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The Effects of Cadmium and Zinc Exposure on the Freshwater Amphipod *Hyalella azteca*

by

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ABSTRACT

Toxic effects of cadmium and zinc on the freshwater amphipod *Hyalella azteca* Saussure were assessed using acute, chronic and behavioural testing. The 96 h LC_{50} for Cd and Zn was determined to be 10 $\mu gCd \cdot L^{-1}$ and 940 $\mu gZn \cdot L^{-1}$, respectively. One and 10 percent of the 96 h LC_{50} for Cd and Zn was used in chronic (12 weeks) testing. Chronic exposure had effects on energy acquisition and displayed the ability of animals to allocate their energy among different physiological components in order to maintain fitness. Pre-exposure of *H. azteca* to sublethal concentrations of Cd and Zn in their early life showed an induced tolerance when they were exposed to the same or different metal later. Behavioural testing displayed the high sensitivity and definite avoidance of *H. azteca* to Cd and Zn contaminated water at all concentrations used. The effect of Cd and Zn on bioenergetics of exposed animals during chronic exposure indicates that current North American water standards are insufficient to protect aquatic life.

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DEDICATION

I dedicate this Thesis to my loving husband, my dearest son and my wonderful parents.

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CHAPTER 1 INTRODUCTION

1.1. Ecotoxicology

Ecotoxicology, as an extension of toxicology (the science of the effects of poisons on individual organisms), is concerned with effects of pollutants on ecosystems (Truhaut, 1977). A pollutant is a substance that occurs in the environment at least in part as a result of human activities (Moriarty, 1983). It causes hazards to human health, harm to living resources and ecological systems (Alloway and Ayres, 1993). The main factors responsible for increased pollution are the combined effects of increased human population size, affluence and technology (Timmermans, 1993). Biotic and abiotic factors in an ecosystem can exert stresses on organisms, potentially changing population size, distribution and abundance, and hence community composition and structure.

If an organism cannot compensate for a change in its environment and suffers a reduction in fitness (relative contribution of genes to future generations), the environmental change is termed a stress (Brett, 1958; Koehn and Bayne, 1989); i.e. a stress limits either the rate of resource acquisition or allocation to growth and reproduction so that fitness is reduced (Grime, 1989). Sibly and Calow (1989) defined stress as an environmental condition that, when first applied, impairs Darwinian fitness or a correlate of fitness (e.g. reduces survivorship and/or fecundity and/or increases the time between life-cycle events). Under stress, some genotypes may be less affected than others and these resistant forms are favoured with the stress acting as a selection pressure (Calow, 1989a). Sibly and Calow (1989) refer to factors that inhibit production and

impair survivorship as growth and mortality stress. The effect of mortality stress may be alleviated to some extent if an organism spends more of its metabolic income on defence (e.g. exclusion, removal or neutralization of toxicants) and the effects of a growth stress may be alleviated by allocating more on growth and less on defence. However, there may be a trade-off between mortality and growth (e.g. energy allocated to defence is not available for growth and vice versa). The presumption is that stress acts as a selection pressure and that, given appropriate circumstances, organisms can respond adaptively (Bradshaw and McNeilly, 1981). Modification of behaviour or energy acquisition and/or allocation can compensate for changes in the environment so that there is no change or reduced change in fitness. While the allocation of energy to stress resistance can potentially increase survival at a species distribution boundary, less energy remains for reproduction and growth (Dunson and Travis, 1991).

While local impacts of human activity are of considerable importance, more concerns are developing regarding regional, landscape and global levels (Alloway and Ayres, 1993). A comparative review of case histories of stress on large ecosystems (e.g. forests, lakes, sea) suggest that there are common symptoms of pathology present in all cases (Rapport, 1989). Some of the most common causes of ecosystem pathology are the circulation and bioaccumulation of man-released persistent organic (e.g. DDT, mineral oils, resin acids) and inorganic (e.g. heavy metals) compounds. These substances are increased by human activity and have deleterious consequences at physiological and population levels (Olsson, 1987).

The effects of chemical toxicants, which challenge biological systems, can be resisted by organisms in numerous ways: avoidance or escape reactions (i.e. behavioural changes), exclusion (e.g. exposed aquatic animals secrete mucus onto exposed surfaces), removal (in-coming toxicants actively pumped out), neutralization (by complexation with protective proteins) and/or excretion and repair of damage caused by toxicants. If these responses fail, there will be irreversible damage, pathological effects, leading ultimately to death (Calow, 1991). There is a large body of evidence that combating the detrimental effects of toxic chemicals are costly for the organism in terms of metabolic resources and especially energy (see review Calow, 1991).

Bioassays play a crucial role in assessing the actual or potential impacts of anthropogenic agents on the environment. They can be used to monitor to what extent the ecosystem has been polluted and to predict the impact of a substance prior to its release. An important role for scientific research is to investigate how the results of a bioassay relate to ecological impact; i.e. what their ecological relevance is (Calow, 1989b). Maltby and Calow (1989) stated that there is a concern with all bioassays because the responses observed in particular systems might not be relevant in general. They considered the possibility of developing a general theoretical infrastructure for bioassays that addresses this problem. Calow and Sibly (1990) showed how physiological response to toxicants at an individual level could be used to predict population-dynamic responses. This can be achieved by providing functional relationships between various physiological responses and survivorship, fecundity and developmental rates.

Ecotoxicology deals with adverse effects of toxicants on the ecosystems using two different approaches: acute and chronic toxicity testing. An acute toxicity test determines the concentration of a pollutant, which has detrimental effects (change in behaviour, death etc.), on a group of test organisms during a short-term exposure. The 96 h median lethal concentration (96 h LC_{50}) resulting in 50% mortality of test animals after 96 h of exposure is most commonly used. Chronic toxicity exposure involves a stress which persists for a relatively long time, generally at least one tenth of the life span (Sprague, 1973) with effects exhibited over a long period of time.

Acute toxicity bioassays provide information on relative toxicity among toxicants and species but were not designed to, and cannot substitute for, definitive chronic tests (Giesy and Graney, 1989). It is recognized that information obtained by acute testing is insufficient to identify "safe" concentrations of toxicants released into aquatic ecosystem over the long term. Therefore chronic toxicity tests are carried out to assess pollutant effects. Chronic tests measuring sublethal effects on growth, reproduction, behaviour, physiology and/or biochemistry (Rainbow and Dallinger, 1993) provide a more sensitive and ecologically meaningful measure of toxicity than acute tests.

When choosing animals from the field for bioassays it is important to know whether animals have been pre-exposed to toxicants in their natural environment. This is especially important when establishing water-quality criteria for the protection of aquatic life. Natural populations in polluted areas are probably subjected to selective pressures for an increased resistance to toxicants (Klerks and Weis, 1987). Tolerance can be achieved either by functioning normally when exposed to pollutants or by metabolizing

and detoxifying pollutants. This can result in the evolution of resistance, which may have important implications on decisions regarding safe ambient toxicant levels. Klerks and Weis (1987) found that the oligochaete *Limnodrilus hofmeisteri* Brinkhurst, collected from cadmium (Cd) and nickel (Ni) polluted sediment was more resistant (96 h LC_{50}) to Cd and Ni than those collected from uncontaminated sediment. The ability to tolerate higher concentrations of metals, along with the ability to exclude, remove and excrete toxicants, can also be achieved with metal binding at sites in cells (Wright, 1980). These cells are involved with the synthesis of specific metal-binding proteins (i.e.metallothioneins) which protect important cellular sites from damage (Kagi and Vallee, 1960; Kagi and Nordberg, 1979; Bebianno et al., 1994; George et al., 1996). Furthermore, the induction of metallothionein synthesis by exposure to one metal can result in an increased resistance to the toxic effect of another metal (Webb, 1972; Brown, 1978; Kito et al., 1982; Polek and Obselkova, 1992; Marr et al., 1995). Couillard et al. (1995a) proposed direct measurement of metallothionein as an indicator of prior exposure to toxic metals.

Stress imposed by toxicants can also cause behavioural changes in animals. Locomotor behaviour in the form of avoidance and preference is the most commonly studied behavioural response to pollutants. Monitoring change in behaviour is especially important because it may occur at toxicant concentrations that are quite different from those causing significant physiological or biochemical effects and may be expressed at concentrations lower than the lethal threshold (Little et al., 1993; Woodward et al., 1995).

1.2. Heavy metals

The term 'heavy metal' is used to indicate 37 elements of the periodic table. It is usually applied to elements such as cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) which are commonly associated with toxicity problems. All these elements occur in the natural environment in very low concentrations and are therefore known as trace elements (Baudo, 1989).

Heavy metals occur naturally in rock-forming and ore minerals. They can be found in soils, sediments, waters and living organisms. Although heavy metals are ubiquitous in most natural materials, anthropogenic sources contribute a significant amount of metal to the environment. They can enter the environment through metalliferous mining (metals utilized in manufacturing are obtained from the mining of ore bodies in the rocks or the recycling of scrap metal), agricultural materials (impurities in fertilizers, pesticides, composts etc.), fossil fuel combustion, metallurgical industries, electronics, batteries, stabilizers etc. Production of all metals has increased significantly over recent time (Alloway and Ayres, 1993).

Some heavy metals are required by living organisms because they are constituents of enzymes and other important proteins (referred to as micronutrients or essential elements) involved in metabolic pathways. Such essential elements are Cu, manganese (Mn), iron (Fe) and Zn which in excess concentrations cause toxicity (Simkiss and Taylor, 1989). Elements with no known essential biochemical function are called non-essential elements and include arsenic (As), Cd, Hg, Pb, plutonium (Pu), antimony (Sb), thallium (Tl) and uranium (U). They cause toxicity at concentrations, which exceed the

tolerance of the organism but do not cause deficiency disorders at very low concentrations like micronutrients. Levels of essential trace metals in organisms can be regulated to some degree (Devineau and Amiard-Triquet, 1985), whereas levels of non-essential elements depend on their concentrations in the medium.

When heavy metals reach the environment after emission and enter aquatic systems through aerosol particles or by being directly released into water, they either react with the constituents of the water or settle to the bottom where they react with the sediments. It has been demonstrated that divalent metals tend to bind to the acid volatile sulphide (Karouna-Renier and Sparling, 1997) and EDTA (Borgmann and Norwood, 1995a) fractions of aquatic sediments, rendering them biologically unavailable with the result that free metal activity and toxicity are reduced. The ionic form of metals is most available to aquatic organisms (Stokes and Campbell, 1985) and the total metal concentration in water does not provide a reliable indication of bioavailability (Laxen, 1984).

Concentrations of anions and chelating ligands in the water as well as its pH, redox status and the presence of adsorbent sediments determine the solubility of metal ions (Alloway and Ayres, 1993). Bioavailability of Cd for *Hyalella azteca* (Saussure) was shown to be affected by the concentration of Cd^{2+} and dissolved organic substances in solution (Stephenson and Mackie, 1989a). The chemical speciation of metals in solution and partitioning of metals between the aqueous and solid phases influence the biological availability and toxicity to organisms as well as the geochemical fate of metals (Wagemann et al., 1994). Bioavailable metals are defined by Campbell and Stokes

(1985) as metals in such a chemical state that they can be taken up by an organism and can react with its metabolic system. For aquatic animals trace metal bioavailability is of greatest importance. Sediments consisting of smaller grain-size fractions (e.g. clay or silt), with high percentages of Fe and Mn and high organic carbon content generally have a low availability of trace metals, irrespective of the total concentration (Timmermans, 1993).

There is no direct link between metal concentration in water or sediments and accumulated concentrations within an organism. In aquatic environments, the concentrations of metals in organisms are usually far higher than in the water and lower than in the sediment (Naqui et al., 1993). On the contrary, a high concentration of heavy metal (the same magnitude as that of the sediment) was reported for a freshwater sponge (Richelle et al., 1995). However, different organisms have different sensitivities to the same metal, and the same organisms may be more or less damaged by different metals.

1.2.1.Cadmium

Cadmium is a toxic, non-essential trace metal which reacts very easily with negatively charged particles and it is released into the global environment by human activities at a rate that far exceeds the natural rate of release (Nriagu, 1981). It is widely used in industry for corrosion prevention, polymer stabilization, electronics, pigment application and as a neutron absorber in nuclear reactors. It is highly toxic and causes kidney, testes and liver disfunction, emphysema, inflammation and degeneration of lungs, reduced growth and skeletal deformities in humans. Intakes of Cd may be higher in

communities living in the vicinity of metal smelters. The principal medium of exposure to Cd is through food, followed by cigarette smoke, air, soil and water (Newhook et al., 1994).

Aqueous Cd concentrations are elevated in many soft-water lakes in central Canada (Stephenson and Mackie, 1988a) and elsewhere (Borg, 1983; Laxen, 1984). The average total concentrations of Cd in unpolluted rivers, streams and lakes generally range from less than 0.001 to 0.08 $\mu g \cdot L^{-1}$ (Nriagu, 1981; Stephenson and Mackie, 1988a). Significantly higher concentrations have been observed near industrial and urban areas. In aquatic environments polluted by mining and manufacturing, Cd concentrations in fresh waters range from 50 $\mu g \cdot L^{-1}$ (Ringwood, 1992) to 1,000,000 $\mu g \cdot L^{-1}$ (McKee and Wolf, 1963). Huckabee and Blaylock (1973), following the transfer of Cd from a terrestrial to an aquatic ecosystem, found that Cd is mostly (94-96%) accumulated in the sediments rather than in the water. Contamination from mining, smelting and industrial activity has produced very high concentrations of Cd in some areas. The highest concentrations in Canada (ranging from 125-3950 $mg \cdot kgDW^{-1}$) were reported in lakes near a Cu/Zn smelter at Flin Flon, Manitoba (Jackson, 1978; Harrison and Klaverkamp, 1990).

Canada is one of the world's leading producers of Cd (Hoskin, 1991). There is great concern regarding the extend and severity of Cd contamination in Canadian ecosystems and the potential impacts of contamination on ecosystem characteristics and functions. Canadian Council of Ministers of the Environment (CCME, 1991) set a concentration of 0.48 $\mu gCd \cdot L^{-1}$ (water hardness 120 mg $CaCO_3 \cdot L^{-1}$) as the maximum

allowable concentration of Cd in Canadian surface freshwaters. This was accepted to be the lowest observed effect concentration (LOEC) to result in any observable adverse effect in aquatic organisms. A new level was set by reviewing the toxicological literature regarding the Cd concentration at which the most sensitive life stage of the most sensitive species exhibited significant toxicity (Outridge et al., 1994). For surface freshwater, the LOEC was identified as 0.17 $\mu g \cdot L^{-1}$ at a hardness of 48.5 $mg \cdot L^{-1}$, based on significant reproductive impairment in the zooplankter *Daphnia magna* Strauss (Biesinger and Christensen, 1972). Because of the significant negative correlation between Cd toxicity and water hardness, a regression equation, for adjusting the LOEC to variations in hardness was proposed (Outridge et al., 1994):

$$\text{LOEC } (\mu g Cd \cdot L^{-1}) = 10^{\{0.9694[\text{Log } 10 \text{ (hardness)}] - 2.404\}} \quad (1.1)$$

The calculated LOEC for Cd in chronic and behavioural testing, using water of hardness 205.7 mg $CaCO3 \cdot L^{-1}$ is 0.8116 $\mu gCd \cdot L^{-1}$. The two concentrations used in these experiments, 0.1 and 1 $\mu gCd \cdot L^{-1}$, are a direct test of whether the calculated LOEC, using this equation, is appropriate.

Cadmium is toxic because it alters the normal cellular biochemistry and physiology of the exposed organisms. After Cd adsorption to epithelia (Zia and McDonald, 1994), Cd^{2+} can be transported across cell membranes via ion-inspecific carrier molecules (Wright, 1995) or uptake is achieved through the calcium (Ca^{2+}) ion pump of cells (Playle and Dixon, 1993). Cadmium can indirectly cause the failure of the ion channel, the regular function of which is Ca^{2+} homeostasis (Simkiss and Taylor, 1989), by inhibiting the influx of Ca from the environment into the organism (Wright,

1995). Within an organism Cd causes a suppression of mitochondrial respiration by inhibiting mitochondrial enzymes through interaction with active SH-groups of the enzyme (Korotkov et al., 1994). Cadmium also causes the displacement of the magnesium ion (Mg^{2+}) associated with the ATPase enzyme (Wright, 1995), inhibiting the function of the Na^{+}/K^{+} pump (Pratap and Wendalaar Bonga, 1993). Changes in this enzyme activity, which drives epithelial ion exchange and osmoregulation, can result in losses of Na^{+} and K^{+} ions from the body (Wright, 1995). Cadmium can also substitute for Zn in metallothionein-like proteins and Zn containing enzymes which can also explain the toxic and carcinogenic properties of Cd (Sunderman, 1990). Although it is generally believed that Cd is a trace metal without any known biological function (Abel and Bärlocher, 1988) it has been shown that under conditions of Zn limitation, low concentrations of Cd act as a nutrient for the marine diatom *Thalassiosira weissflogii* (Grunow) (Lee et al., 1995) and can enhance growth of a variety of marine phytoplankton species (Lee and Morel, 1995).

The concentration of Cd in the tissues of freshwater organisms is positively correlated with Cd levels in the water and negatively correlated with some dissolved ions in the water (Stephenson and Mackie, 1988b; Wang and Evans, 1993). Wright (1980) observed a competitive effect between Cd and Ca in *Gammarus pulex* (L.) and in the freshwater mussel *Elliptio complanata* (Lightfoot) (Wang and Evans, 1993). In *H. azteca* a strong negative effect of Ca on Cd levels was found by Stephenson and Mackie (1988a, 1989b) who suggested competition between Cd ions and Ca for uptake sites on the gill surfaces. Cadmium uptake was lowered by the presence of Zn in concentrations as low

as 10 $\mu g \cdot L^{-1}$ (Howell, 1985) in the freshwater amphipod *Gammarus pulex*. There was a significant decrease in whole-body Cd-content of Cu/Cd co-exposed fish compared to the Cd content of Cd-exposed fish (Pelgrom et al., 1994). Furthermore, injection of Cu prior to Cd injection prevented hepatic and testicular lipid peroxidation induced by Cd in mice (Li et al., 1995).

Despite Cd accumulation in fresh-water organisms, it is generally believed that Cd does not biomagnify in the food chain (Jenkins, 1980; Giesy et al., 1980). However, it has been shown that prey choice may be an important factor for Cd accumulation in predators (Timmermans et al., 1992; Langevoord et al., 1995). The accumulation and allocation of Cd in organisms depends on the way it is ingested, with food or water, and also varies with species and the specific tissues examined. For *Gammarus fossarum* Koch (Abel and Bärlocher, 1988) and for *Asellus aquaticus* (L.) (Hattum et al., 1989) higher mortality was reported for animals taking up Cd through water compared to the animals taking up Cd through food (Abel and Bärlocher, 1988). On the contrary, Cd uptake in water mites and caddisfly larvae through food was greater than the uptake through water (Timmermans et al., 1992). Presing et al. (1993) showed that the soft tissues of the pond snail *Lymnaea stagnalis* (L.) concentrate Cd much more than the shell while gills, livers and kidneys accumulate the highest amounts of Cd in fish (Harrison and Klavercamp, 1990; Zia and McDonald, 1994; Langevoord et al., 1995; Melgar et al., 1997).

In the natural environment, both biological and environmental fluctuations modulate pollutant toxicity. Most studies of pH effects on Cd toxicity indicate elevated

toxicity or bioaccumulation of Cd at high pH because of increased solubility of Cd (Campbell and Stokes, 1985; Schubauer-Berigan et al., 1993; Fjeld et al., 1994). However, for *H. azteca*, toxicity of Cd was greater at pH 4.4 than at pH 6.1 (Stephenson and Mackie, 1988a). Temperature is an important controlling factor of metabolism and can have a marked effect on the toxicity of heavy metals (Roch and Maly, 1979). They found that cold-acclimated rainbow trout showed a greater 10-day lethal threshold concentration and greater survival than warm-acclimated trout. However, toxicity of Cd increased with increasing exposure temperature above acclimation temperature in the freshwater snail *Potamopyrgus antipodarum* (Gray) (Møller et al., 1994).

Cadmium toxicity can also cause changes in the behaviour of test animals. A vigorous curling motion was observed for caddisfly larvae exposed to 85,500 and 238,000 $\mu gCd \cdot L^{-1}$ (Spehar et al., 1978) and uncontrolled body motions were observed in several species of fish (Benoit et al., 1976). Cadmium had an immobilizing effect on *Nephelopsis obscura* Verill (Wicklum, 1995) and *Gliptotendipes pallens* Meigen exposed to Cd for 96 hours, exhibited decreases in activity at 2,500 $\mu gCd \cdot L^{-1}$ (Heinis et al., 1990). There was significant suppression of locomotion evident in a grass shrimp exposed to 560 $\mu gCd \cdot L^{-1}$ (Hutcheson et al., 1985).

1.2.2. Zinc

Zinc is an essential micronutrient for biological activity and it is usually connected with serious deficiency problems in plants and animals. In humans extreme

deficiency can cause short stature and delayed sexual maturity. However, at elevated concentrations it may become toxic. Zinc pollution is often associated with mining and smelting. Mining causes pollution of air, water and soil with fine tailings particles, which ultimately undergo oxidation to release Zn^{2+}. It can also enter aquatic environment as a constituent of industrial by-products as it is extensively used for corrosion protection and galvanizing in the automobile and construction industries.

In freshwater ecosystems, the concentration of Zn ranges from 0.02-1.8 $\mu g \cdot L^{-1}$ in unpolluted areas to over 42,500 $\mu g \cdot L^{-1}$ in water draining a lead mine (Kelly, 1988). Overloads of Zn in Canada (the largest producer of Zn metal in the world) are a major concern. In 1976 the IJC (International Joint Commission) established a limit of 30 $\mu gZn \cdot L^{-1}$ for the Great Lakes. However, in 1980 the USEPA (United States Environmental Protection Agency) adopted a limit of 47.0 $\mu gZn \cdot L^{-1}$ which was also accepted by Manitoba (Williamson, 1983).

The most bioavailable form of Zn is the free ion Zn^{2+} which is often the most abundant of the dissolved forms of Zn (O'Brien et al., 1990). The free-metal-ion concentration, a key variable for the reactivity, bioavailability, and effects of metals (Sunda, 1994), is regulated by complex interactions among trace metal ions, ligands and major ions and particles (Bruland et al., 1991). In lake-waters Xue et al. (1995) found the release of Zn from electrochemically inert complexes upon addition of Cu which suggested direct competition of Cu and Zn for ligands.

The toxicity of Zn to aquatic animals varies greatly and is dependent on numerous factors including life stage, pH, hardness and humic acid levels (Borg, 1983; Campbell

and Stokes, 1985; Amyot and Pinel-Alloul, 1994;). Naylor at al. (1990) showed for *Gammarus pulex* that juveniles and brooding females were the most sensitive life stages to Zn. The acute lethality of dissolved Zn to fish *Salmo gairdneri* Richardson was significantly increased at higher pH and lower hardness levels (Bradley and Sprague, 1985). In *H. azteca* Zn was most toxic at pH 8.3 and least toxic at pH 6.3 (Schubauer-Berigan et al., 1993). They explained this phenomena as being caused by two competing mechanisms: as the pH rises, dissolved Zn becomes increasingly toxic, but at lower pH levels it is replaced by Zn hydroxide precipitates which are of very low toxicity to animals. Also, presence of organic matter decreased the uptake of Zn in rainbow trout *Salmo gairdneri* (Ramamoorthy and Blumhagen, 1984).

Generally, the acute toxicity of Zn is lower in waters with high water hardness (Bradley and Sprague, 1985). Because water hardness influences toxicity of Zn, the USEPA (1980) established a limit for total recoverable Zn based on the following formula:

$$Zn\ (\mu g \cdot L^{-1}) = e^{(0.83[\ln(\text{hardness})]+1.95)} \qquad (1.2)$$

The numerical limit for total recoverable Zn in chronic and behavioural testing in this present study with water hardness of 205.7 is 584.6 $\mu gZn \cdot L^{-1}$. This concentration is much higher than the 9.4 and 94 $\mu gZn \cdot L^{-1}$ tested. The exposure of *H. azteca* to these concentrations will be a direct test of the safe levels stated by USEPA.

Zinc can enter the bodies of aquatic animals by penetrating across the gill epithelium or other permeable surfaces and entering the haemolymph or blood (Xu and Pascoe, 1993). With the circulation of the body fluid, Zn is transported to internal

organs. De March (1988) suggested that Zn^{2+}, at acutely lethal concentrations, inhibited processes of ionic regulation and led to suffocation by precipitating and causing damage to the gill surface in *Gammarus lacustris* (Sars.). Another route of Zn entering aquatic animals is the absorption of the metal from the alimentary canal, mostly via the hepatopancreas or equivalent caeca in crustaceans (Xu and Pascoe, 1993). The hepatopancreatic caeca absorb the majority of Zn taken in from food, possibly binding it in metallothioneins or in granular form and it is the prime site for metal storage, regulation, and detoxification in crustaceans (Morgan et al., 1990).

High concentrations of Zn can be tolerated using different mechanisms such as the sequestering of Zn in amoeboid lymphocytes by the oyster *Ostrea edulis* L. (George et al., 1978) or precipitation of Zn pyrophosphate granules by the barnacle *Elminus modestus* Darwin (Rainbow and White, 1989). Some aquatic organisms are able to regulate essential trace metals, such as Zn. Animals are considered good regulators when they show no significant change in body metal content over time on exposure to a raised metal bioavailability (Rainbow and Dallinger, 1993). Metal regulation requires a regulator to maintain body metal content approximately constant by altering the rate of metal excretion to match the rate of metal uptake.

The Zn regulatory abilities of amphipods differ from species to species. *Hyalella azteca* was unable to completely regulate Zn during chronic exposure (Borgmann et al., 1993) but *Gammarus zaddachi* Sexton could regulate Zn (Amiard et al., 1987) as could *Allorchestes compressa* Dana (Ahsanuellah and Williams, 1991). *Echinogammarus pirloti* Sexton and Spooner did not completely regulate Zn (Rainbow and White, 1989).

Gammarus duebeni Lilljeborg regulated Zn up to external Zn concentrations of 200 $\mu g \cdot L^{-1}$ (Johnson and Jones, 1989) but *Gammarus pulex* demonstrated no Zn regulatory ability (Bascombe et al., 1990).

In general, changes in energy acquisition and allocation are only seen at lethal concentrations of Zn. Zn exposure at low chronic levels for 11 weeks (Gray, 1995) did not cause changes in the growth rate of *Nephelopsis obscura*. *Hyalella azteca* did not show changes in growth or reproduction at concentrations of Zn less than those causing mortality (Borgmann et al., 1993). Similarly the cladoceran *Moina macrocopa* Straus did not exhibit changes in reproductive output (Wong, 1993) and in the Atlantic salmon *Salmo salar* (L.), there was no change in growth rate until Zn reached levels which caused significant mortality (Farmer and Ashifield, 1979).

1.3. Test organisms

Hyalella azteca was chosen for this project because of its high sensitivity to the presence of contaminants in freshwater systems (Borgmann et al., 1991; West et al., 1993; Suedel et al., 1996; Borgmann and Norwood, 1997). Its short life cycle, widespread and abundant distribution and ease of culture also make it a very suitable test species (DeMarch, 1976; Borgmann and Ralph, 1986). A comprehensive knowledge of this species and its reaction to common pollutants would aid to track long term and acute changes in anthropogenically stressed ecosystems.

Hyalella azteca belongs to the family Hyalellidae, superfamily Talitroidea, order Amphipoda, class Crustacea (Holsinger, 1972). It is abundant and widely distributed,

found in almost all unpolluted shallow waters of ponds, lakes and slow moving streams throughout North America. It is an omnivorous deposit feeder, feeding on algae and bacteria associated with the sediments and aquatic macrophytes (Hargrave, 1970) and dead animal and plant material (Cooper, 1965).

Reproduction is sexual with *H. azteca* exhibiting precopula where the male and female swim together, with the male holding the female with his gnathopods, for several days until mating takes place. They then separate and eggs are released into the marsupium, a ventral brood chamber of the female, where they are fertilized. Eggs hatch in the marsupium just prior to female moult when the first instar offspring are released. Developing individuals pass through seven instars before reaching maturity (DeMarch, 1976).

The breeding season of *H. azteca* varies with location. Strong (1972) recorded that the breeding season varied among populations in Oregon, where *H. azteca* from hot springs reproduced year-round whereas populations from mountains and coastal lakes reproduced only during summer. DeMarch (1976, 1977) proposed that the main factors for induction and termination of reproduction are photoperiod and temperature. Edwards and Cowell (1992) showed that warm temperatures are the main factor for continuous reproduction. Differences in life-history features (e.g. size of eggs, young and adults, growth rate) were shown among populations of *H. azteca* from Oregon (Strong, 1972) and biogeographical variation in size-specific fecundity was also reported (France, 1992). Seasonal growth and reproduction, and the presence of predaceous fish influences population dynamics. Wellborn (1994) found that size-selective predation contributed to

overall mortality patterns in different habitats. The observed differences in life-history traits between populations agree with predictions that size-biased mortality influences the evolution of reproductive allocation, size at maturity and egg size. Two distinct *H. azteca* morphotypes among adult individuals were apparent in nine diverse habitats in Michigan (Wellborn, 1995). It was suggested that the regime of size-biased predation experienced by *H. azteca* in each habitat might be the primary determinant of the pattern of association between *H. azteca* morphotype and habitat type. However, positive correlations were observed among growth rates, feeding rates and developmental rates in *H. azteca* (DeMarch, 1977, 1978).

Contaminant accumulation, along with their relative sensitivity to toxicants suggests that amphipods may be one of the most vulnerable groups of organisms to chemical pollution (Borgmann et al., 1989). *Hyalella azteca* has been used in many experiments to evaluate toxicity of different metals and it is particularly sensitive to Cd in both the water and sediments (Borgmann and Munawar, 1989). *Hyalella azteca* is known to readily accumulate Cd to high concentrations (Stephenson and Turner, 1993) with water hardness, total Cd and dissolved organic carbon concentrations being significantly correlated with Cd concentrations in the animal (Stephenson and Mackie, 1988b). It is a benthic organism and can be used for testing sediments and water-born contaminants (Borgmann and Munawar, 1989; West at al., 1993; Ingersoll et al., 1995; Nimmo et al. 1995; Becker et al., 1995; Tomasovic et al., 1995; Borgmann and Norwood, 1997) and it has been shown to be sensitive to Cu (West et al., 1993; Suedel et al., 1996) and Cd

contaminated sediments (Nebeker et al. 1986; Borgman and Munawar, 1989; Borgmann et al., 1991).

Borgmann et al. (1993) observed accumulation, regulation and toxicity of Cu, Zn, Pb and Hg in *H. azteca* but none of the metals resulted in any significant reduction in growth, as judged by wet biomass, or reproduction at any concentration which did not also cause mortality. This agrees with earlier observations on the chronic toxicity of Cd, pentachlorophenol and polyclorobiphenyls (PCBs) to *H. azteca* by Borgmann et al. (1989, 1990). Borgmann and Norwood (1995a) studied the effect of the strong metal-complexing agent EDTA on Cu and Zn concentrations in *H. azteca* to determine if metal concentrations in control amphipods represent minimum physiological levels. Since exposure to EDTA did not reduce metal levels in the animals, they concluded that background metals are firmly bound to *H. azteca*.

Stephenson and Turner (1993) found that the Cd uptake in *H. azteca* is rapid, with equilibrium being approached within 2 weeks. Uptake of Cd mostly derived from food rather than from water and 80% of ingested Cd was assimilated. It was also shown that they are very sensitive on low pH combined with higher temperature (Pilgrim and Burt, 1993). Toxicity of ammonia to the amphipod decreases with increased water hardness and becomes more pH dependent (Ankley et al., 1995). Borgmann and Norwood (1995b) showed that Cu and Zn concentrations in whole body *H. azteca* are independent of body size.

1.4. Objectives

The toxicity of Cd and Zn to aquatic organisms has been extensively investigated. Most toxicity studies have used species (e.g. fish) that are generally more resistant to toxicants than lower trophic level organisms. However, some invertebrates (e.g. *Daphnia*, *Gammarus*, *Hyalella*), which are ecologically important because of their relative abundance and ubiquity, have much higher sensitivity. Therefore, recommended standards for Cd and Zn concentrations in fresh water may not be adequate to protect sensitive macroinvertebrate species. Information concerning the toxicity of pollutants to animals representative of lower trophic groups is sparse (Murphy, 1980) and must be extended if the impact of toxicants on natural freshwater communities is to be fully understood. Furthermore, if the tests are to be ecologically relevant then the most sensitive life stage of test species should be used (Gauss et al., 1985) to estimate the impact of the toxicants on the species.

This study was conducted to investigate the potential effect of two common pollutants of natural waters, Cd and Zn, on the amphipod *H. azteca*. Acute toxicity testing was initially carried out to assess relative sensitivity of the most sensitive stage of *H. azteca*. To determine the effect of heavy metals other than mortality, a chronic toxicity test along with behaviour responses were used. At the same time chronic toxicity was used to investigate the effect of pre-exposure on the tolerance of *H. azteca*.

The objectives of this study were:

1. Determine the lethal concentration of Cd and Zn on *H. azteca* with the acute toxicity (96 h LC_{50}) test.

2. Examine the ecophysiological responses of *H. azteca* during chronic exposure to 10% and 1% LC_{50} for Cd and Zn.
3. Investigate the effect of Cd and Zn on the survival, fecundity, respiration and lipid stores of *H. azteca* after pre-exposure to Zn or Cd.
4. To determine whether pre-exposure to one metal will change tolerance to the toxic effect of the other metal.
5. Determine the effect of Zn and Cd on survival, fecundity and lipid stores of first generation *H. azteca* after pre-exposure of their parents.
6. Observe the preference-avoidance response of *H. azteca* exposed to both concentrations of Cd and Zn and clean (uncontaminated) water.

CHAPTER 2 ACUTE TOXICITY

2.1. Introduction

Despite recent developments in chronic sublethal testing, traditional acute lethality tests are still widely used as a standard ecotoxicological procedure by environmental agencies to assess the potential impact of chemicals on ecological systems. Acute toxicity tests have been designed to provide rapid and relatively inexpensive estimates of the concentration of toxicants that have deleterious effects on test organisms during a short-term exposure under controlled conditions. They are considered ecologically significant, scientifically and legally defensible, modest in predictive capability and have the greatest utility (Macek et al., 1987). There are two standard toxicity tests used for testing toxicant effects on aquatic invertebrates: the 96 h Median Lethal Concentration (96 h LC_{50}) and 96 h Median Effective Concentration (96 h EC_{50}) (Buikema et al., 1982). The most common acute toxicity test is the acute lethality test (LC_{50}) which is used to determine a lethal threshold concentration of a toxicant. This is the concentration that causes 50% mortality in the test organisms during a 96 h exposure time. In some invertebrates, where death is not easily determined, effects such as immobilization and loss of equilibrium in 50% of test animals after 96 h of exposure are used to estimate 96 h EC_{50} (Parrish, 1985).

Acute toxicity tests are useful to establish the relative toxicity of a toxic agent and are the first step in understanding the hazards of toxic materials in aquatic ecosystems. They are also conducted to find the range of concentrations that should be tested to determine if

chronic effects also occur. Comparing LC_{50} for different toxicants with the same test organism and test conditions, or for the same toxicant with different species, can yield information on relative toxicity or organism sensitivity: the higher the LC_{50}, the lower the toxicity or sensitivity.

As a result of industrialization and release of heavy metals, especially Cd and Zn, into the environment, acute toxicity testing has been performed on many aquatic organisms. A great deal of variation in sensitivity to toxicants among different species and age classes has been shown (Table 2.1). Most studies showed that animals exhibit higher sensitivity to toxicants at earlier life stages (Williams et al., 1986; Green et al., 1986; Park et al., 1994; Collyard et al. 1994; Sharma and Sharma, 1995; Wicklum, 1995; Bambang et al., 1995). While populations, communities and ecosystems represent complex interactions among individuals, species and the environment (Giesy and Odum, 1980) it is not possible to predict with great certainty the effect of a toxicant on ecosystems.

When conducting a short-term test, relative to the animal's life span, it is important to study the most sensitive life stage of the test organism. It has been found that the use of critical life stages has resulted in estimates of safe concentrations of toxicants in the environment which are very similar to those determined in chronic exposures (Kenaga, 1977). Ten-day growth assay using the second instar larvae of *Chironomus tentans* Fabricius was sufficient to determine sensitivity of this species to several metals (Nebeker et al., 1984). Therefore, if a test that includes this sensitive stage is conducted, the toxicity of a pollutant can be more accurately predicted.

Table 2.1 A comparison of 96 h LC_{50} values for Cd and Zn ($mgCd \cdot L^{-1}$ or $mgZn \cdot L^{-1}$) of different species of animals at different life-stages under similar temperature, water hardness and pH conditions.

Organism and life stage	**96 h LC_{50}**	**Reference**
Amphipods		
Hyalella azteca Saussure juveniles	0.007 Cd	Collyard et al., 1994
Hyalella azteca adults	0.030 Cd	Mackie 1989
Gammarus pulex L. adults	0.030 Cd	Williams et al., 1985
Gammarus pulex juveniles	2.100 Zn	Naylor et al., 1990
Isopods		
Asellus aquaticus (L.) juveniles	0.080 Cd	Green et al., 1986
Asellus aquaticus embryos	>2.000 Cd	Green et al., 1986
Asellus aquaticus adults	1.000 Cd	Green et al., 1986
Asellus aquaticus juveniles	18.200 Zn	Naylor et al., 1990
Cladocerans		
Moina irrasa (Brehm) juveniles	0.102 Zn	Zou and Bu, 1994
Leeches		
Glossiphonia complanata (Linnaeus) adults	0.480 Cd	Brown and Pascoe, 1988
Nephelopsis obscura Verill juveniles	~0.180 Cd	Wicklum, 1995
Nephelopsis obscura adults	~0.260 Cd	Wicklum, 1995
Dipterans		
Chironomus riparius Meigen larvae	54.000 Cd	Williams et al., 1986
Chironomus riparius adults	300.000 Cd	Brown and Pascoe, 1988
Gastropods		
Potamopyrgus antipodarum (Gray) juveniles	~4.000 Cd	Møller et al., 1994
Polychaetes		
Tubifex tubifex (Muller) juveniles	1.000 Cd	Fargašova, 1994
Fish		
Rivulus marmoratus Poey larvae	0.800 Cd	Park et al., 1994
Rivulus marmoratus juveniles	18.800 Cd	Park et al., 1994
Rivulus marmoratus adults	32.200 Cd	Park et al., 1994
Salmo gairdneri Richardson juveniles	~4.000 Zn	Bradley and Sprague, 1985
Oncorynchus mykiss Walbaum adults	0.093 Zn	Chapman, 1978
Cirrhinus mrigala (Hamilton) fries	7.000 Zn	Sharma and Sharma, 1995
Cirrhinus mrigala fingerlings	0.350 Zn	Sharma and Sharma, 1995
Shrimps		
Penaeus japonicus (Bate) zoae	0.030 Cd	Bambang et al., 1995
Penaeus japonicus postlarvae	3.500 Cd	Bambang et al., 1995
Penaeus japonicus juveniles	5.500 Cd	Bambang et al., 1995

Acute toxicity test in this study was conducted to determine the range of Cd and Zn concentrations that should be used in the chronic toxicity testing. For this purpose, the same amount of food and cotton gauze used in the chronic test was provided in this acute test. To determine the median lethal concentration of Cd and Zn for *Hyalella azteca* only the most sensitive early life stages, determined by Collyard et al. (1994) were used.

2.2. Methods and materials

Test animals

Hyalella azteca used in this study were obtained from cultures of specimens collected from Stephenson's Pond in June and July 1996. Stephenson's Pond (114°16'W; 51°9'N), located in NW Calgary, is a small, shallow hypereutrophic prairie pond with a substrate made up of soft mud and sand with some large rocks and gravel at the eastern end of the pond. The southern shoreline is covered with *Salix* sp. and *Populus balsamifera* L. and the remainder is open grassland.

The animals were maintained in aquaria containing Stephenson's Pond water of 8.0-9.0 pH, hardness 170 mg $CaCO_3L^{-1}$ and cotton gauze in a light- and temperature-controlled incubator at 25°C, 100% oxygen saturation with 16h light: 8h dark photoperiod regime, using fluorescent tubes placed 40 cm above the shelves. These conditions were also used for the toxicity testing. Amphipods were fed *ad libitum* three times a week on a diet of Tetra-Min® fish-food flakes and poplar leaves *Populus balsamifera* inoculated with the fungus *Cladosporium*. Offspring were removed and placed into separate aquaria so that the age of

individuals could be tracked. The age of animals used in the experiment were 10 - 12 days for Cd and 2-4 or 4-6 days for Zn. These stages were shown by Collyard et al. (1994) to be the most sensitive to these two metals.

Food and sediment

The food source used for the test was Tetra-Min® fish-food flakes and poplar (*Populus balsamifera*) leaves inoculated with the fungus *Cladosporium*. Poplar leaves were chosen because they are a major part of the diet in the field, the animals grow well on them and allow easy measurement of food ingestion (Naylor et al., 1989). Fish-food flakes were used because they provide high growth in *H. azteca* (Borgmann et al., 1989).

Poplar leaves, collected around Stephenson's Pond in September 1996, were dried and stored. Before use they were rehydrated, cut into discs and autoclaved to remove microorganisms. The discs were inoculated with the fungus *Cladosporium* and incubated for 10 days. The fungi facilitate the breakdown of the leaf material, making it more palatable (Barlocher and Kendrick, 1975; Chamier and Willoughby, 1986). Before being fed to the animals, the discs and fish food flakes were rehydrated, using experimental solution. This increases palatability and allows toxicants to come into equilibrium with the food (Naylor et al., 1989) as heavy metals tend to adsorb to organic substances (Abel and Bärlocher, 1988).

Cotton gauze was used as an artificial sediment to provide conditions close to those in the field. Borgman et al. (1989) showed that cotton gauze compared to real sediment eases the enumeration process and the ability to observe any differences in behaviour of exposed

animals. Cultivation of *H. azteca* in aquaria with natural sediments from the field or cotton gauze demonstrated that the production of young did not significantly differ between the two substrates (Borgmann et al., 1989). Cotton gauze can also be used to test water-born contaminants, if pre-soaked, because it does not influence toxicant concentration in the water as natural sediments do.

Test solutions

Test organisms were exposed to two different experimental solutions, Cd and Zn. Zinc sulphate ($ZnSO_4$) was used to prepare the Zn solution and cadmium chloride ($CdCl_2$) to prepare the Cd solution. Zinc sulphate and cadmium chloride were recommended by Environment Canada (1990) for use as reference toxicants. These two chemicals were diluted in Stephenson's Pond water, which had been filtered through a sterile Gelman brand capsule filter with 0.45 μm pore size. Before the animals were added into an experimental solution, the water was aerated in order to bring dissolved oxygen into equilibrium with the ambient air. To minimize leaching, dissolution and sorption, the containers used in the experiment were plastic (USEPA, 1990).

Experimental design

To assist the calculation of the LC_{50} and 95% confidence limits for each treatment, geometric dilution series, in which each successive concentration was about 50% of the previous one, was used. For each concentration, 10 animals, the minimum number of animals

that can be used for a static acute test (Parrish, 1985) of the corresponding age group for Cd and Zn were placed in an 800 mL plastic container containing 500 mL of the experimental solution and one 5.0 x 5.0 cm piece of sterile pre-soaked Kendall cotton gauze pad as a substrate. To each container 2.0 mg of pre-soaked ground fish-food flakes and 2.0 mg of pre-soaked leaves were added as food. The water was aerated throughout the test and containers were covered loosely to reduce evaporation. Cotton gauze, food and water were not replaced during the 4 day testing.

The plastic containers used in the experiment were rinsed with the treatment solution prior to use to allow metals to plate the walls of the container and food and cotton gauze were pre-soaked to minimize the loss of heavy metals from the test solutions during the test. There was a control and three replicates for each treatment. The term control refers to animals maintained in clean water, not exposed to Cd or Zn, but otherwise treated exactly the same as exposed animals and originating from the same batch of test animals. Mortality was monitored daily for 4 days and dead animals were removed from the pots and counted.

