ACUTE AND CHRONIC ZINC EXPOSURE ON JUVENILE RAINBOW TROUT.

EFFECTS OF ACUTE AND CHRONIC ZINC EXPOSURE ON JUVENILE RAINBOW TROUT: INFLUENCE OF WATER CHEMISTRY AND BIOTIC LIGAND MODELLING.

By

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Abstract

The acute and chronic effects of waterborne zinc exposure were examined in juvenile rainbow trout (*Oncorhynchus mykiss*), together with the interactive effects of water chemistry.

Exposure of juvenile rainbow trout to 150 μg/L Zn or 450 μg/L Zn in hard water (hardness=120 mg $CaCO_3$/L) or exposure to 50 μg/L Zn or 120 μg/L Zn in soft water (hardness=20 mg $CaCO_3$/L) had no effect on growth or whole body Na^+, Ca^{2+}, or liver and gill Zn^{2+} levels. After 30 d of exposure, all zinc-exposed fish exhibited toxicological acclimation as indicated by 2.2-3.9 fold increases in the 96 h LC_{50} values over control trout. However, there was no effect of zinc exposure on metabolic rate or fixed velocity swimming performance. Critical swimming speed, however, was significantly reduced in zinc-exposed fish. Through the use of radiolabelled ^{65}Zn to distinguish new zinc accumulation, two zinc pools were found in the gills, a fast turnover pool (T½=3-4 h) and a slow turnover pool (T½=days to months). The fast pool was much larger in soft water than in hard water, but at most accounted for <3.5% of the zinc content of the gills. Acclimation to zinc increased the size of the fast pool. The loading rate into the slow pool was more rapid in soft water.

Branchial zinc uptake was reduced by a variety of waterborne cations (Na^+, K^+, NH_4^+, N-methyl-D-glucamine$^+$) in juvenile rainbow trout acclimated to soft water. The magnitude of the reduction was related to the concentration of positive charges in the water, regardless of which element or compound carried the charge. There appears to be non-specific competition between various cations and zinc, for negative sites on the gill

surface which are responsible for initiating zinc uptake. Ca^{2+} was more potent than the other divalent cation tested, Mg^{2+}, likely due to multiple sites of competition since Ca^{2+} and zinc share the same apical transport protein at the gill. Although Na^{+} and Mg^{2+} greatly reduced zinc uptake, they had no effect on zinc toxicity as measured with 96 h LC_{50} tests. This was paralleled by their inability to protect against zinc-induced inhibition of Ca^{2+} uptake. In contrast, Ca^{2+} greatly reduced zinc toxicity by preventing zinc-induced Ca^{2+} uptake inhibition, in addition to its ability to reduce zinc uptake.

Zinc accumulation in the gills of control and 250 μg/L Zn-acclimated trout exhibited saturation kinetics when fish were exposed to a range of zinc concentrations with ^{65}Zn for 0.5 to 72 h. In both groups, affinity of the gill for zinc increased rapidly (K_d decreased) from 0.5 to 3 h and then remained stable from 3 to 72 h. The binding capacity (B_{max}) continually increased in both groups from 0.5 to 72 h. Both K_d and B_{max} were greater in Zn-acclimated trout at all times. The stabilized log *K* binding constant (>3 h) for the gill and zinc was 5.6 in control fish, and 5.3 in Zn-acclimated fish. These log *K* values, together with the B_{max} estimates, provide the basis for the development of a Biotic Ligand Model to predict acute zinc toxicity by zinc-binding to trout gills.

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Everything I need to know I learned in a fish lab...

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One of my favorite escapes was to Catch-22, a novel by Joseph Heller, in which the character Orr is very much alike our fabled Ruban Kanagaratnam. I realize now that is why I liked/tolerated/laughed with him as much as I did.

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Actually, in the end I've really no clue what it's all about, but that is not necessarily a bad thing. However, Fern would argue it is about coffee, chocolate, and cheese (not sex).

Thesis Organization and Format

With the recommendation of my supervisory committee, this thesis has been presented in the "open-faced" format approved by McMaster University. Therefore, this thesis consists of a total of four chapters and a summary. Chapter 1 provides a general introduction to the area of study for this thesis, and a summary of the objectives, findings, and conclusions of each study. Chapters 2-4 are the manuscripts that have been published or submitted for publication in scientific journals. Finally, a brief summary of the findings concludes the thesis.

Chapter 1: General introduction and thesis overview.

Chapter 2: The costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water.

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Chapter 3: The influence of waterborne cations on zinc uptake and toxicity in rainbow trout, *Oncorhynchus mykiss*.

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Chapter 4: A kinetic analysis of zinc accumulation in the gills of juvenile rainbow trout: the effects of prior zinc acclimation and implications for biotic ligand modelling.

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Table of Contents

List of Tables

Chapter 2

Chapter 3

Chapter 4

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

CHAPTER 1

General Introduction and Thesis Overview

The themes of this thesis are the physiology and the toxicology of zinc in freshwater rainbow trout, under chronic and acute waterborne zinc exposures. Zinc is an essential micronutrient and a cofactor for over 300 enzymes (Vallee and Falchuk 1993). It is found at high levels in the tissues of fish and can range from approximately 10 μg Zn/g muscle to upwards of 5000 μg Zn/g in the eye (Hogstrand and Wood 1996). Zinc is primarily acquired from the diet and taken up by the gut, but when waterborne levels of zinc increase or when dietary zinc levels are low, uptake by the gill can increase to a large proportion of total uptake (Spry et al. 1988). If zinc levels in the water become too high, zinc can become toxic to fish.

Canada is one of the world's top producers of zinc. This heavy metal is mainly used in the production of brass and noncorrosive alloys, and in the galvanization of iron and steel products; galvanized products are used primarily in construction materials, automobile parts and household appliances. Of the total zinc that is discharged into the global environment, 96% is due to human activities. Major sources of zinc pollution include smelting and ore processors, electroplaters, drainage from mining operations, domestic and industrial sewage, combustion of fossil fuels and solid wastes, road surface runoff, corrosion of zinc alloys and galvanized surfaces, and erosion of agricultural soils (see Eisler 1997 for a review). A recent example of zinc pollution due to human activities from our area is the Pickering nuclear power plant (ON, Canada) which has discharged an estimated 1000 tonnes of zinc and copper into Lake Ontario over the past 25 years

(Toronto Star, May 17, 1997). The zinc pollution is due to the constant scouring of zinc-copper condensers which cool the steam generated by the plant.

While normal zinc levels in pristine freshwaters are only a few μg/L or less, concentrations of 50 μg/L are routine in industrialized areas. Maximum zinc concentrations in natural surface waters are reported to range from 130 to 1170 μg/L in different areas of Canada (CCME 1995).

Acute zinc toxicity

At extremely high waterborne levels, zinc causes gross morphological alterations at the teleost gill such as epithelial lifting and lamellar clubbing (Skidmore and Tovell 1972). The fish usually dies within a few hours due to tissue hypoxia resulting from impairment of gas exchange at the gill (Spry and Wood 1984; Lappivaara et al. 1995).

At environmentally realistic exposure concentrations, zinc specifically disrupts calcium uptake by the gills (Spry and Wood 1985; Hogstrand et al. 1995; Hogstrand et al. 1996), leading to hypocalcemia which may end with the death of the fish within a few days, depending on the zinc concentration.

The most influential component of water chemistry that modulates acute zinc toxicity is thought to be the concentrations of waterborne Mg^{2+} and Ca^{2+}, which together are termed "water hardness" (CCME 1995). Perhaps the best study that has demonstrated the modulating effect of hardness on acute zinc toxicity to rainbow trout was the work done by Bradley and Sprague (1985). These authors showed that a 10-fold increase in water hardness alone increased the 96 h LC_{50} (lethal zinc concentration that induces mortality in 50% of the population in 96 h) approximately 10-fold. Almost universally,

there has been a general acceptance of the protective effect of water hardness on zinc toxicity. For example, to set Ambient Water Quality Criteria (AWQC) for waterborne zinc in industrial areas, the U.S. EPA (1980) uses an equation based on hardness to calculate a numerical acute limit for total allowable zinc:

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

At a hardness typical of Hamilton tapwater from Lake Ontario (120 mg $CaCO_3$/L), the acute limit for zinc would be 374 μg/L based on this U.S. EPA equation. Similar adjustments for hardness are applied in European criteria (Alabaster and Lloyd 1980). However, the International Joint Commission (1976) limit for Lake Ontario is 30 μg/L, and the Canadian national guideline is also 30 μg/L Zn, regardless of water hardness (CCME 1995). These latter values are designed to protect against both acute and chronic toxicity, and are based on the belief that hardness does not affect chronic toxicity, in contrast to acute toxicity (U.S. EPA 1980). However, there is little direct experimental evidence on this point.

Chronic zinc exposure

If a fish survives the zinc exposure, then the ionic disturbance that can occur may be eventually corrected (McDonald and Wood 1993), as seen with the full recovery of plasma Ca^{2+} levels during a sublethal exposure to zinc (Hogstrand et al. 1995). An increased tolerance (in terms of survival) to the metal may arise upon a threshold exposure. With zinc-exposed trout, this acclimation or increased tolerance was fully acquired within 5 days, with the tolerance having increased 2.5 times compared to unexposed fish as judged by LC_{50} tests (Bradley et al. 1985). Acclimation to zinc, as well

as to other metals, is thought to occur in a variety of ways including changes at the gill, such as alterations to transport proteins, hypertrophy and hyperplasia of mucous and chloride cells, and a general thickening of lamellar and filamental epithelia (Mallatt, 1985; McDonald and Wood 1993).

There have been few reported costs of zinc acclimation to the fish which takes place during the first few days of exposure, or any negative effects of prolonged exposure. Protein synthesis rates in the gills of rainbow trout were elevated after 9 days of exposure to sublethal zinc, a process presumably reflecting damage-repair (McDonald and Wood 1993) which may carry a significant metabolic cost (Hogstrand et al. 1995). However, the protein synthesis rates had subsided to control levels after 18 days (Hogstrand et al. 1995) and 23 days (Hogstrand et al. 1994) of exposure. Growth rates in these fish were temporarily compromised, though the trout were able to regain the lost growth. Indeed, another study demonstrated that the growth of salmon was unaffected by a 3 or 18 month chronic zinc exposure, and may have even been stimulated, perhaps due to the role of zinc as a micronutrient (Chapman 1978). The structural changes of the gills that are thought to occur with acclimation could have effects on the maximum O_2 and CO_2 exchange rates, and in doing so could limit aerobic swimming performance. In studies conducted with other metals, critical swimming speed (U_{Crit}) was significantly depressed upon acclimation to aluminum (Wilson et al. 1994) and to copper, the latter depending on the water composition (Waiwood and Beamish 1978). However, Galvez et al. (1998) found no change in the chloride cell surface area after 15 days of exposure to sublethal zinc, though they did not test whether these fish had acclimated in terms of

increased resistance to zinc. As noted in the previous section, there is little concrete evidence on whether hardness affects chronic zinc toxicity.

Gill binding model

As a result of international regulatory agreements arising from the Rio (1992) "Earth Summit" Conference, the Canadian zinc industry may soon be required to adhere to global regulations for zinc discharge. These global regulations ignore water chemistry and would likely devastate the zinc industry by over-regulating some companies. At the same time, such regulations also stand to harm the environment by under-regulating others. There has been much interest recently in developing a method for predicting heavy metal toxicity to aquatic biota while taking water chemistry into account, due to the realization that water chemistry may exert profound effects on heavy metal toxicity (Playle et al. 1993; Bergman and Doward-King 1997; Renner 1997; Playle 1998; Meyer 1999). As the branchial epithelium appears to be the primary target of acute metal toxicity, it has been proposed that the amount of metal accumulating at the gill could be an indicator of toxicity.

The receptor loading model is one such approach that has been successfully used to predict metal levels in the gills of fish. This method, now generally called the Biotic Ligand Model or BLM, was derived from the original framework proposed by Pagenkopf (1983), and involves using experimentally determined binding constants [affinity (K_d) and capacity(B_{max})] of the gill for the metal and for other ions. Electrolytes such as Ca^{2+}, H^+, and Na^+, which occur naturally in freshwaters, can compete for negative sites on the gill surface and thereby keep the metal off the gill, while natural complexing agents such

as dissolved organic carbon can bind zinc in the water and keep it off the gill. The gill binding constants, together with known water chemistry at a particular site, are then used in an aquatic geochemical programs such as MINEQL+ (Schecher and McAvoy 1994) or MINTEQA2 (Allison et al. 1991) to predict by calculation how much metal will bind to fish gills, and thereby estimate toxicity. This approach has worked for metals such as copper and cadmium (Playle et al. 1993, Hollis et al. 1997), silver (Janes and Playle 1995) and cobalt (Richards and Playle 1998).

Attempts have been made to apply these techniques to zinc (Galvez et al. 1998), but the extremely high levels of zinc found in the gills of all fish, due to its role as a micronutrient, made detecting any accumulation difficult. However, Galvez et al. (1998) had some success by using radiolabelled ^{65}Zn to distinguish newly accumulated zinc in the gills of juvenile rainbow trout from the large pool of native zinc already present. With Michaelis-Menton analysis, they were able to estimate the affinity (K_d) and binding capacity (B_{max}) of the gill for zinc.

Zinc transport

Although the majority of zinc acquired for nutritional requirements is absorbed by the gut from the diet, the gill can be another important pathway that can supply the fish with zinc from the water, especially when waterborne zinc levels are elevated or dietary zinc levels are low (Spry et al. 1988). From the water, zinc crosses the apical membrane into the gill cell via the voltage independent calcium channel (Fig. 1) (Spry and Wood 1985; Hogstrand et al. 1995; Hogstrand et al. 1996). Since it is believed that Ca^{2+} uptake occurs in the chloride cells and not in the respiratory cells (Flik and Verbost 1993), zinc

uptake also probably occurs in this population of cells. Due to Ca^{2+} and zinc's shared apical transport route, it is understandable that when waterborne zinc levels become too high, hypocalcemia is specifically induced, which may lead to the death of the fish.

Once in the gill cell, Ca^{2+} is transported across the basolateral membrane into the blood via an ATPase (Perry and Flik 1988) and a Na^{+}/Ca^{2+} exchange transporter (Flik et al. 1995). It is unknown how zinc crosses the basolateral membrane, except that it is not via the Ca^{2+}-ATPase nor is it via the Na^{+}/Ca^{2+} exchanger (Hogstrand et al. 1996). Zinc is, however, a potent inhibitor of the Ca^{2+}-ATPase (Hogstrand et al. 1996).

Study 1) ***The costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water.***

The goal was to examine the effects of chronic, sublethal, waterborne zinc exposure on a variety of physiological parameters in freshwater rainbow trout, and determine any modifying effects of water hardness on zinc exposure. The parameters of interest included resistance to acute elevated zinc exposure (acclimation), swimming performance, metabolic rate, growth, and tissue zinc burdens and ion concentrations (Na^{+} and Ca^{2+}). In light of the uncertainties mentioned earlier, determining the influence of water hardness on the effects of chronic zinc exposure was of importance due to the proven impact of hardness on acute zinc toxicity (Bradley and Sprague 1985) and general metal bioavailability (Meyer 1999; Meyer et al. 1999). There is also great environmental relevance to studying the effects of zinc in different water chemistries due to the wide range of water compositions in the freshwater lakes of Ontario. For example, the

concentrations of ions such as Ca^{2+} and Mg^{2+} can differ by over two orders of magnitude between the hard water of Lake Ontario and soft water of the Sudbury area lakes.

In addition, to further our understanding of the dynamics of zinc accumulation in the gills for the development of a Biotic Ligand Model, an additional goal was to assess the zinc accumulation/turnover rates in the gills of control and zinc-acclimated trout at the zinc exposure concentrations to which they had been acclimated.

Juvenile rainbow trout were exposed to two zinc levels in both hard water (HW: hardness=120 mg $CaCO_3$/L) and soft water (SW: 20 mg $CaCO_3$/L) for at least 30 days. Based on preliminary toxicity tests, a high zinc level was chosen that would induce some acute mortality (450 μg/L Zn in HW and 120 μg/L Zn in SW), while a low zinc level was chosen that would not induce any mortalities (150 μg/L Zn in HW and 50 μg/L Zn in SW). During the 30 days, growth, mortality, tissue zinc burdens and tissue ion levels (Na^+ and Ca^{2+}) were monitored. After 30 days, resistance to zinc (96 h LC_{50}s), swimming performance (critical swimming speed and fixed velocity swimming performance), metabolic rate, and the accumulation/turnover rate of zinc in the gills of control and zinc-acclimated trout were assessed.

During the exposures, there were no apparent effects of zinc on growth, whole body Na^+ or Ca^{2+} concentrations, or zinc levels in the tissues. After 30 days, 96 h LC_{50} tests were performed and demonstrated that water hardness had a profound impact on acute zinc toxicity. In soft water, zinc was 5.4 times more toxic than in hard water (162 μg/L Zn vs. 869 μg/L Zn). In addition, all zinc-exposed groups had acclimated to zinc as demonstrated by substantial increases in the 96 h LC_{50} (a 2.2-3.9 fold increases in tolerance). Although the fish must have acclimated through physiological and/or

structural changes, there appeared to be no marked effect or cost on the surviving individuals, for there was no effect of zinc exposure on metabolic rate or fixed velocity swimming performance. This absence of effect was seen despite the fact that 26% mortality occurred in the high zinc-exposed group (450 μg/L Zn) in hard water during the first few days of exposure. There was however, a significant decrease in the critical swimming speed with zinc acclimation, indicating that zinc may act as a "limiting stressor"-one which depresses aerobic capacity without necessarily affecting routine metabolism.

^{65}Zn was used to determine zinc turnover rates in the fish. Zinc turnover rate was higher in the gills and other tissues in soft water than in hard water, reflecting the increased bioavailability of zinc in soft water. At least two pools of zinc operating in the trout gill were found. There was a fast exchanging zinc pool which had a time to 50% turnover (T½) of about 3-4 hours. Although the size of the fast pool increased with acclimation to zinc, its absolute size was small, approximately 0.14% of the total gill zinc levels in control trout and 3.5% in zinc-acclimated trout. In addition, there was a much slower exchanging pool which appeared to turn over linearly with time, with a T½ of days to months.

The fast pool is interpreted as a dynamic pool bound to high affinity sites, a pool of zinc which is in the process of being taken up, excreted, detoxified, or stored. The size of the fast turnover pool increased with the concentration of zinc to which the fish were chronically exposed. The slow pool presumably represents incorporation into structural components of the gill (e.g. zinc-dependent proteins) in growing fish, though it could also represent long-term detoxification storage in zinc-binding proteins.

Overall, this study showed that water hardness did influence the few chronic effects of waterborne zinc exposure. The 96 h LC_{50} tests on fish chronically exposed to zinc indicated that at a given zinc concentration, zinc induces a greater acclimatory response from the fish in soft water. In addition, at similar chronic exposure concentrations, the size of the fast turnover pool of zinc in the gills was much greater in soft water than in hard water, and the loading rate into the slow pool was also much faster in soft water. Finally, there was 25% "delayed" mortality (during days 29-32) in the 120 μg/L Zn exposure in soft water, while no mortalities occurred in any other groups in soft or hard water during this time.

Study 2) ***The influence of waterborne cations on zinc uptake and toxicity in rainbow trout.***

An accidental discovery during the course of this thesis research was that zinc uptake was greatly reduced by Na^+ in soft water. This was an unexpected result since Na^+ is not thought to interact with zinc during branchial uptake, in contrast to Ca^{2+} for example, which shares an apical transporter with zinc (Fig. 1). From this finding, the logical next step was to determine the effects of a variety of cations on zinc uptake and toxicity. A particular objective was to compare Ca^{2+} and Mg^{2+}, the two "hardness" cations that are both generally believed to protect aquatic organisms from acute zinc toxicity.

Radiolabelled zinc uptake at a total waterborne concentration of 100 μg/L Zn was determined during short fluxes (7 h) in the presence and absence of a variety of different cations (at concentrations ranging from 0.5 to 2.0 mM). In addition, 96 h LC_{50} toxicity tests for zinc in soft water were conducted in the presence of added Na^+, Mg^{2+}, or Ca^{2+}. In

parallel experiments, effects of these cations in protecting against zinc-induced Ca^{2+} uptake inhibition was examined.

Zinc uptake was reduced by a variety of cations, in direct proportion to the concentration of positive charges, regardless of which ion carried that charge. Thus, 1.0 mM Na^+, K^+, NH_4^+, N-methyl-D-glucamine$^+$ and 0.5 mM Mg^{2+} all reduced Zn^{2+} influx to a similar extent (approximately 50%). These results suggest a relatively non-specific competition for negatively charged sites on the gill surface, including those which are responsible for Zn^{2+} uptake into the gill cells. However Ca^{2+} was one important exception and was much more potent than Mg^{2+} in reducing Zn^{2+} uptake. Ca^{2+} would likely also compete for absorption since Ca^{2+} and Zn^{2+} are known to share the same apical transport channel at the gill. Indeed, 100 μg/L Zn^{2+} inhibited Ca^{2+} uptake by 87%. Although Na^+ and Mg^{2+} were able to markedly reduce Zn^{2+} uptake, they had no effect on Zn^{2+} toxicity, a result paralleled by their inability to restore Ca^{2+} uptake. In contrast, 1 mM Ca^{2+} both reduced Zn^{2+} toxicity (the 96 h LC_{50} increased 18-fold) and restored Ca^{2+} uptake, in addition to reducing Zn^{2+} uptake. These results partially dissociate Zn^{2+} uptake from Zn^{2+} toxicity, implicate disturbed Ca^{2+} uptake as the mechanism of toxicity, and have profound implications for water quality criteria where Ca^{2+} and Mg^{2+} (the two 'hardness' cations) are traditionally considered to be equally protective.

Study 3) ***A kinetic analysis of zinc accumulation in the gills of juvenile rainbow trout: the effects of prior zinc acclimation and implications for biotic ligand modelling.***

Galvez et al. (1998) showed that gill zinc accumulation is saturable during 3 h exposures to a range of waterborne zinc concentrations. In addition, the first study of this

thesis showed that there are two pools of zinc in the gills, a fast and a slow turnover pool. The present study was now designed to examine zinc accumulation in the gills as a function of both waterborne zinc concentration and time. In particular, the primary goal was to determine gill binding constants (K_d, B_{max}, log *K*), and their possible time dependence, for Biotic Ligand Modelling (BLM) of zinc in naïve and zinc-acclimated rainbow trout. Zinc accumulation kinetics in the gills were examined in both control and 250 μg/L Zn-acclimated trout during exposures to a range of zinc concentrations from 0.5 to 72 h in hard water. Radiolabelled ^{65}Zn was used to measure accumulation due to the high levels of cold zinc already present in the gills of unexposed trout (Galvez et al. 1998).

The gill ^{65}Zn accumulation exhibited saturation kinetics, allowing calculation of binding capacity (B_{max}) and affinity (K_d). In both control and Zn-acclimated trout, affinity increased (K_d decreased) from 0.5 to 3 h, and then remained constant up to 72 h. The binding capacity of the gills (B_{max}) increased rapidly from 0.5 to 3 h in both groups, then the rate of increase began to subside but was still increasing from 24 to 72 h. At all times, the K_d of Zn-acclimated fish was higher (i.e. lower affinity) and B_{max} was greater than controls. The stabilized log *K* binding constants (>3 h) were 5.6 and 5.3 in control and Zn-acclimated fish respectively.

Conclusions and implications

There appeared to be few negative physiological consequences of chronic, low level zinc exposure to juvenile rainbow trout. Even after significant acute mortality (during the first few days of exposure) in 25% of the population, there was no effect on

the surviving fish with respect to growth, sprint swimming performance, metabolic rate, tissue zinc levels and whole body Na^+ and Ca^{2+} levels, even though these fish had undergone physiological or structural changes during the process of toxicological acclimation. However, aerobic swimming performance was slightly impaired in zinc acclimated trout, suggesting structural changes had occurred at the gill with acclimation. There were at least two pools of zinc in the trout gills, a fast and a slow turnover pool. The size of the fast pool increased with acclimation to elevated waterborne zinc, perhaps due to increased detoxification capacity or zinc storage (e.g. metallothionein) in the gills. This may be one factor contributing to the toxicological acclimation of the zinc-exposed fish. Water hardness influenced both the acute and chronic effects of zinc. On an acute basis, zinc was 5.4 times more toxic in soft water than in hard water as measured with 96 h LC_{50} tests. After chronic zinc exposure, the fast pool was larger in softwater than in hardwater and the loading rate of zinc into the slow turnover pool was faster in soft water at a given zinc exposure concentration. The decreased levels of Ca^{2+} in soft water may increase the availability of sites for zinc binding. In addition, in terms of increased resistance to zinc, trout acclimated to a greater degree in soft water than in hard water at similar zinc exposure concentrations. Finally, there was 25% "delayed" mortality during days 29-32 in the 120 μg/L Zn exposure in soft water, while no mortalities occurred in any other groups in soft or hard water during this time. Although zinc cannot be singularly implicated for these deaths in soft water, further investigation into the mechanisms behind these deaths would be important in attempting to determine the chronic effects of zinc exposure.

Zinc uptake by the gill was reduced by a variety of waterborne cations. The magnitude of the reduction was directly related to the concentration of positive charges in the water, regardless of which element or compound carried that charge. However, Ca^{2+} was more potent in reducing zinc uptake, likely due to multiple sites of competition with zinc for uptake into the gill, since Ca^{2+} and zinc share an apical transport protein at the gill. In addition, unlike Na^{+} and Mg^{2+}, Ca^{2+} was able to ameliorate acute zinc toxicity through an ability to restore the branchial Ca^{2+} uptake that was inhibited by zinc. This difference in protection offered by Ca^{2+} and Mg^{2+} is not recognized by the U.S. EPA (1980) Ambient Water Quality Criteria for acute toxicity. The U.S. EPA regulates the potential for acute toxicity of zinc based on water "hardness", as the sum of both waterborne Ca^{2+} plus Mg^{2+} (see Eqn. 1 above). Thus the higher the Mg^{2+} levels in the water, the more zinc the EPA would allow to be discharged. However, since Mg^{2+} did not protect against acute zinc toxicity, this could prove harmful to aquatic life. The IJC has set a limit of 30 μg/L Zn in the Great Lakes and this limit has also been adopted as the Canadian national guideline (CCME 1995). This limit is independent of water chemistry, and would appear to be safe in the hard water of Lake Ontario, where the measured 96 h LC_{50} for rainbow trout is 869 μg/L Zn (Study 1/Chapter 2). However, this limit may be too high for the soft water systems of Ontario. Exposure to 50 μg/L Zn in soft water elicited a strong toxicological acclimatory response where the 96 h LC_{50} increased 2.2 fold over the control level. This indicates that the fish could perceive and react to 50 μg/L Zn. If zinc is to be discharged into the aquatic environment, what is required is a strict regulation that takes water chemistry into account and recognizes that Ca^{2+} protects against acute zinc toxicity while Mg^{2+} does not. In addition, since chronic zinc toxicity is

influenced by water hardness, a regulation for chronic zinc exposure is required that takes this into consideration. However, further studies are needed to determine whether the protective effects of hardness on chronic zinc exposure are due to both Ca^{2+} and Mg^{2+}, or just Ca^{2+}.

The affinity of the gill for zinc increased rapidly (K_d decreased) during the first 3 h of exposure, but was stable from 3 h to 72 h in both control and zinc-acclimated trout gills. Prior zinc exposure reduced the affinity of the gill for zinc at all times. For the purposes of Biotic Ligand Modelling, a stable log *K* binding constant of 5.6 for control trout gills and 5.3 for zinc-acclimated trout gills was determined. The log *K* binding constant for zinc is relatively weaker than for most other metals. The log *K* for control fish gills and cadmium is 8.6 (Playle et al. 1993), which indicates cadmium binds to gills 1000-fold better than zinc. In addition, zinc is also relatively less toxic, approximately 100-fold less than cadmium as measured with 96 h LC_{50}s in water of similar hardness (Hollis et al. 1999), demonstrating a relationship between binding affinity and toxicity.

Although the affinity of the gill for zinc stabilized by 3 h, the number of zinc binding sites had not stabilized by 72 h, but instead was continuously increasing with exposure time. The number of binding sites was approximately 20 times greater at 72 h than at 0.5 h in both groups and were always more numerous in zinc-acclimated trout gills.

The use of aquatic geochemical programs such as MINEQL+ (Schecher and McAvoy 1994) to predict gill metal accumulation levels in different water chemistries, requires both a log *K* binding constant and the number of metal binding sites in the gill. Although this thesis has determined the log *K* constants for zinc, it has also shown that

the number of binding sites changes with exposure time. It should be the goal of future Biotic Ligand Modelers to determine at which time the number of binding sites is most appropriate for use in BLMs to predict gill metal accumulation and acute toxicity.

