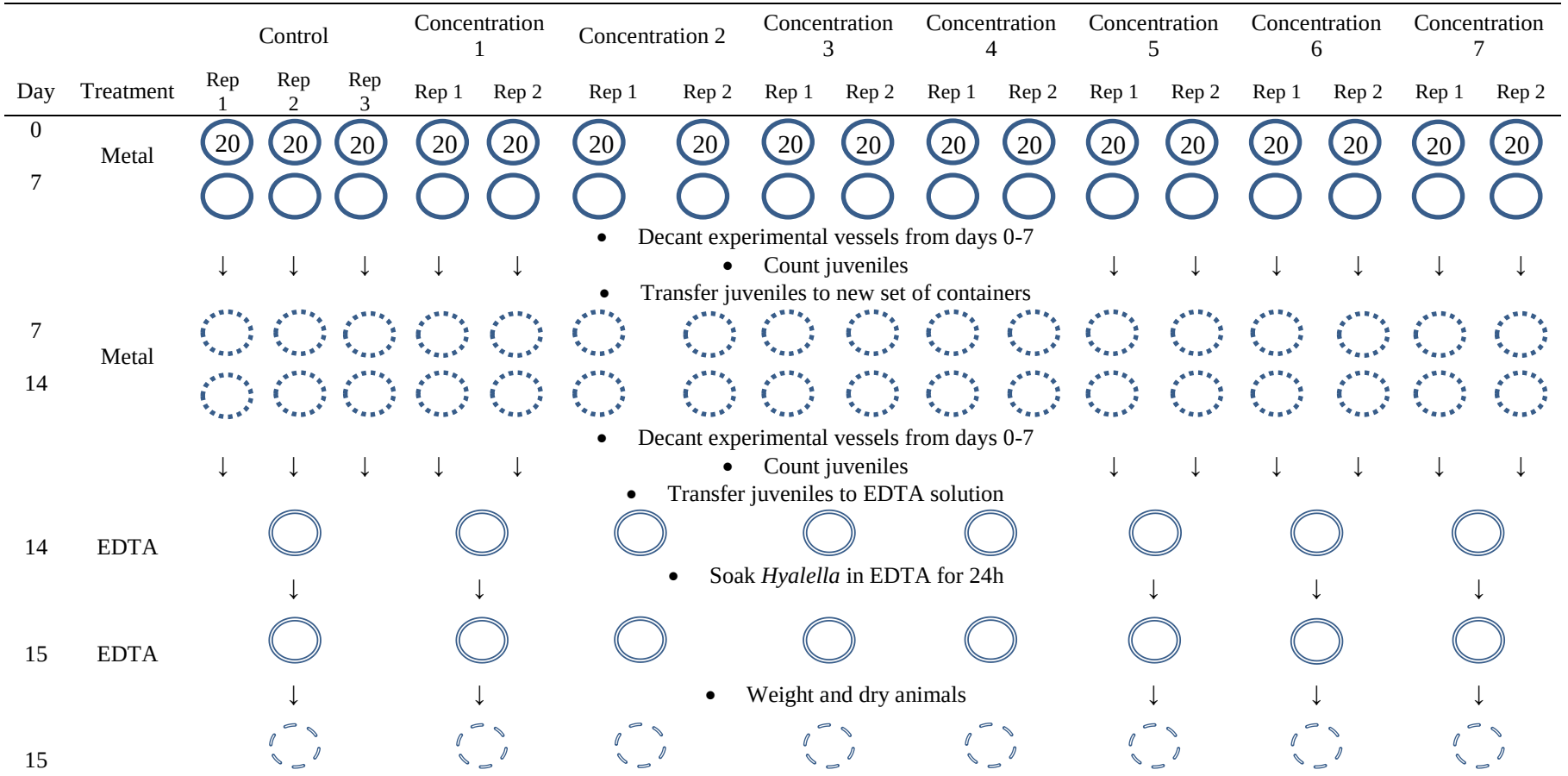

Figure 3-1: The general procedure for a single toxicity experiment for either copper or nickel. This toxicity experiment was performed three times for each clade and metal for a total of 12 experiments. Each concentration had two replicates except the controls, which had three. On day 0, a set of experimental vessels with increasing metal concentrations were prepared. Twenty juveniles were placed into each replicate and exposed to the metal solutions for seven days. On the seventh day, surviving juveniles were counted and transferred to a new set of experimental vessels. These juveniles were exposed to metal once again for another 7 days before being decanted, counted, placed into EDTA for 24 hours, weighed, and dried.

The dried amphipods were digested following the methodology of Stephenson and Mackie (1988) modified according to Borgmann *et al*. (1991) and Norwood *et al*. (2006) using six medium sized animals. Since all total-body samples fell between 0.000-0.750 mg, 13 µL of concentrated nitric acid was added to the *Hyalella*-filled cryovials and digested at room temperature for 6 days, subsequently, hydrogen peroxide (H_2O_2) was added to the digest (10 µL) and incubated for an additional 24 h followed by topping the volumes up to 0.5 mL using Milli-Q® water (Norwood *et al*., 2006). Correspondingly, the CRM for total-body samples, TORT-1 (National Research Council, lobster hepatopancreas), was processed as above and analyzed concurrently with the total-body digests.

To determine the metal concentration that best represented true exposure, subsamples of the test solutions were taken four times throughout the course of the experiment to determine the measured (actual) metal concentrations in media. These samples were taken at the beginning and end of each renewal period. The metal concentration from the first replicate of each treatment was quantified by collecting two 1 mL subsamples of the overlying test solution. One of the 1 mL metal test solution subsample was collected using a Millipore® membrane filter (0.45 µm) to measure the amount of *dissolved* metals within the test solution. The other 1 mL subsample was collected without a filter to measure the *total* metal concentration within the test solution. To correct for contamination, blank samples were acquired each time subsamples of the test solutions were collected. These blank samples are composed of two filtered and non-filtered samples of Milli-Q® water (four blanks in total). These subsamples for test solutions and blank samples (Milli-Q® water) were preserved for metal analyses with 10 µL of pure nitric acid (JT Baker®).

Several test solution samples experienced uneven levels of evaporation since the caps were not sufficiently tightened. The evaporated water from these samples was replaced with Nanopure® and filled until the 1mL mark on the cryovial. Samples of the test solution at higher metal concentrations that exceeded the range of calibration were diluted appropriately with Nanopure® prior to analyses to bring

them in range of the standard curve utilized by the Varian SpectrAA 400 graphite furnace atomic absorption spectrophotometer (GFAAS) with Zeeman background correction.

The GFAAS was used to measure the actual metal concentrations in the test solution. Copper and nickel samples did not require a modifier, and were analyzed using a partition tube in the GFAAS. Ultra-pure argon was used as the carrier gas and blank Milli-Q® samples were analyzed to test for contamination. The test solution samples were compared to a certified drinking water reference material (CRM, from High-Purity Standards Inc.) to ensure the accuracy and precision of the analyses, assessment of metal recovery, and consistency of runs. A calibration curve was generated at the beginning of each run. In addition, the analyses of the standards and blanks were conducted after every five samples in order to compensate for drift and to ensure quality assurance as well as control. The copper and nickel standards (1000 ± 3μg/mL in 2% HNO_3) were diluted to 1ppm and 50ppb, respectively, with Nanopure® water (High Purity Standard from Delta Scientific Laboratory LTD) to produce the instruments standards.

The measured metal concentrations were used instead of the nominal metal concentrations since the latter do not account for evaporation or metals that bind to the sides of the container, food particles, etc. Using the measured metal concentrations was more accurate than the use of nominal concentrations (Hayes, 2008).

Both the filtered and unfiltered 1 mL subsamples of the test solutions were processed to determine whether the filtering excluded some metal in solution. The metal concentrations in the test solutions were corrected for drift using metal standards at each run on the GFAAS. Metal standards and blanks were reanalyzed after every five samples during the analysis on the GFAAS and the CRMs were used to check for precision as well as the average metal recovery. Table 3-2 summarizes the analyses for copper and nickel standards, recoveries, and detection limits.

Table 3-2: Standards, certified reference materials (CRM), and blanks processed by the GFAAS for copper and nickel. Sample sizes, averages, 95% CIs, standard deviations, and detection limits for blanks are given below.

	Percent recovery (%)					
	Copper Standard	Nickel Standard	CRM for Copper	CRM for Nickel	Blank Copper (μg/L)	Blank Nickel (μg/L)
Sample size	128	136	15	12	136	137
Average	97.3	97.0	84.8	87.5	-0.0001	-0.004
95% CI	96.5-98.1	95.5-98.4	80.3-89.3	81.2-93.8	-0.001-0.0001	-0.006--0.002
Standard Deviation	4.6	8.7	8.1	9.9	0.03	0.012
Detection limit	-	-	-	-	0.03	0.036

Samples were corrected with the blank MilliQ® samples and adjusted using the dilution factor. The arithmetic averages for the measured metal concentrations of each experiment represented the filtered and non-filtered metal concentration in the media. Subsamples of the test solution (filtered and non-filtered test solutions) and the total-body samples were tested for normality and homoscedasticity (i.e., the variance among groups are equal) using the Kolmogorov-Smirnov test and Levene's test respectively, using IBM® SPSS® Statistics 22 (IBM Corporation; Armonk, NY). The Kolmogorov-Smirnov test is one of the most sensitive methods for evaluating normality (Lilliefors, 1967). If a significant non-normal distribution was detected, then a probability plot containing the theoretical and actual values was visually assessed to determine normality before transforming the data (Ahad *et al*., 2011; Baccouche *et al*., 2013; Erin Harvey, personal communication). Levene's test is the most robust method to assess the equality of variances (Schultz, 1985). Subsamples of the test solutions and total-body samples were log and square root transformed (respectively) to meet the assumptions of normality and homogeneity of variances (Wardlaw, 1999).

3.3 Results

3.3.1 Toxicity Procedures

Filtered and unfiltered water samples were compared to determine the best metal concentration in each treatment. Nominal and measured metal concentrations were log transformed to meet parametric assumptions of normality and homogeneity of variances.

A two-way ANOVA was conducted to examine the effect of filtered (vs. unfiltered) water samples and nominal metal concentrations on the measured metal concentration in test solutions. There were statistically significant reduction in measured metal concentrations between the filtered and unfiltered water samples for copper [$F(1, 76) = 61.755$, $p < 0.001$] and nickel [$F(1, 76) = 51.724$, $p < 0.001$]. The unfiltered water samples contained higher levels of metals (Figure 3-2). The loss of copper (mean=31%, 95%CI 25-38) and nickel (mean=22%, 95% CI 15-29) from the filtered and unfiltered water samples may be a result of binding to particulates or organic ligands, etc. (Becher *et al.*, 1983; Norwood *et al.*, 2006). Since there was a statistical difference between filtered and unfiltered water samples, parameters determined in this thesis (e.g., bioaccumulation, LC50, IC25, etc.) used only the filtered fraction (dissolved metals) since it provided a better estimate of the bioavailability of the metal (CCME, 2007).