2.3. Statistical analysis

Median lethal 96 h LC_{50} values were estimated by probit analysis using the program developed by Stephen (1977). The program uses probit, moving average and binomial methods to assess LC_{50}. The values from the probit method with 95% confidence limits were reported, because they were recommended by USEPA (1990).

2.4. Results

After 4 days of testing, no mortality was observed in the controls. Generally, in both treatments the proportion of test animals that died in a fixed time increased as the concentration of toxicant increased (Fig.2.1 and 2.2). This dose response curve shows percent mortality rapidly increasing and then levelling out as the concentration increased.

Calculated 96 h LC_{50} for *H. azteca* was 11.4 $\mu g \cdot L^{-1}$ and 943.2 $\mu g \cdot L^{-1}$ for Cd and Zn respectively. The LC_{50} for Cd and Zn with there 95% confidence limits are listed in the Table 2.2.

2.5. Discussion

As expected, survival of *H. azteca* decreased with increasing concentrations of Cd and Zn. The 96 h LC_{50} for Zn in this experiment, using food and cotton gauze was higher than previously observed by other authors. The 96 h LC_{50} value for Zn reported by Collyard et al. (1994) for the most sensitive stages of *H. azteca* was 200 $\mu g\ Zn \cdot L^{-1}$. The 96-h LC_{50} for Zn in this experiment was almost five times higher than the one observed by Collyard et al. (1994) where sediment and food were not present in the experiment. The higher readings in my experiment are probably due to adsorption of metals to the sediment and food particles, which lowered the concentration of bioavailable metals in the water. Also, fed animals have been shown to have higher survival compared to starved animals when exposed to toxicants (Abel and Bärlocher, 1988).

On the contrary, there was no major difference between the 96 h LC_{50} for Cd in this

Fig. 2.1 Geometric dilution series of Zn treatments ($\mu g \cdot L^{-1}$) and corresponding mortality (mean±standard error; n=3) of *Hyalella azteca* after four days of exposure.

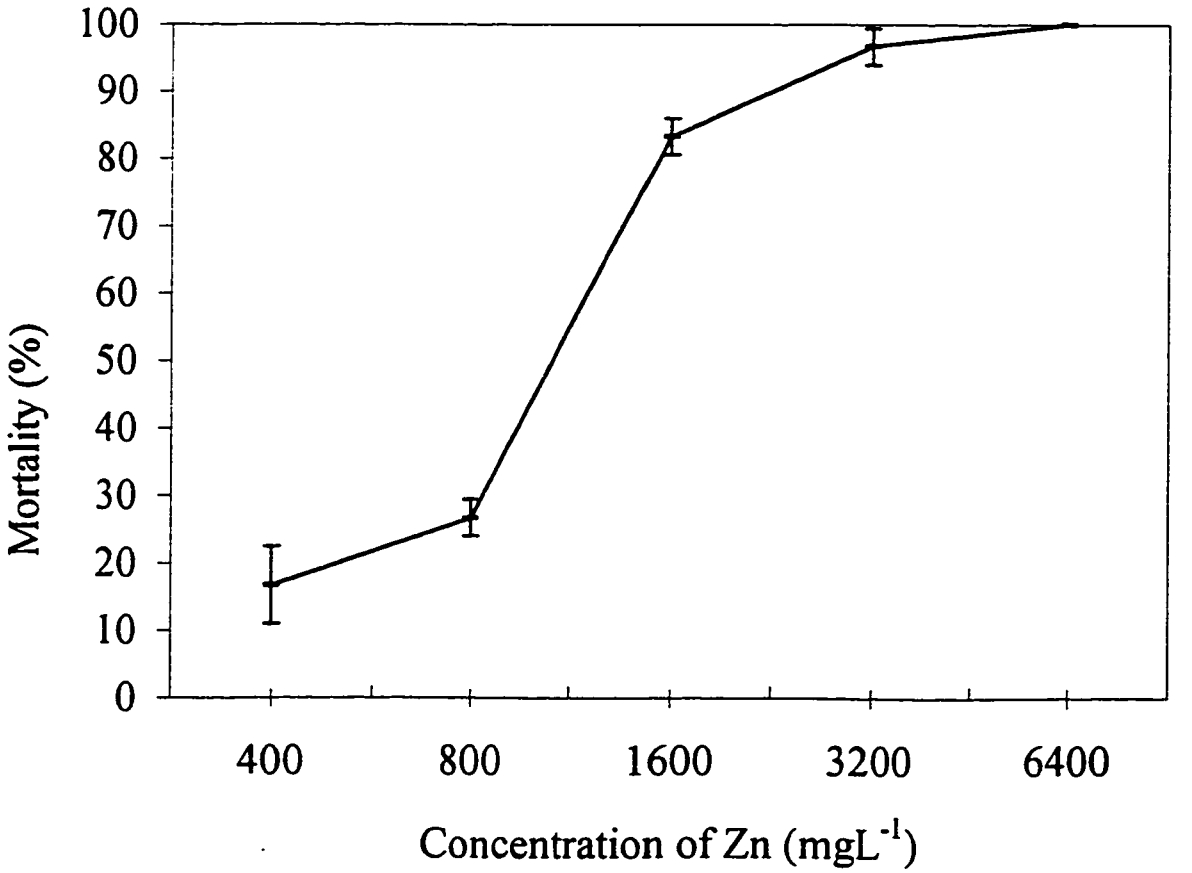

100
90
80
70
60
50
40
30
20
10
0
Mortality (%)
400
800
1600
3200
6400
Concentration of Zn (mgL-1)

Fig. 2.2 Geometric dilution series of Cd treatments ($\mu g \cdot L^{-1}$) and corresponding mortality (mean±standard error; n=3) of *Hyalella azteca* after four days of exposure.

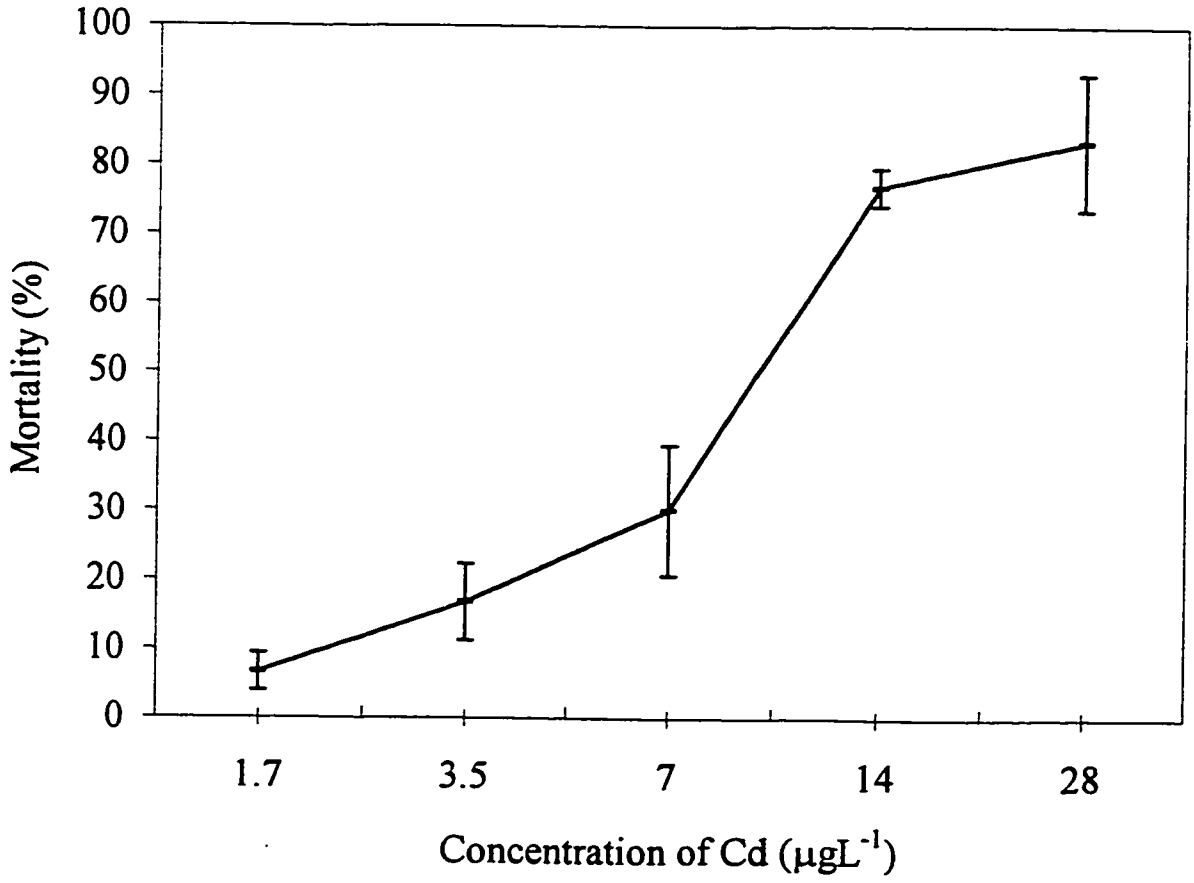

100
90
80
70
60
50
40
30
20
10
0
Mortality (%)
1.7
3.5
7
14
28
Concentration of Cd (μgL-1)

Table 2.2 96 h LC_{50} values ($\mu g \cdot L^{-1}$) for *Hyalella azteca* with 95% confidence limits.

Treatment	LC_{50} ($\mu g \cdot L^{-1}$)	95% confidence limits ($\mu g \cdot L^{-1}$)
Zn	943.2	633.56 and 1338.20
Cd	11.4	6.58 and 20.70

(11.4 $\mu gCdL^{-1}$) and previous experiments. The 96 h LC_{50} values for *H. azteca* reported by Collyard et al. (1994) was 7.0 $\mu gCd \cdot L^{-1}$, in close agreement with Nebeker et al. (1986) who reported a 96 h LC_{50} of 8.0 $\mu gCd \cdot L^{-1}$ for the most sensitive age class of *H. azteca*. The close results, despite using food and sediment in this experiment, could be due to the higher uptake of heavy metals through food and water simultaneously, not changing the response of animals substantially, compared to the conditions where food is not present. Stephenson and Turner (1993) reported that uptake of Cd in *H. azteca* mostly derived from food and 80% of ingested Cd was assimilated. Similar responses were found in water mites and caddisfly larvae where the uptake of Cd through food dominated, whereas for Zn, uptake through water dominated (Timmermans et al., 1992). Also a large increase of Cd concentration in the animals was observed after either dietary or aqueous exposure, whereas increases in Zn concentrations were limited and seemed to be regulated.

The LC_{50} values of the two heavy metals show a higher toxicity of Cd compared to Zn. The difference is most likely due to different regulatory abilities of *H. azteca* exposed to essential (Zn) and nonessential (Cd) heavy metals. It has been shown that the uptake of Zn is commonly implicated in homeostatic regulation in aquatic organisms, while the uptake of nonessential metals such as Cd is determined by exposure concentration (Luoma, 1983). Therefore, organisms using regulatory mechanisms can withstand higher levels of Zn, compared to Cd without lethal effect. Cadmium in this experiment was almost 100 times more toxic than Zn. Similar results have been reported for the leech *Nephelopsis obscura* were Cd was in some cases over 1000 times more toxic than Zn (Wicklum, 1995; Gray,

1995). The 96 h Cd LC_{50} of *Tanytarsus dissimilis* (Johannsen) was 10 times higher than Zn LC_{50} (Anderson et al., 1980).

The different water hardness of the solution, life stage of the animal, different sensitivity and pre-history of test organisms explain much of the variation in acute toxicity values between studies. Hardness is a major modifying factor of Cd and Zn toxicity in aquatic organisms. An increase in water hardness from 31 to 383 mg $CaCO_3 \cdot L^{-1}$ decreased Zn toxicity to rainbow trout *Salmo gairdneri* from LC_{50} being 170 μg $Zn \cdot L^{-1}$ in soft water to 5000 μg$Zn \cdot L^{-1}$ in hard water (Bradley and Sprague, 1985). The values of Zn LC_{50} for the leech *Nephelopsis obscura* in hard water was 20 times greater compared to soft water (Gray, 1995). These studies demonstrate that heavy metals become less toxic as water hardness increases probably due to the competition of hard water cations for binding sites and the reduction of biologically available heavy metals.

A significant positive effect of biomass and consequently life stage of an animal (Table 2.1), on resistance to Cd and Zn was found for many organisms. Wier and Walter (1976) found that immature snail *Physa gyrina* Say were three times more sensitive to Cd than mature snails. The same results were found for freshwater mysids (Birmelin et al., 1995). *Hyalella azteca* was most sensitive to Cd and Zn when immature (Collyard et al., 1994). The younger life stages of the kill fish *Rivulus marmoratus* Poey were nearly 40 times more susceptible to Cd than adult fish (Park et al., 1994). The leech *Nephelopsis obscura* showed a higher sensitivity to Cd at lower biomass (Wicklum, 1995). Generally, younger or smaller specimens are more susceptible to heavy metal toxicity due to the large surface area to body

weight ratio and also due to the greater moulting frequency in earlier life stages of animals, particularly crustaceans.

Considerable variation in sensitivity can occur among different taxonomic groups (Table 2.1). Williams et al. (1985) showed a wide range of species sensitivity among freshwater macroinvertebrates. He showed that members of the class Crustacea were extremely sensitive whilst insect species of the order Plecoptera and Trichoptera exhibited high short-term tolerance to Cd. The most sensitive species, the crustacean *Gammarus pulex* was approximately 2.6×10^4 times more sensitive to Cd than the least sensitive species, the caddis *Hydropsyche angustipennis* (Curtis). Similar observations were made by Brown and Pascoe (1988) on predatory macro invertebrates where the most tolerant species, the hemipteran *Aphelocheirus aestivalis* Fabricius had a 96 h LC_{50} approximately 10,000 times that of the most sensitive species, the leech *Glossiphonia complanata* (L.). Although some species exhibit high short-term tolerance, prolonged exposure to low concentrations can significantly affect survival. Relatively low concentrations of Cd (300 $\mu gCd \cdot L^{-1}$) affect the feeding apparatus of the caddis *Hydropsyche angustipennis* (Williams et al., 1985) and it is likely that prolonged exposure to relatively low concentrations could indirectly kill individuals by inhibiting feeding. Such observations underline the need for chronic toxicity tests over the complete life time.

Higher metal tolerance is a frequent occurrence for organisms from contaminated environments. If the parents of individuals were subjected to heavy metal exposure in their previous lifetime, this can significantly change their sensitivity to toxicants. Bradley et al.

(1985) compared LC_{50} for Zn of pre-exposed and not pre-exposed rainbow trout. The lethal concentration of Zn for pre-exposed animals was 2.5 times higher than that of control animals. There was significantly higher 48 h LC_{50} of Cu observed for crayfish *Cambarus robustus* Girard from a metal-contaminated lake compared to crayfish from an uncontaminated stream (Taylor et al., 1995). *Gammarus pulex* and *Asellus aquaticus* from Zn contaminated sites showed significantly higher 24 h LC_{50} for Zn than animals from clean sites (Naylor et al., 1990).

Although conducted in the laboratory these toxicity tests can be accepted as a conservative estimate of the potential effect of Cd and Zn in the field. The laboratory test gave a conservative estimate of a field effect because of the absence of factors such as sunlight, sediment and bacteria that would decrease or change the toxicity of Cd and Zn in natural conditions. The LC_{50} values of this test were mainly used to decide on the concentrations of the two heavy metals that were to be used in long term chronic testing. This was also the main reason why food and cotton gauze were present in the testing despite Parrish's (1985) assertation that test organisms should not be fed during acute toxicity testing.

CHAPTER 3 CHRONIC TOXICITY TESTING (BIOENERGETICS MODEL)

3.1. Introduction

Organisms in contaminated aquatic ecosystems are often exposed to toxicants for their entire lifetime or large portion of their life cycle. Therefore, chronic experiments give a better reflection of the field situation than do acute experiments (Kraak et al., 1994). There are a number of sublethal responses, including whole-organism and within-organism responses, that have been measured and can be more easily studied in long term chronic testing (Sprague, 1988; Postma et al., 1994). The effect of toxicants on growth, reproduction and respiration has been shown to be more sensitive and often ecologically more relevant than survival (Suter et al., 1987).

Contaminant accumulation, along with relative sensitivity to toxicants, suggests that amphipods may be one of the most sensitive groups of organisms to heavy metal pollution. (Arthur and Leonard, 1970; Sundelin, 1983; Borgmann et al., 1989). The amphipod *Hyalella azteca* is potentially one of the most useful freshwater species for life cycle studies in the laboratory, because they are widely distributed, breed and grow quickly and are easy to culture (Borgmann et al., 1989). Understanding the effects of heavy metals on *H. azteca* is important because elimination or reduction of this species would negatively affect the biodiversity of numerous freshwater ecosystems throughout North America since they are an important food source for fish, amphibians and other invertebrates (Pilgrim and Burt, 1993). Toxicants can have effects on the feeding rate, biomass conversion efficiency and energy requirements of aquatic organisms (Borgmann and Ralph, 1986). Reduced food consumption

due to direct biochemical effects or decreased feeding efficiency results in reduced energy intake (Borgmann and Ralph, 1986) and increases energetic costs to resist a stressor (Giesy et al., 1988). These effects can reduce the overall fitness of the organisms by reducing the scope for growth, the difference between absorbed energy and total metabolic cost (Giesy and Graney, 1989). When an organism is exposed to a toxicant, energy is often reallocated among different physiological processes. Since energy is usually limited there is often competition amongst different processes resulting in physiological trade offs which might change mortality, growth, development time and fecundity (variables that determine population dynamics and which are correlates of fitness) (Sibly and Calow, 1986, Calow and Sibly, 1990). Utilizing a bioenergetic model, which includes all the physiological processes among which the energy has to be partitioned, the allocation and acquisition of energy can be investigated.

Bioenergetic models have been used to provide useful insights into growth processes (Forsman and Lindell, 1991), predator-prey dynamics (Trudel and Boisclair, 1994; Smith and Davies, 1997) and to evaluate physiological responses to environmental stresses (Naylor et al., 1989; Maltby et al., 1990; Carter and Brafield, 1991; Wicklum, 1995; Westcott, 1997; Duguay, 1997). The bioenergetic model used in this study is similar to the one modified by Dratnal and Davies (1990), Reddy et al. (1992), Smith and Davies (1997) and Wicklum and Davies (1996) from Sibly and Calow (1986) energy budget for use with an invertebrate. The model includes the following components:

$$A = I - F - U = G_s + G_r + S + R_t \qquad (3.1)$$

A = energy assimilated

I = energy ingested

F = energy lost in faeces

U = energy lost in excretion (as ammonia wastes)

G_s = energy utilised in somatic growth

G_r = energy utilised in reproductive growth

S = energy stored (as total lipids)

$R_t = (R_a \times T_a) + (R_r \times T_r)$

R_t = total energy utilised in respiration

R_a = energy utilised while active

T_a = proportion of time spent active

R_r = energy utilised while resting (growth, repair, maintenance)

T_r = proportion of time spent resting

This bioenergetic model includes physiological processes among which resources can be partitioned and it has been shown to be very sensitive in measuring the effects of toxicants on aquatic invertebrates (Wicklum, 1995; Duguay, 1997; Westcott, 1997). While evaluating the energetic responses of the organism, stress can be detected earlier, and changes within and among components of a bioenergetic model are likely to be seen prior to the manifestation of overt stress effects such as mortality and fitness loss (Depledge et al., 1995).

This study was designed to evaluate the response of *H. azteca* exposed to Cd and Zn throughout the whole life cycle, measuring the energetic values of each physiological process of the bioenergetic model. All the components of the model were measured every 3 weeks

for 12 weeks.

3.2. Methods and material

Test animals

Hyalella azteca offspring brought into the laboratory from Stephenson's Pond did not reproduce sufficient offspring to run all the experiments. Because a high number of animals of the same age were required for the experiments, animals were obtained from aquarium cultures originally bred from one batch of animals collected at a small pond in Rochester, New York, USA. Before being used in the experiment the animals were acclimated to experimental conditions. At the beginning of the experiment all the animals were 1 week old.

Test solution

The water used to prepare test solutions came from the Glenmore Reservoir, Calgary. This was untreated Elbow River water of pH 8.3, alkalinity 148 $mg \cdot L^{-1}$ and hardness 205 $mg \cdot L^{-1}$, which had a constitution (pH, hardness) similar to Stephenson's pond water. Before use, the water was filtered through a capsule filter, sterile Gelman with 0.45 μm pore size, and aerated. To prepare the Zn solution zinc sulphate was used and cadmium chloride was used to prepare the Cd solution.

To determine the actual concentration of the toxicants to which *H. azteca* was exposed during chronic testing, a computer metal speciation MINTEQA2 V3.0 model was used (USEPA, 1991). This is a geochemical equilibrium speciation model capable of

computing equilibria among the dissolved, adsorbed, solid and gas phases of environmental systems. The free metal-ion concentration, which is a key variable for the reactivity, bioavailability and effects of metals (Sunda, 1994), is regulated by complex interactions among trace metals ions, ligands and major ions and particles (Bruland et al., 1991). To assess the fate and biological effects of trace metals in water systems, it is essential to determine metal speciation and especially to evaluate the free metal-ion concentrations. MINTEQA2 used the chemical composition of the water used in this experiment to predict the speciation of Cd and Zn added to the experimental system (Table 3.1). However, the model cannot predict the adsorption of the metals to the amphipod's tissues or their excretory products. To maintained more constant exposure concentrations of Cd and Zn, the experimental water was replaced weekly.

Based on the chemical composition of the experimental water MINTEQA2 predicted for both Cd treatments (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) that 91% of it is present in the free ion form (Cd^{2+}), with the remainder speciated as: 7% $CdSO_4$ (aq); 1% $CdOH^+$ and 1%$CdCl^+$ (Table 3.2). For both Zn treatments (9.4 and 94 $\mu gZn \cdot L^{-1}$) the model predicts that 58.6% is present in the free ion form (Zn^{2+}), with the reminder speciated as: 26.4% Zn $(OH)_2$ (aq); 1.3% $ZnOH^+$ and 3.6% $ZnSO_4$ (aq) (Table 3.3). The speciation of Cd and Zn is the same for both of their treatment concentrations because complexation depends only on stability constants and concentration of the ion ligands, provided they remain in excess, and not on Cd and Zn concentrations (Gardiner, 1974).

Table 3.1 The ion composition ($mg \cdot L^{-1}$) of the water used for maintaining *Hyalella azteca* and in the experimental treatments (Glenmore Reservoir, 1996 Water Examination Report).

Ions	Concentration ($mg \cdot L^{-1}$)
H^+	0.100
K^+	1.267
Ca^{2+}	5.450
Mg^{2+}	1.543
Na^+	6.267
SO_4^{2-}	5.633
Cl^-	5.667
F^-	0.227
NO_3^-	0.068
NH_4^+	0.013
Fe^{2+}	0.047
Al^{3+}	0.020
Cr^{2+}	0.001
Cu^{2+}	0.004
Pb^{2+}	0.001
Ni^{2+}	0.001

Table 3.2 Cadmium ion speciation in two Cd concentrations (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) predicted by the MINTEQA2 V3.0 computer model

Nominal Cadmium Concentration ($\mu g \cdot CdL^{-1}$)	**Predicted Speciation and Cadmium Ion Concentration ($\mu gCd \cdot L^{-1}$)**			
	Cd^{2+}	**$CdSO_4$ (aq)**	**$CdOH^+$**	**$CdCl^+$**
0.1	0.091	0.007	0.001	0.001
1.0	0.910	0.070	0.010	0.010

Table 3.3 Zinc ion speciation in two Zn concentrations (9.4 and 94 $\mu gZn \cdot L^{-1}$) predicted by the MINTEQA2 V3.0 computer model.

Nominal Zinc Concentration ($\mu gZn \cdot L^{-1}$)	**Predicted Speciation and Zinc Ion Concentration ($\mu gZn \cdot L^{-1}$)**			
	Zn^{2+}	**$Zn(OH)_2(aq)$**	**$ZnOH^+$**	**$ZnSO_4$ (aq)**
9.4	5.508	2.482	1.062	0.338
94.0	55.084	24.816	10.620	3.384

Food and sediment

Food and sediment were provided following the procedures in Chapter 2.

Experiment design

Organisms were exposed to two different concentrations of Cd and Zn: 10% and 1% of the 96 h LC_{50} of each metal. The concentrations for two Cd treatments were 0.1 and 1.0 $\mu gCd \cdot L^{-1}$ and 9.4 and 94 $\mu gZn \cdot L^{-1}$ for Zn treatments. Each treatment was replicated three times and each replicate consisted of 65 animals. Prior to adding the animals, aquaria were rinsed for 1 h with an experimental solution to minimise the amount of Cd and Zn plating from the treatment water to the sides of the container (Batley and Gardner, 1977). To alleviate the problems of heavy metals adsorbing to the body tissues of the animals, food, faeces and cotton gauze, water was replaced every week. Nine litre aquaria were filled with 4 L of experimental solution, covered loosely to minimize evaporation, and kept in a test chamber under conditions as described in Chapter 2. Every 3 weeks for 12 weeks animals were randomly selected from each aquaria for bioenergetic measurements. For most of the measurements, because of the small size of the amphipods, more than one animal had to be used.

3.2.1. Growth

Growth was measured on the same individuals throughout the whole experiment. The animals were placed into separate pots with mesh windows and kept in the aquaria containing the experimental solutions. Every 3 weeks, at least 38 h after feeding, each animal was

removed from its pot using a disposable plastic pipette. It was put onto a 363 μm nylon screen and the water removed by wiping the underside of the screen with a tissue. Capillary action drew the water away from the amphipod through the nylon screen without the animal touching the tissue. Then the animal was carefully moved onto a pre-weighed pan on a balance with a fine paintbrush and its wet biomass (WWT) was determined (±0.001mg). The WWT was converted to dry biomass (DWT) equivalents using the following equations:

$$\text{mg DWT} = \text{mg WWT} \times 0.2042 \quad (3.2)$$

$$\text{mg DWT} = \text{mg WWT} \times 0.2483 \quad (3.3)$$

when they were in the juvenile stage or mature, respectively. There was a significant difference observed in comparing conversion factor of juvenile and mature animals (ANOVA; df=1,10; F=9.26; $p<0.05$) to convert WWT into DWT. Dry biomass was converted to energetic equivalents using the equation:

$$\text{J energy (juvenile)} = \text{mg DWT} \times 14.053\ \text{J}\cdot\text{mg}^{-1} \quad (3.4)$$

$$\text{J energy (mature)} = \text{mg DWT} \times 12.828\ \text{J}\cdot\text{mg}^{-1} \quad (3.5)$$

The conversion factor is the energetic value of the animals, measured using a bomb calorimeter. There was no significant difference observed in comparing energetic values of the control and the treatment animals when immature (ANOVA; df=4,10; F=2.06; $p>0.05$) and when mature (ANOVA; df=4,10; F=2.59; $p>0.05$). However, there was a significant difference observed between immature and mature animals (ANOVA; df=1,28; F=4.93; $p<0.05$).

3.2.2. Ingestion

Following the method described by Naylor et al. (1989) 10 animals were randomly removed from each aquarium, at least 38 h after feeding, and placed in 900 mL plastic pots and provided with a known amount of food (B1). The initial DWT of leaf material was obtained for leaf discs that were dried at 60^0 C for 48 h. Before being fed to the animals, the discs were rehydrated in the experimental solution to increase palatability and to come into equilibrium with toxicants. The unconsumed food left after 4 days was removed, dried at 60^0 C to constant weight and reweighed (B2). To correct for weight lost due to leaching by leaves (D), three control pots were used in which there were leaves but no animals. The amount of energy ingested in $J \cdot mg^{-1} \cdot h^{-1}$ (I), over the 4 day experimental period was calculated by:

$$I = \frac{[((B1 * D) - B2) * E]}{W * 4 * 24} \qquad (3.6)$$

where E is the energy content of the food determined by bomb calorimetry (19.651 $J \cdot mg^{-1}$) and W is the DWT (mg) of the animal. The food correction factor was calculated as: D = [(A2/A1)], where A1 and A2 are the initial and final DWT of the control leaves.

3.2.3. Faeces production

The amount of energy lost in faeces was determined from the same experiment that measured the amount of food ingested. After 4 days of exposure, faecal material was ultrafiltrated onto pre-weighed (±0.001mg) filter paper (P1), 0.45 μm pore size, dried to constant weight in an oven at $60\,^{0}$C and reweighed (P2). There were also five control filters

to correct for any weight change in filters during the drying and filtering process (Cf), where the same volume of water (without faeces) was filtered through the filter paper. The amount of energy lost in faeces in $J \cdot mg^{-1} \cdot h^{-1}$ (F) was calculated as:

$$F = \frac{[(P2 - (P1 * Cf)) * Ef]}{W * 4 * 24} \quad (3.7)$$

where W is the DWT (mg) of the animals and Ef is the energy content of the faeces:

$$\text{Control} = 15.060\ J \cdot mg^{-1} \quad (3.8)$$

$$\text{Cd and Zn treatments} = 12.774\ J \cdot mg^{-1} \quad (3.9)$$

The measured energetic values of faeces, determined by micro bomb calorimetry, of exposed animals were significantly lower than the control's (ANOVA; df=4,15; F=5.55; $p<0.05$). The correction factor for weight changes of filters was calculated as: Cf = [(B2/B1)], where B1 is the initial weight of control filters and B2 is the final weight of control filters.

After measuring ingestion and faeces production Energy absorbed (A) was calculated from:

$$A = I - F \quad (3.10)$$

where I = energy ingested and F = energy lost in faeces.

3.2.4. Ammonia production

Immediately after feeding four animals (less than 5 weeks old) and two animals (more than 5 weeks old), from each replicate were put into 25 mL flasks containing 10 mL of fresh treatment solution. Every 8 h for 80 h, 2.5 mL samples of water were removed from each flask for analysis. The rest of the water was discarded and replaced with the fresh treatment

water. Beginning the measurement of ammonia immediately after feeding and continuing through the 80 h period allowed the measurement of both exogenous excretion (the ammonia produced as a result of absorption of food from the gut) and endogenous excretion (the ammonia produced as a result of assimilation of absorbed energy into new tissue growth). The amount of ammonia produced was determined using the phenolhypochloride method described by Solorzano (1969) with absorption measured at 640 nm with a spectrophotometer (Pharmacia LKB Novaspec II). Absorption was converted to mg $NH_3 \cdot L^{-1}$ using the equation:

$$\text{mg } NH_3 \cdot L^{-1} = -13.4729 + 904.324 \times A_{640} \qquad (3.11)$$

The regression equation was determined by measuring absorption of different concentrations of ammonia following standard methods for ammonia determination (APHA, 1971). To convert the ammonia production into energetic equivalents the following equation (Carter and Brafield, 1991) was used:

$$J \cdot NH_3 \cdot L^{-1} = \text{mg } NH_3 \cdot L^{-1} \times 24.85 \text{ J} \cdot \text{mg}^{-1} NH_3 \qquad (3.12)$$

3.2.5. Fecundity

One male and one female were placed together in a 250 mL plastic container with mesh windows and put into the aquarium containing the experimental solution. Six pairs of animals were monitored for each treatment. Every day, the containers were checked for offspring. When present, the offspring were counted (using a glass 2x magnifier) and measured (using a compound microscope, 60x magnification). Six females from each treatment were used to count and measure eggs. Because the newly born offspring could not

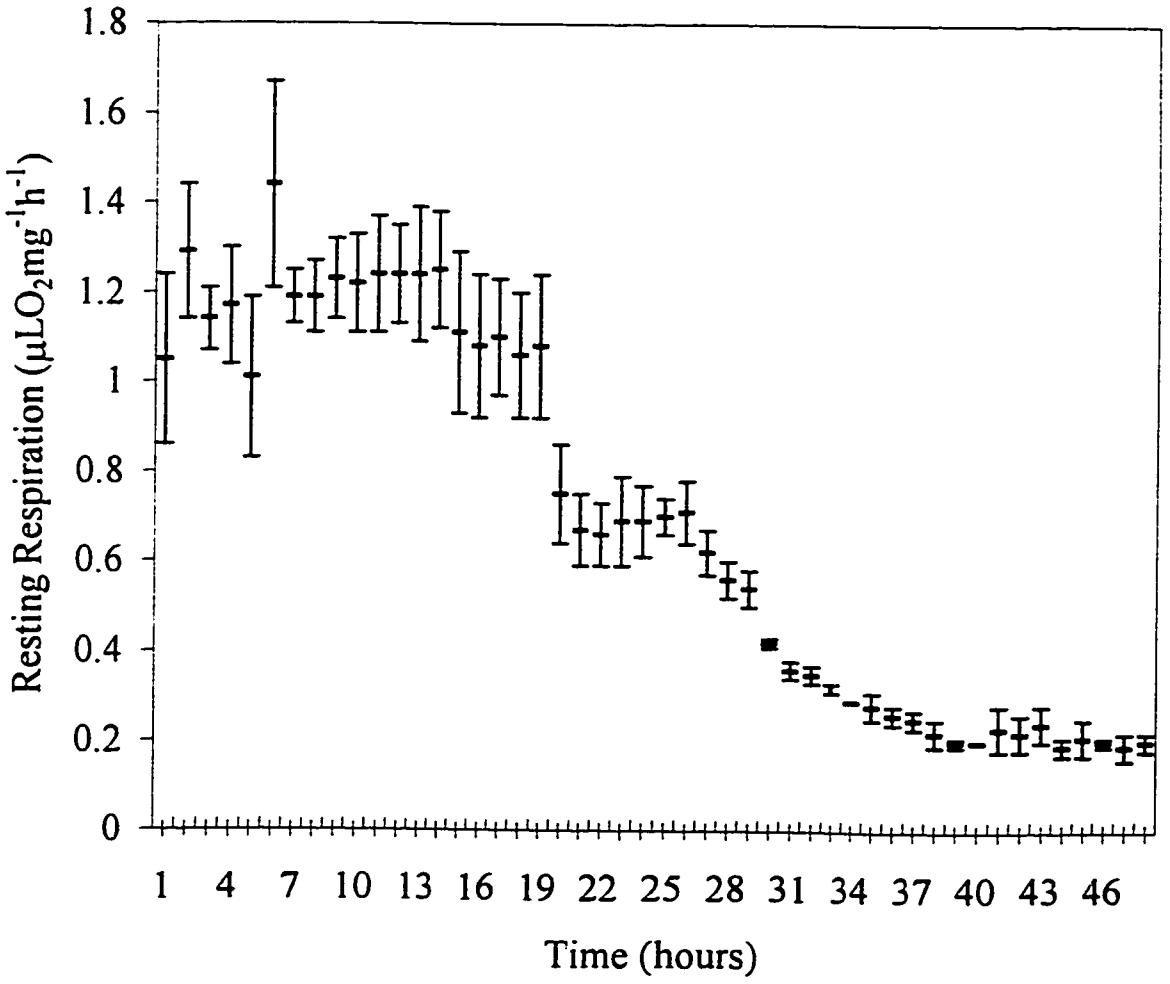
be weighed and too many offspring would have been needed to measure energy values, the weight of offspring was not converted into energy equivalents.

3.2.6. Respiration

Before respiration measurements were taken, the specific dynamic action (SDA) (energy costs of digestion, absorption and biochemical transformation of absorbed food) was determined. Immediately after feeding, single mature animals were put into individual chambers of a computerized flow through respirometer (Davies et el., 1992) for 48 h. Every hour the resting respiration measurements were taken. After feeding the metabolic rate increases and it is measured as an increase in oxygen consumption. When animals finish digesting, absorbing and biochemically transforming the absorbed food, oxygen consumption drops. SDA is calculated as the difference between the energy cost of resting respiration of fed and unfed animals (Kalarani and Davies, 1994; Barber et al., 1994). The SDA was deemed to terminate at the time when the amount of oxygen consumed reached the lowest values and the curve levelled off as illustrated in Fig. 3.1 between hours 38 and 48.

For respiration measurements, animals were removed from the aquarium and placed into plastic cups containing appropriate clean treatment water for at least 38 h in order for them to empty their guts (thereby excluding the energetic costs of SDA). Single animals, when mature, and paired when immature, were placed into the individual respirometer chambers. Respiration measurements began 1 h after introduction to the respirometer to allow the animals to acclimate to the new conditions (Davies et al., 1992). Oxygen consumption when resting (R_r) and active (R_a) was measured but the time spent resting and

Fig. 3.1 Size-specific resting respiration rate ($\mu LO_2 \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* measured every hour over 48 hours.


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Resting Respiration (μLO2mg-1h-1)
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46
Time (hours)

active was not detectable. The lowest 5% of the respiration readings were considered equivalent to the resting respiration and the highest 5% were taken as the active respiration. R_r is the respiratory cost of maintenance, repair, growth, gonad and faeces production and R_a is the respiratory cost of activity and locomotory movements (swimming and crawling). Oxygen consumption was converted to energetic equivalents using the following equation (Elliott and Davison, 1975):

$$J\,O_2 = \mu L\,O_2 \times 0.0202\ J\cdot(\mu L\,O_2)^{-1} \qquad (3.13)$$

Aerobic scope was calculated as the difference R_a - R_r. This specifies the energy available for other aerobic activities (Fry, 1947, 1971).

3.2.7. Total Lipids

Amount of stored energy was determined by measuring total lipids. Male animals were taken from each treatment replicate every 3 weeks and freeze-dried. To measure total lipids in juveniles 3 or 4 individuals were pooled into one sample to ensure sufficient biomass for analysis. Total lipids were quantified using a chloroform-methanol extraction micromethod designed for aquatic invertebrates (Gardner et al., 1985). Lipid content was calculated as a percentage of DWT.

3.3. Statistical analysis

Prior to statistical analysis, all the data were tested for normality and homoscedasticity. Normality was assessed using the Kolmogorov-Smirnov one sample test, while homoscedasticity was examined using plots of studentized residuals. Fecundity data

were analysed using a single-factor analysis of variance (ANOVA) with treatment as the main effect. All the other variables were analysed using a two-factor ANOVA with treatment and time as main effects. When the null hypothesis of equality of treatment means was rejected, the Tukey multiple pairwise comparison test was performed to determine which means differenced. Some data were found to be heteroscedastic, but since ANOVA is robust against departures from homoscedasticity if the design is balanced (Zar, 1984), no remedial action was taken before performing the ANOVA. Some data were not normal, however this was due to platy kurtosis, against which ANOVA is also robust. Significance for all tests was determined at a level of α=0.05. Most statistical analysis were performed using the SAS program (1996), however analysis of fecundity and survival were performed with Systat version 7.0 (Wilkinson, 1997).

3.4. Results

The data are presented graphically and diagrammed. In the Tukey's diagram means which are connected with a line do not differ significantly from each other.

3.4.1. Survivorship

Survivorship (Fig. 3.2) in two Cd treatments (0.1 and 1.0μg Cd$\cdot$L^{-1}), and two Zn treatments (9.4 and 94 μg Zn$\cdot$L^{-1}) showed a significant treatment*time interaction (ANOVA; df=12,40; F=10.7; p>0.05). The animals exposed to all concentrations of Cd and Zn suffered the highest mortality during the first three weeks of exposure. Mortality occurred in all the treatments and the control (Table 3.4).

Fig. 3.2 Survivorship (%; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

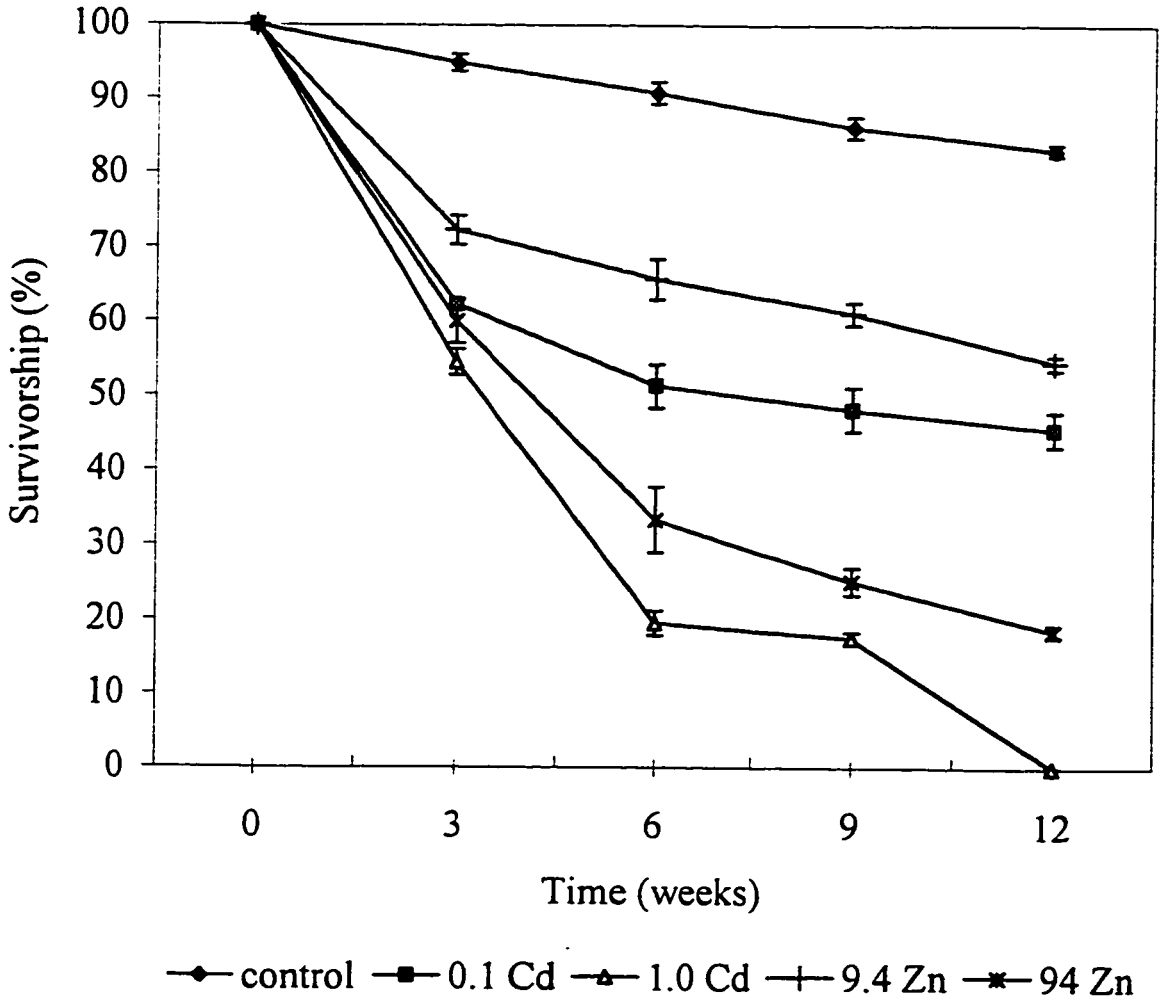

100
90
80
70
60
50
40
30
20
10
0
Survivorship (%)
0
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.4 Tukey's diagram of mean survivorship (%; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Survivorship (%)
Control	3	94.9
Control	6	90.8
Control	9	86.2
Control	12	83.1
9.4 Zn	3	72.3
9.4 Zn	6	65.6
0.1 Cd	3	62.3
9.4 Zn	9	61.0
94.0Zn	3	60.0
1.0Cd	3	54.5
9.4 Zn	12	54.4
0.1Cd	6	51.3
0.1 Cd	9	48.2
0.1 Cd	12	45.5
94.0Zn	6	33.3
94.0Zn	9	25.1
1.0Cd	6	19.5
94.0Zn	12	18.5
1.0 Cd	9	17.4
1.0 Cd	12	0.0

Survival in the control at the end of the 12 week experiment was 83.1 (±0.73)% which was not significantly different from week 3. There was no significant difference in survival in 9.4 $\mu gZn \cdot L^{-1}$ treatments across weeks 3 through 9. However, between weeks 9 and 12 survival dropped significantly. Survival in 0.1 $\mu gCd \cdot L^{-1}$ treatment dropped significantly between weeks 3 and 6 with no significant drop between weeks 6 and 9 and 9 and 12. In the 94 $\mu gZn \cdot L^{-1}$ treatment, survival changed significantly between weeks 3 and 6 and between weeks 6 and 9 with no significant change thereafter. Amphipods exposed to 1.0 $\mu gCd \cdot L^{-1}$ showed significant difference in survivorship from week 3 to 6, with no significant change at week 9, culminating in complete mortality at week 12.

