INTERNAL BIOAVAILABILITY OF ZINC IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FOLLOWING CHRONIC WATERBORNE AND DIETARY EXPOSURE

BY

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ABSTRACT

The principal objective of this thesis was to illuminate the internal bioavailability and the nature of interactions between the branchial and gastrointestinal routes of Zn uptake in rainbow trout (*Oncorhynchus mykiss*). A comprehensive analysis of tissue Zn distribution following waterborne and dietary Zn exposure, singly and in combination, was carried out. We demonstrate differences in Zn accumulation, at the tissue and subcellular levels, and distribution patterns, with the majority of the accumulated Zn being metabolically active (i.e. bioavailable). At the tissue level the carcass acted as the main sink for Zn containing 84-90% of whole-body Zn, with the intestine (3-7%) and gill (4-6%) holding the majority of the remainder. The estimated metabolically active pools were: gill 81-90%; liver 65-78%, and intestine 59-75%. Interestingly, in the two primary tissues of Zn uptake (gill and intestine) the nuclei-cellular debris fraction bound the highest amounts of Zn while in the liver heat-stable proteins were dominant. A shift in Zn distribution between the metabolically active and detoxified pools with Zn exposure was most prominent in the intestine in which the proportion of detoxified Zn increased from 25 to 41%. Interactions between the branchial and gastrointestinal uptake pathways were complex and appeared to be tissue-specific. In plasma, blood cells, and gill, uptake from water drove the Zn accumulation, while uptake from food drove the accumulation of Zn in the intestine. Both pathways appeared to contribute equally to Zn accumulation in the liver, carcass, and kidney. At the subcellular level there was minimal evidence of cross-talk between waterborne and gastrointestinal pathways. Only the branchial heat-stable, hepatic heat-denaturable, and intestinal microsomes-lysosomes fractions showed some evidence of cross-talk. We also found that the intestine was the main site for dietary Zn detoxification, while the gill had a similar role for waterborne Zn. Finally, the mitochondrial fraction was identified as a potential common target site of Zn accumulation independent of tissue and route of exposure. Overall the Zn exposure regimes employed in this research caused minimal toxicity manifested by a transient inhibition of protein synthesis in the waterborne exposure. We concluded that Zn bioaccumulation is very highly regulated in rainbow trout.

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hukam rajaa-ee chalnaa naanak likhi-aa naal.

O Nanak, it is written that you shall obey the Hukam of His Command, and walk in the Way of His Will.

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% – percentage

AA – amino acid

AAT– amino acid transporter

ANOVA – analysis of variance

ATP – adenosine triphosphate

ATPase – class of enzymes that catalyze the decomposition of adenosine triphosphate

AVC – Atlantic Veterinary College

BSA – bovine serum albumin

Bwt – body weight

Ca – calcium

Ca^{2+} ATPase – calcium adenosine triphosphatase

$CaCO_3$ – calcium carbonate

CBR – critical body residue

Cd – cadmium

CRIP – cysteine rich intestinal protein

Cl – chloride

Cu – copper

DNA – deoxyribonucleic acid

DTT – dithiothreitol

ECaC – epithelial calcium channel

FAAS – flame atomic absorption spectroscopy

FIAM- free ion activity model

FRET – fluorescence resonance energy transfer

g – gram/gravitational force

G – guage

GI – gastrointestinal

H^+ – hydrogen ion

Hg – mercury

h – hour

HDP – heat-denaturable protein

HNO_3 – nitric acid

H_2O_2 – hydrogen peroxide

HPLC – high performance liquid chromatography

HSP – heat-stable protein

ICP– MS – inductively coupled plasma mass spectrometry

i.u. – international unit

kg – kilogram

l – litre

LDH – lactate dehydrogenase

Li – ligand

M – molar

m – meter

mM – millimolar

mg – milligram

min – minute

ml – millilitre

mm^3 – cubic millimetre

Mg – magnesium

MAP – metabolically active pool

MDP – metabolically detoxified pool

MT – metallothionein

N – normal

Na – sodium

Na^+ K^+ ATPase – sodium-potassium adenosine triphosphatase

NaOH – sodium hydroxide

ng – nanogram

nm – nanometre

P – phosphorus

pCO_2 – partial pressure of carbon dioxide

pH – potential of hydrogen or negative log of hydrogen ion concentration

ppm – parts per million

PBS – phosphate buffer saline

PMSF – phenylmethylsulfonyl fluoride

RNA – ribonucleic acid

sec – second

SEM – standard error of the mean

TMDA – trace metal direct aspiration

µg – microgram

µl – microlitre

µmole – micromole

WHAM – Windermere humic-aqueous model

ZIP – Zrt, Itr-like protein

ZnT – zinc transporter

Zn – zinc

Zn-Pr – zinc proteins

$ZnCl_2$ – zinc chloride

$ZnCO_3$ – zinc carbonate

ZnO – zinc oxide

ZnS – zinc sulphide

$ZnSO_4$ – zinc sulphate

ZTL – zinc-regulated zinc transporter

1.0 INTRODUCTION

1.1 Overview and scope of the thesis

Zinc (Zn) is an essential trace metal required for the normal metabolism and physiology in fish but it becomes toxic when accumulated in excess (Du Preez and Van Vuren 1994; Hogstrand and Wood 1996; Hogstrand *et al.* 1996; Glover and Hogstrand 2003). As a result, fish have evolved mechanisms to maintain optimal levels (homeostasis) in order to meet the needs for normal metabolism and physiology without toxicity. These mechanisms may involve regulating influx and efflux at the uptake sites, excretion, and sequestration by metal binding proteins e.g. metallothionein (MT) and glutathione (Masons and Jenkins 1995; Wallace *et al.* 2003; Giguere *et al.* 2006). Due in part to the sequestration mechanisms, not all the Zn present within fish tissues contributes to metabolic needs or toxicity. Therefore the key to the understanding of Zn homeostasis and toxicity in fish is the characterization of internal bioavailability or subcellular compartmentalization of accumulated Zn burden. A second feature that further complicates the understanding of Zn homeostasis and toxicity in fish is the presence of multiple routes of uptake. There is currently limited data on the homeostatic and toxicological interactions resulting from multiple routes of metal uptake in fish. Interactions between waterborne and dietary copper (Cu) uptake studied by Kamunde *et al.* (2001, 2002) in rainbow trout (*Oncorhynchus mykiss)* provided the first evidence that homeostatic interaction occurs between the two routes of metal uptake. The objective of this research was to address two issues that are central to the understanding of Zn homeostasis and toxicity in fish. Specifically, juvenile rainbow trout were chronically exposed to waterborne and dietary Zn, alone and in combination, and the internal

speciation (bioavailability) of Zn in key target tissues, as well as the cross-talk between waterborne and dietary pathways of uptake, was assessed.

1.2 Occurrence, sources, transport and properties of Zn

Zinc is fairly abundant in nature being dispersed extensively in all environmental compartments including air, soil and water (Kelly 1988; Li and Robinson 1996; Sanders *et al.* 1999). Soluble Zn salts like $ZnCl_2$ and $ZnSO_4$ or insoluble precipitates of $ZnCO_3$, ZnO and ZnS are found commonly in industrial wastes. Zinc in nature can be found associated with other metals of which iron (Fe) and cadmium (Cd) are the most common (Dallas and Day 1993). The release of Zn into the environment occurs by natural (e.g. weathering) and anthropogenic (e.g. mining, steel production and burning of waste) activities. Upon release into the environment, Zn is attached to dust particles in the air and may be transported long distances by wind before settling to the ground by atmospheric fallout. Although the greater proportion of Zn remains bound to soil and sediment particles, some Zn eventually moves into ground water, lakes, streams, and rivers where it combines with other inorganic or organic matter. In aquatic ecosystems Zn occurs in two oxidative states, namely metallic and ionic forms. The aquatic biota are potentially at risk from both the waterborne and sediment-borne Zn.

Anthropogenic activities mainly contribute to increased Zn levels in inland waters whereas leaching from rocks and other natural processes contribute relatively smaller amounts (Birch *et al.* 1996; Sanders *et al.* 1999). Due to its chemical and metallurgical properties, Zn is generally used in industrial and mining processes (Holcombe *et al.*

1979; Hellawell 1986) and therefore becomes a common component of mining effluents (Holcombe *et al.* 1979) that ultimately end up in aquatic ecosystems. Within the aquatic environment, the majority of the Zn settles at the bottom of water bodies and a relatively small amount remains either dissolved in water or as fine suspended particles (Kelly 1988; Sanders *et al.* 1999). Zinc, like all metals, is not degradable and is therefore highly persistent in the environment. The measured background Zn concentrations in air, freshwater, seawater, sediments and soil are 300 ng m^{-3}, 20 μg l^{-1}, 10-600 ng l^{-1}, up to 100 mg kg^{-1} dry weight and 10–300 mg kg^{-1} dry weight, respectively. Increased total Zn concentrations in freshwater, estuarine waters and soil mainly due to anthropogenic contamination have been reported to be up to 150, 20-30,000 μg l^{-1} and 35000 mg kg^{-1} dry weight, respectively (Handy 1996).

1.3 Essentiality of Zn for normal physiology of fish

Zinc is essential for normal physiology in fish (Watanabe *et al.* 1997) and is a required supplement in the nutrition of aquaculture fish (Li and Robinson 1996) for normal growth and development. For example, channel catfish (*Ictalurus punctatus)* require 15-90 mg kg^{-1} supplemental Zn in the diet for maximum Zn deposition in bones to promote optimum growth (Li and Robinson 1996) while Atlantic salmon (*Salmo salar*) require 37-67 mg Zn kg^{-1} dry diet to maintain the normal range of whole-body and serum Zn concentrations (Maage and Julshamn 1993). Zinc is a component of more than 1000 proteins including >300 enzymes important for normal physiology (Berg and Shi 1996; Maret 2001, 2003). These enzymes include alkaline phosphatase, carbonic anhydrase, alcohol dehydrogenase, superoxide dismutase (Rainbow 2002), carboxypeptidase and

several hydrogenases (Malik *et al.* 1998). Table 1 shows representative Zn metalloproteins and their physiological functions.

1.4 Zinc uptake in fish

Generally metal uptake in aquatic organisms occurs through several routes including the body surface, across the gills, and via the gut lining (Bryan 1971, 1979; Koli *et al.* 1978; Memmert 1987). In fish, the key pathways of Zn uptake are the gills (waterborne Zn) and the intestinal tract (dietary Zn) (Handy 1996; Wood 2001). Figure 1 outlines the mechanisms of branchial and intestinal uptake of Zn. These mechanisms represent the generic epithelial Zn uptake pathways in teleost fish (Bury *et al.* 2003) which are described in detail under uptake of waterborne and dietary Zn.

1.4.1 Uptake of waterborne Zn

The mechanisms of waterborne Zn uptake through the gills are fairly well understood (Wood 2001) and are shown in Figure 2. It is widely accepted that Zn uptake at the gills occurs via chloride cells in the form of Zn^{2+} (Wood 2001). Specifically, Ca^{2+} and Zn^{2+} share a common uptake pathway with Zn^{2+} competing with Ca^{2+} for apical branchial lanthanum-sensitive Ca^{2+}channels (ECaC) Figure 1. This competitive interaction between the two elements leads to decreased uptake of Zn in the presence of Ca^{2+}. In addition Mg^{2+}, Na^{+} and H^{+} also compete for Zn binding sites at the gill (Farag *et al.* 1994; Alsop and Wood 2000). However, Mg^{2+} and Na^{+} have lower affinity for the binding sites and, as a result, non-specific competition occurs between Mg^{2+}, Na^{+} and Zn^{2+} (Alsop and Wood 2000). Several factors, including water hardness (Ca^{2+} and Mg^{2+} concentration), pH, dissolved organic matter and acclimation, affect this uptake. For example increased

Table 1. Zinc metalloproteins and their physiological functions

Zinc containing protein	Physiological function	Reference
Alcohol dehydrogenase	Facilitate the interconversion between alcohol and aldehydes or ketones (breakdown of alcohols)	Vallee and Hoch 1957; Coleman 1992; Rainbow 2002; Eide 2006
Alkaline phosphatase	Dephosphorylation	Coleman 1992; Rainbow 2002
Carbonic anhydrase	Maintain acid base balance in blood and other tissues	Prasad 1979; Stahal *et al.* 1989; Rainbow 2002
Carboxypeptidase	Regulate biological processes (e.g. food digestion, maturation of proteins, growth factor production)	Prasad 1979; Stahl *et al.* 1989; Coleman 1992
Glutathione	Important role in Zn uptake	Jiang *et al.* 1998
Metallothionein	Zinc homeostasis	Brady 1982; Karin 1985
Phospholipase C	Catalyzes the splitting of phospholipid molecule by addition of water	Hough *et al.* 1989; Coleman 1992
P1 nuclease (Nuclease 5'-nucleotidehydrolase, 3'-phosphohydrolase)	Hydrolyzes single stranded DNA and RNA completely to the level of mononucleoside 5'-monophosphates	Volbeda *et al.* 1991; Coleman 1992
Zinc-albumin	Blood Zn transport	Favier *et al.* 1985
Zinc-CRIP (cysteine rich intestinal protein)	Diffusable intracellular transport protein	Hempe and Cousins 1992
Zinc-superoxide dismutase	Catalyze the dismutation of superoxide radical to hydrogen peroxide	Iscan *et al.* 1995; Tjalkens *et al.* 1998
Zinc-transferrin	Blood Zn transport	Favier *et al.* 1985
ZIP family of transporters (Zrt, Irt-like protein)	Zinc uptake transporter (facilitate cellular Zn uptake)	Lioumi *et al.* 1999; Cragg *et al.* 2002; Qiu and Hogstrand 2005
Zinc transporter-1 (ZnT-1)	Zinc export from intestine into the blood stream	Cousins and McMahon 2000