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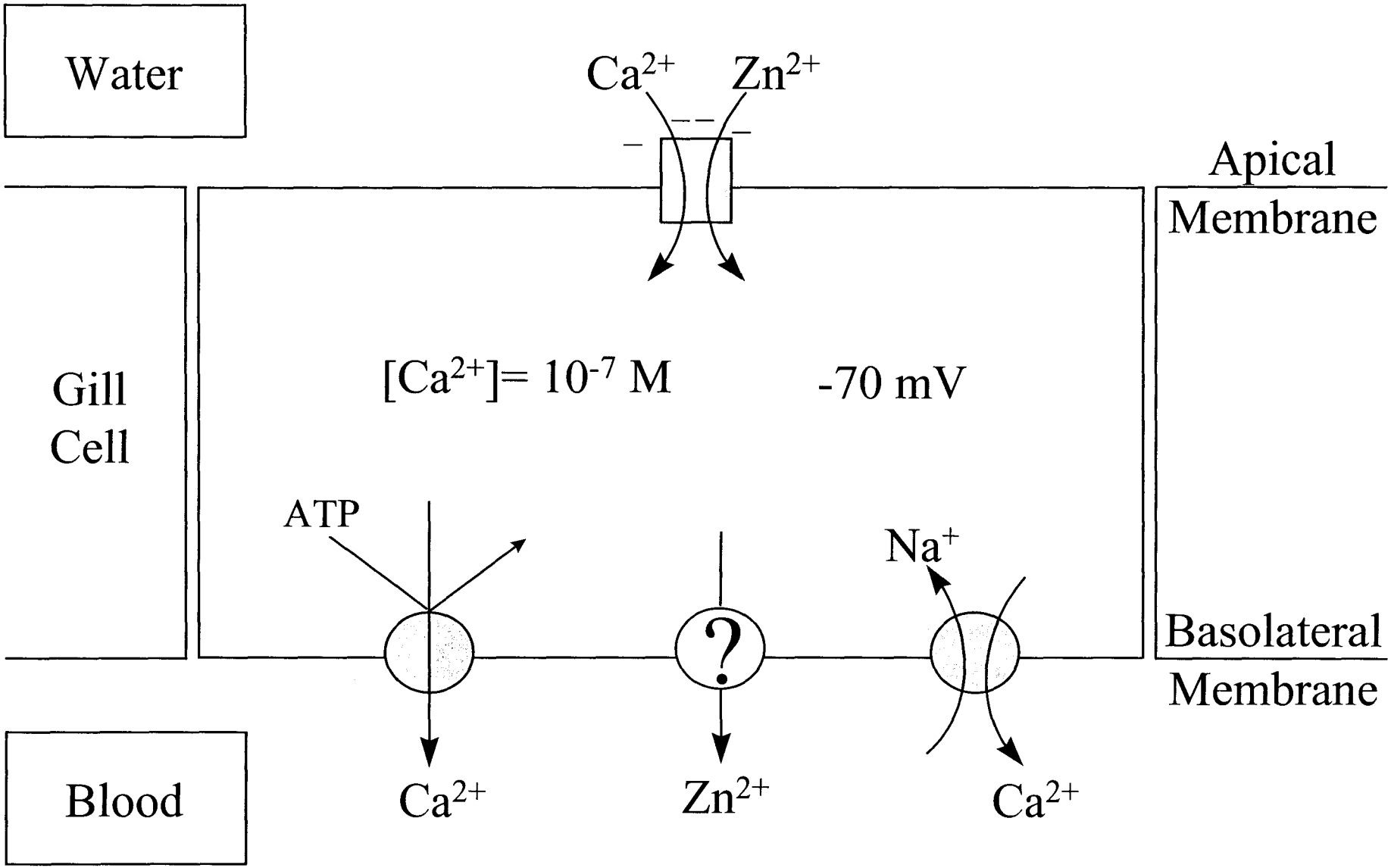
Figure 1-1. In freshwater, calcium and zinc enter the gill cell through the voltage independent calcium channel down the electrochemical gradient. Once in the cell, calcium is transported across the basolateral membrane into the blood via a high affinity Ca^{2+}-ATPase or a sodium/calcium exchanger against the electrochemical gradient. It is unknown how zinc crosses the basolateral membrane, though it is not via the Ca^{2+}-ATPase or sodium exchanger. However, zinc is a potent inhibitor of the Ca^{2+}-ATPase.


Water
Ca^{2+}
Zn^{2+}
Apical
Membrane
Gill
Cell
$[Ca^{2+}]= 10^{-7}$ M
-70 mV
ATP
Na^{+}
?
Basolateral
Membrane
Blood
Ca^{2+}
Zn^{2+}
Ca^{2+}

CHAPTER 2

The costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water.

Abstract- Juvenile rainbow trout were exposed to zinc in both moderately hard water (hardness=120 mg $CaCO_3$/L, pH=8.0, Zn=150 μg/L or 450 μg/L) and soft water (hardness=20 mg $CaCO_3$/L, pH=7.2, Zn=50 μg/L or 120 μg/L) for 30 days. Only the 450 μg/L Zn exposed fish experienced significant mortality (24% in the first two days). There was no effect of zinc exposure on growth rate, but growth affected tissue zinc levels. Whole body zinc levels were elevated, but gills and liver showed no consistent increase relative to controls over the 30 days. Therefore, tissue zinc residues were not a good indicator of chronic zinc exposure. After the 30 day exposure, physiological function tests were performed. Zinc was 5.4 times more toxic in soft water (control 96 h-LC_{50}s in hard and soft water were 869 μg/L and 162 μg/L respectively). All zinc-exposed trout had acclimated to the metal, as seen by an increase in the LC_{50} 2.2-3.9 times over control fish. There appeared to be few physiological costs to acclimation. Zinc exposure had no effect on whole body Ca^{2+} or Na^{+} levels, resting or routine metabolic rate, or fixed velocity sprint performance. However, critical swimming speed (U_{Crit}) was significantly reduced in zinc-exposed fish, an effect which persisted in zinc-free water. Using radioisotopic techniques to distinguish new zinc incorporation, the gills were found to possess two zinc pools, a fast turnover pool (T½=3-4 h) and a slow turnover pool (T½=days to months). The fast pool was much larger in soft water than in hard water, but at most accounted for <3.5% of the zinc content of the gills. The size of the slow pool is

unknown, but its loading rate was faster in soft water. Chronic zinc exposure was found to increase the size of the fast pool, and to increase the loading rate of the slow pool.

Keywords- Rainbow trout, Zinc, Acute/Chronic toxicity, Acclimation, Gill metal-binding model.

Introduction

Zinc is an essential micronutrient and a cofactor of over 300 enzymes [1], but becomes toxic at increased waterborne levels. At extremely high waterborne levels, zinc causes gross morphological alterations at the teleost gill such as epithelial lifting and lamellar clubbing [2]. The fish usually dies within a few hours due to tissue hypoxia resulting from impairment of gas exchange at the gill [3]. At lower waterborne concentrations more realistic of contaminated environments, zinc specifically disrupts calcium uptake across the gills [4-6], leading to hypocalcemia which may end with the death of the fish within a few days, depending on the zinc concentration.

Fish have been shown to acclimate to metals during sublethal waterborne exposure in two respects; first, if a fish survives the metal exposure, then the ionic disturbance may be eventually corrected [7], as seen with the full recovery of plasma Ca^{2+} during a sublethal exposure to zinc [6]. Secondly, an increased tolerance (in terms of survival) to the metal may arise upon a threshold exposure. With zinc-exposed trout, this acclimation or increased tolerance was fully acquired within 5 days with the tolerance having increased 2.5 times compared to unexposed fish as judged by LC_{50} tests [8].

Acclimation to metals is thought to occur in a variety of ways including changes at the gill, such as alterations to transport proteins, hypertrophy and hyperplasia of mucous and chloride cells, and a general thickening of lamellar and filamental epithelia

[7,9]. These structural changes could have effects on the maximum O_2 and CO_2 exchange rates, and in doing so would limit aerobic swimming performance. In previous studies, critical swimming speed (U_{Crit}) was significantly depressed upon acclimation to aluminum [10] and to copper, the latter depending on the water composition [11].

Zinc toxicity is dependent not only on the zinc concentration, but also on the presence of other dissolved ions. Water hardness (as mg $CaCO_3$/L) is considered to be the most influential component of water chemistry modifying toxicity. For example, a decrease in water hardness of approximately 10 times was shown to increase zinc toxicity by 10 times [12]. By analogy to work done with other metals [13-15], it is thought that in soft water there is less competition from other ions, most importantly Ca^{2+}, for zinc binding sites on the gill surface. There are also fewer ligands in the water that keep zinc from binding to the gill sites.

The objectives of this study were to determine the effects of chronic zinc exposure on juvenile rainbow trout and how they are modified by water hardness. Fish were exposed to 150 μg/L and 450 μg/L Zn at a water hardness of 120 mg $CaCO_3$/L, and to 50 μg/L and 120 μg/L Zn at a hardness of 20 mg $CaCO_3$/L. During the exposures, mortality and growth were recorded and tissue levels of zinc were measured. After the 30-day zinc exposure, the performance of the fish was evaluated in various physiological function tests that included two types of swimming performance, acclimation as assessed by zinc tolerance (96 h-LC_{50}), metabolic rate and the turnover rates of zinc in various tissues.

The kinetics of zinc turnover in the gills were a particular focus because of recent interest in using gill metal-binding models to predict site-specific toxicity [14,16]. A

recent study has demonstrated that because zinc is a micronutrient present at high background levels in gill tissue, zinc binding to gills can only be detected if radioisotopic ^{65}Zn is employed [17]. This being the case, it is essential to understand the kinetics of turnover detected by the radioisotope, and to determine whether these kinetics change during chronic sublethal zinc exposure.

Materials and methods

Chronic zinc exposures were performed in two water qualities, moderately hard Hamilton tap water from Lake Ontario, and synthetic soft water.

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Rainbow Springs Trout Hatchery (Thamesford, Ontario), initially held in aerated 500L tanks supplied with 3 L/min of dechlorinated Hamilton tap water ("hard water" ionic composition; Ca^{2+}, 1.0 mM; Mg^{2+}, 0.2 mM; Na^{+}, 0.6 mM; Cl^{-}, 0.7 mM; hardness, 120 mg $CaCO_3$/L; alkalinity, 95 mg $CaCO_3$/L; dissolved organic matter (DOM), 3 mg/L; pH 8.0), and allowed to acclimate for one week. The fish were then slowly brought to the appropriate water chemistry if required and temperature over 7 days (up to 14-18°C, and reduced hardness in the soft water exposure). "Soft water" (ionic composition; Ca^{2+}, 0.13 mM; Mg^{2+}, 0.04 mM; Na^{+}, 0.13 mM; Cl^{-}, 0.1 mM; hardness, 20 mg $CaCO_3$/L; alkalinity 15 mg; $CaCO_3$/L; DOM, 0.4 mg/L; pH 7.2) was synthesized by mixing one part hard water to six parts ion-reduced water, the latter produced by reverse osmosis (Anderson Water Systems), a procedure which unavoidably raised the temperature to 18°C, relative to 14°C in the hard water exposure. The fish were allowed to acclimate to their new

water conditions for at least three weeks prior to the experiment. Fish were fed twice daily a commercial ration totaling 2% body mass/day during this holding period [fish food composition (partial analysis only): crude protein (min), 52%; crude fat (min), 17%; crude fibre (max), 2.5%; water, 12%; Ca^{2+}, 1.4%; Na^{+}, 0.4%; zinc (measured), 0.02% (173 μg/g)].

One week prior to each experiment, fish (N=1620) were non-selectively transferred to one of six identical 211L tanks (270 fish per tank, mean fish weight 1.68 ± 0.16g in the hard water exposure and 5.27 ± 0.06g in the soft water exposure). Water flow into each tank was >0.75 L/min; Po_2 was maintained at > 90% air saturation through continuous aeration of the tanks. Feces and organic debris were siphoned out of the tanks daily. Photoperiod was set to 10-12 h light/balance dark to a mimic natural photoperiod.

Exposures

At the start of each exposure, the six tanks were non-selectively assigned to one of the three zinc exposure concentrations (two tanks/Zn exposure concentration). Hard water; control (<1 μg/L Zn), low zinc (150 μg/L Zn) and high zinc (450 μg/L Zn). Soft water; control (<1 μg/L Zn), low zinc (50 μg/L Zn) and high zinc (120 μg/L Zn). The levels were chosen on the basis of rangefinder toxicity tests that were performed prior to each exposure. A high zinc exposure level was chosen to produce slight acute mortality, while the low exposure was intended to cause no mortality. To begin the exposure, flow from a Mariotte bottle of concentrated zinc solution ($ZnSO_4.7H_2O$, Anachemia with the addition of 1 ml concentrated HNO_3/L de-ionized water, trace metal analysis grade, BDH Chemicals) was started into a head tank where it mixed with inflowing fresh water by vigorous aeration. Zinc was also added directly to the exposure and head tanks to rapidly

bring each one up to the desired level. Zinc levels in the hard water experiment ranged from 129-165 μg/L (mean 157 μg/L) in the low zinc exposure and 425-465 μg/L (mean; 458 μg/L) in the high exposure. In soft water, the ranges were 45-67 μg/L (mean; 53 μg/L) in the low zinc exposure and 109-138 μg/L (mean; 118 μg/L) in the high exposure.

After the two exposures were completed, a supplementary third series was conducted in hard water in exactly the same manner as the first. This time however, there was only one control tank and one 250 μg/L zinc exposure tank. The fish were acclimated for one month to investigate the effects of zinc acclimation on oxygen consumption of individual fish in respirometers and aerobic swimming performance (methods described below).

For each exposure, fish were fed three 1% body mass meals per day totaling 3% per day. Each meal was calculated as 1% of the bulk weight of each tank, and the meal amount was modified with each bulk weighing. Throughout the exposure, mortalities were recorded and removed daily, weighed and feeding quantities adjusted as needed.

Sampling

At one day prior to the start of the experiment, and at days 2 (hard water) or 5 (soft water), 10, 20 and 30 after exposure initiation, fish (N=6 per treatment) were removed and quickly sacrificed with a blow to the head. The gills and liver were excised and frozen in liquid nitrogen along with the remaining carcass. Whole fish (N=6) were also removed, sacrificed and frozen.

Zinc levels in the tissues were determined by digestion in 5 volumes of 1 N HNO_3 (trace metal analysis grade, BDH chemicals) for 3 h at 80°C. Samples were vortexed, allowed to settle for 24 h; 100 μl of supernatant was diluted to 1 ml with deionized water

(Barnstead, NANOpure II) and analyzed by atomic absorption spectroscopy (Varian AA-1275, using an air/acetylene flame). Whole body Ca^{2+} and Na^{+} concentrations were measured from dilutions of the whole body acid digest in the same manner. Water samples were collected throughout the exposures (20 ml water + 50 μL concentrated HNO_3). Water samples were analyzed by atomic absorption spectroscopy for Zn^{2+}, Ca^{2+} and Na^{+}.

On the day prior to the start of the experiment as well as every 6-7 days during the exposure, all the fish in each tank were bulk-weighed using a removable sieve. The specific growth rate (SGR) or % increase in body mass/day (with 95% C.L.) was calculated from these bulk weight measurements by linear regression of ln weight vs. time using the statistical package SPSS.

Acclimation tests

After 30 days, the zinc-exposed fish were tested for acclimation to zinc using a 96 h-LC_{50} test. Fifty fish from each treatment were removed and divided into five 18 L tanks (10 fish/tank) with each tank receiving 150 ml/min of control water for one hour prior to the start of the LC_{50} trial. After the hour, the tanks were randomly assigned to one of five zinc concentrations plus a control group (0-4000 μg/L Zn for hard water groups and 0-1250 μg/L Zn for the soft water groups). The LC_{50} test was then started in the same manner as the exposures; the concentrated zinc solution flows were started at the same time zinc was added to each tank (apart from the control tanks) to bring them up to the chosen zinc level. Mortalities were recorded over 96 h. Water samples were taken daily and acidified for later zinc analysis. The 96 h-LC_{50}s ± 95% C.L. were calculated by log probit analysis of mortality vs. measured waterborne zinc concentration [18].

Oxygen consumption

1) Routine oxygen consumption

Routine oxygen consumption was measured in-tank after 30 days of exposure, in two tanks for each treatment in both exposures. Rates were measured over 1 h periods starting at 2 h after the second feeding of the day and again at 6 h after the final feeding of the day. The surface of the tank was sealed with a tight-fitting, transparent lid of heavy plastic and both the aeration and the flow of fresh water to the tanks were stopped. The tank water was then recirculated at 10 L/min by means of a pump (Little Giant Company) which drew water from the bottom and returned it back into the upper region of the tank. Po_2 levels were measured over the hour by taking water samples at 20 minute intervals from each tank and injecting them into a Cameron E101 oxygen electrode thermostatted to the experimental temperature and connected to a Cameron OM-200 O_2 meter. Water Po_2 levels never dropped below 70% of the air saturation values.

The following formula was used to calculate the absolute O_2 consumption rate (Mo_2) from changes in Po_2 levels:

$$Mo_2 = \Delta\, Po_2(\text{torr}) \times \alpha o_2(\mu\text{mol/L/torr}) \times \text{vol(L)} / \text{mass(g)} \times \text{time(h)} \qquad \text{(Eqn. 1)}$$

where ΔPo_2 is the measured change in Po_2 values between the beginning and end of each 1 h test period, vol is the volume of water in each tank (211 L), mass is the total mass of fish in the tank and αo_2 is the solubility constant for O_2 in water at the experimental temperature [19].

2) Resting oxygen consumption

Oxygen consumption was also measured in individual small-volume (3.23 L), Blazka-type respirometers similar to those described by Beamish et al. [20], using control

fish and trout exposed to 250 μg/L Zn for 30 days in the supplementary hard water series (see *Exposures*). The fish were fasted for two days prior to the measurement. The control fish were tested with control hard water and the zinc-exposed fish were tested with 250 μg/L Zn added to the water. Water velocity was set to 5 cm/s, just enough to circulate the water, but slow enough that the fish could rest on the bottom of the respirometer without having to swim. Water samples for Mo_2 measurements were taken at the beginning and the end of a 1 h time period when the respirometers were closed off. Po_2 analyses and Mo_2 calculations were performed as in the routine oxygen consumption test (Eqn. 1) using individual fish weights and respirometer volumes (3.23L).

Swimming performance tests

1) Fixed velocity test

Fixed velocity swimming, a test of sprint performance, was evaluated after 30 days of exposure using the protocol of McDonald et al. [21]. Fish were not fed the day of the test. Two sets of 10 fish were tested from each treatment in both the hard and soft water experiments. Ten fish were removed from one treatment and placed in a flume with control water (no zinc added). The fish were given 5 minutes to settle at a current velocity of 10 cm/s, and then were brought up to the test velocity of approximately 7 body lengths/s (57 cm/s in hard water and 63 cm/s in soft water) over a period of 2 minutes. At the end of the 2 minute 'ramp-up', the clock was started and fish were timed until exhaustion. Fish were deemed exhausted when they became impinged on the rear screen and would not swim after being reintroduced manually into the current. Once exhausted, fish were removed, blotted dry, weighed to the nearest 0.01 g and fork length was measured to the nearest mm. Times to fatigue were corrected to a reference body

length of 7 cm in the hard water exposure and 10 cm in the soft water exposure. The time to 50% fatigue (± 95% C.L.) was calculated by linear regression of probit fatigue vs. log time with SPSS [21].

2) Critical swimming speed (U_{Crit})

The critical swimming speed tests (U_{Crit}) [22] were performed on the control and 250 µg/L Zn acclimated fish from the supplementary hard water series (see *Exposures*). A modified 100 L Beamish-style swimming tunnel calibrated prior to use with a Kent Miniflo Type 265 propeller-style flow meter was employed. The fish were not fed for 2 days prior to the U_{Crit} tests. Control fish (N=11) were tested in the control hard water while separate batches of the 250 µg/L Zn exposed fish were tested in the presence (N=13) and absence (N=10) of 250 µg/L Zn. Fish were allowed an initial 45 minute settling time in the swimming tunnel with the current set at 10 cm/s. The U_{Crit} test was then performed by increasing the water velocity by 10 cm/s increments every 45 minutes, until the fish became exhausted. Fish were considered exhausted once they impinged on the rear screen and would not swim after being manually reintroduced into the current. After exhaustion, the fish were blotted dry, weighed and measured as in the stamina test.

The critical swimming speed (U_{Crit}) was determined for each fish using the equation given by Brett [22]:

$$U_{Crit} = V_f + [(T/t) \times dV] \qquad \text{(Eqn. 2)}$$

where U_{Crit} is in cm per second, V_f is the velocity prior to the velocity at which exhaustion occurred (the last velocity which was swum for the entire 45 minute period), dV is the velocity increment (10 cm/s), t is the time swum at each velocity (45 minutes),

and T is the time swum at the final velocity before exhaustion. U_{Crit} was then converted to body lengths/s by dividing by the fork length of the fish.

Zinc turnover tests

After 30 days of exposure, a short term (14h) radiolabelled ^{65}Zn exposure was performed with hard water, 150 μg/L Zn exposed fish. Fish were placed in a 25 L tank containing 150 μg/L Zn in hard water. Added to the tank was 25 μCi of radiolabelled ^{65}Zn (as $ZnCl_2$, specific activity=1.97 mCi/mg, NEN Life Science Products, Boston, MA, USA), an amount which had negligible influence on the total Zn concentration of the water. At the sampling times of 0.5, 1, 2, 4, 8.5 and 14 h, water samples were taken and fish were quickly removed (N=5) and sacrificed with a blow to the head. The gills were excised and rinsed vigorously for 10 s in double distilled water, blotted dry, weighed and assayed for ^{65}Zn activity in a γ-counter (MINAXI γ Auto-Gamma 5000 Series, Canberra-Packard). Water samples were similarly assayed for ^{65}Zn activity, as well as for total Zn concentration by atomic absorption spectrophotometry, so as to allow calculation of the specific activity of the waterborne zinc (Eqn. 3).

A similar experiment with more extensive tissue sampling was conducted over a longer time period, with all treatments in both exposures. As in the short term test, fish were exposed to the same water quality and zinc concentration to which they had been previously exposed. Sampling occurred three times (N=5-6) between 24-75 h. A blood sample was taken from the caudal vein and the gills were excised and rinsed vigorously in double distilled water for 10 s. The liver and gall bladder (with bile content intact) were also excised and the remaining carcass was rinsed in 25 mg/L Zn in deionized water solution for 30 s to displace any surface bound ^{65}Zn. Each tissue was weighed and

assayed for ^{65}Zn along with quadruple water samples; the latter were also analyzed for total zinc concentration using atomic absorption spectrometry.

Again, a similar experiment was performed, exposing control and 250 μg/L Zn acclimated fish to a high level of waterborne zinc (1125 μg/L), and sampling the gills in the same manner described above over a 48 h period.

The appearance of ^{65}Zn from the water in the various tissues was calculated by first determining the mean specific activity (S.A.) of zinc in the water over the time period in question:

$$S.A.=(cpm/ml)/[Zn] \quad \text{(Eqn. 3)}$$

where cpm are the γ-counts per minute and [Zn] is the concentration of zinc in μg/ml. In practice, Zn total concentrations and specific activities underwent negligible change during these tests. Total zinc appearance in the tissues was then calculated as:

$$\text{Total Zn Appearance} = (cpm/\text{tissue weight})*(1/S.A.) \quad \text{(Eqn. 4)}$$

where total zinc appearance is in μg Zn/g tissue.

For the long term ^{65}Zn turnover experiments (24-75 h), linear regressions were performed on the relationships between tissue zinc appearance and time. By extrapolating the labelling of this slow turnover pool back to time=0, the size of the fast turnover pool (in μg Zn/g tissue) could be estimated (y-intercept) ± 95% C.L. by SPSS.

Statistics

Data have been expressed as means ± standard error (N) except for the specific growth rates, 96 h-LC_{50}s, fixed velocity performance times, fast turnover pool sizes and slow turnover pool rates where means ± 95% confidence limits (C.L.) have been

reported. For the latter, values were considered significantly different if the 95% C.L. did not overlap.

For other data, significant differences were tested with a one-way analysis of variance. If the F value indicated significance, then a Student-Newman-Keuls test for multiple comparisons was applied to test for significant differences among treatments. A Student's *t*-test (two-tailed, unpaired) was used to test for significant differences in the resting oxygen consumption and U_{Crit} experiments. A 5% significance level was employed throughout.

Results

Exposure mortality and growth

Fish exposed to zinc showed little acute mortality apart from the 450 μg/L Zn hard water group (24% mortality in the first two days). By the end of the 30 day exposure, the 450 μg/L Zn group had experienced 25.8% mortality, the 150 μg/L Zn group 2.5% and the control group 0.7%. In the soft water exposure, there were no mortalities during the first 11 days in any of the groups. By the end of 30 days however, the 120 μg/L Zn group had experienced 10.5% mortality, the 50 μg/L Zn group no mortality and the control group 2.8% mortality.

Even after 25.8% mortality in the 450 μg/L Zn fish, there appeared to be no effect of zinc exposure on the specific growth rate (SGR= % increase in mass per day). In hard water, the SGR for control, 150 μg/L Zn and 450 μg/L Zn was 3.47 ± 0.36, 3.59 ± 0.21 and 3.59 ± 0.21 respectively (mean ± 95% C.L.). This was also the case in the soft water exposure where the SGR was 3.33 ± 0.28, 3.25 ± 0.24 and 3.10 ± 0.25 in control, 50 μg/L

Zn and 120 μg/L Zn exposed fish respectively. Growth rate was not significantly different in hard vs. soft water ($P<0.05$).

Zinc toxicity and acclimation tests

Zinc was much more toxic in soft water, where the control 96 h-LC_{50} was 5.4 times less than in hard water (869 μg/L vs. 162 μg/L). Both the high and low zinc exposed groups, in both hard and soft water, exhibited significant acclimation to zinc as demonstrated by the 96 h-LC_{50} measurements (Fig. 1). In hard water, the increase in the LC_{50} was 2.3 times in the 150 μg/L Zn group and 2.7 times in the 450 μg/L Zn group over controls. In soft water the increase was 2.2 times in the 50 μg/L Zn group and 3.9 times in the 120 μg/L Zn group over controls.

Costs and consequences of chronic zinc exposure

There was no effect of zinc exposure on the in-tank routine oxygen consumption rates measured after 30 days of exposure in either hard or soft water (Table 1). There was, however, a 37% higher Mo_2 in the soft water experiment, probably reflecting the effect of the 4°C higher temperature. Resting Mo_2 values measured on individual fish in respirometers also showed no effect of chronic exposure to 250 μg/L Zn on metabolic rate in hard water (Table 1). Resting Mo_2 was not measured in soft water.

There was no effect of zinc exposure on the fixed velocity swimming performance in either the hard or soft water experiments (Fig. 2A). However, there was a significant 8.4% decrease in critical swimming speed (U_{Crit}) when 250 μg/L Zn exposed fish were tested in 250 μg/L Zn zinc in hard water. This performance remained significantly depressed even when 250 μg/L Zn fish were tested in control, Zn-free hard water (Fig. 2B).

The zinc exposure in hard water had no effect on whole body Ca^{2+} and Na^{+} levels which averaged 86.69 ± 2.96 μmol/g and 43.78 ± 0.98 μmol/g respectively (N=30). In soft water, zinc exposure had no effect on whole body Ca^{2+} which was again constant at 83.27 ± 1.43 μmol/g (N=30) over the 30 day exposure. Zinc exposure had a small effect, however, on whole body Na^{+} on day 5 where the 120 μg/L Zn exposed fish had a significantly lower level of 30.02 ± 2.34 μmol/g (N=6) compared to the control levels of 37.39 ± 1.39 μmol/g (N=6). This effect did not persist at later times. However, the more striking effect on whole body Na^{+} came with time where levels decreased progressively in all treatments in soft water from the day -1 level of 41.34 ± 2.97 μmol/g to 29.32 ± 0.53 μmol/g by day 30, a decrease of 29% (N=18).

Zinc was present in relatively high levels in all tissues that were measured, in all treatments including controls (Fig. 3A&B, Table 2). There were only a few significant increases in zinc levels in the gills or livers of the zinc-exposed fish relative to simultaneous control levels in either the hard or soft water. However, levels in the whole bodies of 450 μg/L Zn exposed fish in hard water were significantly higher than controls at days 20 and 30. In soft water, the 50 μg/L Zn exposed fish had significantly elevated zinc levels in the liver and whole body by day 30, as did the 120 μg/L Zn exposed fish in the gills, liver and whole body on day 30, and in whole body on day 20 (Table 2). The zinc levels in the gills of all three treatments (i.e. including the controls) in the hard water exposure increased with time by approximately 70% over the 30 days (Fig. 3A). This did not occur in the soft water experiment, where fish were larger and absolute levels were much higher in the gills at the start of the soft water trial (Fig. 3B). Interestingly, in the

soft water exposure, whole body zinc levels of the controls continually decreased during the 30 days, becoming significantly lower on days 20 and 30 (Table 2).

Zinc turnover in the gills

There appear to be two pools of zinc in the trout gill. The fast turnover pool was characterized by a hyperbolic loading curve (Fig. 4), with a half time (T½) of 3-4 h. The slow turnover pool loaded linearly (Fig. 5A&B) for up to 75 h. The rate of appearance of ^{65}Zn in the slow pool (i.e. slope of the line) was higher in chronically zinc exposed fish in both hard and soft water and increased in proportion to exposure concentration (Fig. 5A&B, Fig. 6B). The rates of ^{65}Zn appearance in the slow pool were much greater in soft water than in hard water. For example, the rate of appearance in the soft water group chronically exposed to 120 μg/L Zn was 8.8 times greater than it was in the hard water group chronically exposed to 150 μg/L Zn (Fig. 6B).

By extrapolating the regression of the slow turnover pool to time=0, the size of the fast turnover pool was estimated (Fig. 5A&B). The fast turnover pool in the gills increased with exposure concentration and was also much greater in soft water than in hard water (Fig. 6A). For example, the fast pool in the gills was 4.3 times greater in soft water at 120 μg/L Zn than it was at 150 μg/L Zn in hard water (Fig. 6A). The size of the fast turnover pool was a very small percentage of the total zinc present in the gill ranging from 0.14% to 1.18% in hard water and 0.36% to 3.45% in soft water (cf. Table 2). The size of the slow turnover pool could not be estimated from the current data, because the ^{65}Zn accumulation showed no indication of levelling off.

Zinc turnover in other tissues

There also appeared to be a two pool system for zinc operating in the blood (Fig. 7A), with the same patterns as seen in the gill. The two pool system, however, did not appear to apply to the carcass (Fig. 7B), liver or whole gall bladder (Table 3), where the y-intercepts were not significantly different from zero (i.e. no fast turnover zinc pools). The slow pools still followed the same patterns as the gill: an increasing rate of appearance of ^{65}Zn with increasing zinc exposure, and a greater rate in soft water than in hard water (Table 4).

When control and 250 μg/L Zn acclimated fish were exposed to 1125 μg/L Zn in hard water (close to lethal levels, cf. Fig. 1), there was a larger fast pool of zinc in the gills in 250 μg/L Zn acclimated fish compared to control fish (0.991 ± 0.327 μg/g vs. 0.269 ± 0.115 μg/g respectively). It was also apparent that zinc-acclimated fish took up new zinc more quickly into the slow turnover pool in the gills than did control fish. The rate of new Zn incorporation into the slow pool of the gills of 250 μg/L Zn acclimated fish was 0.071 ± 0.010 μg/g/h vs. 0.039 ± 0.004 μg/g/h in control fish (Fig. 8).

Discussion

Overview

On an acute basis, zinc was 5.4 times more toxic to trout in soft water than in hard water. Zinc turnover rate, as measured by ^{65}Zn, was also higher in the gills and other tissues in soft water than in hard water reflecting the increased bioavailability of zinc in soft water. Only the 450 μg/L Zn group in hard water experienced significant mortality which largely ceased after the first few days of exposure. After 30 days, all zinc-exposed groups had acclimated to zinc as demonstrated by substantial increases in the LC_{50}. Although the fish must have acclimated through physiological and/or structural changes,

there appeared to be no marked effect or cost of long term zinc exposure on growth, whole body Na^+ or Ca^{2+} concentrations, zinc levels in the tissues, metabolic rate or fixed velocity swimming performance. There was however, a significant decrease in the critical swimming speed with zinc acclimation, which persisted in zinc-free water. Acclimation to zinc also involved an increase in the size of the fast turnover pool of zinc in the gills and blood.