There were significant differences in survivorship between the control and Cd and Zn treatments at all sampling times. Survivorship of animals exposed to 1.0$\mu gCd \cdot L^{-1}$ treatment differed significantly from the rest of the treatments at week 3. At weeks 6, 9 and 12 survivorship differed significantly among all the treatments, with the exception of 94 $\mu gZn \cdot L^{-1}$ and 1.0 $\mu gCd \cdot L^{-1}$ treatments at week 9.

3.4.2. Growth

Changes in body size, as measured by energy content of the amphipods, had a significant treatment*time interaction effect (ANOVA; df=11,38; $F=3.32$; $p<0.05$), which is indicated by the divergence of the lines in Figure 3.3. There was a significant increase in energy content of the control, 0.1 $\mu gCd \cdot L^{-1}$ and 9.4 $\mu gZn \cdot L^{-1}$ treatment animals between all sampling times (Table 3.5). The energy content of 94 $\mu gZn \cdot L^{-1}$ treatment animals increased

Fig. 3.3 Energy content (μJ; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

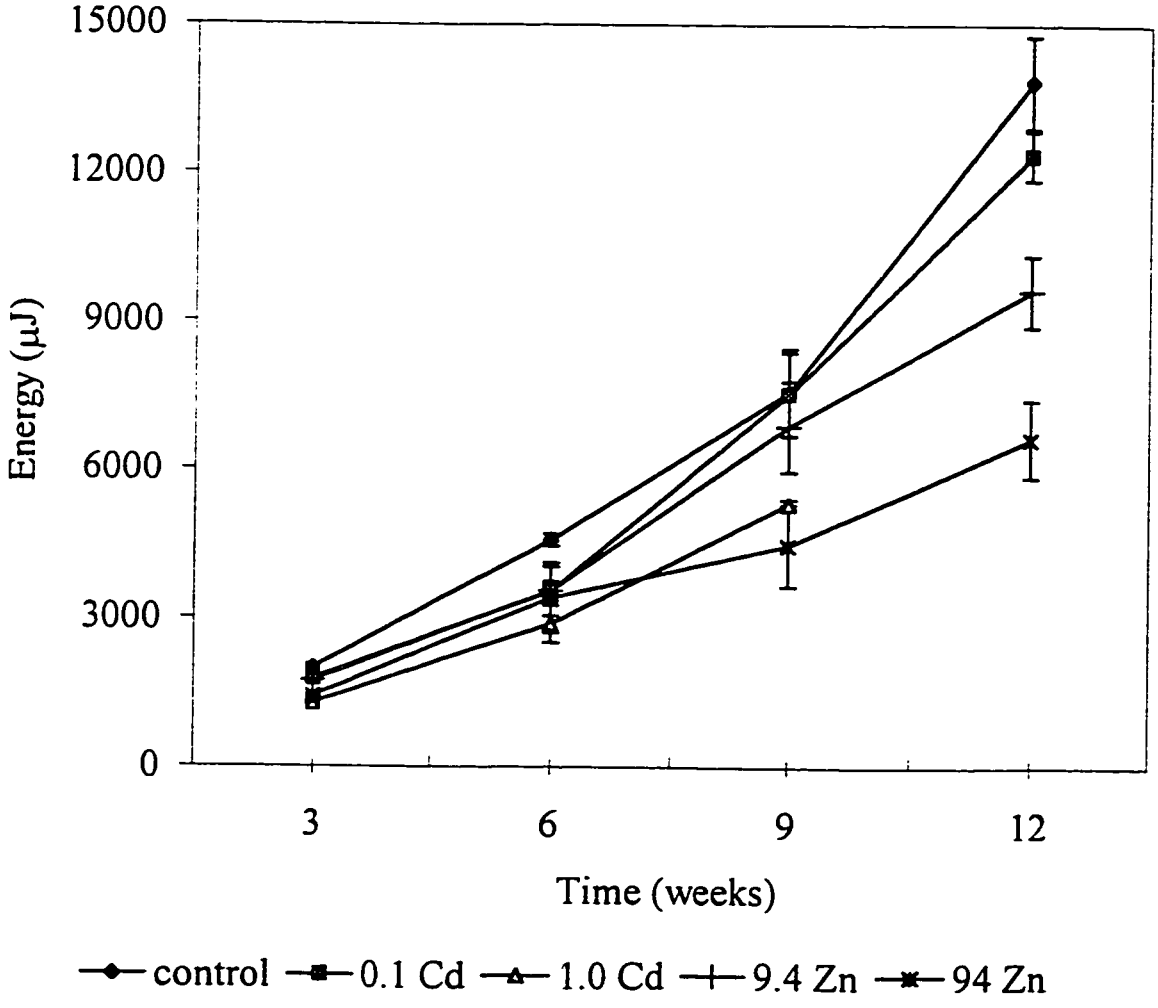

15000
12000
9000
6000
3000
0
Energy (μJ)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.5 Tukey's diagram of mean energy content (μJ; n=3) of *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Energy content (μJ)
Control	12	13834.4
0.1 Cd	12	12347.9
9.4 Zn	12	9608.7
Control	9	7570.1
0.1 Cd	9	7527.7
9.4 Zn	9	6869.4
94.0Zn	12	6614.6
1.0 Cd	9	5298.0
Control	6	4363.7
94.0Zn	9	4459.3
9.4 Zn	6	3546.2
0.1 Cd	6	3535.6
94.0Zn	6	3408.2
1.0 Cd	6	2877.3
Control	3	1989.6
0.1 Cd	3	1779.2
9.4 Zn	3	1731.3
94.0Zn	3	1425.2
1.0 Cd	3	1281.8

from a mean of 1425.2 (±28.2) μJ at week 3 to a mean of 6614.6 (±777.7) μJ at week 12 with significant increase between weeks 3 and 6 and no significant change thereafter. In the 1.0 μgCd·L^{-1} treatment the energy content of exposed animals increased from a mean of 1281.8 (±43.5) μJ at week 3 to a mean of 5298.0 (±88.0) μJ at week 9. No significant increase occurred between weeks 3 and 6, or between weeks 6 and 9.

There were no significant differences between the control and 0.1 μgCd·L^{-1} amphipod groups at any sampling times. Animals exposed to the 1.0 μgCd·L^{-1} treatment had significantly lower energy content than the control animals at week 6 and 9 with no survivors at week 12. The only significant difference between 9.4 μgZn·L^{-1} animals and the control was at week 12. The energy content of animals in 94 μgZn·L^{-1} treatment was significantly lower than the control at weeks 9 and 12.

At week 3 there was no significant difference observed among the treatments. At week 6 animals in 1.0 μgCd·L^{-1} treatment had significantly lower energy content than the rest of the treatments. Energy contents of animals in 0.1 μgCd·L^{-1} and 9.4 μgZn·L^{-1} treatments were significantly higher than the 1 μgCd·L^{-1} and 94 μgZn·L^{-1} treatments at week 9. For the last sampling time, animals in 0.1 μgCd·L^{-1} treatment had the highest energy content which was significantly higher than the animals exposed to 9.4 and 94 μgZn·L^{-1}.

3.4.3. Ingestion

There was a significant treatment*time interaction effect (ANOVA; df=11,38; $F=10.54$; $p<0.05$) on the size-specific ingestion rates of amphipods exposed to Cd and Zn

treatments. The interaction is visible as the non-parallel lines in Figure 3.4. Ingestion in all the treatments was the highest in the juvenile stage (week3) and dropped significantly by week 6, with the exception of 1.0μgCd·L^{-1} (Table 3.6).

Ingestion in the control dropped significantly from 176.427 (±4.730) μJ·mg^{-1}·h^{-1} at week 3 to 74.025 (±7.435) μJ·mg^{-1}·h^{-1} at week 9 with no significant change at week12. There was a significant difference between weeks 3 and 6 and also 6 and 9. Ingestion of animals treated with 9.4μgZn·L^{-1} had a highest mean of 166.905 (±2.365) μJ·mg^{-1}·h^{-1} at week 3 and the lowest at week 9 (49.232±7.851 μJ·mg^{-1}·h^{-1}). There was a significant difference in ingestion among all sampling times, with the exception of weeks 6 and 12. Significantly lower ingestion in the 9.4 μgZn·L^{-1} treatment compared to control was observed at weeks 6 and 9. Animals in the 0.1 μgCd·L^{-1} treatment ingested significantly less food at week 6 (55.743±6.433 μJ·mg^{-1}·h^{-1}) compared to week 3 (121.194±5.746 μJ·mg^{-1}·h^{-1}) with no significant change thereafter. Compared to the control, there was a significant difference in ingestion observed at weeks 3 and 6. Ingestion in the 1.0 μgCd·L^{-1} treatment was the highest at week 3 (78.434±9.720 μJ·mg^{-1}·h^{-1}) with a significant drop by week 9 (42.020±1.883 μJ·mg^{-1}·h^{-1}). Control animals had significantly higher ingestion rates than 1 μgCd·L^{-1} treatment animals at all sampling times. Amphipods exposed to 94 μgZn·L^{-1} had the highest ingestion at week 3 (66.001±3.023 μJ·mg^{-1}·h^{-1}) with a significant decrease by week 6 (47.280±7.963μJ·mg^{-1}·h^{-1}) with no significant change thereafter. Animals in this treatment had significantly lower ingestion compared to the control at all sampling times.

Among treatments, the ingestion by amphipods at week 3 was least impaired in the

Fig. 3.4 Size-specific ingestion ($\mu J \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

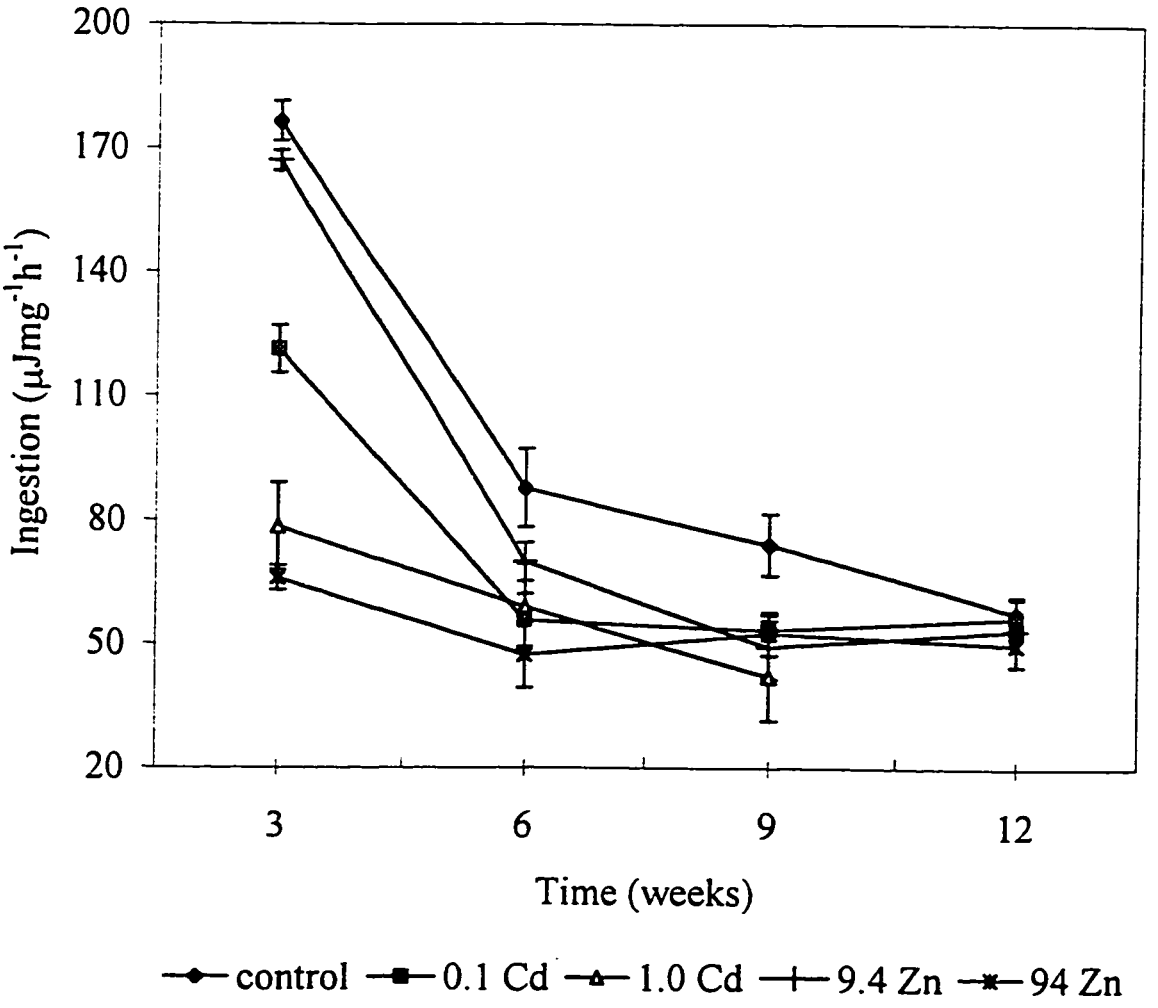

200
170
140
110
80
50
20
Ingestion (μJmg-1h-1)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.6 Tukey's diagram of mean ingestion rate ($\mu J \cdot mg^{-1}h^{-1}$; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Food ingested ($\mu J \cdot mg^{-1}h^{-1}$)
Control	3	176.427
9.4 Zn	3	166.905
0.1 Cd	3	121.194
Control	6	87.650
1.0 Cd	3	78.434
Control	9	74.025
9.4 Zn	6	69.866
94.0Zn	3	66.001
1.0 Cd	6	58.940
Control	12	57.500
0.1 Cd	12	56.451
0.1 Cd	6	55.743
9.4 Zn	12	53.464
0.1 Cd	9	53.313
94.0Zn	9	52.501
94.0Zn	12	49.927
9.4 Zn	9	49.232
94.0Zn	6	47.280
1.0 Cd	9	42.020

9.4μgZn·L^{-1} treatment which was significantly different from the 0.1 μgCd·L^{-1} treatment and both were significantly different from the 1.0 μgCd·L^{-1} and 94 μgZn·L^{-1} treatments. At week 6, ingestion was lowest in animals exposed to 94 μgZn·L^{-1} and it was significantly different from all the other treatments. At week 9, ingestion was lowest in the 9.4 μgZn·L^{-1} and 1.0 μgCd·L^{-1} treatments and significantly different from 94 μgZn·L^{-1} and 0.1 μgCd·L^{-1} treatments. In the final week, ingestion was significantly lower in the 94 μgZn·L^{-1} treatment compared to the 9.4 μgZn·L^{-1} and 0.1 μgCd·L^{-1} treatments.

3.4.4 Faeces production

The size-specific faeces production (Fig. 3.5) had a significant treatment*time interaction effect (ANOVA; df=11,38; F=148.23; $p<0.05$). In all the treatments, faeces production was the highest at week 3 and decreased significantly in the following weeks. The lowest faeces production was observed at week 9 (Table 3.7).

At week 3 the control animals produced significantly more faeces (1.883±0.129 μJ·mg^{-1}·h^{-1}) than thereafter. At week 9 faeces production reached the lowest point with a mean of 0.122(±0.031)μJ·mg^{-1}·h^{-1}. Animals in the 9.4 μgZn·L^{-1} treatment at week 3 produced 5.663(±0.055)μJ·mg^{-1}·h^{-1} of faeces. Then faeces production dropped significantly being the lowest at week 9 (0.173±0.028 μJ·mg^{-1}·h^{-1}). Faeces production in 0.1 μgCd·L^{-1} treatment was the highest at week 3 (7.839±0.174 μJ·mg^{-1}·h^{-1}) and the lowest at week 9 (0.146±0.013 μJ·mg^{-1}·h^{-1}). The highest faeces production at week 3 was also observed in the 94 μgZn·L^{-1} (7.911±0.146 μJ·mg^{-1}·h^{-1}) and 1.0 μgCd·L^{-1} (9.598±0.150 μJ·mg^{-1}·h^{-1}) treatments and the

Fig. 3.5 Size-specific faeces production ($\mu J \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

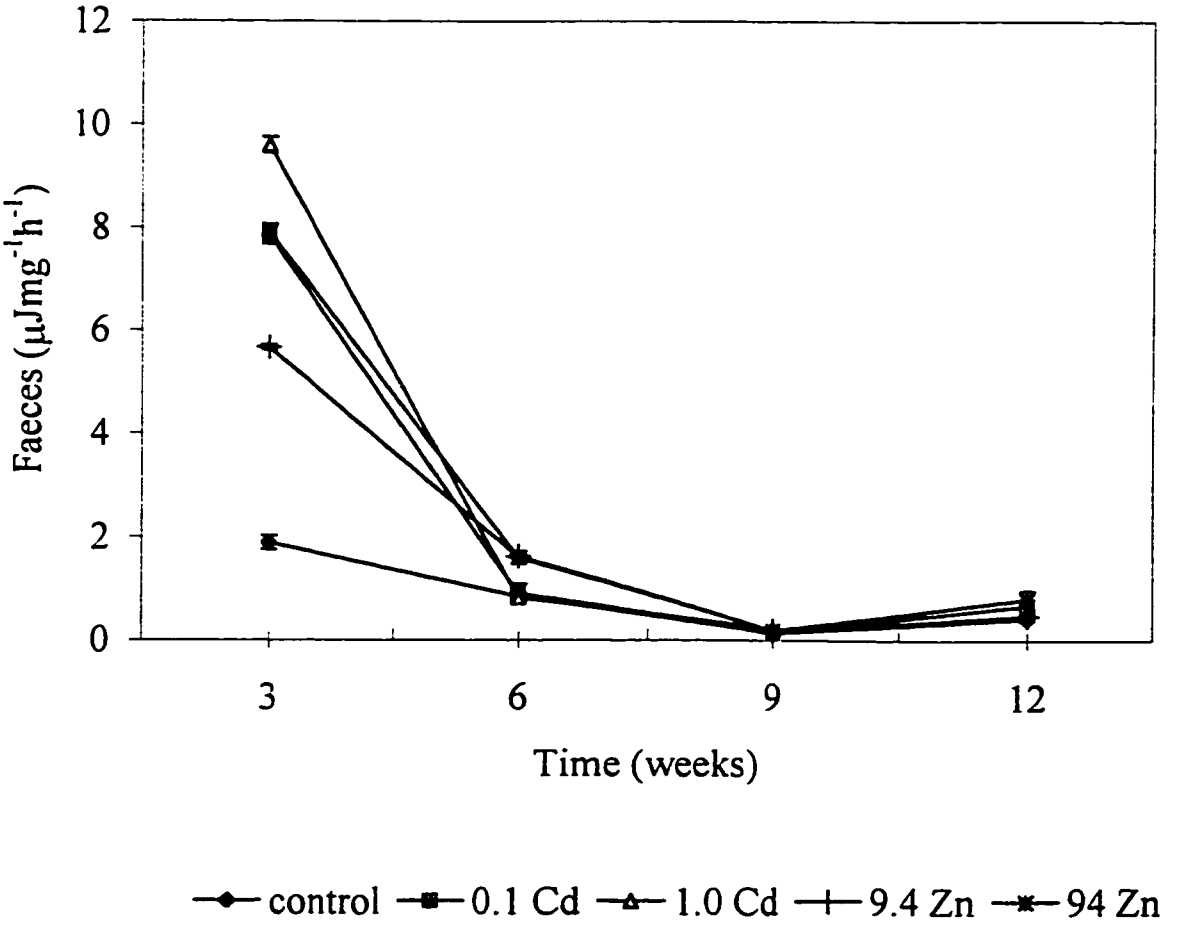

12
10
8
6
4
2
0
Faeces (µJmg-1h-1)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.7 Tukey's diagram of mean faeces production ($\mu J \cdot mg^{-1}h^{-1}$; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Faeces production ($\mu J \cdot mg^{-1}h^{-1}$)
1.0 Cd	3	9.598
94.0Zn	3	7.911
0.1 Cd	3	7.839
9.4 Zn	3	5.663
Control	3	1.883
9.4 Zn	6	1.622
94.0Zn	6	1.601
0.1 Cd	6	0.913
Control	6	0.837
1.0 Cd	6	0.815
94.0Zn	12	0.789
0.1 Cd	12	0.669
9.4 Zn	12	0.457
Control	12	0.415
1.0 Cd	9	0.212
9.4 Zn	9	0.173
94.0Zn	9	0.170
0.1 Cd	9	0.146
Control	9	0.122

lowest at week 9 (0.170±0.023 $\mu J \cdot mg^{-1} \cdot h^{-1}$ and 0.212±0.015 $\mu J \cdot mg^{-1} \cdot h^{-1}$), respectively.

Animals exposed to the metal treatments produced significantly more faeces at week 3 than the control. At week 6 only animals in the 9.4 and 94 $\mu gZn \cdot L^{-1}$ treatments produced significantly more faeces than the control. No significant difference between the treatments and the control was observed at weeks 9 and 12.

Among all the treatments at week 3, animals in 1.0 $\mu gCd \cdot L^{-1}$ produced significantly more faeces than animals in the rest of the treatments. At week 6 significantly higher faeces production was detected in the 9.4 and 94 $\mu gZn \cdot L^{-1}$ treatments than in the 0.1 and 1.0 $\mu gCd \cdot L^{-1}$ treatments. Faeces production at weeks 9 and 12 did not differ among all the treatments.

3.4.5. Absorption Efficiency

The size-specific absorption efficiency (Fig. 3.6) had a significant treatment*time interaction effect (ANOVA; df=11,38; F=18.24; $p<0.05$). In all the treatments, absorption efficiency was the lowest in the juvenile stage and increased when animals reached maturity (Table 3.8).

Absorption efficiency of control animals did not change significantly over the course of the experiment with 98.9 (±0.1)% efficiency at week 3 being the low and 99.8 (±0.0)% at week 9 being the high. Animals exposed to 9.4 $\mu gZn \cdot L^{-1}$ showed their lowest absorption efficiency at week 3 (96.6±0.0%) and increased significantly by week 9 (99.6±0.0%) with no significant change between weeks 3 and 6, nor between 9 and 12. Absorption efficiency of the 94 $\mu gZn \cdot L^{-1}$ treatment animals increased significantly from week 3 (88.0±0.4%) to week

Fig. 3.6 Absorption efficiency (%; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

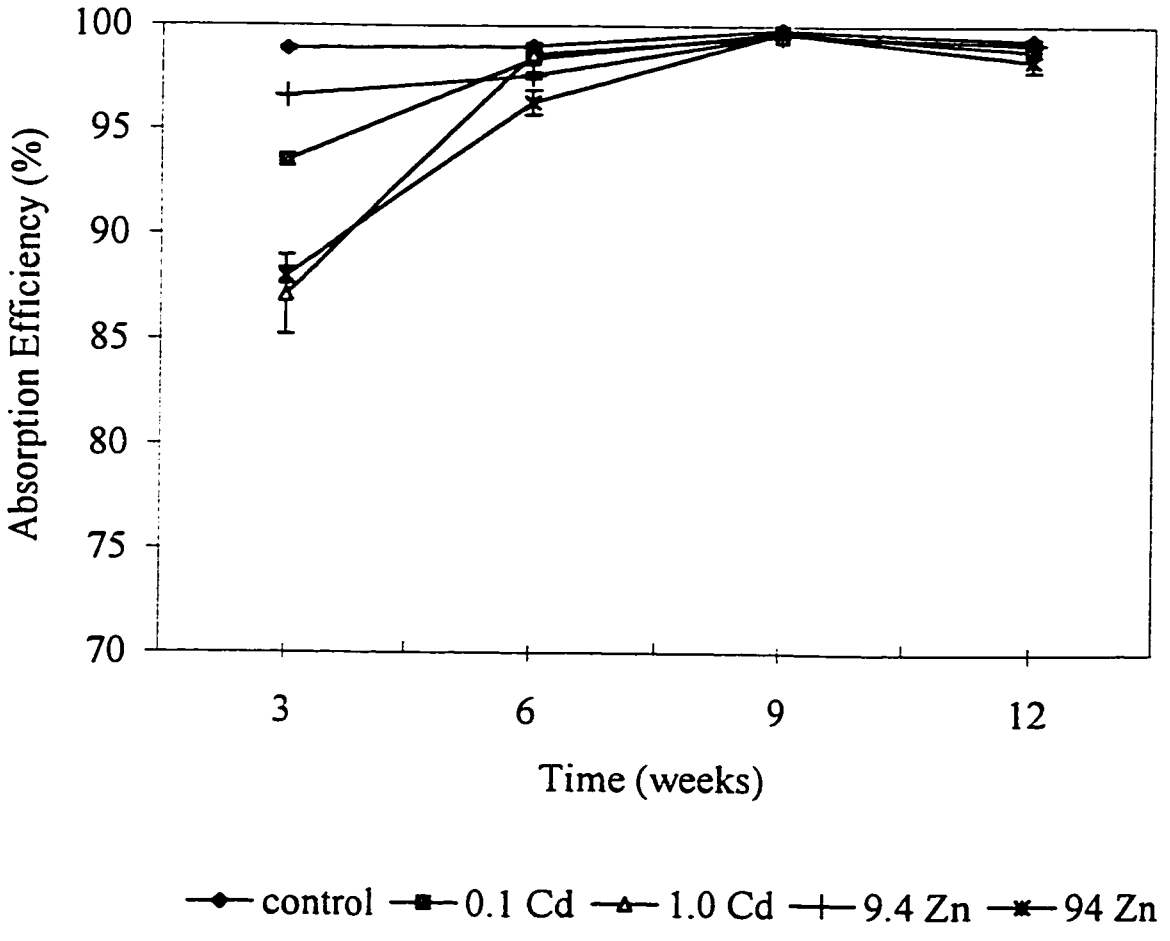

100
95
90
85
80
75
70
Absorption Efficiency (%)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.8 Tukey's diagram of mean absorption efficiency (%; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Absorption efficiency (%)
Control	9	99.8
0.1 Cd	9	99.7
94.0Zn	9	99.7
9.4 Zn	9	99.6
1.0 Cd	9	99.5
Control	12	99.3
9.4 Zn	12	99.1
Control	6	99.0
Control	3	98.9
0.1 Cd	12	98.8
1.0 Cd	6	98.6
0.1 Cd	6	98.4
94.0Zn	12	98.3
9.4 Zn	6	97.7
9.4 Zn	3	96.6
94.0Zn	6	96.3
0.1 Cd	3	93.5
94.0Zn	3	88.0
1.0 Cd	3	87.1

6 (96.3±0.6%) and again to week 9 (99.7±0.1%), with no significant change thereafter. Animals exposed to 0.1 and 1.0 $\mu gCd \cdot L^{-1}$ followed the same trend with absorption efficiency being the lowest at week 3 (93.5±0.3% and 87.1±1.9%), increasing significantly by week 6 (98.4±0.1% and 98.6±0.1%) and no significant change thereafter.

There was a significant difference between all the treatments and the control at week 3. The only significantly different absorption efficiency, compared to the control, at week 6 was detected for the 94 $\mu gZn \cdot L^{-1}$ treatment animals. At weeks 9 and 12 there was no significant difference observed between the control and the treatments.

Among all the treatments at week 3, 94 $\mu gZn \cdot L^{-1}$ and 1.0 $\mu gCd \cdot L^{-1}$ treatment animals had significantly lower absorption efficiency than animals exposed to 0.1 $\mu gCd \cdot L^{-1}$ which was also significantly lower from absorption efficiency of animals exposed to 9.4 $\mu gZn \cdot L^{-1}$. The lowest absorption efficiency at week 6 was observed for 94 $\mu gZn \cdot L^{-1}$ treatment animals, which was significantly lower from the rest of the treatments. In weeks 9 and 12 there was no significant difference observed among the treatments.

3.4.6. Ammonia Production

The size-specific total ammonia production of the animals exposed to Cd and Zn treatments over time (Fig. 3.7) had significant treatment*time interaction effect (ANOVA; df=11,38; F=2.74; $p<0.05$) (Table 3.9). Control animals produced 0.410 (±0.010) $\mu J \cdot mg^{-1} \cdot h^{-1}$ at week 3 with no significant change over the course of 12 weeks. Animals exposed to 0.1 $\mu gCd \cdot L^{-1}$ had the highest ammonia production at week 3 (0.599±0.033 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and showed a significant drop to week 6 (0.338±0.001$\mu J \cdot mg^{-1} \cdot h^{-1}$) and no significant change

Fig. 3.7 Size-specific total ammonia production ($\mu J \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

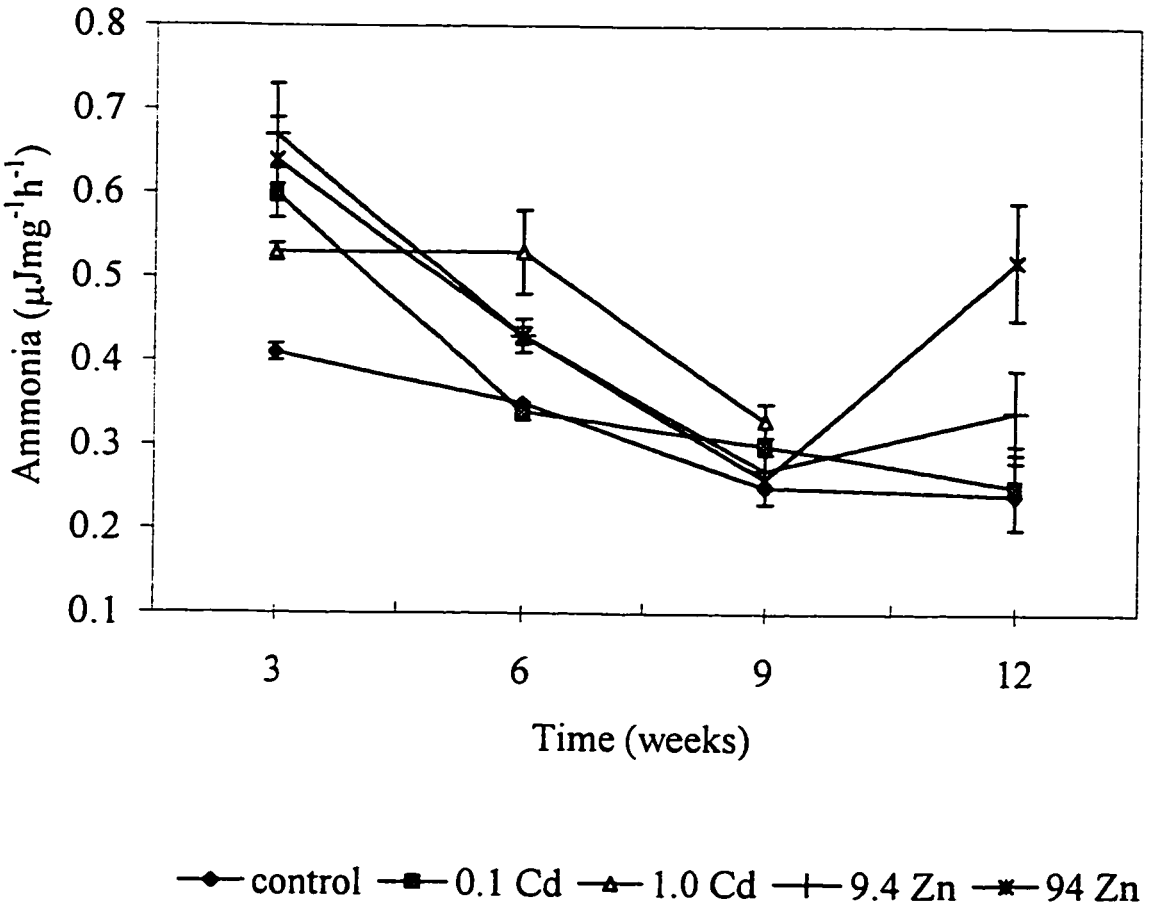

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Ammonia (μJmg-1h-1)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.9 Tukey's diagram of mean ammonia production ($\mu J \cdot mg^{-1}h^{-1}$; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Total ammonia ($\mu J \cdot mg^{-1}h^{-1}$)
9.4 Zn	3	0.665
94.0Zn	3	0.642
0.1 Cd	3	0.599
1.0 Cd	6	0.528
1.0 Cd	3	0.525
94.0Zn	12	0.520
94.0Zn	6	0.432
9.4 Zn	6	0.432
Control	3	0.410
Control	6	0.347
9.4 Zn	12	0.342
0.1 Cd	6	0.338
1.0 Cd	9	0.333
0.1 Cd	9	0.303
9.4 Zn	9	0.265
94.0Zn	9	0.258
Control	9	0.247
0.1 Cd	12	0.245
Control	12	0.239

thereafter. Ammonia production of animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ was the highest at week 3 (0.525±0.001 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and 6 (0.528 ±0.052 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and dropped significantly to a mean of 0.333 (±0.025) $\mu J \cdot mg^{-1} \cdot h^{-1}$ at week 9. Animals in the 9.4 $\mu gZn \cdot L^{-1}$ treatment had the highest ammonia production at week 3 (0.665 ± 0.058 $\mu J \cdot mg^{-1} \cdot h^{-1}$) with no significant change at week 6. At week 9 ammonia production dropped significantly to a mean of 0.265 (±0.040) $\mu J \cdot mg^{-1} \cdot h^{-1}$, with no significant change thereafter. The animals exposed to 94 $\mu gZn \cdot L^{-1}$ showed no significant differences in ammonia production among weeks 3,6 and 12. The lowest ammonia production was observed at week 9 (0.258 ± 0.032 $\mu J \cdot mg^{-1} \cdot h^{-1}$) which was significantly lower than weeks 3,6 and 12.

Compared to the control, there was significantly higher ammonia production observed in all the treatments at week 3. At week 6 ammonia production was significantly higher in the 9.4 and 94 $\mu gZn \cdot L^{-1}$ and 1.0 $\mu gCd \cdot L^{-1}$ treatments compared to the control. There was no significant difference observed between the treatments and the control at week 9. At week 12 only the animals exposed to 94 $\mu gZn \cdot L^{-1}$ had significantly higher ammonia production then the control animals.

Among the treatments there was no significant difference observed at weeks 3 and 9. At week 6 animals exposed to 0.1 $\mu gCd \cdot L^{-1}$ had significantly lower ammonia production than the rest of the treatments. At week 12 the only significantly higher ammonia production, compared to the other treatments, was observed for the animals exposed to 94 $\mu gZn \cdot L^{-1}$.

3.4.7. Fecundity

There was a significant treatment effect (ANOVA; df=4,25; F=13.22; $p<0.05$) on egg production (Fig. 3.8). There was no significant difference observed among the Cd and Zn treatments, however they were all significantly lower then the control. However, there was no difference observed in the size of eggs.

There was also a significant treatment effect (ANOVA; df=4,25; F=7.22; $p<0.05$) on the number of offspring (hatchlings) produced (Fig. 3.9). Again no significant difference was observed among the Cd and Zn treatments, however they were all less than the control. Also, no difference in size of offspring was observed between the treatments and the control.

3.4.8. Respiration

3.4.8.1. Resting Respiration

The size-specific resting respiration of the *H. azteca* exposed to Cd and Zn treatments over time (Fig. 3.10) showed a significant treatment*time interaction effect (ANOVA; df=11,38; F=10.70; $p<0.05$) (Table 3.10). The resting respiration of control animals did not change significantly over time. Animals in the 0.1μgCd$\cdot$L^{-1} treatment had significantly higher resting respiration at weeks 3 (0.733±0.121 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and 12 (0.754±0.068 $\mu J \cdot mg^{-1} \cdot h^{-1}$) compared to weeks 6 and 9 which did not differ. Animals exposed to 9.4 μgZn$\cdot$L^{-1} had the highest resting respiration at week 3 with a mean of 1.132 (±0.079) $J \cdot mg^{-1} \cdot h^{-1}$ which dropped significantly at week 6 to a mean of 0.435 (±0.052) $\mu J \cdot mg^{-1} \cdot h^{-1}$ with no significant change thereafter. A similar response was observed for the animals exposed to 1.0 μgCd$\cdot$L^{-1} with the highest resting respiration at the first sampling time (1.610±0.015 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and a

Fig. 3.8 Eggs produced (number; mean±standard error; n=6) by *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations.

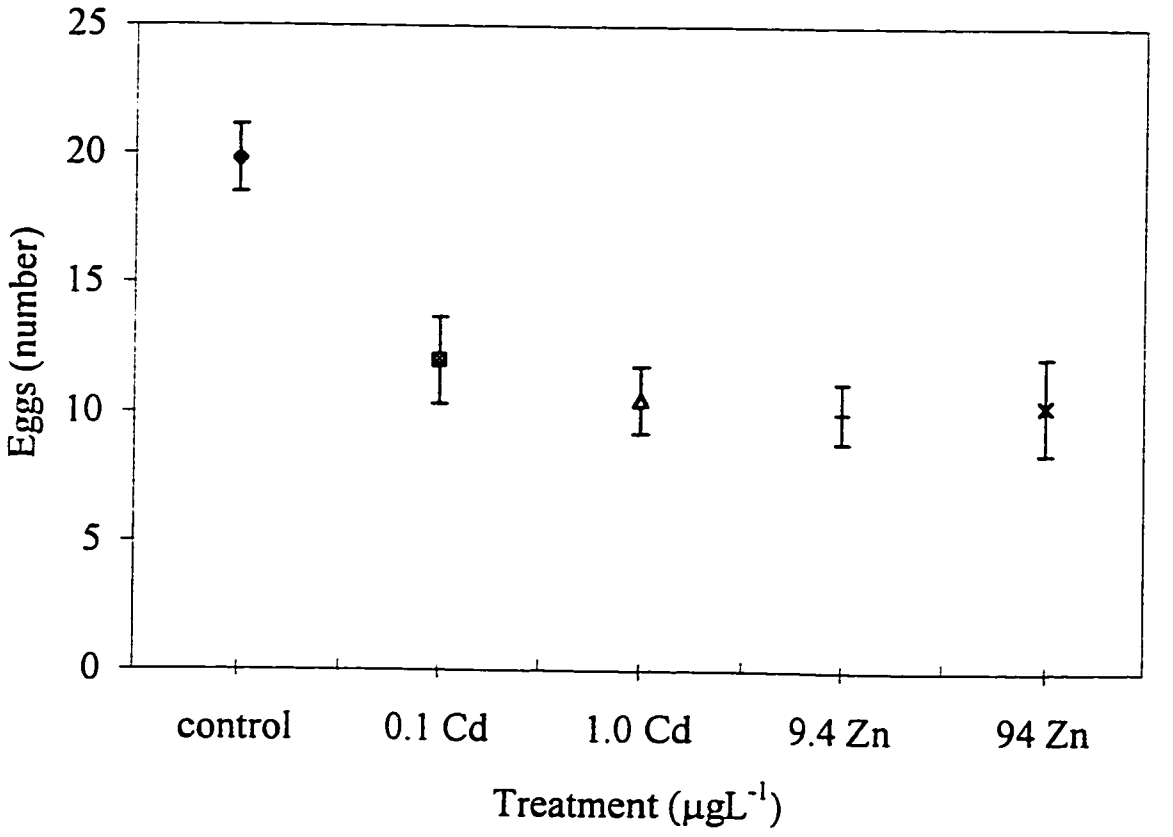

25
20
15
10
5
0
Eggs (number)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (µgL-1)

Fig. 3.9 Offspring born (number; mean±standard error; n=6) to *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations.

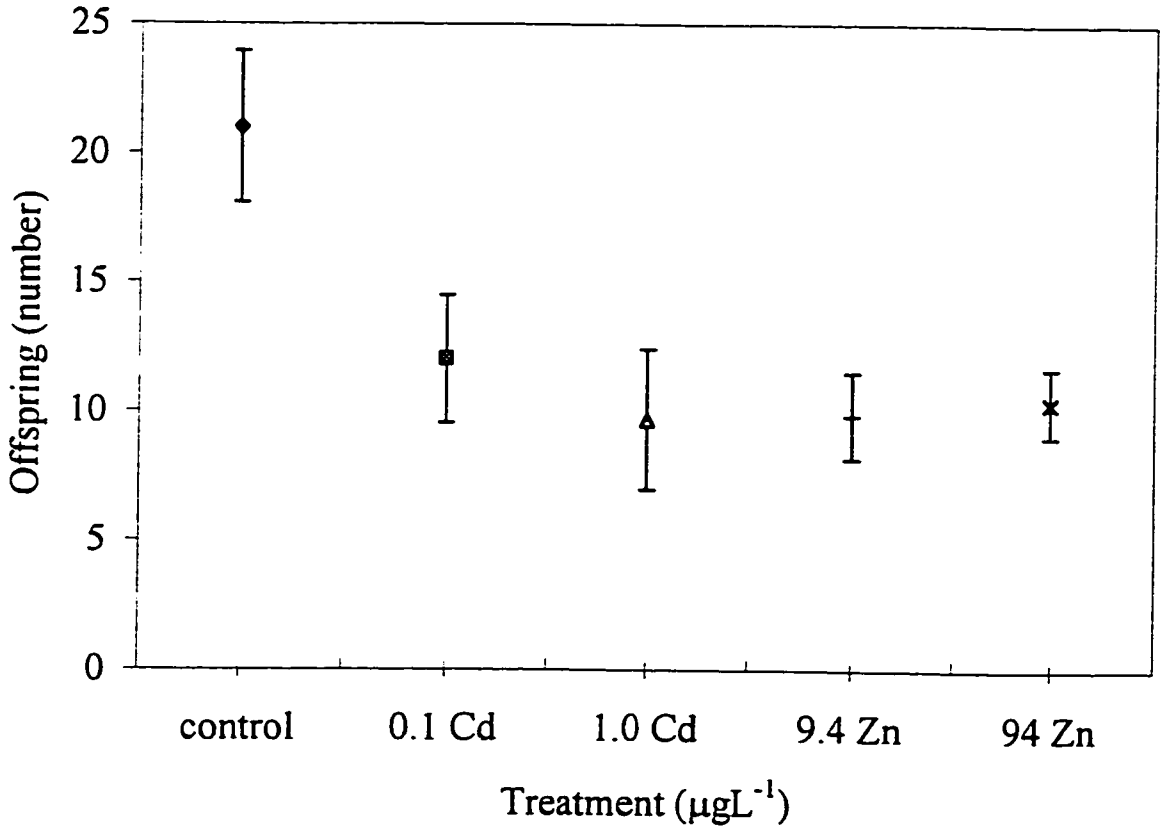

25
20
15
10
5
0
Offspring (number)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

Fig. 3.10 Size-specific resting respiration rate ($\mu J \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

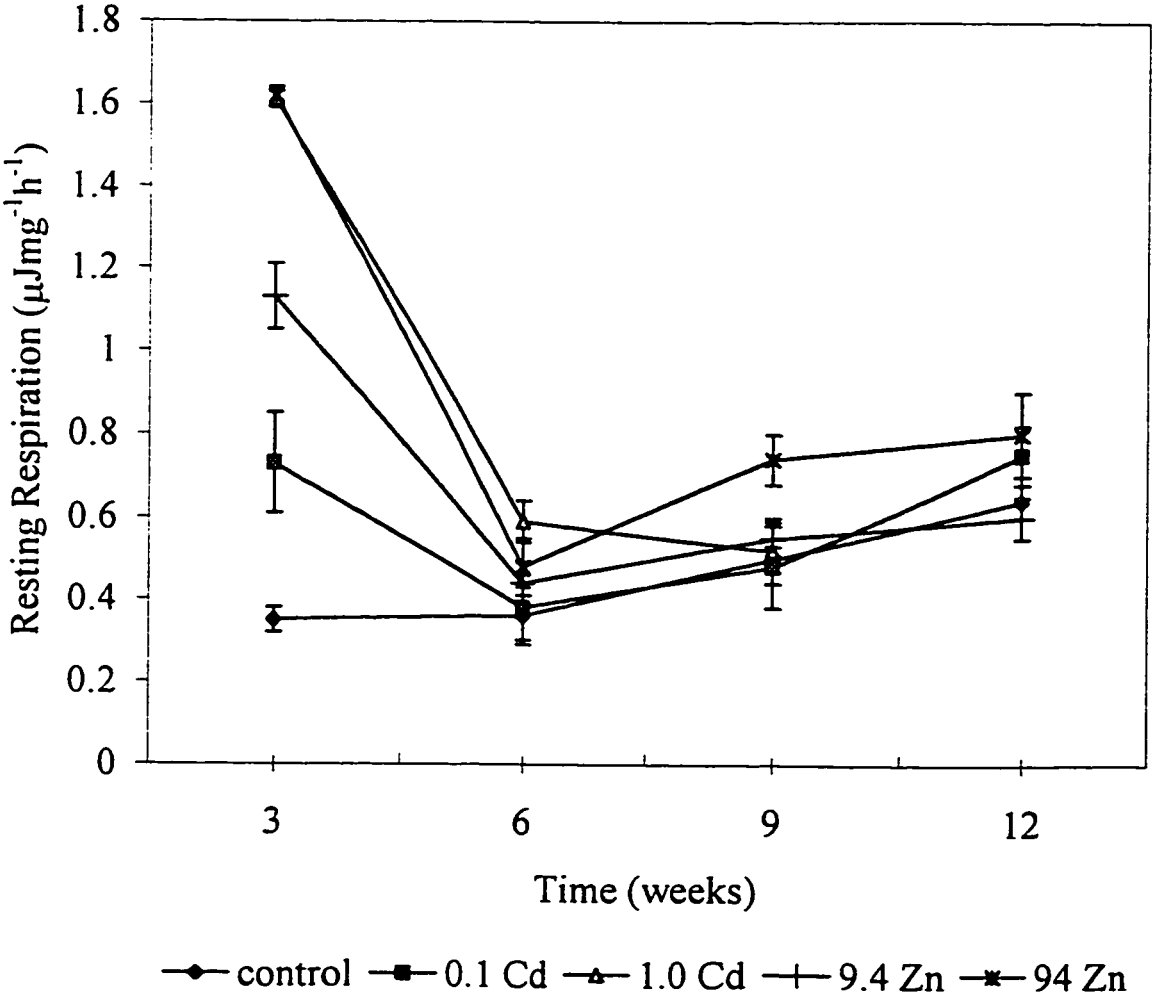

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Resting Respiration (μJmg-1h-1)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.10 Tukey's diagram of mean resting respiration rate ($\mu J \cdot mg^{-1}L^{-1}$; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Resting respiration ($\mu J \cdot mg^{-1}L^{-1}$)
94.0Zn	3	1.615
1.0 Cd	3	1.610
9.4 Zn	3	1.132
94.0Zn	12	0.795
0.1 Cd	12	0.754
94.0Zn	9	0.735
0.1 Cd	3	0.733
Control	12	0.639
1.0 Cd	6	0.593
9.4 Zn	9	0.552
1.0 Cd	9	0.515
Control	9	0.502
94.0Zn	6	0.484
0.1 Cd	9	0.477
9.4 Zn	12	0.461
9.4 Zn	6	0.435
0.1 Cd	6	0.384
Control	6	0.357
Control	3	0.348

significant drop at week 6 (0.593±0.049 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and no change thereafter. Also, the highest resting respiration observed for the animals exposed to 94 $\mu gZn \cdot L^{-1}$ was at week 3 (1.615±0.021 $\mu J \cdot mg^{-1} \cdot h^{-1}$). Followed by a significant decrease at week 6 (0.484±0.074 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and a significant rise again at week 9 (0.735±0.064 $\mu J \cdot mg^{-1} \cdot h^{-1}$) with no significant change thereafter.