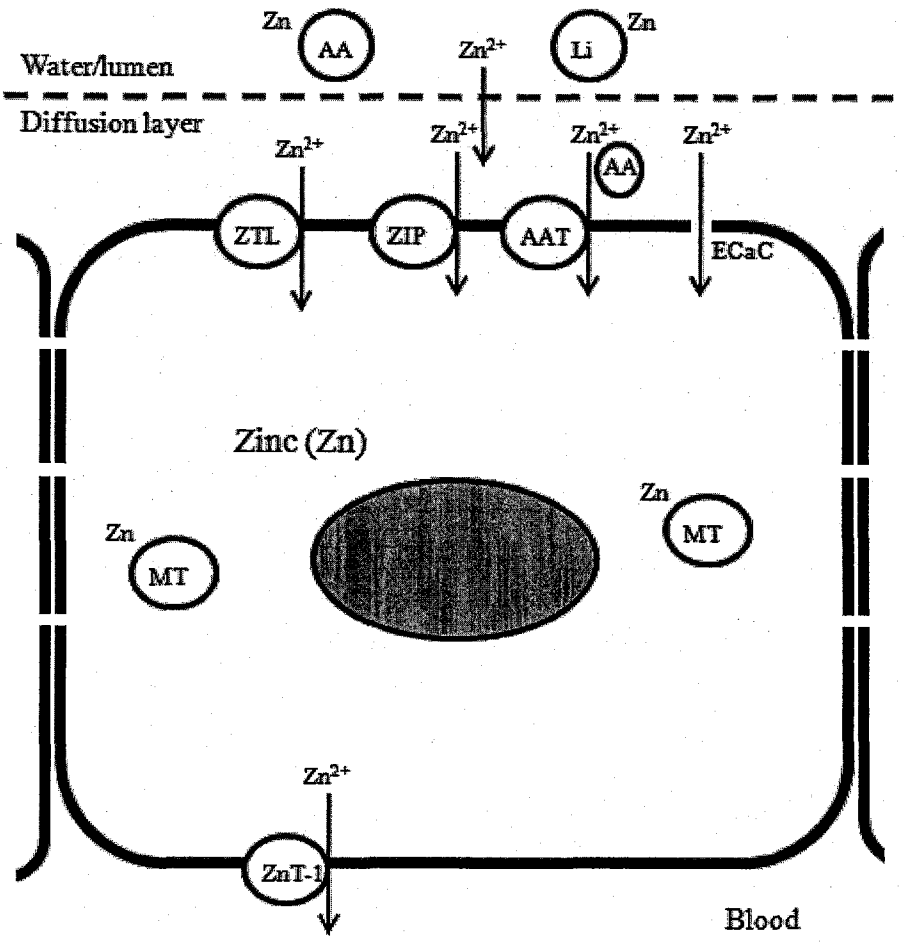


Figure.1. Schematic representation of cellular Zn uptake pathways in fish combining data from gill and intestine (Bury *et al.* 2003). Li: aquatic ligand; AA: amino acid; AAT: amino acid transporter; ECaC: lanthanum sensitive epithelial calcium channel; MT: metallothionein; ZIP: Zrt, Irt-like protein; ZNT-1: zinc transporter 1; ZTL: zinc-regulated zinc transporter. This figure briefly depicts that zinc enters *via* either a putative calcium channel, a ZIP-'like' transporter, ZTL a zinc transporter similar to Zn transporter 1 (ZnT-1), or bound to an amino acid (i.e. histidine), *via* an amino acid transporter. Excess cytoplasmic zinc is bound to metallothionein. Basolateral transfer is *via* a ZnT-1.

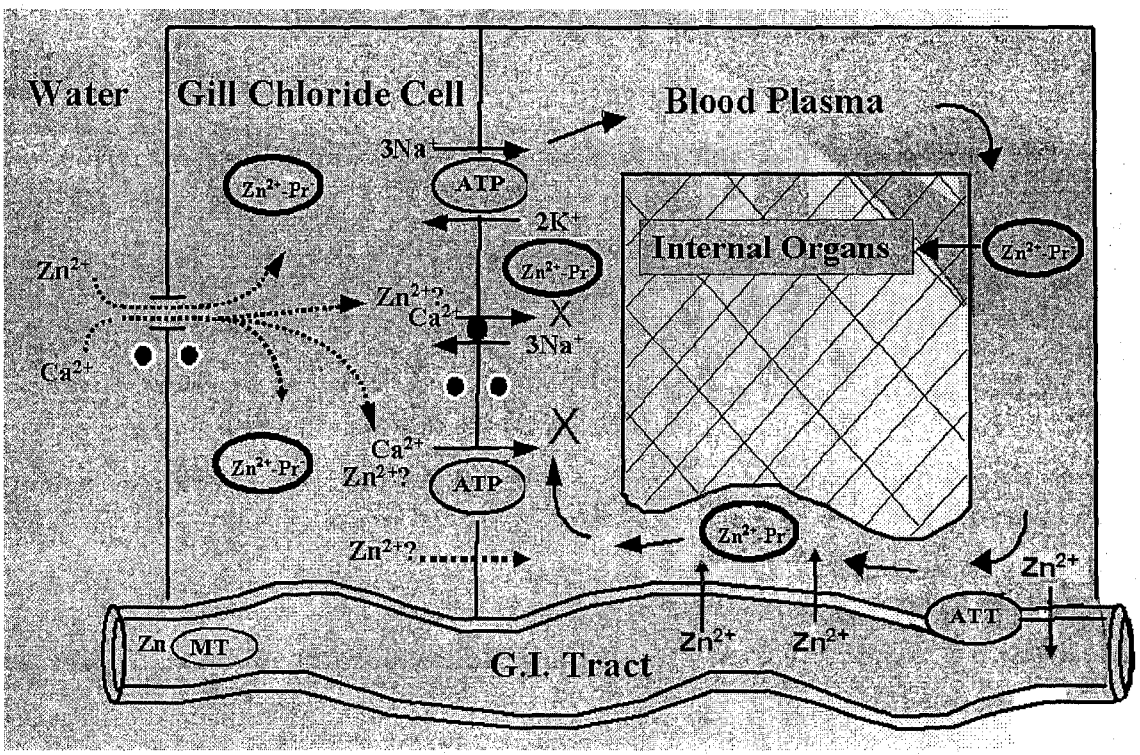


Figure 2. Summary of the mechanisms of Zn uptake, transport and distribution in plasma, branchial and gastrointestinal tract of freshwater fish. Zinc enters through the apical surface of branchial cells (e.g. gill chloride cell) via calcium channels and gets distributed in the cells by combining with Zn proteins or enzymes. It is not clearly understood if Zn leaves the branchial cells via diffusion or some other pathway into the blood plasma. In the plasma it combines with various Zn binding proteins (Zn^{2+}– Pr^{-}) for its distribution and transport into the other internal organs. The entry of Zn in intestinal cells occurs through various transporters e.g. amino acid transporters (ATT). Excess amounts of Zn bind to metallothionein (MT) in epithelial cells and basolateral transfer occurs via Zn transporters or diffusive pathways. No study to date has clearly determined the interactions occurring between the waterborne and dietary Zn.

water hardness decreases waterborne Zn uptake in rainbow trout (Alsop and Wood, 1999). Zinc uptake is, however, increased under conditions of low pH, low alkalinity, low dissolved O_2 and elevated temperature. According to Alsop and Wood (2000) gills possess two pools of Zn, a fast and a slow turnover pool. The size of the fast pool increases with acclimation (pre-exposure) to elevated waterborne Zn concentrations.

It is worth noting that recently the ZIP family of metal transporters (Qui *et al.* 2007) was shown to be expressed in the gill, suggesting a role in specific uptake of Zn akin to the gastrointestinal (GI) tract (see below).

1.4.2 Uptake of dietary Zn

Shears and Fletcher (1983) extensively studied GI uptake of Zn in the winter flounder (*Pseudopleuronectes americanus*) and Glover and Hogstrand (2002a, b) studied dietary Zn uptake in the freshwater rainbow trout. These authors demonstrated that Zn uptake occurs through saturable and diffusive pathways. The fish gut possess Zn transporters [ZIP (Zrt and Irt-related protein) family of transporters or Zn transporter-1] or low molecular mass ligands (MT and glutathione), which play important roles in Zn uptake (Cousins and McMahon 2000; Bury *et al.* 2003; Liuzzi and Cousins 2004). Gastrointestinal Zn uptake is affected by the presence of phosphate, phytate, Ca and amino acids in the diet (Clearwater *et al.* 2002). High levels of phosphate in the diet of rainbow trout lower Zn absorption while the ratio of Ca to P influences Zn bioavailability (Satoh *et al.* 1993; 1996). Elevated levels of phytate in diet decreases Zn absorption whereas amino acids modulate Zn absorption by their interaction with Zn luminally at the

uptake surface, and post-epithelially (Glover and Hogstrand 2003). It is believed that amino acids form complexes with Zn thus increasing its uptake. At high dietary Zn concentrations the capacity to assimilate Zn decreases. For example, Brafield and Koodie (1991) observed decreased assimilation efficiency from 85% in the control group to 75-78% in fish exposed to high dietary Zn.

Overall, Zn uptake into the gut tissue is rapid, but its distribution to the blood and internal organs is slow (Glover and Hogstrand 2002a, b). It has been shown in the common carp (*Cyprinus carpio*) that dietary Zn concentrations higher than 1000 ppm allow Zn to first accumulate in the digestive tract, then in skeletal tissues, and eventually in skin and muscle (Jeng and Sun 1981). Although the entire digestive tract is capable of absorbing Zn, the anterior intestine possesses the greatest, while the stomach has the least, absorptive capacity (Farag *et al.* 1994; Glover and Hogstrand 2002a, b).

1.5 Interactions between waterborne and dietary Zn uptake

When rainbow trout were exposed to dietary and waterborne Zn simultaneously, Zn uptake from the water was independent of the uptake from the diet (Spry *et al.* 1988). At any dietary Zn exposure, increasing the waterborne Zn concentration resulted in increased whole-body Zn (Spry *et al.* 1988). However, in a study carried out by Mount *et al.* (1994), rainbow trout exposed to various combinations of waterborne and dietary Zn showed increased tissue metal concentrations from water and/or dietary exposure. In terms of the relative importance, the affinity of Zn for gills is more than that for the gut but the gut has a greater capacity for Zn uptake. Thus Zn is acquired primarily from the

diet and uptake through gills becomes important at low dietary Zn levels, which has led to the suggestion that gills supplement absorption when dietary Zn decreases or waterborne Zn increases (Clearwater *et al.* 2002). Generally the relative proportion of metal uptake from each route varies with the bioavailability of the metal in water and diet (Wang and Fisher 1999; Rainbow and Wang 2001). Thus if the bioavailability of Zn increases either in water or in diet, there is a proportional increase in the rate of uptake, which leads to metal accumulation.

To the best of our knowledge there are no studies to date examining the interactions and relative contribution of waterborne and dietary Zn at lower (subcellular) levels of biological organization. A key objective of this thesis research is to begin to bridge this knowledge gap through specific studies aimed at resolving the cross-talk between branchial and GI uptake of Zn in rainbow trout.

1.6 Zinc homeostasis in fish

The maintenance of a constant concentration of Zn with varying external bioavailability is essential for fish survival. At both the organismal and cellular levels Zn status is tightly controlled to guard against deficiency and toxicity (Bury *et al.* 2003). This maintenance of Zn balance may involve the regulation of uptake, distribution, detoxification and excretion. Excess Zn is either excreted through bile (Chowdhury *et al.* 2003), intestinal sloughing (Handy 1996) or the gills (Hardy *et al.* 1987), while similar to humans (King *et al.* 2000), urinary loss of Zn in fish is minimal (Spry and Wood 1985). It has been proposed that excretion through the GI tract is the main mechanism by which fish

maintain whole-body Zn homeostasis (Hardy *et al.* 1987). Nonetheless, branchial Zn accumulation is also regulated when rainbow trout are exposed to increased concentrations of waterborne Zn. Reductions in Zn uptake rates occur during prolonged exposure of fish to elevated waterborne Zn levels thus limiting the accumulation of Zn in the gill (Hogstrand *et al.* 1994, 1996, 1998). Similarly, Zn absorption from the diet decreases as dietary Zn load increases (Hardy *et al.* 1987; Glover and Hogstrand 2002a, b), suggesting that the mechanisms involved in the uptake of dietary Zn are down-regulated (Bury *et al.* 2003).

1.7 Toxicity of waterborne Zn in fish

Zinc toxicity is commonly observed in fish, with the early life stages being extremely sensitive. Generally effects produced by environmental chemical contaminants such as Zn occur at various levels of biological organization, extending from the biochemical level to the physiology of individual organisms and ultimately to the level of populations, communities and the ecosystem (Giguere *et al.* 2006). Alterations occurring in the biochemical systems would underlie and precede effects at higher levels of organization, rendering them potentially useful as early indicators of toxicity (Stegeman *et al.* 1989). The toxic effects of Zn can be categorized as acute or chronic depending on the exposure concentration and duration.

On the one hand, acute toxicity denotes the adverse effects resulting from a single short-term exposure or multiple exposures in a short time to a toxic substance. The mechanisms of acute waterborne Zn toxicity are well understood (Wood 2001). Upon acute

waterborne exposure, Zn impairs branchial ionoregulatory and gaseous exchange processes. Zinc concentrations of 1250-40,000 μg l^{-1} lead to hypoxemia due to gross morphologic damage of gills. *In vitro*, Zn is a potent inhibitor of carbonic anhydrase leading to increased arterial pCO_2. At more environmentally realistic exposure concentrations (450 μg l^{-1} – 4000 μg l^{-1}), Zn competitively inhibits the active uptake of Ca^{2+} across the gill epithelium leading to hypocalcaemia, which may eventually result in death of the fish (Hogstrand *et al.* 1995). Zinc ions also inhibit the basolateral Ca^{2+}-ATPase which powers transbranchial Ca^{2+} transport (Wood 2001).