Environmental relevance

The levels of zinc used (150 and 450 μg/L in hard water, 50 and 120 μg/L in soft water) are of environmental and regulatory significance. While normal zinc levels in pristine freshwaters are only a few μg/L or less, concentrations of 50 μg/L are routine in industrialized areas. Maximum zinc concentrations in natural surface waters are reported to range from 130 to 1170 μg/L in different areas of Canada [23]. There is general acceptance of the principle that acute toxicity is related to hardness [23]. For example, the U.S. EPA [24] employs an equation based on hardness (in mg $CaCO_3$/L) to calculate a numerical limit of total allowable waterborne zinc:

$$[Zn]=e^{(0.83[\ln(\text{hardness})]+1.95)}\ \mu g/L \qquad \text{(Eqn. 5)}$$

In the waters used in the present study, the acute limit for zinc would be 374 μg/L in hard water (120 mg $CaCO_3$/L) and 85 μg/L in soft water (20 mg $CaCO_3$/L) based on this equation. The hard water value may be too high, based on the 24 % mortality of trout in the first few days at 450 μg/L Zn; these fish likely died from hypocalcemia [4,6]. On the other hand, chronic toxicity is generally believed to be unrelated to hardness, and in most jurisdictions, chronic limits around 30 μg/L have been established, independent of hardness [23-25].

Acute toxicity and water chemistry

The acute toxicity of zinc was 5.4 times greater in soft water than hard water, a result in general accord with the literature [12,26,27]. The slightly higher temperature (18°C vs. 14°C) in the soft water experiments probably had little effect on the toxicity of zinc. Hodson and Sprague [28] found little difference in zinc toxicity (LC_{50}) with Atlantic salmon acclimated to 11°C and 19°C.

The greater toxicity of zinc in soft water is likely explained by the presence of fewer ions to offer competition to zinc for binding sites on the gill, and fewer ligands that could complex with the zinc ion in the water. In hard water, 60% of the zinc was in the free ion form (Zn^{2+}) with the remainder complexed with DOC and carbonate. In soft water however, 100% of the zinc was in the free ion form as determined with the aquatic geochemical program MINEQL+ [29].

In soft water, the 120 µg/L exposed fish suffered 10.5% mortality on days 29 and 30. Further investigation into the mechanisms behind these deaths in soft water, particularly the role of zinc, if any, would be extremely important in attempting to determine the long term effects of zinc exposure.

Acclimation

Independent of water hardness or chronic exposure concentration, all zinc-exposed fish acclimated to the metal with a 2.2-3.9 times increase in tolerance (96 h-LC_{50}), comparable to the results of Chapman [30] and Bradley et al. [8]. For metals such as zinc which kill fish through their actions on the gills, three mechanisms have been suggested that may provide increased tolerance: (i) alterations to gill barrier properties such that the rate of metal entry is reduced; (ii) increased metal storage and

detoxification; and (iii) increased resistance of metal-sensitive processes to metal poisoning [7].

Zinc and calcium competitively inhibit the uptake of each other across the gill, and share, at least partially, a common uptake pathway [5,31,32]. A series of studies [32,33] in which the actual influx rates of calcium and zinc into the fish were measured (rather than the pool turnover rates as in the present investigation) demonstrated that an interesting combination of mechanisms (i) and (iii) certainly applies, at least for rainbow trout chronically exposed to 150 μg/L Zn in hard water. In zinc-acclimated fish, the affinity of the shared branchial transport system was greatly reduced for both calcium and zinc (i.e. K_m's were increased) with little change in maximum transport rates. Because of the very different concentrations of calcium and zinc in the water relative to the respective K_m values, calcium uptake rate was little affected, but zinc uptake rate was thereby substantially reduced in acclimated fish. In a related study, Galvez et al. [17] characterized zinc binding to the low-affinity, relatively non-specific, binding sites on the gill surface in comparably treated trout. Calcium more readily displaced zinc from these sites in zinc-acclimated fish.

The results of the present study suggest that mechanism (ii) may also contribute. The size of the "fast zinc pool" in the gills increased markedly as a result of acclimation (Fig. 6A). Presumably, this is either a storage, excretion, or detoxification pool, as discussed in greater detail below. In addition, there is abundant evidence [8,34,35] for the induction of metallothionein and other specific metal-detoxification proteins in the gills and other tissues during chronic sublethal zinc exposure.

Costs and consequences

Although the fish underwent a physiological change and acclimated to zinc, there was little detectable long-term cost of the acclimation process. There was no effect of zinc exposure on growth, in either hard water or soft water, for these fish maintained on a fixed ration of 3% body mass/day. This finding is in accord with other studies where effects of zinc on growth were either absent or stimulatory [6,30,36]. There was also negligible influence of zinc exposure on whole body Na^+ or Ca^{2+} concentrations. After 30 days of zinc exposure, there was no evidence of an increased maintenance cost-of-living seen in either routine 'in-tank' Mo_2 for the whole group of fish, or in resting Mo_2 for individual fish in respirometers where activity level was controlled. However, given the known time course of acclimation in other sublethal metal studies (generally 5-15 days [7,8,10]), it is quite possible that our metabolic measurements after day 30 would have missed the major initial costs, and that remaining costs would no longer be expressed in maintenance metabolism at this time. For example, at day 9 of an exposure to 150 μg/L Zn in hard water, protein synthesis rates in the gills of exposed trout were significantly elevated, but the rates dropped to control levels or below by days 18 and 23 [6,32]. The lack of a persistent effect on maintenance metabolism indicates that zinc did not act as a "loading stressor" [37].

However, the depressed U_{Crit} of zinc-acclimated fish (Fig. 2B) indicates that zinc may well have acted as a "limiting stressor" - one which depresses aerobic capacity without necessarily affecting routine metabolism [37]. This inhibitory effect occurred independently of whether or not zinc was present in the test water. Thus the depressed swimming performance was not due to the presence of zinc, but rather to the physiological changes which the fish had undergone as a result of the zinc exposure.

With acclimation to aluminum, the "limiting stressor" effect has been attributed to a thickening of the respiratory epithelium due to mucous cell and chloride cell hyperplasia [9,10]. However, this may not be the case with zinc acclimation because Galvez et al. [17] found no change in the gill's chloride cell density or surface area after acclimation of trout to 150 μg/L Zn in hard water. Changes in the viscosity of mucus that arise from metal exposure may have been a contributing factor here [38].

Although aerobic swimming performance was depressed, there was no effect of zinc acclimation on swimming stamina, as measured by the fixed velocity test (Fig. 2A). This type of swim test is thought to involve both aerobic and anaerobic components [21], and may place less overall demand on the cardio-respiratory system than the U_{Crit} test. Overall, the result suggests that anaerobic capacity was not impacted by chronic zinc exposure.

Zinc was present in substantial concentrations in the gills, liver and whole body in both control and zinc exposure treatments throughout both the hard and soft water experiments (Table 2, Fig. 3A&B). This reflects the role of zinc as an essential micronutrient, important as a cofactor for the function of numerous proteins [1]. Relative to these high background levels in control fish, there was no consistent increases in total zinc levels in the gills or liver of zinc-exposed fish, in either hard water or soft water. Modest increases occurred in whole body zinc concentrations. Three previous studies have reported comparable results with rainbow trout chronically exposed to approximately 150 μg/L Zn in Hamilton hard water [6,32,36]. Furthermore, Bradley and Sprague [39] reported modest elevations (40%) in gill zinc concentration, and no change

in liver concentration, in trout exposed for 20 days to over 2000 μg/L Zn in extremely hard, alkaline water.

Recently, there has been great interest in using tissue metal burdens, especially those in gills, as predictors of acute mortality and indicators of chronic exposure in freshwater fish (e.g. [16]). However the present and previous data (cited above) all clearly indicate that concentrations of this essential metal are subject to remarkable homeostasis in rainbow trout in the face of environmental challenge. Indeed growth-related changes in zinc tissue content are much more obvious than those resulting from chronic zinc exposure (Fig. 3A). Because of this physiological homeostasis, coupled with high background zinc levels in non-exposed fish, regulatory strategies based on measuring *total* tissue metal burdens will not work for zinc; clearly alternate strategies for assessing gill-zinc binding are needed.

Zinc turnover in the gills

This conclusion was also reached by Galvez et al. [17], who found that it was impossible to determine zinc-binding kinetics to trout gills by measuring total tissue zinc concentration, the approach which has been successfully used with other metals such as copper, cadmium, and silver [14,40]. Instead, Galvez et al. [17] employed the radiotracer ^{65}Zn with some success in short-term (up to 3 h) binding experiments.

Using the studies of Galvez et al. [17] as a point of departure, in the present investigation we employed much longer exposures to ^{65}Zn in an attempt to characterize the zinc pool(s) in the gills and other tissues, and their kinetics of turnover. We purposely used low concentration levels which would recruit only high affinity sites (those with affinities in the micromolar zinc range), rather than high concentration levels which

would also recruit the relatively non-specific low affinity sites (those with affinities in the millimolar zinc range)[17]. Therefore, rather than looking at concentration-dependence within each exposure group, we elected to expose the fish to the radiotracer at the total zinc concentration to which they had been acclimated, and employed time as the principal variable. With this technique, at least two pools of zinc operating in trout gill were found. The fast exchanging zinc pool had a time to 50% turnover (T½) of about 3-4 hours (Fig. 4). The slower exchanging pool appeared to turn over linearly with time (Fig. 5A&B). The size of the slow pool could not be determined from the present data. However, if we assume it is the total measured zinc content of the gills, then T½ was clearly in the range of days to months or more. In fact, T½ for the slow pool of control fish in hard water was estimated at 3 years! The size of the fast turnover pool could be estimated by extrapolation of the slow pool line back to time zero. For control fish in hard water, this yielded a value of about 0.1 μg Zn/g gill tissue, only 0.14% of the total zinc content of the gill, or about 20% of the "high affinity sites" determined by Galvez et al. [17]. This difference is explained at least partially by the difference in technique; Galvez et al. [17] attempted to measure the pool when all the high affinity sites were saturated (i.e. maximum binding capacity), whereas our technique measures the pool size simply at the exposure level.

We interpret the fast pool as a dynamic pool bound to high affinity sites, one which is in the process of being taken up, excreted, detoxified, or stored. Clearly the size of the fast turnover pool increased with the concentration of zinc to which the fish were chronically exposed (Fig. 6A). Taken together with the finding that zinc flux rates into the fish (measured in the range of the exposure concentrations) are reduced during

chronic exposure [6,32,33; see above], a simple interpretation is that the increased size of the fast pool is due to increased detoxification or temporary storage (e.g. metallothionein). In comparisons at similar exposure concentrations (0 vs. 0 μg/L Zn or 120 vs. 150 μg/L Zn), the fast pool size was clearly much greater in soft water than in hard water (Fig. 6A). A simple interpretation here is that the paucity of calcium in soft water increases the availability of sites for zinc-binding [13].

The slow pool presumably represents incorporation into structural components of the gill (e.g. zinc-dependent proteins) in growing fish, though it could also represent long-term detoxification storage in zinc-binding proteins. Without knowledge of the true size of the slow pool or its T½, it is difficult to interpret the higher turnover rates in zinc-exposed fish, beyond the fact that they increase with concentration as expected (Fig. 6B). However, when compared at similar concentrations in soft water vs. hard water (i.e. 0 vs. 0 μg/L Zn, or 120 vs. 150 μg/L Zn), the labelling of this slow pool was clearly much faster in the soft water fish, probably explained by increased access through the fast pool and reduced calcium in the water (Fig. 6B).

This approach may provide a practical tool for modeling zinc-binding in the fast pool. For example, it is of interest to know whether the binding capacity (i.e. total available site number in the fast pool) or affinity changes as a result of acclimation. This can be done by exposing the fish to radiotracer at total zinc levels different from those used during acclimation, and then extrapolating back to time 0 at each new concentration to determine the pool size. Fig. 8 illustrates one such example where the test concentration (1125 μg/L Zn) was much higher than the acclimation (250 μg/L Zn) or control concentrations (0 μg/L Zn) so as to estimate the maximum binding capacity of

the fast pool. The results indicate that the maximum binding capacity was expanded almost 4 times as a result of chronic zinc acclimation. This conclusion is in accord with recent findings, using very different techniques, on trout acclimated to both cadmium [41] and copper (Taylor L, personal communication). Increased maximum binding capacity by high affinity sites in the gills may be a common feature of acclimation to different metals.

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41. Hollis L, McGeer JC, McDonald DG, Wood CM. 1998. Cadmium accumulation, gill Cd binding, acclimation, and physiological effects during longterm sublethal Cd exposure in rainbow trout. *Aquat Toxicol*, in press.

Figure 2-1. The 96 h-LC_{50} in hard water (open columns) and soft water (filled columns, italics) measured after 30 days of exposure. The asterisk (*) denotes a significant increase in the LC_{50} for the zinc exposed fish groups over the control treatment of the same water chemistry. An (a) indicates a significant increase in the LC_{50} of the high zinc exposed trout (hard water=450 µg/L Zn, soft water=120 µg/L Zn) over the low zinc exposed trout (hard water=150 µg/L Zn , soft water=50 µg/L Zn) of the same water chemistry. Values expressed as means ± 95% C.L.

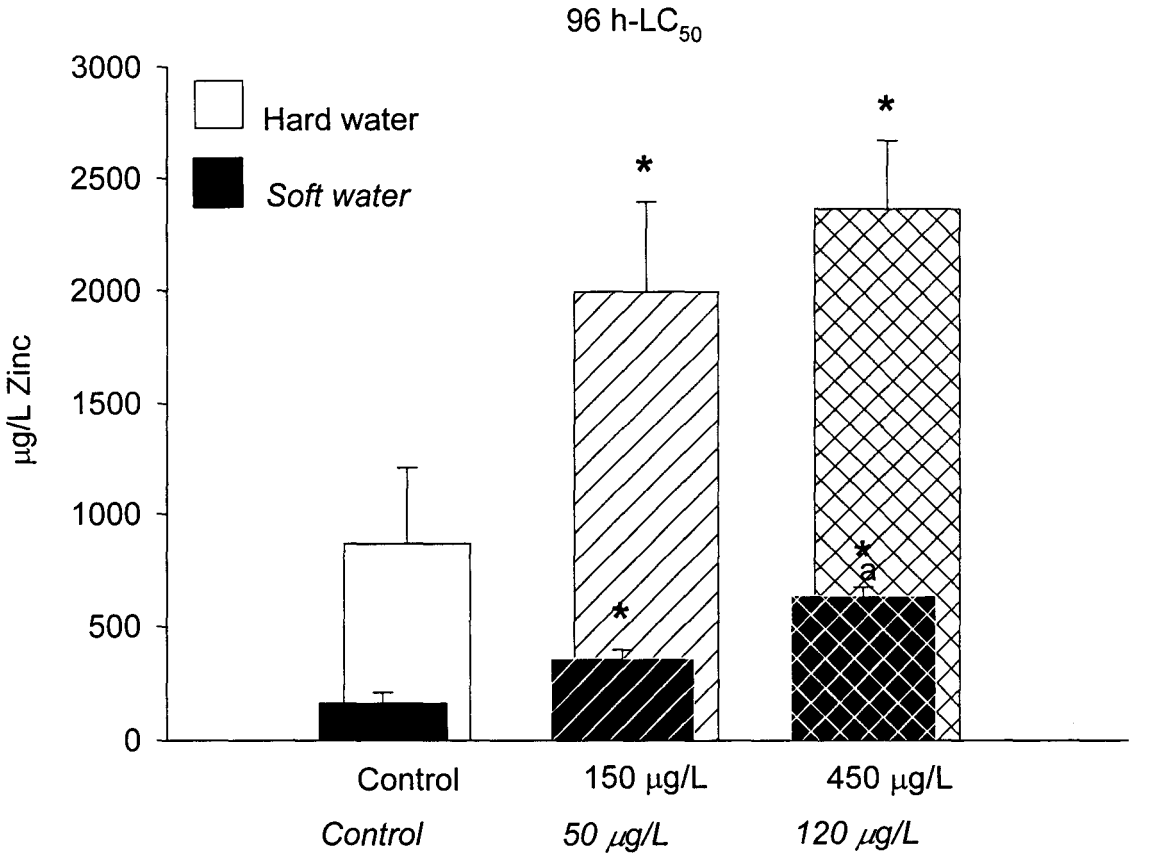
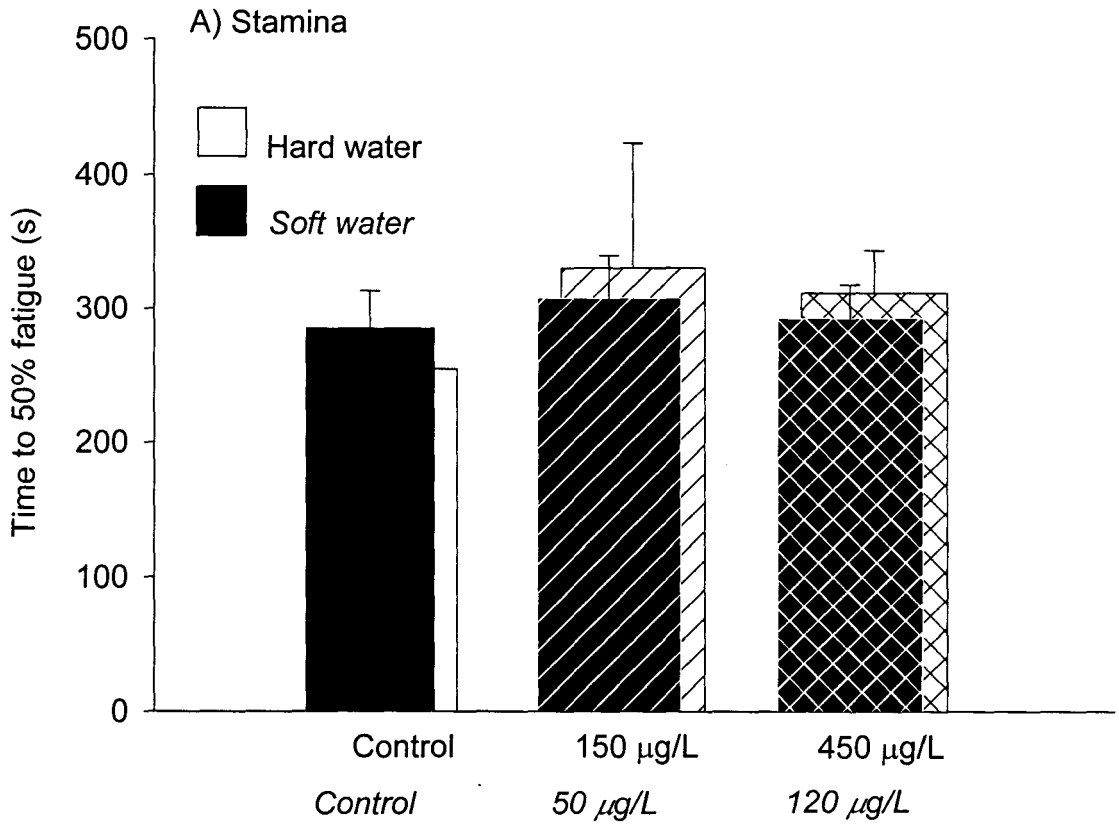

96 h-LC50
3000
2500
2000
1500
1000
500
0
µg/L Zinc
Hard water
Soft water
*
*
*
*a
Control
150 µg/L
450 µg/L
Control
50 µg/L
120 µg/L

Figure 2-2. A) Time to 50% exhaustion during a fixed velocity swimming test. Rainbow trout were tested at approximately 7 BL/s in hard water (open columns) and soft water (filled columns, italics). Times were corrected to a body length of 7 cm in hard water and 10 cm in soft water. There were no significant differences between or within treatments. B) Critical swimming speed for control, and zinc-acclimated rainbow trout. The zinc-acclimated trout were tested in water both containing and free of the zinc levels to which they had been acclimated. The asterisk (*) denotes a significant difference from the control value as determined by a Students *t*-test. $P<0.05$. Values expressed as means ± SEM. (N=10-13).


A) Stamina
Hard water
Soft water
Time to 50% fatigue (s)
500
400
300
200
100
0
Control
150 µg/L
450 µg/L
Control
50 µg/L
120 µg/L

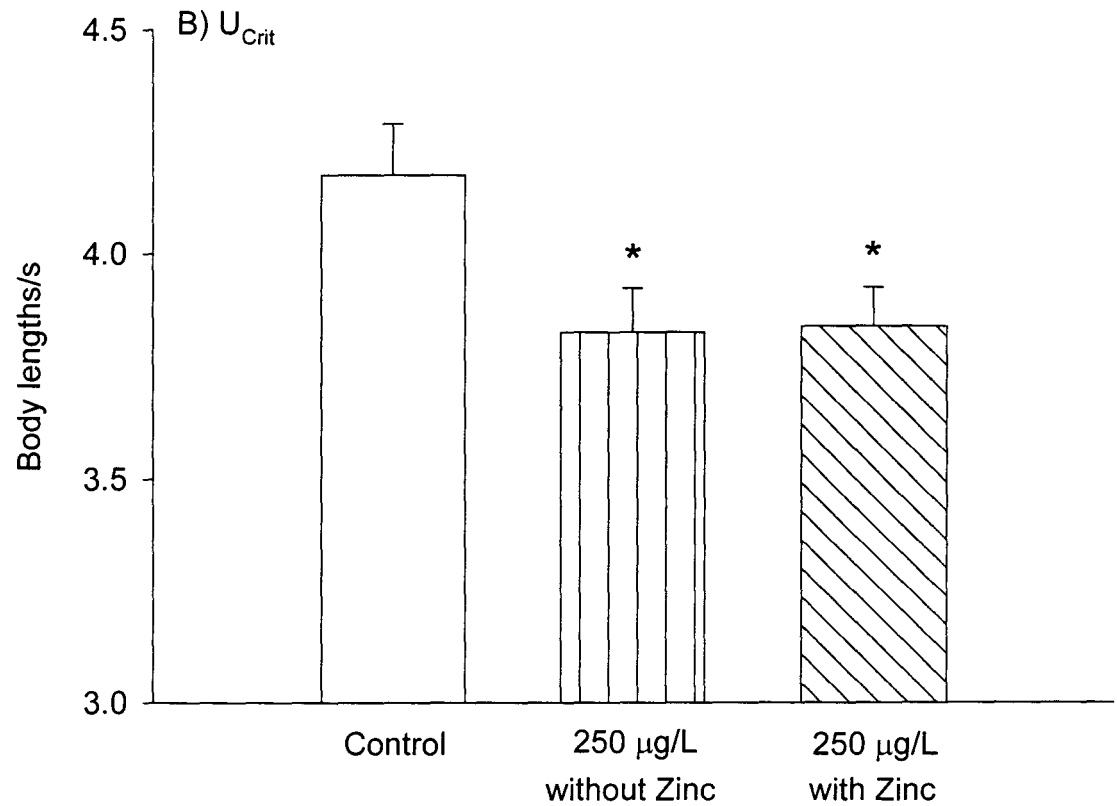

B) U_{Crit}
Body lengths/s
4.5
4.0
3.5
3.0
*
*
Control
250 µg/L
without Zinc
250 µg/L
with Zinc

Figure 2-3. Total zinc levels in rainbow trout gills at three zinc treatments in A) hard water and B) soft water during the 30-day exposures. The asterisk (*) indicates a significant difference (ANOVA followed by a Student-Newman-Keuls multiple comparisons test, $P<0.05$) from the control zinc level that day, while the plus (+) indicates a significant difference from the control zinc levels measured on day -1.

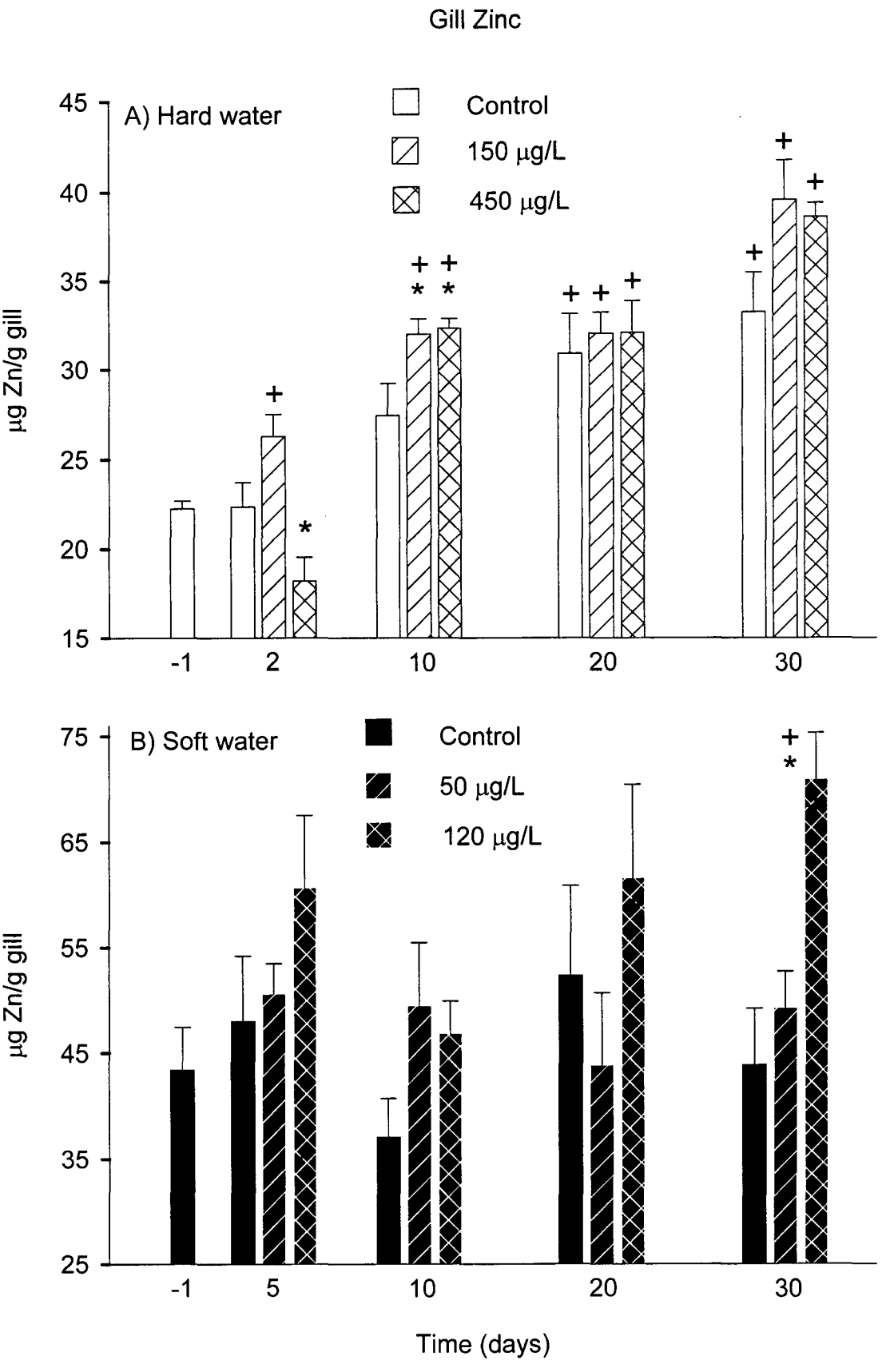

Gill Zinc
A) Hard water
Control
150 µg/L
450 µg/L
µg Zn/g gill
45
40
35
30
25
20
15
-1
2
10
20
30
B) Soft water
Control
50 µg/L
120 µg/L
µg Zn/g gill
75
65
55
45
35
25
-1
5
10
20
30
Time (days)

Figure 2-4. The appearance of radiolabelled ^{65}Zn in the gills of rainbow trout, previously acclimated for 30 days to 150 μg/L Zn in hard water. The trout were exposed for 15 h to a zinc concentration equal to what they had been exposed to for the previous 30 days. ^{65}Zn was also added to the water as a tracer which had a negligible effect on the total zinc concentration of the water. Values expressed as means ± SEM. (N=5).

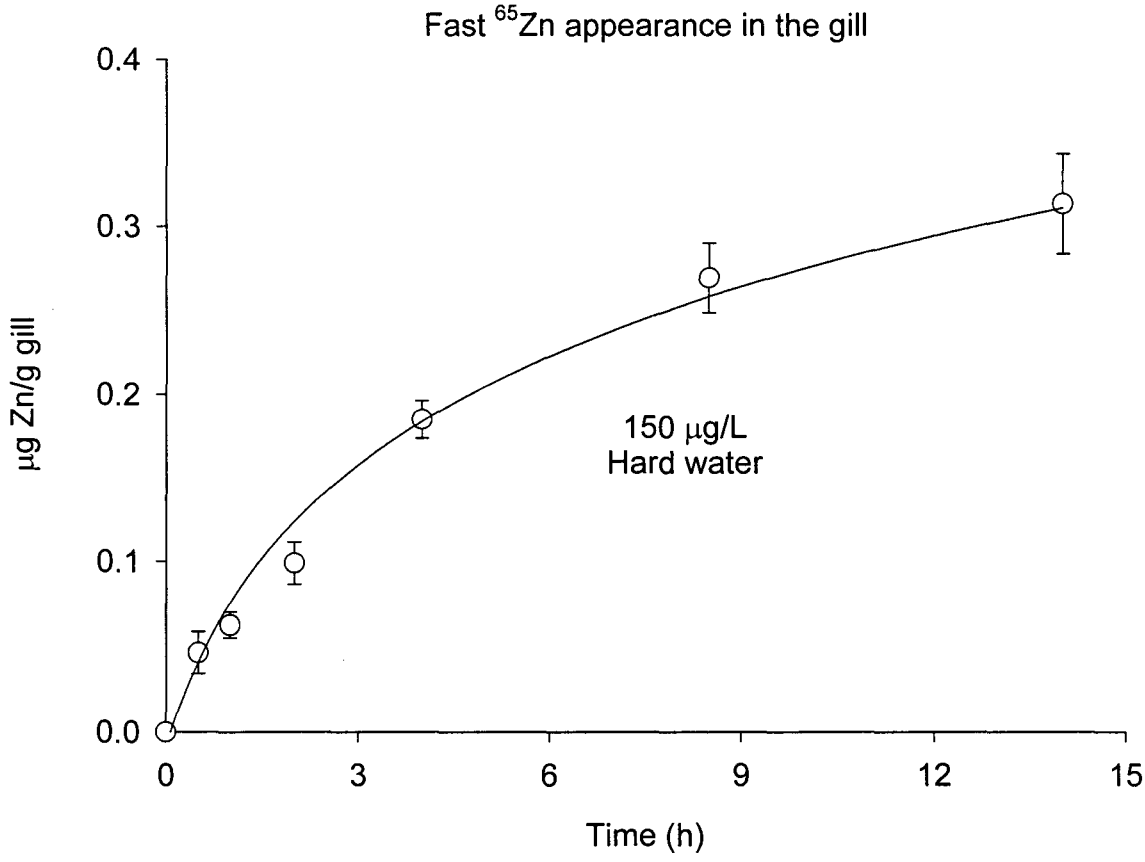

Fast 65Zn appearance in the gill
0.4
0.3
0.2
0.1
0.0
µg Zn/g gill
150 µg/L
Hard water
0
3
6
9
12
15
Time (h)

Figure 2-5. The appearance of radiolabelled ^{65}Zn in the gills of A) hard water and B) soft water acclimated rainbow trout. Trout were exposed to a zinc concentration with ^{65}Zn equal to the zinc concentration which they had been exposed to for the previous 30 days. Values expressed as means ± SEM. (N=5-6).