At week 3 animals exposed to 1.0 $\mu gCdL^{-1}$, 9.4 $\mu gZn \cdot L^{-1}$ and 94 $\mu gZn \cdot L^{-1}$ had significantly higher resting respiration rates than the control, though no significant difference compared to the control was observed for the animals exposed to 0.1 $\mu gCd \cdot L^{-1}$. There was no significant difference between the treatments and the control at week 6. Significantly higher resting respiration rate compared to the control at week 9 was observed for the animals exposed to 94 $\mu gZn \cdot L^{-1}$. At week 12 significantly higher resting respiration rates were observed for the animals exposed to 94 $\mu gZn \cdot L^{-1}$ and 0.1 $\mu gCd \cdot L^{-1}$ compared to the control with no difference between the 9.4 $\mu gZn \cdot L^{-1}$ treatment and the control.

The resting respiration rate of the animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ and 94 $\mu gZn \cdot L^{-1}$ was significantly higher than those animals exposed to 9.4 $\mu gZn \cdot L^{-1}$ and 0.1 $\mu gCd \cdot L^{-1}$ at week 3. There was no significant difference observed among the treatments at week 6. The only significantly higher resting respiration rate at week 9 was observed for the animals exposed to 94 $\mu gZn \cdot L^{-1}$ compared to the rest of the treatments. At the last sampling time, a significantly higher resting respiration rate was observed for the treatment 0.1 $\mu gCd \cdot L^{-1}$ and 94 $\mu gZn \cdot L^{-1}$ animals compared to the animals exposed to 9.4 $\mu gZn \cdot L^{-1}$.

3.4.8.2. Active Respiration

The size-specific active respiration of the amphipods exposed to the Cd and Zn treatments over time (Fig. 3.11) showed a significant treatment*time interaction effect (ANOVA; df=11,38; F=2.67; $p<0.05$). All the treatments and the control had the highest active respiration rate at week 3 followed by a drop to week 6 with no significant change thereafter (Table 3.11).

The active respiration of the control animals dropped from a mean of 2.919 (± 0.065) $J \cdot mg^{-1} \cdot h^{-1}$ at week 3 to a mean of 0.747 (±0.135) $\mu J \cdot mg^{-1} \cdot h^{-1}$ at week 6. Animals exposed to 9.4 and 94 $\mu gZn \cdot L^{-1}$ had significantly higher active respiration rates with means of 2.036 (±0.376) $\mu J \cdot mg^{-1} \cdot h^{-1}$ and 2.045 (±0.053) $\mu J \cdot mg^{-1} \cdot h^{-1}$, respectively at the first sampling time compared to week 6, where the rates dropped to means of 1.053 (±0.179) $\mu J \cdot mg^{-1} \cdot h^{-1}$ and 1.494 (±0.397) $\mu J \cdot mg^{-1} \cdot h^{-1}$, respectively. The active respiration rates of the animals exposed to 0.1 and 1.0 $\mu gCd \cdot L^{-1}$ dropped significantly from means of 2.232 (±0.076) $\mu J \cdot mg^{-1} \cdot h^{-1}$ and 3.749 (±0.244) $\mu J \cdot mg^{-1} \cdot h^{-1}$, respectively at week 3 to means of 1.034 (±0.230) $\mu J \cdot mg^{-1} \cdot h^{-1}$ and 1.231 (±0.336) $\mu J \cdot mg^{-1} \cdot h^{-1}$, respectively at week 6.

The active respiration rate of the control animals was significantly higher than the active respiration rate of the animals exposed to 0.1 $\mu gCd \cdot L^{-1}$, 9.4 $\mu gZn \cdot L^{-1}$ and 94 $\mu gZn \cdot L^{-1}$ at week 3 but no difference was observed between the control and the animals exposed to 1.0 $\mu gCdL^{-1}$. When animals levelled off at week 6 there were no significant differences observed between the control and the treatment animals thereafter.

The only significant difference among treatments was detected at week 3 where

Fig. 3.11 Size-specific active respiration rate ($\mu J \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

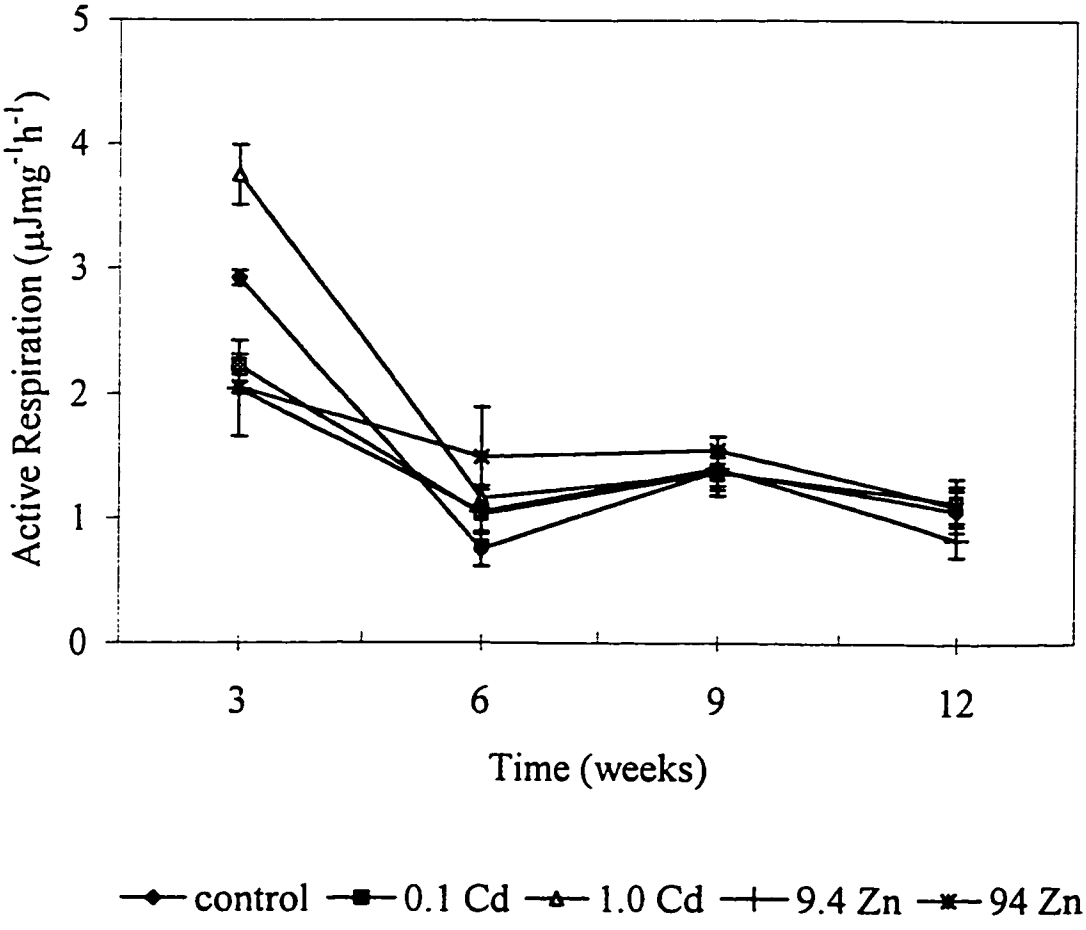

Active Respiration (µJmg-1h-1)
5
4
3
2
1
0
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.11 Tukey's diagram of mean active respiration rate ($\mu J \cdot mg^{-1}h^{-1}$; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Active respiration ($\mu J \cdot mg^{-1}h^{-1}$)
1.0Cd	3	3.749
Control	3	2.919
0.1Cd	3	2.232
94.0 Zn	3	2.045
9.4 Zn	3	2.036
94.0 Zn	9	1.555
94.0 Zn	6	1.494
9.4 Zn	9	1.403
Control	9	1.377
0.1Cd	9	1.367
1.0Cd	9	1.363
1.0Cd	6	1.231
0.1Cd	12	1.134
94.0 Zn	12	1.098
Control	12	1.056
9.4 Zn	6	1.053
0.1Cd	6	1.034
9.4 Zn	12	0.828
Control	6	0.747

animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ had a higher active respiration rate compared to the other treatments. Animals exposed to Cd and Zn treatments and the control did not differ observably in activity throughout the experiment. They exhibited active behaviour such as swimming, pairing, foraging and avoidance responses to tactile stimuli such as prodding at all sampling periods.

3.4.8.3. Aerobic Scope

The size-specific aerobic scope of the amphipods exposed to Cd and Zn over time (Fig. 3.12) had a significant treatment*time interaction effect (ANOVA; df=11,38; F=9.20; $p<0.05$). Aerobic scope of animals exposed to all the treatments, except in the 94 $\mu gZn \cdot L^{-1}$ treatment followed the same trend with aerobic scope being the highest at the first sampling time (Table 3.12).

Aerobic scope of control animals dropped significantly from a mean of 2.572 (±0.078) $\mu J \cdot mg^{-1} \cdot h^{-1}$ at week 3 to a mean of 0.390 (±0.079) $\mu J \cdot mg^{-1} \cdot h^{-1}$ at week 6. Aerobic scope at week 9 (0.875 ±0.069 $\mu J \cdot mg^{-1} \cdot h^{-1}$) was significantly higher than week 6 and dropped again at week 12. Animals in the 1.0$\mu gCd \cdot L^{-1}$ treatment also had the highest aerobic scope at the first sampling time (2.139 ±0.241 $\mu J \cdot mg^{-1} \cdot h^{-1}$) which dropped significantly by week 6 (0.637 ±0.047 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and increased significantly by week 9 (0.849±0.156 $\mu J \cdot mg^{-1} \cdot h^{-1}$). Aerobic scope of 0.1 $\mu gCd \cdot L^{-1}$ treatment animals was the highest at week 3 (1.499±0.097 $\mu J \cdot mg^{-1} \cdot h^{-1}$), dropped significantly by week 6 (0.649±0.165 $\mu J \cdot mg^{-1} \cdot h^{-1}$) which was not significantly different from week 9. At week 12, aerobic scope reached the lowest point with

Fig. 3.12 Aerobic scope ($\mu J \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.

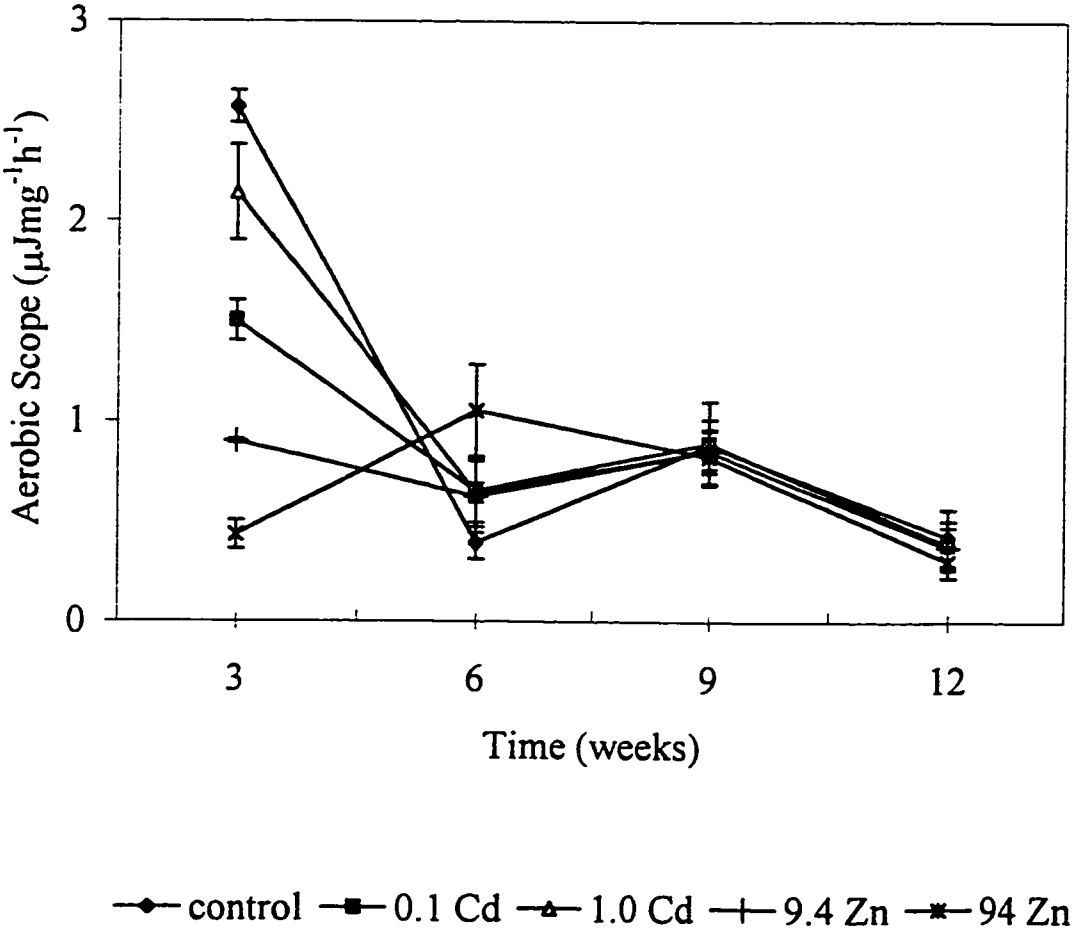

3
2
1
0
Aerobic Scope (μJmg-1h-1)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

Table 3.12 Tukey's diagram of mean aerobic scope ($\mu J \cdot mg^{-1}h^{-1}$; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Aerobic scope ($\mu J \cdot mg^{-1}h^{-1}$)
Control	3	2.572
1.0 Cd	3	2.139
0.1 Cd	3	1.499
94.0Zn	6	1.053
9.4 Zn	3	0.901
0.1 Cd	9	0.891
Control	9	0.875
9.4 Zn	9	0.851
1.0 Cd	9	0.849
94 Zn	9	0.820
0.1 Cd	6	0.649
1.0 Cd	6	0.637
9.4 Zn	6	0.617
94.0Zn	3	0.430
Control	12	0.417
Control	6	0.390
0.1 Cd	12	0.380
9.4 Zn	12	0.366
94.0Zn	12	0.303

a mean of 0.380 (±0.124) $\mu J \cdot mg^{-1} \cdot h^{-1}$. Animals exposed to 9.4 $\mu gZn \cdot L^{-1}$ had significantly higher aerobic scope at weeks 3 (0.901±0.012 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and 9 (0.851±0.110 $\mu J \cdot mg^{-1} \cdot h^{-1}$) compared to the weeks 6 (0.617±0.181 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and 12 (0.366±0.099 $\mu J \cdot mg^{-1} \cdot h^{-1}$). Aerobic scope of the 94 $\mu gZn \cdot L^{-1}$ treatment animals was significantly lower at week 3 (0.430±0.074 $\mu J \cdot mg^{-1} \cdot h^{-1}$) compared to weeks 6 (1.053±0.230 $\mu J \cdot mg^{-1} \cdot h^{-1}$) and 9 (0.820±0.063 $\mu J \cdot mg^{-1} \cdot h^{-1}$). At week 12 aerobic scope reached its lowest point with a mean of 0.303 (±0.079) $\mu J \cdot mg^{-1} \cdot h^{-1}$.

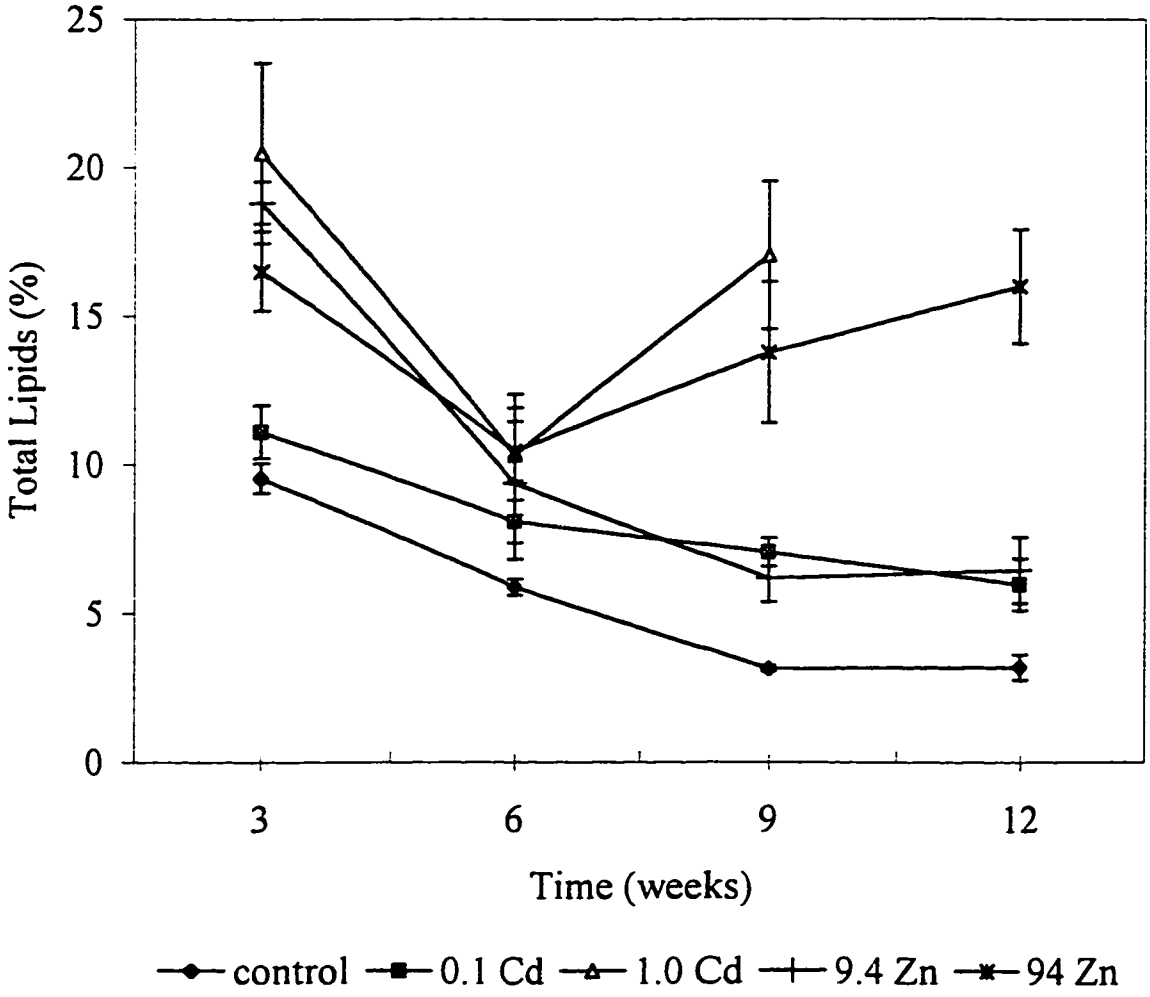
At week 3 animals in the 0.1 $\mu gCd \cdot L^{-1}$, 9.4 and 94 $\mu gZn \cdot L^{-1}$ treatments had significantly lower aerobic scope than the control. At the next sampling time only the 94 $\mu gZn \cdot L^{-1}$ treatment animals had significantly higher aerobic scope than the control. There was no significant difference observed between the treatments and the control at weeks 9 and 12.

Among all the treatments, at week 3, the 0.1 and 1.0 $\mu gCd \cdot L^{-1}$ treatment animals had significantly higher aerobic scope than the animals exposed to 9.4 $\mu gZn \cdot L^{-1}$ which was also significantly higher from the 94 $\mu gZn \cdot L^{-1}$ treatment animals. At week 6 animals exposed to 94 $\mu gZn \cdot L^{-1}$ had significantly higher aerobic scope than the rest of the treatments. There was no significant difference observed among all the treatments at weeks 9 and 12.

3.4.9. Total Lipids

The size-specific total lipid content of *H. azteca* (Fig. 3.13) exposed to Cd and Zn had a significant treatment*time interaction effect (ANOVA; df=11,38; F=2.20; $p<0.05$). All the treatments and the control had the highest total lipid content at the first sampling time at

Fig. 3.13 Total lipid content (%; mean±standard error; n=3) of *Hyalella azteca* exposed to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations measured every three weeks for 12 weeks.


25
20
15
10
5
0
Total Lipids (%)
3
6
9
12
Time (weeks)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn

3 weeks of exposure (Table 3.13).

Total lipid content of control animals was the highest at week 3 (9.5±0.5%) and showed a non-significant decline to a mean of 3.2 (±0.4)% at week 12. Animals exposed to 9.4 $\mu gZn \cdot L^{-1}$ and 0.1 $\mu gCd \cdot L^{-1}$ had the highest total lipid content at the first sampling time (18.8±0.7% and 11.1±0.9%, respectively) followed by a significant drop at week 6 (9.4±2.5% and 8.1±0.7% respectively) and no significant change thereafter. Total lipid content of animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ at week 3 (20.5±3.0%) and 9 (17.0±2.5%) was significantly higher than at week 6 (10.3±2.0%). A similar response was observed for animals exposed to 94 $\mu gZn \cdot L^{-1}$ with the animals having the highest total lipid content at week 3 (16.5±1.3%). At week 6 total lipid content of exposed animals dropped significantly to a mean of 10.5 (±1.0)% and increased significantly again to a mean of 13.8 (±2.4)% at week 9 with no significant change at week 12.

Animals exposed to all Cd and Zn treatments had significantly higher total lipid content compared to the control at week 3. There was no significant difference between the treatments and the control at week 6. At week 9 the 94 $\mu gZn \cdot L^{-1}$ and 1 $\mu gCd \cdot L^{-1}$treatment animals had significantly higher total lipid content than the control animals. The only significantly higher total lipid content compared to the control at week 12 was observed for the animals exposed to 94 $\mu gZn \cdot L^{-1}$.

There was no significant difference observed among the animals in Cd and Zn treatments at weeks 3 and 6. At week 9 the animals exposed to 94 $\mu gZnL^{-1}$ and 1.0 $\mu gCdL^{-1}$ had the highest total lipid content and for those exposed to the 94 $\mu gZnL^{-1}$ treatment, total lipid content stayed significantly higher at week 12.

Table 3.13 Tukey' diagram of mean total lipid content (%; n=3) by *Hyalella azteca*

Treatment ($\mu g \cdot L^{-1}$)	Time (weeks)	Lipid content (%)
1.0 Cd	3	20.5
9.4 Zn	3	18.8
1.0 Cd	9	17.0
94.0Zn	3	16.5
94.0Zn	12	16.0
94.0Zn	9	13.8
0.1 Cd	3	11.1
94.0Zn	6	10.5
1.0 Cd	6	10.3
Control	3	9.5
9.4 Zn	6	9.4
0.1 Cd	6	8.1
0.1 Cd	9	7.1
9.4 Zn	12	6.4
9.4 Zn	9	6.2
0.1 Cd	12	6.0
Control	6	5.9
Control	12	3.2
Control	9	3.2

3.5. Discussion

This chronic toxicity study demonstrates the high sensitivity of *H. azteca* to sublethal concentrations (10 and 100 times less than the LC_{50}) of the two heavy metals, Cd and Zn. All the bioenergetic variables measured during the course of the experiment were affected by heavy metal exposure with some being more affected than the others. The early life stages of *H. azteca* were more sensitive to of heavy metals.

One of the most important effects of Zn and Cd exposure on *H. azteca* was on survival. This is consistent with other studies where substantially lower concentrations of toxicants than those causing acute responses caused significant reductions in survival during chronic exposure. Increased mortality of *H. azteca* and *Gammarus fasciatus* Say was observed during chronic exposure to 1.0 $\mu gCd \cdot L^{-1}$ and 3.2 $\mu gCd \cdot L^{-1}$, respectively (Borgmann et al., 1989). The experimental concentration of 57 $\mu gCd \cdot L^{-1}$ decreased survival of the fathead minnow *Pimphales promelas* Rafinesque (Pickering and Gast, 1972). Brown trout *Salmo trutta* L. suffered considerable mortality in a 29.1 $\mu gCd \cdot L^{-1}$ treatment (Brown et al., 1994). Mortality of the midge *Chironomus riparious* (Meigen) was increased by Cd concentration of 2.0 $\mu gCd \cdot L^{-1}$ (Postma et al., 1994) and an experiment with the leech *Nephelopsis obscura* culminated in 100% mortality of the population after 14 weeks of exposure to 10 $\mu gCd \cdot L^{-1}$ (Westcott, 1997). On the contrary, exposure to Zn caused mortality of the zebra mussel *Dreissena polymorpha* (Pallas) only at higher concentrations (1266-2739 $\mu gZnL^{-1}$) (Kraak et al., 1994).

Hyalella azteca in this study suffered the highest mortality in the immature and

juvenile stages during the first 3 weeks of exposure. The principal reason for the high mortality was the extreme sensitivity of the early instars, confirming the results of acute tests with these life cycle stages (Collyard et al., 1994). The influence of size classes was also found for copepods (van Leeuwen et al., 1985), chironomids (Pascoe et al., 1988), fish (Pickering and Gast, 1972; Brown et al., 1994) and leeches (Westcott, 1997). A possible reason for higher susceptibility of smaller animals is a higher surface area to volume ratio compared to larger sized animals (Rainbow and Moore, 1986; Amyot and Pinel-Alloul, 1994). Another reason could be higher sensitivity of amphipods immediately after moulting, which is more frequent when animals are immature. Higher sensitivity may result from the increased permeability of the new exoskeleton prior to tanning and calcification. This was found to be the main cause of increased Cd and Zn uptake (Rainbow and White, 1989).

The definitive cause of death of *H. azteca* following exposure to Cd and Zn is not known. Cadmium disturbs many biological systems such as protein and nucleic acid synthesis (Patnaik and Gantayat, 1975), causing reduction in the total protein contents (Rathore at al., 1979) or abnormal protein formation (Jungmann et al., 1993). It also has an inhibitory effect on ATPases (Muiño et al., 1990) reducing oxygen availability (Hutcheson, et al., 1985; Reddy et al., 1989) and is involved in a shift from aerobic to anaerobic pathways (Forbes and Depledge, 1992). Zinc causes metabolic disturbances which lead to impaired energy transformation (Bengtsson, 1974) and has been known to alter the incorporation of inorganic phosphate into tissue and to inhibit oxygen uptake in mitochondria (Hiltibran, 1971). Exposure of air-breathing catfish *Heteropneustes fossilis* (Bloch) to acute concentration of zinc chloride (75 $\mu gZn \cdot L^{-1}$: 96 h LC_{50}) caused severe toxicopathological damage to gills and

to accessory respiratory organs (Hemalatha and Banerjee, 1997). Zinc is also thought to exert acute toxic effects on crustaceans primarily by interfering with ionic regulation and damaging gill structure (Xu and Pascoe, 1993).

Coping with environmental stress can involve a reduction in growth as available energy is channelled into stress-resisting processes (Calow, 1989c; Holloway at al., 1990). Exposure to metals has been associated with decreased growth in many studies, which suggest that exposure may affect the synthesis of protective proteins (Bradley et al, 1985) and cause decreases of ingestion (Lett et al., 1976). It has also been reported that, in general, the first indication of toxicity is a marked reduction in growth of animals and that this occurs at concentrations lower than those causing mortality. On the contrary, growth of chironomids was impaired only at Cd and Zn concentrations greater than the average LC_{50} (Anderson et al., 1980). Reduction in growth rate in the present study, evaluated in terms of animal biomass, following exposure to stress was significant in the animals exposed to higher concentrations of Cd (1.0 $\mu gCd \cdot L^{-1}$) and Zn (94 $\mu gZn \cdot L^{-1}$) while in low concentrations it was affected only at the end of exposure (9.4 $\mu gZn \cdot L^{-1}$) or not at all (0.1 $\mu gCd \cdot L^{-1}$). Similar results were found for the leech *Nephelopsis obscura*, where a decrease in size was apparent at the end of the exposure to 1.0 $\mu gCd \cdot L^{-1}$, while severe degrowth was experienced by the leech exposed to 10 $\mu gCd \cdot L^{-1}$ (Westcott, 1997). Results of this experiment agree with previous studies where growth rate decreased with increasing Cd and Zn concentrations (Watling, 1982; Woodward et al., 1995; Wicklum and Davies, 1996). Decrease in growth rate was found for mysids exposed to 1.0 $\mu gCd \cdot L^{-1}$, but ingestion was not affected (Birmelin et al., 1995). Exposure for 24 days to just 2.0 $\mu gCd \cdot L^{-1}$ reduced the growth rate of *Daphnia*

magna (Baird et al., 1990). Growth rate of the snail *Hydrobia ulvae* (Pennant) was substantially reduced by exposure to 100 $\mu gCd \cdot L^{-1}$ for 3 weeks, but no effect on feeding rate was detected (Forbes and Depledge, 1992). Brungs (1969) demonstrated reduced growth of fathead minnows *Pimphales promelas* exposed to 2800 $\mu gZn \cdot L^{-1}$, but yearlings of the minnow *Phoxinus phoxinus* L. suffered reduced growth when exposed for 30 days to concentrations as low as 130 $\mu gZn \cdot L^{-1}$ (Bengtsson, 1974). Growth in wet weight was reduced after 134 days of exposure to 607 $\mu gZn \cdot L^{-1}$ in guppies *Poecilia reticulata* Peters but it remained uniform for the first 28 days of exposure (Pierson, 1980). Zinc concentrations ranging from 96-640 $\mu gZn \cdot L^{-1}$ in soft water and 960-6400 $\mu gZn \cdot L^{-1}$ in hard water did not impaired the growth rate of the leech *Nephelopsis obscura* (Gray, 1995). However, Pickering (1968) reported that low concentrations of Zn had a stimuling effect on the growth of the bluegill, *Lepomis macrochirus* Rafinesque and under conditions of Zn limitation Cd can enhance the growth of variety of marine phytoplankton species (Lee et al., 1995).

Energy ingested is one of the basic factors, which determines how much energy can be allocated to growth, storage and reproduction (Smith and Davies, 1997). The apparent cause of growth retardation in this study was a decrease in food ingestion by the animals exposed to Cd and Zn, a common toxic response. The growth rate being slower in higher concentrations of Cd and Zn in water has its parallels in ingestion rate being lower by animals exposed to higher concentrations. Although the toxicants had an affect on ingestion rate at all concentrations it was more drastic at higher concentrations of toxicants. Bodar et al. (1988) reported that feeding rate, body weight and assimilation efficiency of *Daphnia magna* were significantly reduced at Cd concentrations as low as 5.0 $\mu gCd \cdot L^{-1}$, exposed for 14 days,

as compared to an 24 h LC_{50} of 970 $\mu gCd \cdot L^{-1}$. Ingestion rate and energy absorbed by *Gammarus pulex* was significantly effected at 300 $\mu gZn \cdot L^{-1}$ (25 times less than 24 h LC_{50}) after 6 days of exposure (Maltby and Naylor, 1990). There was no effect found on leech ingestion rate at concentrations as high as 6400 $\mu gZn \cdot L^{-1}$ after 11 weeks of exposure (Gray, 1995), while exposure to 10 $\mu gCd \cdot L^{-1}$ for 14 weeks caused a significant decrease in ingestion rate (Westcott, 1997). Decrease in pumping rate was observed by the mussel *Mytilus edulis* L. exposed to 230 $\mu gZn \cdot L^{-1}$ and 7.5 $\mu gCd \cdot L^{-1}$ (Redpath and Davenport, 1988) and by the zebra mussel *Dreissena polymorpha* exposed to 382 $\mu gZn \cdot L^{-1}$ (Kraak et al., 1994) and 399 $\mu gCd \cdot L^{-1}$ (Kraak et al., 1992). The decrease in ingestion rates of exposed animals was apparently due to Cd and Zn toxicity. Morphological changes of mouth parts or digestive system could be one of the reasons for lower ingestion rates. Foster et al. (1966) suggested that the damage to taste receptors caused by sublethal concentrations of detergents disturbed feeding behaviour in the flagfish *Jordanella floridae* Goode. A similar mechanism was proposed to be responsible for the decreased food intake by the minnow *Phoxinus phoxinus* treated with Zn (Bengtsson, 1974). When *Daphnia magna* was exposed to Cd (12.0 $\mu gCd \cdot L^{-1}$) and Zn (2000 $\mu g\ Zn \cdot L^{-1}$) their gut diverticula, which are the sites of secretion of enzymes, digestion and absorption, became shrunken and paralysed (Griffiths, 1980).

Following exposure to Cd and Zn, increased faecal elimination of *H. azteca* was detected. This was more pronounced at the juvenile stage of the animals. Consequently, because of the lower ingestion rate and higher faeces production, the absorption efficiency of the animals at this stage was low. The lowest absorption efficiency was found for the animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ and 94 $\mu gZn \cdot L^{-1}$ indicating more stressful conditions.

Elimination of heavy metals through excretion is one of the mechanisms of regulating Cd and Zn accumulation (Forbes and Depledge, 1992; Posthuma et al., 1992; Chan and Rainbow, 1993). Higher faecal production by stressed animals was also reported for chironomids exposed to 0.1, 0.5 and 1.0 $\mu gCd \cdot L^{-1}$ (Heinis et al., 1990) and for leeches exposed to 960, 3200 and 6400 $\mu gZn \cdot L^{-1}$ in hard water (Gray, 1995). In contrast, exposure of leeches to 10 $\mu gCd \cdot L^{-1}$ (Westcott, 1997) and 96, 320 and 640 $\mu gZn \cdot L^{-1}$ in soft water (Gray, 1995) resulted in a reduction of faecal production.

There was a clumping of faeces noticed in all the treatments, but it was more pronounced in the treatments with higher concentrations of Cd and Zn. This could possibly be due to higher mucus excretion of more stressed animals. Because mucus binds to heavy metals, including Cd and Zn (Pärt and Lock, 1983), it has been suggested that the hyper-secretion of mucus in response to metal exposure reduces metal binding to epithelia, tissue exposure and bioaccumulation (Meyer et al., 1991; de Boeck et al., 1995). Higher levels of mucus production were found in *Mytilus edulis* exposed to Cd (Sunila, 1984) and in *Nephelopsis obscura* exposed to 10 and 50 $\mu gCd \cdot L^{-1}$ (Westcott, 1997; Wicklum and Davies, 1996) and 3200 and 6400 $\mu gZn \cdot L^{-1}$ in hard water (Gray, 1995). Detoxification, sequestration, elimination and repair mechanisms of animals exposed to toxicants are energetically costly, but elimination of heavy metals through mucus and faeces excretion can reduce the need for these energetically expensive mechanisms. Increased total ammonia excretion resulted from exposure to Cd and Zn at all concentrations and was higher at the first two sampling times. This indicates higher sensitivity of younger stages, which are trying to cope with stressful conditions by using excretion as a major route for heavy metal elimination. Excretion provides

an important detoxification mechanism for binding and removing toxic substances from circulation (Sperling, 1984). Proportionately more energy use for ammonia excretion was also found for *Cyprinus carpio* L. exposed to sublethal levels of Cu (de Boeck et al., 1995) and *Nephelopsis obscura* exposed to 1.0 and 10 μgCd·L $^{-1}$ (Westcott, 1997).

High ammonia production in animals, in which ingestion dropped, due to the stress conditions, could indicate use of other sources of energy to compensate for repair and detoxification demands. The only other available source of energy that animals could use was energy stored in the form of lipids, glycogen or proteins. Since the levels of lipids stayed relatively high in animals exposed to Cd and Zn it is believable that the metabolism of glycogen and proteins were the only source to make up the difference in energy required. Although glycogen and/or lipids are generally utilized first in stressful conditions, proteins can become an important energy source (Bayne, 1973; Reddy et al., 1989). During starvation of invertebrates proteins are utilized as energy sources (Claybrook, 1983) and have been shown to be mobilized under other stressful conditions (Bayne, 1973). Similarly, the increased energy demand associated with stress resulted in depletion in glycogen reserves (Bhagyalakhsmi et al., 1984). Increased ammonia production and decreased protein content was found in the crab *Scylla serrata* (Forskal) exposed to 2500 μg Cd·L^{-1} (Reddy and Bhagyalakshmi, 1994) and in *Daphnia magna* exposed to Cd, with protein catabolism increasing with increasing Cd concentration (Barber et al., 1990). Increase in ammonia excretion in *H. azteca* during the prolonged exposure to heavy metals in this experiment indicates an increased catabolism and turnover of body protein due to higher energy demands. Animals exposed to stressful conditions eventually expend all of their energy reserves, pass

into the exhaustion stage and ultimately die. This was the case in the animals exposed to 1.0 $\mu gCd \cdot L^{-1}$.

Number of eggs produced and the number of offspring released were negatively affected by Cd and Zn exposure at all concentrations compared to the control. However, there was no effect on the size of eggs or offspring. Maltby and Naylor (1990) proposed possible ways in which stressors may influence the reproductive efforts of females. Stressors can have a direct lethal effect on the current brood resulting in a decrease in the number of viable offspring produced; they can cause a reduction in the amount of energy available to provision eggs resulting in offspring being smaller; or they can have a lethal effect on the eggs whilst they are being provisioned resulting in the offspring being reduced in number but of normal body size. How toxicants affect the reproduction of aquatic organisms is still not well understood. Effects of toxicants on processes like oogenesis, embryogenesis and hormone regulation express themselves directly on reproduction, while malfunctioning of feeding, digestion and resorption caused by toxic agents, results in a lack of essential reproduction components, thus affecting reproduction indirectly (Bodar et al., 1988). In this experiment, feeding rates of exposed animals were reduced which was likely the major reason insufficient energy was available to be allocated to reproduction. In *Gammmarus pulex* Zn stress caused an increase in the number of broods aborted but had no effect on the size or number of offspring released (Maltby and Naylor, 1990). Fecundity and egg size were significantly decreased in white sucker *Catostomus commersoni* (Lacepede) exposed to 13-15 $\mu gCu \cdot L^{-1}$ and 209-253 $\mu gZn \cdot L^{-1}$ (Munkittrick and Dixon, 1988). After 70 days of exposure of the guppy *Poecilia reticulata* to 173 $\mu gZn \cdot L^{-1}$ significantly fewer females had matured and gave

birth (Pierson, 1980). Zebra fish *Brachydanio rerio* Hamilton-Buchanan showed a delay in spawning and produced significantly fewer viable eggs than the control when exposed to 5.0 $\mu gZn \cdot L^{-1}$ for a 9-day period (Speranza et al., 1977). Reproduction of *Daphnia magna* was completely inhibited after 14 days at 3.2 $\mu gCd \cdot L^{-1}$ (van Leeuwen et al., 1985). Eggs obtained from rainbow trout *Oncorhynchus mykiss* Walbaum exposed to 1.8 and 3.4 $\mu gCd \cdot L^{-1}$ failed to develop to the fry stage (Brown et al., 1994). Ovarian maturation of the red swamp crayfish *Procambarus clarkii* (Girard) was significantly inhibited by Cd and mercury exposure (Reddy et al., 1997). In *H. azteca* reproduction is size dependent with smaller females producing fewer offspring (Cooper, 1965). This was possibly one of the reasons for a reduced number of offspring born to the females exposed to higher concentrations of Cd and Zn.

Energy allocation to production decreases as a result of a reduction in assimilation and/or an increase in respiration (Baird et al., 1990). Resting (R_r) and active (R_a) respiration were used in this study to indicate respiratory responses to stress. However, the total amount of energy respired while active or resting, obtained by the product of time spent active*R_a or time spent resting*R_r respectively, were not assessed in this study. It was not feasible to measure active or resting times because the juvenile animals were paired within a respiration chamber and it was not possible to determine the activity of each. Higher resting respiratory costs, compared to the control, were detected for juveniles exposed to all concentrations of Cd and Zn. These resting respiratory costs include basal metabolism (that level of metabolism below which the animal cannot survive) cellular repair, excretion, somatic and reproductive growth. Decreases in somatic and reproductive growth observed in *H. azteca* exposed to Cd

and Zn was caused presumably by lower contribution of energy to tissue and gamete production despite the increase in faeces and ammonia production which contributed in part to the increased resting respiration. The removal or neutralization of toxicants and repair of cellular damage caused by Cd and Zn are the most likely causes of the increased resting respiration rate, although the relative contributions of these processes to resting respiration are not known (Calow, 1989c).

At first sampling time, less (0.1 $\mu gCd \cdot L^{-1}$, 9.4 and 94 $\mu gZn \cdot L^{-1}$) or the same (1.0 $\mu gCd \cdot L^{-1}$) amount of energy was used by animals exposed to Cd and Zn compared to the control when animals were active. Lower energetic cost of active respiration of exposed animals could be due to animals preferring to move slower. Elevated energy expenditure of animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ while active could possibly be due to agitation by toxicant causing involuntary movements (body displacement or swimming) of the animals. Aerobic scope is the difference between R_a and R_r. In all the treatments, resting respiration was higher and active respiration lower than the control's (except the 1.0 $\mu gCd \cdot L^{-1}$ treatment) and therefore the aerobic scope was lower in all the treatments compared to the control. This could be explained by lower activity of animals or a partial shift to anaerobic respiratory pathways due to the toxic effect of the heavy metals.