The effects of acute waterborne Zn exposure on physiological and biochemical parameters in African catfish (*Clarias lazera*) and red belly tilapia (*Tilapia zilli*) were investigated by Hilmy *et al.* (1987) and significant increases were observed in liver and serum proteins, serum alkaline phosphatase, erythrocyte count, hematocrit or packed cell volume, and hemoglobin concentrations. Zinc exposure also reduced the activity of liver and serum acid phosphatase as well as liver alkaline phosphatase.

The major physicochemical factors that modify acute waterborne Zn toxicity are the carbonate alkalinity, hardness (Ca^{2+} and Mg^{2+}) and pH of the water (Spear 1981; Bradley and Sprague 1985; Alsop and Wood 1999). For example a 10-fold decrease in water hardness increases acute Zn toxicity to rainbow trout by a factor of 10 (Bradley and Sprague 1985). Similarly, Alsop *et al.* (1999) showed that a six-fold reduction in water hardness increases toxicity by about six times in rainbow trout. In addition the amount of

Zn accumulation in the gills is decreased when water pH is low (Bradley and Sprague 1985).

On the other hand, chronic toxicity is the ability of a substance to cause adverse effects from repeated long-term exposures, which are often at lower doses than those required to cause acute effects. Sublethal concentrations of waterborne Zn affect gill ion transport functions (Farag *et al.* 1994). Specifically the unidirectional Na^+ and Cl^- influx and efflux rates increase due to displacement of Ca^{2+} by Zn^{2+} from the paracellular pathway and/or from entry sites on the apical membrane of ionocytes (Wood 1992). Sublethal chronic Zn exposure also alters the biochemical composition of rainbow trout and brown trout (*Salmo trutta*) tissues by increasing glycogenolysis resulting in decreased calorific value of muscle and liver glycogen (Malik *et al.* 1998). Other toxic effects reported during chronic waterborne Zn exposure include elevated lipid content in liver due to increased mobilization of fat and its transport to the liver (De Schamphelaere and Janssen 2004). Additionally, sublethal Zn exposure can induce changes in fish behavior. For example alterations in respiration, locomotion, social organization, reproduction, feeding, and predator avoidance all have been reported (Henry and Atchison 1990).

Lastly, chronic waterborne Zn exposure can lead to scale loss and mortality in the rainbow trout (Farag *et al.* 1994). Mortality occurs mainly due to damage to gill epithelium (Malik *et al.* 1998). In zebra fish, long-term exposure to low concentration of waterborne Zn resulted in stress, which induced adverse effects i.e. damage to gill epithelial cells which eventually led to death. Interestingly, survival was observed to be a

more sensitive endpoint of chronic Zn toxicity than growth (De Schamphelaere and Janssen 2004). In the same study, these authors demonstrated that, similar to acute Zn exposure, elevating the concentrations of Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} in the exposure water reduced chronic Zn toxicity.

1.8 Toxicity of dietary Zn in fish

In contrast to waterborne Zn, acute toxicity as a result of increased dietary Zn exposure has not been clearly demonstrated so far. Rainbow trout can tolerate more than 3 mg g^{-1} Zn in the food without adverse effects (Clearwater *et al.* 2002). It is believed that fish reject food containing excessively high concentrations of Zn or regurgitate it once ingested, thus limiting exposure. However, dietary Zn does accumulate in the gut, gills, liver, and blood (Clearwater *et al.* 2002).

The toxic effects of chronic dietary Zn exposure have also been investigated albeit to a lesser extent than the chronic effects of waterborne Zn exposure. Elevated dietary Zn levels ranging from 34 to 1000 mg kg^{-1} fed for 20 weeks to rainbow trout affected the activity of various enzymes (Knox *et al.* 1984). It reduced the activity of manganese superoxide dismutase and increased the activity of Cu-Zn superoxide dismutase. A recent review by Clearwater *et al.* (2002) suggests that long-term exposure to dietary Zn in the concentration range of 30-3000 mg Zn kg^{-1} is non-toxic to fish. However, despite the seemingly high Zn concentrations in the diets, the actual Zn doses delivered (after taking into account the feeding rates) in most studies have generally been below 30 mg Zn kg^{-1} body weight d^{-1}. It is probably because of these relatively low dose exposures that dietary

Zn toxicity has rarely been demonstrated in fish. However, even studies that used higher doses of dietary Zn (60-90 mg Zn kg^{-1} body weight d^{-1}) during chronic exposure were unsuccessful in revealing toxicity; survival and growth were not affected (Wekell *et al.* 1983, 1986; Mount *et al.* 1994; Kock and Buchner 1997). It has recently been suggested that the failure to demonstrate dietary Zn toxicity could be due to feeding of Zn contaminated artificial feed rather than metal in natural diets (Clearwater *et al.* 2002). Natural diets with metals 'biologically incorporated' into prey organisms may contain forms of metal that are easily absorbed and hence these diets may be more toxic (Clearwater *et al.* 2002).

1.9 Acclimation

When fish are chronically exposed to waterborne Zn, they are able to acclimate physiologically, and this acclimation involves a progressive reduction in the branchial influx rate of Zn^{2+} and restoration of plasma Ca^{2+} concentrations (Hogstrand *et al.* 1994, 1995). According to Niyogi and Wood (2006), under chronic waterborne Zn exposure in freshwater fish, gills play an important role in maintaining Ca homeostasis and mediating acclimatory responses. A 'damage-repair' model has been described to understand the general process of acclimation during chronic sublethal waterborne exposure to metals (McDonald and Wood 1993). According to this model three phases are involved in acclimation to metals: an initial 'shock' phase, recovery phase, and acclimation. In the latter phase tolerance to metal increases and persists indefinitely during continued exposure. During the initial 'shock' phase there is physical damage, which mainly occurs at the gill, and disturbances in physiological homeostasis (McGeer *et al.* 2000). The

damage phase usually lasts for a few days after which recovery starts. During the recovery phase, there is upregulation of biosynthetic processes to counteract or repair the damage and the physiological disturbances. Ultimately, a new equilibrium is established during the period of increased tolerance or the internal physiological conditions of the animal returns to the pre-exposure level. Under these conditions, harmful effects of chronic sublethal exposure may be minimal (McGeer *et al.* 2000; Taylor *et al.* 2000).

1.10 Metal speciation

Speciation is the partitioning of a metal among specific chemical forms (species), defined by isotopic composition, oxidation or electronic state, complex or molecular structure (Templeton *et al.* 2000). Because interactions among these chemical forms involve weak coordinate bonding or changes in oxidation states, the speciation of a metal is driven by the chemical composition of the medium in which it is found. In natural systems metal ions generally form complexes with naturally occurring complexing agents or ligands and these metal complexes are then mobilized and transported in environmental and biological systems (Fernando 1995). According to Campbell (1995) complexation of a metal with one or more ligands in solution leads to a decrease in its bioavailability. This is the principle behind the free-ion activity model (FIAM, Campbell *et al.* 1995) which has been effective in explaining the central role of the free-ion concentration (or activity) as a regulator of interactions (at the uptake or toxicity levels) between metals and aquatic organisms. It explains that the biological effects of metals are best predicted by the activity of the free metal ion, rather than by total metal concentration.

1.10.1 Metal speciation in the external environment

Following recent advancements in trace chemical analysis it has become clear to aquatic toxicologists that the toxicity of metals is poorly correlated with total metal concentration in the water (Paquin *et al.* 2002). This provided the impetus for identifying and measuring the metal species that correlated with toxicity. At present the study of metal speciation in the external environment is achieved by equilibrium calculations using thermodynamic modeling programs such as MINEQL+ (Schecher and McAvoy 1994), WHAM (Tipping 1994), and MINTEQA2 (Brown and Allison 1987). The application of these models to metal speciation and environmental regulation of metals has recently been reviewed (DiToro *et al.* 2001; Paquin *et al.* 2002). Briefly, it is now accepted that metal speciation in the external environment is a function of the chemical characteristics of the medium including but not limited to salinity, pH, water hardness, and dissolved organic matter. More importantly, speciation is the single most important factor determining metal bioavailability and, by extension, toxicity.

1.10.2 Metal speciation in the internal environment

Similar to the external environment, metal bioavailability is variable within the organism. However, relative to the external environment, the internal environment (i.e. the cell) is dynamic and much more complex (Finney and O' Halloran 2003) and not amenable to equilibrium calculations. According to Campbell *et al.* (2005, 2006) and Vijver *et al.* (2004) subcellular fractionation of tissues after homogenization resembles metal speciation in the external environment in that it allows the estimation of the internal bioavailable metal fraction in much the same way as equilibrium modeling estimates the

bioavailable metal fraction in the external environment. The conceptual idea behind the subcellular fractionation protocol is based on the principles of analysis of metal speciation in sediments (Campbell *et al.* 2005).

In this model, metals entering the body in a reactive form are first captured by reversible binding to proteins and other ligands, followed by localization to targets that have stronger affinity for metals (Vijver *et al.* 2004). Overall the internal metal partitions into a metabolically detoxified pool (MDP) presumed to be inactive and of no toxicological implications, and a metabolically active pool (MAP) consisting of metal that can undergo inappropriate binding to metal-sensitive cellular sites resulting in toxicity. The MDP comprise the metal ions bound by inducible metal binding proteins e.g. MT or precipitated in insoluble metal-rich granules, while the MAP consist of metal associated with heat-sensitive proteins and enzymes, small peptides, nucleic acids, membranes, and organelles (mitochondria, nuclei and endoplasmic reticulum) and can cause deleterious effects (Mason and Jenkins 1995). Note that toxicity of metals is currently based on the spillover hypothesis (Hamilton and Merhle 1986; Campbell et al. 2005) whereby metal ions are envisaged to initially partition into MDP before inappropriate binding (spillover) to metal-sensitive cellular compartments. Therefore isolating and quantifying metals binding to subcellular target sites can obtain a more definitive link between metal accumulation and toxicity. Indeed it is apparent that through employment of speciation analysis to the internal environment, physiological effects and toxicity of trace metals strongly depend on their intracellular localization. For example Cd toxicity is related to its binding to the mitochondria (Sokolova *et al.* 2005).

1.11 Critical body residue (CBR)

The most common dose metric in aquatic toxicology is the exposure dose which defines the substance concentration in the medium to which an organism is exposed. Due to the lack of correlation between exposure dose and effect, organism-based dose metrics, such as the CBR, have been recommended as better surrogates of the toxic dose (McCarty and Mackay 1993). The CBR defines the threshold concentration of a substance accumulated in an organism that corresponds to adverse toxic effects in either individuals or populations of a defined age or stage of development (Wim Ma 2005). The CBR concept assumes that the total body concentration of a chemical is proportional to its concentration at the target or receptor site (McCarty and Mackay 1993; Vijver *et al.* 2004). Because it integrates internal transport, metabolic processes and toxicity at specific sites of toxic action (McCarty and Mackay 1993; Hickie *et al.* 1995), environmental characteristics which modify chemical bioavailability are surmounted. Application of the CBR concept for narcotic organic toxicants has been successful in demonstrating that the toxic concentration for diverse groups of chemicals with similar mechanism of toxic action is relatively constant among diverse groups of animals (McCarty and Mackay 1993).

For metals, however, interpretation of toxicity on a body residue-basis is challenging due to the complex often species- and metal-specific mechanisms of accumulation and toxic action (McCarty and Mackay 1993; Wood 2001; Vijver *et al.* 2004). This is in part due to the diverse physiological metal handling strategies (e.g. sequestration by MT or metal-rich granules) which render, in a species-specific manner, proportions of accumulated

metals unavailable to cause toxicity (Rainbow 2002). Furthermore the mechanisms of toxic action vary among metals (Wood 2001). Thus unlike narcotic organic toxicants, the toxic body concentration of metals vary widely (see for example Crommentuijn *et al.* (1994) for a discussion of Cd toxicity in invertebrates). Even more tenuous is the utility of CBR for the assessment of toxicity of essential trace metals such as Zn and Cu due to lack of adequate knowledge about confounding physiological factors relating to essentiality and homeostasis (Wim Ma 1995). Therefore, in order to apply the CBR concept to metals, a clearer understanding of metal handling strategies, physiological homeostasis and the partitioning of total body burdens is warranted for precise quantification of the proportion of accumulated metal that is biologically available to interact with target sites of toxic action. Although CBR for any metal is highly variable among species, we speculate that the concentration of accumulated metal directly related to toxicity is similar in all organisms.

1.12 Approaches for measuring internal bioavailability of metals

Various techniques including ultrastructural observations, size-exclusion chromatography (Olsson and Hogstrand 1987; Micallef *et al.* 1992; Graham and Rickwood 1997) and differential centrifugation (Wallace *et al.* 2003) have been used to study internal metal partitioning in aquatic organisms. The latter procedure has the advantage that a range of cellular compartments can be studied at a time leading to evaluation of their relative contributions to metal sequestration under real-world exposure conditions. In addition, the quantification of metal binding to both sensitive particulate components (e.g.

organelles) and heat-sensitive proteins provides more complete information regarding the onset of toxicity than a study limited to cytosolic ligands alone (Wallace *et al.* 2003).