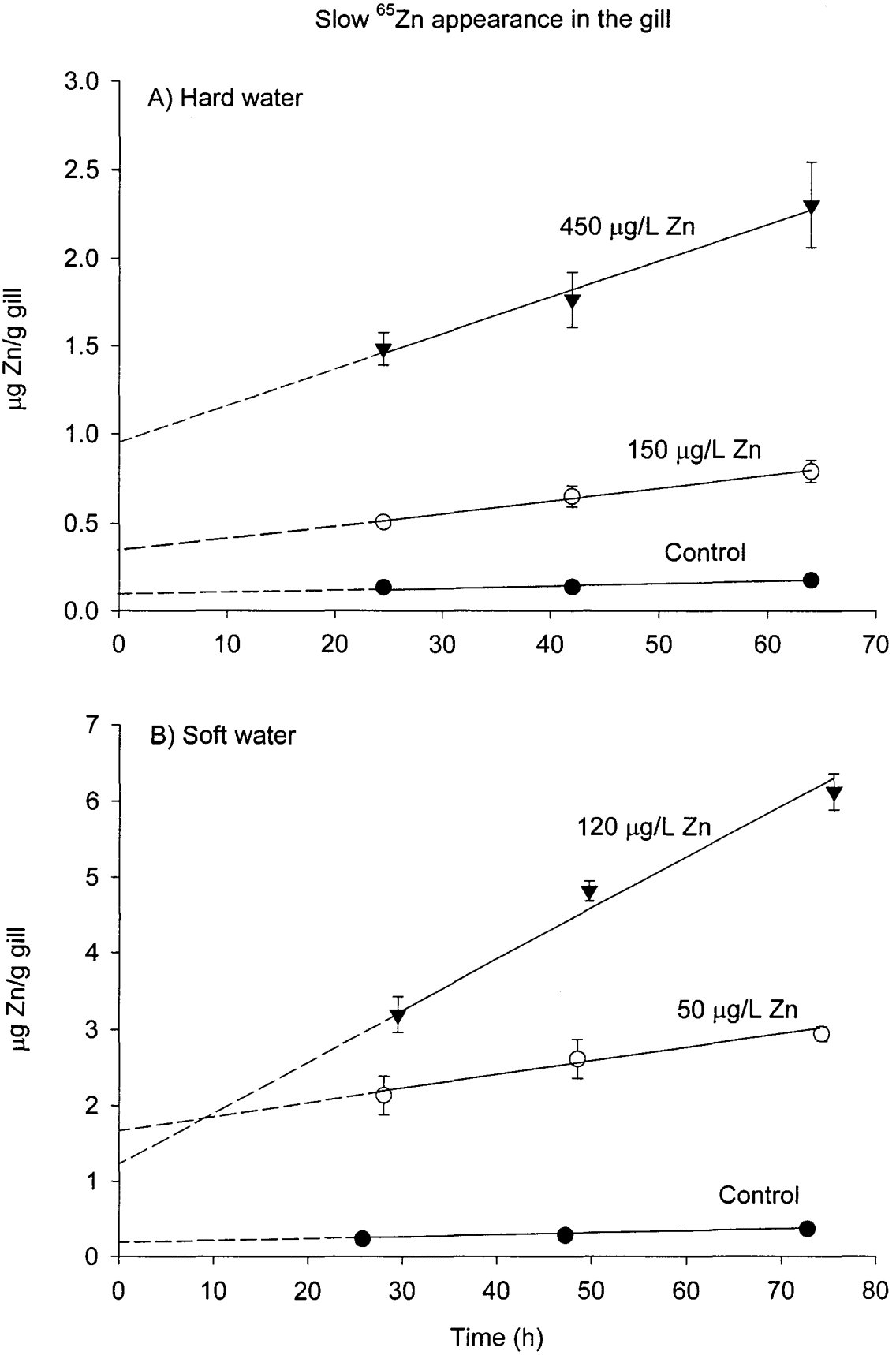

Slow 65Zn appearance in the gill
A) Hard water
450 µg/L Zn
150 µg/L Zn
Control
µg Zn/g gill
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
B) Soft water
120 µg/L Zn
50 µg/L Zn
Control
µg Zn/g gill
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
80
Time (h)

Figure 2-6. The calculated sizes of the A) fast turnover zinc pools and B) the rates of turnover of the slow zinc pools of the gills after 30 days of exposure in hard water (open columns) and soft water (filled columns, italics). The asterisk (*) denotes a significant difference from the control values from the same exposure. An (a) indicates a significant increase in the zinc pool size or turnover rate of the high zinc exposed trout (hard water=450 μg/L Zn, soft water=120 μg/L Zn) over the low zinc exposed trout (hard water=150 μg/L Zn , soft water=50 μg/L Zn) of the same water chemistry.

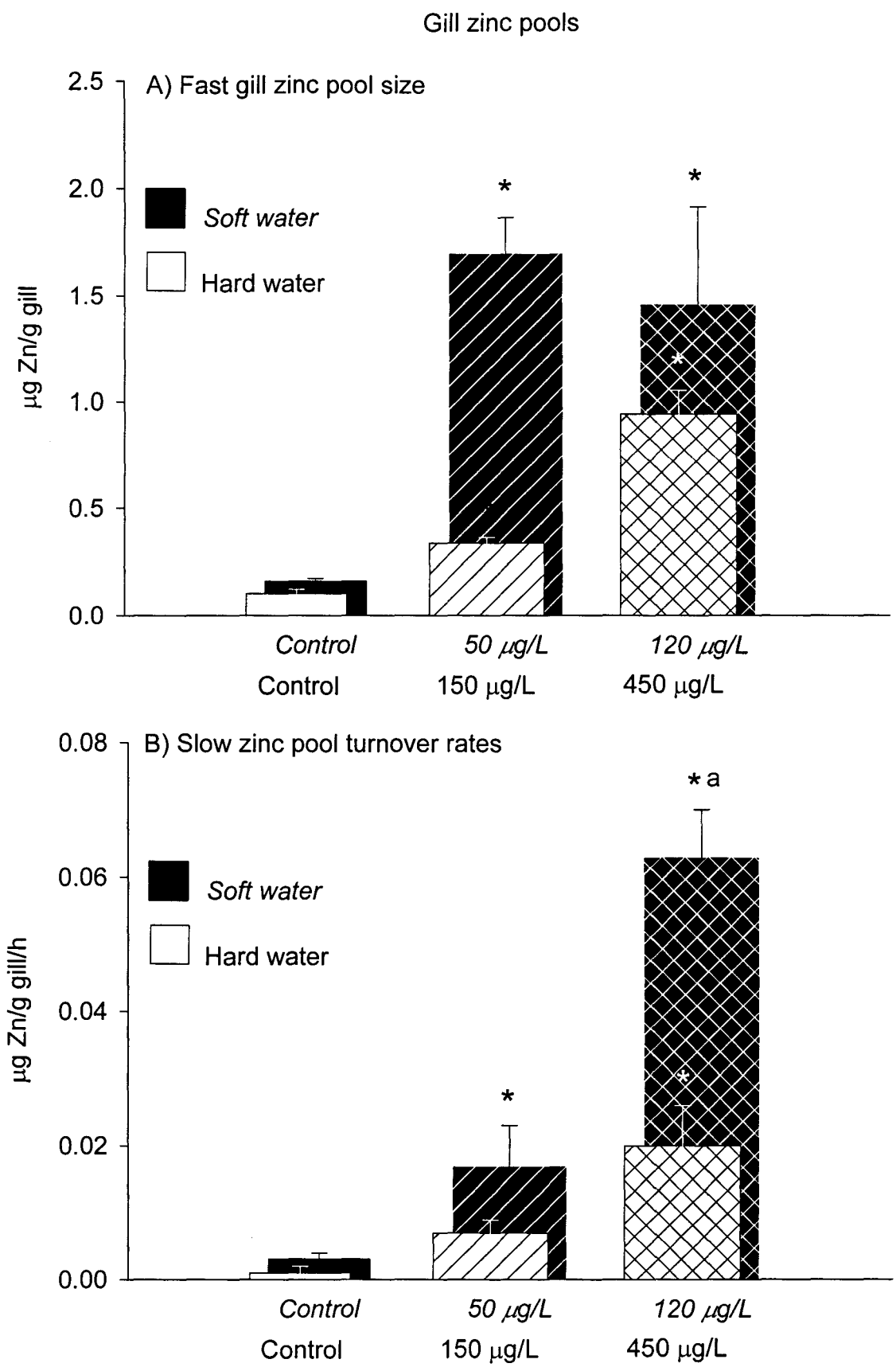

Gill zinc pools
A) Fast gill zinc pool size
2.5
2.0
1.5
1.0
0.5
0.0
µg Zn/g gill
Soft water
Hard water
*
*
*
Control
50 µg/L
120 µg/L
Control
150 µg/L
450 µg/L
B) Slow zinc pool turnover rates
0.08
0.06
0.04
0.02
0.00
µg Zn/g gill/h
Soft water
Hard water
* a
*
*
Control
50 µg/L
120 µg/L
Control
150 µg/L
450 µg/L

Figure 2-7. The appearance of radiolabelled ^{65}Zn in the A) blood and B) carcass of soft water acclimated rainbow trout at the three zinc exposure concentrations. The carcass was that portion of the rainbow trout remaining after the gills, liver and gall bladder were excised. The fish were exposed to a radiolabelled ^{65}Zn concentration equal to the zinc concentration which they had been exposed to for the previous 30 days. Values are expressed as means ± SEM. (N=5).

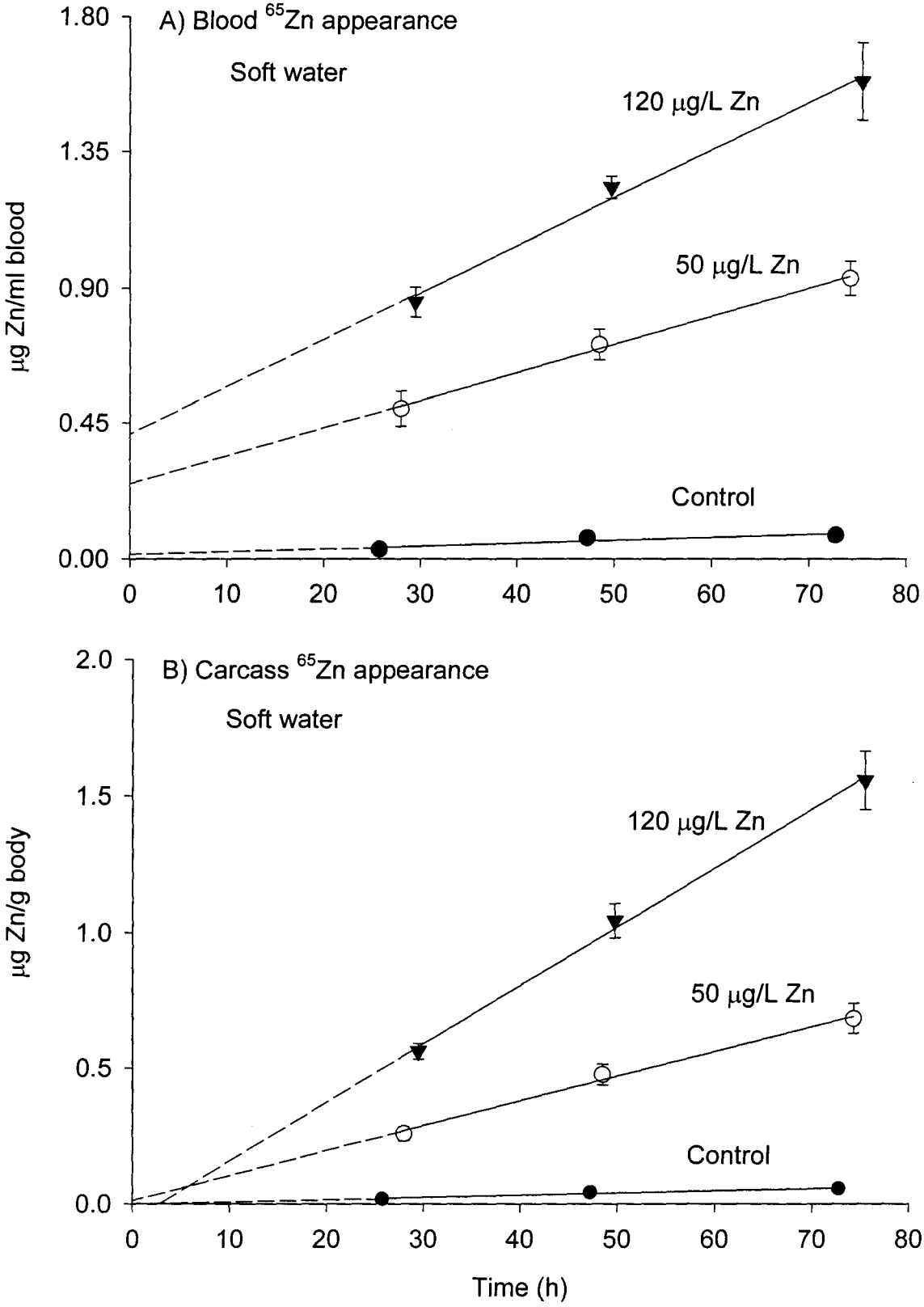

A) Blood 65Zn appearance
Soft water
120 µg/L Zn
50 µg/L Zn
Control
µg Zn/ml blood
1.80
1.35
0.90
0.45
0.00
0
10
20
30
40
50
60
70
80
B) Carcass 65Zn appearance
Soft water
120 µg/L Zn
50 µg/L Zn
Control
µg Zn/g body
2.0
1.5
1.0
0.5
0.0
Time (h)

Figure 2-8. ^{65}Zn appearance in the gills of 250 μg/L Zn acclimated and non-acclimated rainbow trout over time at an exposure concentration of 1125 μg/L Zn. Values are expressed as means ± SEM. (N=6).

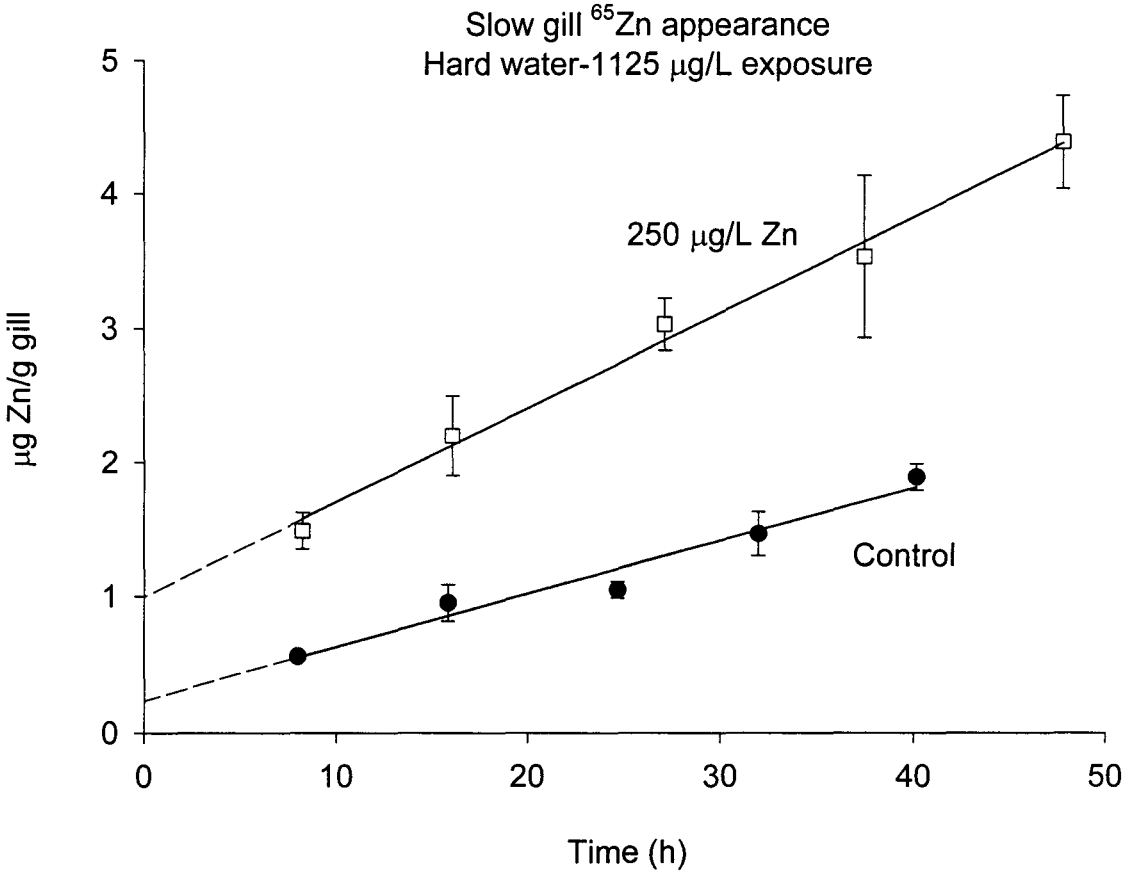

Slow gill 65Zn appearance
Hard water-1125 µg/L exposure
250 µg/L Zn
Control
µg Zn/g gill
Time (h)
0
1
2
3
4
5
0
10
20
30
40
50

Table 2-1. Routine in-tank oxygen consumption (Mo_2) and resting Mo_2 measured on individual rainbow trout in respirometers, after 30 days of zinc exposure. Values are expressed as means ± SEM. (Routine N=2 tanks of trout, control resting N=19 trout, 250 µg/L Zn resting N=16 trout).

Zinc Exposure Level -measurement condition	Hard Water µmol/g/h ± SEM	Soft Water µmol/g/h ± SEM
Control-routine	11.8 ± 0.4	16.2 ± 0.5
Low[a]-routine	10.8 ± 0.3	16.3 ± 0.6
High[b]-routine	11.9 ± 0.5	14.7 ± 0.5
Control-resting	8.0 ± 0.6	-
250 µg/L-resting	7.1 ± 0.6	-

[a]-Hard water=150 µg/L Zn, soft water=50 µg/L Zn.
[b]-Hard water=450 µg/L Zn, soft water=120 µg/L Zn.

Table 2-2. Liver and whole body zinc burdens (μg Zn/g tissue) in rainbow trout over 30 days of zinc exposure in hard and soft water. An asterisk (*) denotes a significant difference from the control measurement on that day. The plus (+) denotes a significant difference from the control value on Day -1 (ANOVA followed by a Student-Newman-Keuls multiple comparisons test, $P<0.05$). Values are expressed as means ± SEM. (N=6).

		Hard water					Soft water				
		Day -1	Day 2	Day 10	Day 20	Day 30	Day -1	Day 5	Day 10	Day 20	Day 30
Liver	Control	21.37 ±0.59	15.86 ±2.43	19.56 ±2.90	20.02 ±0.94	20.02 ±0.69	27.59 ±1.43	26.15 ±1.92	22.68^{+} ±0.81	24.24 ±0.91	23.12^{+} ±0.39
	Low[a]		20.60 ±0.77	25.27 ±1.74	24.09* ±1.15	21.10 ±0.71		31.03 ±2.21	27.45 ±2.32	22.29 ±0.75	24.67* ±0.47
	High[b]		22.12 ±2.62	19.89 ±0.94	25.20*$^{+}$ ±1.30	21.46 ±0.32		29.62 ±4.73	27.98 ±1.11	25.20 ±1.16	27.93* ±0.61
Whole body	Control	32.57 ±1.94	28.79 ±1.05	35.11 ±3.88	30.44 ±1.14	28.06 ±1.18	30.90 ±2.47	28.29 ±2.91	25.50 ±1.44	21.39^{+} ±0.87	19.17^{+} ±0.91
	Low[a]		30.64 ±1.36	36.06 ±1.38	31.44 ±1.15	30.90 ±1.37		28.07 ±1.50	28.16 ±2.76	24.41^{+} ±1.84	25.61*$^{+}$ ±1.00
	High[b]		38.55* ±3.02	36.57 ±1.30	36.60* ±0.57	37.28* ±1.28		26.81 ±2.23	31.81 ±3.20	28.28* ±1.32	33.31* ±1.39

[a]-Hard water=150 μg/L Zn, soft water=50 μg/L Zn.
[b]-Hard water=450 μg/L Zn, soft water=120 μg/L Zn.

Table 2-3. Fast zinc turnover pool sizes (μg Zn/ml blood, μg Zn/g tissue) in the blood, liver and gall bladder of control, low and high zinc exposed rainbow trout after 30 days of zinc exposure in hard and soft water. An asterisk (*) denotes a significant difference from the control value. Values are expressed as means ± SEM. (N=15).

	Hard water			Soft water		
	Control	150 μg/L	450 μg/L	Control	50 μg/L	120 μg/L
Blood (μg Zn/ml)	0.0012 ±0.025	0.208 ±0.116	0.511* ±0.185	0.014 ±0.021	0.242* ±0.028	0.411* ±0.078
Liver (μg Zn/g)	-0.019 ± 0.013	-0.037 ± 0.065	0.128 ± 0.084	-0.016 ± 0.009	-0.087 ± 0.097	-0.542 ± 0.018
Gall Bladder (μg Zn/g)	0.010 ± 0.004	0.041 ± 0.016	0.075 ± 0.039	0.001 ± 0.004	0.013 ± 0.058	0.105 ± 0.108

Table 2-4. Slow zinc pool turnover rates (μg Zn/ml blood/h, μg Zn/g tissue/h) in the blood, liver and gall bladder of control, low and high zinc exposed rainbow trout after 30 days of zinc exposure in hard and soft water. An asterisk (*) denotes a significant difference from the control value. An (a) indicates a significant increase in the turnover rate of the high zinc exposed trout (hard water=450 μg/L Zn, soft water=120 μg/L Zn) over the low zinc exposed trout (hard water=150 μg/L Zn , soft water=50 μg/L Zn) of the same water chemistry. Values are expressed as means ± SEM. (N=15).

	Hard water			Soft water		
	Control	150 μg/L	450 μg/L	Control	50 μg/L	120 μg/L
Blood (μg Zn/ml/h)	0.002 ± 0.001	0.004 ± 0.003	0.019* ± 0.005	0.0010 ± 0.0002	0.0094* ± 0.0016	0.0157* ± 0.0025
Liver (μg Zn/g/h)	0.002 ± 0.0003	0.007* ± 0.001	0.014* ± 0.002	0.0022 ± 0.0003	0.0218* ± 0.0027	0.0550*[a] ± 0.0057
Gall Bladder (μg Zn/g/h)	0.0001 ± 0.0001	0.0004 ± 0.0004	0.002 ± 0.001	0.0003 ± 0.0001	0.004* ± 0.001	0.008* ± 0.002

CHAPTER 3

The influence of waterborne cations on zinc uptake and toxicity in rainbow trout, *Oncorhynchus mykiss*.

Abstract

The effects of waterborne cations on radiolabelled Zn^{2+} uptake, Zn^{2+} toxicity, and possible relationships with calcium uptake were examined in juvenile rainbow trout (*Oncorhynchus mykiss*) acclimated to soft water (Ca^{2+}=0.02-0.06 mM; Mg^{2+}=0.01-0.02 mM). Whole body Zn^{2+} uptake (at a total waterborne [Zn]=100 μg/L=1.5 μM) was greatly reduced by a variety of positively charged ions. The addition of Na^+ to the water (as NaCl) at 0.5, 1.0 and 2.0 mM reduced Zn^{2+} uptake by 26%, 48% and 87% respectively. The magnitude of the reduction was directly related to the concentration of positive charges, regardless of which element or compound carried that charge (except for Ca^{2+} and H^+). Thus K^+, NH_4^+ and N-methyl-D-glucamine$^+$ reduced Zn^{2+} uptake to a similar extent as Na^+, whereas divalent Mg^{2+} was approximately twice as potent on a molar basis. These results suggest a relatively non-specific competition for negatively charged sites on the gill surface, including those which are responsible for Zn^{2+} uptake into the gill cells. Ca^{2+} had an even greater effect in reducing Zn^{2+} uptake than Mg^{2+}, likely because Ca^{2+} would also compete for absorption since Ca^{2+} and Zn^{2+} are known to share the same apical transport channel at the gill. Indeed, 100 μg/L Zn^{2+} inhibited Ca^{2+} uptake by 87%. Although Na^+ and Mg^{2+} were able to markedly reduce Zn^{2+} uptake, they had no effect on Zn^{2+} toxicity, a result paralleled by their inability to restore Ca^{2+} uptake. In contrast, 1 mM Ca^{2+} both reduced Zn^{2+} toxicity (the 96 h LC_{50} increased from 103

μg/L to 1800 μg/L) and restored Ca^{2+} uptake, in addition to reducing Zn^{2+} uptake. These results partially dissociate Zn^{2+} uptake from Zn^{2+} toxicity, implicate disturbed Ca^{2+} uptake as the mechanism of toxicity, and have profound implications for water quality criteria where Ca^{2+} and Mg^{2+} (the two 'hardness' cations) are traditionally considered to be equally protective.

Introduction

Zinc (Zn^{2+}) is an essential micronutrient which can be absorbed by fish via the gill from the aquatic environment, as well as from the diet (Spry et al. 1988). The pathway of branchial Zn^{2+} uptake (as well as other cations) begins with the adsorption of the cation to a negatively charged site on the gill surface (Pagenkopf 1983), followed by absorption into the gill cell (Hogstrand et al. 1996). Handy and Eddy (1991) showed that Na^{+} adsorption (and consequently absorption into the gill) was reduced by competition from other waterborne cations such as H^{+} and Ca^{2+} for binding sites.

If waterborne Zn^{2+} concentrations become high enough, too much adsorption/absorption may ultimately lead to toxic effects. Zn^{2+} specifically disrupts Ca^{2+} uptake at the gill which can lead to hypocalcemia and even death of the fish (Spry and Wood 1985; Hogstrand et al. 1995). Zn^{2+} toxicity is not only dependent on Zn^{2+} concentration, but also on the presence of other ions in the water. In water quality regulations (e.g. U.S. EPA 1980), water hardness (the sum of Mg^{2+} and Ca^{2+} expressed as mg/L $CaCO_3$) is the only component of water quality which is taken into account as a modifying agent for Zn^{2+} toxicity. For example, a decrease in water hardness of approximately 10 times was shown to increase Zn^{2+} toxicity to juvenile rainbow trout by

10 times (Bradley and Sprague 1985). Similarly, Alsop et al. (1999) reported that a six fold reduction in hardness increased toxicity approximately six times. The toxic effects of Zn^{2+}, like most metals, are therefore of particular concern in ion-poor water.

Other ions may also compete with Zn^{2+} binding to the gill, and any ions which impede the initial adsorption of Zn^{2+} to the gill might be expected to reduce its toxicity (Simkiss and Taylor 1989). For example, Pagenkopf (1983) predicted that H^+ ions would compete with cationic metals for binding sites on the gill, leading to lower toxicity at sublethal low pH. While data were not available for Zn^{2+} at the time, the protective effect of sublethal low pH was subsequently shown experimentally by both Bradley and Sprague (1985) and Cusimano et al. (1986). In contrast, Pagenkopf (1983) discounted the protective effect of other monovalents such as Na^+ and K^+ because of their "less extensive" interactions with the gill.

One aim of the present study on freshwater rainbow trout acclimated to soft water was to look at the effect of a range of different cations on radiolabelled Zn^{2+} (^{65}Zn) uptake and the relative potencies of their inhibition. The use of the radioisotope ^{65}Zn was required to measure Zn^{2+} accumulation (Hogstrand et al. 1998; Galvez et al. 1998) due to the high levels of Zn^{2+} already present in fish tissues, which reflect its role as a micronutrient. We predicted that Ca^{2+} would have the largest influence on Zn^{2+} uptake due to the fact that not only would Ca^{2+} compete with Zn^{2+} for binding sites on the gill surface, but it would also compete for uptake. Recent evidence indicates that Ca^{2+} and Zn^{2+} share the same apical transport channel in the gill (reviewed by Hogstrand and Wood 1996). Another goal of this study was to determine whether reductions in Zn^{2+} uptake by various cations translate into a protective effect against Zn^{2+} toxicity. Of

particular interest was the relative effectiveness of Ca^{2+} and Mg^{2+}, the two major components of water "hardness", in influencing both Zn^{2+} uptake and Zn^{2+} toxicity. Finally, since inhibition of branchial Ca^{2+} uptake may be the mechanism of Zn^{2+} induced mortality, we examined whether protection against acute Zn^{2+} toxicity (i.e. increased 96 h LC_{50}), by a particular cation was associated with a restoration of Ca^{2+} uptake.

Materials and methods

Fish care

Juvenile rainbow trout (*Oncorhynchus mykiss*, 3-10 g) were obtained from Rainbow Springs Trout Hatchery (Thamesford, Ontario), and initially held in aerated 500 L tanks supplied with 3 L/min of dechlorinated Hamilton tap water (Table 1), for one week. The fish were then slowly acclimated to "soft water" over the next two weeks by mixing hard water with increasing amounts of ion-reduced water (produced by reverse osmosis; Anderson Water Systems, Dundas, ON, Canada) until the desired water chemistry was reached (Table 1). The fish were allowed to acclimate to this soft water for at least one month before experimentation. Soft water was favored for Zn^{2+} uptake experiments, for 100% of waterborne zinc would be in the free ionic form (Zn^{2+}) as predicted with the aquatic geochemical program MINEQL+ (Schecher and McAvoy, 1994). Trout were fed to satiation three times per week with commercial trout feed [fish food composition (partial analysis only): crude protein (min), 52%; crude fat (min), 17%; crude fibre (max), 2.5%; water, 12%; Ca^{2+}, 1.4%; Na^{+}, 0.4%; zinc (measured), 0.02% (173 μg/g)]. Water temperature was 10°C ± 1°C during the experiments.