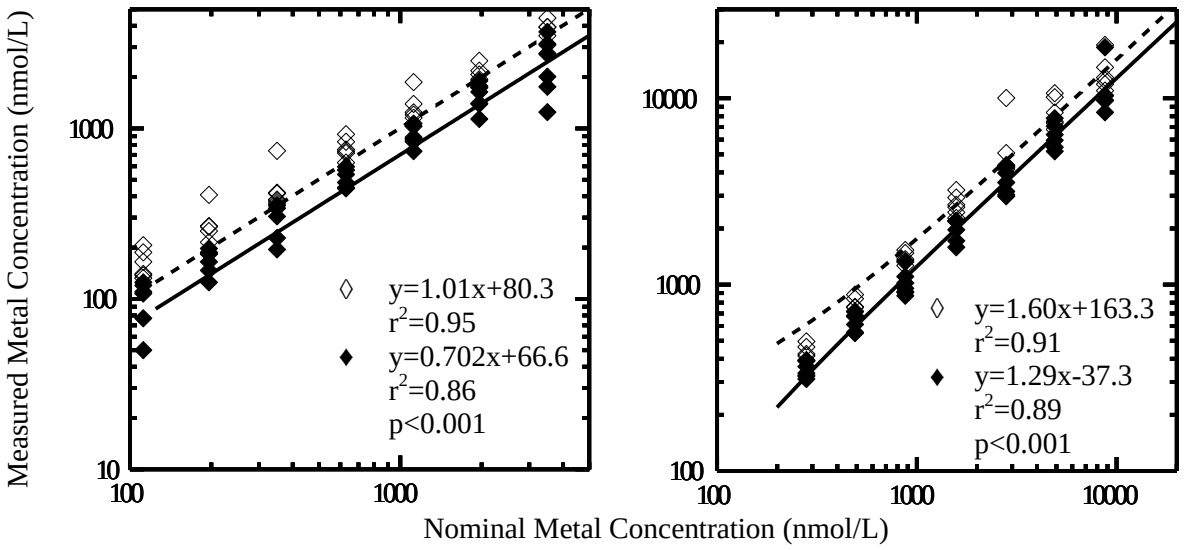

Figure 3-2: The relationship between nominal and measured metal concentrations from the 14 day copper and nickel toxicity tests conducted using *H. azteca*. The linear regression represents the line of best fit for the data. Filtered test solutions (n=48, ♦) and unfiltered test solution (n=48, ◊) for copper and nickel.

3.3.2 Bioaccumulation of Copper

Copper accumulation by clades 1 and 8 demonstrated a positive correlation with increasing dissolved copper concentration in solution (Figure 3-3). The opposite trend was observed for clade 1, experiment A (data not presented). Experiment A from clade 1 had considerably high variability that led to its exclusion and may be a result of contamination.

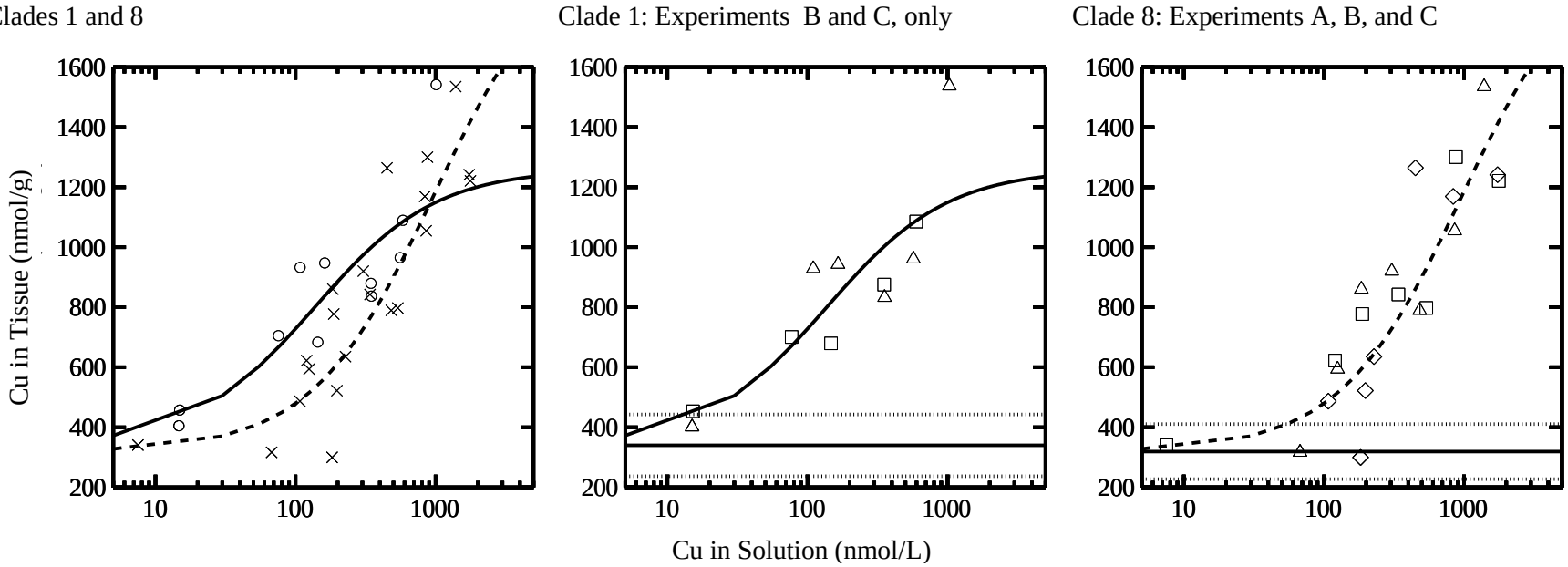

Figure 3-3: Copper accumulated on a dry weight basis by clades 1 (○) and 8 (×) after 14 days of exposure to increasing measured copper concentration in solution. The solid and dashed line represents the best fit nonlinear regressions for clades 1 and 8 (respectively) using the saturation bioaccumulation model. The solid horizontal line represents the estimated mean background body concentration with its 95% CI (dotted lines). Bioaccumulation data for clade 1 included experiments B (□) and C (Δ) only. Data for clade 8 included all experiments: A (◊), B (□), and C (Δ).

The nonlinear regression fitted the data well with r^2 values greater than 0.74 (Table 3-3). Copper accumulated in the tissues of amphipods from clade 1 reached a maximum body concentration (*max*) at 920 (95% CI 696-1144) nmol/L and 1690 (95% CI 786-2594) nmol/L for clade 8 (Table 3-3). Although the *max* value for clade 8 appeared to be almost two times greater than for clade 1, the confidence intervals were overlapped and there were no significant differences. The background term (C_{BK}), *k* and *max/k* values between clades were similar based on overlapping confidence intervals (Table 3-3).

Table 3-3: Number of data points (n), background copper concentration (C_{BK} ± 95% CI), maximum copper accumulation (*max* ± 95% CI), the half saturation constant (*K* ± 95% CI), and model fit (r^2) for copper accumulation fit to a saturation bioaccumulation curve.

Clade	Exp	*n*	C_{BK} (nmol/g)	±	*max* (nmol/g)	±	*K* (nmol/L)	±	*max/K* (L/g)	±	r^2
1	B and C Only	17	340	103	920	224	137	131	6.71	5.47	0.86
8	Combined	22	319	91.7	1690	904	956	965	1.77	0.988	0.74

3.3.3 Bioaccumulation of Nickel

Nickel concentrations in tissues increased with increasing exposure in solution (Figure 3-4). A positive background concentration for nickel was detected in controls. To facilitate the fit of the nonlinear regression to the data, the average nickel concentration of the three experiments was used (Borgmann *et al.*, 2004). Estimates of *max* or *k* could not be obtained individually since nickel bioaccumulation data did not reach a saturation point in these experiments. Instead, the term *max/k* was determined using the "funpar" command in Systat (Equation 3-2). The *max/k* terms were 0.271 (95% CI 0.187-0.355) L/g for clade 1 and 0.313 (95% CI 0.207-0.419) L/g for clade 8. The *max/k* values were similar between clades based on overlapping confidence intervals.

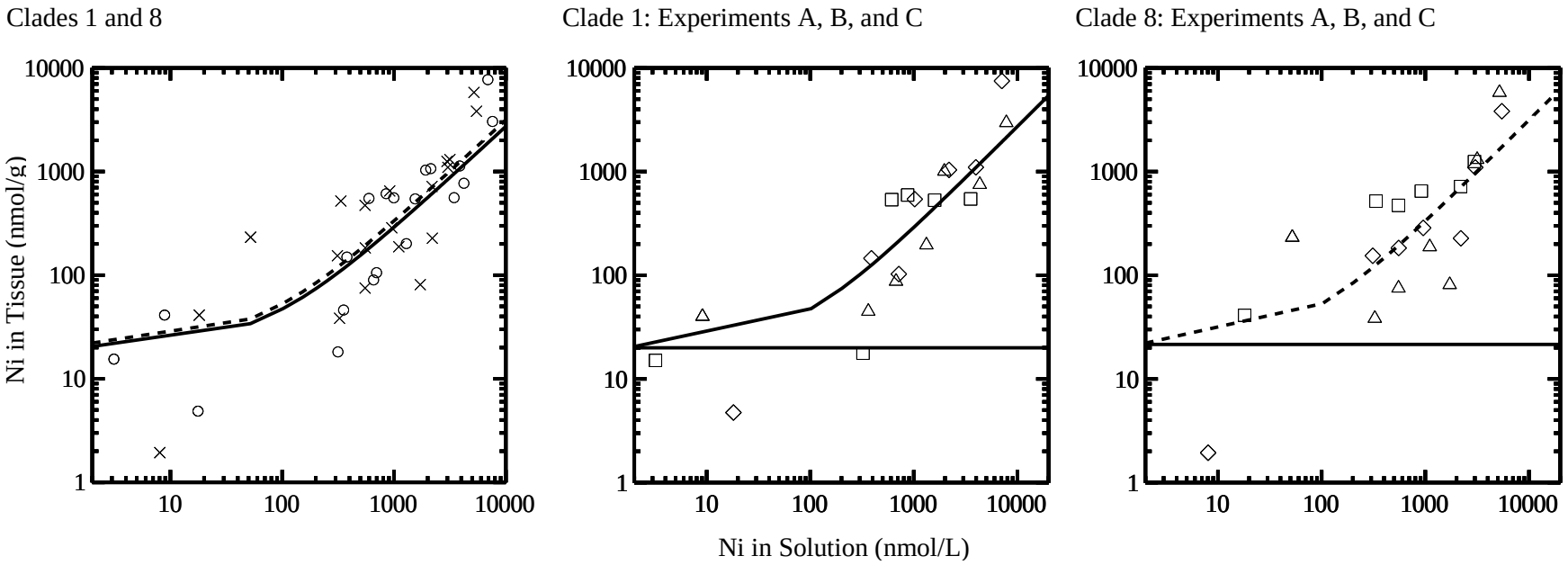

Figure 3-4: Nickel accumulated on a dry weight basis by clades 1 (○) and 8 (×) after 14 days of exposure to increasing measured nickel concentration in solution. The solid and dashed line represents the best fit nonlinear regressions for clades 1 and 8 (respectively) using the saturation bioaccumulation model. The solid horizontal line represents the set mean background body concentration. Bioaccumulation data for clade 1 and 8 included all respective experiments: A (◊), B (□), and C (Δ).

Table 3-4: Number of data points (n), the set background nickel concentration (C_{BK}), the ratio of the maximum nickel accumulation relative to the half saturation constant (*max/K* ± 95% CI), and model fit (r^2) for nickel accumulation fit to a saturation bioaccumulation curve.

Clade	Exp	*n*	C_{BK} (nmol/g)	±	*max/K* (L/g)	±	r^2
1	Combined	21	20.0	Set	0.271	0.0838	0.69
8	Combined	21	21.5	Set	0.313	0.106	0.55

3.4 Discussion

Copper concentrations for 4-6 week (non gut-cleared) control amphipods from 1-week toxicity tests were reported to be 1170 nmol/g (Borgmann and Norwood, 1995b). This is ~3 times greater than the background copper concentrations for clades 1 and 8 in this study. Similarly, the *max* copper body concentration for both clades is 2.1-3.9 times lower than that reported by Borgmann *et al*. (2004), which was 3600 (95%CI 3210-3990) nmol/g. This difference in copper background concentrations and *max* may be due to differences in toxicity protocols executed. In particular, Borgmann and Norwood (1995b) used 4-6 week adults that were gut-cleared compared to the 2-9 day old gut-cleared juveniles.