The first requirement when developing methods for subcellular fractionation is a proper understanding of ultrastructural details of the tissue or cell which is to be separated (Graham and Rickwood 1997). It is possible to divide intracellular structures into various compartments like nuclei, mitochondria, lysosomes, microbodies, secretion granules, unbroken or partially broken cells, and microsomes. Different cell organelles differ in size, shape and density. These differences result in different rates of sedimentation in centrifugal fields. In practice there is always an overlap in the sizes of the different types of cell organelles. Simple differential pelleting and rate-zonal sedimentation separate particles on the basis of their size. Differential pelleting results in very impure preparations. Rate-zonal sedimentation is ideally suited as a second stage in fractionation following differential pelleting. During separation by density there is risk of damage to cell organelles by hyperosmotic media and the centrifugation times are much longer (Graham and Rickwood 1997). An alternative approach for separating organelles that overlap in size and density is to specifically perturb the density of one of the components (density perturbation). However, results obtained by density perturbation are difficult to interpret and the properties of the organelle concerned are often affected.

Non-centrifugal procedures such as size exclusion chromatography, partition between liquid phases and immunoisolation or electrophoresis also have been developed for the separation of subcellular organelles. Immunoisolation has been more widely used for the

separation of living cells than for subcellular separations as the amounts of material separated are usually small. With regard to electrophoresis, the necessary equipment required is expensive and separation techniques based on the less expensive free flow electrophoresis appear less reliable then those based on density gradient centrifugation. Moreover, free flow electrophoresis is regarded as a specialized technique rather than a routine method for separating cell organelles (Graham and Rickwood 1997).

This thesis employed differential centrifugation to study Zn speciation and bioavailability in rainbow trout tissues. Although initially developed for invertebrate tissues (Rainbow 2002; Wallace *et al.* 2003), this technique also has been applied successfully on a variety of fish tissues (e.g. Giguere *et al.* 2003, 2006; Campbell *et al.* 2005; Kraemer *et al.* 2005; Kamunde and Macphail 2007).

1.13 Characterization of subcellular compartments

To increase the significance of toxicological inferences made using data from subcellular fractions, it is important to ascertain the purity of the separated fractions. Subcellular fractions may be identified either by examination under an electron microscope or by biochemical techniques. The biochemical approach entails the use of marker enzymes to determine organelle content and purity of the subcellular fractions obtained (Graham and Rickwood 1997).

Enzymes are largely confined to a single subcellular compartment. Hence measurement of characteristic enzymes may be used to determine the composition of a cell fraction.

Although ideally marker enzymes for each cell type of tissue should be validated, it is possible to rely on enzymes whose fundamental role means that they are present in most cell types. Marker enzymes for the major organelles, mitochondria, lysosomes, and peroxisomes are perhaps the best characterized. The common marker enzymes for mitochondria are succinate dehydrogenase, monoamine oxidase, citrate synthase, cytochrome c oxidase and adenylate kinase. Acid phosphatase is the most commonly used marker enzyme for lysosomes while catalase is a marker enzyme for both large peroxisomes and microperoxisomes. Lactate dehydrogenase activity (LDH), which is a cytosolic marker enzyme, can be detected in any organelle fractions and is used to assess the level of contamination of organelle fractions with cytoplasm.

It is important to emphasize that deleterious effects of metals can be associated with elevated marker enzyme activity in fractions where the enzymes would not normally occur at high enough levels. Moreover the subcellular fractions are operational in nature and the convenient grouping of the fractions into metal sensitive (HDP, mitochondria, microsomes) and detoxified (metal-rich granules and MT-bound) categories is likely an oversimplification (Giguere *et al.* 2006). Indeed subcellular centrifugation does not yield perfectly clean separations among organelles (De Duve 1975) and some level of overlap among the fractions is inevitable.

1.14 Linking bioaccumulation and toxicity

Subcellular partitioning of metals in aquatic organisms reflects internal processing that occurs during metal accumulation and can provide valuable information about metal

toxicity and tolerance (Jenkins and Mason 1988; Wallace *et al.* 2003). Quantifying the binding of metals to sensitive cellular components (e.g. organelles and enzymes) could reveal potential mechanisms of toxicity and help in predicting whether or not the accumulated metals will induce deleterious effects (Masons and Jenkins 1995; Wallace *et al.* 2003). Therefore by isolating and quantifying metal binding to subcellular targets, a link between accumulation and toxicity can be established. Moreover, knowledge of the biochemical basis of interactions of metal ions and intracellular targets helps in understanding the function of metals in tissues, cells and their constituents (Borovansky 1994). For essential metal Zn, the balance between essentiality and toxicity is regulated through binding to specific cellular sites and hence knowledge concerning the intracellular biochemical speciation cannot be overemphasized.

1.15 Metallothioneins: role in metal homeostasis and toxicity

Cellular mechanisms involved in the accumulation, regulation and immobilization of trace metals are diverse among aquatic organisms and include metal-binding proteins such as MT, metal-rich granules and membrane-bound vesicles (Wallace *et al.* 2003; Campbell *et al.* 2005). Among the metal-binding proteins described to date, MTs are probably the most important and widely studied. MTs are ubiquitous low molecular mass (6000-7000 Da) proteins characterized by remarkable heat-stability, lack of histidine and aromatic amino acids, and very high cysteine content (Klaassen *et al.* 1999; Coyle *et al.* 2002). They play an important role in the regulation of loosely bound cytosolic essential metals, such as Zn and Cu, and in the sequestration and detoxification of non-essential metals such as Hg and Cd (Roesijadi 1992; Roesijadi and Robinson 1994; Mason and

Jenkins 1995; Roesijadi 1996; Campbell *et al.* 2005; Eroglu *et al.* 2005). Metallothionein synthesis is known to be induced by both essential and non-essential metals though the capacity of induction may differ among metals (Hogstrand and Haux 1990; Wu *et al.* 1999; Atli and Canli 2003). In the aquatic environment, MTs could be a sensitive indicator of metal contamination (Hogstrand and Haux 1990; Dallinger *et al.* 1997) and their induction as a measure of response to metal exposure in aquatic organisms has been widely investigated (Langston *et al.* 2002).

Metallothioneins show high affinity for group I B and II B metal ions of the periodic table (Wang *et al.* 1999). Their involvement in metal metabolism is based on their capacity to complex metals, effectively buffering free-metal ion concentrations in the intracellular environment (Klaassen *et al.* 1999; Bonneris *et al.* 2005). As a result, the subcellular distribution of several metals including Cd, Cu, Hg and Zn is highly influenced by MTs. Because the level of MT induction varies among tissues it is feasible that metal binding would depend on the tissue. For example Van Campenhout *et al.* (2004) observed very strong differences in MT induction and binding of Cu, Cd and Zn in common carp tissues. Moreover, more than 30% of cytosolic Zn was MT-bound in the liver compared with only 2% in the kidneys despite considerably higher total cytosolic levels in kidney. Even within a tissue MT induction varies with cell type. In the gill for example the induction occurs primarily in the chloride cells and to a lesser extent in other cell types (Burkhardt-Holm *et al.* 1999; Dang *et al.* 2000).

During subcellular fractionation MTs and MT-like proteins are isolated in the heat-stable proteins (HSP) fraction. It has been demonstrated that MTs are the major heat- stable ligand in HSP (Giguere *et al.* 2006). Metallothionein concentrations can in fact be calculated from the concentrations of Cu, Cd and Zn in HSP (explained in methodology section). More specific measurement of MT concentration involves enzyme-linked immunosorbent assays, metal-saturation assays and pulse polarography, but these approaches also fail to provide information on induction of different MT isoforms and differential binding of metals to them (Lobinski *et al.* 1998). Alternatively high performance liquid chromatography coupled to inductively-coupled-plasma-mass spectrometry can be used for speciation studies of metal-binding proteins including MTs (Mason and Storms 1993; Welz 1998).

1.16 Research hypothesis

The central hypothesis of this research was that exposing fish to elevated concentration of Zn in water and/or in diet would increase its uptake and proportionately increase the MAP of cellular Zn. It is envisaged that the initial partitioning would occur in the MDP before inappropriate binding to metal-sensitive compartments.

1.17 Research objectives

The overall objective was to characterize the internal bioavailability of Zn in key tissues of rainbow trout to illuminate the mechanisms of Zn homeostasis in fish. We aimed to isolate and quantify the metabolically active and detoxified pools of Zn in fish through a

comprehensive analysis of its subcellular distribution in gill, liver and intestinal tissues following chronic waterborne and dietary Zn exposure. The specific objectives were:

1) To isolate and quantify the binding of Zn to subcellular compartments of gill, liver and intestine of rainbow trout during chronic waterborne and dietary Zn exposure with a view to elucidating the mechanisms of intracellular Zn regulation and toxicity.
2) To identify a common subcellular target that responds to Zn exposure irrespective of the tissue and route of uptake.
3) To assess if spillover of Zn occurs to metal-sensitive compartments during chronic Zn exposure.
4) To illuminate the interactions at the subcellular and tissue levels, resulting from branchial and GI Zn uptake.
5) To determine to what extent chronic Zn exposure induces MT in rainbow trout gill, liver and intestine.

2.0 MATERIALS AND METHODS

2.1 Fish sources and management

Juvenile (mean initial weight 14g) rainbow trout (*Oncorhynchus mykiss)* were obtained from Ocean Trout Farm, Brookvale, PE and acclimated to laboratory conditions for 1 month at the Atlantic Veterinary College (AVC) Aquatic Research Facility. Laboratory conditions consisted of holding fish in a single 250-l tank supplied with aerated flow-through well water (AVC, Charlottetown, PE) containing: Na 47.1, Cl 137.3, Ca 58.8, Mg 27.6, hardness 260 (as $CaCO_3$) and dissolved organic carbon 1.5, all in mg l^{-1}. The water pH and temperature were 7.89 ± 0.08 (n = 30) and 11.68 ± 0.02°C (n = 40), respectively. The measured background concentrations of Zn and Cu in the water were 34.71 ± 14.13 and 0.60 ± 0.03 μg l^{-1} (n = 10), respectively while the concentration of Cd was below the limit of detection of our method. During the pre-experimental and acclimation period, fish were fed 2% daily ration (dry feed/wet bwt) of commercial granulated 3.0 grade dry trout chow (Corey Feed Mills Ltd., Fredericton, NB) containing crude protein 46% (minimum), crude fat 26% (minimum), crude fibre 1.7% (maximum), Ca 1.3% (actual), phosphorous 1.0% (actual), Na 0.6% (actual), vitamin A 4400 i.u. kg^{-1} (minimum), vitamin D3 3200 i.u. kg^{-1} (minimum), and vitamin E 2000 i.u. kg^{-1} (minimum). Measured concentrations of Zn, Cu and Cd in the feed were 228 ± 6, 26 ± 1 and 0.78 ± 0.08 mg kg^{-1} (n = 6), respectively.

2.2 Chemicals, reagents and assay kits

All chemicals and reagents used during the experiment and analysis were of the highest available grade. Heparin lithium salt, zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), phenylmethylsulfonyl fluoride (PMSF), DL-dithiothreitol (DTT), hydrogen peroxide (H_2O_2), and acid phosphatase (CS0740) assay kit were obtained from Sigma-Aldrich, Oakville, ON. Sodium dihydrogen orthophosphate, monobasic (NaH_2PO_4) was purchased from BDH Inc., Toronto, ON. Sodium hydroxide (NaOH) and nitric acid (HNO_3, trace metal grade) were obtained from Fisher Scientific, Nepean, ON. For the protein analysis, Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard and Bio-Rad dye reagent concentrate, were purchased from Bio-Rad Laboratories, Mississauga, ON. Purified water used throughout the analysis was obtained by in-house filtration and deionization, using a Milli-Q Gradient A10 system (Millipore, Fisher Scientific, Nepean, ON).

2.3 Experimental diets

Experimental diets were made in-house by supplementing commercial trout chow with the required amount of Zn calculated to deliver nominal 30 and 120 μg Zn per g fish per day in the low and high dietary Zn groups, respectively. This was achieved by making diets containing nominal Zn concentrations of 1000 and 4000 mg kg^{-1}, respectively (Table 2). For preparation of the diets, unpelleted commercial trout chow was mixed with the appropriate amount of $ZnSO_4 \cdot 7H_2O$. Zinc was initially dissolved in 40% diet

Table 2: Nominal and actual dietary Zn concentrations and total Zn dose delivered per g fish per day during the experiment. Values are mean ± SEM, n = 5.

Treatment Group	Diet Zn concentration (mg kg^{-1})		Feeding rate (% bwt)	Total Zn dose (µg per g fish per day)	
	Nominal	Actual		Nominal	Actual
Control diet	0	228 ± 6	3	0	6.8
Low waterborne	0	228 ± 6	3	0	6.8
High waterborne	0	228 ± 6	3	0	6.8
Low dietary	1000	1441 ± 76	3	30	43
High dietary	4000	4820 ± 200	3	120	145

weight of deionized water, added to an appropriate amount of unpelleted trout chow and mixed in a pasta maker (Kamunde *et al.* 2001, 2002) for 30 min. This ensured homogenous distribution of the Zn throughout the food. Thereafter, an additional 20% weight/volume of deionized water was added (bringing the total volume of water added to 60% diet weight) and mixed for a further 15 min. The food was subsequently extruded via a 3 mm die of the pasta maker, air-dried, and broken into small pellets (approximately 3 mm^3) by hand. The control diet was prepared in the same manner except that no Zn was added. The experimental fish feed was kept at -20°C until use. The actual Zn concentrations of the diets (mean ± SEM, n = 5) as determined by flame atomic absorption spectroscopy (FAAS) were 228 ± 6, 1441 ± 76 and 4820 ± 200 mg kg^{-1} for the control, low and high Zn diets, respectively. The nominal and actual Zn concentrations of the diets including the oral dose of Zn administered are also shown in Table 2. These dietary levels of exposure were chosen to fall within environmentally relevant levels (Clearwater *et al.* 2002).