Zn^{2+} uptake

Twenty L buckets served as ^{65}Zn flux units and were lined with polypropylene bags. The buckets were filled with 14 L of soft water (ionic composition, Table 1) and placed in a wet table surrounded by flowing water for temperature regulation. A stock Zn^{2+} solution was added to each flux unit (as $ZnSO_4 \cdot 7H_2O$, Anachemia) to bring each one to 100 μg/L Zn (1.53 μmol/L). In addition, 10 μCi of ^{65}Zn (as $ZnCl_2$, specific activity=1.97 mCi/mg, NEN Life Science Products, Boston, MA, USA) was added to each flux unit, an amount which had negligible influence on the total Zn concentration of the water. Each trial was run with a control, and with the addition of 0.5, 1.0 and 2.0 mM of a test salt, unless otherwise noted. Once the flux units were prepared and equipped with gentle aeration, 28 fish were removed from their holding tank and divided evenly between the 4 flux units (control, 0.5, 1.0 and 2.0 mM test salt; N=7). Fluxes lasted 7 h, with triplicate 5 ml water samples taken just before the termination of the flux. At the end of the flux period, the fish were removed and rinsed for 1 min in a bath containing a lethal amount of anesthetic (0.5 g/L MS222) and 40 mg/L Zn^{2+} (as $ZnSO_4 \cdot 7H_2O$) to displace any surface-bound ^{65}Zn. After rinsing, trout were blotted dry and weighed to the nearest 0.01 g. Fish were assayed for ^{65}Zn activity in a γ-counter (MINAXI γ Auto-Gamma 5000 Series, Canberra-Packard). Water samples were similarly assayed for ^{65}Zn activity, as well as for total Zn^{2+} and the concentration of the cation being tested (Na^+, K^+, Mg^{2+} or Ca^{2+}) by atomic absorption spectrophotometry. NH_4^+ concentrations were determined by the method of Verdouw et al. (1978).

The following salts were tested; NaCl, $NaNO_3$, KCl, NH_4Cl, $MgCl_2$, and $CaCl_2$ (BDH Chemicals) and N-methyl-D-glucamine chloride. For the latter, a stock solution of N-methyl-D-glucamine (Sigma) was first prepared and titrated to pH 7.0 with

concentrated HCl. $MgCl_2$ and $CaCl_2$ were tested solely at 0.5 mM against 0.5 mM NaCl and a control. In addition, the effects of pH on ^{65}Zn uptake were investigated. Two tanks were set up as described above, a control tank (pH 5.6) and a tank at pH 3.7 (addition of ~0.2 mM H^+). pH levels were achieved by the addition of concentrated HCl to the water, and levels were constantly monitored during the flux and adjusted if needed.

To determine if acclimation to the test salts had an effect on their inhibition of Zn^{2+} uptake, 20 fish were acclimated to soft water plus 1 mM Na^+ (as NaCl), and 20 fish to soft water plus 1 mM Ca^{2+} (as $CaCl_2$) for three weeks. During acclimation, trout were kept in 30 L tanks and 90% of the water was replaced every 2 days. Fish were fed to satiation as usual. Flux tests were then performed in 1 mM NaCl and 1 mM $CaCl_2$ with acclimated and non-acclimated fish, against a soft water control with non-acclimated fish.

Finally an experiment was performed to examine the distribution of radiolabelled Zn^{2+} accumulation during the flux tests in the absence (control) and presence of 1.0 mM Na^+. The flux test was performed in the normal manner. At the end of 7 h, fish were rinsed and lethally anaesthetized for 1 minute as above, then blood was collected in heparinized capillary tubes by caudal severance, the gills were removed and blotted dry, and a piece of skin was removed from the side of the fish underneath the dorsal fin. Any musculature adhering to the skin was scraped off. The height of the blood collected in capillary tubes was measured in mm (internal radius of tubes=1.15 mm) to determine its volume, and the other tissues and remaining carcass were weighed to the nearest 0.001 g. All samples were assayed for ^{65}Zn. For calculations of partitioning, the blood was assumed to account for 5% of the body mass (Olson 1992), the skin was assumed to

weigh 0.014g/cm^2, and the total skin was assumed to equal 1.36 cm^2/g fish based on measurements carried out by M. Grosell (personal communication, Dept. of Biology, McMaster University).

Expressed concentrations of Zn^{2+} and cations are nominal, but were always within 10% of the desired concentrations.

Toxicity testing

To test whether inhibition of Zn^{2+} uptake had an effect on acute Zn^{2+}-induced mortality, 96 h LC_{50} tests were conducted. The four test conditions were soft water (Table 1), soft water plus 2.0 mM Na^+, soft water plus 1.0 mM Mg^{2+}, and soft water plus 1.0 mM Ca^{2+}.

Forty trout were removed and divided into five 18 L tanks (8 fish/tank, with each tank receiving 200 ml/min soft water) and allowed to settle for 1 h. After the hour, the tanks were randomly assigned to one of five Zn^{2+} concentrations including a control group (0-350 μg/L Zn, 0-3000 μg/L Zn for the Ca^{2+} trial). To begin the LC_{50}, flow of toxicant from a Mariotte bottle was started into a head tank where it mixed with inflowing fresh water by vigorous aeration. The toxicant consisted of $ZnSO_4 \cdot 7H_2O$, with the appropriate concentration of cationic salt if required, dissolved in deionized water acidified with 0.2 ml/L of concentrated HNO_3 (trace metal analysis grade, BDH Chemicals). At the same time, Zn^{2+} and the appropriate cation were added to each tank (apart from the control tanks) to immediately bring them up to the desired Zn^{2+} concentrations. Mortalities were recorded over 96 h. Water samples were taken daily and acidified for later analysis. The 96 h-LC_{50}s ± 95% confidence limits (C.L.) were

calculated by log-probit analysis of mortality *vs.* measured waterborne Zn^{2+} concentration (Finney 1971).

Ca^{2+} uptake

The effect of various ions on the ability of Zn^{2+} to disrupt Ca^{2+} uptake, was investigated in an attempt to determine their protective mechanism. Six flux units were set up as for the ^{65}Zn fluxes above. The tanks were designated as 1) control soft water pH=5.8, 2) soft water plus 100 μg/L Zn^{2+} pH=5.8, 3) soft water plus 100 μg/L Zn^{2+} with pH adjusted to 4.0 with HCl, 4) soft water plus 100 μg/L Zn^{2+} plus 2 mM Na^{+}, 5) soft water plus 100 μg/L Zn^{2+} plus 1 mM Mg^{2+}, and 6) soft water plus 100 μg/L Zn^{2+} plus 1 mM Ca^{2+}, all as chloride salts. Fifteen μCi of ^{45}Ca (as $CaCl_2$; NEN Life Science Products) was added to each flux unit and the fish (N=8) were exposed for 7 h. In a separate experiment, the time course of the effect of low pH on Zn^{2+}-induced inhibition of Ca^{2+} uptake was investigated. Trout were exposed to ^{45}Ca in soft water, soft water plus 100 μg/L Zn^{2+} and soft water plus 100 μg/L Zn^{2+} at pH 4.1, all for 5 h. Sixteen fish were also held in 100 μg/L Zn^{2+} and 100 μg/L Zn^{2+} at pH 4.1 for 8 h and 30 h without ^{45}Ca, and then exposed to ^{45}Ca for 5 h.

At the termination of the ^{45}Ca fluxes, water samples were taken and fish were removed and rinsed for 1 min in 0.5 g/L MS222 and 5 mM $CaCl_2$ to displace any surface bound ^{45}Ca. Fish were weighed and digested in a vial with 5 times their weight of tissue solubilizer (TS-2, Research Products International, Mount Prospect, IL, USA). Digestion was accelerated by heating to 55°C for 36 h. Triplicate 0.5 ml samples of the digest media and water were assayed for ^{45}Ca by scintillation counting using an LKB 1217

Rackbeta (Hamilton, ON, Canada). Differences in counting efficiency between digest media and water were corrected by internal standardization.

Calculations and statistics

The appearance of ^{65}Zn from the water into the fish was calculated from the ^{65}Zn activity of the whole body and the specific activity of Zn^{2+} in the water. The mean specific activity (S.A.) of Zn^{2+} in the water over the flux period was calculated as:

$$S.A.=(cpm/ml)/[Zn] \quad \text{(Eqn. 1)}$$

where cpm are the γ-counts per minute and [Zn] is the concentration of Zn^{2+} in $\mu g/ml$. In practice, Zn^{2+} total concentrations and specific activities underwent negligible change during these tests. Total Zn^{2+} appearance in the body was then calculated as:

$$\text{Total } Zn^{2+} \text{ Appearance= (cpm/body weight)*(1/S.A.)} \quad \text{(Eqn. 2)}$$

where total Zn^{2+} appearance was in μg Zn/g body, which was then divided by 7 h (the duration of the exposure) to yield a final uptake rate in μg Zn/g body/h. Ca^{2+} uptake rates using ^{45}Ca were calculated in an analogous fashion.

Data have been expressed as means ± standard error (N) except for the 96 h-LC_{50}s where means ± 95% C.L. have been reported. For the ^{65}Zn and ^{45}Ca uptake experiments, significant differences from the control uptake rate were tested with a one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons to determine significant differences among treatments. LC_{50}s were compared by means of the Bonferroni adjustment to the independent two-tailed Student's t-test. The limit of significance was 5%, 1%, and 0.1% (indicated on the graphs as *, **, and *** respectively).

Results

Zn^{2+} uptake

Rainbow trout exposed to 100 μg/L Zn^{2+} had a control whole body uptake rate of 0.033 μg Zn/g body/h (Fig. 1A). Na^+, as NaCl, at 0.5, 1.0 and 2.0 mM reduced the control whole body Zn^{2+} uptake by 26%, 48% and 87% (Fig. 1A). As well, Na^+ as $NaNO_3$, at 0.5, 1.0 and 2.0 mM also reduced Zn^{2+} uptake by 33%, 67% and 86% respectively showing that the effect was independent of the anion (Fig. 1B). The chloride salts of K^+ reduced Zn^{2+} uptake by 42%, 75% and 93% (Fig. 2A), NH_4^+ by 53%, 52% and 80% (Fig. 2B) and N-methyl-D-glucamine by 37%, 52% and 37% (Fig. 2C), all at 0.5, 1.0 and 2.0 mM concentrations, very similar effects to those of Na^+. N-methyl-D-glucamine was tested because it is generally considered to be impermeable across fish gills.

The addition of 0.5 mM Na^+ (monovalent), 0.5 mM Mg^{2+} (divalent) and 0.5 mM Ca^{2+} (divalent), all as chloride salts, decreased Zn^{2+} uptake by 20%, 53% and 85% respectively from a control uptake rate of 0.039 μg Zn/g body/h (Fig. 3). The effect of 0.5 mM Ca^{2+} was significantly greater than that of 0.5 mM Mg^{2+}, which in turn was significantly greater than that of 0.5 mM Na^+.

When pH was decreased by ~2 pH units from 5.6 to 3.7 (a 100-fold increase in H^+ ion concentration using HCl), Zn^{2+} uptake was decreased by 74% (Fig. 4). As pH 3.7 represents the addition of approximately 0.2 mM H^+, protons were clearly more potent than the other cations tested in antagonizing Zn^{2+} uptake.

Acute *versus* chronic effects of cation exposure were evaluated. Whether trout were acclimated to soft water plus 1.0 mM Na^+ for three weeks prior to testing or were acutely exposed to 1.0 mM Na^+ only at the time of the experiment, the presence of 1.0

mM Na^+ in the water had the same effect in decreasing Zn^{2+} uptake (Fig. 5). This was also the case for Ca^{2+}, where acclimation to 1.0 mM Ca^{2+} had the same effect on reducing the uptake rate of Zn^{2+} as did acute exposure to 1.0 mM Ca^{2+}, in comparison to control trout (Fig. 5).

In the test to determine the internal distribution of radiolabelled Zn^{2+} taken up from the water over the 7 h period, the majority (71%) was in the carcass in the control treatment (Fig. 6B). The gills, which accounted for only 2.0% of the body weight, held 17% of the Zn^{2+}; the blood, which accounted for 5% of the body weight (Olson 1992) held 10% of the Zn^{2+}, and the skin which accounted for 0.7% of the body weight held only 2.5% of the Zn^{2+} (Fig. 6B). In this experiment, the presence of 1 mM NaCl reduced overall Zn^{2+} uptake by 30% (Fig. 6A). By far, the largest absolute reduction was in the carcass (29%), with most of the remaining reduction occurring in the gills. There was no significant reduction in the blood or skin components. This result shows that the presence of the competing cation inhibits mainly internal Zn^{2+} accumulation, and is not just acting by displacing Zn^{2+} from the body surface.

Toxicity testing

The addition of 2 mM NaCl or 1 mM $MgCl_2$ to the water had no effect on acute Zn^{2+} toxicity as evaluated by 96 h LC_{50} tests (Fig. 7). In contrast, the addition of 1 mM $CaCl_2$ greatly reduced Zn^{2+} toxicity, as indicated by the 18 fold increase in the LC_{50} from a control value of 103 μg/L to 1800 μg/L (Fig. 7).

Ca^{2+} uptake

Exposure to 100 μg/L Zn^{2+} in control soft water significantly reduced Ca^{2+} uptake by 87% (Fig. 8). The addition of 2 mM NaCl, 1 mM $MgCl_2$ or 0.2 mM HCl (pH 4.0; data

not shown) did not diminish the effect of Zn^{2+} on Ca^{2+} uptake over 7 h. However, the addition of 1 mM $CaCl_2$ significantly increased Ca^{2+} uptake back to 54% of the control rate, a value which was not significantly different from the control (Fig. 8). In addition, short term (0-5 h) or long term (up to 35 h) exposure to Zn^{2+} with the addition of 0.2 mM HCl (pH 4.1), did not restore Ca^{2+} uptake (Table 2).

Discussion

Zn^{2+} uptake

Whole body Zn^{2+} uptake was reduced in the presence of a variety of positively charged waterborne ions. The magnitude of the reduction was directly related to the concentration of positive charges in the water (e.g. equivalents), regardless of which element or compound carried that charge (except for Ca^{2+} and H^+, as discussed below). Thus on a molar basis, divalent Mg^{2+} was approximately twice as potent as the monovalent cations. We interpret the slight differences amongst different monovalent cations on inhibition of Zn^{2+} uptake as variation of the effect as opposed to real differences in inhibition potency (except for Ca^{2+} and H^+, as discussed below). For example, 0.5 mM Na^+ decreased Zn^{2+} uptake from control treatments by 26% (Fig. 1A), 33% (Fig. 1B) and 20% (Fig. 3) in three different trials. Free Zn^{2+} ion activity would not have changed significantly with the addition of each salt as a result of ionic strength effects as predicted by the Debye-Hückel equation for the range of concentrations tested in the present study (up to 2 mM). Significant reductions in activity would occur when salts exceed ~10 mM (Stumm and Morgan 1981).

Significant reductions of Zn^{2+} uptake were observed at environmentally realistic freshwater concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} (see Table 1 for typical freshwater Lake Ontario ionic composition, "hardwater"). The Zn^{2+} exposure concentrations in the uptake studies were also in the range of environmental relevance. While normal Zn^{2+} levels in clean freshwaters are only a few μg/L or less, concentrations of 50 μg/L are found in industrialized areas. Maximum Zn^{2+} concentrations in natural surface waters are reported to range from 130 to 1170 μg/L in different areas of Canada (CCME 1995). The Zn^{2+} concentration used in the uptake studies was the same as our measured 96 h LC_{50} (~100 μg/L; Fig. 7). The Zn^{2+} uptake rates in the present study were comparable to those reported by Hogstrand et al. (1998) at 100 μg/L Zn in juvenile rainbow trout in very soft water (Ca^{2+} free). They were also comparable to uptake rates at total Zn^{2+} concentrations near the LC_{50} [~1000 μg/L (Alsop et al. 1999)] in Hamilton hard water (Hogstrand et al. 1998). Nevertheless, the Zn^{2+} uptake rate did vary over the course of the present study from a high of 0.039 μg/g/h (Fig. 2B and 3) to a low of 0.007 μg/g/h (Fig. 4) in different control series. The lower rates occurred towards the end of the study when the fish were larger. Notably, Wagner et al. (1985) found that branchial Ca^{2+} uptake cycled between periods of low uptake and high uptake in rainbow trout. The difference in Ca^{2+} uptake rates varied on the order of 5-fold while the duration of the cycles varied from 7 to 17 days; hormonal changes were thought to be involved, and Zn^{2+} uptake may follow a similar trend.

Ca^{2+} had a much greater effect inhibiting Zn^{2+} uptake than the other divalent cation tested, Mg^{2+} (Fig. 3). This is likely because Zn^{2+} and Ca^{2+} would not only compete on the gill surface for binding sites (adsorption), but would also compete for absorption

since they share the same apical transport channel (Hogstrand et al. 1996). Zn^{2+} and Ca^{2+} may also interact with each other inside the gill cell, where for example, Zn^{2+} has been shown to decrease Ca^{2+} uptake by both the endoplasmic reticulum and mitochondria (Verbost et al. 1996). Zn^{2+} is also a potent inhibitor of the basolateral CaATPase transporter in the trout gill (Hogstrand et al. 1996). Increasing waterborne Ca^{2+} may also elicit stanniocalcin release into the blood which would cause closure of the apical Ca^{2+}/Zn^{2+} channels in the gill and decrease both Zn^{2+} and Ca^{2+} uptake (Wendelaar Bonga and Pang 1991).

The ammonium ion (NH_4^+) had the same effect in reducing Zn^{2+} uptake as the other monovalent cations that were tested (Fig. 2B). This indicates that not only can cations from the water affect Zn^{2+} uptake, but cations which are excreted by the fish at the gills may also affect uptake. Zn^{2+} uptake may therefore be reduced during times of high ammonia excretion which can occur for example after feeding (Kaushik 1980; Beamish and Thomas 1984; Alsop and Wood 1997).

Cationic competition most probably occurs at the apical surface of the gill. N-methyl-D-glucamine$^+$ is a molecule with a single positive charge that is impermeant to biological membranes, so any effects from the presence of that molecule can only occur in the water or at the gill surface. Therefore the reduction in Zn^{2+} absorption (Fig 2B) was due to decreased Zn adsorption to the gill surface due to competition with the charge carried by N-methyl-D-glucamine$^+$.

Decreasing the pH to 3.7 (adding 0.2 mM H^+) reduced Zn^{2+} uptake to a greater extent than did the other monovalent ions tested (Fig.4). With the drop in pH over this range, there would be no change in Zn^{2+} speciation as determined by the aquatic

geochemical program MINEQL+ (Schecher and McAvoy 1994), which predicts 100% of the Zn^{2+} to exist in the "free" form in both control and decreased pH test waters. H^+ is not known to be transported through the Ca^{2+}/Zn^{2+} channel and therefore direct competition should not be occurring there. Handy and Eddy (1991) found an increased potency of H^+ in comparison to Ca^{2+} in reducing Na^+ adsorption and absorption in rainbow trout. They attributed this to the increased mobility of H^+ due to its decreased 'charge density' in comparison to Ca^{2+}. An alternative mechanism may be direct acid damage to the Zn^{2+}/Ca^{2+} transport system or an indirect effect, such as stimulation of mucus secretion by low pH (McDonald 1983). Mucus production may slough Zn^{2+} off before it can be taken up into the gill and once the mucus is sloughed off, it may even bind Zn^{2+} in the water, keeping it off the gill.

Pagenkopf (1983) hypothesized that H^+ would compete for the negative sites on the gill surface, forming Lewis acid-base complexes, unlike Na^+ and K^+. Playle et al. (1993) showed that H^+ had a high affinity for cadmium binding sites on the gills, with log *K* stability constants for Cd^{2+}, H^+, and Ca^{2+} of 8.6, 6.7, and 5.0 respectively. Like Zn^{2+}, Cd^{2+} disrupts Ca^{2+} homeostasis (Verbost et al. 1989). Galvez et al. (1998) estimated the log *K* value for Zn^{2+} in trout gills to be about 5.5. Richards and Playle (1998) found a similar pattern of potency in their study of cobalt (Co^{2+}) binding to rainbow trout gills, and Co^{2+} is also known to interfere with Ca^{2+} uptake (Comhaire et al. 1994). Richards and Playle (1998) reported that H^+ was the most potent cation reducing Co^{2+} accumulation in the gills, followed by Ca^{2+} then Na^+.

Toxicity testing

From the present study, it appears any cation may possess the capability of reducing the uptake of Zn^{2+} at trout gills. Simkiss and Taylor (1989) stated that anything which obstructs the initial adsorption of a metal will reduce its toxicity. The present study showed that this is not always the case. Although Na^{+} and Mg^{2+} were able to reduce Zn^{2+} uptake, presumably through decreased adsorption to the gill (Pagenkopf 1983) (Figs. 1A, 1B and 3), they did not ameliorate Zn^{2+} toxicity (Fig. 7). In contrast, Ca^{2+} strongly reduced both Zn^{2+} uptake and Zn^{2+} toxicity (Figs. 3 and 7).

One explanation may be that Na^{+} and Mg^{2+} may indeed reduce Zn^{2+} uptake by decreasing adsorption to Zn^{2+} uptake sites, but these may not be the sites related to Zn^{2+} toxicity. Ca^{2+} may reduce adsorption to both non-toxic Zn^{2+} uptake sites and to a separate population of toxic Zn^{2+} binding sites. Another explanation may be that cations such as Na^{+} are reducing non-toxic Zn^{2+} uptake, while Ca^{2+} reduces both toxic and non-toxic Zn^{2+} uptake (as opposed to binding). A third possibility relates to effects on Ca^{2+} uptake. Zn^{2+} specifically induces hypocalcemia by inhibiting branchial Ca^{2+} uptake (Spry and Wood 1985; Hogstrand et al. 1995) and increased waterborne Ca^{2+} may simply reduce the symptoms of Zn^{2+} toxicity by preventing this effect. Indeed, we found that 2 mM Na^{+} and 1 mM Mg^{2+} did not restore Zn^{2+}-induced Ca^{2+} uptake inhibition (Fig. 8), nor did they reduce Zn^{2+} induced mortality (Fig. 7). However, 1 mM Ca^{2+} was able to restore branchial Ca^{2+} uptake that had been inhibited by 100 μg/L Zn^{2+} (Fig. 8), a Zn^{2+} concentration that was equivalent to the 96 h LC_{50} in control soft water (Fig. 7). In addition, 1 mM Ca^{2+} simultaneously increased the LC_{50} to 1800 μg/L Zn^{2+}, a Zn^{2+} concentration that was probably high enough to inhibit branchial Ca^{2+} uptake again.

These results partially dissociate Zn^{2+} uptake from acute Zn^{2+} toxicity, and reinforce the view that Zn^{2+}-induced inhibition of Ca^{2+} uptake is the direct cause of toxicity.

Both Bradley and Sprague (1985) and Cusimano et al. (1986) observed a protective effect of decreased pH (increased H^+) against waterborne Zn^{2+} toxicity. Cusimano et al. (1986) speculated that H^+ interference with metal uptake at the gill was the primary mechanism that decreased metal toxicity (interestingly, they found that H^+ also protected against copper and cadmium toxicity). In the present study however, H^+ (pH 4.0) did not restore Ca^{2+} uptake over 7 h of exposure. Hobe et al. (1984) found H^+ (pH 4.0-4.2) alone initially reduced Ca^{2+} uptake in rainbow trout by two-thirds from 0-12 h, but Ca^{2+} uptake was fully restored by 18-24 h. We suspected that if pH was protective, it may require a longer period of time. However, we found no effect of H^+ (pH 4.1) on restoring Zn^{2+}-induced Ca^{2+} uptake inhibition again over short term (0-5 h) or longer term (up to 35 h) exposures (Table 2). If H^+ is truly protective against Zn^{2+} toxicity, it may protect via a different mechanism than does Ca^{2+}.

With respect to zinc, there is also a general acceptance of the principle that acute toxicity is related to the levels of waterborne Ca^{2+} and Mg^{2+} which is termed hardness (CCME 1995). For example, the U.S. EPA (1980) employs an equation based on hardness (in mg $CaCO_3$/L) to calculate a numerical limit of total allowable waterborne zinc:

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

The present study has shown that while Ca^{2+} protects against acute Zn^{2+} toxicity, Mg^{2+} does not. Therefore, Eqn.1 would overestimate safe Zn^{2+} levels in waters where a significant percentage of the total "hardness" was contributed by the Mg^{2+} ion (e.g.

coastal fresh waters), and aquatic life would not be adequately protected. The higher the Mg^{2+} levels of the water, the more Eqn.1 would overestimate the total allowable Zn^{2+} levels. For example, Eqn. 1 would calculate that in the control soft water used in the LC_{50} trials (Table 1), 40 μg/L Zn^{2+} would be the total allowable limit, and presumably protective against acute Zn^{2+} toxicity to aquatic life. The 96 h LC_{50} was determined to be 103 μg/L Zn^{2+} in soft water (Fig. 7), and in this case, Eqn. 1 may be protective. With the addition of 1.0 mM Ca^{2+} to soft water, Eqn. 1 would calculate a total allowable limit to be 343 μg/L Zn^{2+} while the LC_{50} was 1800 μg/L Zn^{2+}, evidently protective. However, with the addition of 1.0 mM Mg^{2+}, Eqn. 1 would similarly calculate a total allowable limit to be 343 μg/L Zn^{2+}. This is 5.5-fold greater than the measured LC_{50} of 63 μg/L Zn^{2+} (Fig. 7). Clearly, this may pose harmful consequences to aquatic life.

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Table 3-1. Water composition of hard water (Hamilton tap water from near shore Lake Ontario), and artificial soft water of the Zn^{2+} and Ca^{2+} uptake experiments and LC_{50} trials. N.D.= not detectable.

	Hard water	Soft water Zn^{2+} uptake	Soft water Zn^{2+} LC_{50}/ Ca^{2+} uptake
Ca^{2+} (mM)	1.0	0.02	0.06
Mg^{2+} (mM)	0.2	0.01	0.02
Na^{+} (mM)	0.6	0.04	0.05
K^{+} (mM)	0.05	N.D.	0.01
Cl^{-} (mM)	0.7	0.04	0.05
Hardness (as mg $CaCO_3$/L)	120	5	10
pH	8.0	5.6	5.8

Table 3-2. Whole body Ca^{2+} uptake (μg Ca/g/h) in control soft water and during exposure to 100 μg/L Zn^{2+} at pH 5.8 (control pH) or 100 μg/L Zn^{2+} at pH 4.1 over 35 h. For the 8-13 h and 30-35 h ^{45}Ca exposures, fish were initially held in identical Zn^{2+} and pH waters without ^{45}Ca until they were used in the ^{45}Ca flux. Values are expressed as means ± standard error. N=8. Refer to Figure 8 caption for more details.

Time (h)	Control (μg Ca/g body/h)	100 μg/L Zn^{2+} (μg Ca/g body/h)	100 μg/L Zn^{2+} at pH 4.1 (μg Ca/g body/h)
-5-0 h	3.05 ± 0.24		
0-5 h		0.40 ± 0.17***	0.22 ± 0.01***
8-13 h		0.21 ± 0.01***	0.18 ± 0.01***
30-35 h		0.20 ± 0.01***	0.17 ± 0.002***

Figure 3-1. Effects of A) NaCl, and B) $NaNO_3$ on whole body Zn^{2+} uptake measured with ^{65}Zn as a tracer in juvenile rainbow trout. The total Zn^{2+} concentration of the water was 100 μg/L. Values are expressed as means ± standard error. N=7. An asterisk (*) denotes a significant decrease in Zn^{2+} uptake from the control treatment as determined by a one way ANOVA followed by Tukey's HSD test, $p<0.05$. Two asterisks (**) denote $p<0.01$ and three (***) denote $p<0.001$.

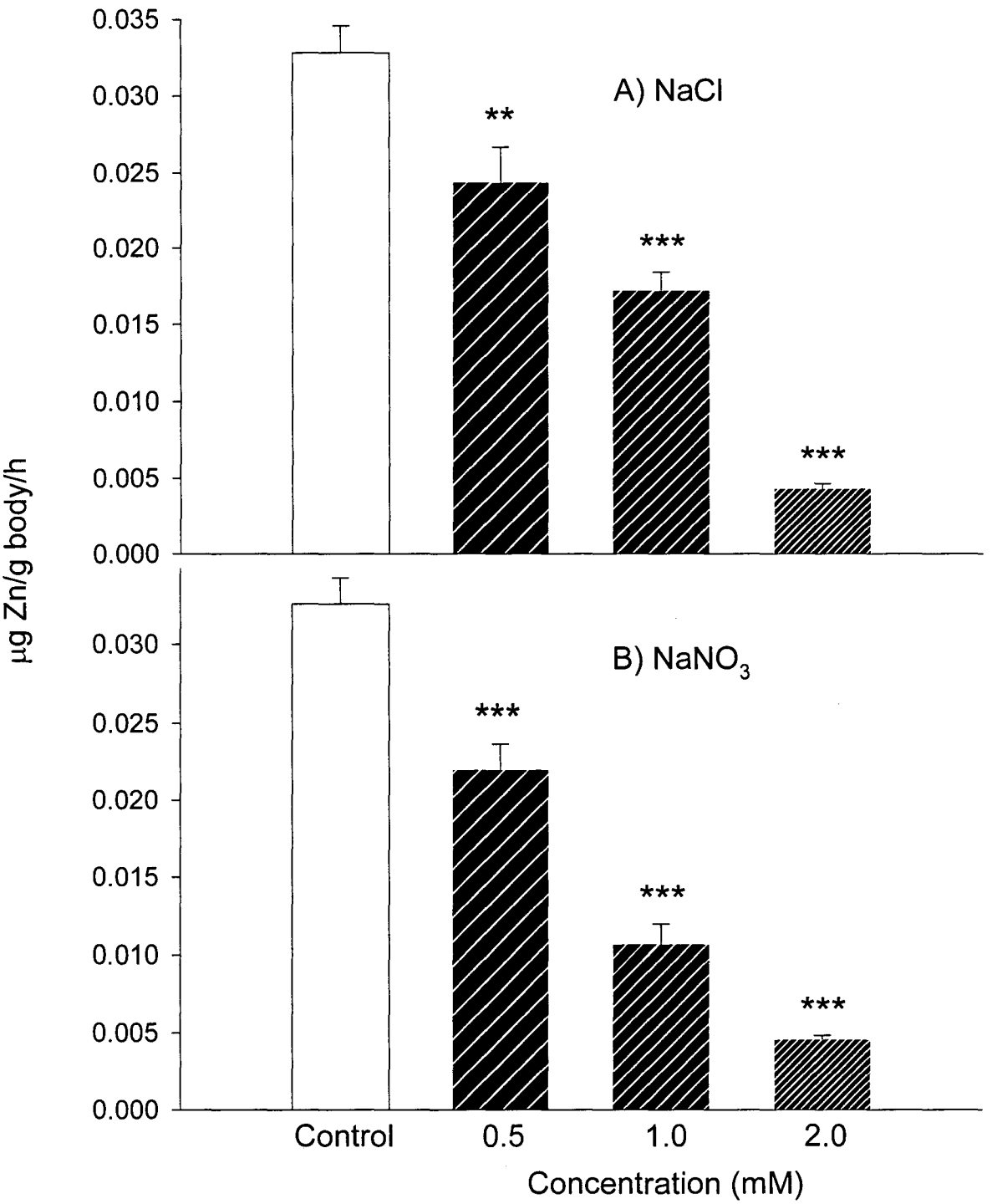

0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
A) NaCl
**
***
***
0.030
0.025
0.020
0.015
0.010
0.005
0.000
B) NaNO3
***
***
***
µg Zn/g body/h
Control
0.5
1.0
2.0
Concentration (mM)

Figure 3-2. Effects of A) KCl, B) ammonium (NH_4Cl) and C) N-methyl-D-glucamine chloride (with a single positive charge) on whole body Zn^{2+} uptake. The total zinc concentration of the water was 100 μg/L. Values are expressed as means ± standard error. N=7. Refer to Figure 1 caption for more details.