The effect of toxicants on energy expenditure, quantified by measuring respiration, of aquatic organisms is difficult to compare among studies because of the different methods used to quantify oxygen uptake. Respiration rates can be measured in closed systems, measuring the decrease of dissolved oxygen concentration (Hawkins et al., 1982; Wolf et al., 1985; Barber et al., 1990; Mule and Lomte, 1994; Barber et al., 1994; Hartman and Brandt,

1995; Musko et al., 1995; Chapelle and Peck, 1995; Eriksen and Iversen, 1997) or in open flow-through systems, providing a constant oxygen tension to the test organisms and continuously removing metabolites (Ultsch et al., 1980; Bridges and Brand, 1980; Wrona and Davies, 1984; Davies et al., 1992; Wicklum, 1995; Kedwards at al., 1996; Westcott, 1997). The problems associated with closed systems are progressively increasing hypoxia and metabolite accumulation, both of which are potentially stressful and will affect the measured rate of respiration.

Other limiting factors for accurate comparison of respiration rates among different animals exposed to toxicants is that many studies do not distinguish between resting and active respiration (Barber et al., 1990) or the animals are not provided with the same acclimation time when placed into the respirometer chambers (Chapelle and Peck, 1995). All these limiting factors and different degrees of sensitivity of different species give variable responses when measuring respiration. Barber et al. (1990) demonstrated an increase in oxygen consumption of daphnid neonates when exposed to 5.0 $\mu gCd \cdot L^{-1}$ for 48 hours, possibly due to the cost associated with protein turnover (i.e. maintenance). Oxygen consumption of *Crassostrea virginica* Gmelin was unchanged after exposure to 100.0 $\mu gCd \cdot L^{-1}$ but increased when exposed to 300 $\mu gCd \cdot L^{-1}$ (Engel and Fowler, 1979). *Gammarus pulex* exposed to 10, 20 and 40 $\mu gCu \cdot L^{-1}$ for 10 days exhibited significantly elevated respiratory rates compared with control animals (Kedwards et al., 1996). Since respiration in these cases was not divided into resting and active states, it is not possible to conclude with certainty that these measurements detected stress-related metabolic changes. In contrast, total respiration of *Nephelopsis obscura* increased when exposed to 1.0 and 10 $\mu gCd \cdot L^{-1}$,

which was significantly correlated with an increase in resting respiration (Westcott, 1997).

Lower oxygen consumption due to heavy-metal exposure has also been reported. The rate of oxygen consumption declined significantly in the freshwater snail *Thiara tuberculata* (Muller) after acute (72 hour) and chronic (20 day) exposure to Cu and Hg (Mule and Lomte, 1994). Brkovic-Popovic and Popovic (1977) found that acutely toxic concentrations lowered the metabolic rate of *Tubifex tubifex* (Muller), while concentrations below that raised it. However, Naylor et al. (1989) reported no respiratory changes in *Gammarus pulex* after 6 day exposure to Zn and there was no significant effect on R_a, R_r and aerobic scope of *Nephelopsis obscura* exposed to 5, 10 and 50 $\mu gCd \cdot L^{-1}$ for 12 weeks (Wicklum, 1995).

The mode of action of heavy metal ions on the invertebrate respiratory system is not well understood. It has been reported that accumulation of metals occurs in gills, interfering with the osmoregulatory and respiratory functions of these organs. Bubel (1976) showed that exposure to heavy metals eventually culminates in a breakdown of gill epithelial cells and causes many ultrastructural changes including distension of microvilli and mitochondria. Some toxicants can also act on the respiratory system itself by interfering with oxygen uptake and affecting phosphorylation (Slater, 1963).

The total lipid content of exposed animals was significantly affected by heavy metals. Lipid levels of animals exposed to Cd and Zn at all concentrations were higher than the controls' at all sampling times. The total lipid content of control animals (3-10%) is close to that found for two other amphipods, *Gammarus pulex* (5.9%) and *Carinogammarus* sp (7.7%) (Geng, 1925). Arts et al. (1995) also reported total lipid content of male *H. azteca* being in the range from 2.5-10%. Higher lipid contents have been reported only for species

that are either carnivorous or/and planktonic, but not for herbivorous and detritivorous benthic crustaceans (Green, 1971).

Elevated total lipid levels of exposed animals from this experiment are apparently due to the toxic effect of Cd and Zn. Higher total lipid content of stressed animals shows that proportionally more energy was allocated to storage than to somatic and reproductive growth (the stressed animals grew slower and produced fewer offspring than the control animals). It is also possible that total lipid levels stayed high because animals used proteins and glycogen to fulfil their energy demands. Therefore, due to protein and glycogen catabolism, ammonia production of exposed animals was elevated. In agreement with this experiment's results, total lipid levels of *Daphnia magna* increased after 7 days of exposure to 1.0 $\mu gCd{\cdot}L^{-1}$, while protein and glycogen levels decreased (Bodar et al., 1988). In contrast, some studies show a decrease in total lipid content of stressed animals. Munkittrich and Dixon (1988) proposed that exposure to toxic contaminants may result in the catabolism of energy reserves to combat decreased energy intake or increased energetic costs. Low levels of energy stored were detected for *Mytilus edulis* exposed to hydrocarbons (Lowe and Pipe, 1986). High utilization of stored energy was reported for *Gammarus pulex* exposed to oil (Carr and Linden, 1984). Lower lipid levels were observed in the white sucker *Catostomus commersoni* from lakes containing elevated levels of Cu and Zn (Munkittrick and Dixon, 1988). However, the amount of phospholipids and cholesterol in the organs of control and experimental molluscs did not differ significantly, when exposed to high Cd (500 $\mu g{\cdot}CdL^{-1}$) concentrations (Evthushenko et al., 1986). The total lipid content of aquatic animals has been shown to vary with season (Dratnal and Davies, 1990; Reddy et al., 1992; Adare and

Lasenby, 1994; Arts et al., 1995; Cavaletto et al., 1996), sex (Arts et al., 1995), type of food ingested (Green, 1971; Adare and Lasenby, 1994) and with exposure to toxicants (Munkittrick and Dixon, 1988; Bodar et al., 1988).

Weight specific energy content of animals in the juvenile and mature stages differed significantly, being higher when animals were in the juvenile stage. This agrees with the overall lipid content of the animals which was highest in the juvenile stage of exposed and nonexposed animals. However, there was no significant difference observed between control and exposed animals. The close size specific energy content of control and treatment animals, despite low lipid levels in nonexposed animals, was probably due to compensation with higher carbohydrate and protein levels in the control animals. As was suggested before, animals exposed to Cd and Zn probably used glycogen and proteins to fulfil their energetic demands but kept their lipids high. This could consequently lead to the same energetic values of control and exposed animals. The size specific energy content of juvenile (14.053 $J \cdot mg^{-1}$) and mature (12.828 $J \cdot mg^{-1}$) *H. azteca* in this experiment is in close agreement with the results obtained by Wissing and Hasler (1968) which found the value of 14.393 $J \cdot mg^{-1}$ for *H. azteca* from Lake Mendota, Wisconsin. Similar results were also reported by Green (1971), who found a value of 15.062 $J \cdot mg^{-1}$ for *H. azteca* from West Blue Lake, Manitoba. The slightly higher values for animals found in these previous studies is possibly due to their animals coming from the field compared to laboratory reared animals from this experiment.

The above analysis of the variables of the bioenergetic model shows that this approach is extremely valuable in detecting metal-pollution problems. The high sensitivity of *H. azteca* favours the use of these animals as an indicator of elevated heavy metal levels in the

environment. Toxicants used in this experiment had an overall effect on the energetic state of exposed animals. The most important effects were a reduction in energy ingestion and absorption efficiency, and increases in faeces and ammonia production. This resulted in less energy available for other physiological processes and consequently resulted in lower somatic and reproductive growth. Fecundity was reduced by a reduced proportion of energy being channelled into gamete production. More energy was probably allocated to maintenance, which includes detoxification processes and repair. These effects were greater in animals exposed to higher concentrations of toxicants.

The most sensitive energetic responses exhibited in response to lower concentrations of Cd and Zn were ingestion, lipid production and reproduction. In animals exposed to higher concentrations of two heavy metals, all the variables measured showed significant differences at almost all sampling times. Survivorship was significantly affected in all the treatments.

The animals affected most were the ones exposed to 1.0 $\mu gCd \cdot L^{-1}$. Due to an initial drop in energy acquisition, animals responded by changing energy allocation patterns, enabling them to survive the first 9 weeks of exposure. Energy was mostly allocated to excretion and respiration, presumably including detoxification and repair processes, at the expense of growth and fecundity. Elevated ammonia production indicated catabolism of energy stored, presumably proteins and glycogen, to compensate for the decrease in energy acquisition and increase in energy demands. Eventually when organisms expended all of their energy reserves, maintenance of repair and detoxification mechanisms could no longer function and this ultimately led to mortality of the amphipods.

CHAPTER 4 TOLERANCE TESTING

4.1. Introduction

Organisms subjected to toxic pollutants may develop a tolerance to their effects. Tolerance refers to an organism's ability to survive when exposed to certain environmental conditions (Fry, 1947; Duncan and Klaverkamp, 1983). Calow (1989a) proposed that these stressors may be a selection pressure so that traits (e.g. survivorship, reproductive output, developmental time) least affected or not affected in some individuals will proportionally increase in the surviving population, leading to the evolution of tolerance. Organisms may acquire tolerance by physiological acclimation during exposure to sublethal concentrations at some prior period of their development or they may evolve genetically based resistance, through the action of natural selection (Klerks and Weis, 1987). The continued presence of a species in a polluted environment may thus be due to animals becoming resistant to pollutants (Klerks and Levinton, 1993). Therefore it is important to know if animals used in testing from the field did or did not experience pre-exposure to toxicants. Difference in results will have important implications for decisions regarding safe ambient toxicant levels (Klerks and Weis, 1987).

Metal tolerance is a frequent occurrence in invertebrates from contaminated environments. Laboratory experiments indicate that chronic exposure to elevated metal concentrations results in the development of metal tolerance (Kito et al., 1982; Krantzberg and Stokes, 1989; Bebianno et al., 1994). Tolerance to Zn is achieved either through regulation or internal storage in a detoxified form and may result in higher Zn concentrations

in tissues of tolerant compared to nontolerant populations (Frazier and Georg, 1983). Cadmium tolerance occurs, but is less frequently associated with the capability of regulation and/or elimination (Krantzberg and Stokes, 1989).

The deleterious effects of Cd and Zn contamination in fresh water organisms result from accumulations within specific tissues (eg. gills, liver, kidney, digestive glands, hepato-pancreas). However, most organisms have developed subcellular detoxification processes such as the synthesis of metallothioneins (Bebianno et al. 1992, 1994). These are low-molecular weight cysteine-rich proteins capable of binding heavy metals (reviewed by Kagi and Schaeffer, 1988). In juvenile and adult fish, synthesis is strongly induced by exposure to Cd and Zn and the metals are sequestered by the newly synthesized protein (George at al., 1992, 1996; George and Olson, 1994).

In contrast it has been demonstrated by Mishima et al. (1997) that Zn induced a tolerance to Cd cytotoxicity in different cell types even when metallothionein was not increased by Zn. When the extracellular level of Zn approximates the normal physiological concentration or is at levels under the threshold of metallothionein induction, tolerance to Cd cytotoxicity appears to be induced by the non-metallothionein mechanisms rather than metallothionein induction at the cell level. The protective mechanisms other than metallothionein, could be 1) an increase in the level of intracellular Zn physicochemically prevents the entrance of Cd into the cells (Mishima et al., 1997), 2) the Zn that had entered cells during pre-treatment protected membrane integrity (Bettger and O'Dell, 1981) or 3) the intracellular Zn served as an antioxidant (Bray and Bettger, 1990). However, metallothionein induction appears to be the most effective mechanism, since pre-treatment of liver cells with

high levels of Zn induced a stronger tolerance to Cd cytotoxicity which paralleled an increase in intracellular metallothionein (Mishima et al., 1997).

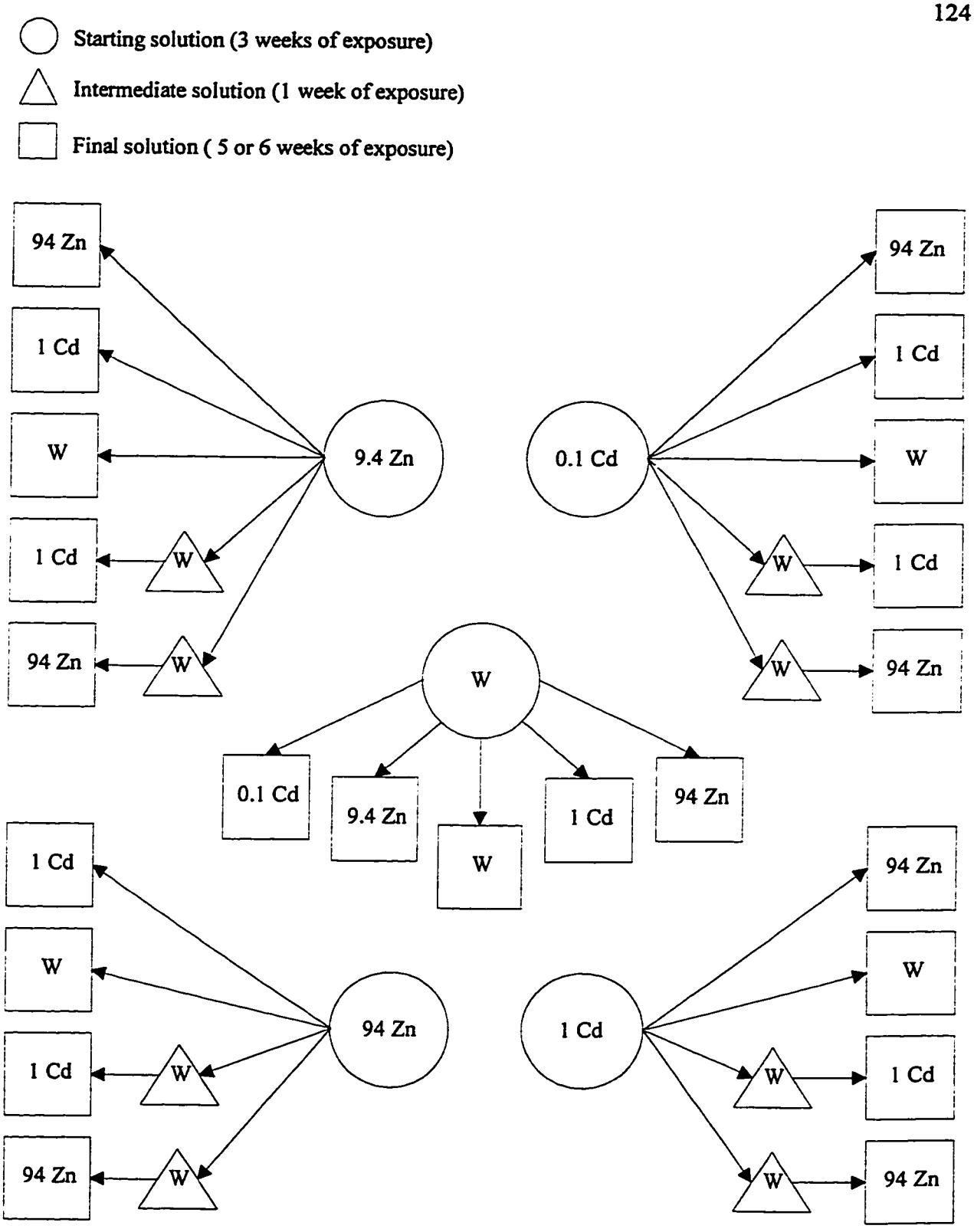
Although short- and long-term laboratory experiments indicate that organisms are sensitive to heavy metal pollution, tolerance may vary over the life cycle and among animals from different populations depending upon their history of exposure. The objectives of this study were:

1. to determine if the pre-exposure to one metal changes energetics which would increase tolerance to the toxic effects of exposure to another metal, and;
2. to determine if the pre-exposure of parents alters the effect of heavy metals on fecundity and total lipid stores of the next generation.

4.2. Methods and material

Two separate experiments were run to test the tolerance of *Hyalella azteca* to two heavy metals, Cd and Zn. In the first experiment animals were tested for acquired tolerance to Zn or Cd through the pre-exposure to the same or different heavy metal. Animals were exposed to 23 different combinations (Fig. 4.1). The animals from all treatment combinations were compared with the control animals that were not exposed to any of the metals during their lifetime (W-W). The animals from the treatments which were exposed to clean water in their immature stage and to high concentrations of Cd and Zn (W-1Cd and W-94Zn) in their mature stage were compared with animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ or 94 $\mu gZn \cdot L^{-1}$ in their mature stage after they had been pre-exposed. Mortality rates during the 3 week pre-exposure were the same as those during chronic testing (Chapter 3) for all concentrations of

Fig. 4.1 Treatment combinations for tolerance testing in first generation *Hyalella azteca*.


Starting solution (3 weeks of exposure)
Intermediate solution (1 week of exposure)
Final solution (5 or 6 weeks of exposure)
94 Zn
1 Cd
W
1 Cd
94 Zn
W
W
W
9.4 Zn
0.1 Cd
94 Zn
1 Cd
W
W
1 Cd
W
94 Zn
W
0.1 Cd
9.4 Zn
W
1 Cd
94 Zn
1 Cd
W
1 Cd
94 Zn
W
W
W
94 Zn
1 Cd
94 Zn
W
1 Cd
W
94 Zn

toxicants. The mortality of animals exposed to 1.0 $\mu gCd \cdot L^{-1}$ or 94 $\mu gZn \cdot L^{-1}$ in their mature stage, after they had been pre-exposed, was compared to the mortality of animals that had been continuously exposed to 1.0 $\mu gCd \cdot L^{-1}$ or 94 $\mu gZn \cdot L^{-1}$ for 9 weeks. All the combinations and the controls had three replicates with 20 animals per replicate. When animals were 1 week old they were put into the starting solution for 3 weeks. Next they were placed into either clean water, or a solution containing the other metal or a solution containing the same metal but at a higher concentration for 6 weeks. In eight cases after the animals were first exposed to one of the toxicants for 3 weeks, they were exposed for 1 week to clean water and then again exposed to the same heavy metal of the same or different concentration, or were put into a solution with the other metal for 5 weeks. To assess if pre-exposure enhances tolerance, resting respiration was measured just before and 6 weeks after the toxicants were interchanged, survivorship and reproduction were monitored (offspring and eggs count and measured) and total lipids quantified.

The second experiment, which had two parts, was initiated to test whether the tolerance of the second generation was affected by the prehistory of their parents. The first part of the experiment tested the responses of offspring born to animals exposed to the toxicant until the offspring appeared. Here eggs and embryos of the second generation were pre-exposed to the toxicant and the offspring were raised in water containing the same contaminant that was experienced by their parents (Fig. 4.2). The second part tested animals whose parents were removed from the contaminated solution before the eggs and embryos developed (Fig. 4.3) thus eggs and embryos were not pre-exposed. Soon after the offspring were born they were put into the solution containing the same toxicant, at the same

Fig. 4.2 Treatment combinations for tolerance testing of the second generation *Hyalella azteca* where eggs and embryos were pre-exposed.

Solution to which parents were exposed.

Solution to which offspring were exposed.

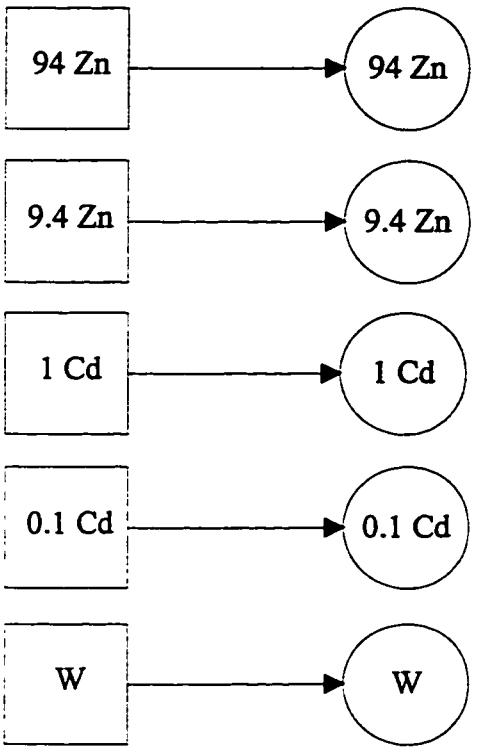
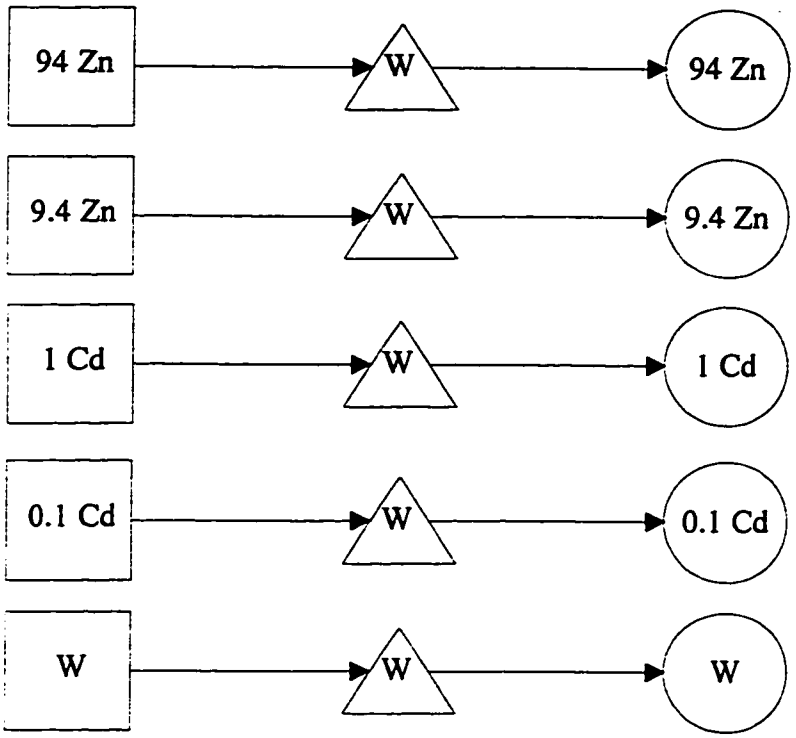


Fig. 4.3 Treatment combinations for tolerance testing of the second generation *Hyalella azteca* where eggs and embryos were not pre-exposed.

Solution to which parents were exposed.

Solution into which parents were put before eggs developed and offspring appeared.

Solution to which offspring were exposed.



concentration, experienced by their parents. When the animals matured, reproduction was monitored and the animals were freeze-dried to determine total lipid levels. There was a control and three replicates for each combination of treatments.

The animals used in this experiment were obtained from the same cultures used in the chronic testing. Test solutions were prepared and food and cotton substrate were provided as described in Chapter 2. During the experiment animals were kept in a light- and temperature-controlled incubator under the same conditions as described in Chapter 2.

4.3. Statistical analysis

The data were first tested for normality using the Kolmogorov-Smirnov one sample test on studentized residuals. The Fmax test was used to test for heteroscedasticity. To test the effect of Cd and Zn on survivorship, fecundity, respiration and lipid stores, data were analysed using a single-factor ANOVA. When the null hypothesis of equality of treatment means was rejected, the Tukey multiple pairwise comparison test was performed to determine between which treatment means differences existed. The statistical analysis were performed using Systat version 7.0 (Wilkinson, 1997).

In many cases there was an overall pattern of treatment responses being consistently larger or smaller than the control, however ANOVA did not have sufficient power to detect many significant differences. The overall pattern was analysed by using a binomial expansion to estimate the probability of getting the observed pattern or one more extreme under the null hypothesis that the probability of a reading being higher than the control was equal to the probability of it being lower than the control. Significance for all tests was determined at a

significance level of α=0.05.

4.4. Results (First Generation)

4.4.1. Survivorship

The overall survival of pre-exposed animals was significantly affected by Cd and Zn exposure (ANOVA; df=22,46; F=3.41; $p<0.05$). In 20 out of 22 treatment combinations survival was not significantly different from the W-W control (Table 4.1). The significant differences were observed in only the W-1.0 $\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ treatment combinations which are the specimens which did not receive pre-exposure.

In all cases in which organisms received a metal pre-exposure followed by a 1.0 $\mu gCd \cdot L^{-1}$ or 94 $\mu gZn \cdot L^{-1}$ exposure (Fig. 4.4, 4.5) survival was intermediate between the W-W control and the W-1.0$\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ treatments. The binomial expansion indicates that the probability of all 14 pre-exposed treatments being less than control is $6 \cdot 10^{-5}$. The probability of these treatments being greater than the W-1.0 $\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ treatments is $6 \cdot 10^{-5}$. It therefore appears that pre-exposure increased survival, however protection was not complete.

In all the cases where organisms received a metal pre-exposure followed by 1.0 $\mu gCd \cdot L^{-1}$ or 94 $\mu gZn \cdot L^{-1}$, mortality was significantly (ANOVA; df=15, 128; F=56.6; $p<0.05$) lower than in the treatments where animals were continuously exposed to 1.0 $\mu gCd \cdot L^{-1}$ or 94 $\mu gZn \cdot L^{-1}$ (Fig. 4.6, 4.7). Thus pre-exposure decreased mortality of pre-treated animals.

Table 4.1 Survivorship (%; mean± standard error; n=3) of *Hyalella azteca* after changing toxicant . * Significantly different from W-W control; $p<0.05$

Treatment ($\mu g \cdot L^{-1}$)	**Survivorship (%)**
W-W	90.0 (±2.7)
W-1.0 Cd	70.0 (±2.4)*
W-94 Zn	70.0 (±2.4)*
W-0.1 Cd	81.7 (±3.6)
W-9.4 Zn	81.7 (±1.4)
9.4 Zn-94 Zn	75.0 (±2.4)
9.4 Zn-1.0 Cd	80.0 (±2.4)
9.4 Zn-W	88.3 (±1.4)
9.4 Zn-W-1.0 Cd	83.3 (±2.7)
9.4 Zn-W-94 Zn	86.7 (±1.4)
94 Zn-1.0 Cd	86.7 (±4.9)
94 Zn-W	81.7 (±1.4)
94 Zn-W-1.0 Cd	81.7 (±1.4)
94 Zn-W-94 Zn	83.3 (±3.6)
0.1 Cd-1.0 Cd	81.7 (±1.4)
0.1 Cd-94 Zn	83.3 (±3.6)
0.1 Cd-W	91.7 (±1.4)
0.1 Cd-W-94 Zn	83.3 (±3.6)
0.1 Cd-W-1.0 Cd	83.3 (±3.6)
1.0 Cd-94 Zn	86.7 (±1.4)
1.0 Cd-W	91.7 (±1.4)
1.0 Cd-W-94Zn	81.7 (±2.7)
1.0 Cd-W-1.0 Cd	86.7 (±1.4)

Fig. 4.4 Survivorship (%; mean±standard error; n=3) of *Hyalella azteca* after six weeks exposure to 1.0 $\mu gCd \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-1.0 $\mu gCd \cdot L^{-1}$ are included for comparison.

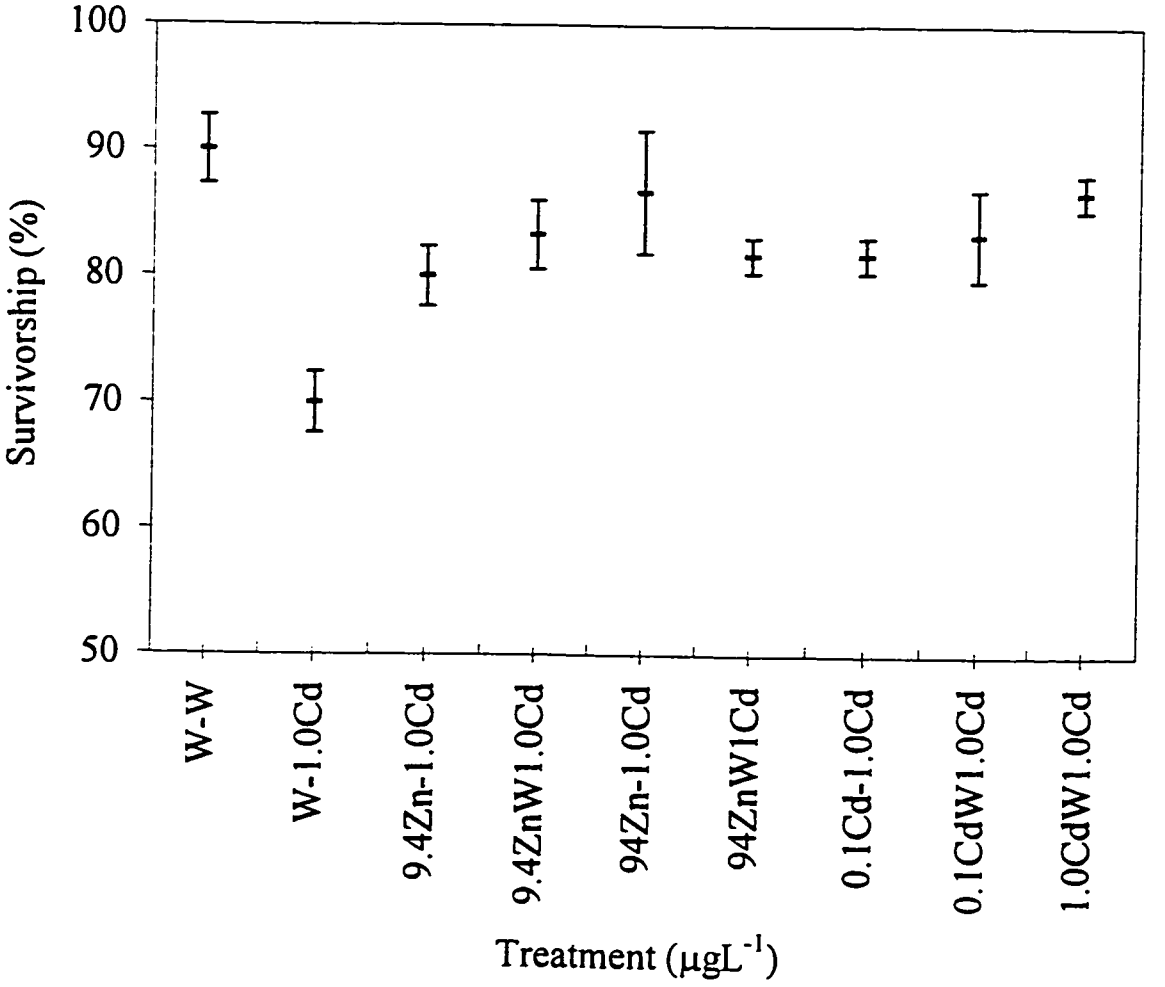

100
90
80
70
60
50
Survivorship (%)
W-W
W-1.0Cd
9.4Zn-1.0Cd
9.4ZnW1.0Cd
94Zn-1.0Cd
94ZnW1Cd
0.1Cd-1.0Cd
0.1CdW1.0Cd
1.0CdW1.0Cd
Treatment (µgL-1)

Fig. 4.5 Survivorship (%; mean±standard error; n=3) of *Hyalella azteca* after six weeks exposure to 94 $\mu gZn \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-94 $\mu gZn \cdot L^{-1}$ are included for comparison.

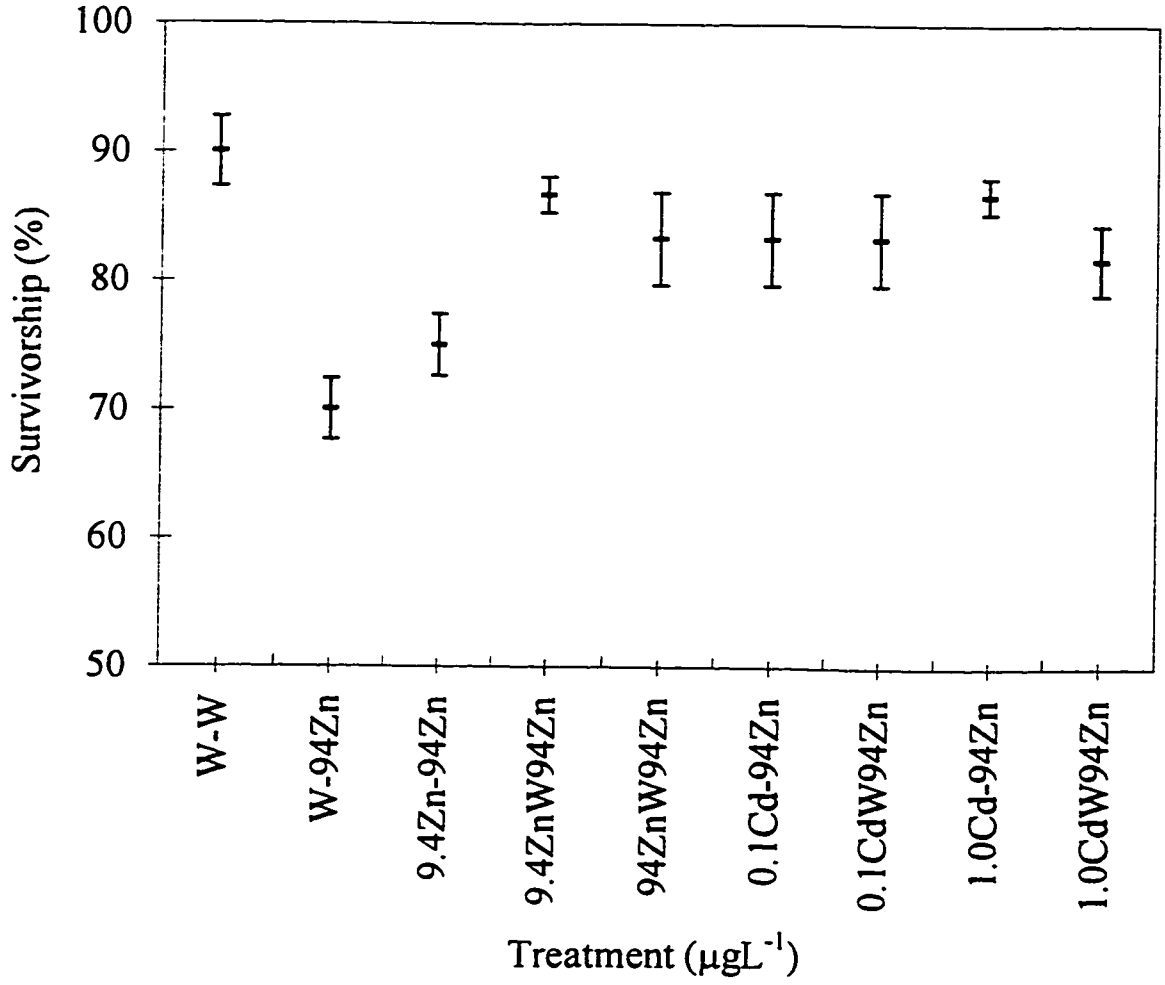

100
90
80
70
60
50
Survivorship (%)
W-W
W-94Zn
9.4Zn-94Zn
9.4ZnW94Zn
94ZnW94Zn
0.1Cd-94Zn
0.1CdW94Zn
1.0Cd-94Zn
1.0CdW94Zn
Treatment (μgL-1)

Fig. 4.6 Mortality (%; mean±standard error; n=3) of *Hyalella azteca* after six weeks exposure to 1.0 $\mu gCd \cdot L^{-1}$ following a heavy metal pre-exposure. Mortality of *H. azteca* continuously exposed to 1.0 $\mu gCd \cdot L^{-1}$ (1.0 Cd-1.0 Cd) for nine weeks is included for comparison.

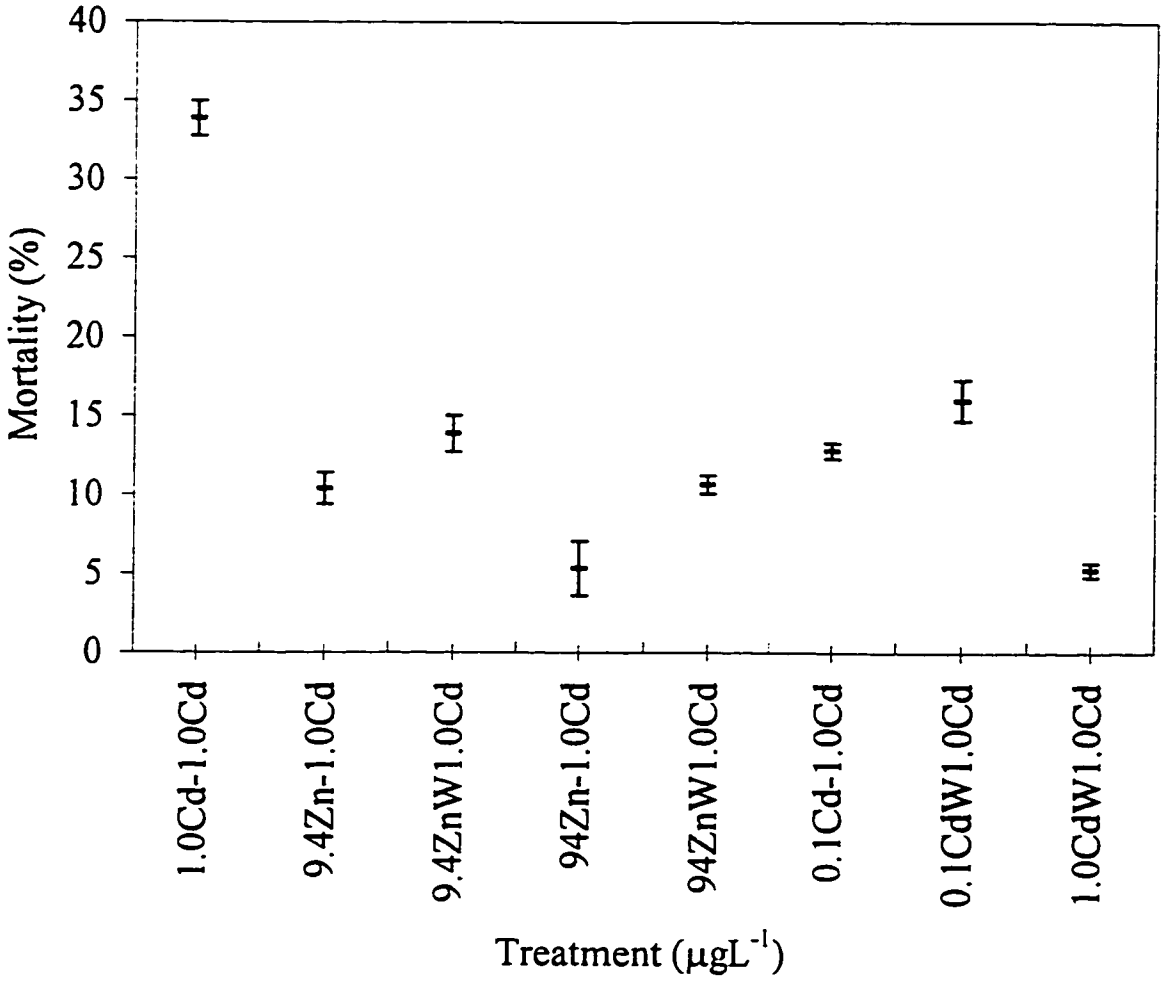
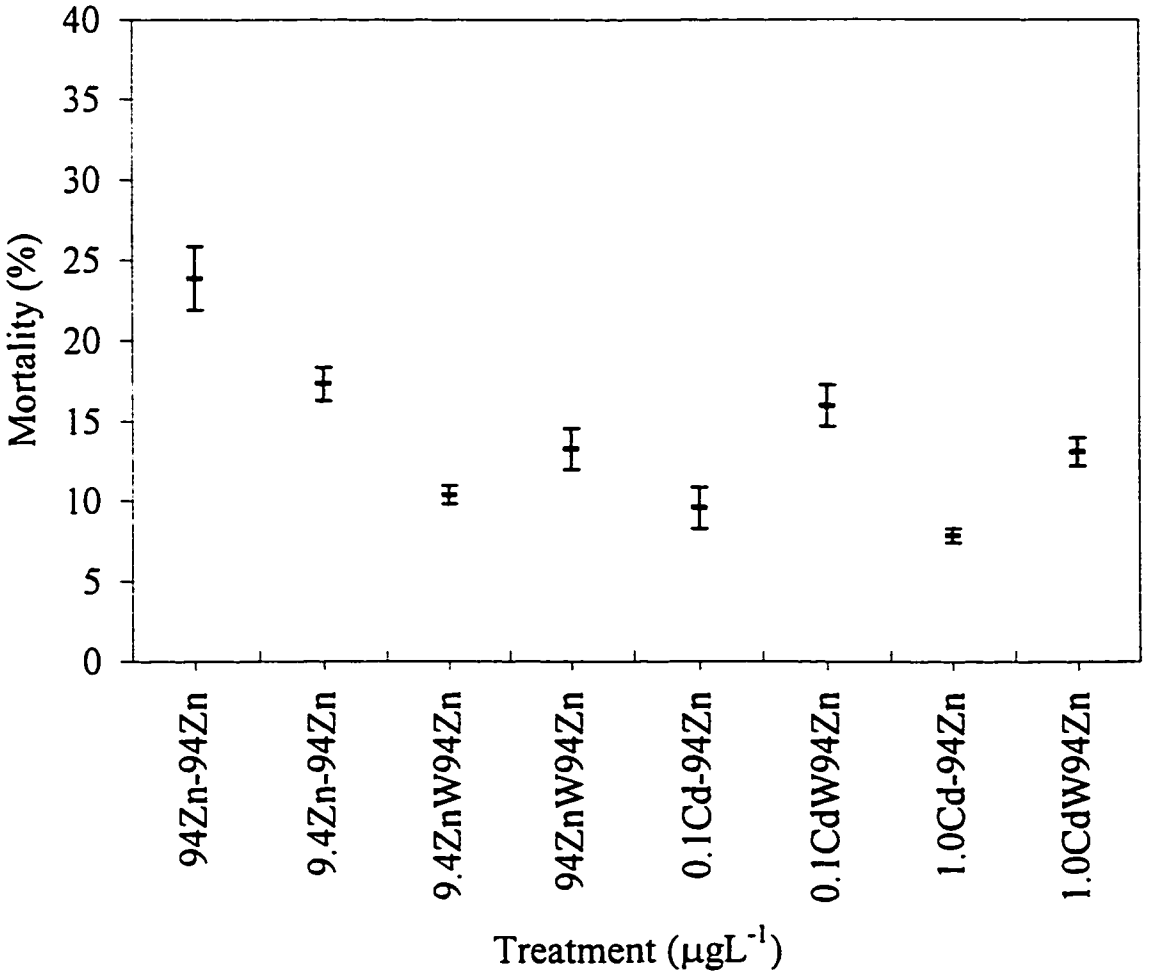

40
35
30
25
20
15
10
5
0
Mortality (%)
1.0Cd-1.0Cd
9.4Zn-1.0Cd
9.4ZnW1.0Cd
94Zn-1.0Cd
94ZnW1.0Cd
0.1Cd-1.0Cd
0.1CdW1.0Cd
1.0CdW1.0Cd
Treatment (μgL-1)

Fig. 4.7 Mortality (%; mean±standard error; n=3) of *Hyalella azteca* after six weeks exposure to 94 $\mu gZn \cdot L^{-1}$ following a heavy metal pre-exposure. Mortality of *H. azteca* continuously exposed to 94 $\mu gZn \cdot L^{-1}$ (94Zn-94Zn) for nine weeks is included for comparison.


40
35
30
25
20
15
10
5
0
Mortality (%)
94Zn-94Zn
9.4Zn-94Zn
9.4ZnW94Zn
94ZnW94Zn
0.1Cd-94Zn
0.1CdW94Zn
1.0Cd-94Zn
1.0CdW94Zn
Treatment (μgL-1)

4.4.2. Resting Respiration

The size-specific resting respiration of the amphipods exposed to Cd and Zn treatments before changing the toxicant showed a significant treatment effect (ANOVA; df=4,10; F=54.80; $p<0.05$). There was a significant difference observed between the control and all the treatments (Fig. 4.8).

The resting respiration of animals after changing the toxicant showed a significant treatment effect (ANOVA; df=22,46; F=4.78; $p<0.05$). In all but three cases resting respiration of pre-exposed animals did not differ significantly from the W-W control (Table 4.2). Significantly increased resting respiration was observed in the animals exposed to following combinations of the treatments: W-94 $\mu gZn \cdot L^{-1}$, 0.1 $\mu gCd \cdot L^{-1}$-W-1.0 $\mu gCd \cdot L^{-1}$ and W-1.0 $\mu gCd \cdot L^{-1}$.