2.4 Experimental protocol and feeding

The experimental design consisted of three waterborne Zn levels (control, low and high), three dietary Zn levels (control, low and high) and one combined exposure group with low waterborne and low dietary Zn levels. The exposure system consisted of 18 tanks having triplicates of six treatments of combinations of waterborne and dietary Zn concentrations: control waterborne Zn + control dietary Zn, low waterborne Zn + control dietary Zn, high waterborne Zn + control dietary Zn, control waterborne Zn + low dietary Zn, control waterborne Zn + high dietary Zn, and low waterborne Zn + low dietary Zn.

Following the one-month laboratory acclimatization period, fish were equally (n = 16-17) and non-selectively distributed into eighteen 15-l experimental tanks. Fish were randomly picked from the holding tank and distributed to the treatment tanks without bias using a random number set. Exposure to 150 and 600 μg l^{-1} waterborne Zn was achieved via a constant drip of 3.0 ml min^{-1} of a stock solution containing, respectively 50 and 200 mg l^{-1} Zn (as $ZnSO_4 \cdot 7H_2O$) from Mariotte bottles into head tanks receiving 1000 ml min^{-1} of well water. Uniform distribution of Zn in head tanks was achieved by constant vigorous aeration. There were three head tanks (low, high and combined waterborne) and each of them supplied three experimental tanks at the flow rate of 333 ml min^{-1}. Actual concentrations of Zn in the low, high waterborne and combined exposure experimental tanks were 179.90 ± 9.43 (n = 49), 649.50 ± 14.80 (n = 53) and 189.60 ± 10.60 μg l^{-1} (n = 49), respectively. Measured in-tank Zn concentrations in the control, low dietary and high dietary experimental tanks were 34.71 ± 14.13 (n = 32), 31.23 ± 9.37 (n = 32) and 36.27 ± 12.35 μg l^{-1} (n = 30), respectively.

During the experiment fish were fed the designated diets (control, low Zn or high Zn) at half the total ration (1.5% wet bwt) twice a day, once in the morning (09:00-10.00 h) and again in the evening (21:00-22.00 h). The fish were allowed to feed for 1 h, after which fecal material was siphoned off. Visual examination during the feeding revealed that all fish readily ingested the diets. Water samples collected after 1 h of feeding were analyzed for Zn concentrations in all the treatments, and no significant leaching of Zn into the water was recorded in any treatment. The complete ingestion of the ration by the fish together with the siphoning of fecal material and continuous flushing by the flow through

system ensured that leaching did not occur. Bulk fish weights were obtained weekly for each group and were used to calculate the ration for the following week.

The experimental set-up was monitored daily to ensure that vital water quality parameters were within the acceptable or expected ranges. Water flow rate to all the experimental tanks were set and maintained at approximately 330 ml min^{-1}. Dissolved oxygen levels measured with a handheld dissolved oxygen meter (YSI Model 550A, Macalaster Bicknell Co, New Haven, CT) and pH measured with pH Meter Lab M220 (Radiometer Analytical, CO) were 7.89 ± 0.08 (n = 30) and 9.95 ± 0.06 mg l^{-1} (n = 42) respectively, throughout the experimental period. Ammonia levels (Nessler Method, Hach DR/4000V Spectrophotometer, Hach Company, CO) measured during the experiment were 0.44 ± 0.04 mg l^{-1} (n = 30).

2.5 Sampling

Sampling was done at the start of the exposure (day 0) and subsequently at days 14, 28 and 40 to assess tissue and whole-body Zn status. A sampling interval of 12-14 days was used to allow acclimation of the fish to each exposure regime. Prior to sampling, fish were starved for two days to purge the gut of ingesta and bulk-weighed on a per tank basis. During the starvation period, fecal material was siphoned from the tanks every 12 h to minimize coprophagy and leaching of the fecal Zn into experimental water. During each sampling interval, four fish per replicate (12 fish per treatment) were randomly netted from the experimental tanks and were euthanized with an overdose of 1.5% tricaine-methane sulfonate (MS-222). Weight, length and general appearance of the fish

were recorded individually. To measure plasma Zn levels, blood samples were obtained by caudal puncture into heparinised 1-ml syringes fitted with 18-G needles and transferred into the pre-labelled Eppendorf tubes (Flat top 1.5 ml microcentrifuge tube, Fisher Scientific). Each blood sample was immediately placed on ice and then centrifuged for 5 min at 10,000 × g, 4°C (IEC Micromax RF Refrigerated Microcentrifuge, Thermo Electron Corporation, Milford, MA) to separate plasma, which was stored at -20°C until analysis. Thereafter the fish were dissected. Gills, liver, intestine, and kidney were flash-frozen in liquid nitrogen and stored at -80°C (Revco, Kendro Laboratory Products, Asheville, NC) until further processing. The rest of the carcass for each fish was collected into a pre-weighed 50 ml tube and preserved at -20°C until further processing.

2.6 Subcellular fractionation

For subcellular fractionation, tissue samples (gills, liver and intestine) were removed from the -80°C freezer and gently thawed on ice for homogenization. Three individual tissues of each type were pooled together to derive n = 4 composite samples for each tissue at each sampling time. Phosphate buffered saline (PBS) containing (all in mM) 137 NaCl, 4.3 KCl, 4.3 Na_2HPO_4, 1.4 NaH_2PO_4, 0.1 PMSF and 1 DTT, was added in a ratio of 1:3 (tissue: buffer) to each sample to be homogenised. The tissues were handled on ice throughout to minimize autolysis.

The tissues were initially manually homogenised using a 2 or 5 ml Potter-Elvehjem homogeniser (Cole-Parmer, Anjou, QC) equipped with a polytetrafluoroethylene pestle.

Optimum numbers of turns for homogenization (i.e. five for liver, eight for gills and twelve for intestine) were determined by running a trial for each tissue to avoid disruption of cellular organelles. The tissue homogenates obtained were then subjected to successive differential centrifugations in a step-by-step procedure modified from the protocol described by Wallace *et al.* (2003) and Giguere *et al.* (2006). This procedure produced seven subcellular fractions, namely nuclei + cellular debris, NaOH-resistant granules, mitochondria, microsomes and lysosomes, cytosol, heat-stable proteins (HSP) and heat-denaturable proteins (HDP). The subcellular fractionation protocol is summarised in Figure 3. Note that the subcellular fractions obtained using this protocol are operationally defined and some level of overlap is inevitable (Walace *et al.* 2003; Giguere *et al.* 2006). Weights of all the samples and fractions were recorded during the entire fractionation process.

Immediately following the homogenisation, a representative sub-sample of each tissue homogenate was taken for total tissue Zn concentration and protein analysis and frozen at -80°C. The remaining homogenate was vortexed for 5-10 sec (VELP Scientifica Vortex Mixer, Mandel, Guelph, ON) and then centrifuged at 800 × g, 4°C, for 15 min to obtain unbroken cells, membranes, nuclei and granules. To isolate granules, this first pellet was resuspended in de-ionized water (500 µl) and heated to 100°C in a heating block (Isotemp 205, Fisher Scientific, Nepean, ON) for 2 min. To this hot pellet an equal volume of 1N NaOH was added and the mixture was incubated in a water bath at 60-70°C for 60 min before a final centrifugation in Eppendorf Mini-Spin Plus (Brinkmann Instruments Inc., VWR, Toronto, ON) at 10,000 × g, 20°C for 30 min, to isolate NaOH-

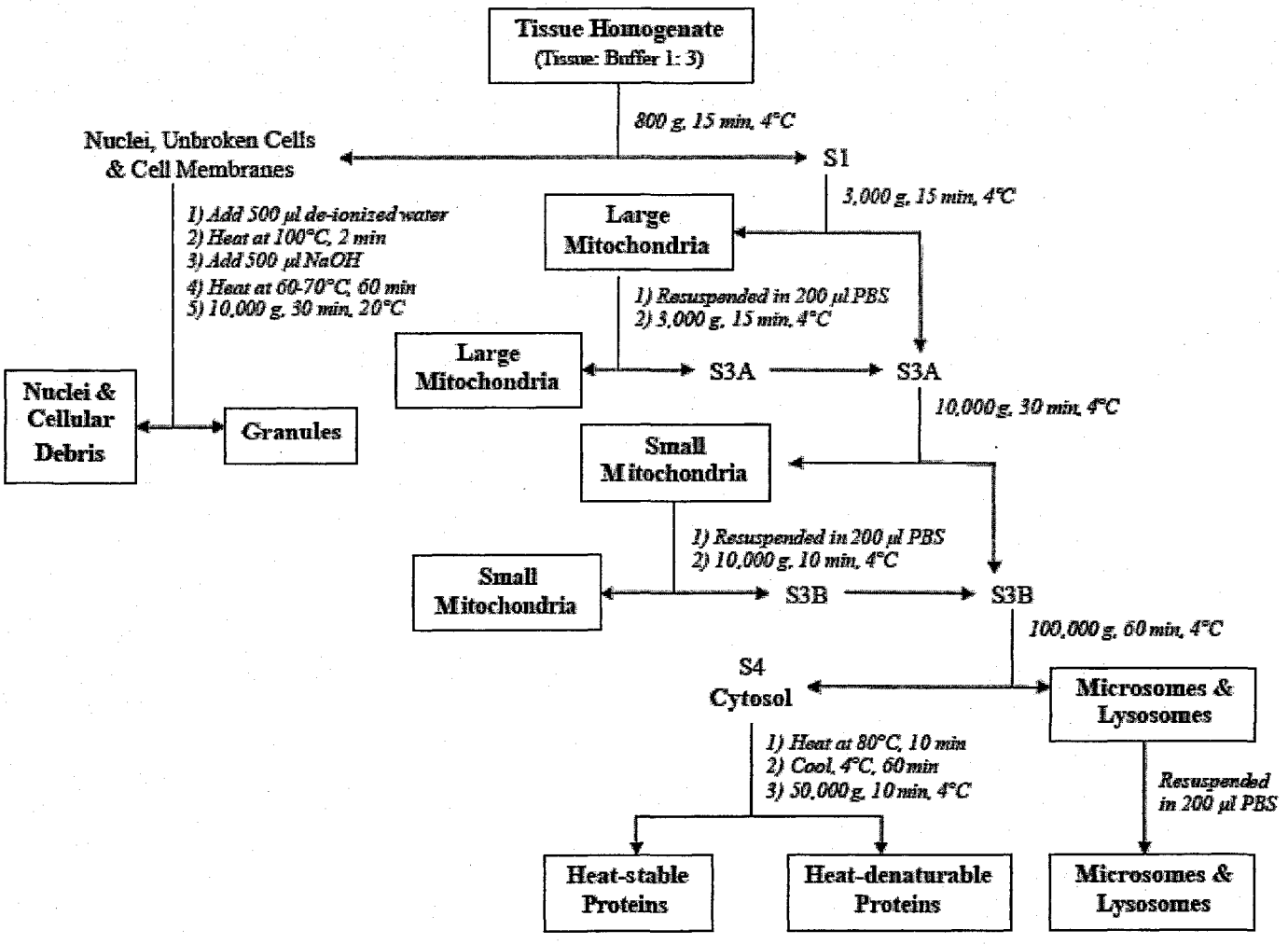


Figure 3. Protocol followed to separate various subcellular fractions from fish gill, liver and intestine. Modified from Walace *et al.* (2003) and Giguere *et al.* (2006).

resistant granules. The resulting supernatant containing nuclei and cellular debris was stored at -20°C and the pellet containing the NaOH-resistant granules was kept at -80°C until further processing.

A 20-µl sub-sample of the supernatant obtained from the initial 800 × g separation was frozen at -80°C for protein analysis. The remaining supernatant was vortexed for 5-10 sec and recentrifuged at 3,000 × g, 4°C for 15 min to obtain large mitochondrial fraction and a supernatant (S3A). The large mitochondrial pellets were then resuspended in 200 µl PBS buffer. The resuspended large mitochondrial pellets were further centrifuged at 3,000 × g, 4°C for 10 min and the supernatants were transferred to the original S3A supernatants and used to isolate small mitochondrial fraction. The large mitochondrial pellets were stored at -80°C for future processing and Zn analysis.

The supernatants for isolation of small mitochondrial fraction (i.e. S3A) were vortexed for 5 to 10 sec and centrifuged at 10,000 × g, 4°C for 30 min. The supernatant obtained (S3B) was transferred to 4-ml Beckman ultraclear centrifuge tubes (Beckman Instruments, Inc. Palo Alto, CA) and used to isolate microsomes and lysosomes as described below. Similar to the large mitochondrial pellets, the small mitochondrial pellets were also resuspended in 200 µl PBS buffer. The resuspended small mitochondrial pellets were further centrifuged at 10,000 × g, 4°C, for 10 min and the supernatants were transferred to appropriate 4-ml Beckman ultraclear tubes containing the original S3B supernatants. The small mitochondrial pellets obtained were stored at -80°C for future processing and Zn analysis.

The Beckman ultraclear tubes containing the S3B supernatants were then filled with the PBS buffer and centrifuged in a pre-cooled ultracentrifuge (Beckman L8-60M Ultracentrifuge, Beckman Instruments, Inc.) at 100,000 × g, 4°C, for 60 min. This step yielded the microsomes-lysosomes fraction and a supernatant (S4, cytosol). Two separate sub-samples of the cytosol, 50 and 100 µl, were taken and stored at -80°C for measurement of protein and marker enzymes and total cytosolic Zn, respectively. The microsome-lysosome pellets were resuspended in 200 µl of PBS buffer and 40-µl sub-samples were obtained and frozen at -80°C for protein and enzyme analysis. The remaining resuspended microsomal-lysosomal fraction was frozen at -80°C until further processing and Zn analysis.

The Beckman ultraclear tubes containing S4 were filled with PBS buffer and heated to 80°C for 10 min in a water bath. They were then cooled for 60 min, 4°C and subsequently centrifuged at 50,000 × g, 4°C for 10 min in order to obtain HDP and a final supernatant of HSP which were frozen at -80°C and -20°C, respectively, until further processing and Zn analysis.