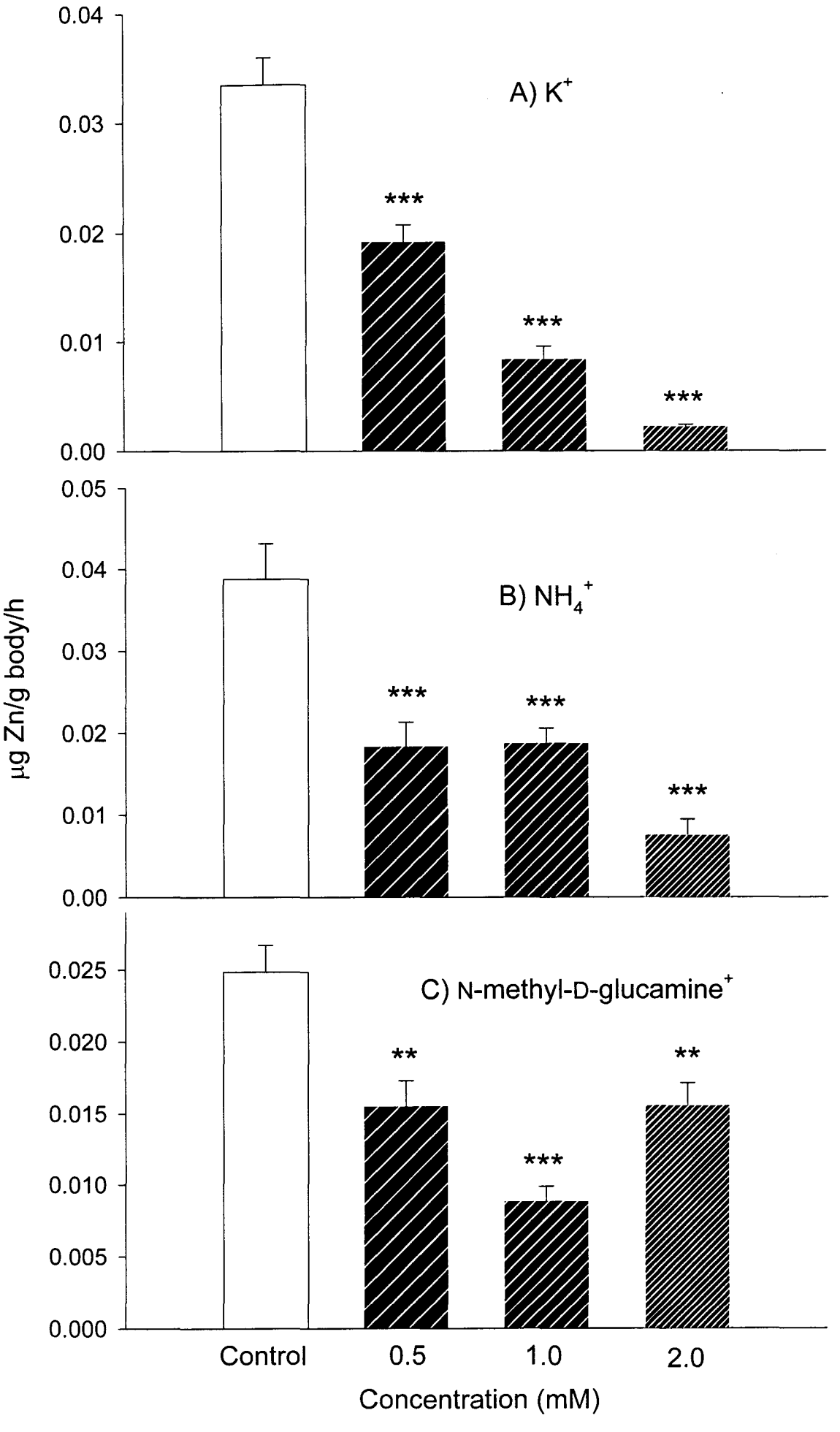

A) K+
B) NH4+
C) N-methyl-D-glucamine+
μg Zn/g body/h
0.04
0.03
0.02
0.01
0.00
0.05
0.04
0.03
0.02
0.01
0.00
0.025
0.020
0.015
0.010
0.005
0.000
***
**
Control
0.5
1.0
2.0
Concentration (mM)

Figure 3-3. Effects of 0.5 mM NaCl, $MgCl_2$ and $CaCl_2$ on whole body Zn^{2+} uptake. The total Zn^{2+} concentration of the water was 100 μg/L. Values are expressed as means ± standard error. N=7. Refer to Figure 1 caption for more details.

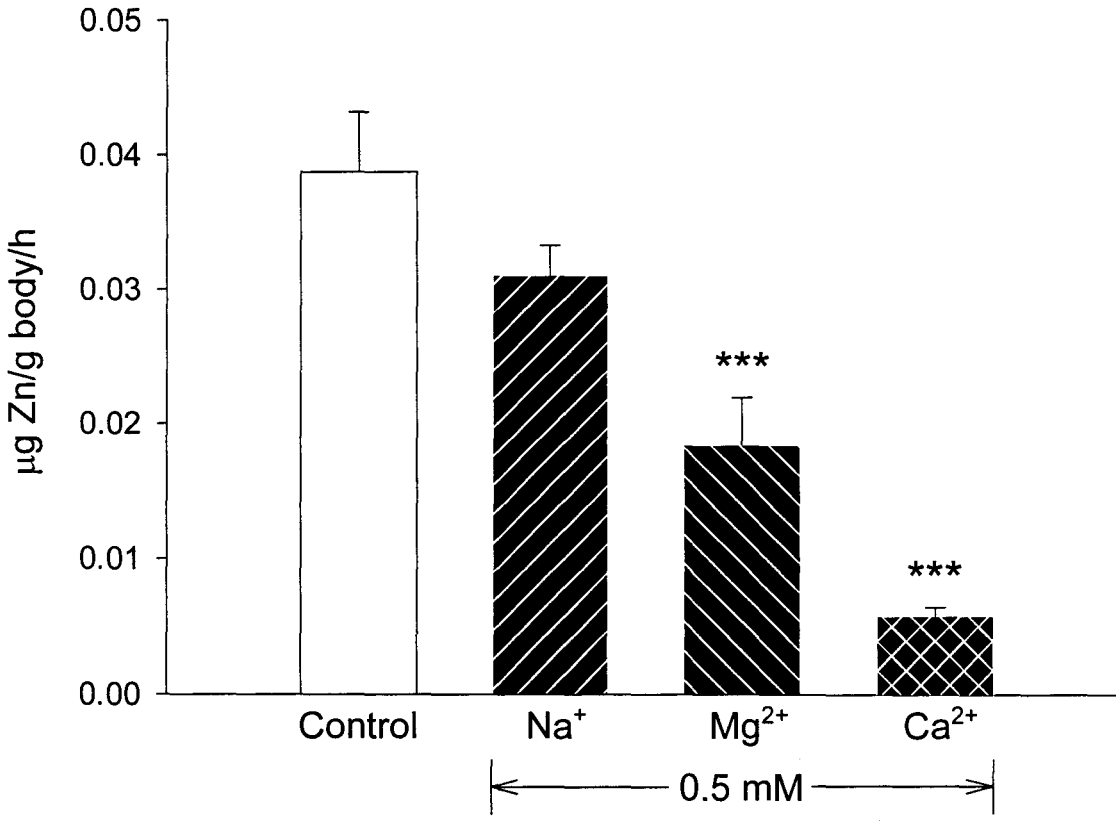

0.05
0.04
0.03
0.02
0.01
0.00
µg Zn/g body/h
***
***
Control
Na+
Mg2+
Ca2+
0.5 mM

Figure 3-4. Effects of pH on whole body Zn^{2+} uptake. The total Zn^{2+} concentration of the water was 100 μg/L. Values are expressed as means ± standard error. N=7. Refer to Figure 1 caption for more details.

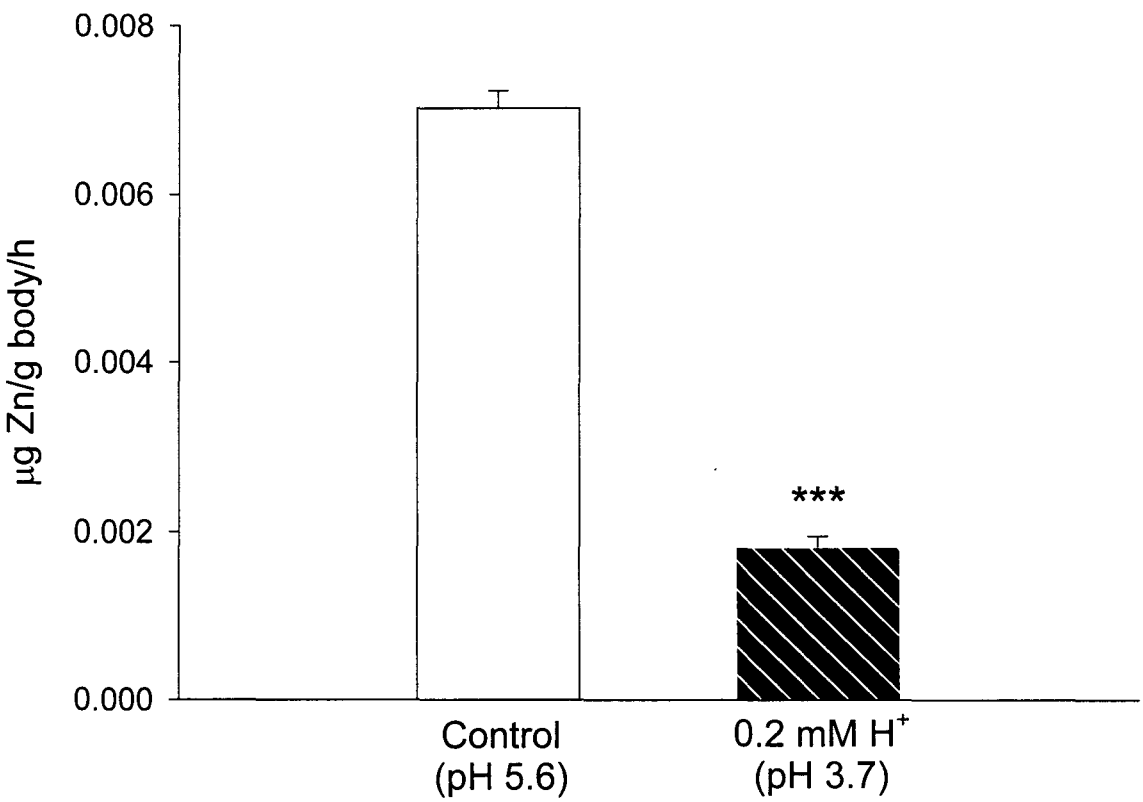

0.008
0.006
0.004
0.002
0.000
µg Zn/g body/h
***
Control
(pH 5.6)
0.2 mM H+
(pH 3.7)

Figure 3-5. Effects of 1 mM NaCl and 1 mM $CaCl_2$ on whole body Zn^{2+} uptake in 1 mM NaCl and 1 mM $CaCl_2$ acclimated (3 weeks) and non-acclimated trout. The total Zn^{2+} concentration of the water was 100 μg/L. Values are expressed as means ± standard error. N=7. Refer to Figure 1 caption for more details.

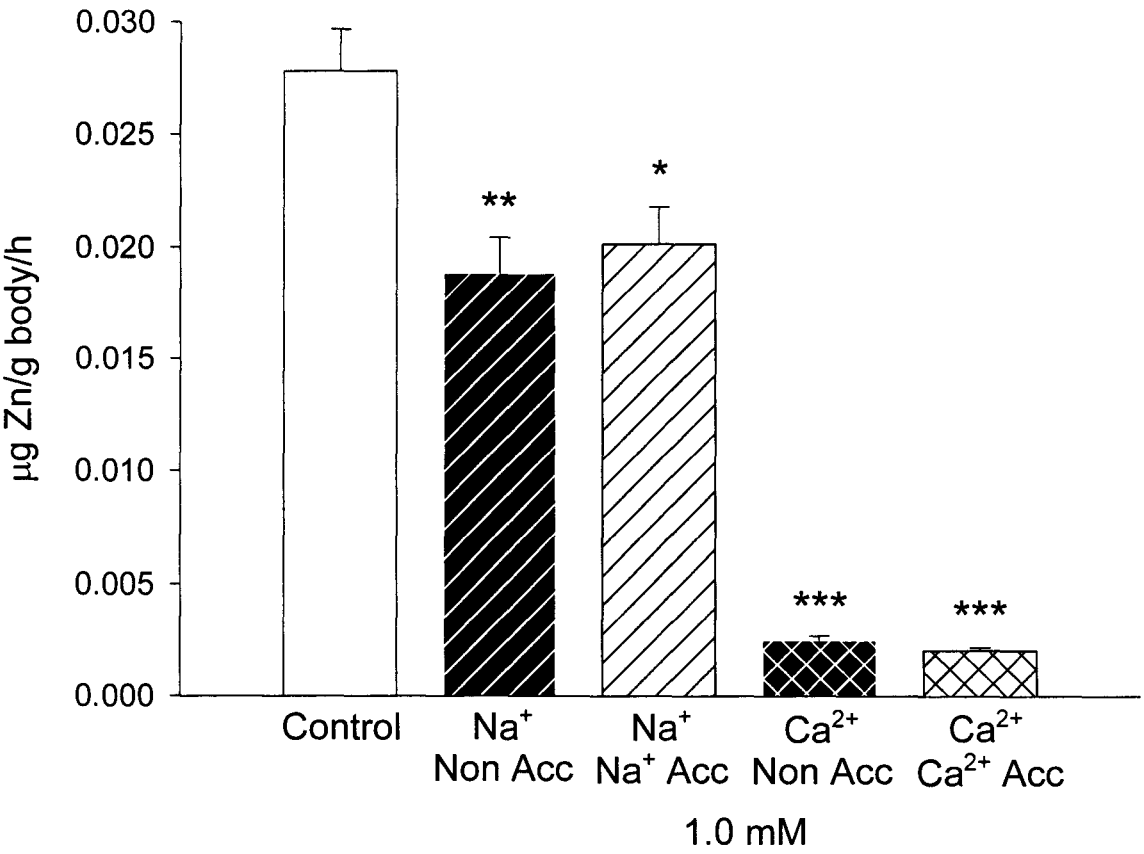

0.030
0.025
0.020
0.015
0.010
0.005
0.000
µg Zn/g body/h
**
*
***
***
Control
Na+ Non Acc
Na+ Na+ Acc
Ca2+ Non Acc
Ca2+ Ca2+ Acc
1.0 mM

Figure 3-6. A) The effect of 1 mM NaCl on Zn^{2+} uptake into the whole fish. B) The partitioning of ^{65}Zn into the gills, skin, blood and remaining carcass. Uptake was significantly reduced in the gills and remaining carcass of trout exposed to 1 mM Na^+. 1 mM Na^+ did not have an effect on the amount of ^{65}Zn bound to the exterior of the fish. The total Zn^{2+} concentration of the water was 100 µg/L. Values are expressed as means ± standard error. N=8. Refer to Figure 1 caption for more details.

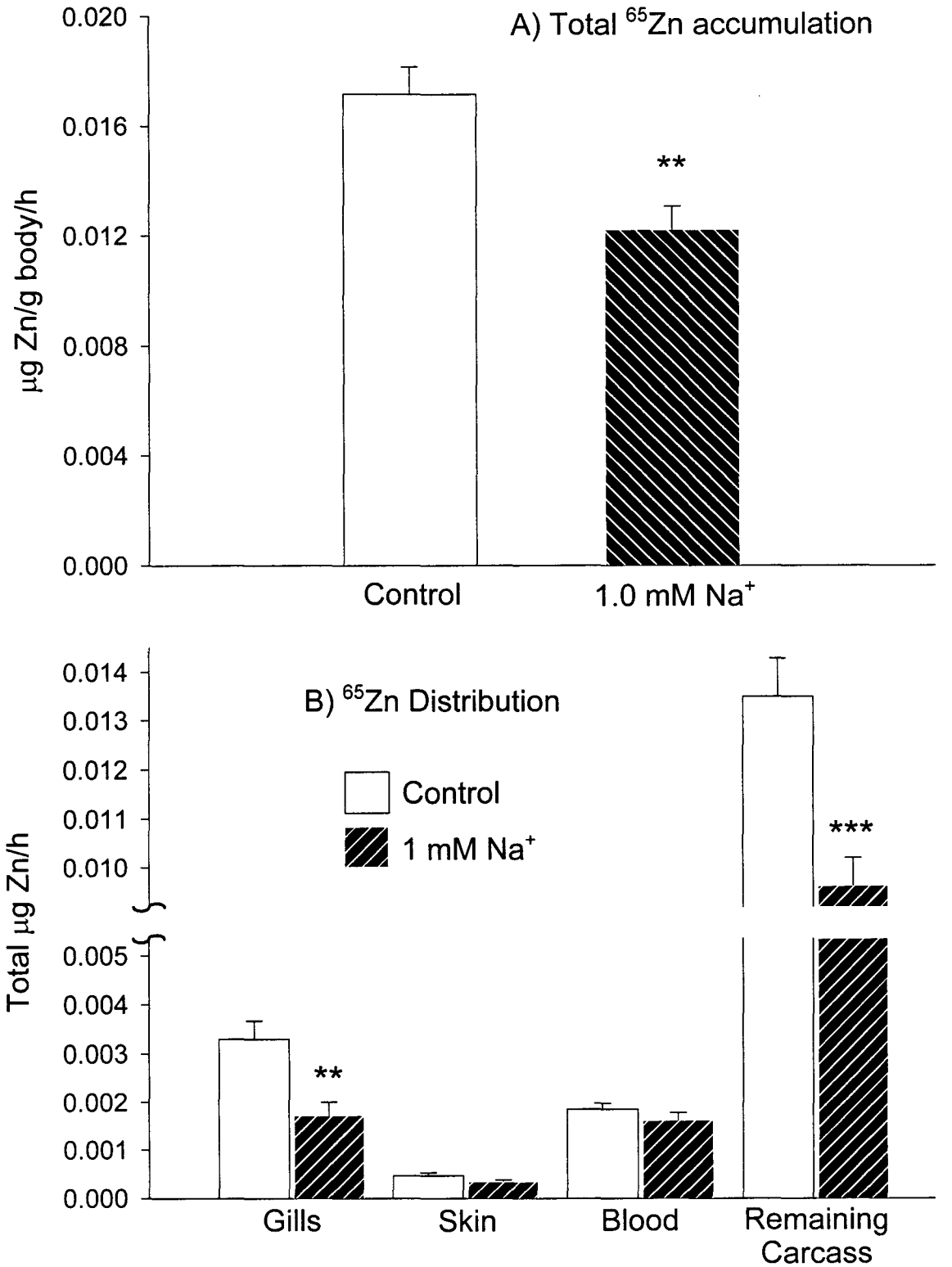

A) Total 65Zn accumulation
0.020
0.016
0.012
0.008
0.004
0.000
µg Zn/g body/h
**
Control
1.0 mM Na+
B) 65Zn Distribution
Control
1 mM Na+
0.014
0.013
0.012
0.011
0.010
0.005
0.004
0.003
0.002
0.001
0.000
Total µg Zn/h
**
***
Gills
Skin
Blood
Remaining Carcass

Figure 3-7. 96 h LC_{50}s in control water plus the addition of 2 mM NaCl, 1 mM $MgCl_2$ or 1 mM $CaCl_2$. Values are expressed as means ± the average of the upper and lower 95% C.L. Refer to Figure 1 caption for more details.

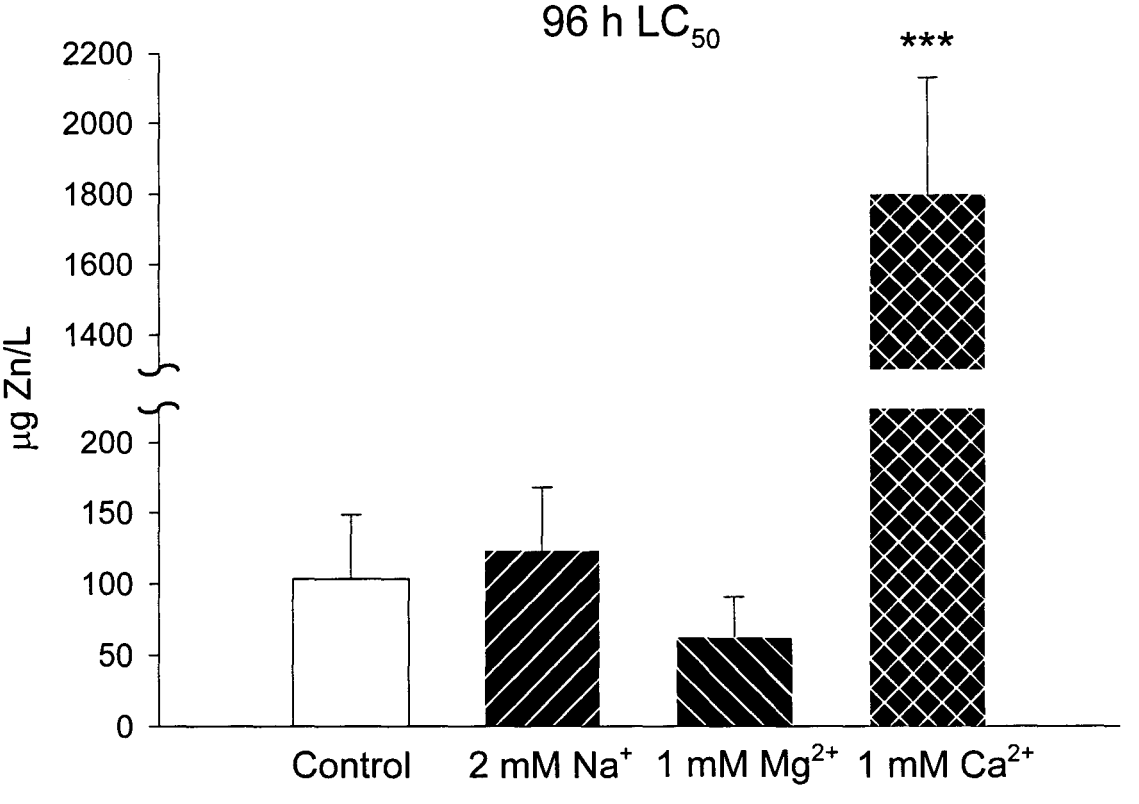
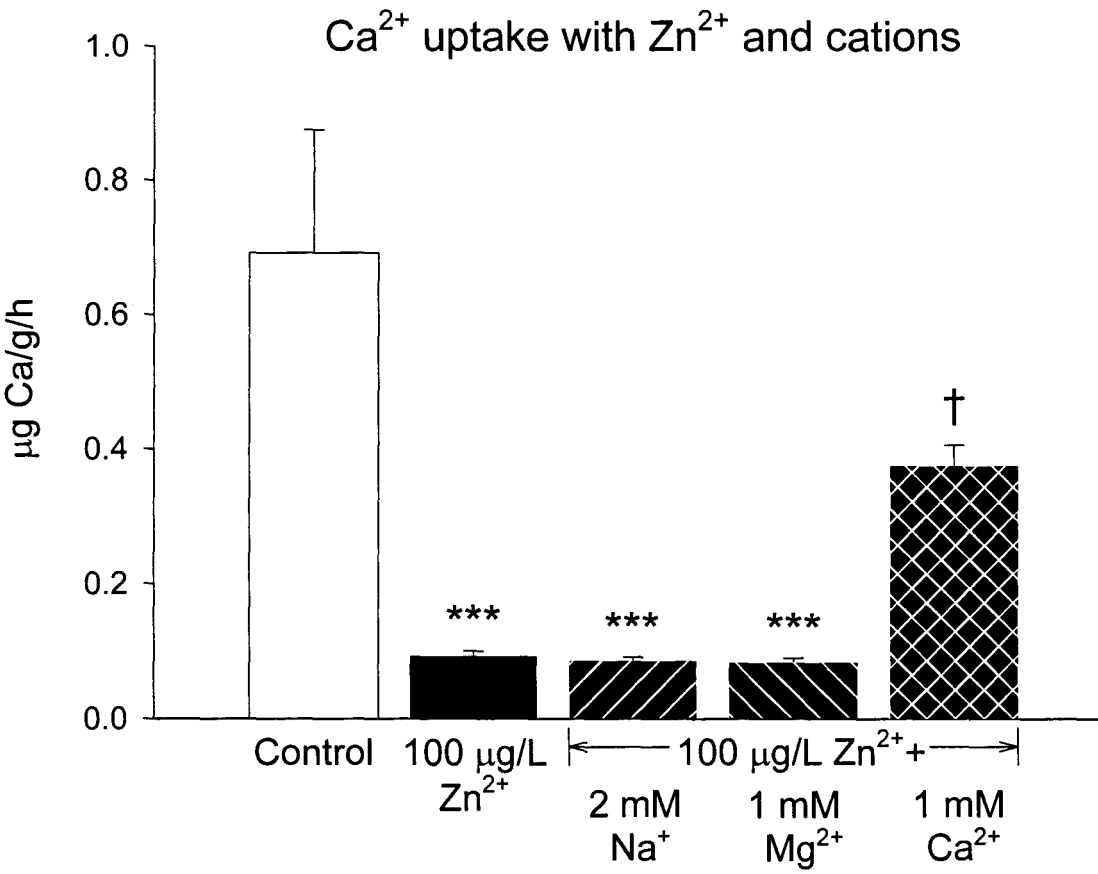

96 h LC_{50}
***
2200
2000
1800
1600
1400
200
150
100
50
0
µg Zn/L
Control
2 mM Na^+
1 mM Mg^{2+}
1 mM Ca^{2+}

Figure 3-8. Ca^{2+} uptake in control soft water plus the addition of 100 μg/L Zn^{2+}, or either 2 mM NaCl, 1 mM $MgCl_2$ or 1 mM $CaCl_2$ in addition to 100 μg/L Zn^{2+}. Values are expressed as means ± standard error. N=8. An asterisk (*) denotes a significant decrease in Ca^{2+} uptake from the control treatment as determined by a one way ANOVA followed by Tukey's HSD test, $p<0.05$. Two asterisks (**) denote $p<0.01$ and three (***) denote $p<0.001$. A dagger (†) indicates a significant increase in Ca^{2+} uptake over the 100 μg/L Zn^{2+} treatment ($p<0.001$). Note that the 1 mM $CaCl_2$ mean was not significantly different ($p>0.05$) from the control mean.


Ca2+ uptake with Zn2+ and cations
µg Ca/g/h
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
†
Control
100 µg/L Zn2+
100 µg/L Zn2+ +
2 mM Na+
1 mM Mg2+
1 mM Ca2+

CHAPTER 4

A Kinetic Analysis of Zinc Accumulation in the Gills of Juvenile Rainbow Trout: The Effects of Acclimation and Implications for Biotic Ligand Modelling

Abstract

Juvenile rainbow trout were acclimated to hard water (Ca^{2+}=1.0 mM, Mg^{2+}=0.2mM) and hard water plus 250 μg/L Zn (3.8 μM). After 30 d of exposure, there was no difference in the total Zn levels of the gills of Zn-exposed and control fish (~70 μg Zn/g gill). Exposure of both groups to a range of Zn concentrations (0-2900 μg/L Zn) for up to 7 d also had no effect on the measured total Zn levels in the gills. However, using radiolabelled ^{65}Zn, measurement of new Zn appearance in the gills was possible. Trout were exposed to a range of Zn concentrations (with ^{65}Zn) and the gills were sampled at times ranging from 0.5 to 72 h. The fast turnover pool of Zn in the gills increased with increasing acute Zn exposure concentration, while the maximum size of the fast pool was about 9-fold larger in Zn-acclimated fish (4.14 μg Zn/g gill) vs. control fish (0.45 μg Zn/g gill). At all sampling times, gill ^{65}Zn accumulation exhibited saturation kinetics, allowing calculation of binding capacity (B_{max}) and affinity (K_d). In both control and Zn-acclimated trout, K_d decreased rapidly (affinity increased) from 0.5 to 3 h, and then remained constant up to 72 h. B_{max} increased rapidly from 0.5 to 3 h in both groups, then the rate of increase began to subside but was still increasing from 24 to 72 h. At all times, the K_d of Zn-acclimated fish was higher (i.e. lower affinity) and B_{max} was greater than controls. The stabilized K_ds (>3 h) were approximately 280 μg/L total Zn (log K=5.6 as Zn^{2+}) and 575 μg/L total Zn (log K=5.3 as Zn^{2+}) in control and Zn-acclimated fish

respectively. The B_{max} of control fish at 0.5 h was 0.37 μg Zn/g gill and increased to 8.63 μg Zn/g gill by 72 h. The B_{max} of Zn-acclimated fish increased from 0.70 to 11.61 μg Zn/g gill over the same time period. Pre-exposure to 250 μg/L Zn appeared to have little effect on acute zinc toxicity, though the 96 h LC_{50}s for both groups were relatively high (~3000 μg/L Zn) in comparison to previous measurements. The relationship between gill binding constants for different metals and relative toxicity is critically assessed with respect to Biotic Ligand Modelling.

Introduction

Zinc (Zn) is an essential micronutrient which is found at high levels in the tissues of fish [1,2], and is primarily acquired from the diet. However, with increasing waterborne Zn levels or deficient dietary level, uptake at the gill can increase to a significant proportion of total uptake [3]. If waterborne levels become too high, Zn can become toxic to fish.

While normal Zn levels in pristine freshwaters are only a few μg/L or less, concentrations of 50 μg/L are routine in industrialized areas. Maximum Zn concentrations in contaminated surface waters are reported to range from 130 to 1170 μg/L in different areas of Canada [4]. At these levels, Zn specifically disrupts calcium uptake by the gills [5-7], leading to hypocalcemia which may end with the death of the fish within a few days, depending on the Zn concentration.

A frequently reported observation that accompanies chronic, sublethal exposure to Zn (and other metals) is physiological acclimation. If a fish survives the metal exposure, then the ionic disturbance that can occur may be eventually corrected [8], as seen with the full recovery of plasma Ca^{2+} levels during a sublethal exposure to Zn [6]. In addition, an increased tolerance (in terms of survival) to the metal may arise upon a threshold exposure. With Zn-exposed trout, this toxicological acclimation or increased tolerance was fully acquired within 5 days, with the tolerance having increased 2.5 times compared to unexposed fish as judged by LC_{50} tests [9].