The food source (Tetramin®) may also explain some differences in background copper concentrations observed in the literature. Norwood *et al.* (2006) digested Tetramin® and reported copper concentrations to be 168 nmol/g, a value lower than the background concentrations from either publications. Although Tetramin® was used in the previously mentioned publication and in this study, the different lengths of test duration or the use of various Tetramin® products may have influence the background copper concentration.

Nickel background concentration also varied in a publication by Borgmann *et al.* (2004), but differences may be a result of different test protocols (e.g., contaminated sediment, dechlorinated tap water, test duration, etc.). Borgmann *et al.* (2004) reported a background nickel concentration of 7.55 nmol/g, which was ~3 times less than the average concentration determined in this study (20.0 nmol/g for clade 1; 21.5 nmol/g for clade 8). The background concentration of nickel determined by Borgmann *et al.*

(2004) was not actually measured in tissue samples, but was estimated by the nonlinear regression that used re-evaluated data from 4-week sediment toxicity tests performed by Borgmann *et al.* (2001). In contrast, the background nickel concentration in this study could not be estimated by the model and was set instead. The measured background nickel concentrations in this study for both clades ranged from 2-41 nmol/g. Despite different experimental methods, the measured background nickel concentrations in tissues from this study compared to the C_{bk} estimated by Borgmann *et al.* (2004) were similar.

Like Borgmann *et al.* (2004), individual values for *max* and *k* could not be obtained by the model in this study. Hence the *max/k* values were compared instead. The nickel *max/k* for clades 1 and 8 were similar. However, these values were nearly two times lower than the 0.70 L/g reported by Borgmann *et al*. (2001), and were likely a result of differences in experimental methods.

Regardless of metals, both clades had very similar background concentrations as well as their *max*, *k*, and *max/k* values (if applicable), which were comparable based on overlapping CI. My results suggested that the bioaccumulation patterns after 14-day exposure to copper or nickel may not suitable for determining differences in sensitivity between clades within a cryptic species complex. However, only two clades within the *Hyalella azteca* cryptic species complex were compared. More clades within the complex should be examined for their bioaccumulation pattern.

Martin *et al.* (2009) also faced a similar challenge to those that other researchers encounter with cryptic species complex. This group studied two species of *Chironomus*, which are morphologically difficult to distinguish to their species level: *Chironomus tigris* and *Chironomus staegeri.* However, with the aid of a specialist, one can distinguish either species by inspecting the salivary gland (Butler *et al.*, 1995). Although these two species of *Chironomus* were morphologically similar and inhabited the same area, they had very different bioaccumulation patterns (Martin *et al.*, 2009). Cadmium concentrations were more than eight times greater between species, but zinc and copper concentrations were similar.

The difference in bioaccumulation patterns between the two *Chironomus* species may be due to physiological and behaviour differences (Rainbow 2002; Buchwalter and Luoma 2005). It is interesting to note that these two species are closely related: 1.49% nucleic sequence divergence at the COI gene (Proulx *et al.*, 2013). In this chapter, clades 1 and 8 have very similar bioaccumulation patterns for copper and nickel, yet they are 26% divergent at the COI gene. Clade 1 and 8 may have different bioaccumulation patterns if exposed to metal-contaminated sediment instead of dissolved metals in solution. Furthermore, natural populations of either clade do not overlap in North America (see chapter 2). Although there are few studies, the bioaccumulation pattern among morphologically similar species is complex.

3.5 Conclusion

The metal exposure and its accumulation in the two clades were estimated by the saturation bioaccumulation model. Clades 1 and 8 had similar background concentrations and *max/K* ratios when exposed to copper as well as nickel. The *max* terms were also not significantly different between clades for copper.

4. Chapter 4 – Mortality and Growth Responses for Two Members of the *Hyalella azteca* Cryptic Species When Exposed to Copper and Nickel

4.1 Introduction

Hyalella azteca has been used in toxicity assays since the mid-1980s due to its broad distribution, sensitivity to contaminants, short generation time, as well as the fact that it is easy to sex, age, measure, and culture (USEPA 1994; Environment Canada, 1997, 2013). However, the interpretation of toxicity results using *H. azteca* has become complicated since this popular test organism is in fact a cryptic species complex, a group of genetically distinct species that are morphologically similar, but erroneously grouped together (Witt *et al*., 2000). Currently, 85 provisional species within the *H. azteca* complex have been identified in surveys across North America (Witt and Wellborn, in preparation); yet only two clades are being used among 17 institutes that had submitted their animals for sequencing: clades 1 and 8 (Major *et al*., 3013; Weston *et al*., 2013).

In this study, the two lineages common in laboratories (clades 1 and 8) were exposed to copper and nickel in three 14-day toxicity tests. Mortality and growth were assessed to determine different sensitivities between these two clades. Specifically, differences between clades for mortality relative to exposure (LC50 and LC25) and mortality relative to metal tissue concentrations (LBC50 and LBC25) were determined using the saturation mortality model (Norwood *et al*., 2007). Similarly, the general growth model (Norwood *et al*., 2007) was used to determine differences in the dry weight relative to metal exposure (IC25) and the dry weight relative to metal concentration in tissue (IBC25). Clades 1 and 8 were hypothesized to have different mortality and growth endpoints upon exposure to identical copper or nickel toxicity conditions.

Differences observed in adult mortality between clades (1, 3, and 8) in chapter 2 had several confounding variables (e.g., variable age of amphipods, number of amphipods in each container, etc.). Since the confounding variables from chapter 2 were controlled for in the toxicity experiments, the

control treatments of toxicity experiments after 14 days were used to determine differences in dry weights and mortality between clades 1 and 8. Mortality and growth responses between clades 1 and 8 when exposed to copper or nickel were also compared. Both clades are also hypothesized to have similar mortality and growth responses in control containers.

4.2 Materials and Methods

Collection, identification and culturing procedures are outlined in Chapter 2. Clades 1 and 8 were exposed to increasing copper or nickel concentrations for 14 days. At the end of the experiment, the amphipods were enumerated, gut cleared, dried, weighed, and digested to determine the amount of metal in their tissues. These total-body samples were compared to the measured metal concentration in test solution using the saturation bioaccumulation model (Equation 3-1). Details for the toxicity procedures are outlined in Chapter 3.

4.2.1 Control Mortality of Each Clade after 14 Days

To compare mortality between clades, survival data from the control containers were examined at the end of the 14-day toxicity test. Survival was converted to mortality data using Equation 1 in Chapter 2. Since the test was conducted over 14 days or a two week span, the mortality rate was calculated using Equation 4-1, where t is time in weeks, N is the number of survivors, and N_o is the initial number of test organisms. An independent-samples t-test compared the mortality rates of clades 1 and 8 from the control.

Equation 4-1: Conversion of survival to mortality rate.

$$m = \frac{-ln\left(\frac{N}{N_o}\right)}{t}$$

4.2.2 Copper and Nickel Induced Mortality

Nonlinear regressions were fit to mortality and growth data in Systat 10: saturation based mortality model and general growth model, respectively (Norwood *et al*., 2007). These models were used instead of other methods (e.g., probit, logit, etc.) to calculate the endpoints since nonlinear regressions provided better

accuracy and precision (Meddings *et al*., 1989). The measured metal concentration and total-body samples were equivalent and interchangeable when used by the regressions (Norwood *et al*., 2007). The 95% confidence intervals (CI) for each parameter (e.g., constants, coefficients, exponents, etc.) were determined by using the Wald calculation ("funpar" command in Systat), which measured the variance around the data in the regression analysis (Piegorsch and Bailer, 1997).

Survival was modified to facilitate the calculation of unknown parameters by using the saturation based mortality model. If 20 animals survived at the end of the 14-day toxicity test, then 19.5 animals were assumed to have survived. When 100% mortality had occurred in only one of the two replicates of a treatment, 0.5 of an animal was assumed to survive in that replicate. If both replicates had 100% mortality in a treatment, then 0.25 animals were assumed to have survived in each replicate. This correction was only applied to the lowest metal concentration with 0% survival in both replicates within the same treatment.

Mortality rates (*m*) was computed by regressing ln (survival) against time (Equation 4-1) for weeks 0, 1, and 2 (Borgmann *et al*., 1998). Regressing mortality rates over multiple weeks produced a larger number of partial effects than using only survival at week 2; thus, parameters could be more accurately determined (Borgmann *et al*., 1998). The mortality rates were fourth root transformed to better fit the saturation based mortality model in Systat 10 (Norwood *et al*., 2007).

In order to describe mortality relative to the metal concentrations in the test solutions, the Equation 4-2 was applied to provide estimates for the constants, coefficients, exponents, LC50 and the LC25 (the lethal metal concentration causing 50% and 25% mortality, respectively). Equation 4-2 is the mortality model for metal concentrations in test solutions,

Equation 4-2: The saturation-based mortality model for metal concentrations in test solutions (Norwood *et al*., 2007).

$$m = m' + \frac{ln2}{t} \times \left[C_w \frac{\left(LC50^{-1} + K_w^{"\,-1} \right)}{\left(1 + C_w K_w^{"\,-1} \right)} \right]^{nw}$$

in which, *m* is the overall mortality rate; *m'* is the control or background mortality rate; *n* is a constant; K_w'' is the metal concentration in the test solution when the contaminant induced mortality was half the maximum; C_w is the metal concentration from the test solutions; and finally, *t* is the exposure time (in weeks). In the situation where *n* was greater than 100, the constant was arbitrarily set to 100.

The relationship between mortality as a function of total-body concentration was determined by Equation 4-3. Estimates for the constants, coefficients, exponents, LBC50 and LBC25 (the lethal body concentration resulting in 50% or 25% that produced an inhibitory response) were fit to the below model:

Equation 4-3: The nonlinear regression mortality saturation model for body concentrations (Norwood *et al*., 2007).

$$m = m' + \frac{ln2}{t} \times \left[C_{TBX} \frac{\left(LBC50_x^{-1} + K_{TBX}''^{-1} \right)}{\left(1 + C_{TBX} K_{TBX}''^{-1} \right)} \right]^{nb}$$

K_{TBX}' represents the metal concentration in the body at half the maximum of metal induced mortality and C_{TBX} is the metal concentration in the body that was background corrected. All other parameters are as in Equation 4-2 (Norwood *et al*., 2007). These two equations evolved from the empirically derived allometric model, which also describes the relationship between accumulated metal within the body and the metal present in the environment (McGeer *et al.*, 2003).

4.2.3 Control Dry Weights of Each Clade after 14 Days

Dry weights of the amphipods within each clade were measured at the end of the 14-day experiment. The dry weights of animals in the control containers were compared to determine whether clades were significantly different after 14 days despite identical conditions, age, etc. An independent-samples t-test was used to compare the average dry weight between the two groups.

4.2.4 Copper and Nickel Induced Growth Response

The measurements for dry body weights were square root transformed to normalize and equalize variances, which were tested using the Kolmogorov–Smirnov and Levene's test respectively in SPSS

12.0. The following nonlinear regression determined growth by using the relationship for dry weights against metal concentration from test solutions or total-body metal concentration (Borgmann *et al*., 1998). Equation 4-4 estimated constants and exponents, which were used by Equation 4-5 to determine the IC25 or IBC25, respectively (Norwood *et al*., 2007). The IC25 (or IBC25) represented the concentration of a compound in solution (or within the tissue) at which the organism exhibited a 25% inhibition of a biological measurement, such as growth.

Equation 4-4: The nonlinear regression or general growth model from Borgmann *et al*. (1998).

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

where *W* is the total wet weight; *W'* is the wet weight of the control animals; *C* is the metal concentration from the test solution or total-body samples, and the constants are *a* and *n*.

Equation 4-5: Exponents *a* and *n* from Equation 4-4 were used to determine the IC25 or IBC25.

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

4.3 Results

4.3.1 Control Mortality of Each Clade after 14 Days

The mean mortality rates for each clade were compared using an independent-sample t-test. The mortality rate was fourth root transformed to normalize data and equalize variances. Clade 1 and 8 expressed no significant differences [t (10) = 0.729, p = 0.483)]. The mean fourth root transformed mortality rates for clades 1 and 8 were 0.522 (95% CI 0.367-0.677) and 0.475 (95% CI 0.412-0.538), respectively.