2.7 Tissue drying and lyophilization

All of the carcasses were dried in a gravity convection oven (Precision Scientific Inc, Chicago, IL) at 80°C for 96 h whereas the blood cells and kidneys were dried at 80°C for 48 h. The pellets obtained from subcellular fractionation were lyophilized (Labconco Lyophilizer 75035, Labconco Corporation, Kansas City, MI) overnight and their dry

weights were recorded. Supernatants were not lyophilized; they were instead directly acidified and analyzed for Zn (see below).

2.8 Tissue digestion

The carcasses were digested overnight with 10 times their weight of 1N HNO_3 in an oven at 60-70°C while the blood cells and kidney were digested with 10 times their volume of 70% HNO_3 + 30% H_2O_2 at room temperature for 48 h.

All of the pellets resulting from the fractionation were digested with 500 µl of 70% HNO_3 + 30% H_2O_2 at room temperature for 24 h. Due to their large size, the lyophilized sub-sample pellets were transfered to 15 ml Eppendorf tubes for overnight digestion with 2 ml 70% HNO_3 + 30% H_2O_2 at room temperature for 24 h.

For quality control procedural blanks and a certified reference material (bovine liver, standard reference material 1577b, National Institute of Standards and Technology Gaithersburg, MD) were digested and analyzed in the same manner as all the tissues and the pellets. Blanks indicated negligible contamination (concentrations below limit of detection), and the recoveries of Zn, Cu and Cd from bovine liver were in the range of 91-102%.

2.9 Analysis

2.9.1 *Atomic absorption spectrophotometry*

Following digestion, all the samples were analyzed for Zn by FAAS (AAnalyst 800, Perkin Elmer Instruments, Norwalk, CT) and results were expressed as concentrations in $\mu g\ g^{-1}$ dry wt or $ng\ mg^{-1}$ protein. Initial runs using a cross-section of samples for each tissue/fraction were performed to determine dilution factors. Plasma samples and supernatants obtained from the fractionation process were analyzed for Zn without digestion, after appropriate dilution with 0.2% HNO_3. Digested carcasses, blood cells, kidneys, pellets and sub-samples obtained from the fractionation process were analyzed for Zn after appropriate dilution with deionized water.

Copper and Cd concentrations were measured in HSP supernatants using graphite furnace AAS equipped with a transversely heated graphite atomizer (Perkin-Elmer, AAnalyst 800) and an autosampler (Perkin-Elmer, AS-800). The supernatants were diluted with 0.2% HNO_3 in ratios of 1:5 for gills and intestine, and 1:50 for liver for Cu analysis. For Cd analysis in liver, gills and intestine, the HSP supernatants were diluted in the ratio of 1:2 with 0.2% HNO_3. Procedural blanks and certified reference materials (TMDA 54.4 and 54.3, National Water Research Institute, Burlington, ON) were also analyzed for metals (Zn, Cu and Cd) during each analytical run. The measured metal concentrations in the reference materials were consistently within the certified range, with recoveries of Zn, Cu and Cd being in the range of 90-98%. Concentrations in the blanks were below the method limits of detection for all the metals.

2.9.2 *Metallothionein concentrations*

Theoretical MT concentrations were calculated from the concentrations of Zn, Cu and Cd in the final supernatant, HSP. This calculation takes into account the fraction of the HSP metals bound to the MT fraction for the different exposure groups by using the following equation:

$$MT_{theor} = Fr_{Zn} \cdot [Zn] / K_{Zn} + Fr_{Cu} \cdot [Cu] / K_{Cu} + Fr_{Cd} \cdot [Cd] / K_{Cd}$$

where [Zn], [Cu] and [Cd] are the HSP concentrations of the metals in $\mu g\ g^{-1}$ dry wt in the individual samples, Fr is the fraction, K is the specific capacity of each metal to bind to MT by metal thiolate linkages which is 7 for Zn and Cd, and 12 for Cu (Van Campenhout *et al.* 2004; Giguere *et al.* 2006).

2.9.3 *Measurement of total protein*

Total protein concentrations were measured in the aliquots of homogenate, microsomes-lysosomes, and cytosol, according to the method of Bradford (1976) using Bio-Rad protein assay kit with BSA as the standard. Samples were initially thawed on ice and appropriately diluted with deionized water. The BSA standard was reconstituted in deionized water to a concentration of 1 mg ml^{-1} and 50 µl working aliquots were prepared and kept at -20°C. For measurement of protein in 26 samples, a 50 µl aliquot of BSA standard was thawed and standards of 0, 50, 100, 200, 400 and 600 $\mu g\ ml^{-1}$ concentrations were prepared in triplicate in clean microcentrifuge tubes and gently mixed. A working dye solution was prepared by diluting the concentrated dye reagent in

a ratio of 1:5 with deionized water. Subsequently 10 µl of BSA standards were added to wells of a 96-well plate assigned to the standards while to the other wells 10 µl of the unknown samples were added. To all the wells 200 µl of the dilute dye reagent were added and the plate was mixed by tapping and allowed to sit for 5 min before reading at 595 nm (Spectra Max Plus Microplate Spectrophotometer, Molecular Devices, Sunnyvale, CA). Note that the unknown samples and standards were analyzed in triplicate. Procedural accuracy was assessed from the linearity of the standard curve. Unknown sample values were accepted if they fell within the standard range (i.e. 0-600 µg ml^{-1}) and repeated after additional dilution if they were above this range. The results obtained are expressed as mg protein g^{-1} dry wt of tissue. Note that dry weights for gill, liver and intestine were calculated using correction factors obtained following lyophilization of whole tissue homogenate sub-samples.

2.9.4 *Marker enzyme assay*

To assess the purity and potential cross-contamination of the fractions acid phosphatase, a biochemical marker for lysosomes, was measured in the microsomes-lysosomes fraction and in the cytosol.

2.9.4.1 *Acid phosphatase assay*

This enzyme was measured, using a commercial acid phosphatase assay kit, in the aliquots of microsomes-lysosomes and cytosol samples which were stored at -80°C following the centrifugation process. In the first step, protein was measured in all the aliquots in order to calculate the dilution factor. The samples were then diluted in

deionized water to 0.2 μg μl^{-1} and kept on ice. The substrate solution was prepared by dissolving 1 tablet of 4-nitrophenyl phosphate in 2.5 ml of 0.09 M citrate buffer solution and 50 μl of this substrate solution was pipetted into each microplate well, except for the standard wells. The mixture was then warmed for 5-10 min in a spectrophotometer set at 37°C. Substrate solution (50 μl) and 50 μl of sample were then added to the test wells. Similarly, 50 μl of substrate solution and 50 μl of citrate buffer were added to the blank wells, while 300 μl of standard solution were added to the standard wells. For the positive control 50 μl of substrate solution, 2 μl of control acid phosphatase and 48 μl of citrate buffer was added. After shaking, the plate was placed in the spectrophotometer at 37°C for an incubation of 10 min. The reaction was stopped by adding 0.2 ml of stop solution (0.5 N NaOH) to all wells except the standard wells, and the absorbance was measured at 405 nm. The results obtained are expressed as units ml^{-1}.

Acid phosphatase activity was calculated using the following equation:

$$\text{Units ml}^{-1} = \frac{(A_{405}\,[\text{sample}] - A_{405}\,[\text{blank}]) \times 0.05 \times 0.3 \times DF}{A_{405}\,[\text{standard}] \times \text{Time} \times \text{Venz}}$$

Where:

A_{405} [sample] = the absorbance of the sample at a wavelength of 405 nm

A_{405} [blank] = the absorbance of the blank

A_{405} [standard] = the absorbance of the standard

DF = dilution factor of the original sample

Time = time of incubation at 37°C in min

Venz = volume of enzyme sample added to the assay in ml

0.05 = concentration (μmole ml^{-1}) of 4-nitrophenol in the standard solution

0.3 = 0.3 ml, the total assay volume, including the stop solution.

Unit definition: one unit of acid phosphatase hydrolyzes 1 μmole of 4-nitrophenyl phosphate per minute at pH 4.8 at 37°C.

Figure 4 shows the acid phosphatase activity in the microsomes-lysosomes fraction and cytosol of the gill. The enzyme activity was measured only in the controls to avoid any potential effect of Zn exposure. The microsomes-lysosomes fraction was highly enriched with lysosomes as indicated by high acid phosphatase activity. On the other hand, the cytosol displayed very low activity of this enzyme indicating minimal contamination with a lysosomal enzyme. Enzyme profiles in the liver and intestine were also characterized by high activities in microsomes-lysosomes fractions and low activities in the cytosol (data not shown).

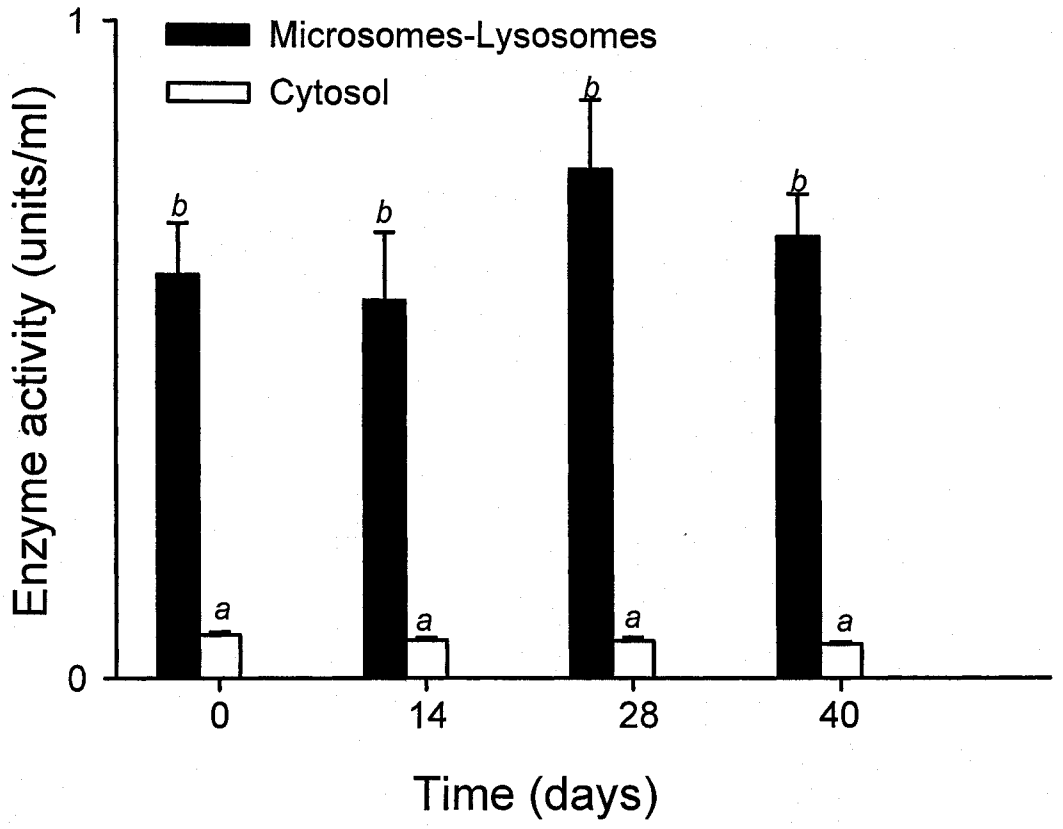


Figure 4. Acid phosphatase activity in microsomes-lysosomes fraction and cytosol of gill. Bars with different letters are significantly different, Student's t-test, $n = 4$, $p < 0.05$.

2.10 Statistical analysis

All the data are presented as mean ± SEM. All the data were initially checked for normality of distribution and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests and then analyzed using two-way factorial analysis of variance (ANOVA, Statistica version 5.1, Statsoft, Inc., Tulsa, OK) with time and treatment as independent variables. In addition, all proportional data were initially arcsine-transformed before analysis with ANOVA. Subsequently a *post hoc* Tukey's honest significant difference test was used to delineate differences among the mean values of the measurements. The enzyme activity data were statistically analyzed using a two-tailed Student's t-test. Mean values were considered different at $p < 0.05$.

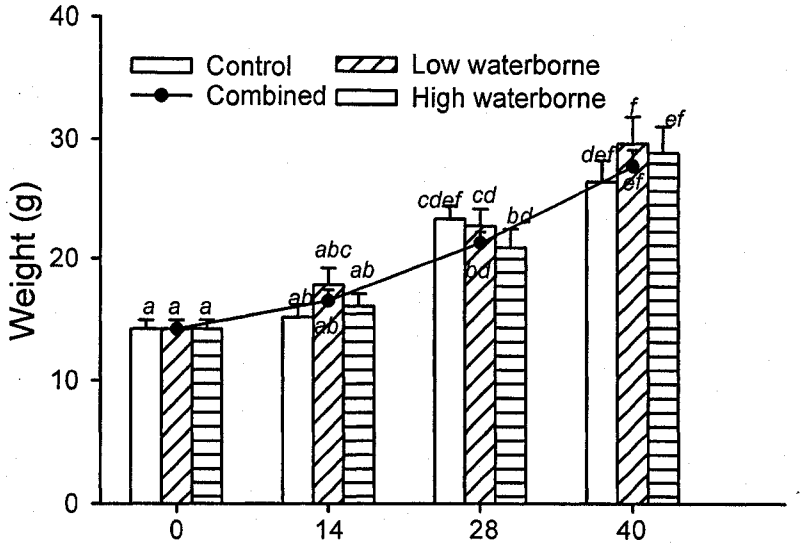
3.0 RESULTS

3.1 Toxicity and growth

Chronic exposures of juvenile rainbow trout to 150 (low water treatment) and 600 (high water treatment) μg l^{-1} waterborne Zn, 1000 (low diet treatment) and 4000 (high diet treatment) mg kg^{-1} dietary Zn singularly, or combined (150 μg l^{-1} + 1000 mg kg^{-1} Zn) were well tolerated. Out of a total of 300 fish, only 6 (2 each in control and high diet treatments and 1 each in the low water and combined treatments) died over the entire experimental period. These mortalities were not related to the plane of Zn exposure.