There has been much interest recently in developing a method, based on geochemical modelling applied to the gill, for predicting heavy metal toxicity to aquatic biota in different water chemistries [10-15]. With the gill as the primary target organ of

acute metal toxicity, it has been proposed that the amount of metal accumulating at the gill could be used as a direct indicator of toxicity. The gill receptor loading model (one form of the Biotic Ligand Model, BLM) is one such approach. By first experimentally determining binding constants of the gill for metals and other ions, aquatic geochemical programs such as MINTEQA2 [16] and MINEQL+ [17] can then be used to predict metal bound to gills in a range of different water chemistries. This approach has been successful for metals such as copper and cadmium [10,18], silver [19], and cobalt [20]. Attempts have been made to apply these techniques to Zn [21], but the extremely high levels of Zn found in the gills of unexposed fish made detecting any accumulation difficult.

Galvez et al. [21] had some success by utilizing radiolabelled ^{65}Zn to distinguish newly accumulated Zn in the gills of juvenile rainbow trout from the large pool of native Zn already present. They were able to calculate the affinity (K_d) and binding capacity (B_{max}) of the gill for Zn. Alsop et al. [15] used a temporal approach with ^{65}Zn exposure for different periods at one concentration as opposed to varying the ^{65}Zn concentration at fixed sample times. From this, they were able to determine that the gills possessed at least two pools of Zn, a fast and a slow turnover pool. The fast turnover pool had a time to 50% turnover ($t_{½}$) of about 3-4 hours while the slow pool appeared to turn over linearly with time from 24 to 72 h with an overall $t_{½}$ of days to months. The size of the fast pool in control fish was found to be only 0.14% of the total Zn in the gills, and increased in size with acclimation to increasing waterborne Zn concentrations.

Using Galvez et al. [21] and Alsop et al. [15] as points of departure, the present study was designed to define both the temporal and concentration-dependent effects of

^{65}Zn exposure on accumulation in the rainbow trout gill. In addition, we were interested in determining if the accumulation patterns were predictive of acute Zn toxicity. The effects of a chronic pre-exposure to sublethal Zn on toxicity and gill accumulation kinetics were also evaluated. The results will be of use for future development of a BLM for zinc in freshwater fish.

Materials and Methods

Fish care

1600 juvenile rainbow trout (*Oncorhynchus mykiss*; 3-5 g) were purchased from Humber Springs Trout Hatchery (Orangeville, Ontario), and divided equally into two aerated 500L tanks supplied with 3 L/min of dechlorinated Hamilton tap water (ionic composition; Ca^{2+}, 1.0 mM; Mg^{2+}, 0.2 mM; Na^{+}, 0.6 mM; Cl^{-}, 0.7 mM; hardness, 120 mg $CaCO_3$/L; alkalinity, 95 mg $CaCO_3$/L; dissolved organic matter (DOM), 3 mg/L; pH 7.5). Fish were fed to satiation once daily with a commercial ration [fish food composition (partial analysis only): crude protein (min), 52%; crude fat (min), 17%; crude fibre (max), 2.5%; water, 12%; Ca^{2+}, 1.4%; Na^{+}, 0.4%; zinc (measured), 0.02% (173 μg/g)]. Feces and organic debris were siphoned out of the tanks daily. Photoperiod was set to mimic natural photoperiod. Fish were held under these conditions for two weeks prior to the commencement of chronic, sublethal Zn exposure. Water temperature was maintained at 13°C ± 1°C during holding and experiments.

Zn acclimation

After the two weeks of holding, one of the two tanks was randomly designated as the chronic Zn exposure group (250 μg/L Zn). To the head tank of this group, flow from a Mariotte bottle of concentrated Zn solution ($ZnSO_4.7H_2O$, Anachemia with the addition of 1 ml concentrated HNO_3/L de-ionized water, trace metal analysis grade, BDH Chemicals) was started where it mixed with inflowing fresh water by vigorous aeration. Measured water Zn levels in the exposed group ranged from 204-268 μg/L (mean 244 μg/L; 3.76 μM). No mortalities were recorded during the Zn-acclimation period. Trout were exposed for at least 30 d before experiments began, and tests were performed over the subsequent 90 d period.

Elevated Zn exposure and toxicity testing

At the start of these continuous flow tests, 200 Zn-acclimated and 200 non-acclimated trout were removed and divided into six 18 L tanks (40 fish/tank), with each tank receiving a flow of 250 ml/min. Then, flow from a Mariotte bottle of concentrated Zn solution ($ZnSO_4.7H_2O$, with the addition of 1.0 ml concentrated HNO_3/L de-ionized water) was started into a head tank where it mixed with inflowing fresh water by vigorous aeration. At the same time, Zn was added to each tank (apart from the control tanks) to immediately bring them up to the chosen Zn concentration. Nominal concentrations were 0, 250, 700, 1200, 1900, and 2900 μg/L Zn. At 24, 48, 72 and 144 h, 7 fish from each tank were removed and sacrificed with a blow to the head. Their gills were removed, blotted dry and weighed to the nearest 0.001g. Mortalities were recorded over 144 h. Water samples were taken daily and acidified for later analysis of actual Zn concentration. Zn levels in the gills were determined by digestion in 5 volumes of 1 N

HNO_3 (trace metal analysis grade, BDH chemicals) for 24 h at 60°C. Samples were vortexed, allowed to settle for 24 h; 100 µl of supernatant was diluted to 1 ml with deionized water (Barnstead, NANOpure II) and analyzed by atomic absorption spectroscopy (AAS; Varian AA-1275, using an air/acetylene flame).

Surprisingly, there was insufficient mortality to determine 96 h or 144 h LC_{50} values, so a second 96 h LC50 test was performed three weeks later using a higher range of Zn concentrations. In this second exposure, groups of 8 fish from both groups were exposed to six Zn concentrations (0, 500, 1000, 2000, 3000, and 4500 µg/L Zn, nominal values) as in the first exposure for 96 h. Mortalities were recorded and the 96 h LC_{50}s ± 95% C.L. were calculated by log probit analysis of mortality *vs.* measured waterborne Zn concentration [22].

Long term ^{65}Zn exposure

After 30 days of exposure, 250 µg/L Zn-acclimated and non-acclimated trout were exposed to a range of radiolabelled ^{65}Zn levels for 72 h. Twenty-one 250 µg/L Zn-acclimated trout were placed in each of the 25 L tanks containing 50, 100, 150, 350, 850, 1250 or 1750 µg/L Zn (by the addition of $ZnSO_4.7H_2O$, Anachemia) plus 30 µCi ^{65}Zn per tank (as $ZnCl_2$, specific activity=1.97 mCi/mg, NEN Life Science Products, Boston, MA, USA), an amount which had negligible influence on the total Zn concentration of the water. Similarly, 21 non-acclimated trout were placed in each of the 25 L tanks containing 50, 100, 200, 400 or 800 µg/L Zn plus 30 µCi ^{65}Zn per tank. At the sampling times of approximately 24, 48 and 72 h, water samples were taken and 7 fish from each tank were quickly removed and rinsed in a bath for 1 minute containing a lethal amount

of anesthetic (0.5 g/L MS222) and 40 mg/L Zn to displace any ^{65}Zn loosely bound to the gill surface. After rinsing, the gills were excised, blotted dry and weighed to the nearest 0.001g. Gills were assayed for ^{65}Zn activity in a γ-counter (MINAXI γ Auto-Gamma 5000 Series, Canberra-Packard). Water samples were similarly assayed for ^{65}Zn activity, as well as for total Zn by atomic absorption spectrophotometry, so as to allow calculation of the specific activity of the waterborne Zn.

Short term ^{65}Zn exposure

A similar experiment was conducted over a shorter time period. Non-acclimated and 250 μg/L Zn-acclimated trout were exposed to a range of radiolabelled ^{65}Zn levels for 3 h. Twenty-one 250 μg/L Zn-acclimated trout were placed in each of five 15 L tanks containing 250, 500, 900, 1400 or 2100 μg/L Zn plus 15 μCi ^{65}Zn per tank. Similarly, 21 non-acclimated trout were placed in each of five 15 L tanks containing 75, 150, 250, 500 or 1000 μg/L Zn plus 15 μCi ^{65}Zn per tank. Trout were sampled as above at 0.5, 1.25, and 3 h. A final experiment was carried out as above with the trout sampled at 7.75 h.

Calculations and statistics

The K_d and B_{max} of the gill ^{65}Zn accumulation were calculated via a non-linear regression for line of best fit to a Michaelis-Menten equation, using SigmaPlot 4.0. Log *K* binding constants were calculated in the same manner, however, gill Zn accumulation was plotted against the calculated free Zn^{2+} ion concentration at each exposure. Based on measured water chemistry, the free Zn^{2+} ion concentrations in our test water were

approximately 60% of the total Zn concentrations using the aquatic geochemistry program MINEQL+ [17].

Data have been expressed as means ± standard error (N) except for the 96 h-LC_{50}s where means ± 95% confidence limits (C.L.) have been reported.

Results

Gill Zn levels

After 30 days, the gills of 250 μg/L Zn-exposed trout had similar total Zn levels to unexposed controls (68.0 ± 13.6 μg Zn/g gill and 72.0 ± 7.7 μg Zn/g gill for control and Zn-acclimated trout gills respectively, Fig.1) as determined by AAS. There was no detectable increase or trend in gill Zn levels when both control and Zn-acclimated fish were exposed to a range of water Zn concentrations (from 0 to 2900 μg/L Zn) for 7 d (Fig. 1A and 1B).

Gill ^{65}Zn accumulation

As fish were acutely exposed to ^{65}Zn at a variety of total water Zn concentrations for up to 3 h, accumulation was initially rapid, but began to level off by 3 h at the higher Zn exposure concentrations (Fig. 2A and 2B). ^{65}Zn accumulation was greater with increasing Zn exposure concentrations. When fish were exposed to a range of Zn concentrations and gills were sampled from 24 to 72 h, accumulation occurred in an approximately linear fashion over time (Fig. 3A and 3B). In our previous study [15], this linear accumulation was termed the slow turnover of Zn in the gills. Linear regression analyses against time demonstrated that the rate of ^{65}Zn appearance in the slow pool (i.e.

slope) was greater with increasing Zn exposure concentration (Table 1). However, at a given Zn exposure concentration, ^{65}Zn appearance in the slow pool was considerably slower in the Zn-acclimated fish than in the control fish (Table 1). By extrapolating back to 0 h (cf. Fig. 3A and 3B), the size of the fast turnover pool at different Zn exposure levels could be estimated [15]. This analysis indicated that the fast turnover pool appeared to be saturable in both control and Zn-acclimated fish (Fig. 4A and 4B). The absolute size of the fast turnover pool of Zn in the gills was clearly much larger in the Zn-acclimated trout. The maximum size of the fast pool was calculated to be 0.45 ± 0.16 μg Zn/g gill and 4.14 ± 0.91 μg Zn/g gill for control and Zn-acclimated fish respectively. In addition, affinity appeared to be lower (i.e. 16 fold higher K_d) in the Zn-acclimated trout, although the difference was not significant, reflecting variability in the data set.

^{65}Zn accumulation kinetics

At each sampling time from 0.5 h to 72 h, ^{65}Zn accumulation showed saturation kinetic trends (Figs. 5 and 6). Absolute values of B_{max} and K_d are tabulated in Table 2, while trends over time are summarized in Fig. 7A and 7B. B_{max} increased with time of ^{65}Zn exposure: the rate of increase was rapid at first, after which, it began to subside (Fig. 7A). The B_{max} of Zn-acclimated fish was always higher than that of unexposed controls. For both Zn-acclimated and unexposed fish, the K_d decreased rapidly from 0.5 h to 3 h, where it remained constant up to the last sampling time of 72 h. In all cases, K_d was greater (decreased affinity) in Zn-acclimated fish in comparison to unexposed controls.

Zn toxicity

Significant mortality occurred in the long term ^{65}Zn exposures. One-third of the control fish did not survive at the 400 μg/L Zn exposure, and all died at exposures greater than 400 μg/L Zn. All Zn-acclimated trout died at exposures greater than 1250 μg/L Zn.

However, classic 96 h LC_{50}s performed at a later date showed the trout to be much less sensitive. The LC_{50}s were 2615 μg/L Zn (2083-3326 μg/L, 95% C.L.) for control trout and was slightly higher for Zn-acclimated fish at 3340 μg/L Zn (2758-4141 μg/L, 95% C.L.), though this difference was not significant.

Discussion

Gill Zn levels

Acclimation to 250 μg/L Zn for at least 30 days had no effect on the total Zn levels of the gills, measured by atomic absorption spectrophotometry, compared to unexposed controls (~70 μg/g gill, Fig. 1). In addition, exposure of both Zn-exposed and unexposed groups to up to 2900 μg/L Zn for 7 d had no apparent effect on the gill Zn levels (Fig. 1). A similar result was found previously (Alsop et al. 1999) where juvenile rainbow trout were exposed to 150 μg/L Zn and 450 μg/L Zn in the same water chemistry as the present study and did not accumulate any Zn over 30 days. These data reconfirm the difficulties encountered when attempting to look at tissue accumulation of a nutrient metal already present in high concentrations in unexposed fish, and reinforce the conclusion that the use of radiotracer (^{65}Zn) is essential to detect gill Zn uptake.

Temporal patterns of gill ^{65}Zn accumulation

^{65}Zn appearance was rapid during the first 3 h of exposure (Fig. 2A and 2B). However, at the higher Zn exposure concentrations, the rate of appearance had begun to curtail by 3 h. This rapid appearance occurred in what has been termed the fast turnover pool of Zn in the gills [15]. In addition, from 24 h to 72 h, ^{65}Zn appearance in the gills was linear over time (Fig. 3A and 3B), and this appearance occurred in the slow turnover pool of Zn in the gill [15]. Within a treatment group, the loading rate into the slow turnover pool increased with increasing Zn exposure concentration. However, at a given Zn concentration, the loading rate into the slow turnover pool was consistently faster in control fish gills than 250 μg/L Zn-acclimated fish gills (Table 1). This is contrary to our first study [15] where we found the loading rate was faster in 250 μg/L Zn-acclimated fish than in control fish when both were exposed to 1125 μg/L Zn. The apparent discrepancy could be due to the fact that in Alsop et al. [15], only a single concentration was tested, while the present results at a range of concentrations (Table 1) indicate that the rates can be quite variable in Zn-acclimated fish, especially at higher concentrations.

When the linear regressions of the gill ^{65}Zn levels from 24 to 72 h are extrapolated back to 0 h, an estimate of the size of the fast turnover pool at different exposure concentrations can be determined [15]. With increasing Zn exposure concentration, the size of the fast pool increased, but could only expand to a limit, leveling off at higher concentrations (Fig. 4A and 4B). The maximum size of the fast pool estimated by this approach was approximately 9 times larger in the Zn-exposed trout than in the control trout (0.45 vs. 4.14 μg Zn/g gill). The maximum size of the fast pool amounted to 0.66% and 5.75% of the total Zn present in the gills of control and 250 μg/L Zn-acclimated fish respectively. These results confirm and extend our previous study

[15] where pool size measurements by this approach were made only at the Zn exposure concentration the fish had been acclimated, and not a range of concentrations as in the present study.

^{65}Zn accumulation kinetics

At every point in time the gills were sampled, the accumulation of ^{65}Zn showed saturation kinetics as a function of exposure concentration, and the affinity (K_d) and binding capacity (B_{max}) of the gill for Zn could be calculated (Fig. 5 and 6). B_{max} values determined by this approach up to 7.75 h exhibited good quantitative agreement with the maximum size of the fast pool by the linear regression extrapolation approach (Fig. 5) in the control group, and at least qualitative accord in the Zn-acclimated group (Table 2).

The K_ds decreased rapidly from 0.5 h to 3 h in both control and 250 μg/L Zn-acclimated trout, whereas from 3 h to 72 h they remained constant (Fig. 7B). It appears that at first, Zn binds to lower affinity sites at the gill while it takes more time for Zn to access and bind to the higher affinity sites (3 h). At times greater than 3 h, the K_ds have stabilized and Zn is continuously able to bind to a set of sites with higher affinities than those at the onset of the exposure.

Unlike K_d, the B_{max} increased continually with ^{65}Zn exposure time in both groups (Fig. 7A). The rapid increase at first would represent mainly ^{65}Zn appearance in the fast turnover pool, whereas from 24 h to 72 h, the slower increase would represent entry into the slow pool. The B_{max} (binding capacity) of Zn-acclimated fish was always greater than that of control fish (Fig. 7A). This is also seen in the size of the fast turnover pools of Zn in the gill (Fig. 4). In addition, Zn-acclimated fish exhibited a higher K_d (decreased

affinity) than control fish at all the time points that were tested (Fig. 7B), again in accord with the trends seen in the fast turnover pool analysis (Fig. 4).

Hogstrand et al. [23] similarly found a decrease in the branchial affinity for Zn transport (K_m) with sublethal Zn acclimation, which was apparent by the first day of exposure. Note that Hogstrand et al. [23] determined these Zn transport values by uptake of ^{65}Zn into the whole body over 24 h, whereas we determined Zn binding to the gills by branchial accumulation of ^{65}Zn from 0.5 to 72 h. The absolute values of Hogstrand et al. [23] for K_m were very close to the long term (i.e. 24-72 h) K_d values for Zn gill binding in the present study: control fish ~350 μg/L Zn [23] *vs* ~280 μg/L Zn (present study); 150 μg/L Zn-acclimated fish ~700 μg/L Zn [23] *vs*. 250 μg/L Zn-acclimated fish ~550 μg/L Zn (present study). Our branchial K_d is also in good agreement with Spry and Wood [24] who found the K_m for Zn transport in control fish to be 240 μg/L Zn. The average K_d determined by Galvez et al. [21], with 3 h exposures in the same manner as the present study, was more than 2-fold greater (650 μg/L Zn) than our measurement (270 μg/L Zn). However, the K_d value in Galvez et al. [21] varied greatly over the 15 d period during which the measurements were made. In addition, Galvez et al. [21] found that acclimation to 150 μg/L Zn had no effect on the K_d or B_{max}, contrary to Hogstrand et al. [23] and the present study. All studies were performed in the same water quality (dechlorinated Hamilton tapwater from Lake Ontario).

The toxic action of Zn is a specific disruption of branchial calcium uptake [5-7], which leads to hypocalcemia. It has been proposed that the changes in binding affinity (increased K_m or K_d) that occur with Zn exposure, are adaptive to help minimize Zn uptake while protecting calcium uptake [23]. The increase of B_{max} (binding capacity) that

occurs with Zn exposure may be due to the induction of detoxification or temporary storage mechanisms in the gills such as Zn-binding proteins (e.g. metallothionein, MT). MT levels in the gills of 150 μg/L Zn-exposed trout were elevated after 30 d of exposure in one study [6], though MT was not elevated after 60 d of exposure to the same Zn concentration in another study [25].

Zn kinetics and toxicity

Although the gill accumulation characteristics were different between control and 250 μg/L Zn-exposed trout, there was no significant difference between their respective LC_{50}s. However, the sensitivity to Zn appears to change with time. In the first test (which also yielded the data of Fig. 1), resistance was high in both groups (survived up to 2900 μg/L Zn for 144 h) and 96 h LC_{50}s could not be determined. In the second test three weeks later, both 96 h LC_{50}s were in the range of 3000 μg/L Zn, though slightly higher in the 250 μg/L Zn-acclimated trout. However, the same batch of fish appeared to be much more sensitive to Zn during the long term ^{65}Zn exposures where control fish died at exposures greater than 400 μg/L Zn, and Zn-acclimated fish died at exposures greater than 1250 μg/L Zn, perhaps suggesting the fish had acquired increased tolerance to Zn. The absolute levels of the 96 h LC_{50}s in the present study (~3000 μg/L Zn) were relatively high in comparison to our previous work when toxicological acclimation had occurred (869 μg/L Zn for control trout *vs.* ~2200 μg/L Zn, Zn-exposed; [15]). It appears that fish may not show increased tolerance with sublethal exposure at times of low sensitivity to Zn. Sensitivity and toxicological acclimation to Zn may be lower during

periods of reduced Ca^{2+} uptake in the cycles described by Wagner et al. [26], and greater during periods of high Ca^{2+} uptake.

Previous studies in our lab [15] have shown that rainbow trout exhibited toxicological acclimation when chronically exposed to Zn levels of either 150 μg/L Zn or 450 μg/L Zn for 30 days in the same water chemistry as the present study (96 h LC_{50}s increased by 2.5 fold). The 2.5-fold increase in tolerance is very similar to the 2.1-fold increase in K_d (decrease in affinity) determined in the present study. This decrease in affinity for Zn may be the basis of waterborne Zn acclimation. Alternatively or additionally, the larger increase in maximum binding capacity (B_{max}) may be involved as a detoxification mechanism.

Implications for the Biotic Ligand Model

The gill receptor loading model is one form of the Biotic Ligand Model (BLM) and has been successfully employed to predict metal levels in the gills of fish, using experimentally determined binding constants (affinities and capacities) of the gill for the metal and for other ions [13]. This method has worked for metals such as copper and cadmium [10,14,18], silver [19] and cobalt [20].

The binding constants determined by Playle and coauthors for the metals mentioned above are summarized in Table 4, and were derived based on 2-3 h exposures to a range of metal concentrations. Playle et al. [10] concluded that this was an appropriate time, based on cadmium levels in the gills of minnows having saturated by 2.5 h during exposure to a single concentration of cadmium. The present study, however,

is the first to critically assess the influence of exposure time on the gill binding kinetics (K_d and B_{max}) of a heavy metal (i.e. at a range of concentrations).

It appears that with Zn, a 3 h exposure would be the minimum time required to obtain a stable K_d and hence log *K* (Table 2 and 3). Our stable log *K* (>3 h) for Zn binding to control trout gills of 5.6 is slightly higher than the log *K* of Galvez et al. [21] who determined a value of 5.1 using a 3 h exposure. It is also similar to that of Cusimano et al. [27] who determined a theoretical gill binding constant of 5.4 based on 96 h and 168 h Zn LC_{50} studies rather than gill metal accumulation. Although acclimation to 250 μg/L Zn increased the stable K_d more than 2-fold (i.e. decreased affinity; Table 2), our stable log *K* value only dropped from 5.6 to 5.3, reflecting the fact that log *K* values are expressed on a logarithmic scale. Again, the decrease in affinity of the gill for Zn may be a basis of the reduced toxicity of acute Zn exposure to pre-exposed fish.

The BLM assumes that there are a set number of binding sites at the gill. Depending on the time of Zn exposure, this number can change greatly. For example, at 0.5 h, B_{max} was 0.37 μg Zn/g gill, or 5.7 nmol Zn binding sites/g gill (Table 2). By 72 h, this number had grown to 8.63 μg Zn/g gill or 132.0 nmol Zn binding sites/g gill, an increase of 23 fold. In addition, prior Zn exposure increased the number of Zn binding sites in comparison to control, at every time point (Table 2; Fig. 7A). The difference was more apparent at the shorter Zn exposure times; Zn-acclimation increased the number of binding sites by 150% when the gills were sampled at 1.25 h, while at 72 h, the number of sites were increased only 35%. Our 3 h binding site number for control trout gills (8.3 nmol/g gill; Table 2) is similar to that of Galvez et al. [21] who reported 8.6 nmol/g gill at 3 h.

The reported binding constants (log *K*) and number of binding sites (B_{max}) of five different heavy metals binding to fish gills are compiled in Table 4; all values were determined with 2-3 h exposures. In addition, acute LC_{50} concentrations determined in previous studies in our lab are shown. Consistent with chronic prior exposure reducing the affinity and toxicity of Zn, it is obvious that those metals which have a greater affinity for fish gills, are also those which are much more toxic. At first glance, the toxicity data are less consistent with the number of metal binding sites in the gills. However, the number of binding sites appears to decrease with metals of greater toxicity when they are grouped by mechanism of toxic action. For example, both silver and copper disrupt Na^+ uptake [28,29], but silver is more toxic and has fewer binding sites. Similarly, cadmium [30], zinc [6], and cobalt [31] all disrupt Ca^{2+} uptake. Cadmium is the most toxic and has the fewest binding sites whereas cobalt is least toxic and has the greatest number of binding sites. Thus both the affinity (log *K*) and capacity (B_{max}) of the gill to bind metals appear to be directly related to metal toxicity. The challenge of future research is to determine the molecular basis of these differences.

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Table 4-1. ^{65}Zn loading rates into the slow turnover pool of Zn in the rainbow trout gill. Values are given as means ± S.E. N=18-21, except for control 400 μg/L Zn where N=12.

Control: Zn exposure level	^{65}Zn appearance (μg Zn/g gill/h)	Zn-acclimated: Zn exposure level	^{65}Zn appearance (μg Zn/g gill/h)
50 μg/L	0.0224 ± 0.002	50 μg/L	0.0064 ± 0.002
100 μg/L	0.0361 ± 0.006	100 μg/L	0.0095 ± 0.003
200 μg/L	0.0542 ± 0.008	150 μg/L	0.0351 ± 0.005
400 μg/L	0.0932 ± 0.024	350 μg/L	0.0211 ± 0.005
		850 μg/L	0.0849 ± 0.019
		1250 μg/L	0.0297 ± 0.017

Table 4-2. Affinity (K_d; expressed in terms of total water Zn concentrations) and binding capacity (B_{max}) of Zn in rainbow trout gills over 72 h. Values are means ± SEM. For 0.5 to 7.75 h N=35. For control trout at 24 and 48 h N=28 and at 72 h N=21. For Zn-acclimated trout at 24 and 48 h N=42 and at 72 h N=37.

	Control		250 µg/L Zn-acclimated	
Time (h)	K_d (µg/L Zn)	B_{max} (µg Zn/g gill)	K_d (µg/L Zn)	B_{max} (µg Zn/g gill)
0.5	939.9 ± 296.2	0.37 ± 0.07	1040.7 ± 292.3	0.70 ± 0.09
1.25	611.9 ± 130.3	0.48 ± 0.07	1049.8 ± 212.6	1.21 ± 0.12
3	309.9 ± 68.4	0.54 ± 0.05	563.1 ± 125.6	1.57 ± 0.13
7.75	301.1 ± 110	0.64 ± 0.09	448.0 ± 108.2	1.52 ± 0.12
24	297.0 ± 141.8	4.51 ± 1.17	735.8 ± 125.7	7.03 ± 0.59
48	289.9 ± 78.6	8.29 ± 1.20	541.8 ± 148.2	9.28 ± 1.11
72	205.3 ± 59.3	8.63 ± 1.51	579.8 ± 199.0	11.61 ± 1.92

Table 4-3. Log *K* binding constants for Zn in control and 250 μg/L Zn-acclimated rainbow trout gills over 72 h. Values are calculated as the free Zn ion (Zn^{2+}) concentrations. See text for details.

Time (h)	Control log *K*	Zn-acclimated log *K*
0.5	5.06	5.02
1.25	5.25	5.02
3	5.55	5.29
7.75	5.54	5.38
24	5.56	5.17
48	5.57	5.30
72	5.72	5.21

Table 4-4. Binding constants (log *K*) and number of binding sites (B_{max}) for five heavy metals binding to control (previously unexposed) fish gills. All experiments were conducted in a similar manner with 2-3 h metal exposures. In addition, approximate LC_{50} values (96-144 h) determined previously with juvenile rainbow trout in our laboratory are reported, which were all performed in the same water chemistry. The cobalt LC_{50}, however, is estimated based on toxicity tests performed by Diamond et al. [34] in water of similar chemistry.

Metal	log *K*	Binding sites (B_{max}; nmol/g gill)	LC_{50} in Hamilton tapwater (μM)
Silver	10.0 [19]	6.1 [19]	0.083 [32]
Cadmium	8.6 [10]	2 [10]	0.196 [14]
Copper	7.4 [10]	30 [10]	1.10 [33]
Zinc	5.6 *	8.3 *	13.29 [15]
Cobalt	5.1 [20]	88 [20]	> 50 [34]

*Present study

Figure 4-1. Total Zn levels in the gills measured by atomic absorption spectrophotometry of A) control and B) 250 μg/L Zn-acclimated trout over 7 days of exposure to a range of Zn concentrations. Note the large amount of Zn in the gills prior to exposure even in control fish, and the similarity in gill Zn levels between control trout and trout that had been chronically exposed to 250 μg/L Zn for at least 30 days. Values expressed as means ± SEM. (N=7).

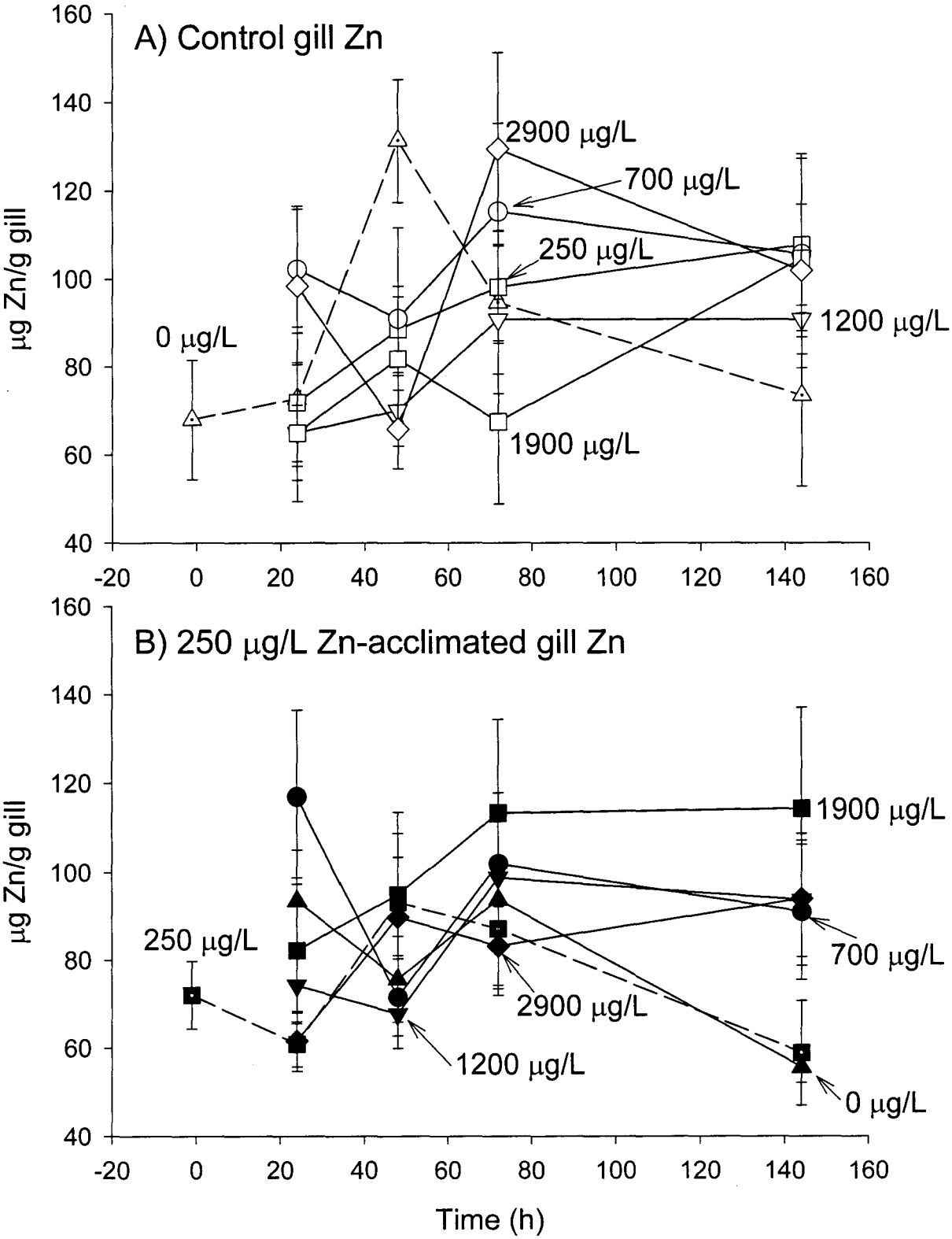

A) Control gill Zn
μg Zn/g gill
2900 μg/L
700 μg/L
250 μg/L
0 μg/L
1200 μg/L
1900 μg/L
B) 250 μg/L Zn-acclimated gill Zn
μg Zn/g gill
1900 μg/L
250 μg/L
700 μg/L
2900 μg/L
1200 μg/L
0 μg/L
Time (h)

Figure 4-2. Radioisotopically (^{65}Zn) determined Zn accumulation in the gills of A) control and B) 250 μg/L Zn-acclimated trout exposed to a range of Zn concentrations up to 3 h. Note the 3-fold difference in y-axis scales between panels A and B. Values expressed as means ± SEM. (N=7).