In all cases in which organisms received a metal pre-exposure followed by a 1.0 $\mu gCd \cdot L^{-1}$ or 94 $\mu gZn \cdot L^{-1}$ exposure (Fig. 4.9, 4.10) resting respiration was intermediate between the W-W control and the W-1.0 $\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ treatments. The binomial expansion indicates that the probability of all 14 pre-exposed treatments being greater than control is $6 \cdot 10^{-5}$ and the probability of all these treatments being lower than the W-1.0 $\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ is also $6 \cdot 10^{-5}$. It therefore appears that pre-exposure lowers resting respiration, however this protection is not sufficient to keep it at the level of unstressed animals.

Fig. 4.8 Size-specific resting respiration ($\mu LO_2 \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* after three weeks of exposure to clean water (control) and two Cd (0.1 and 1.0 $\mu gCd \cdot L^{-1}$) and two Zn (9.4 and 94 $\mu gZn \cdot L^{-1}$) concentrations.

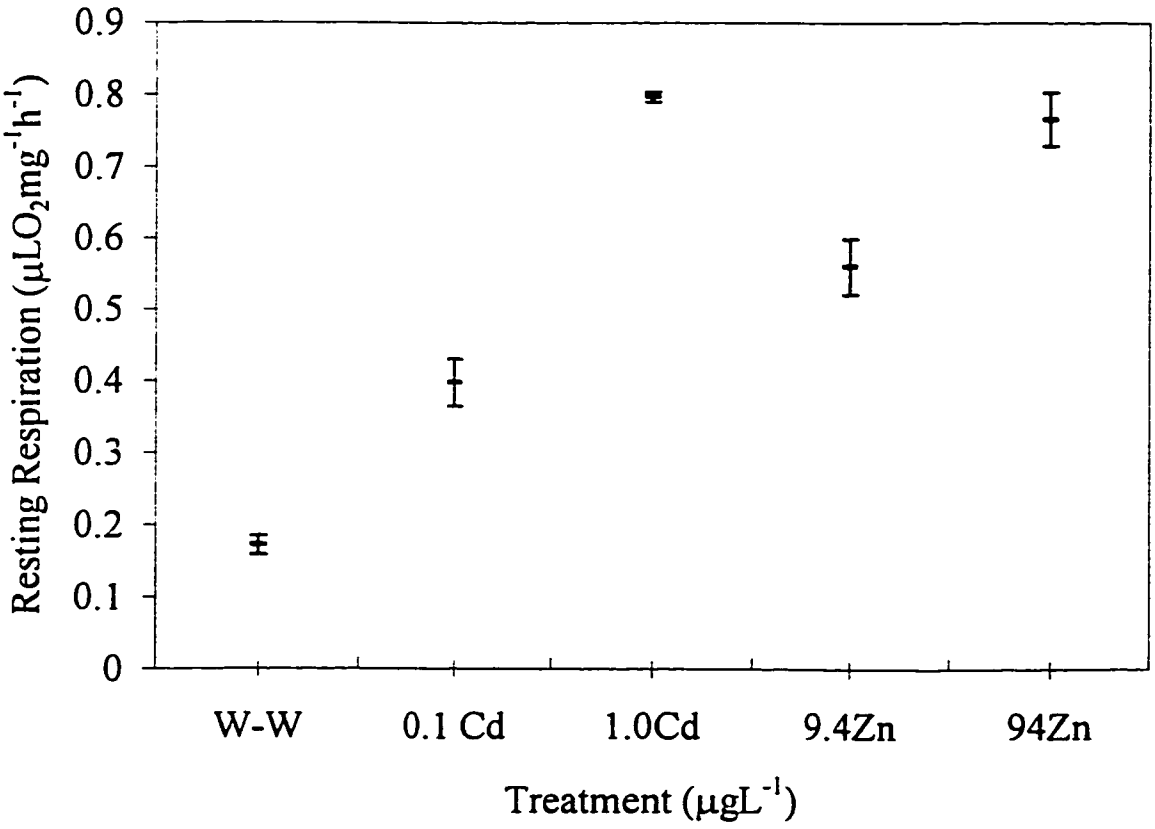

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Resting Respiration (μLO2mg-1h-1)
W-W
0.1 Cd
1.0Cd
9.4Zn
94Zn
Treatment (μgL-1)

Table 4.2 Resting respiration rate ($\mu LO_2 \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* after changing toxicant. *Significantly different from W-W control; $p<0.05$

Treatment ($\mu g \cdot L^{-1}$)	Resting respiration ($\mu LO_2 \cdot mg^{-1} \cdot h^{-1}$)
W-W	0.143(±0.021)
W-1.0 Cd	0.507(±0.033)*
W-94 Zn	0.501(±0.036)*
W-0.1 Cd	0.137(±0.024)
W-9.4 Zn	0.230(±0.036)
9.4 Zn-94 Zn	0.233(±0.005)
9.4 Zn-1.0 Cd	0.320(±0.052)
9.4 Zn-W	0.303(±0.020)
9.4 Zn-W-1.0 Cd	0.160(±0.005)
9.4 Zn-W-94 Zn	0.410(±0.057)
94 Zn-1.0 Cd	0.213(±0.057)
94 Zn-W	0.260(±0.049)
94 Zn-W-1.0 Cd	0.167(±0.017)
94 Zn-W-94 Zn	0.230(±0.008)
0.1 Cd-1.0 Cd	0.250(±0.056)
0.1 Cd-94 Zn	0.233(±0.043)
0.1 Cd-W	0.360(±0.061)
0.1 Cd-W-94 Zn	0.307(±0.052)
0.1 Cd-W-1.0 Cd	0.487(±0.069)*
1.0 Cd-94 Zn	0.257(±0.066)
1.0 Cd-W	0.343(±0.067)
1.0 Cd-W-94Zn	0.227(±0.014)
1.0 Cd-W-1.0 Cd	0.170(±0.009)

Fig. 4.9 Size-specific resting respiration ($\mu LO_2 \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* after six weeks exposure to 1.0 $\mu gCd \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-1.0 $\mu gCd \cdot L^{-1}$ are included for comparison.

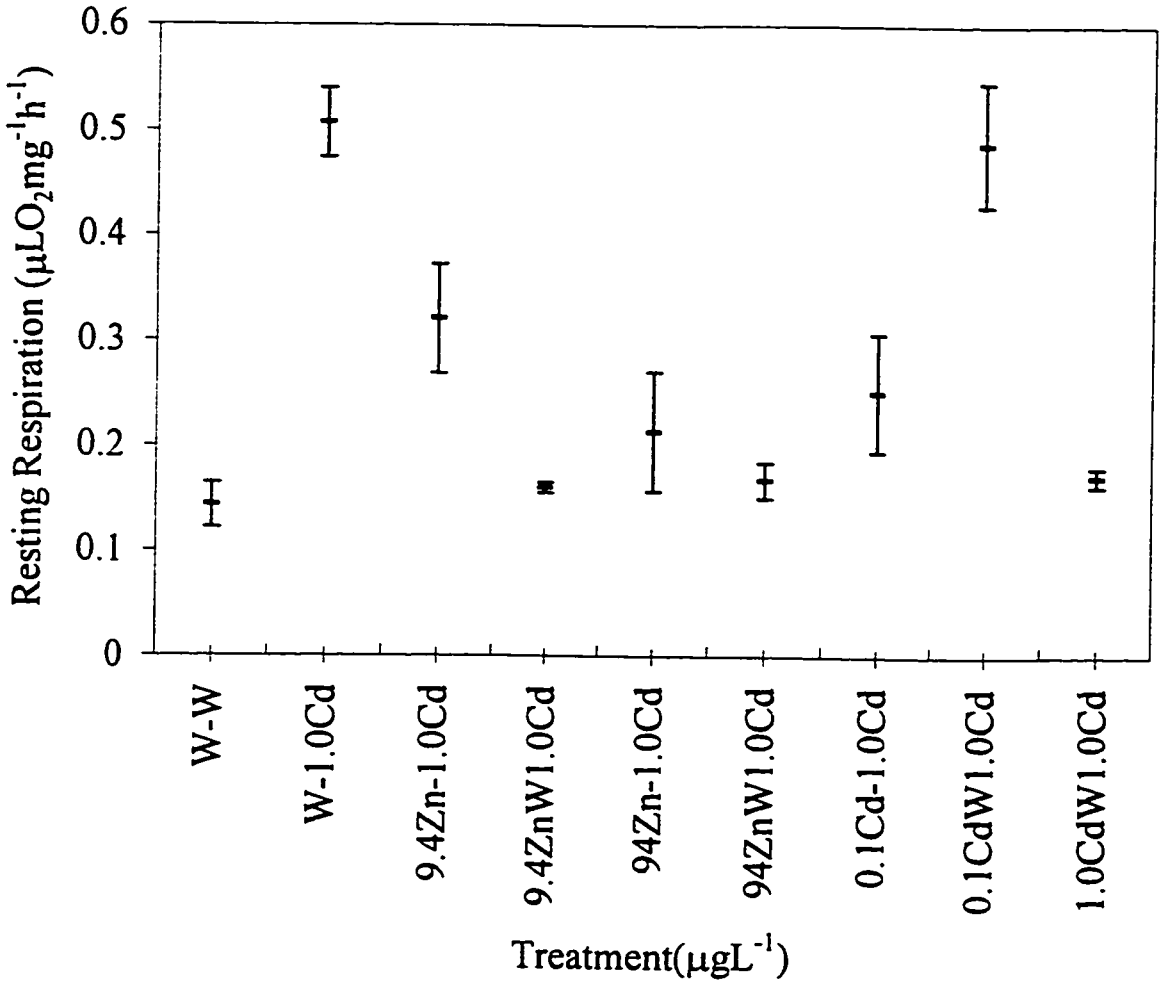
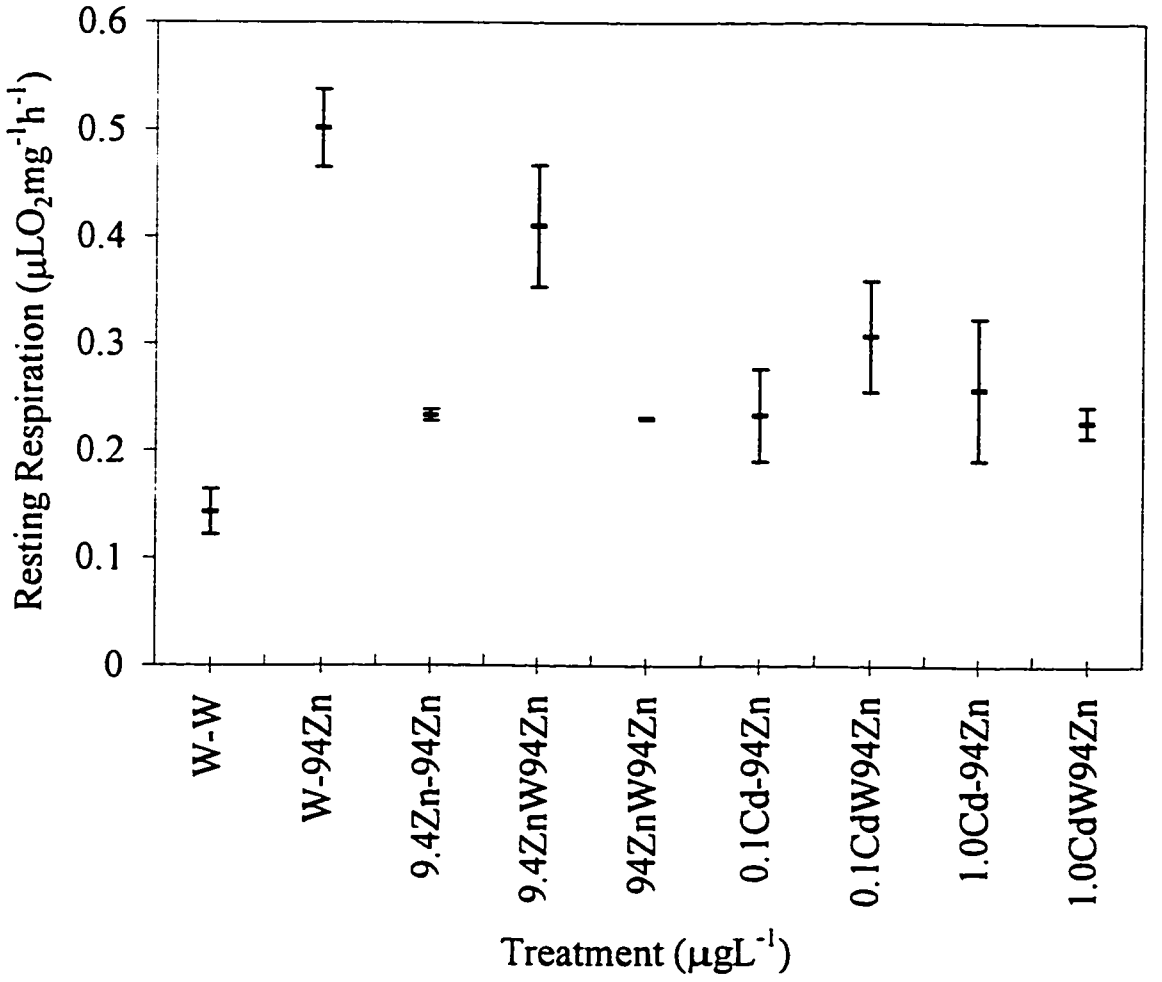

Resting Respiration ($\mu LO_2 mg^{-1} h^{-1}$)
0.6
0.5
0.4
0.3
0.2
0.1
0
W-W
W-1.0Cd
9.4Zn-1.0Cd
9.4ZnW1.0Cd
94Zn-1.0Cd
94ZnW1.0Cd
0.1Cd-1.0Cd
0.1CdW1.0Cd
1.0CdW1.0Cd
Treatment(μgL^{-1})

Fig. 4.10 Size-specific resting respiration ($\mu LO_2 \cdot mg^{-1} \cdot h^{-1}$; mean±standard error; n=3) of *Hyalella azteca* after six weeks exposure to 94 $\mu gZn \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-94 $\mu gZn \cdot L^{-1}$ are included for comparison.


Resting Respiration ($\mu LO_2 mg^{-1}h^{-1}$)
0.6
0.5
0.4
0.3
0.2
0.1
0
W-W
W-94Zn
9.4Zn-94Zn
9.4ZnW94Zn
94ZnW94Zn
0.1Cd-94Zn
0.1CdW94Zn
1.0Cd-94Zn
1.0CdW94Zn
Treatment (μgL^{-1})

4.4.3. Fecundity

There was an overall significant effect of Cd and Zn on the number of eggs produced (ANOVA; df=22; 115; F=3.62; $p<0.05$), but significant decreases were only observed in the following cases: 9.4 $\mu gZn \cdot L^{-1}$-94 $\mu gZn \cdot L^{-1}$, 0.1 $\mu gCd \cdot L^{-1}$- 1.0$\mu gCd \cdot L^{-1}$, 1.0$\mu gCd \cdot L^{-1}$-94 $\mu Zn \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ (Table 4.3).

The pattern for egg production is similar to the pattern for survival and respiration, with treatments that received pre-exposure being intermediate to the W-W control and the treatments with no pre-exposure (Fig. 4.11, 4.12). All 14 pre-exposed treatments had lower egg production than the W-W control with probability of $6 \cdot 10^{-5}$. The number of eggs produced in the W-1.0$\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ treatments was reduced in all but one case compared to the 14 corresponding treatments with pre-exposure. The binomial expansion showed that the probability of this pattern, or one more extreme was $9 \cdot 10^{-4}$. Thus, exposure to 1.0$\mu gCd \cdot L^{-1}$ and 94 $\mu gZn \cdot L^{-1}$ without pre-exposure significantly reduces the number of eggs produced. There was no difference observed in the size of eggs in any of the treatments.

ANOVA showed an overall significant effect of Cd and Zn on the number of offspring born (ANOVA; df=22; 115; $p<0.05$), but significant decreases from the W-W control were observed only in the following treatment combinations: 9.4 $\mu gZn \cdot L^{-1}$-94 $\mu gZn \cdot L^{-1}$, 9.4 $\mu gZn \cdot L^{-1}$- W-94 $\mu gZn \cdot L^{-1}$, 0.1 $\mu gCd \cdot L^{-1}$-1.0 $\mu gCd \cdot L^{-1}$, 1.0 $\mu gCd \cdot L^{-1}$-94 $\mu gZn \cdot L^{-1}$, W-1.0$\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ (Table 4.4). The number of offspring born in the W-1.0 $\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ was reduced in all but one case compared to the 14

Table 4.3 Mean number of eggs produced (±standard error; n=6) by *Hyalella azteca* after changing toxicant. *Significantly different from W-W control; $p<0.05$

Treatment ($\mu g \cdot L^{-1}$)	Eggs (number)
W-W	16.6 (±1.1)
W-1.0 Cd	9.5 (±2.8)
W-94 Zn	8.2 (±1.1)*
W-0.1 Cd	12.5 (±1.9)
W-9.4 Zn	17.8 (±2.2)
9.4 Zn-94 Zn	8.8 (±0.6)*
9.4 Zn-1.0 Cd	12.3 (±2.4)
9.4 Zn-W	13.0 (±1.8)
9.4 Zn-W-1.0 Cd	9.5 (±0.7)
9.4 Zn-W-94 Zn	13.8 (±2.2)
94 Zn-1.0 Cd	14.2 (±3.6)
94 Zn-W	16.0 (±2.2)
94 Zn-W-1.0 Cd	14.0 (±1.5)
94 Zn-W-94 Zn	12.0 (±1.5)
0.1 Cd-1.0 Cd	7.8 (±0.9)*
0.1 Cd-94 Zn	10.8 (±1.7)
0.1 Cd-W	13.3 (±2.3)
0.1 Cd-W-94 Zn	11 2 (±1.4)
0.1 Cd-W-1.0 Cd	13.7 (±1.2)
1.0 Cd-94 Zn	8.2 (±1.3)*
1.0 Cd-W	11.7 (±1.7)
1.0 Cd-W-94Zn	14.3 (±2.1)
1.0 Cd-W-1.0 Cd	13.0 (±1.3)

Fig. 4.11 Eggs produced (number; mean±standard error; n=6) by *Hyalella azteca* after six weeks of exposure to 1.0 $\mu gCd \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-1.0 $\mu gCd \cdot L^{-1}$ are included for comparison.

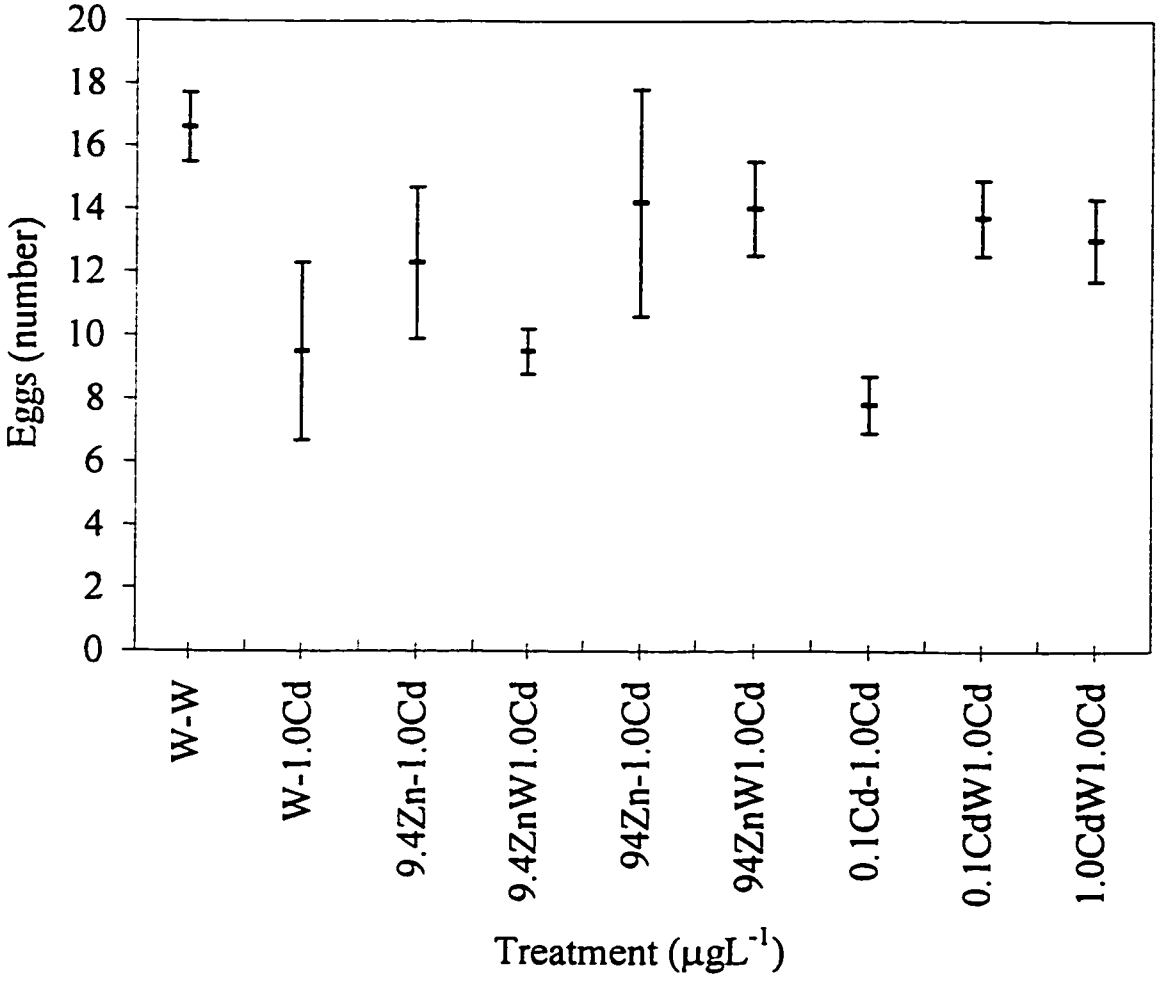

20
18
16
14
12
10
8
6
4
2
0
Eggs (number)
W-W
W-1.0Cd
9.4Zn-1.0Cd
9.4ZnW1.0Cd
94Zn-1.0Cd
94ZnW1.0Cd
0.1Cd-1.0Cd
0.1CdW1.0Cd
1.0CdW1.0Cd
Treatment (μgL-1)

Fig. 4.12 Eggs produced (number; mean±standard error; n=6) by *Hyalella azteca* after six weeks of exposure to 94 $\mu gZn \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-94 $\mu gZn \cdot L^{-1}$ are included for comparison.

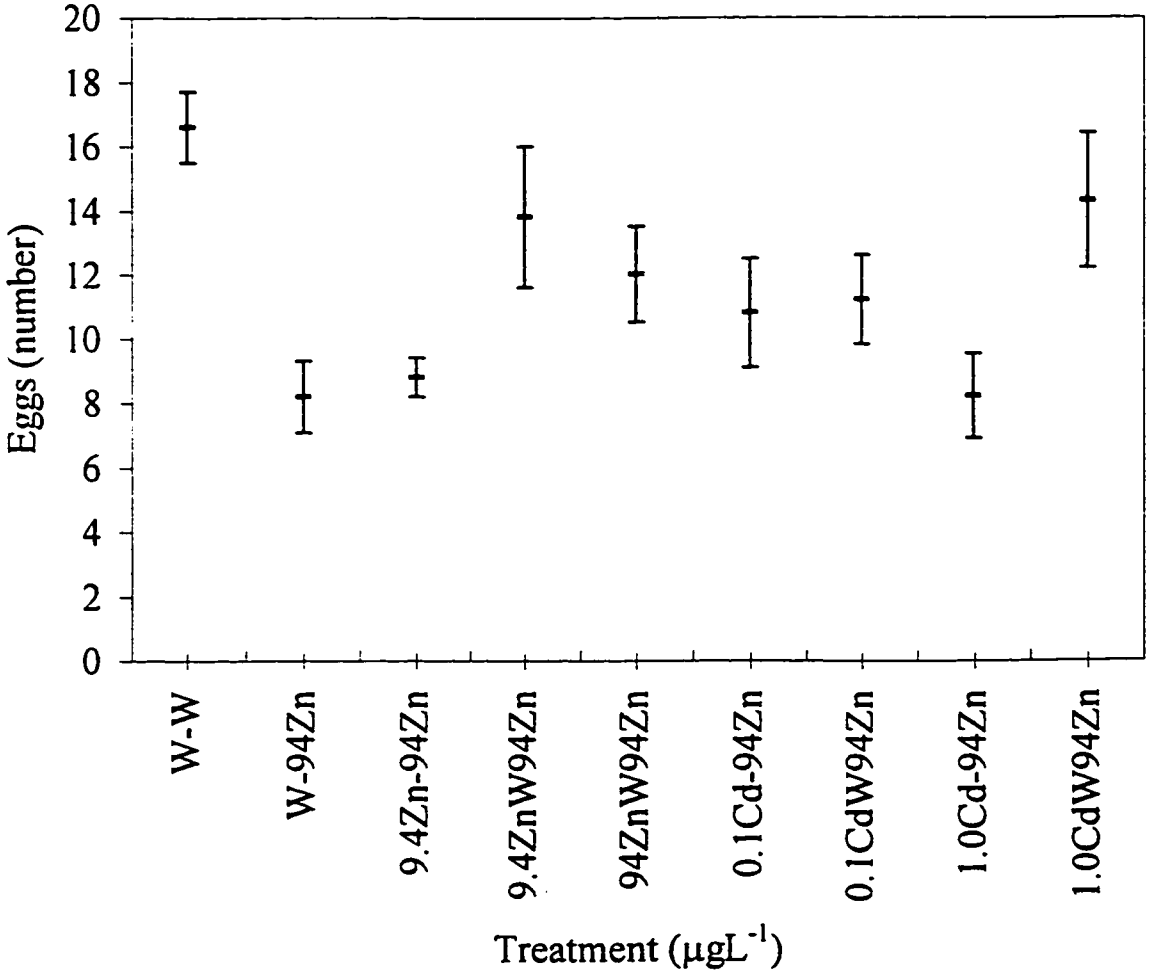

Eggs (number)
0
2
4
6
8
10
12
14
16
18
20
W-W
W-94Zn
9.4Zn-94Zn
9.4ZnW94Zn
94ZnW94Zn
0.1Cd-94Zn
0.1CdW94Zn
1.0Cd-94Zn
1.0CdW94Zn
Treatment (μgL-1)

Table 4.4 Mean number of offspring born (±standard error; n=6) to *Hyalella azteca* after changing toxicant. * Significantly different from W-W control; $p<0.05$

Treatment ($\mu g \cdot L^{-1}$)	Offspring (number)
W-W	13.7 (±1.9)
W-1.0 Cd	7.0 (±2.2)*
W-94 Zn	6.7 (±1.8)*
W-0.1 Cd	11.7 (±2.5)
W-9.4 Zn	15.8 (±2.7)
9.4 Zn-94 Zn	8.7 (±0.8)*
9.4 Zn-1.0 Cd	10.0 (±2.0)
9.4 Zn-W	10.2 (±2.2)
9.4 Zn-W-1.0 Cd	9.5 (±1.0)
9.4 Zn-W-94 Zn	8.0 (±0.6)*
94 Zn-1.0 Cd	11.5 (±1.8)
94 Zn-W	14.7 (±1.2)
94 Zn-W-1.0 Cd	9.7 (±2.4)
94 Zn-W-94 Zn	10.0 (±1.2)
0.1 Cd-1.0 Cd	7.5 (±0.6)*
0.1 Cd-94 Zn	9.0 (±1.7)
0.1 Cd-W	8.7 (±1.2)
0.1 Cd-W-94 Zn	10.5 (±1.5)
0.1 Cd-W-1.0 Cd	10.8 (±1.3)
1.0 Cd-94 Zn	5.3 (±1.2)*
1.0 Cd-W	11.7 (±1.7)
1.0 Cd-W-94Zn	9.3 (±2.0)
1.0 Cd-W-1.0 Cd	11.8 (±1.2)

corresponding treatments (Fig. 4.13, 4.14). The binomial expansion showed the probability of this pattern, or one more extreme was $9 \cdot 10^{-4}$. All 14 pre-exposed treatments were lower than the W-W control with a probability of $6 \cdot 10^{-5}$. Thus, the exposure to 10% LC_{50} of Cd and Zn in the mature stage lowers the number of offspring born to animals that have not been pre-exposed, but any amount of exposure reduces fecundity relative to unexposed animals (W-W). No difference was observed between the treatments and the control when comparing the size of the offspring.

4.4.4. Total Lipids

There was a significant overall treatment effect (ANOVA; df=22,46; F=15.94; $p<0.05$) on the size specific total lipid content of exposed animals. The Tukey analysis showed that the lipid content was not significantly different from the W-W control in 72% of the cases (Table, 4.5). A significant increase in lipid content was found in the animals exposed to the following treatments: 9.4 $\mu gZn \cdot L^{-1}$-94 $\mu gZn \cdot L^{-1}$, 9.4 $\mu gZn \cdot L^{-1}$-W-94 $\mu gZn \cdot L^{-1}$, 0.1 $\mu gCd \cdot L^{-1}$-1.0 $\mu gCd \cdot L^{-1}$, 1.0 $\mu gCd \cdot L^{-1}$-94 $\mu gZn \cdot L^{-1}$, W-1.0 $\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$.

The size-specific lipid content of the 14 pre-exposed treatments increased relative to the W-W control in all cases (Fig. 4.15, 4.16). The binomial expansion showed that the probability of this pattern was $6 \cdot 10^{-5}$. The total lipid content of animals in W-1.0 $\mu gCd \cdot L^{-1}$ and W-94 $\mu gZn \cdot L^{-1}$ treatments was higher in all but one case from animals in the 14 corresponding treatments. The binomial expansion showed that the probability of this pattern, or one more extreme was $9 \cdot 10^{-4}$. It appears that the exposure of animals without pre-treatment to higher concentrations of Cd and Zn increases lipid levels of exposed animals.

Fig. 4.13 Offspring born (number; mean±standard error; n=6) to *Hyalella azteca* after six weeks of exposure to 1.0 $\mu gCd \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-1.0 $\mu gCd \cdot L^{-1}$ are included for comparison.

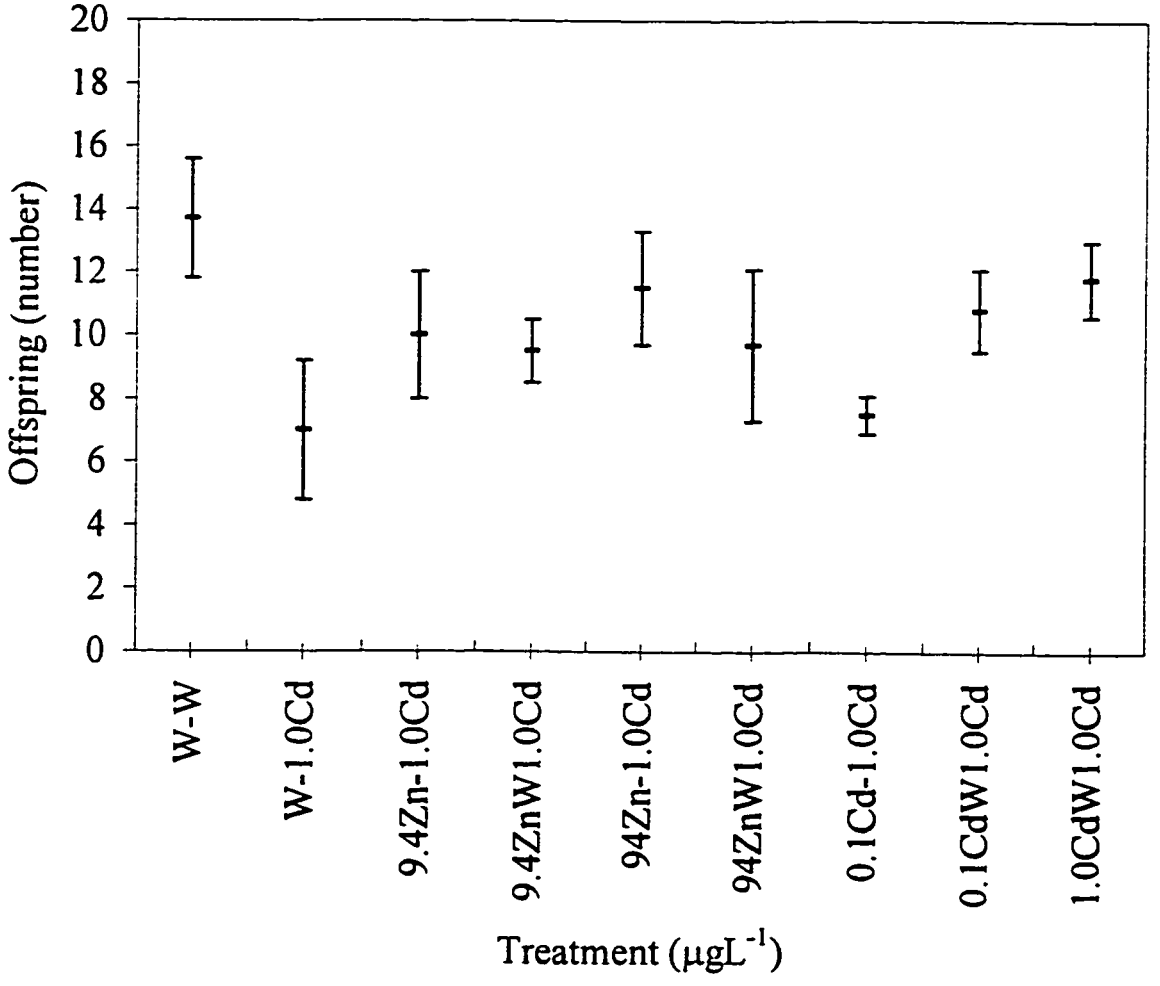

20
18
16
14
12
10
8
6
4
2
0
Offspring (number)
W-W
W-1.0Cd
9.4Zn-1.0Cd
9.4ZnW1.0Cd
94Zn-1.0Cd
94ZnW1.0Cd
0.1Cd-1.0Cd
0.1CdW1.0Cd
1.0CdW1.0Cd
Treatment (μgL-1)

Fig. 4.14 Offspring born (number; mean±standard error; n=6) to *Hyalella azteca* after six weeks of exposure to 94 $\mu gZn \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-94 $\mu gZn \cdot L^{-1}$ are included for comparison.

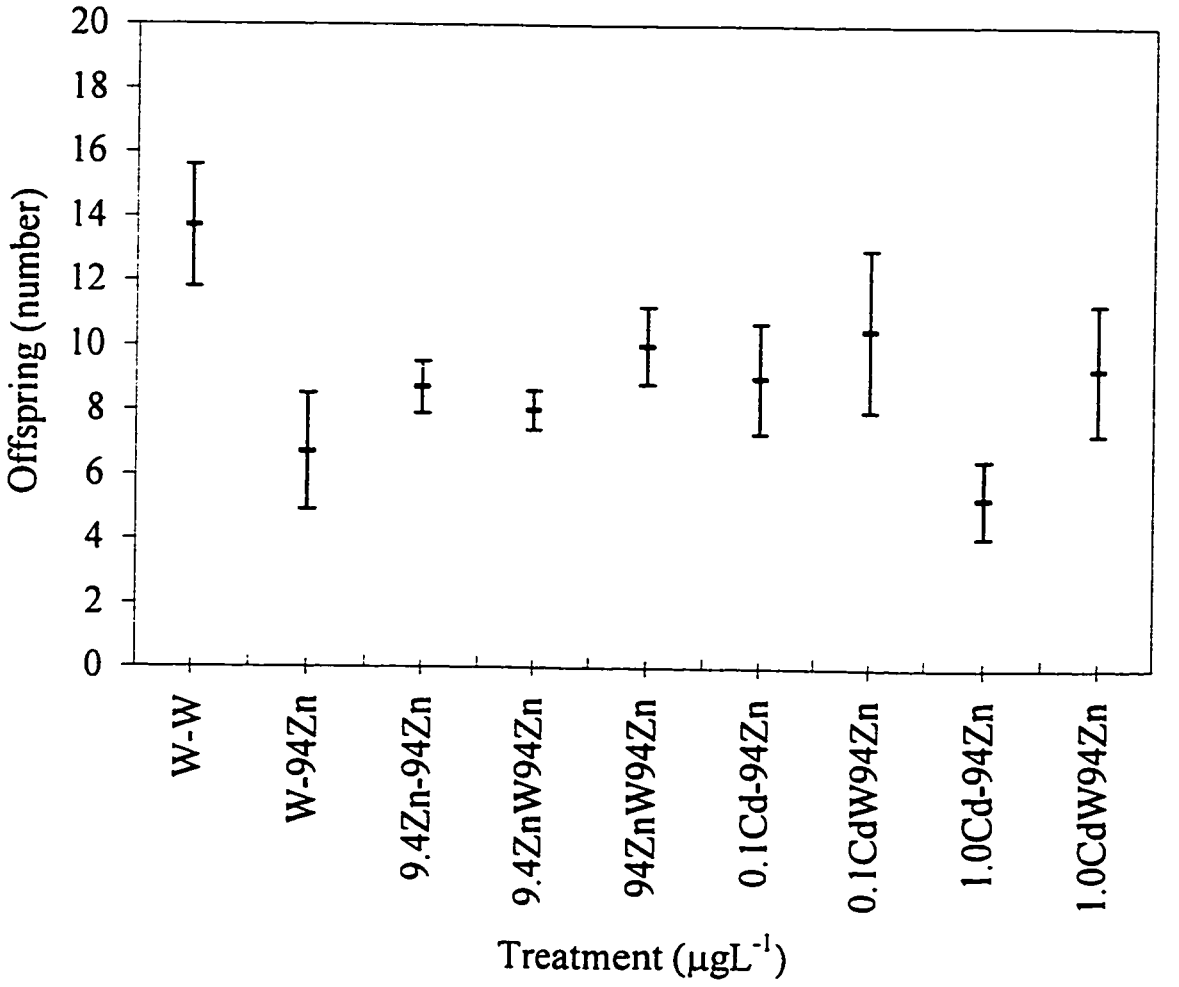

Offspring (number)
0
2
4
6
8
10
12
14
16
18
20
W-W
W-94Zn
9.4Zn-94Zn
9.4ZnW94Zn
94ZnW94Zn
0.1Cd-94Zn
0.1CdW94Zn
1.0Cd-94Zn
1.0CdW94Zn
Treatment (μgL-1)

Table 4.5 Mean total lipid content (%; ±standard error; n=3) of *Hyalella azteca* after changing toxicant. *Significantly different from the W-W control; $p<0.05$.

Treatment ($\mu g \cdot L^{-1}$)	**Total lipid content (%)**
W-W	3.4 (±0.3)
W-1 Cd	16.6 (±0.5) *
W-94 Zn	12.2 (±1.1) *
W-9.4 Zn	5.2 (±0.8)
W-0.1 Cd	5.9 (±0.5)
9.4 Zn-94 Zn	10.4 (±0.8) *
9.4 Zn-1 Cd	6.7 (±0.7) *
9.4 Zn-W	6.0 (±1.1)
9.4 Zn-W-1Cd	4.4 (±0.5)
9.4 Zn-W-94 Zn	8.7 (±0.1) *
94 Zn-1Cd	6.1 (±1.2)
94 Zn-W	6.3 (±1.3)
94 Zn-W-1 Cd	4.3 (±0.5)
94 Zn-W-94 Zn	6.2 (±0.6)
0.1 Cd-1 Cd	15.2 (±0.4) *
0.1 Cd-94 Zn	6.2 (±0.1)
0.1 Cd-W	6.9 (±0.9)
0.1 Cd-W-94 Zn	7.5 (±0.2)
0.1 Cd-W-1 Cd	7.8 (±0.9)
1Cd-94 Zn	16.0 (±0.7) *
1Cd-W	5.8 (±0.1)
1 Cd-W-94Zn	5.8 (±0.7)
1 Cd-W-1 Cd	5.4 (±0.9)

Fig. 4.15 Total lipid content (%; mean±standard error; n=3) of *Hyalella azteca* after six weeks of exposure to 1.0 $\mu gCd \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-1.0 $\mu gCd \cdot L^{-1}$ are included for comparison.

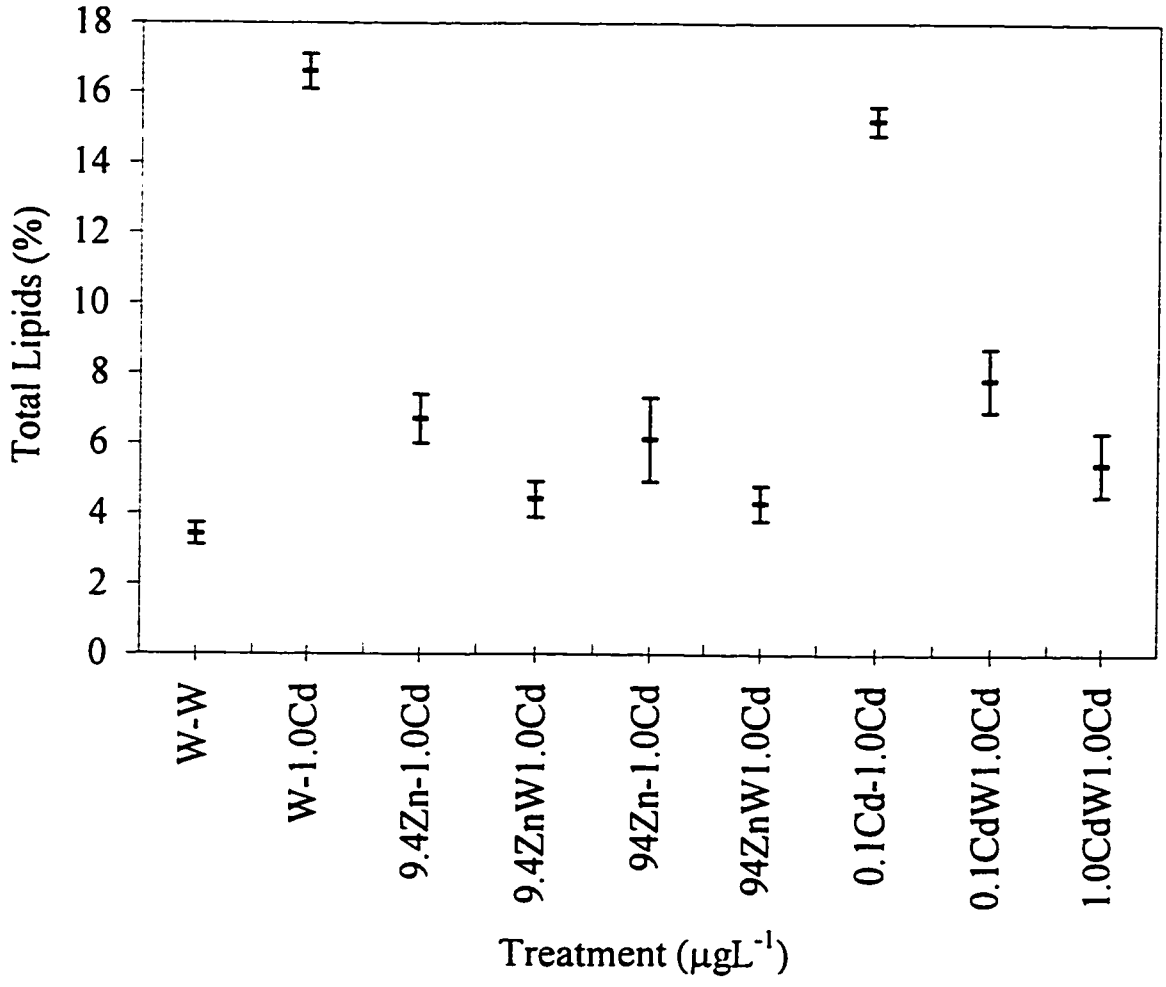
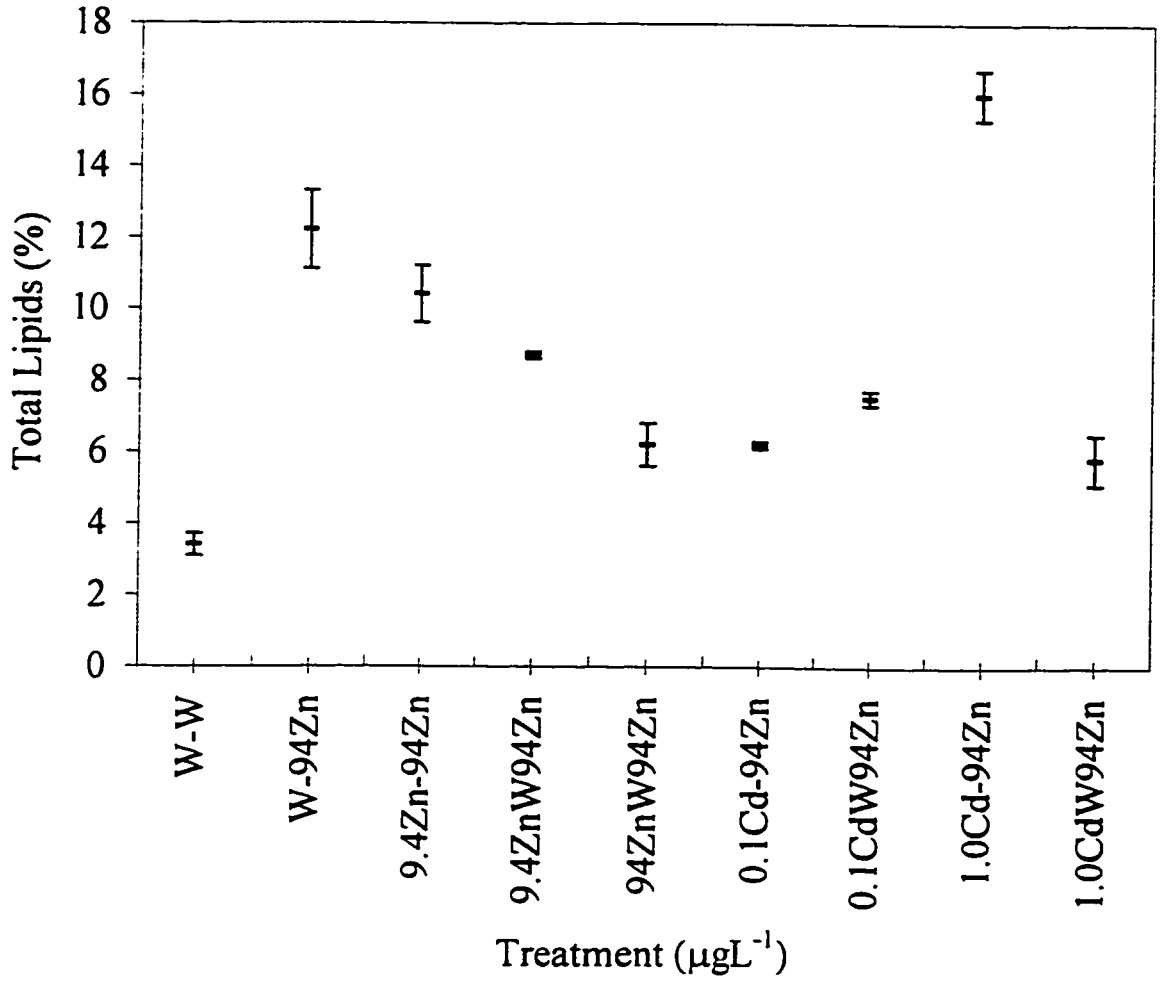

Total Lipids (%)
0
2
4
6
8
10
12
14
16
18
W-W
W-1.0Cd
9.4Zn-1.0Cd
9.4ZnW1.0Cd
94Zn-1.0Cd
94ZnW1.0Cd
0.1Cd-1.0Cd
0.1CdW1.0Cd
1.0CdW1.0Cd
Treatment (μgL-1)

Fig. 4.16 Total lipid content (%; mean±standard error; n=3) of *Hyalella azteca* after six weeks of exposure to 94 $\mu gZn \cdot L^{-1}$ following a heavy metal pre-exposure. W-W control and W-94 $\mu gZn \cdot L^{-1}$ are included for comparison.