During the 40-day experimental period, fish significantly increased in weight from an initial weight of about 14 to about 30 g (Fig. 5). There was a highly significant effect of time ($p < 0.001$) with fish from all of the experimental groups being significantly larger than the day 0 controls from day 28 onward. Waterborne and dietary Zn exposure significantly ($p < 0.05$) affected fish growth. Generally, waterborne Zn exposure appeared to stimulate, while dietary Zn exposure inhibited growth. The consequence of this contrasting exposure route-dependent effect of Zn on growth was that the high Zn diet fish were significantly smaller than the low water Zn fish at days 14 and 28, while the low Zn diet fish were significantly smaller than both the low water and high water Zn fish at day 40. In addition, the most significant differences across time occurred between the waterborne and dietary Zn-exposed fish. The fish condition factor (Table 3) ranged from 0.95 to 1.08 and did not change with time or the Zn exposure. No significant time-dependent or treatment-dependent differences in the condition factor were found.

A



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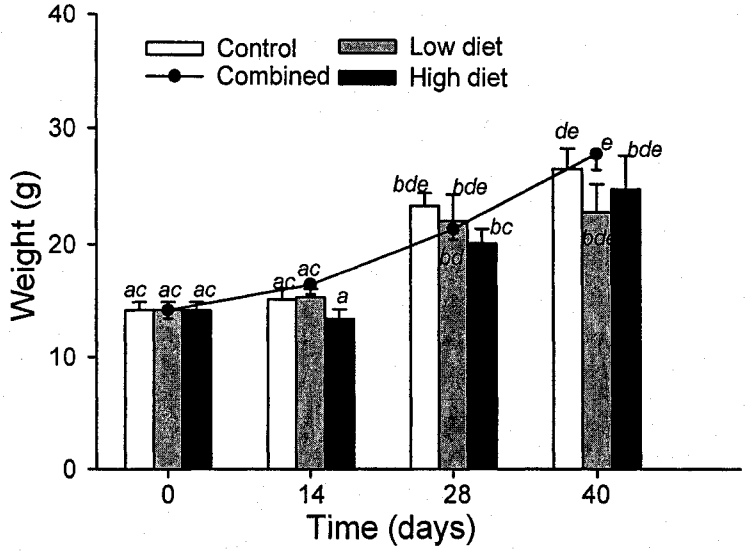


Figure 5. Effect of Zn exposure on growth of juvenile rainbow trout. All values are mean ± SEM, n = 12 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

Table 3: Condition factor of juvenile rainbow trout. All values are mean ± SEM, $n = 12$ per group.

DAY	TREATMENT					
	Control	**Low Waterborne**	**High Waterborne**	**Low Dietary**	**High Dietary**	**Combined**
0	0.95 ± 0.02	0.95 ± 0.02	0.95 ± 0.02	0.95 ± 0.02	0.95 ± 0.02	0.95 ± 0.02
14	0.98 ± 0.04	0.97 ± 0.02	0.97 ± 0.02	1.01 ± 0.04	1.10 ± 0.04	0.99 ± 0.03
28	1.02 ± 0.03	1.01 ± 0.02	1.01 ± 0.01	1.05 ± 0.02	1.08 ± 0.04	1.00 ± 0.02
40	1.02 ± 0.02	1.02 ± 0.01	1.06 ± 0.02	1.04 ± 0.03	1.07 ± 0.04	1.02 ± 0.02

3. 2 Protein concentrations in gill, liver and gut tissue

Protein concentrations in the gill, liver and intestinal tissue were measured both as a potential biomarker of effects and as a reference parameter for Zn concentration in subcellular fractions and respective tissue. There was a significant decrease in concentrations of protein in gills ($p < 0.05$) of the high waterborne group. Overall the gill protein concentrations ranged between 290 and 475 mg g^{-1} in the waterborne exposures (Fig. 6A). It was apparent that fish exposed to dietary Zn had higher gill protein concentrations on day 14 than those exposed to waterborne Zn (Fig. 6A vs. 6B). For example, the low diet group registered the highest protein concentration of 560 mg g^{-1} on day 14; this value was statistically different ($p < 0.05$) from the concentration recorded for the high water treatment on the same day. In the combined exposure group, the protein concentration remained relatively stable throughout the experimental period suggesting that there was no interaction between the two exposure pathways.

Liver protein concentrations were not statistically different from the gill and ranged from 427 to 588 mg g^{-1} (Fig. 7). Main effects analysis revealed that the protein concentration increased significantly with time ($p < 0.05$). A significant decrease in protein concentration was observed in the day 40 high waterborne group as compared to day 14 control. In the dietary exposure there was a significant increase in concentration of proteins in the day 40 high dietary group as compared to day 0, 40 controls, day 28 high diet, and the day 14 and 40 combined exposure groups . There was no interaction ($p = 0.71$ for waterborne and $p = 0.13$ for dietary) between the two main factors for both exposure routes. Similar to the gill, the protein concentration in the combined exposure was not different from that in comparable singular waterborne and dietary Zn exposure.

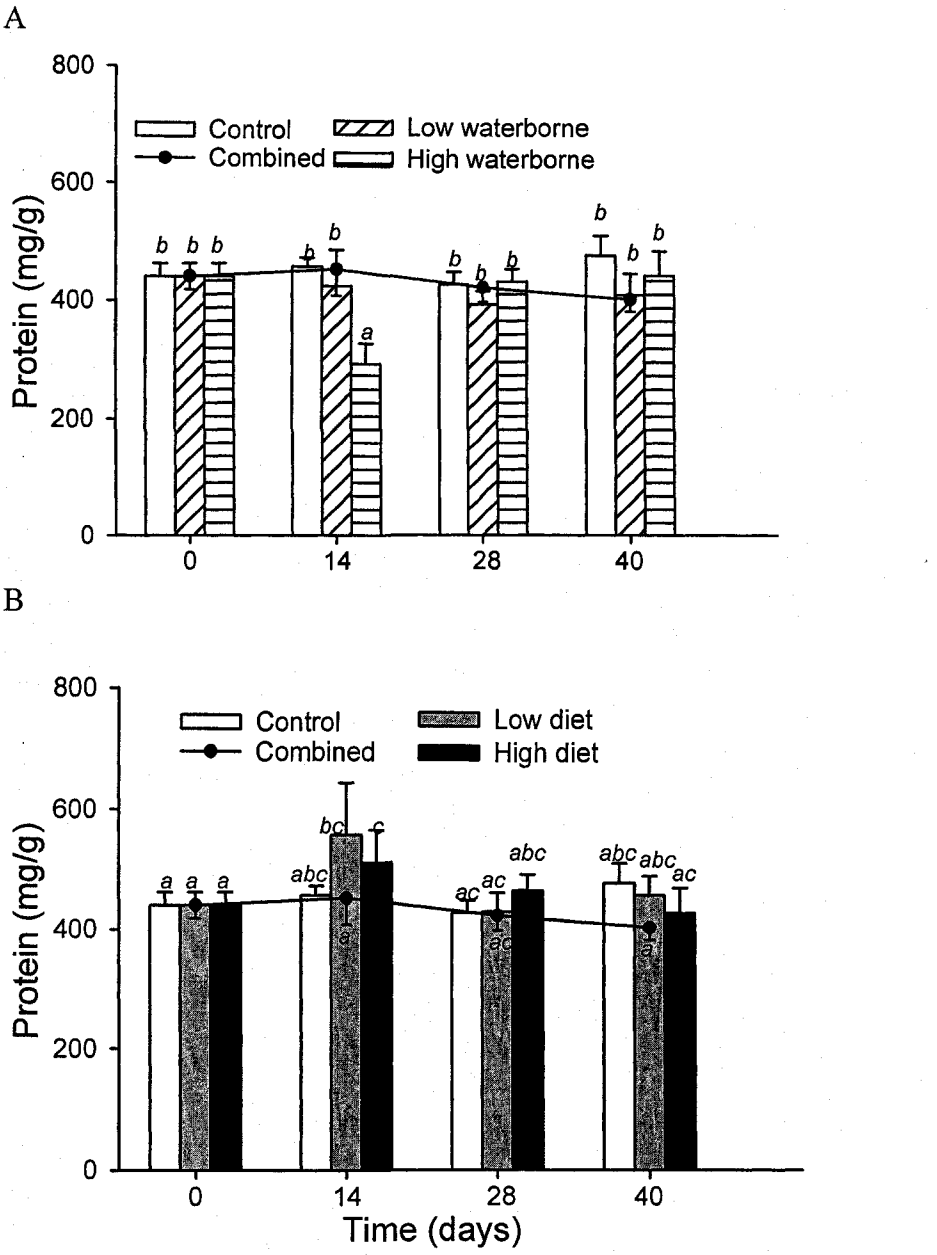


Figure 6. Total protein concentrations in juvenile rainbow trout gill. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

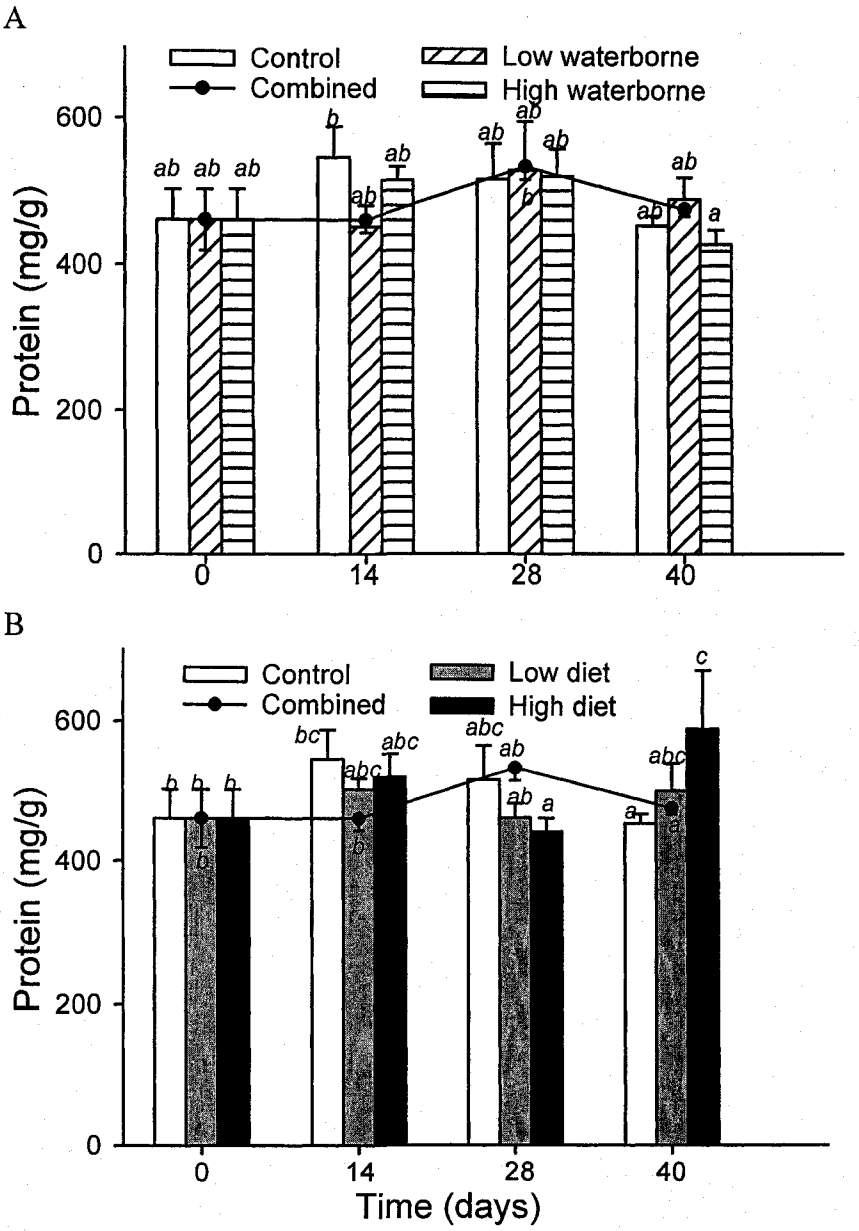


Figure 7. Total protein concentrations in juvenile rainbow trout liver. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

Protein concentrations in the intestine (Fig. 8) were about half those in the gill and the liver. In addition, these concentrations increased in a time-dependent manner from about 200 mg g^{-1}on day 0 to 350 mg g^{-1}on day 40. Main effects analysis revealed a significant effect of time ($p < 0.05$) on the concentrations. Significant increases in protein concentrations were observed on day 28 controls, day 40 low waterborne and high waterborne groups as compared to day 14 combined exposure. Similarly in the dietary exposure there was a significant increase in the concentration of proteins in day 28 controls, low dietary and day 40 low dietary groups as compared to day 14 and day 40 combined exposure. The combined exposure revealed no interaction between waterborne and dietary Zn exposure pathways.

The protein concentrations were tested statistically across the tissues i.e. gills, liver and intestine. Overall the concentration increased with time ($p < 0.001$) but there was neither an effect of Zn exposure ($p = 0.99$) nor an interaction ($p = 0.99$) between the two main effects for both exposure routes. However, intestinal protein concentrations were significantly lower ($p < 0.001$) than those of the liver and gill.