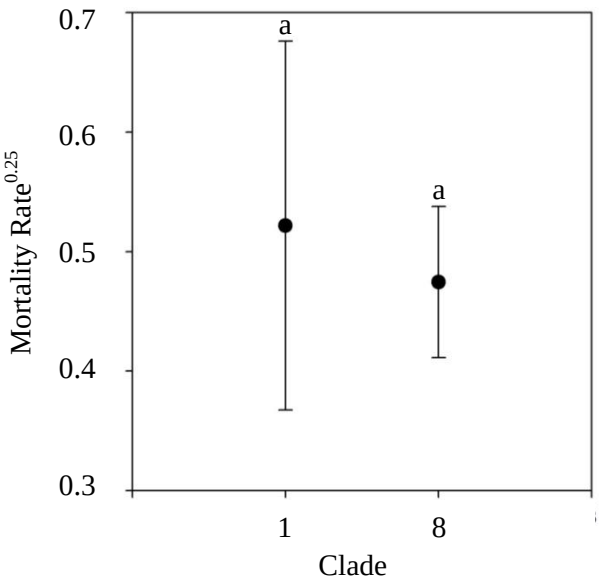

Figure 4-1: Fourth root transformed mortality rates (mean ± 95% CI) of control amphipods from clades 1 (n=6) and 8 (n=6) at the end of the 14-day toxicity tests. Letters "a" over the error bar plot indicate no significant differences.

4.3.2 Copper and Nickel Induced Mortality

Experiments A, B, and C were combined since the estimated LC50 and LC25s for the individual experiments had overlapping confidence intervals. Hence, the three experiments data (A, B, and C) were pooled to determine the (combined) LC50s and LC25s. The relationships for the measured or *dissolved* copper concentration in solution relative to mortality using the individual experiments (A, B, and C) and the combined experiments are presented in Figure 4-2 and Table 4-1.

Mortality rates generally increased with increasing copper concentration in the test solutions (Figure 4-2). The mortality rates for clade 1 and 8 did not increase until the copper concentrations started to exceed 300 nmol/L. However, the rate of increase for the latter was more gradual than for the former (Figure 4-2). The 95% CIs for the estimated mortality rates for the controls overlapped.

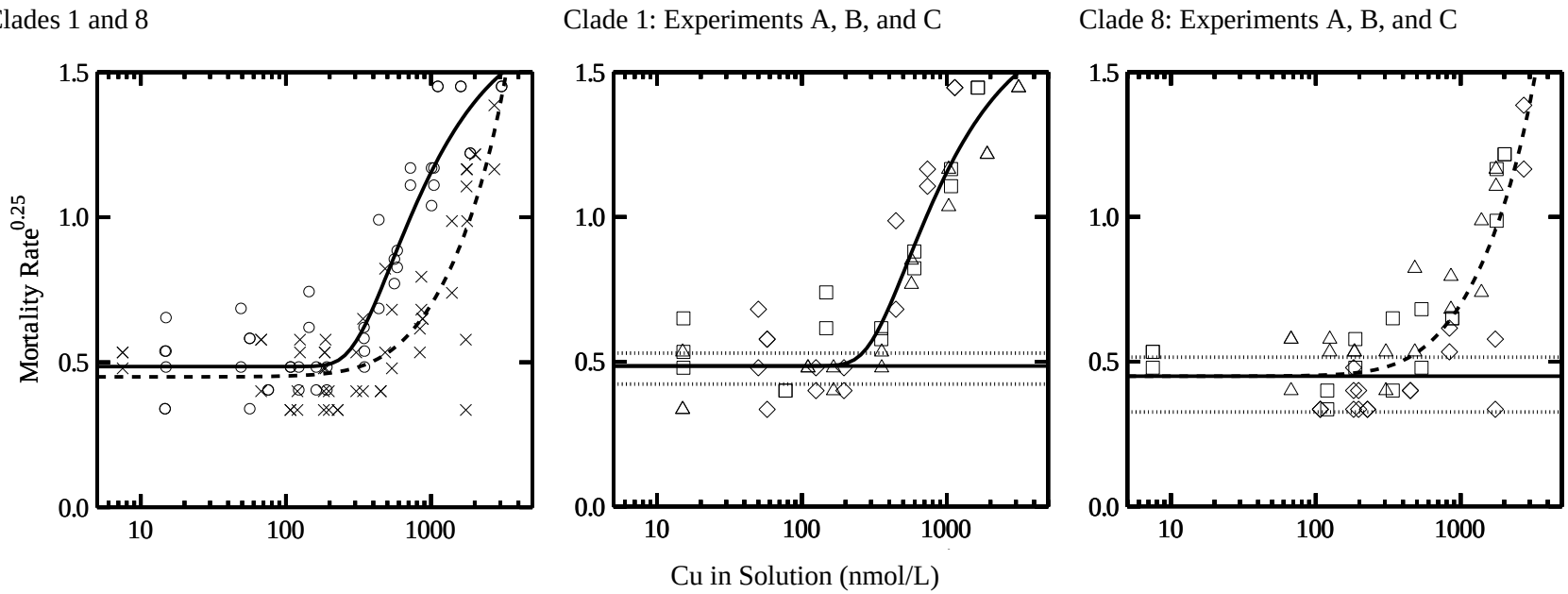

Figure 4-2: The relationship between fourth root transformed mortality rates and the measured copper concentration in solution from the 14-day toxicity tests conducted using clades 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the saturation-based mortality model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). Clades 1 and 8 included all three experiments: A (◊), B (□), and C (Δ).

If exponents (n_w) estimated by the nonlinear regression were greater than 100, then they were set to 100; otherwise, the exponents could not be determined accurately. The mortality model fit the data for clade 1 with a range of 0.90-0.97. The regression estimated the copper LC50 to be 491 (95% CI 423-559) and the LC25 to be 383 (95% CI 324-442) using the combined data for clade 1.

Clade 8 did not require the n_w value to be set to 100. The copper mortality model fit the data with r^2 values that ranged from 0.70-0.92. The copper LC50 and LC25 for clade 8 were 1260 (95%CI 998-1522) nmol/L and 876 (95%CI 585-1167) nmol/L, respectively (Table 4-1). Based on the non-overlapping confidence intervals, these differences were significant with the copper LC50 and LC25 for clade 8 to be 2.6 and 2.3 times (respectively) larger than clade 1.

Table 4-1: Combined and individual experiments (A, B, C) for clades 1 and 8 were used to estimate the control mortality (m'), exponent (n_w), half saturation constant (K''_w), LC50 and LC25s (their respective 95% CIs), as well as model fit (r^2) using mortality rate as a function of measured copper concentration in test solutions. Bold faced values indicate significance differences between clades 1 and 8 based on non-overlapping CI.

Clade	Exp	m'	±	n_w	±	K''_w	±	LC50 (nmol/L)	±	LC25 (nmol/L)	±	r^2
1	Combined	0.0554	0.0234	100	Set	15.8	3.59	**491**	**68.3**	**383**	**59.0**	0.90
1	A	0.0576	0.0380	100	Set	18.1	8.12	435	98.0	356	92.6	0.93
1	B	0.0789	0.0553	2.63	4.38	10,100	188,000	571	158	402	194	0.94
1	C	0.0343	0.0182	100	Set	17.5	3.79	578	83.0	445	69.5	0.97
8	Combined	0.0409	0.0296	2.16	2.61	-9820	56,700	**1260**	**262**	**876**	**291**	0.70
8	A	0.000100	0.171	0.383	2.01	-2730	5.29	2710	403	2510	1220	0.92
8	B	0.0513	0.0374	1.81	2.65	-3270	5930	1200	343	864	331	0.92
8	C	0.0569	0.102	0.945	1.55	-2010	1090	1160	381	702	662	0.83

The relationship between mortality and copper concentration in tissue was similar to those for copper exposure. The mortality rate for clade 1 did not increase until copper tissue concentrations exceeded 400 nmol/g. Although the mortality rate for clade 8 increased starting at 300 nmol/g, the mortality rate was much more gradual than that for clade 1.

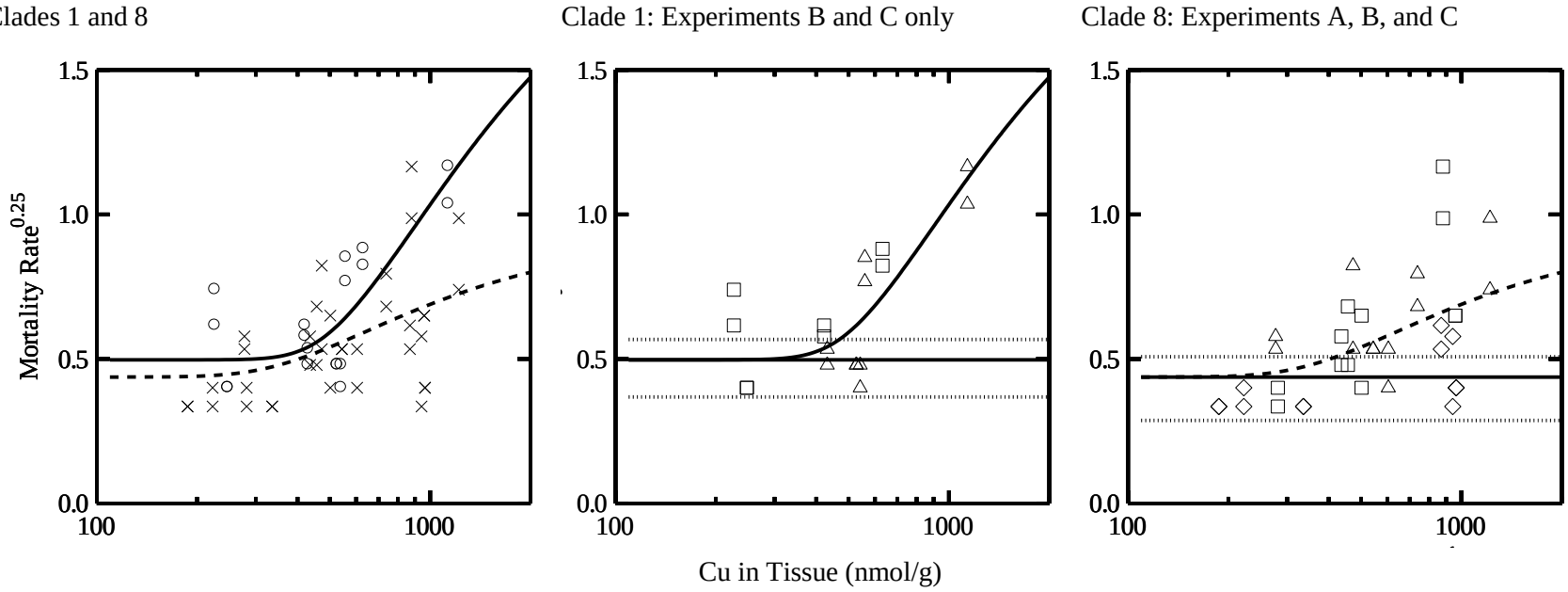

Figure 4-3: The relationship between fourth root transformed mortality rates and the measured copper concentration in tissue samples from the 14-day toxicity tests conducted using clades 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the saturation-based mortality model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). Data for clade 1 included experiments B (□) and C (Δ) only; data for clade 8 included all experiments: A (◊), B (□), and C (Δ).