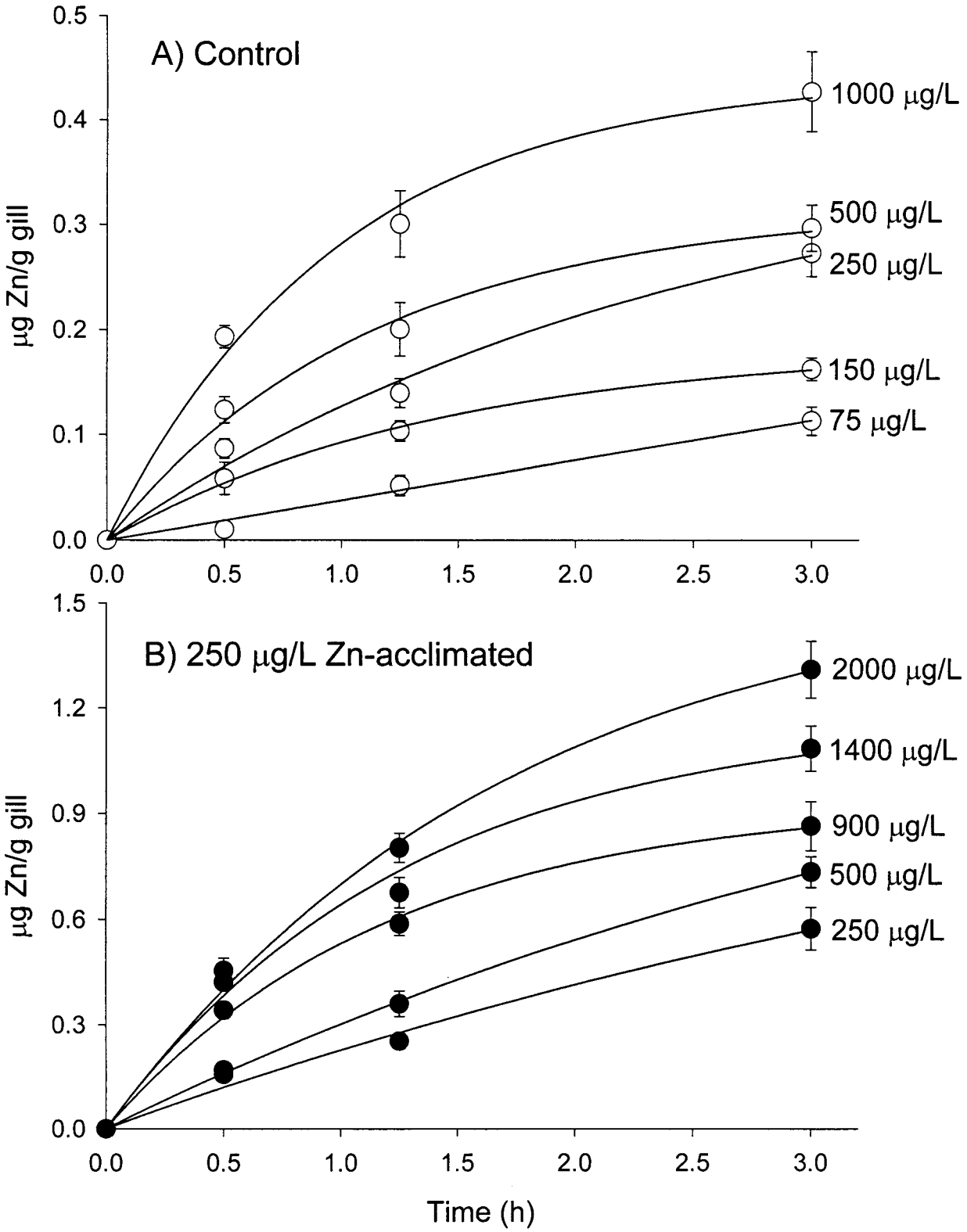

A) Control
1000 µg/L
500 µg/L
250 µg/L
150 µg/L
75 µg/L
µg Zn/g gill
0.0
0.1
0.2
0.3
0.4
0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
B) 250 µg/L Zn-acclimated
2000 µg/L
1400 µg/L
900 µg/L
500 µg/L
250 µg/L
µg Zn/g gill
0.0
0.3
0.6
0.9
1.2
1.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (h)

Figure 4-3. Radioisotopically (^{65}Zn) determined Zn accumulation in the gills of A) control and B) 250 μg/L Zn-acclimated trout exposed to a range of Zn concentrations up to 72 h. Note the 2-fold difference in y-axis scales between panels A and B. Values expressed as means ± SEM. (N=6-7). The extrapolations of the linear regressions to 0 h provide estimates of the sizes of the fast turnover pool of Zn in the gills (y-axis intercept), which are displayed in Fig. 4-4.

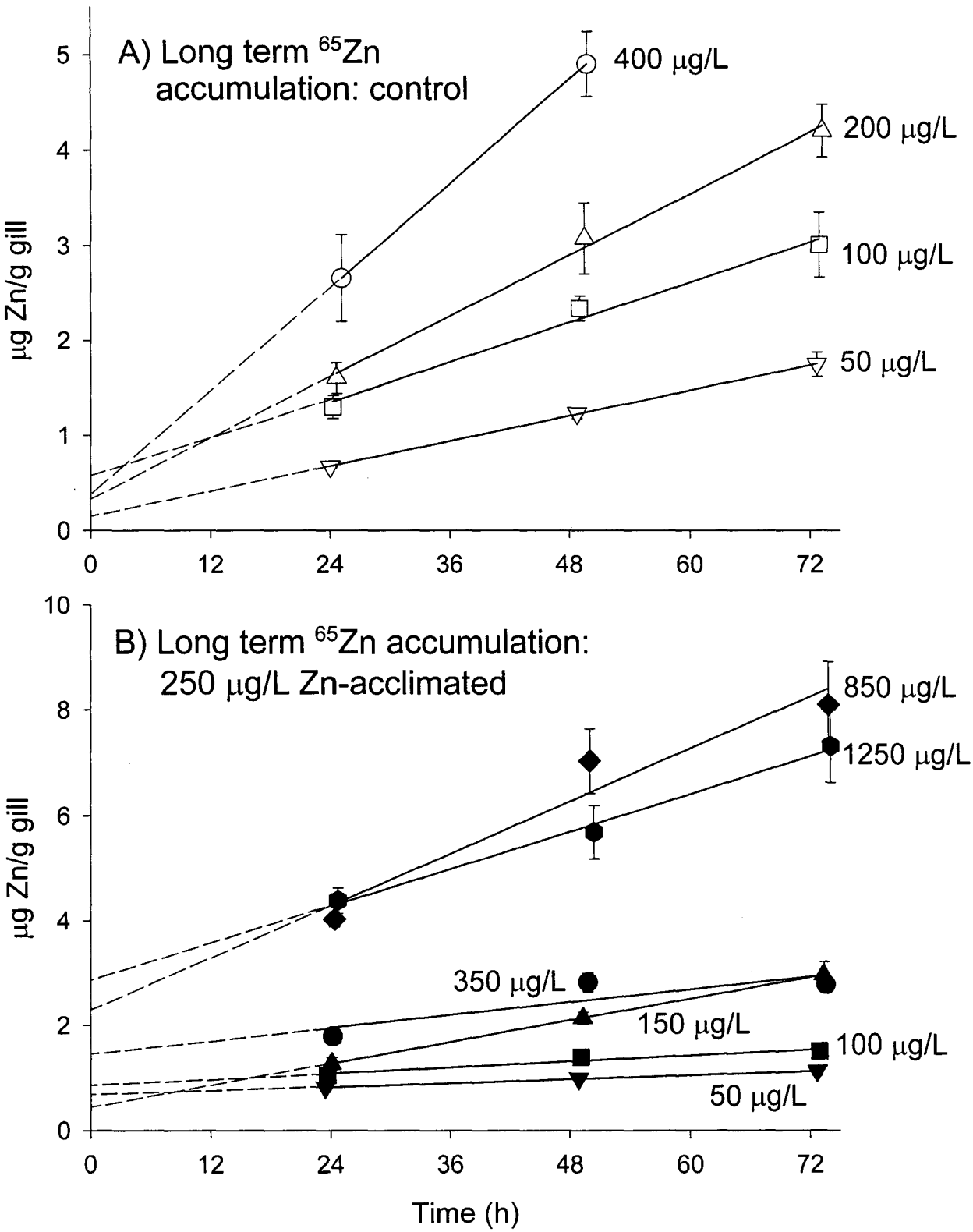

A) Long term 65Zn accumulation: control
400 µg/L
200 µg/L
100 µg/L
50 µg/L
µg Zn/g gill
0
1
2
3
4
5
0
12
24
36
48
60
72
B) Long term 65Zn accumulation: 250 µg/L Zn-acclimated
850 µg/L
1250 µg/L
350 µg/L
150 µg/L
100 µg/L
50 µg/L
µg Zn/g gill
0
2
4
6
8
10
0
12
24
36
48
60
72
Time (h)

Figure 4-4. The calculated sizes of the fast turnover Zn pools as a function of water Zn concentration in the gills of A) control and B) 250 μg/L Zn-acclimated trout over a range of waterborne Zn concentrations. Note the difference in axis scales. Values expressed as means ± SEM. Values were determined by the extrapolation of the linear regressions in Fig. 4-3 to 0 h (y-axis intercept). The K_d and B_{max} were calculated via a non-linear regression for line of best fit to a Michaelis-Menten equation.

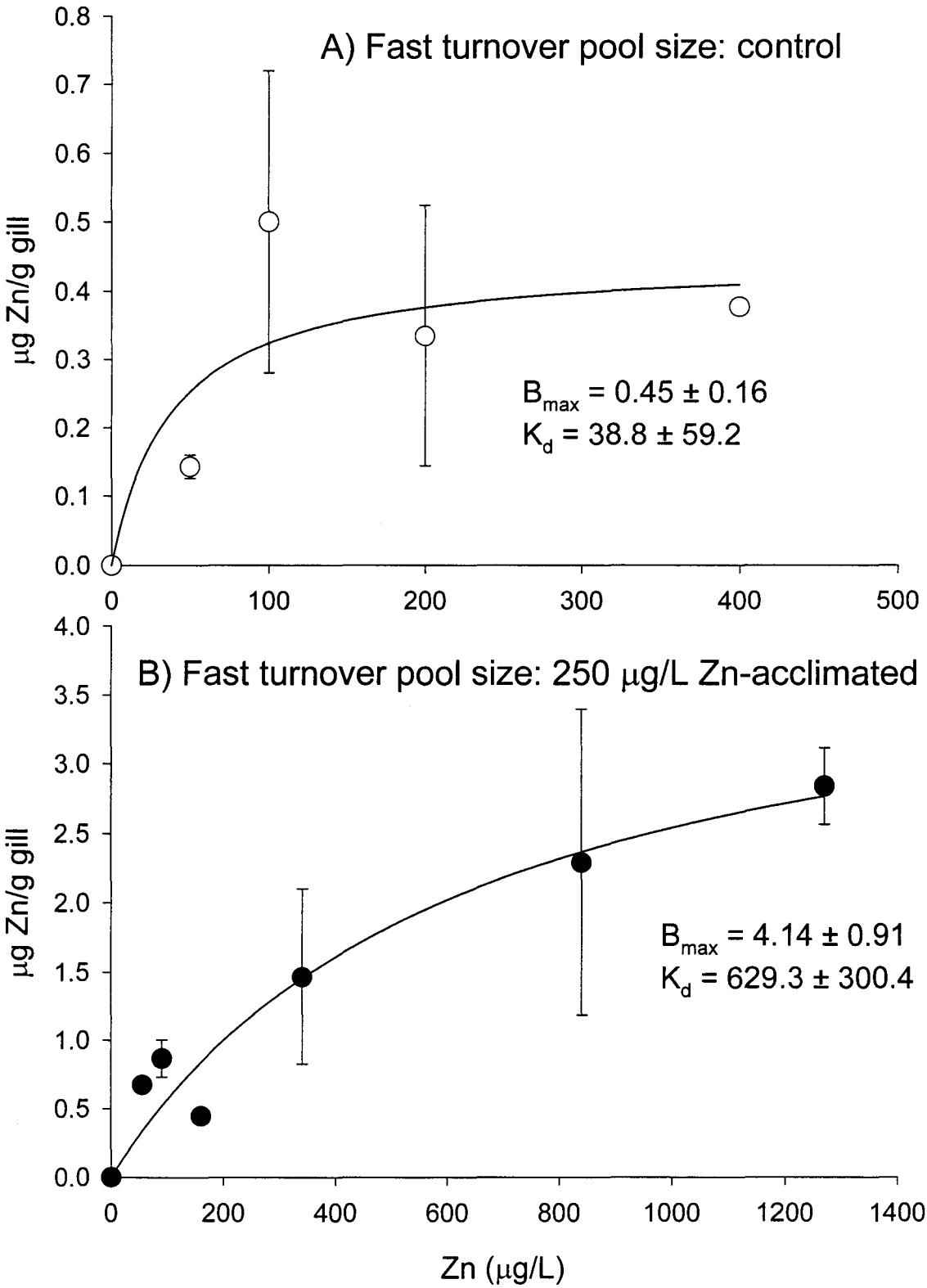

A) Fast turnover pool size: control
μg Zn/g gill
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
B_{max} = 0.45 ± 0.16
K_d = 38.8 ± 59.2
B) Fast turnover pool size: 250 μg/L Zn-acclimated
μg Zn/g gill
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
1200
1400
Zn (μg/L)
B_{max} = 4.14 ± 0.91
K_d = 629.3 ± 300.4

Figure 4-5. A kinetic analysis of radioisotopically (^{65}Zn) determined Zn accumulation in the gills of A) control and B) 250 μg/L Zn-acclimated trout exposed to a range of Zn concentrations for 0.5 h (upside-down triangles), 1.25 h (squares), 3 h (circles) and 7.75 h (triangles). Values expressed as means ± SEM. (N=7). Note the differences in scales of the axis scales between panels A and B. The K_d and B_{max} of the gill ^{65}Zn accumulation were calculated via a non-linear regression for line of best fit to a Michaelis-Menten equation.

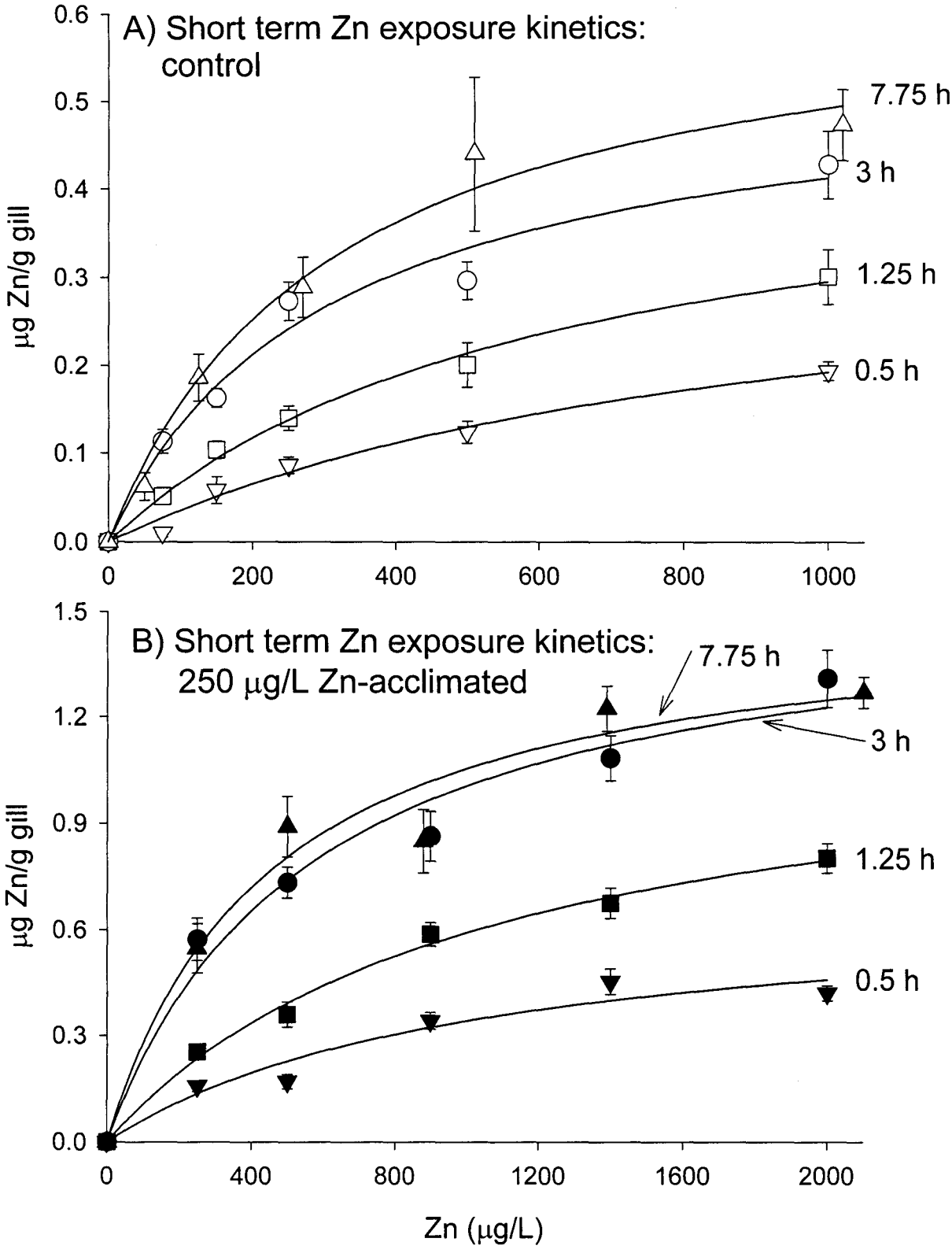

A) Short term Zn exposure kinetics:
control
7.75 h
3 h
1.25 h
0.5 h
µg Zn/g gill
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
B) Short term Zn exposure kinetics:
250 µg/L Zn-acclimated
7.75 h
3 h
1.25 h
0.5 h
µg Zn/g gill
1.5
1.2
0.9
0.6
0.3
0.0
0
400
800
1200
1600
2000
Zn (µg/L)

Figure 4-6. A kinetic analysis of radioisotopically (^{65}Zn) determined Zn accumulation in the gills of A) control and B) 250 μg/L Zn-acclimated trout exposed to a range of Zn concentrations at 24 h, 48 h and 72 h. Values expressed as means ± SEM. (N=6-7). Note the differences in scales of the axis scales between panels A and B. The K_d and B_{max} of the gill ^{65}Zn accumulation were calculated via a non-linear regression for line of best fit to a Michaelis-Menten equation.

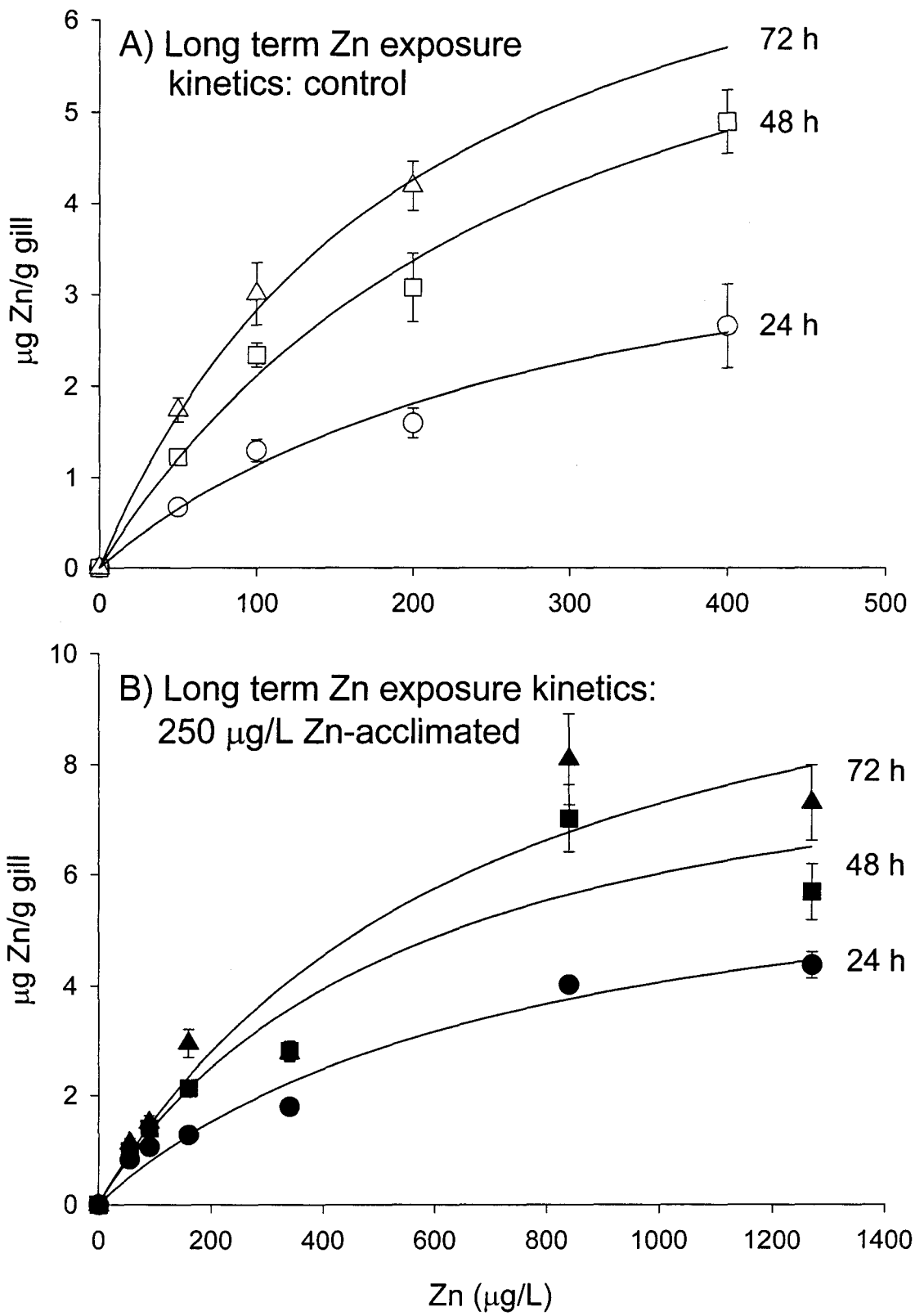
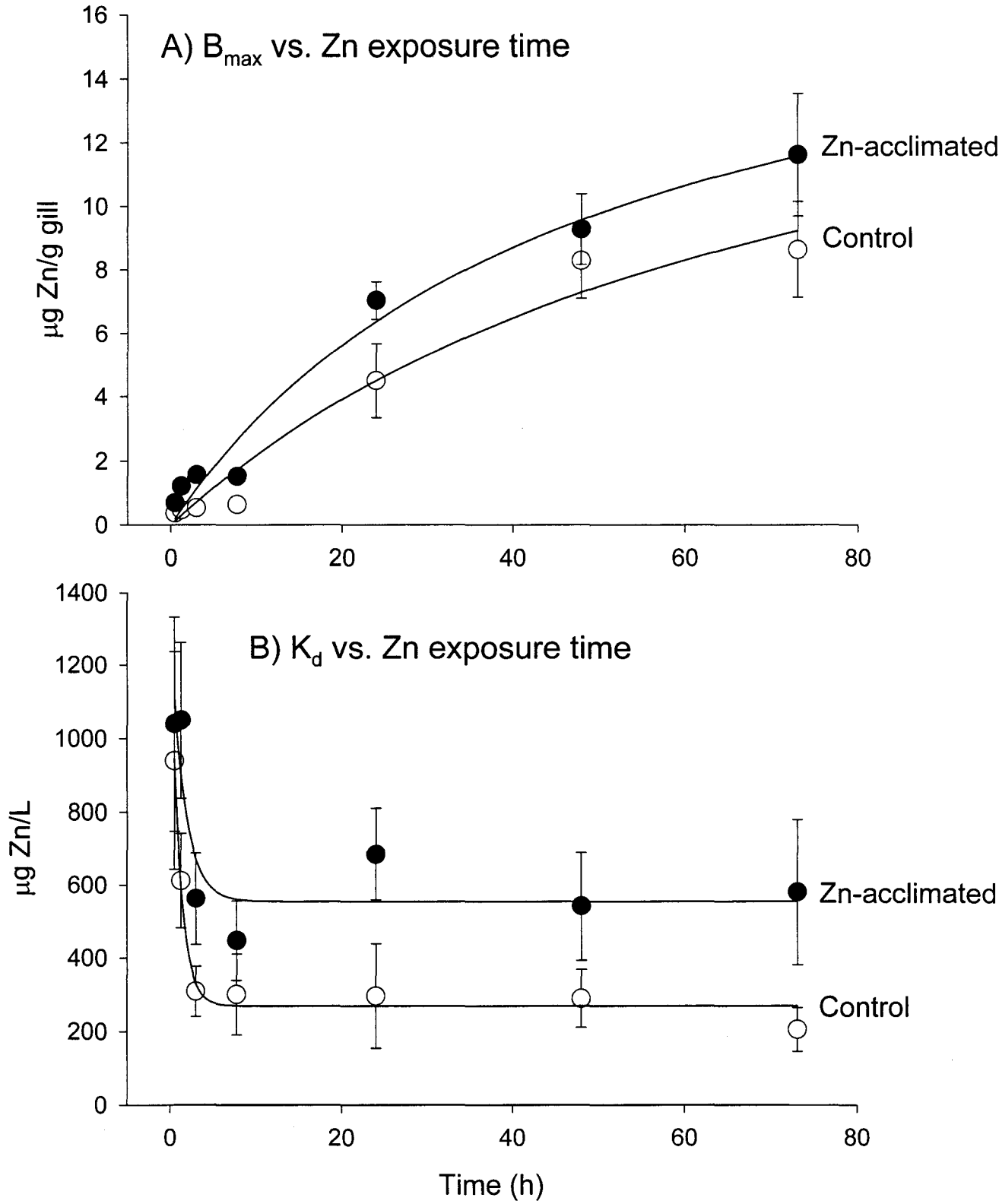

A) Long term Zn exposure kinetics: control
72 h
48 h
24 h
µg Zn/g gill
0
1
2
3
4
5
6
0
100
200
300
400
500
B) Long term Zn exposure kinetics: 250 µg/L Zn-acclimated
72 h
48 h
24 h
µg Zn/g gill
0
2
4
6
8
10
0
200
400
600
800
1000
1200
1400
Zn (µg/L)

Figure 4-7. A) B_{max} (binding capacity) and B) K_d (affinity) of radioisotopically (^{65}Zn) determined Zn accumulation in the gills of control (open circles) and 250 μg/L Zn-acclimated trout (filled circles) up to 72 h.


A) B_{max} vs. Zn exposure time
µg Zn/g gill
Zn-acclimated
Control
B) K_d vs. Zn exposure time
µg Zn/L
Zn-acclimated
Control
Time (h)

Summary

- There appeared to be few consequences of chronic zinc exposure. Acute resistance (acclimation) increased 2.2-3.9 fold and critical swimming speed was reduced, but there was no effect on growth, metabolic rate, tissue zinc, sodium, or calcium levels, or sprint swimming performance.

- There were at least two pools of zinc in the trout gills, a fast and a slow turnover pool. The size of the fast pool increases with acclimation to elevated waterborne zinc and is larger in softwater than hardwater at a given zinc exposure concentration.

- Water hardness influenced the few chronic effects of waterborne zinc exposure. The toxicological acclimatory response was greater in soft water than in hard water at similar zinc exposure concentrations. In addition, the size of the fast turnover pool of zinc in the gills was greater in soft water than in hard water, and the loading rate into the slow pool was also much faster in soft water at similar zinc exposures. Finally, there was some chronic mortality during exposure to 120 μg/L in soft water, while there were no chronic mortalities recorded during exposure to 150 μg/L in hard water.

- Branchial zinc uptake was greatly reduced by a variety of positively charged, waterborne ions such as Na^+ and Mg^{2+}. The magnitude of the reduction was directly related to the concentration of positive charges, regardless of which element or compound carried that charge.

- However, Ca^{2+} was more potent in its reduction of zinc uptake, and was the only cation tested that was able to ameliorate acute zinc toxicity, through restoring Ca^{2+} uptake that was disturbed by the zinc exposure.

- The affinity of the gill for zinc increased rapidly (K_d decreased) during the first 3 h of exposure in both control and Zn-acclimated fish. However, the K_d was stable from 3 to 72 h. The number of zinc binding sites in the gills changed dramatically with time of exposure and was always greater in zinc-acclimated fish.

- A stable log *K* binding constant of 5.6 was determined for control rainbow trout gills. Affinity for zinc decreased with acclimation to 250 μg/L Zn, as seen in the decrease in binding constant to 5.3.