Total Lipids (%)
0
2
4
6
8
10
12
14
16
18
W-W
W-94Zn
9.4Zn-94Zn
9.4ZnW94Zn
94ZnW94Zn
0.1Cd-94Zn
0.1CdW94Zn
1.0Cd-94Zn
1.0CdW94Zn
Treatment (μgL-1)

4.5. Results (Second Generation)

4.5.1. Survivorship

Survivorship in *H. azteca* exposed to Cd and Zn treatments, where eggs and embryos were not pre-exposed (Fig. 4.17), showed a significant treatment effect (ANOVA; df=4,10; F=42.21; $p<0.05$). The Tukey analysis indicated that all metal treatments were significantly different from control. In *H. azteca*, where eggs and embryos were pre-exposed (Fig. 4.18), survivorship did not differ significantly from the control (ANOVA; df=4,10; F=2.26; $p>0.05$).

4.5.2. Fecundity

There was a significant treatment effect (ANOVA; df=4,25; F=10.09; $p<0.05$) on egg production of the *H. azteca* where eggs and embryos were not pre-exposed (Fig. 4.19). Egg production was significantly reduced from the control in all the metal treatments. There was also a significant treatment effect (ANOVA; df=4,25; F=8.52; $p<0.05$) on the number of offspring born (Fig. 4.20). A significant decrease was observed between all the treatments and the control, although there was no difference observed in the size of eggs or the size of offspring from the control and from the treatments.

The effect of Cd and Zn on egg production of the amphipods, where eggs and embryos were pre-exposed (Fig. 4.21), was significant (ANOVA; df=4,25; F=5.64; $p<0.05$). The significance was due to the 0.1 $\mu gCd \cdot L^{-1}$ treatment, where the egg production was significantly higher than the control. However, there was no significant difference observed between the control and the rest of the treatments. There was no significant difference observed in the number of offspring produced (Fig. 4.22) (ANOVA; df=4,25; F=0.62;

Fig. 4.17 Survivorship (%; mean±standard error; n=3) of the second generation *Hyalella azteca* where eggs and embryos were not pre-exposed.

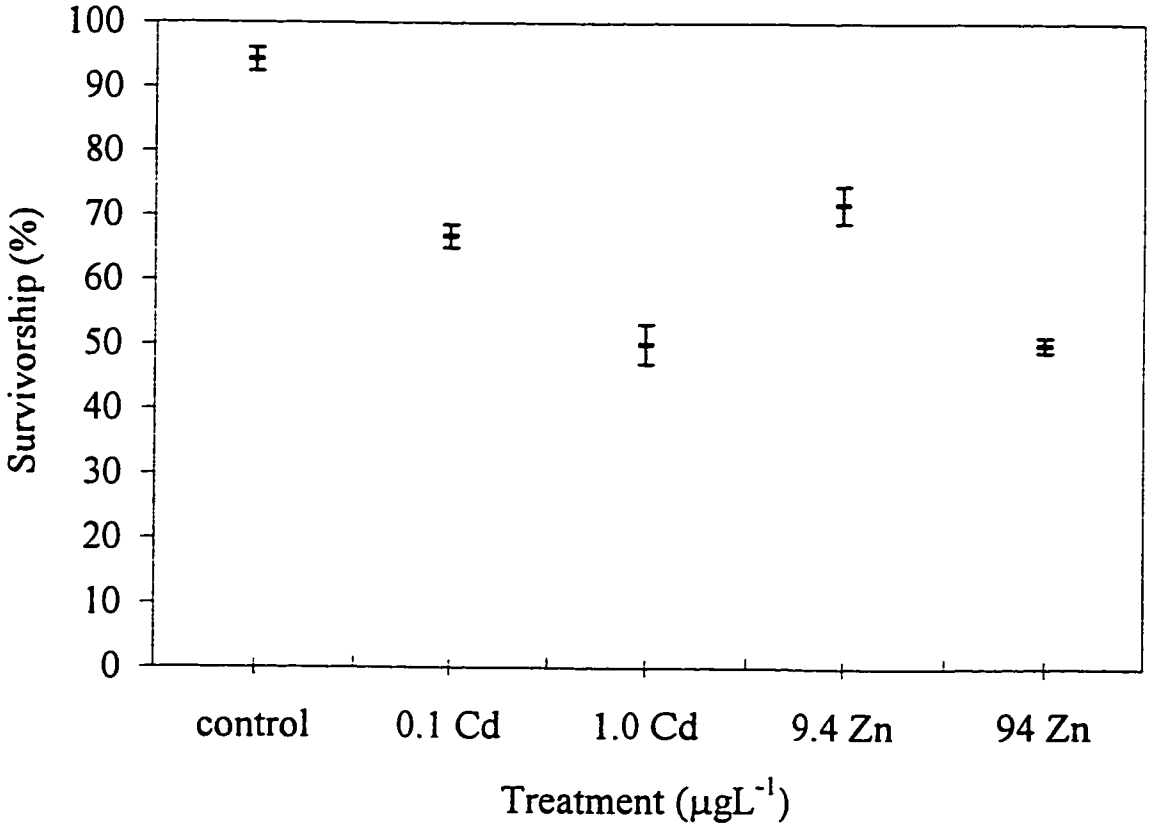

100
90
80
70
60
50
40
30
20
10
0
Survivorship (%)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

Fig. 4.18 Survivorship (%; mean±standard error; n=3) of the second generation *Hyalella azteca* where eggs and embryos were pre-exposed.

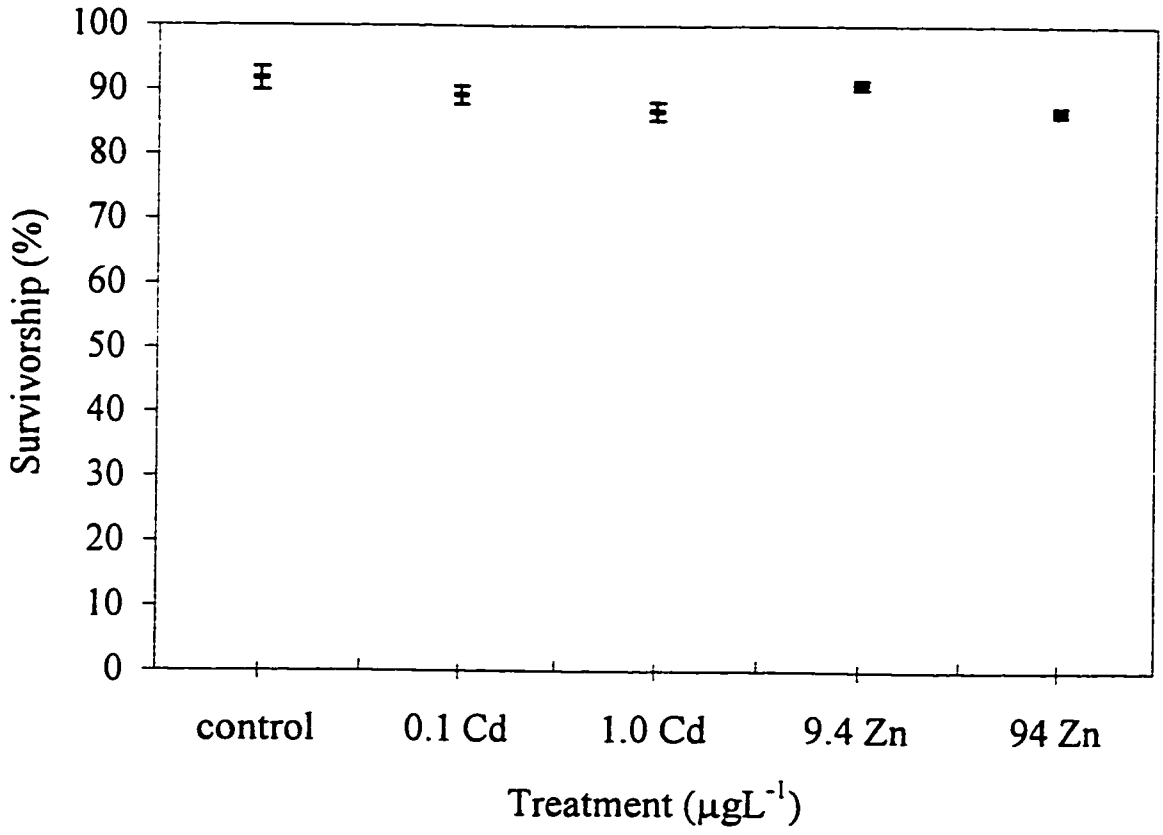

100
90
80
70
60
50
40
30
20
10
0
Survivorship (%)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

Fig. 4.19 Eggs produced (number; mean±standard error; n=6) by the second generation *Hyalella azteca* where eggs and embryos were not pre-exposed.

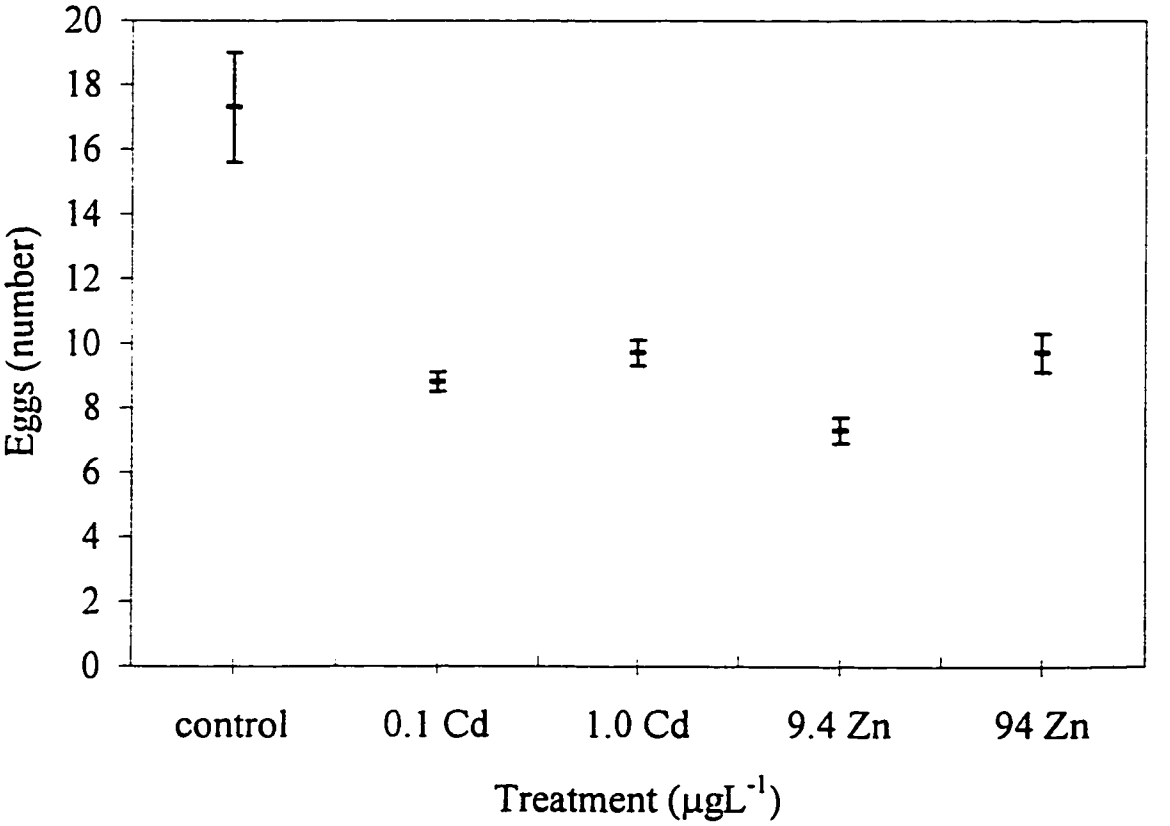

20
18
16
14
12
10
8
6
4
2
0
Eggs (number)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

Fig. 4.20 Offspring born (number; mean±standard error; n=6) to the second generation *Hyalella azteca* where eggs and embryos were not pre-exposed.

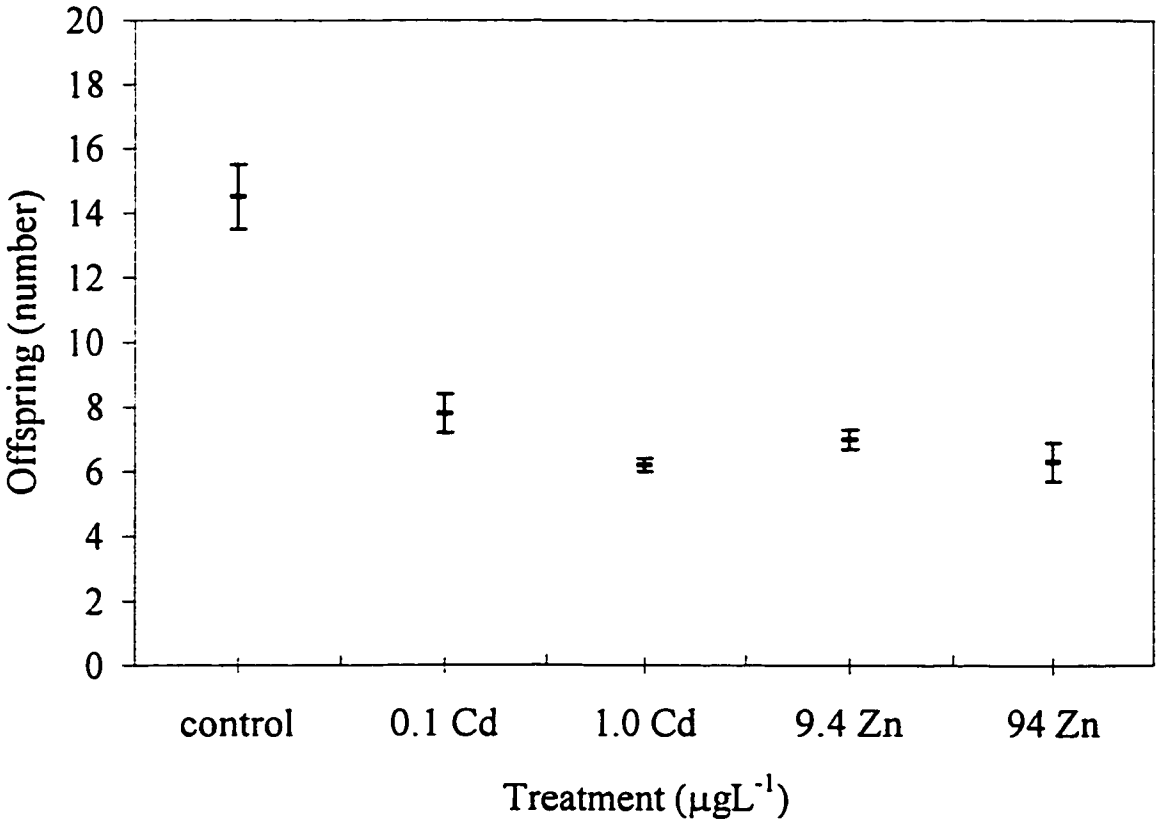

20
18
16
14
12
10
8
6
4
2
0
Offspring (number)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

Fig. 4.21 Eggs produced (number; mean±standard error; n=6) by the second generation *Hyalella azteca* where eggs and embryos were pre-exposed.

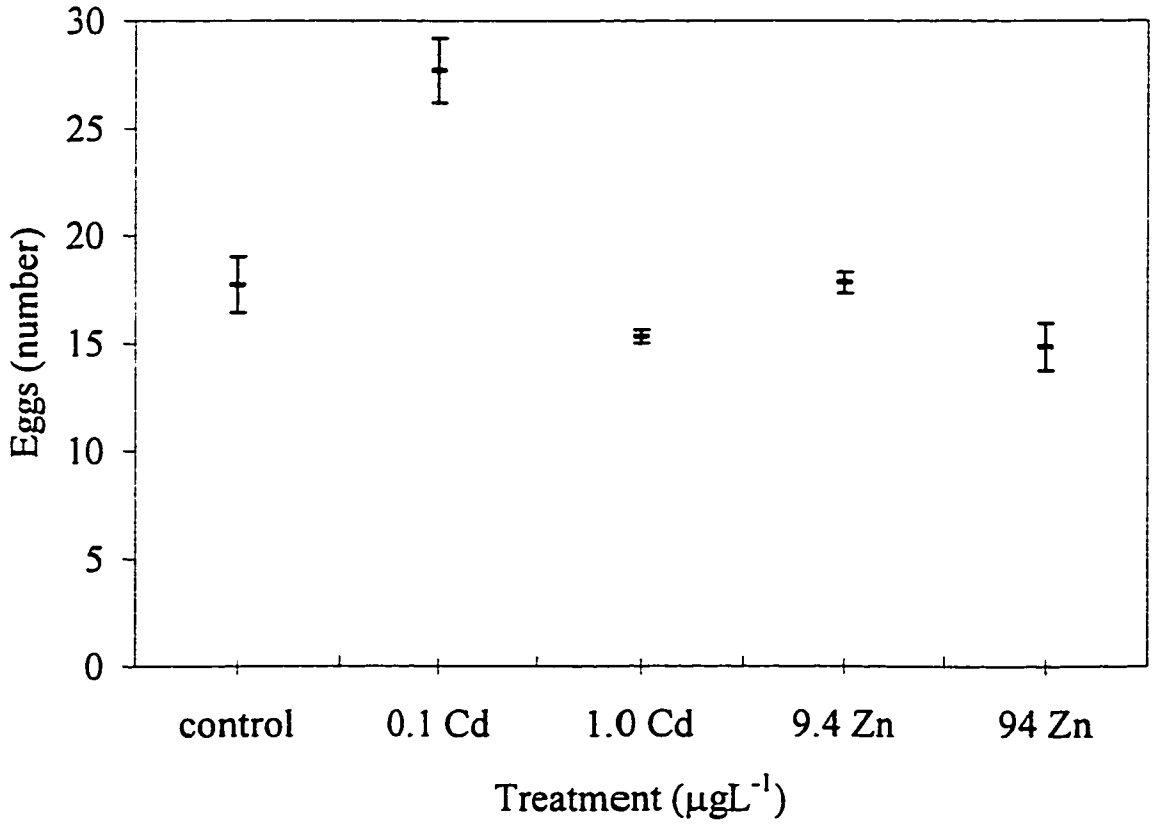
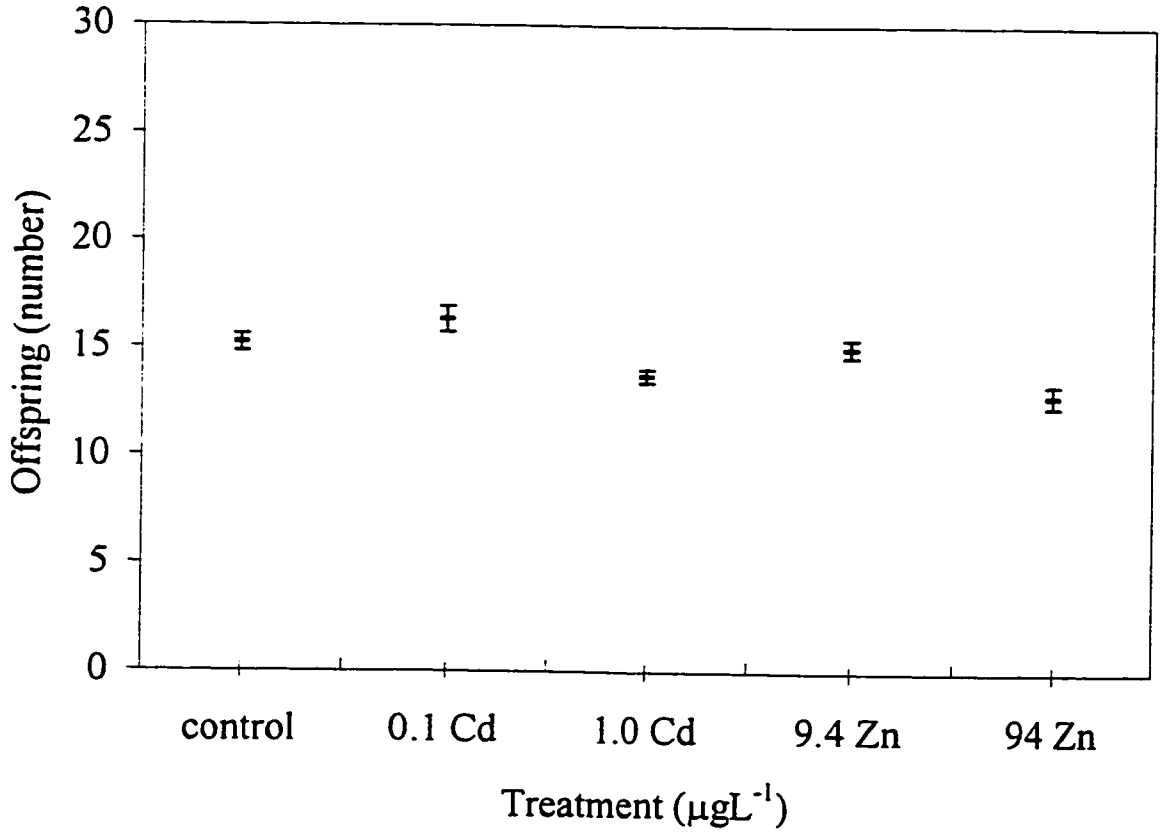

30
25
20
15
10
5
0
Eggs (number)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

Fig. 4.22 Offspring born (number; mean±standard error; n=6) to the second generation *Hyalella azteca* where eggs and embryos were pre-exposed.


30
25
20
15
10
5
0
Offspring (number)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

p=0.65). Also, no difference was observed between the treatments and the control comparing the size of the eggs and the offspring.

4.5.3. Total Lipids

The total size-specific lipid content of the second generation *H. azteca*, where eggs and embryos were not pre-exposed (Fig., 4.23) had a significant treatment effect (ANOVA; df=4,10; F=25.75; $p<0.05$). Lipid content of exposed animals was significantly higher than the control. Treatments did not significantly effect the lipid content of the second generation *H. azteca* (Fig. 4.24) when eggs and embryos were pre-exposed (ANOVA; df=4,10; F=1.11; $p>0.05$).

4.6. Discussion

Pre-exposure of *H. azteca* to sublethal concentrations of Cd and Zn in their early life stages resulted in an increase in tolerance to these metals when animals were exposed to the same or a different metal later in their life time. Although ANOVA indicated that significant differences were observed only between some treatments and the W-W control, the binomial expansion showed a significant pattern. This was especially evident when comparing treatments in which animals did not experienced pre-exposure, with treatments where animals were pre-exposed in their immature stage. When animals were exposed to 10% of Cd or Zn LC_{50} in only their mature stage reproduction was reduced and resting respiration and lipid stores were elevated. Since animals, which were pre-exposed did not allocate as much energy, in their mature stage, for resting respiration, which includes maintenance, repair and

Fig. 4.23 Size-specific total lipid content (%; mean±standard error; n=3) of the second generation *Hyalella azteca* where eggs and embryos were not pre-exposed.

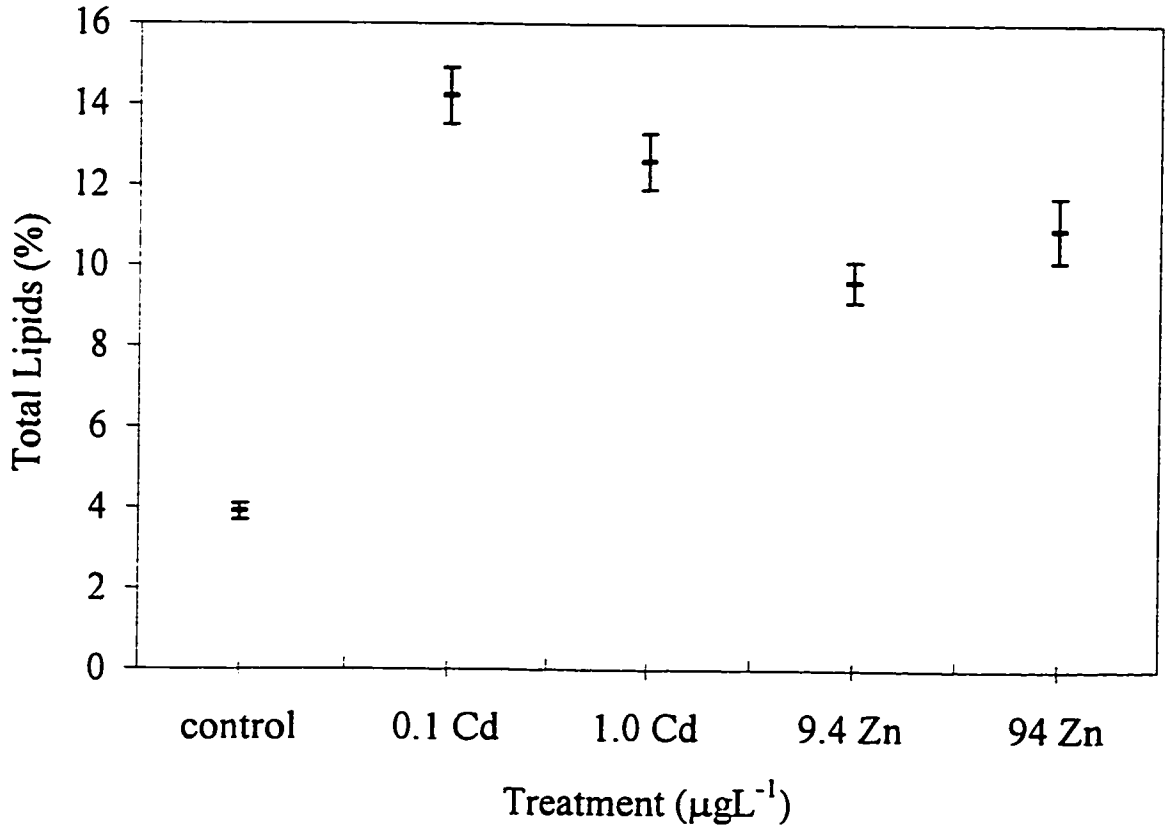
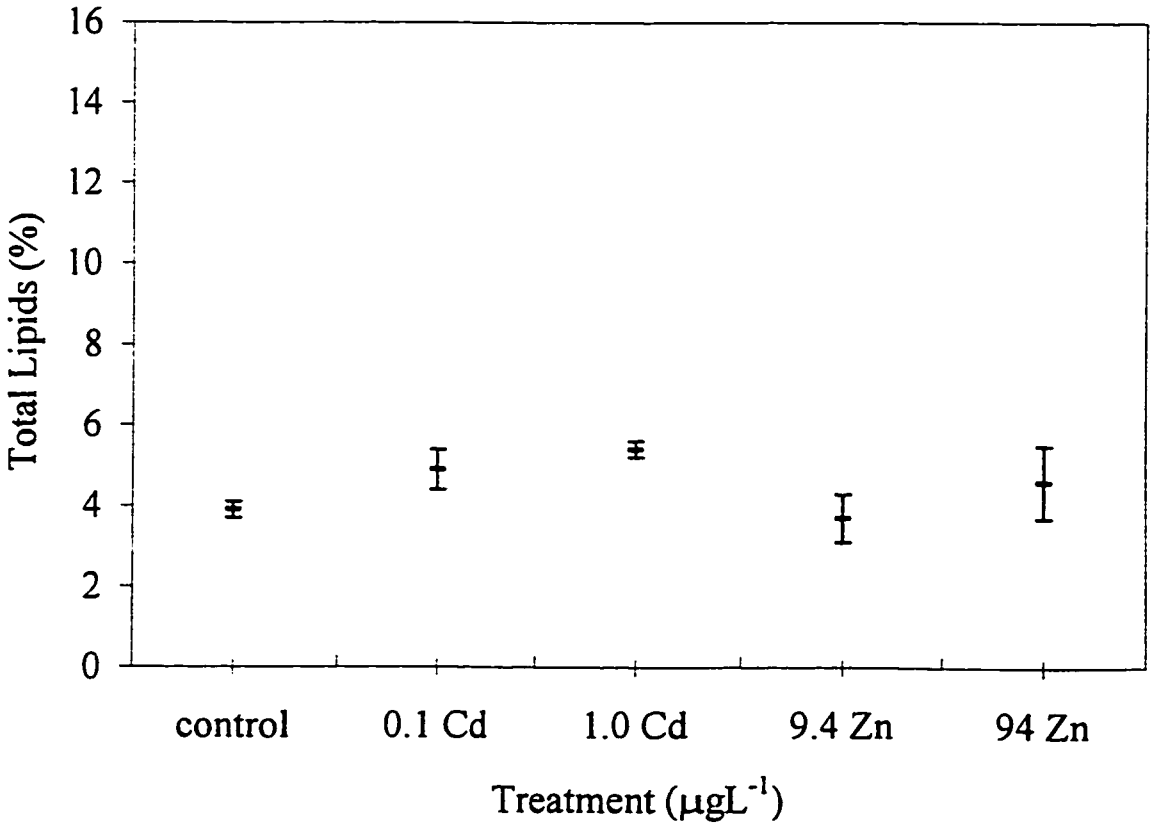

16
14
12
10
8
6
4
2
0
Total Lipids (%)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

Fig. 4.24 Size-specific total lipid content (%; mean±standard error; n=3) of the second generation *Hyalella azteca* where eggs and embryos were pre-exposed.


16
14
12
10
8
6
4
2
0
Total Lipids (%)
control
0.1 Cd
1.0 Cd
9.4 Zn
94 Zn
Treatment (μgL-1)

detoxification processes or in lipid stores and invested more energy in reproduction, this shows on less stress being imposed on these animals. Also survival increased in all the treatments that experienced pre-exposure. This was especially evident when mortality of pre-exposed and continuously exposed animals were compared. This was most likely due to an increase in tolerance supporting the suggestion that tolerant forms are not stressed or less stressed by conditions originally stressful (Calow, 1992).

While the variables measured show a higher sensitivity in animals that were not exposed to toxicants in their early stages, this suggests that pre-exposure did increase tolerance in amphipods exposed to heavy metals. However, survival, respiration, lipids and reproduction were not impaired in animals that were not pre-exposed but were later exposed to lower concentrations (1% LC_{50}) of Cd and Zn. This was probably due to the older animals being less sensitive than the early life stages.

Enhanced tolerance due to prior exposure to sublethal levels of heavy metals has been demonstrated by many authors (Goering and Klaassen, 1984; Polek and Obselkova, 1992; Maltby and Crane, 1994; Grosell et al., 1996). In agreement with the results from this experiment Webb (1972) and Goering and Klaassen (1984) demonstrated that exposure of rats in early life to low concentrations of Zn conferred an increased resistance to Cd poisoning when the animals where exposed to this metal as adults. Pre-treatment of *Bufo arenarum* Hensel embryos with low Cd/Zn concentrations allowed the frogs to tolerate Cd concentrations, which were originally lethal (Herkovits and Perez-Coll, 1995). The exposure of *Gammarus pulex* to 10 $\mu gZn \cdot L^{-1}$ for 2 weeks resulted in a decreased susceptibility to Cd poisoning (Howell, 1985). The LC_{50} of *H. azteca* pre-exposed to Pb was higher than the

LC_{50} of animals not pre-treated with Pb (MacLean et al., 1996). The mortality of a caterpillar significantly decreased when they were injected with low doses of Cd and Zn prior to the following injection of a concentration of Cd lethal without pre-exposure (Polek and Obselkova, 1992). Pre-treatment of rats with Zn *in vivo* induced stress proteins which acted together with metallothionein to protect cells against Cd toxicity *in vivo* and/or *in vitro* (Liu et al., 1996).

Positive evidence for increased tolerance in organisms obtained from metal contaminated environments has also been reported in many studies. Survival and feeding rate of *Gammarus pulex* from a polluted site were significantly higher when compared to the animals from a clean site (Maltby and Crane, 1994). The Zn LC_{50} of *Gammarus pulex* and *Asellus aquaticus* from populations exposed to pollution was significantly higher compared to populations not pre-exposed in the field (Naylor et al., 1990). Phillips and Rainbow (1989) proposed that an increase in tolerance could be due to mechanisms based either on reducing body burdens (e.g. exclusion and depuration) or on decreasing the toxicity of accumulated material (e.g. detoxification and sequestration). Also Duncan and Klaverkamp (1983) suggested a number of mechanisms to explain the increased Cd tolerance in pre-exposed animals. This could be due to decreased uptake or increased excretion of Cd, a redistribution of the Cd to tissues less sensitive to the metal and a redistribution of the Cd ion to less sensitive biochemical storage sites in the same tissue (e.g. metallothionein).

Lower uptake rates of animals from polluted sites compared to the clean sites were reported also. Amphipods from a polluted site had significantly lower uptake rates for Zn, Cu and Pb compared to the clean site, suggesting that acquired tolerance can be due to their

reduced metal uptake (Wright, 1986). Chironomid larvae from an uncontaminated site showed retarded growth and accumulated higher concentrations of heavy metals than the animals from a polluted site when exposed to contaminated sediments (Krantzberg and Stokes, 1989). Glynn and Olsson (1991) reported that Cd pre-exposure decreased the uptake of Cd into the gills of minnows *Phoxinus phoxinus* during Cd exposure later on in their lifetime. The presence of tolerant forms can be used as an indicator that the community has been subjected to stress (Blanck et al., 1988).

A number of researchers have suggested that increased metal tolerance in pre-exposed organisms may coincide with enhanced metallothionein and metallothionein-like protein synthesis (George et al., 1992; Bryan and Langston, 1992; Kaji et al., 1993; Bebianno et al., 1994; Park et al., 1994; Couillard et al., 1995b; George et al., 1996; Koropatnick and Zalups, 1997). This group of metal-binding proteins is believed to provide a protective role against the toxic effects of a number of elements, including Cd and Zn (Rainbow and Dallinger, 1993). The primary function of these proteins appears to be concerned with the homeostatic metabolism of physiologically essential elements, such as Zn, and as such, they occur naturally in trace amounts in tissues of different organisms (Karin, 1985). In addition, metallothionein bound Zn accumulates when animals are exposed to excess Zn and is depleted under conditions of Zn deficiency, suggesting that metallothionein serves as a means of sequestering excess Zn as well as a Zn reservoir that can be utilized when Zn is deficient (Kelly et al., 1996). Goering and Klaassen (1984) reported that tolerance following Cd pre-treatment resulted from an altered hepatic subcellular distribution of Cd, such that less Cd binds to critical organelles and macromolecules and instead binds preferentially to metallothionein in

the cytosol, where it is less toxic. However, exposure to sublethal doses of Cd and Zn, is known to accelerate the synthesis of these proteins to enhance metal tolerance (Kaji et al., 1992). It has also been suggested that tolerance to Cd toxicity following pre-treatment with metals results from an altered organ distribution of Cd due to preformed metallothionein sequestering a higher percentage of the toxic dose in the liver, and subsequently decreasing the amount available for distribution to other target organs such as kidneys (Suzuki and Yoshikawa, 1974). Huang and Ling (1989) suggested that metallothionein may be recognized as a stress protein as its gene expression can be well correlated with cellular defence towards metals. The induced tolerance to Cd and Zn in this experiment was attributed, at least in part, to the induction of these metal-binding proteins.

Antagonism between Cd and Zn has also been shown to reduce the toxicity of Cd (Howell, 1985; Pavičič et al., 1994). Howell (1985) found that low levels of Zn acted antagonistically with Cd, reducing the toxicity of Cd to *Gammarus pulex*. This could possibly be the case in the Zn pre-treated animals in this study, which were later exposed to Cd. Animals that were exposed to higher concentrations of Cd did not suffer significantly when they were pre-treated with Zn contrasting the results from the experiment described in Chapter 3. Kaji et al. (1992) and Mishima et al. (1995) suggested that the mechanism by which Zn protects cells from Cd toxicity is a decrease in the intracellular Cd accumulation. It has also been suggested that a decrease in the intracellular accumulation of Cd is the major mechanism for protection against Cd toxicity when metallothionein is not induced by Zn (Mishima et al., 1997). They speculated that an increase in the level of intracellular Zn might physicochemically prevent the entrance of Cd into cells.

Animals, which were first exposed to one of the toxicants and then moved into uncontaminated water for 7 days, in most cases, showed an enhanced tolerance to the toxicant to which they were exposed later. One week was presumably long enough for animals to recover and eliminate certain heavy metal. Seven days was shown to be long enough for depuration of accumulated Pb and to reach background levels in *H. azteca* (MacLean et al., 1996). When the animals were exposed to the first toxicant they presumably invested some energy in synthesis of metallothionein-like metal binding proteins. After elimination of the first heavy metal, previously induced metallothionein with free thiol groups could bind the subsequent metal. In this case animals did not have to spend much more energy to invest in metal binding proteins synthesis. More energy was therefore allocated to reproduction and somatic growth. Somatic growth of the tolerant animals was most likely not effected by toxicants since there was no significant difference observed in the number and size of the offspring compared to the control. Since reproduction was shown to be size dependent in *H. azteca* (Cooper, 1965) it could be concluded that exposed and control animals did not differ in size. Also animals that survived the exposure to the lower concentrations of Cd and Zn while immature recovered when they were moved into uncontaminated water and left there until the experiment terminated.

It has been demonstrated in many studies that the concentration of the metal to which animals are exposed plays an important role in induction of tolerance. Bradley et al. (1985) showed an increase in tolerance to Zn when rainbow trout *Salmo gairdneri* was pre-exposed to 30% and 50% of the lethal concentration of Zn. When fish were exposed only to 18% LC_{50} no increase in tolerance occurred. It was suggested that these concentrations were too

low to induce synthesis of metalothionein. Similar results were found for white suckers pre-exposed to 19% of Cd LC_{50} which showed no change in tolerance of this metal, while higher levels of pre-exposure induced enhanced tolerance (Duncan and Klaverkamp, 1983). The relatively high concentrations of heavy metals compared to this experiment needed to induce tolerance in other studies were probably due to shorter pre-exposure (1 week) compared to 3 weeks in this experiment. Also, Enderson et al. (1983) have revealed, that the degree of stability of Cd and Zn tolerance is dependent on the capacity of cells to perform immediate new synthesis of large amounts of metallothionein on reexposure to Cd or Zn which can differ within different organisms.

The variables measured for the animals first exposed to Zn and then to Cd did not differ significantly from the W-W control in any of the cases. This shows an induced tolerance to Cd due to pre-treatment with Zn. The possible causes for acquired tolerance could be that Zn that had entered cells during pre-treatment, protected membrane integrity by stabilizing the membrane structure (Bettger and O'Dell, 1981) and that the intracellular Zn served as an antioxidant (Bray and Bettger, 1990) or Zn increased intracellular glutathione content and then protected against Cd induced lipid peroxidation (Manca et al., 1991). Alternatively, the intracellular Zn may have competed with Cd directly at the sites where Cd exhibits its toxicity within the cells (Mishima et al., 1997) or Zn may have blocked the entry of Cd via calcium channels (Hinkle and Osborne, 1994).

The second experiment showed that the exposure of parents to heavy metals did not enhanced Cd and Zn tolerance of the offspring. When eggs and embryos were not exposed to toxicants, survival, reproduction and lipid stores of the offspring were impaired in all the

treatments. However, prior exposure of eggs and embryos of the second generation *H. azteca* to sublethal levels of Cd and Zn significantly enhanced their tolerance. This suggests that induced tolerance was not passed from parents to progeny but it was due to the pre-exposure of eggs and embryos of the subsequent generation. This experiment showed that chronic toxicity to *H. azteca* is dependent upon the stage of the amphipod's life cycle during which exposure begins. This result is in an agreement with that found by Sinley et al. (1974). They showed that rainbow trout *Salmo gairdneri* hatched from Zn-treated eggs were four times more Zn tolerant than were fish which eggs were not pre-exposed. Pascoe and Beattie (1979) also showed that pre-treatment of eggs of rainbow trout with low levels of Cd provided alevins with higher tolerance to higher levels of this metal.

Results with offspring, which did not experienced exposure to toxicants as eggs or embryos are similar to those described in Chapter 3. Animals suffered significant mortality, reduction in eggs and offspring production and they had a significantly higher lipid content compared to the control. It appears that the heavy metal exposure of parents did not enhanced tolerance of their progeny. It could be concluded from this experiment that the increased tolerance exhibited by animals exposed to toxicants as eggs and embryos was probably due to physiological acclimation and not genetical adaptation. This agrees with Klerks and Weis (1987) who proposed two reasons why organisms may be resistant to a pollutant. Firstly, organisms may acquire a degree of tolerance by physiological acclimation during exposure to sublethal concentrations at some period of their lifetime and as such, it cannot be passed down onto progeny, who must also be pre-exposed to acquire it. Secondly, exposed organisms may evolve genetically based resistance through the action of natural

selection on genetically based individual variation in resistance, which can be passed on the subsequent generation. Tolerance to heavy metals of the first generation laboratory animals, reared from animals coming from the field, has been suggested to have a genetic basis with natural selection playing its role in the contaminated sites (Postma et al., 1995).