Data from clade 1, experiment A, was not included in the model since the relationship between mortality and metal concentration in tissue was opposite to that of experiments B and C. This trend may have been due to contaminated or mislabelled samples. The estimated control mortality data for both clades were similar (0.0609 and .0366, respectively). Exponents (n_b) for both clades were set to 100. The r^2 value for clade 1 was 0.64, which was two times higher than the regression fit for clade 8 (0.28). The regression estimated a copper LBC50 for clade 1 to be 717 (95%CI 596-838) nmol/g and 1810 (-871-4490) nmol/g, respectively. The LBC25 for clade 1 and 8 were 588 (95%CI 474-702) nmol/g and 834 (95%CI 515-1153) nmol/g, respectively (Table 4-2). These copper LBC50s and LBC25s were not significantly different based on their overlapping CI.

Table 4-2: Only experiments B and C for clade 1 and the combined data for 8 were used to estimate the control mortality (m'), exponent (n_b), half saturation constant (K''_{TB}), LBC50 and LBC25s (their respective 95% CIs), as well as model fit (r^2) using mortality rate as a function of measured copper concentration in tissue samples.

Clade	Exp	m'	±	n_b	±	K''_{TB}	±	LBC50 (nmol/L)	±	LBC25 (nmol/L)	±	r^2
1	B and C only	0.0609	0.0425	100	Set	29.9	15.1	717	121	588	114	0.64
8	Combined	0.0366	0.0298	100	Set	13.8	15.4	1810	2680	834	319	0.28

Both clades tolerated much higher concentrations of nickel relative to copper. The relationship between mortality rates and nickel concentrations in test solutions followed an increasing trend, but increased gradually as nickel exposure exceeded 700 nmol/L. The mortality rates for clade 1 appeared to plateau at concentrations greater than 7000 nmol/L. This observation was in contrast to the best fit regression for clade 8, which continued to increase in an exponential fashion, but there were no more measured mortality rates past 10,000 nmol/L of nickel (Figure 4-4).

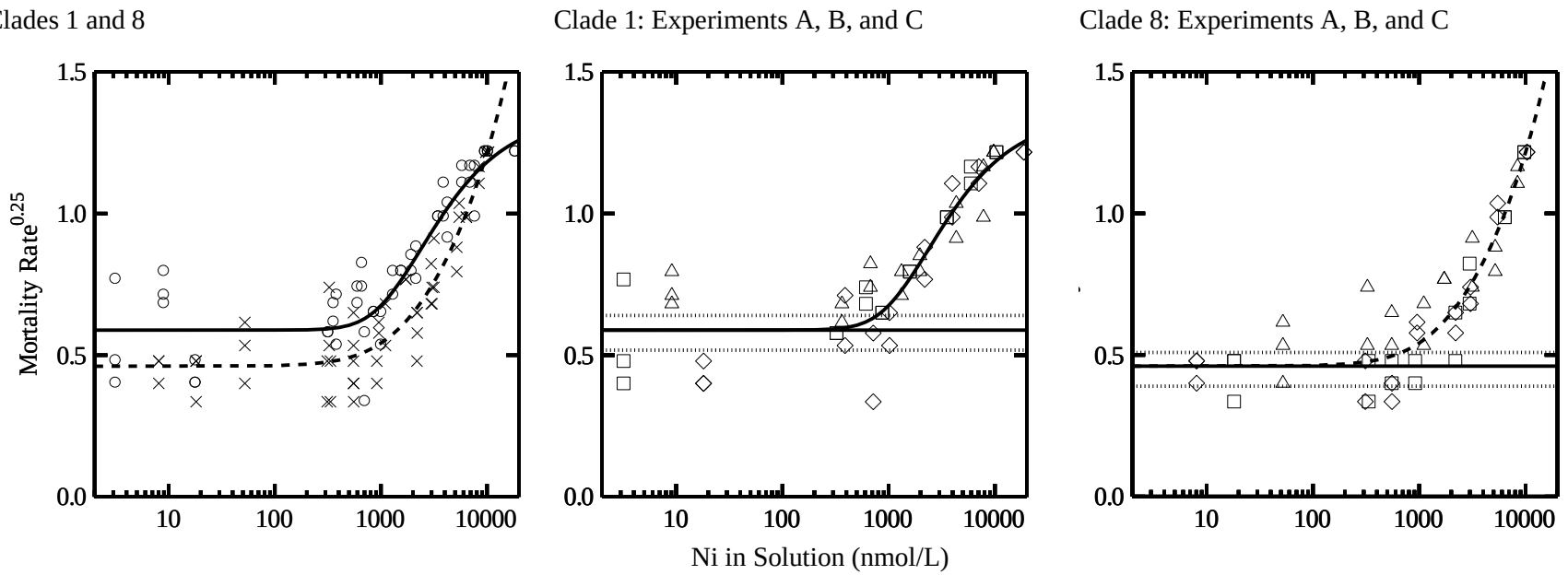

Figure 4-4: The relationship between mortality rates and the measured nickel concentration in solution from the 14-day toxicity tests conducted using clades 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the saturation-based mortality model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). The data for clade 1 and 8 included experiments A (◊), B (□), and C (Δ).

The nickel mortality models for clades 1 and 8 fitted the data with r^2 values ranging from 0.84-0.95 (Table 4-3). The nonlinear regression estimated a nickel LC50 and LC25 for clade 1 to be 1980 (95%CI 1487-2473) nmol/L and 1250 (95%CI 797-1703) nmol/L, respectively. The exponent (n_w) for both clades range from 0.486-3.80, with the exception of clade 1 (experiment A) that was set to 100. These parameters were significantly lower than those for clade 8 based on non-overlapping CIs. The nickel LC50 was 3550 (95%CI 2924-4176) nmol/L for clade 8, which was 1.8 times greater than that of clade 1. Similarly, the nickel LC25 was 1.7 times greater than that for clade 1, which was 2110 (95% CI 1549-2671) nmol/L.

Table 4-3: Combined and individual experiments (A, B, C) for clades 1 and 8 were used to estimate the control mortality (m'), exponent (n_w), half saturation constant (K''_w), LC50 and LC25s (their respective 95% CIs), as well as model fit (r^2) using mortality rate as a function of measured nickel concentration in test solutions. Bolded values indicate significance differences between combined experiments for clades 1 and 8 based on non-overlapping CI.

Clade	Exp	m'	±	n_w	±	K''_w	±	LC50 (nmol/L)	±	LC25 (nmol/L)	±	r^2
1	Combined	**0.120**	**0.0481**	3.80	7.59	1570	4596	**1980**	**493**	1250	453	0.84
1	A	0.0572	0.0383	100	Set	41.1	17.6	1940	597	1360	455	0.92
1	B	0.0925	0.0672	1.67	2.05	6460	23672	1660	673	887	549	0.91
1	C	0.249	0.109	1.35	2.14	-150,000	6,180,000	3030	1490	1590	1540	0.89
8	Combined	**0.0450**	**0.0219**	1.64	1.12	-99,600	1,200,000	**3550**	**626**	2110	561	0.87
8	A	0.0337	0.0228	2.42	3.05	13,800	65,200	3400	736	2200	650	0.95
8	B	0.0318	0.0183	3.44	6.71	6910	33,120	3700	802	2550	601	0.95
8	C	0.0336	0.165	0.486	0.660	-9040	2920	3230	2660	754	2210	0.84

Mortality rates increased gradually as nickel concentrations in tissues also increased. The mortality rate for clade 1 did not increase until 100 nmol/g of nickel in tissue. After which, there was a gradual increase. In contrast, when nickel concentrations in tissue reached 500 nmol/g, clade 8 exhibited a sharp increase in mortality rates. The data for clade 8, experiment C, was not included since the control nickel tissue concentration was contaminated.

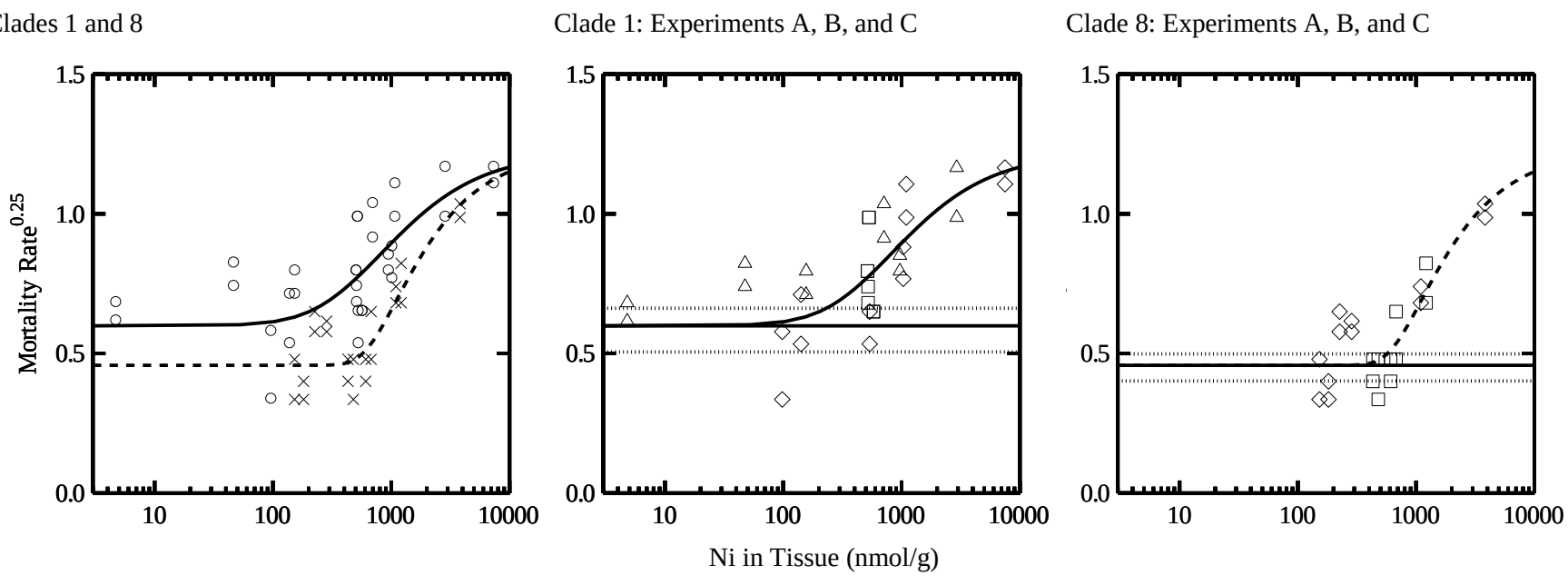

Figure 4-5: The relationship between mortality rates and the measured nickel concentration in tissue samples from the 14-day toxicity tests conducted using clades 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the saturation-based mortality model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). Bioaccumulation data for clade 1 and 8 included experiments A (◊), B (□), and C (Δ).

The nonlinear regression fitted the data with an r^2 value of 0.59 and 0.77 for clades 1 and 8 respectively (Table 4-4). The exponent (n_b) for clade 1 was 2.25. Since the n_b for clade 8 was greater than 100, it was set to 100. The mortality model estimated the LBC50 for clade 1 and 8 to be 710 (95%CI 420-1000) nmol/g and 1490 (95%CI 1137-1843) nmol/g. These LBC50s were significantly different based on non-overlapping CIs. The LBC25s were estimated to be 379 (95%CI 88-670) nmol/g for clade 1 and 1010 (95%CI 767-1253) nmol/g, which were also significantly different. The nickel LBC50 and LBC25 for clade 8 were 2.1 and 2.7 times greater than for clade 1, respectively.

Table 4-4: Combined experiments (A, B, C) for clades 1 and 8 were used to estimate the control mortality (m'), exponent (n_b), half saturation constant (K''_{TB}), LBC50 and LBC25s (their respective 95% CIs), as well as model fit (r^2) using mortality rate as a function of measured nickel concentration in tissue samples.

Clade	Exp	m'	±	n_b	±	K''_{TB}	±	LBC50 (nmol/L)	±	LBC25 (nmol/L)	±	r^2
1	Combined	0.129	0.0634	2.25	6.53	866	4200	**710**	**290**	**379**	**291**	0.59
8	Combined	0.0438	0.0179	100	Set	28.2	11.7	**1490**	**353**	**1010**	**243**	0.77

4.3.3 Control Dry Weights of Each Clade after 14 Days

An independent-samples t-test was conducted to compare the dry weight of individuals from clades 1 and 8 that were not exposed to any metals. The dry weights for both clades were normally distributed and variances were homoscedastic. Clade 1 control animals were significantly larger than clade 8 [t (10) = 2.928, p = 0.015] with a mean dry weight of 0.0619 (95% CI 0.0487-0.0751) mg/indv. Clade 8 had a mean dry weight of 0.0411 (95% CI 0.0284-0.0538) mg/indv.

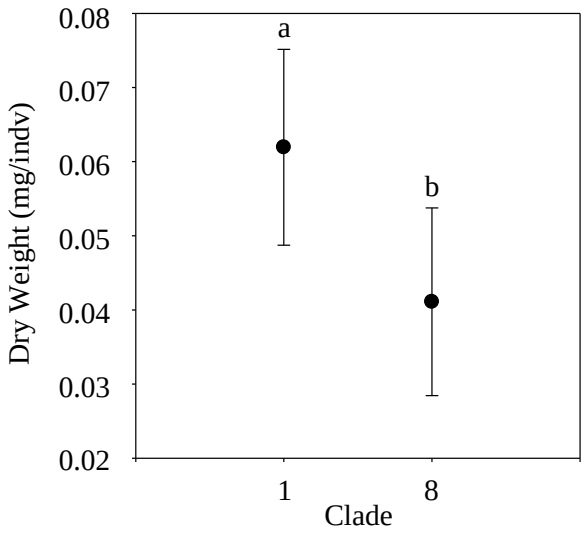

Figure 4-6: Dry weights (mean ± 95% CI) of control amphipods from clades 1 (n=6) and 8 (n=6) at the end of the 14-day toxicity tests.

4.3.4 Copper and Nickel Induced Growth Response

There was a gradual decrease in dry weight of an individual as copper exposure increased. However, the dry weight for clade 1 appeared to be consistently higher than those for clade 8. This was consistent with Figure 4-6, which also indicated that clade 1 control juveniles were significantly larger than those of clade 8 that were the same age.

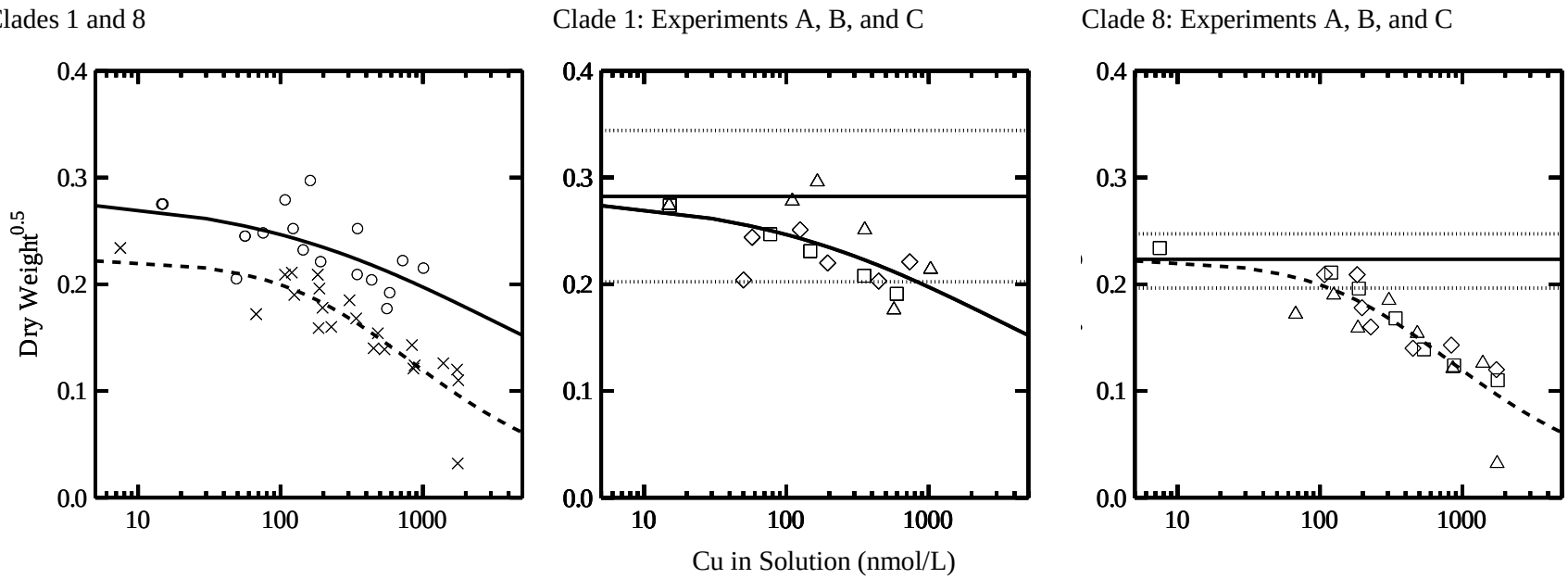

Figure 4-7: The relationship between square root transformed dry weight and the measured copper concentration in solution from the 14-day toxicity tests conducted using clade 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the general growth model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). Clades 1 and 8 included all three experiments: A (◊), B (□), and C (Δ).

The model fit was extremely variable with r^2 values ranging from 0.0042-1.00. This variability was evident (especially for clade 1) with the estimated control dry weights having wide 95% CI (Figure 4-7 and Table 4-5). The general growth model estimated a copper IC25 of 114 (95% CI -359-587) nmol/L for clade 1 and 132 (95%CI 12-230) nmol/L for clade 8. There were no significant differences between the clades for the copper IC25 based on overlapping CI.

Table 4-5: Combined and individual experiments (A, B, C) for clades 1 and 8 were used to estimate the control dry weights (*W'*), exponents (*a* and n_w), IC25 (their respective 95% CIs), as well as model fit (r^2) using dry weight as a function of measured copper concentration in test solutions.

Clade	Exp	*W'* (mg/indv)	±	*a*	±	n_w	±	IC25 (nmol/L)	±	r^2
1	Combined	0.0797	0.0388	2.75E-02	1.52E-01	0.527	0.714	114	473	0.43
1	A	0.0597	Set	1.66E-01	6.56E-01	0.0657	0.718	40,300	2,320,000	0.0042
1	B	0.0751	Set	1.08E-02	2.46E-03	0.718	0.0390	118	7.76	1.00
1	C	0.0748	Set	1.02E-04	1.07E-03	1.33	1.60	432	356	0.55
8	Combined	0.0499	0.0113	2.59E-03	6.44E-03	0.995	0.334	132	120	0.77
8	A	0.0435	Set	6.07E-03	1.27E-02	0.797	0.326	153	100	0.80
8	B	0.0546	Set	2.86E-03	3.27E-03	0.986	0.180	124	38.3	0.96
8	C	0.0295	Set	2.91E-10	4.94E-09	3.18	2.37	702	354	0.79

Only experiments B and C were represented for clade 1 since experiment A had the opposite trend. This was likely a result of contamination or mislabelled samples. Similar to the relationship between growth and exposure, the trend for dry weight decreased as the copper concentrations in tissue increased. Clade 1 was consistently larger than clade 8 (Figure 4-8).

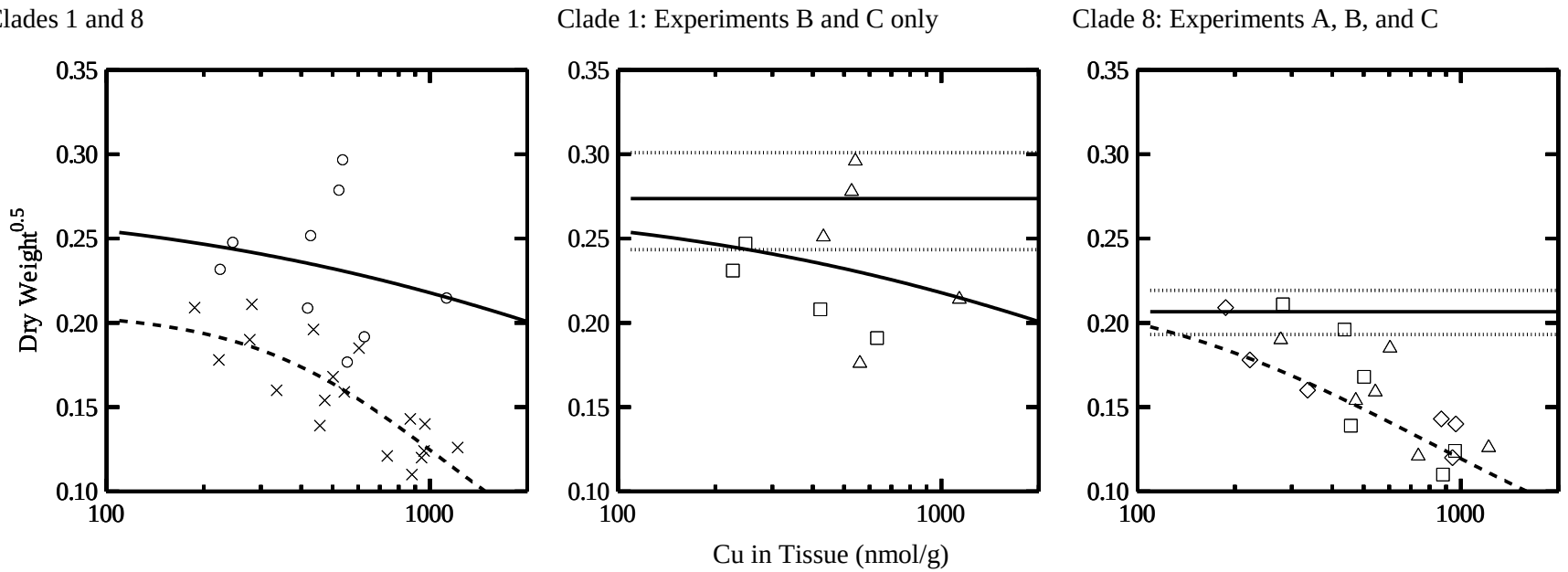

Figure 4-8: The relationship between square root transformed dry weight and the measured copper concentration in tissue samples from the 14-day toxicity tests conducted using clades 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the general growth model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). Clade 1 included experiments B (□) and C (Δ) only; clade 8 included all three experiments: A (◊), B (□), and C (Δ).

The model fits were 0.27 and 0.70 for clades 1 and 8 respectively. The IBC25 for clade 1 was 380 (95%CI -331-1091) nmol/g and 349 (95%CI 201-497) nmol/g for clade 8. There were no significant differences between either clade based on overlapping CIs.

Table 4-6: Experiments B and C for clade 1 and combined experiments (A, B, C) for clade 8 were used to estimate the control dry weights (*W'*), exponents (*a* and n_b), IBC25 (their respective 95% CIs), as well as model fit (r^2) using dry weight as a function of measured copper concentration in tissue samples.

Clade	Exp	*W'* (mg/indv)	±	*a*	±	n_b	±	IBC25 (nmol/L)	±	r^2
1	B and C only	0.0749	0.0157	1.13E-02	8.64E-02	0.5694	1.1733	380	711	0.27
8	Combo	0.0427	0.00540	3.27E-05	1.38E-04	1.5763	0.6191	349	148	0.70

Similar to copper, nickel exposure also caused the dry weight per individual to decrease as the metal concentration increased. Clade 1 consistently had higher dry weight per individual than clade 8, but reduction in dry weight relative to controls began at 100 nmol/L of nickel in solution for both clades (Figure 4-9).