Maintenance of a tolerance mechanism may be energetically costly (Posthuma and Van Straalen, 1993). Lowered fitness (Weis and Weis, 1989) and larval growth rate (Postma et al., 1995) of the tolerant individuals could indicate such cost. However, the larval growth rate of the Cd tolerant population of the midge *Chironomus riparius* did not differ from a reference population (Postma et al., 1995). These results are in concordance with the findings from this experiment. In the tolerant amphipods reproduction, resting respiration and lipid stores did not differ from the control in most of the cases. However, the cases were survival improved with pre-exposure but reproduction, maintenance or lipid stores differed from the control could indicate higher energetic cost for acquired tolerance. Results of the second-generation animals, where eggs and embryos were pre-exposed, do not give any conclusive sign of increased costs causing lower reproduction.

CHAPTER 5 PREFERENCE-AVOIDANCE

5.1. Introduction

Behavioural change is one of many ways that aquatic animals can respond to stresses. Behaviour is a whole animal response incorporating many changes at the physiological and biochemical levels (Little et al., 1985; McNicol and Scherer, 1991) and it is often the first response upon exposure to a toxicant (Fernandez-Casalderrey et al., 1994).

Assessment of potential chemical hazard to aquatic life and establishment of water-quality criteria relies primarily on evidence gathering by standard acute and chronic laboratory toxicity tests (USEPA, 1985) measuring physiological effects, such as growth, survival and reproduction at the individual and population levels. These tests do not assess sublethal alterations in ecologically important behaviour (Sandheinrich and Atchison, 1989) which commonly occurs at or below concentrations affecting physiology variables. Many studies on the effects of metals on fish behaviour showed that metal concentrations near or below the lowest observed effect concentration (LOEC), derived from standardized tests, significantly altered the animal's behaviour (Sandheinrich and Atchison, 1989,1990; Bryan et al., 1995). Therefore, sensitivity of behavioural toxicity tests suggests that these tests could be added to the current chemical hazard evaluation process, because behavioural studies are a particularly promising means of detecting sublethal effects of contaminants (Little et al., 1985; McNicol et al., 1996).

Aquatic organisms have many different behavioural patterns, which can serve as a basis for toxicity testing. Spatial selection by animals (preference-avoidance) has become a

popular tool in behavioural toxicology because it is relatively easy to perform and reactions may be monitored and quantified (McMurtry, 1984; Korver and Sprague, 1989; Døving, 1991; Scherer, 1992; McNicol and Scherer, 1993; Lauridsen and Lodge, 1996; Duguay, 1997; Wicklum et al., 1997). The ability to detect environmental factors and to select favourable conditions is of critical importance for survival. With the advent of pollutants these preference-avoidance responses became a very useful tool to measure the direct response of aquatic organisms to toxicants. These tests are an important means of determining whether species can detect and, if so, avoid a pollutant. Furthermore, a particular substance at a certain concentration may be more hazardous if it is neither avoided nor detected or it appears as an "attractant" (Scherer, 1992).

When performing these tests the organism is given a choice between contaminated and uncontaminated areas and then observed to determine its response. However, exposing a specimen to a gradient of several toxicant concentrations may alter the response due to toxic action having already taken place during the course of the test. Scherer (1976) therefore proposed a design that provides a reliable separation between pure and toxicant contaminated water, with a device for tracing and documenting the specimen's movements into and out of the toxicant.

Many contaminants induce avoidance reactions whereas others apparently "attract" aquatic organisms, most likely due to a reduction in locomotion so they remain longer in contaminated areas. Zinc elicits avoidance reactions in several species of fish, both in the laboratory and in the field (Sprague 1964, 1968; Brown et al., 1982; Korver and Sprague, 1989). The extent to which a compound is avoided may vary with concentration.

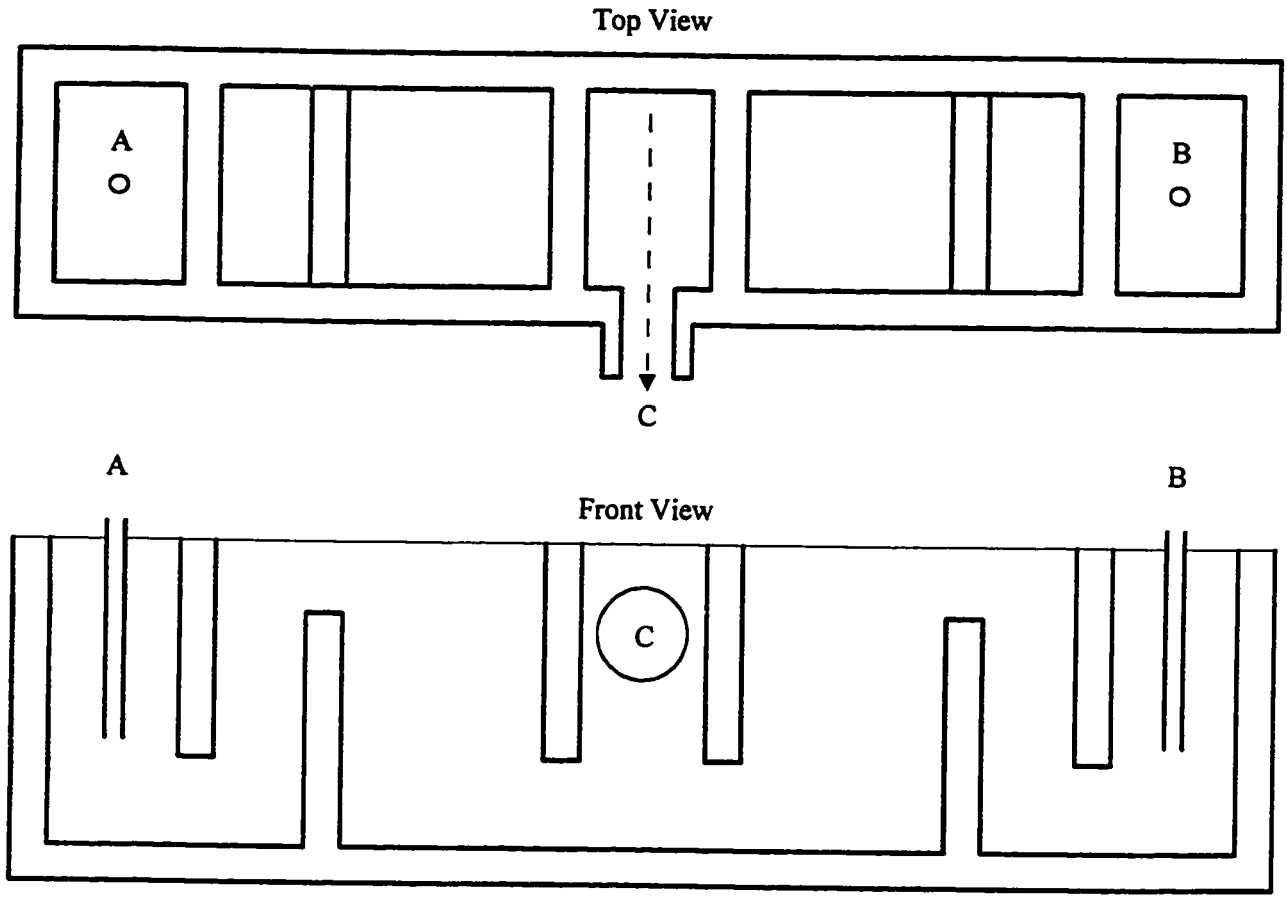
Maciorowski et al. (1976) showed that amphipods avoided copper sulphate at low concentrations (150 and 460 $\mu g \cdot L^{-1}$) but stayed longer in solutions containing lethal concentrations (12,300 and 30,000 $\mu g \cdot L^{-1}$). Also, the freshwater predatory leech *Nephelopsis obscura* spent significantly more time in 200 $\mu gCd \cdot L^{-1}$ water compared to the 50 and 100 $\mu gCd \cdot L^{-1}$ (Wicklum et al., 1997). Although avoidance or preference responses can be detected in avoidance-preference bioassays, the physiological and biochemical mechanisms causing these responses usually are unknown (Little at al., 1985).

The objective of this study was to determine if *Hyalella azteca* exhibits a preference-avoidance response to low concentrations (10% and 1% LC_{50}) of two heavy metals Cd and Zn.

5.2. Methods and materials

The ability of *H. azteca* to detect and avoid Zn and Cd in solution was investigated using a choice chamber based on that described by Scherer (1976). This consisted of a horizontal clear plexiglass trough 45 cm long, 6 cm high and 4 cm wide (Figure, 5.1) into which water flowed from both ends, discharging from a central outlet. Water entering one end contained the pollutant under test while water entering the other end was control water (clean, uncontaminated water). Water was pumped into the choice chamber using a dual-action pump set at the flow rate of 1.0 $mL \cdot s^{-1}$. In the middle of the tank, where water discharged, was a very sharp boundary between the two solutions and prior to each run it was visually assessed using red food dye added to the contaminated side. To avoid biases produced by the apparatus or preferred side, the end into which contaminated water was

Fig. 5.1 Preference-avoidance tank with water containing Cd or Zn pumped into one end (A) and the control (uncontaminated) water pumped into the other (B). A sharp boundary (C) is formed at the middle where the water exits the tank.


Top View
A
B
C
A
B
Front View
C

pumped was changed after each run.

The *H. azteca* used in this behavioural testing came from the same culture of animals that were used in the chronic testing and bred in the laboratory under conditions described in Chapter 2. For the experiments, only mature (more than 5 weeks old) animals were used (mean biomass 4.3±0.8mg) which had never been exposed to any of the toxicants. Preference-avoidance reaction was tested for 30 minutes in the same concentrations of heavy metals, Cd (0.1 and 1.0$\mu g \cdot L^{-1}$) and Zn (9.4 and 94 $\mu g \cdot L^{-1}$) used in chronic testing. The test solutions were prepared as described in Chapter 3. Prior to each trial initiation, one animal was put into the trough and acclimated for 15 minutes. To ensure that animal experienced two different qualities of water, only animal that crossed the concentration boundary at least twice during its acclimation time was used in the experiment. Throughout the 30 minutes trial the animal was observed and videotaped. The time each animal spent in contaminated or clean water was determined and analysed. Five animals were tested individually for each concentration of the metals.

Due to the obvious preference of the animals for clean water, statistical analysis of the experimental data was not necessary.

5.3. Results

Hyalella azteca was found to detect and avoid heavy metals in all Zn and Cd treatments. Animals spent most of their time in the end of the choice chamber containing clean water, only occasionally entering the polluted water for very brief periods (1 minute maximum). The average cumulative time spent by amphipods in control water was much

higher than the amount of cumulative time spent in Cd or Zn contaminated solutions at all concentrations (Fig. 5.2; 5.3). There was also no difference among the treatments in the total amount of time spend in the clean water.

5.4. Discussion

The *H. azteca* in this study displayed high sensitivity and definite avoidance of Cd and Zn contaminated water and a strong preference for clean water. These preference-avoidance results agree with those of Abel and Green (1981) who observed that *Gammarus pulex* avoided concentrations of Zn at least down to 1,000 $\mu g \cdot L^{-1}$ and it could also detect and avoid concentrations of 320-318,000 $\mu gCu \cdot L^{-1}$ (Costa, 1966). The lake whitefish *Coregonus clupeaformis* Mitchill showed avoidance behaviour when exposed to 0.2 $\mu gCd \cdot L^{-1}$ in a 0.25 h test in soft water (McNicol and Scherer, 1991). Other authors using preference-avoidance tests have shown that aquatic invertebrates may both avoid or "prefer" lethal concentrations of a toxicant depending upon the specific concentration and their life stage. Gray (1995) observed that small (4-50 mgWWT) leeches *Nephelopsis obscura* stayed longer in Zn contaminated water at concentrations of 320, 3,200 and 9,600 $\mu gZn \cdot L^{-1}$ and large (410-425 mgWWT) leeches avoided the highest concentration. Different responses were noted for *Nephelopsis obscura* exposed to Cd (Wicklum, 1995). Small (<200 mgWWT) leeches did not avoid low (20 $\mu gCd \cdot L^{-1}$) concentrations of Cd but did avoid higher (100 and 200 $\mu gCd \cdot L^{-1}$) concentrations while large (>450 mgWWT) leeches spent proportionately more time in the solution containing higher concentrations of Cd.

In this study observation of animal's reactions, when given a choice of clean and toxic

Fig. 5.2 Time (seconds) spent in clean water for *Hyalella azteca* given the choice between clean water and Cd (0.1 or 1.0 $\mu gCd \cdot L^{-1}$) contaminated water.

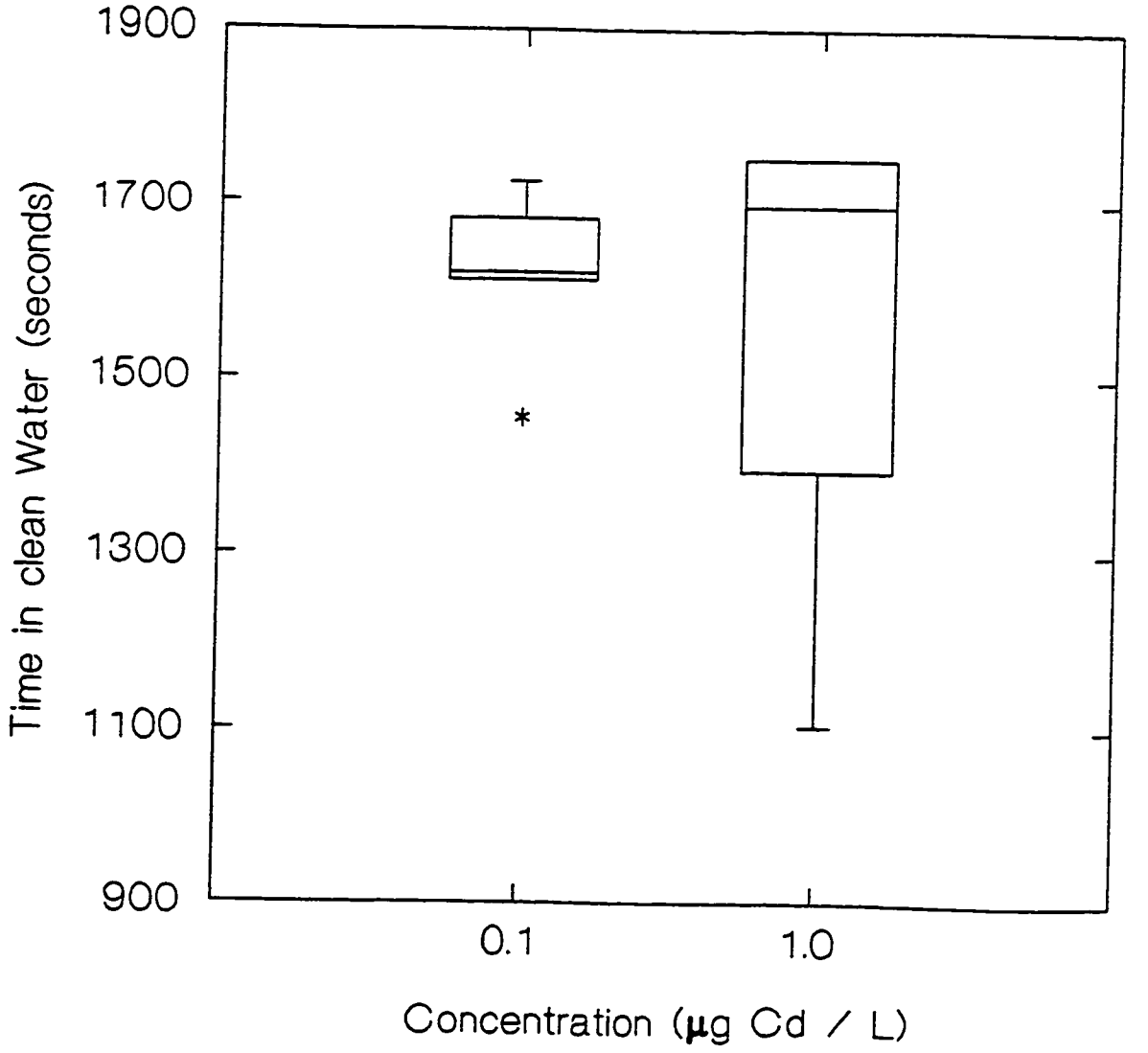
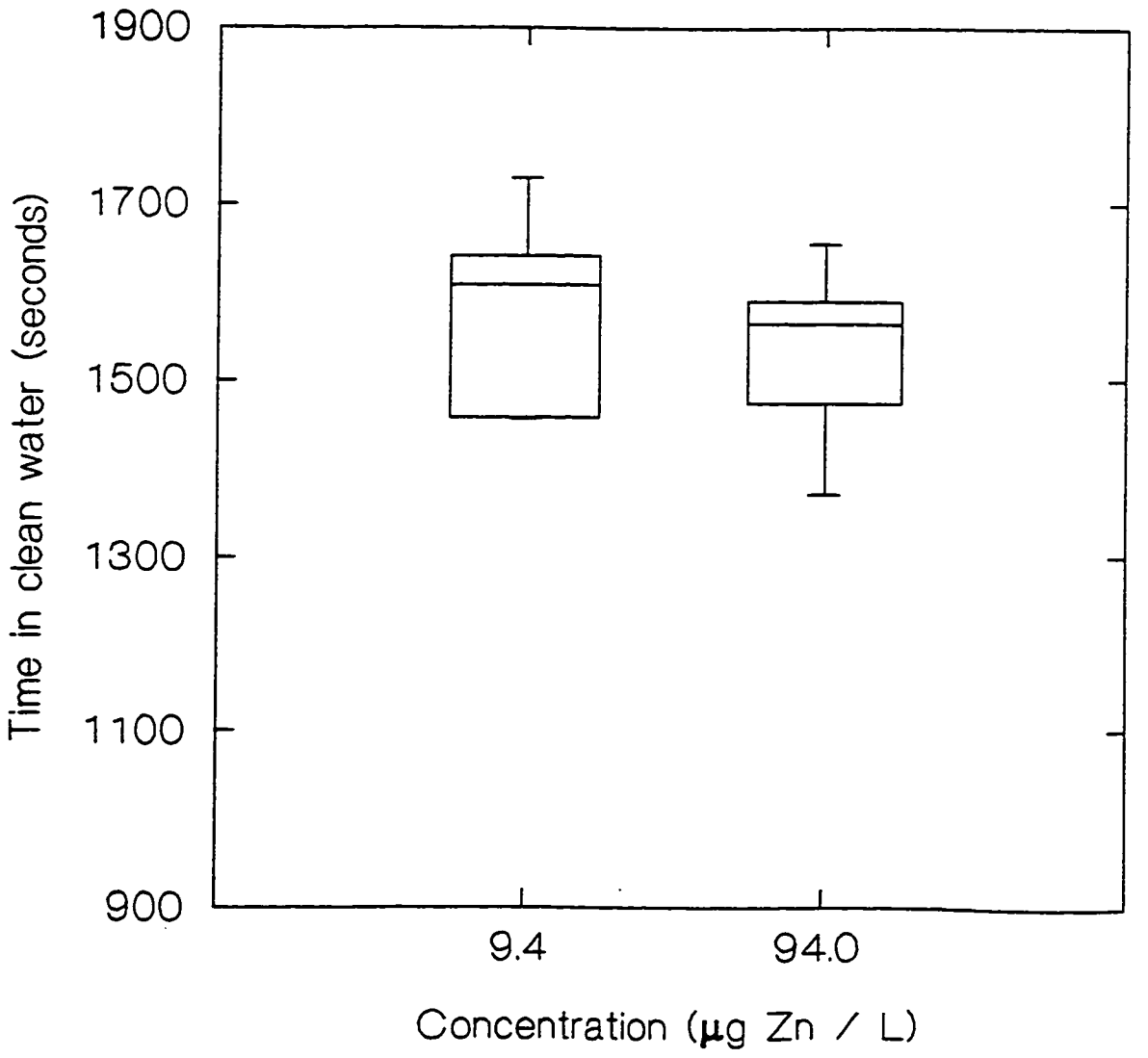

1900
1700
1500
1300
1100
900
Time in clean Water (seconds)
0.1
1.0
Concentration (µg Cd / L)

Fig. 5.3 Time (seconds) spent in clean water for *Hyalella azteca* given the choice between clean water and Zn (9.4 or 94 $\mu gZn \cdot L^{-1}$) contaminated water.


1900
1700
1500
1300
1100
900
Time in clean water (seconds)
9.4
94.0
Concentration (µg Zn / L)

areas, showed real preference to the uncontaminated site. Animals crossing the boundary most of the time swam only half way into the end of the tank containing contaminated water and returned quickly (less than 10 seconds) into the clean water. These trips were more frequent at the beginning of the trial and became less frequent as the experiment continued. Similar observations were noted in the fathead minnow *Pimphales promelas* exposed to 284 $\mu gZn \cdot L^{-1}$ in a preference-avoidance study with a surprise response to the steep gradient upon first encounter. Fish made repeated forays back and forth between clean and dosed sides before spending most of their time in the clean water (Korver and Sprague, 1989).

Presumably, amphipods in these experiments did not spend enough time in the contaminated site to be affected by the toxicants. *Hyalella azteca* are very mobile animals that can avoid stressful situations quickly compared to other species that move more slowly or are sessile and thus have a greater chance of being exposed for longer periods of time. When animals stay longer in the solution containing higher concentrations of toxicants they are often getting immobilized or lethargic and unable to leave the contaminated area and thus they are not showing a preference. This situation was reported for *Nephelopsis obscura* exposed to Cd (Wicklum, 1995) and Zn (Gray, 1995), ten-spined stickleback *Pygosteus pungitius* (L.) exposed to 500 and 400 $\mu gCd \cdot L^{-1}$ (Jones, 1947), *Oncorhynchus mykiss* exposed to 1.0 $\mu gHg \cdot L^{-1}$, 100 $\mu gCu \cdot L^{-1}$ (Black and Birge, 1980) and 300 and 390 $\mu gCu \cdot L^{-1}$ (Giattina et al., 1982) and chironomid larva *Glyptotendipes pallens* exposed to 5,000 and 10,000 $\mu gCd \cdot L^{-1}$ (Heinis et al., 1990).

Concentrations of Cd and Zn used in these experiments were relatively low, only 10% and 1% of 96-h LC_{50}. Based on the results of this study, *Hyalella azteca* actively avoids low

concentrations of these two heavy metals, but a prediction cannot be made as to whether avoidance will occur at higher concentrations. The amphipod *Gammarus lacustris* avoided Cu concentrations one to two times greater than 96 h LC_{50} but did not avoid concentrations 40 to 300 times greater than the 96 h LC_{50} (Maciorowski et al., 1976). Rainbow trout *Salmo gairdneri* avoided lethal concentrations of chlorine (Sprague and Drury, 1969), while stickleback *Gasterosteus aculeatus* L. avoided low concentrations of Pb nitrate but not high concentrations (Jones, 1947). The whitefish *Coregonus clupeaformis* showed avoidance to low concentrations of Cu, but spending more time in higher concentrations, no avoidance was observed for sublethal or lethal concentrations of Cd and strong avoidance was shown for high concentrations of Hg (Brown et al., 1982).

For hololimnic animals with the entire life cycle in the aquatic environment (hololimnic), chemoreception plays a major role in behaviour such as feeding, defence, reproduction, schooling, migration and orientation (Hara, 1975; Scherer, 1992; Jachner, 1995) and it is of most importance for survival (Bryan et al., 1995; Lauridsen and Lodge, 1996). It has been assumed that pollutants can interfere with proteins and lipids in chemoreceptive membranes, which are the probable receptor sites for chemical stimuli (Brown et al., 1982). Binding of stimulant molecules with the receptor is translated into membrane permeability changes, which results in the generation of nerve impulses, conducting to the central nervous system where the appropriate behavioural response is initiated. The physiological importance, chemical reactivity and exposed location of chemosensory membranes make them prime targets for interaction with toxicants (Brown et al., 1982; Scherer, 1992).

Throughout this experiment no motionless or hyperactivity responses were noticed in *H. azteca*. Hyperactivity followed by suppressed activity is a common response of some organisms to pollutants (Miller et al., 1982; Little et al., 1985; Drummond and Russom, 1990). Fitness can be impaired by hyperactivity of an organism if energy intake does not increase to compensate for the increased costs of activity while hypoactivity can result in higher mortality due to lower ability to escape predation or reduced foraging (Miller et al., 1982). Locomotor activity, as well as efficiency of activity of aquatic organisms is obviously of ecological significance, influencing feeding success, finding a mate and predator avoidance. Suppressed activity was reported for the shrimp *Palaemonetes pugio* Holthusis exposed to 300 and 560 $\mu gCd \cdot L^{-1}$ which caused higher susceptibility to predation and reduction in feeding success (Hutcheson et al., 1985). Foraging behaviour of bluegill *Lepomis macrochirus* was impaired at sublethal concentrations of Cu (Sandheinrich and Atchison, 1989) and at sublethal (37.3 $\mu gCdL^{-1}$) concentrations of Cd (Bryan et al., 1995). *Hydropsyche contubernalis* McLachlan larvae exposed to Cd concentrations of 12 $\mu gCd \cdot L^{-1}$ expended significantly less time foraging for food than did the control larvae (Vuory, 1994).

Although escape by aquatic organisms from contaminated areas can be beneficial to the individual at that time, displacement from preferred habitats may result in increased mortality through predation, decreased growth or impaired reproduction (Little et al., 1985). A possible tendency of aquatic organisms to move away from polluted areas or move deeper into the sediment could affect the whole food web in the aquatic ecosystem. Significant reduction of preferred food items could lead to food limitations for some species, which could ultimately alter entire communities and ecosystems (Carman and Todaro, 1996).

The predictive potential of behavioural tests can be improved by selecting species for behavioural analysis that are potentially affected and most sensitive to the contaminants (Little et al., 1985). Aquatic invertebrates, especially crustaceans are well suited organisms for behavioural testing in assessing effects of contaminants because they are very sensitive to pollution and are important as a food source for other organisms. The high sensitivity of *H. azteca* in this experiment illustrates the usefulness of these species in behavioural testing in assessing whether animals respond to heavy metal exposure with avoidance, getting immobilized or do not show response. Behavioural measurements together with biochemical or physiological responses can provide a comprehensive evaluation of hazards posed by contaminants (Little et al., 1985).

CHAPTER 6 SUMMARY AND CONCLUSIONS

This study was conducted to evaluate the potential hazards associated with the releases of two heavy metals, Cd and Zn, into fresh water ecosystems using *Hyalella azteca*, a widely distributed and sensitive model species. The toxicity of metals was evaluated using acute, chronic and behavioural tests.

Acute toxicity testing results showed that *H. azteca* was less sensitive to the heavy metals when sediment and food were present compared to 96 h LC_{50} from other studies where sediment and food were excluded. Presumably, the presence of sediment and food lowered the concentration of biologically available metals in the water and thus toxicity was reduced. This was more evident in animals exposed to Zn treatments where the measured LC_{50} was almost five times higher than that for *H. azteca* reported in earlier studies. There was not a big difference observed for Cd LC_{50} but a much higher LC_{50} detected for Zn. This could be due to Zn being commonly implicated in homeostatic regulation in aquatic organisms, while the uptake of nonessential metals, such as Cd is determined by exposure concentration. Also, Cd uptake from food was found to be the major mode of Cd accumulation while Zn mostly accumulated through water uptake.

The chronic bioenergetic toxicity test (12 weeks) using sublethal concentrations of Cd and Zn (1% and 10% of the LC_{50}) showed a significant effect on survival and reproduction of animals exposed to all the treatments. Growth was seriously impaired in the treatments containing high concentrations of metals. Significantly higher faeces, mucus and ammonia excretion, resting respiration and lipid content of exposed animals were also observed. The

most important toxic effect of these two metals was the decrease in food consumption, which led to deficiency of available energy for physiological processes and consequently affected the fitness of exposed animals. The decrease in energy acquisition was possibly due to pathological effects of Cd and Zn, affecting epithelium and pharynx integrity, constraining the ability of *H. azteca* to forage for and ingest food. *Hyalella azteca* responded by changing energy allocation patterns. To compensate for the decrease in energy ingested the animals presumably increased catabolism of protein stores to meet the increased energy demands of heavy metal exposure. This was evident as higher ammonia production. More energy was allocated to compensatory responses such as faeces and mucus production and respiratory costs, including detoxification mechanisms and repair of cellular damage. Less energy was available for tissue and gamete production, which resulted in slower somatic growth, and fewer offspring produced. However, *H. azteca* showed flexibility through bioenergetic allocation and reproductive strategies in an effort to maintain fitness. The lower acquisition of energy of the stressed animals resulted in a change of the allocation of available energy and enabled them to survive and eventually reproduce, although at a lower fecundity. This study demonstrated that changes in energy acquisition and allocation have broad ecological implications.

Chronic tolerance testing showed that the pre-exposure of young *H. azteca* to sublethal concentrations of Cd and Zn enhanced the tolerance of mature animals. In animals that acquired tolerance, survival, reproduction, resting respiration and lipid stores did not differ significantly from the W-W control. In contrast, animals that did not experience exposure to the metals in their early life stage showed substantially higher sensitivity when

exposed to the toxicants as mature animals. The improvement of the tested physiological variables in this experiment is most likely due to an acquired tolerance of pre-exposed animals. This agrees with the definition of tolerance as the ability of individuals to cope with the stresses associated with exposure to metal concentrations that are inhibitory or lethal to non-tolerant individuals (Mulvey and Diamond, 1991). The concentrations of metals used in tolerance testing has been shown to significantly impaired physiology and survival of non-tolerant animals during chronic exposure (Chapter 3) and in animals that did not experience pre-exposure in their early stage. The enhanced tolerance of pre-exposed animals was probably due to a physiologically based component for metal tolerance and cytoplasmic-metal detoxification, the metallothionein-like metal-binding proteins.

Testing the tolerance of the second generation showed that when parents were exposed to toxicants during their lives and removed from contaminated water before eggs and embryos developed, the offspring did not gain any tolerance. Survival, reproduction and lipid stores of the second generation were impaired similarly to animals whose parents did not experienced exposure to toxicants (Chapter 3). On the contrary, when parents were not removed from the contaminated water during the period when eggs and embryos developed, the second generation *H. azteca* showed a higher tolerance to the toxicant to which they were exposed. Survival, reproduction and lipid levels did not differ from the control in any of the treatments. This suggests that enhanced tolerance was not passed from parents to progeny, but it was acquired because of the pre-exposure of the offspring to the toxicants in their very early life-stages.

Preference-avoidance testing showed a definite avoidance of *H. azteca* to Cd and Zn

contaminated water at all concentrations with strong preference for clean water. Animals were able to detect toxicants quickly, not spending too much time in the contaminated water. At the beginning of the exposure, animals made more frequent forays back and forth between the clean and dosed side until they spent most of their time in the clean water. The animals appeared to be able to detect toxicants soon enough to move away from the contaminated site, before being affected by the toxicants. Detectable behaviour, such as motionless or hyperactivity, due to toxicant effects, were not observed during the course of the experiment.

The chronic testing in this study was a direct test of the Environment Canada and United States Environmental Protection Agency water quality standards. The concentrations of 0.1 and 1.0 $\mu gCd \cdot L^{-1}$ were the values lower and higher than the predicted LOEC calculated for the water hardness used in this study. The results indicate that the LOEC for Cd of 0.8116 $\mu gCd \cdot L^{-1}$ is not sufficient to protect all aquatic organisms from Cd toxicity. Significant adverse effects were found for *H. azteca* after chronic exposure to both concentrations of Cd but it was more pronounced at the exposure of 1.0 $\mu gCd \cdot L^{-1}$ and this is closer to the LOEC value calculated. The same results were found for Zn toxicity, where both concentrations of Zn used in the testing had a significant adverse effect on reproduction and survival of the exposed animals. All the physiological variables measured were seriously impaired by Zn exposure, especially in higher treatment concentrations. Both concentrations used in chronic testing were lower than the limit proposed by USEPA for allowable total Zn in aquatic systems. While fitness decreased in *H. azteca* exposed to all concentrations of Cd and Zn, long term exposure to LOEC would result in population-level changes.

These results support the use of chronic toxicity testing of sensitive species when

determining water quality standards to protect freshwater life. Crustaceans, especially cladocerans and amphipods, have been shown to be the most sensitive group of invertebrates to heavy metal toxicity (Biesinger et al., 1986; Borgmann et al., 1991; Outridge, 1994). Also, the salmonoid fish showed a similar sensitivity as cladocerans and amphipods (Cusimano et al., 1986; McNicol and Scherer, 1991). Therefore, the use of these sensitive species is recommended in assessing water quality and in developing water-quality standards with respect to heavy-metal toxicants, Cd and Zn in conjunction with chronic toxicity testing. While acute toxicity testing upon which the assessment of water quality criteria has traditionally been based, provides insufficient evidence for protecting aquatic ecosystems (van Leeuwen et al., 1985), chronic toxicity effects on for example survival, fecundity, energy acquisition and allocation should be taken into consideration.

Information generated from various toxicity tests can be used in the management of pollution for the purposes of (a) prediction of environmental effects of a waste, (b) comparison of toxicants, animals, and test conditions, or (c) regulation of discharge. While the long-term or chronic effects on survival, growth and reproduction cannot always be directly measured, the chronic effects are often estimated from observations made during short-term or acute field or laboratory studies. When we deal with substances with delayed toxicity or protracted toxicity and bioaccumulative substances, using acute toxicity tests may not be appropriate method to predict the long term effect of pollutants on organisms and consequently ecosystem (Davis, 1978; Rainbow and Dallinger, 1993). Mortality and growth of *H. azteca* became increasingly sensitive with increasing exposure time to Cd (Suedel et al., 1997). Zinc and Pb were shown to increase their toxicity to the zebra mussel *Dreissena*

polymorpha with increasing exposure time (Kraak et al., 1994). The capacity of the mussel to regulate the body concentration of the essential metal Zn decreased when the exposure time was increased, whereas the non-essential metal Pb could not be regulated. This may explain the results from acute and chronic testing. *Hyalella azteca* was able to survive relatively high concentrations of Zn during the acute toxicity testing. When lower concentrations, relative to 96 LC_{50} were used in chronic testing animals suffered significant mortality and lower fecundity. On the contrary, animals exposed to Cd suffered high mortality at relatively low concentrations in acute testing and during chronic exposure, not showing any ability for regulation of this non-essential metal. These results show that the relationship between short-term and long-term toxicity differs for specific metals. The long-term effects of Zn in this study could not be predicted solely by short-term acute toxicity testing.

The main goal of toxicity testing is to monitor and predict the effects of pollutants not only on individual organisms but also on populations, communities and ecosystems. When an organism is exposed to stressful conditions this is projected at an individual level as a reduction in survival and/or growth and/or fecundity (Calow, 1989a). At a lower level, stress can cause the structure and functioning of cellular and molecular systems to become impaired (Koehn and Bayne, 1989) and at higher levels, it will cause a change in population density and in the structural and functional attributes of communities (Underwood, 1989). *Hyalella azteca*, as one of the most common freshwater amphipods in North America (Bousfield, 1958) is an important component of shallow-water ecosystems (Borgman et al., 1991) and can be a dominant food in the diet of fish, waterfowl and some aquatic invertebrates

(Wellborn, 1994, Borgmann, 1996). As these amphipods are prey, any changes to their fitness may also affect their predators. Decrease in a population of a preferential food source, may potentially affect species diversity at higher trophic linkages and interactions at these levels. Other benthic organisms, with the same preferences for a food source may have an advantage over a stressed population of *H. azteca* for acquisition of resources, which could affect the population distribution further. The accumulation of heavy metals within an exposed organism, which is a potential prey, will push the transfer of these metals up the food web. When toxicants impose stress on exposed organisms their performance can be reduced to the point where the community is replaced by a new one, composed of tolerant species or ultimately life in the area gets severely reduced or exterminated entirely.

Bioenergetic data from this study suggest changes to the population dynamics of *H. azteca* would occur indicating that chronic exposure to 94 $\mu gZn \cdot L^{-1}$ and especially 1.0 $\mu gCd \cdot L^{-1}$ will result in extinction or reduction of the population. Concentrations such as 9.4 $\mu gZn \cdot L^{-1}$ and 0.1 $\mu gCd \cdot L^{-1}$ will also have effects at the population level, due to decreased fecundity and survival and increased maintenance and repair costs. While metal bioaccumulation increases with concentration and exposure time, most likely due to an increasing proportion of Cd and Zn binding on metal-binding proteins, this could potentially be the cause of heavy metal transfer from *H. azteca* to their predators and subsequently affecting the whole food web of the aquatic system. In addition, behaviour testing demonstrated that all concentrations of Cd and Zn elicited an avoidance response what could also directly affect the community composition of the polluted areas. On the other hand, tolerance testing showed the possibility of acclimation in exposed organisms, where animals

become more tolerant during their lifetime due to pre-exposure to toxicants. This could be one of the reasons a species continues to survive in polluted environments, which may not necessarily imply that pollutants are not having an affect on the species. Therefore, the continued presence of certain species in the polluted areas should not be a conclusive factor in determining the effects of toxicants. This has obvious implications for long-term effects of both local pollution and global environmental changes.

All toxicological studies, used to predict the potential hazard of toxicants, should explicitly deal with organisms that had not been previously exposed to pollutants. The assumption made by Outridge et al.(1994), that the most Cd-sensitive organisms have limited capabilities for increasing their tolerance through pre-treatment and that the occurrence of inducible tolerance does not affect the derivation of LOEC, proved to be wrong. In this study, pre-exposure of the very sensitive species *H. azteca* to heavy metal poisoning, when pre-exposed to Cd, especially in the very early stage (eggs and embryos) showed an inducible tolerance.

When studying the toxic effects of certain heavy metals the relationship between toxicity and aqueous speciation of a metal should be quantified. The total metal concentration in water is seldom sufficient itself to elucidate the effects on organisms, or explain biological availability of metals. The chemical speciation of metals in solution and partitioning of metals between the aqueous and solid phases influence the biological availability and toxicity to organisms (Wagemann et al., 1994). Factors influencing the bioavailability of waterborne chemicals include the exposure concentration, the presence of particulates or dissolved organic matter, water quality (e.g. pH, water hardness) and molecular size and shape of the

chemical (Xue et al., 1995). The aqueous speciation of Cd and Zn in this study was calculated from the water quality data using the geochemical MINTEQA2 model. The results show that a high portion of Cd in the water is in Cd^{2+} ion form but less Zn^{2+} was in free form. Free metal-ion concentration is the key variable for reactivity and bioavailability and this could possibly explain the higher toxicity of Cd, as more Cd is available in the free ion form. At the same time this may explain in parallel with the regulatory ability of essential metals, the ability of organisms to withstand higher concentrations of Zn compared to Cd during chronic testing.

In conclusion, the combination of acute, chronic and behavioural testing allowed for a comprehensive understanding of the effects of two heavy metals, Cd and Zn on *H. azteca*. Acute testing established relative toxicity of these two metals to *H. azteca*. Measuring variables of the bioenergetic model used in chronic testing provided an insight into the impacts of the toxicant and their effect on the acquisition and allocation of energy. Tolerance testing showed that animals that are exposed to toxicants for their entire life span can acquire tolerance. Definite avoidance to relatively low concentrations of Cd and Zn proved the high sensitivity of *H. azteca* to heavy metal toxicity and calls for broader use of these organisms when conducting toxicity studies.

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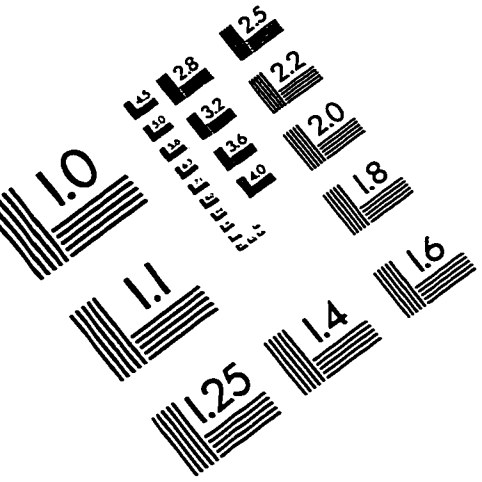
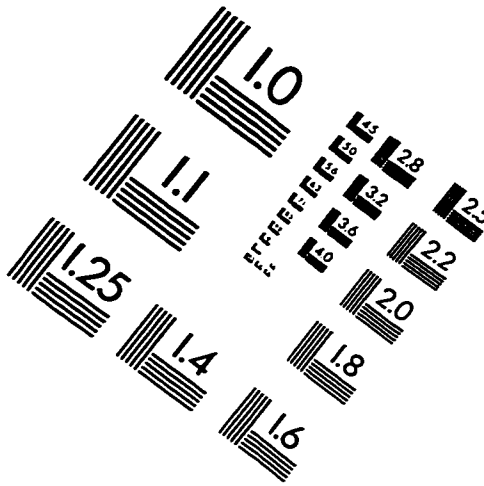
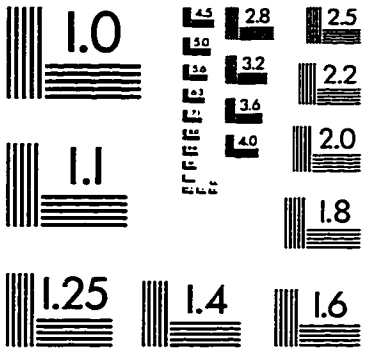
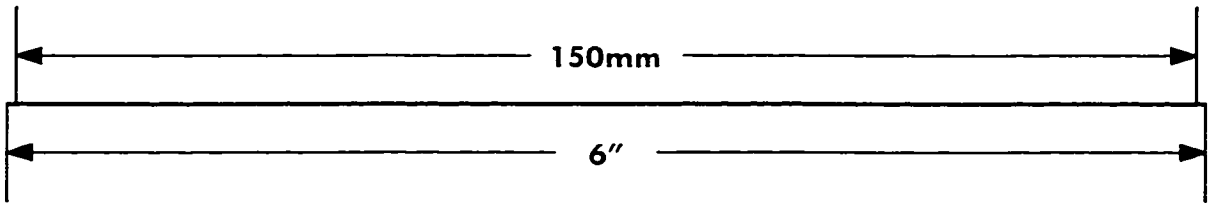
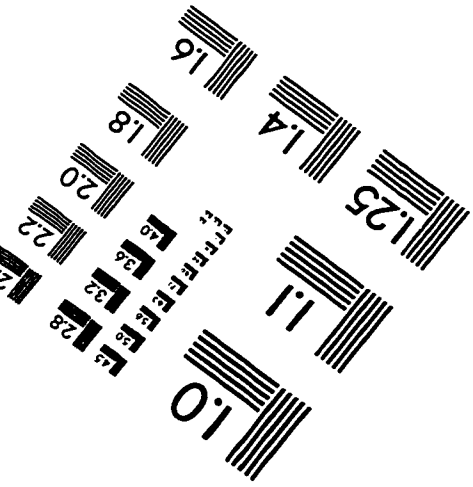
IMAGE EVALUATION
TEST TARGET (QA-3)

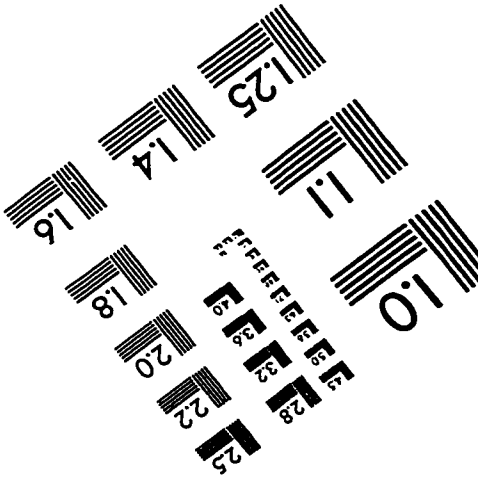












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