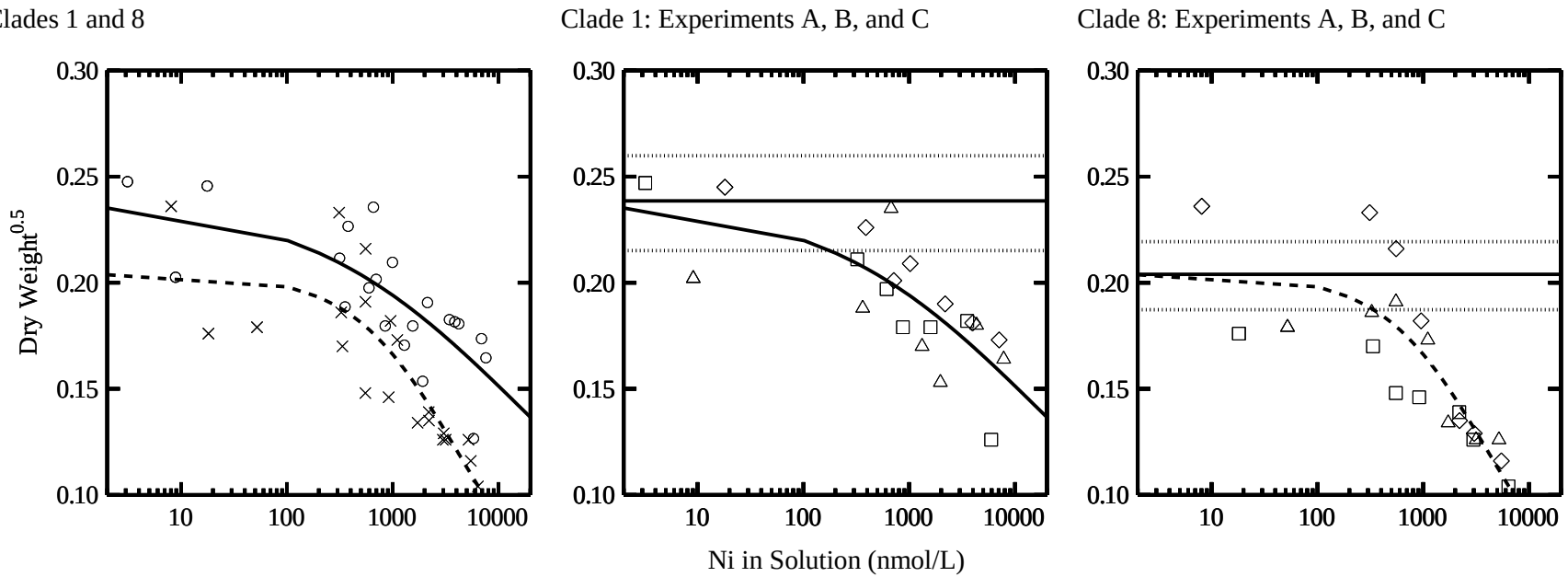

Figure 4-9: The relationship between square root transformed dry weight and the measured nickel concentration in solution from the 14-day toxicity tests conducted using clades 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the general growth model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). Clades 1 and 8 included all three experiments: A (◊), B (□), and C (Δ).

The data was fitted to the general growth model for both clades with r^2 values ranging from 0.25-0.94. The IC25 for clades 1 and 8 were 398 (95%CI -382-1178) nmol/L and 638 (95% CI 80-1196) nmol/L, respectively (Table 4-7). Based on the overlapping CIs, the clades were not significantly differences.

Table 4-7: Combined and individual experiments (A, B, C) for clades 1 and 8 were used to estimate the control dry weights (W'), exponents (a and n_w), IC25 (their respective 95% CIs), as well as model fit (r^2) using dry weight as a function of measured nickel concentration in test solutions.

Clade	Exp	W' (mg/indv)	±	a	±	n_w	±	IC25 (nmol/L)	±	r^2
1	Combined	0.0569	0.0106	2.08E-02	5.17E-02	0.464	0.272	398	780	0.62
1	A	0.0600	Set	1.51E-02	1.45E-02	0.481	0.122	624	262	0.90
1	B	0.0610	Set	1.69E-02	3.08E-02	0.547	0.241	234	233	0.74
1	C	0.0409	Set	1.83E-03	1.57E-02	0.644	1.034	3250	4340	0.25
8	Combined	0.0416	0.00652	8.34E-04	2.71E-03	0.928	0.386	638	558	0.70
8	A	0.0559	Set	1.93E-04	4.18E-04	1.166	0.280	600	217	0.94
8	B	0.0311	Set	2.08E-03	3.07E-03	0.769	0.187	737	266	0.92
8	C	0.0319	Set	2.41E-05	1.67E-04	1.284	0.856	1680	957	0.75

Dry weight per individual decreased as nickel concentrations in tissue increased. The decrease in dry weight in clade 1 was more gradual than the reduction observed for clade 8 (Figure 4-9), which experienced a sharp decrease at 70nmol/g of nickel. The controls could not be included in Figure 4-10 since the concentration of tissue was background corrected. In addition, experiment C for clade 8 was not included due to contamination in the controls.

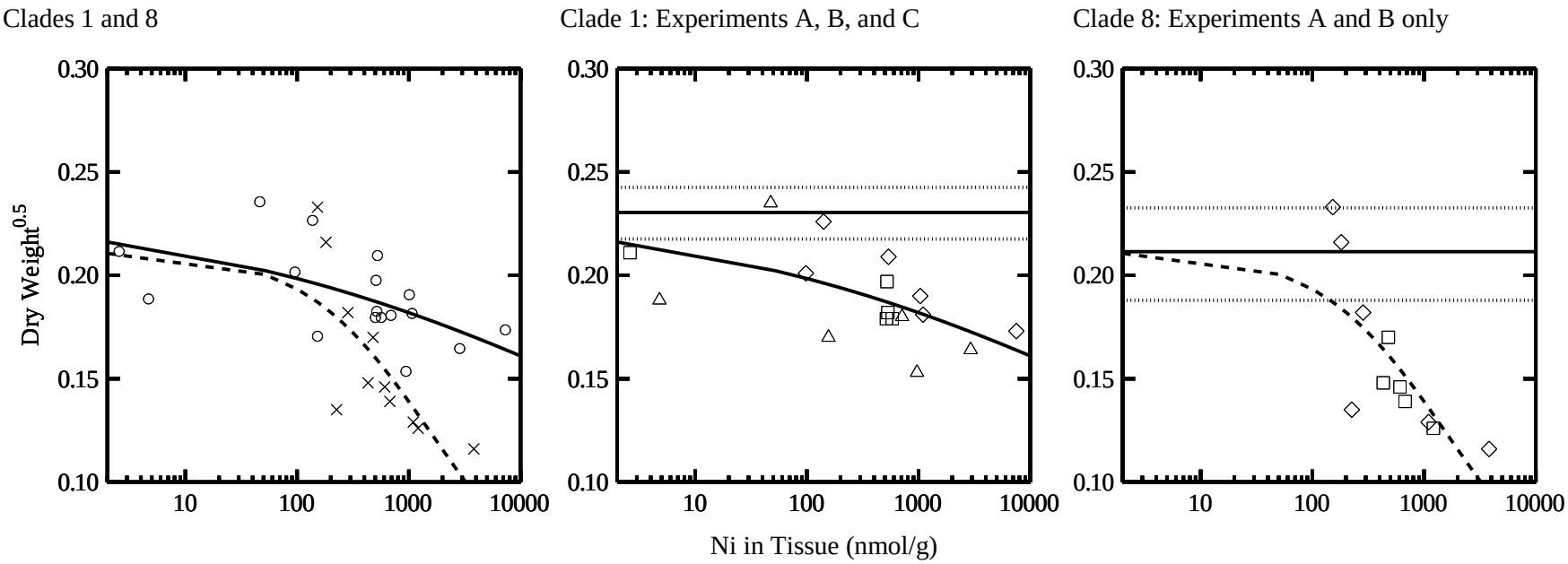

Figure 4-10: The relationship between square root transformed dry weight and the measured nickel concentrations in solution from the 14-day toxicity tests conducted using clades 1 (o) and 8 (×). The non-linear best fit regressions for clades 1 and 8 are indicated by a solid line and dashed line, (respectively) using the general growth model. The solid horizontal line represents the estimated control mortality with its 95% CI (dotted lines). Clade 1 included all three experiments; clade 8 included experiments A (◊) and B (□), only.

The general growth model resulted in r^2 values greater than 0.55. The model also estimated IBC50s of 83.3 (95%CI -118.7-285.3) nmol/g and 190 (95% CI -36-416) nmol/g for clades 1 and 8, respectively. Based on overlapping CI, the nickel IBC50s for clades 1 and 8 were not significant.

Table 4-8: Combined experiments (A, B, C) for clade 1 and only experiments A and B for clade 8 were used to estimate the control dry weights (*W'*), exponents (*a* and n_b), IC25 (their respective 95% CIs), as well as model fit (r^2) using dry weight as a function of measured nickel concentration in tissue samples.

Clade	Exp	*W'* (mg/indv)	±	*a*	±	n_b	±	IBC25 (nmol/L)	±	r^2
1	Combo	0.0531	0.00574	1.16E-01	1.39E-01	0.239	0.157	83.3	202	0.55
8	A and B only	0.0447	0.00940	4.35E-03	1.43E-02	0.827	0.463	190	226	0.60

4.4 Discussion

To the author's knowledge, this thesis is the first to publish copper and nickel toxicity results for genetically characterized *H. azteca* that belonged to clade 1 and 8. Based on a four week toxicity test, Borgmann *et al*. (1993) reported LC50s and LC25s for copper to be 718 (95% CI 545-946) nmol/L and 411 (95% CI 261-746) nmol/L for their amphipods. The 14-day copper LC50 and LC25 for clade 1 reported in this study were 491 (95% CI 423-559) nmol/L and 383 (95% CI 324-442) nmol/L, respectively. Despite the different test durations, the copper LC50 and LC25 reported by Borgmann *et al.* (1993) were similar to the parameters estimated for clade 1 in this chapter. Although the copper LC50 and LC25 for clade 8 were 2.6 and 2.3 times greater than clade 1, both sets of data overlapped in Figure 4-2. Since the mortality data of one clade was not distinct from the other, there may not be any actual toxic significance despite the non-overlapping CI.

The four-week copper LBC50 and LBC25 reported by Borgmann *et al*. (1993) were 2560 (95%CI 2370-2770) nmol/g and 2170 (95%CI 1760-2670) nmol/g respectively. These values were much lower than the copper LBC50 and LBC25 reported in this thesis for clade 1. The higher copper LBC50 and LBC25 observed in the four-week copper toxicity test conducted by Borgmann *et al.* (1993) was not expected since one would expect that a lower concentration of metal would be required to affect 50% of the population over a longer period time. However, this trend may be a result of *Hyalella* being able to regulate copper after a longer period of time (Borgmann *et al.*, 1993). In addition, the animals used by Borgmann *et al.* (1993) were from a 28-day toxicity test and were not gut cleared, which may have resulted in the higher LBC50s and LBC25s. Regardless of differences between the results from this study and Borgmann *et al.* (1993) for copper LBC50 and LBC25, clades 1 and 8 were similar based on overlapping CI and data points (Figure 4-3). The r^2 values estimated in this study for both clades were lower than those published for other metals, such as Norwood *et al.* (2007) with r^2 values that ranged from 0.76-0.90. The lower r^2 values exhibited by clades 1 and 8 in this study may be due to lower sample sizes per experiment.

In relation to nickel, the LC50s were significantly different based on non-overlapping CIs. Clade 8 had a significantly higher nickel LC50 than clade 1, making the former 1.8 times more tolerant to nickel exposure than the latter. However, when comparing clades 1 and 8, the nickel LC25 was not significantly different.

The four-week nickel exposure conducted by Borgmann *et al*. (2001) resulted in a LC50 and LC25 of 576 (95% CI 504-659) nmol/L and 400 (95%CI 325-493) nmol/L, respectively. These parameters were much lower than those reported in this study for either clade. The differences in test duration, test media, feeding regime, and method of metal exposure between experiments likely influenced this variability.

Unlike copper, the nickel LBC50 and LBC25 for clade 8 were 2.1 and 2.7 times greater than that for clade 1. Although the control mortality for clade 1 is 2.9 times higher than that for clade 8, differences in LBC50 and LBC25 were likely to be significant since the nonlinear regression for clade 8 is much steeper than that for clade 1 (Figure 4-5). The four-week nickel exposure conducted by Borgmann *et al*. (2001) produced LBC50 and LBC25 that were 405 (95%CI 355-463) nmol/g and 281 (228-347 95%CI) nmol/g, respectively. These nickel concentrations in tissue were similar to those reported in this study despite differences in test protocols. This may indicate that LBC50s and LBC25s were more consistent of a measure between different laboratory tests (Table 4-4).

The effects of copper and nickel on growth were similar for both clades on a metal exposure and total-body concentration basis. Neither the copper nor nickel IC25s and IBC25s were significantly different in a comparison between clades. The growth inhibition trends were similar for the two clades. However, clade 1 was larger than clade 8 and the independent-samples t-test determined differences in dry weights between lineages were significant (Figure 4-6). Interestingly, clades 1 and 8 are associated with the large-bodied lineages (Wellborn and Broughton, 2008), but the former was significantly larger than the latter.

The different sensitivity observed in this study was consistent with other work that had also examined responses among members of the *H. azteca* cryptic species complex (Soucek *et al*., 2013; Weston *et al*., 2013). A growing number of studies observed different responses using other cryptic species complexes. Notably, Rocha-Olivares *et al*. (2004) identified different responses to a metal mixture from contaminated sites for the two copepods within the *Cletocamptus deitersi* complex. The percent survival was 1.5 times different between the two species of *Cletocamptus* when exposed to a mix of heavy metals. However, they reported similar tolerances for polycyclic aromatic hydrocarbons in their 96-hour bioassays. Using pesticides, Feckler *et al*. (2012) documented comparable trends between two cryptic *Gammarus fossarum* lineages. They observed a 50% decline in feeding for one of the two lineages that were exposed to tebuconazole (a fungicide), but not when both clades were subjected to thiacloprid (an insecticide). These studies clearly indicated that a protocol for proper identification of clades is necessary for cryptic species complexes and that only characterized test organisms should be used in toxicity tests.

The results presented in this chapter, along with those derived from other studies that investigated toxicity responses within the *H. azteca* cryptic species complex, provide evidence that the use of genetically characterized species is necessary. The proper identification of test organisms is essential and should be considered in standardized tests.

4.5 Conclusion

The most sensitive indicator of different responses between clades was determined using mortality rate relative to metal exposure. Clade 1 was 2.3-2.6 times more sensitive to copper exposure than clade 8 relative to mortality. Likewise, clade 8 was 1.8 times more tolerant to nickel exposure than clade 1. Mortality rate relative to nickel concentration in tissue indicated that clade 8 was 2.1-2.7 time more tolerant than clade 1. However, differences in LBC50 and LBC25 were not detected for copper (possibly since copper is regulated by both clades). The effects of metals on growth were not significantly different between clades. Although clade 1 was 1.7-2.6 times more sensitive to copper or nickel exposure than

clade 8, the latter was significantly larger than the former. The results of this chapter indicated that toxicity experiments should be executed with properly identified test organisms.

5. Chapter 5 – Overall Implications, Conclusion, and Future Directions

In this study, the phylogenetic analyses of the mitochondrial COI gene revealed four evolutionary divergent members of the *Hyalella azteca* cryptic species complex: clades 1, 3, 6 and 8. The COI nucleotide sequence divergence among these four clades was >27.4%, which was consistent with interspecific differences. These results were similar to those reported by Witt and Hebert (2000), who also employed the 637 bp COI fragment to determine the average nucleotide sequence divergence in clades 1, 3, and 6.

Varying life history traits in three members of the *H. azteca* cryptic species complex were observed. Clades 1, 3, and 8 were raised in identical culture conditions and monitored for two laboratory-relevant life history traits: the rate of mortality and the number of juveniles produced per adult. The two large-bodied clades (1 and 8) had significantly lower rates of mortality and produced more juveniles than clade 3, which is a small-bodied ecomorph. As a result of the poor survival and juvenile production by clade 6, this lineage was not included in this study, but demonstrated a similar trend observed with clade 3. These observations indicated that survival as well as juvenile production may be clade dependant and potentially a result of physiological, metabolic, and behavioural factors. Variations in culture protocols may be partially explained by the inherent differences in life history traits since parameters, such as food, substrate, water, etc., may impact their physiology (Sherratt *et al*., 1999).

Interestingly, although clades 1 and 8 were classified to be large-bodied ecomorphs (Wellborn and Broughton, 2008), the dry weight of the former was significantly larger than the latter (Chapter 4). Dry weights, length of reproductive adults and their offspring should be performed to determine whether the size differences between clades were significant instead a result of confounding factors (such as the size of reproducing female, crowding in culture containers, etc.).

Regardless of the size discrepancy, the clades used in this study for toxicity testing were representative of the *Hyalella* that had been sequenced in 17 laboratories (Major *et al*., 2013; Weston *et*

al., 2013). Clades 1 and 8 were exposed to identical 14 day copper and nickel tests that demonstrated different sensitivities existed in terms of mortality relative to exposure. There were no significance differences between mortality and metal concentration in tissue. Growth parameters were also similar relative to metal exposure or metal concentration in tissue.

Although clade 8 was more tolerant to copper and nickel exposure than clade 1 relative to mortality, bioaccumulation patterns between both clades were similar. However, more bioaccumulation data for lineages within a cryptic species complex should be collected; different sensitivities yet similar bioaccumulation patterns imply that clade 8 may have a method to regulate copper in tissue that was more efficient than clade 1. Clade 8 may have metallothioneins or metallothionein-like proteins that could be quickly upregulated or that were more effective at controlling/expelling metal than clade 1. Metallothioneins may be important for metal detoxification, as was suggested by Geffard *et al*. (2010), who also determined that this class of protein likely plays a major role in nickel removal for the amphipod, *Gammarus fossarum.* Future studies that include metal exposures and adaptation within a population may involve studying different metallothionein alleles and their frequency.

The difference in sensitivities (1.8-2.7) determined in this study between clades 1 and 8 may be as a result of safety factors already built into regulations. For example, the Biotic Ligand Model (BLM) has been used for predicting the effects of metals to aquatic organisms by accounting for the metal bioavailability to the organism (MPCA, 2010). This model accurately predicted copper LC50s for *Pimephales promelas* and *Daphnia magna* within a factor of 2 (USEPA, 2007; Ryan *et al.*, 2009). Furthermore, based on suggestions by Renwick (1993), the WHO (2005) included a safety factor of 10 for extrapolation between animals and humans (the interspecies safety factor). This safety factor was used in conjunction with the interindividual differences within the human population (factor of 10). These factors of 10 (for interspecies differences and for human variability) have been used to set guidance values for exposure to the general population. Although this safety factor was based on chronic toxicity data related

to the steady-state body burden, the analysis that determined the respective safety factors were derived from a limited number of chemicals (mostly pharmaceuticals) and may not be representative of metals.

Although the variability between clades 1 and 8 may be included in the error margins of regulatory guidelines, it is important to note that only two members of the 85 lineages within the *H. azteca* species complex have been tested for their relative sensitivity. The relative sensitivities of other clades in the wild still remain unknown. However, since clades 1 and 8 within the *H. azteca* species complex have different sensitivities to copper and nickel, the interpretation of toxicity results using uncharacterized lineages should be made cautiously. A standardized clade or clades may be needed to facilitate the comparison of responses among laboratories and to better extrapolate meaningful and ecologically relevant results from toxicity tests. In agreement with Major *et al*. (2013), who noted that the two laboratory lineages may not accurately predict the responses in wild populations across North America, geographically relevant clades should be used for toxicity tests. For instance, populations of clade 1 are widely distributed from New Brunswick to Alaska and as far south as Nevada (Witt and Hebert, 2000; Witt *et al*., 2003; M. Hrycyshyn, PhD thesis in preparation). In contrast, clade 8 has been reported in areas in southeastern United States: Oklahoma, Nebraska, Kansas, Arkansas, Mississippi, Tennessee, Alabama, Georgia, and Florida (Wellborn and Broughton, 2008; Major *et al*., 2013; M. Hrycyshyn, PhD thesis in preparation). Hence, the development of toxicity protocols to include clades relevant to particular regions would provide a better reflection of local species sensitivity and/or site-specific conditions, as well as improving endpoints between laboratories.

In addition to incorporating geographically relevant clades in a standardized toxicity protocol, the two phenotypic classes of *Hyalella* should also be considered. Clades 1 and 8 are large-bodied ecomorphs that are present in habitats that lack or have weak fish predation (Wellborn and Broughton, 2008). The use of large-bodied clades may not necessarily be representative of amphipods that are also associated with fish. Small-bodied clades, which occur in water bodies with strong fish predation (Wellborn *et al*., 2005) should be considered for use in laboratories if the purpose of toxicity results is to

be extrapolated to higher organisms that prey on *Hyalella.* As a result, several different clades of *Hyalella*, including small- and large-bodied lineages, should be considered for use in toxicity tests. However, a culture protocol first needs to be developed for the small-bodied clades before assessing their response to contaminants.

A clade's behaviour and their association with sediment may also be considered when determining a standardized clade (or clades). A lineage's fossorial behaviour and/or location in specific regions of a lake may increase its vulnerability to the toxins in the sediment that would affect exposure levels (Wang *et al*., 2004; Wellborn and Cothran, 2007). Measurements using stable sulphur isotopes may help distinguish between clades of *Hyalella* that occur in the water column from those associated with the sediment (Croisetière *et al*., 2009). Consequently, standardized clades may be chosen according to their association with sediment and/or site-specificity within a contaminated area.

Although members of the *H. azteca* complex were genetically delineated in this study, the relative sensitivities according to their specific genotypes were not determined and monitored. Differences in genotypes are a potentially confounding variable since they can modify the relationship between metals and their toxic effects. Indeed, several laboratory studies have assessed and demonstrated a strong relationship between genotypes and environmental stressors in test organisms that were exposed to a contaminant (Lavie and Nevo, 1982; Newman *et al*., 1989; Diamond *et al*., 1989; Gillespie and Guttman, 1989; Benton and Guttman, 1990; Schlueter *et al*., 1995, 1997).

Additionally, a lack of research addressing the implications of contaminants on the genetics of a population over multiple generations may have consequences for risk assessments in field sites where toxic stress occurs on a long term basis and local, metal resistant ecotypes may exist. Some populations may not adapt, but rather have sufficient phenotypic plasticity for long term survival (Morgan *et al*., 2007). Consequently, exposing multiple generations of *Hyalella* to certain toxins is recommended to

verify whether genetic adaptation is the cause of more resistant amphipods or resistance is due to phenotypic plasticity.

Coupled with multiple generational tests, genomic techniques such as transcriptomics (analysing genes), proteomics (their products), and metabolomics (metabolites) are recommended in order to quantify and qualify toxicity response on an individual basis. Monitoring a series of biomarkers can provide sensitive endpoints and some understanding of the mechanism of toxicity (Snape *et al*., 2004). Recently, Mainković *et al*. (2012) compared aspects of the life history of *Chironomus* that were exposed to four toxicants with transcriptomics responses and revealed that the gene expression was more sensitive than growth or survival. The use of genomic techniques may have important implications for toxicity tests, chemical screenings, environmental monitoring, and environmental risk assessments (Poynton *et al*., 2008). Other biomarkers that may be of use in aquatic invertebrates are metallothieonines for metal contamination, which are becoming more popular in environmental monitoring programs (Amiard *et al*., 2006).

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