3.3 Bioaccumulation and distribution of Zn in tissues

3.3.1 *Overview*

The Zn bioaccumulation data in tissues are expressed on a dry weight basis except for the plasma, gill, liver and intestine. Tissue dry weights were obtained by drying wet tissues to a constant weight at 80°C in a convection oven. The moisture contents of the tissues were then calculated and results are shown in Tables 4 and 5. The moisture contents were

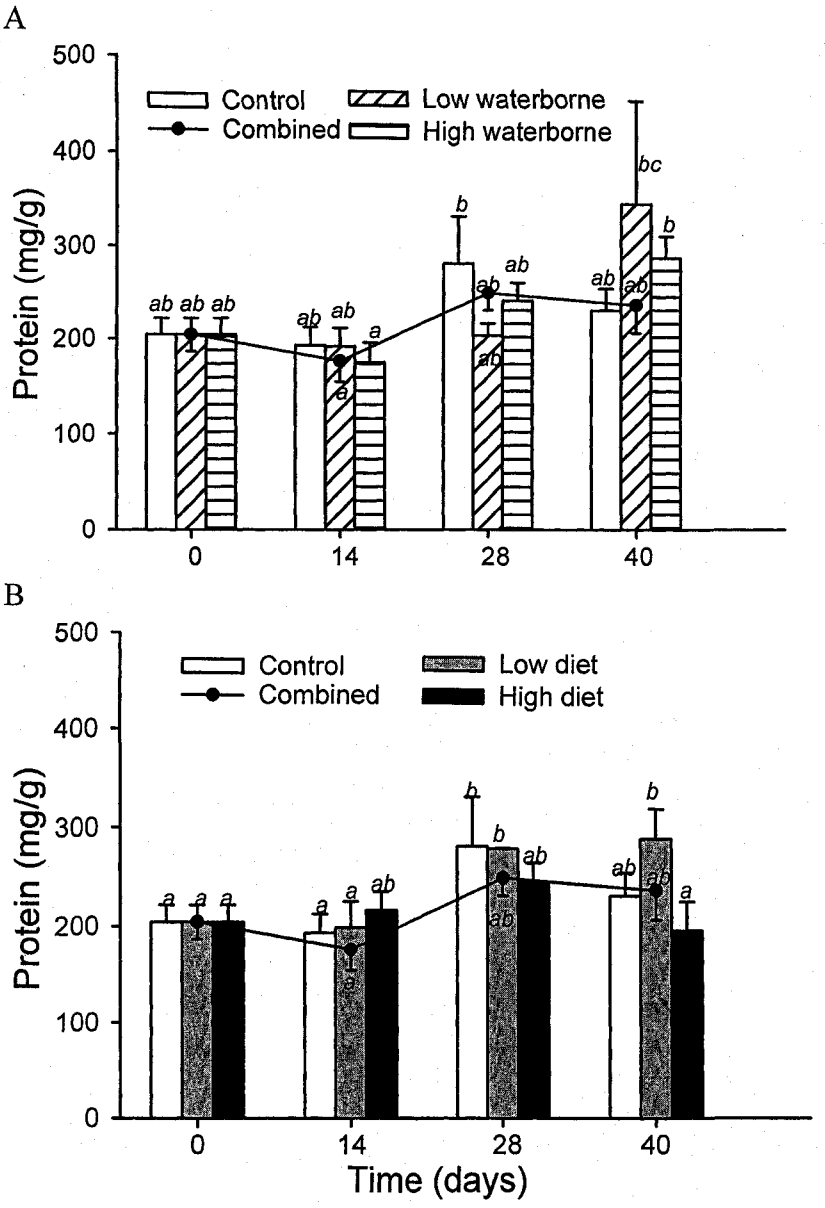


Figure 8. Total protein concentrations in juvenile rainbow trout intestine. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

Table 4: Moisture content in carcasses of juvenile rainbow trout. No significant time-dependent or treatment-dependent difference in the values were found in any of the treatments. All values are mean ± SEM, n = 12 per group.

DAY	TREATMENT					
	Control	Low Waterborne	High Waterborne	Low Dietary	High Dietary	Combined
0	77.58 ± 0.56	77.58 ± 0.56	77.58 ± 0.56	77.58 ± 0.56	77.58 ± 0.56	77.58 ± 0.56
14	77.88 ± 0.44	77.24 ± 0.27	77.59 ± 0.27	78.66 ± 0.36	74.96 ± 4.15	78.03 ± 0.45
28	76.79 ± 0.29	76.26 ± 0.19	77.36 ± 0.46	76.82 ± 0.25	77.06 ± 0.41	78.01 ± 0.73
40	76.10 ± 0.18	76.28 ± 0.32	76.05 ± 0.26	77.19 ± 0.29	76.49 ± 0.74	76.44 ± 0.32

Table 5: Moisture content in kidneys of juvenile rainbow. No significant time-dependent or treatment-dependent difference in the value were found in any of the treatments. All values are mean ± SEM, $n = 12$ per group.

DAY	TREATMENT					
	Control	**Low Waterborne**	**High Waterborne**	**Low Dietary**	**High Dietary**	**Combined**
0	80.92 ± 0.25	80.92 ± 0.25	80.92 ± 0.25	80.92 ± 0.25	80.92 ± 0.25	80.92 ± 0.25
14	81.35 ± 0.47	81.28 ± 0.27	81.49 ± 0.28	81.91 ± 0.36	79.89 ± 2.57	81.65 ± 0.33
28	80.58 ± 0.34	80.18 ± 0.21	80.15 ± 0.18	80.27 ± 0.28	81.04 ± 0.27	80.53 ± 0.20
40	80.23 ± 0.22	80.65 ± 0.30	79.62 ± 0.18	80.75 ± 0.37	80.71 ± 0.80	79.97 ± 0.17

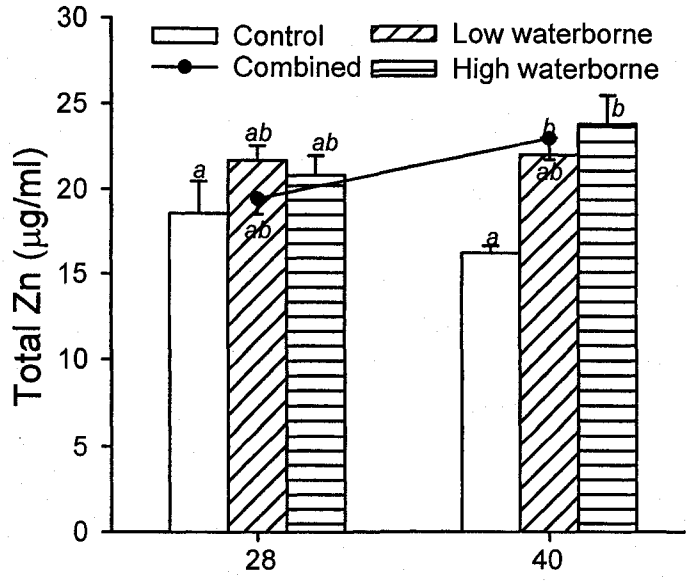
between 76-78 % and 79-81% in carcass and kidney, respectively. Neither the time nor waterborne or dietary Zn exposures altered the percentage of moisture content in carcasses and kidney.

3.3.2 *Zn concentrations in plasma and blood cells*

Plasma and blood cells Zn concentrations are reported for days 28 and 40 because small fish size on days 0 and 14 prevented collection of adequate blood for analysis. Plasma Zn concentrations increased over time ($p < 0.001$) and were significantly altered by both dietary and waterborne Zn exposure (treatment terms, $p < 0.001$). Overall the concentration increased from approximately 14 $\mu g\ ml^{-1}$ to approximately 24 $\mu g\ ml^{-1}$ on day 40 (Fig. 9A and 9B). Moreover the interaction term between the time and treatment was significant ($p < 0.001$). Waterborne Zn exposure increased the plasma Zn concentration much more than the dietary exposure with the highest plasma Zn accumulation being observed in the high waterborne Zn group, i.e. approximately 24 $\mu g\ ml^{-1}$ on day 40. The combined exposure showed that Zn accumulation in plasma was dominated by waterborne uptake. Specifically, the plasma Zn concentrations in the combined exposure were comparable to the respective waterborne exposure alone and dietary Zn exposure did not result in additional accumulation, instead the concentrations declined.

In blood cells, Zn concentrations ranged from approximately 20 to 80 $\mu g\ g^{-1}$ dry wt (Fig. 10) with the lowest concentration being observed on day 28 in the high Zn diet group, while the highest concentration occurred on day 28 in the low waterborne Zn group.

A



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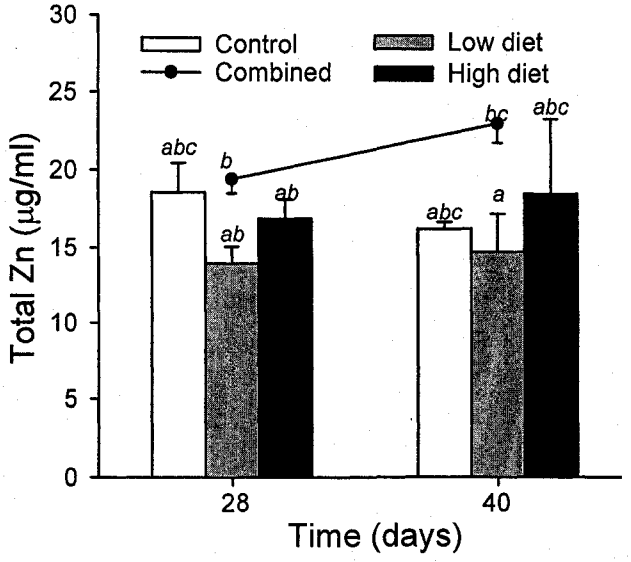
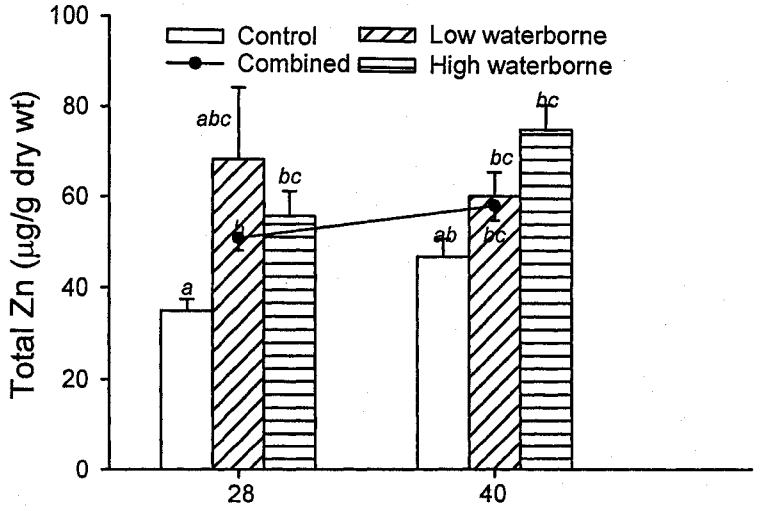


Figure 9. Zinc concentrations in plasma of juvenile rainbow trout. All values are mean ± SEM, n = 12 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

A



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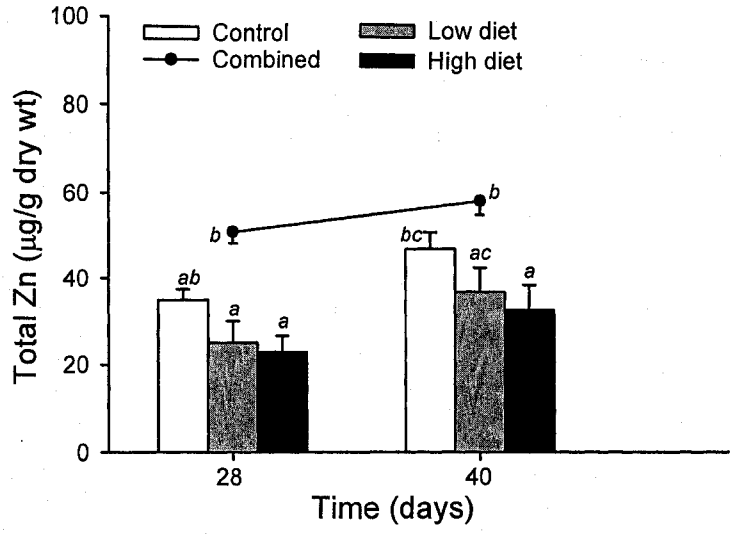


Figure 10. Zinc concentrations in blood cells of juvenile rainbow trout. All values are mean ± SEM, n = 12 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

Whereas no time ($p = 0.71$ for waterborne and $p = 0.052$ for dietary) or waterborne Zn exposure ($p = 0.22$) dependent effects were observed, blood cells zinc decreased in a dose-dependent manner in dietary exposures. In addition the interaction term was insignificant ($p = 0.41$ for waterborne and $p = 0.20$ for dietary) in the waterborne and dietary exposures. It was apparent from the combined exposure that waterborne uptake dominated Zn accumulation in blood cells. Whereas Zn concentrations in the combined exposure were comparable to the respective waterborne exposure alone, dietary Zn exposure did not result in additional accumulation; instead the concentrations declined.

3.3.3 *Zn accumulation in carcass and kidney*

Figure 11 shows the Zn concentrations in the carcass. Carcass Zn concentrations ranged from 120 to 190 $\mu g\ g^{-1}$ dry wt in all the treatments and significantly increased with time ($p < 0.001$ in the waterborne and dietary Zn exposures). Specifically, Zn concentrations increased with time in a dose-dependent manner from day 0 to day 40. In the dietary exposures, concentrations in the high diet group were significantly higher than the respective controls on days 28 and 40. The interaction term between time and Zn exposures was, however, not significant ($p = 0.25$ for waterborne and $p = 0.41$ for dietary). The combined exposure revealed no additional accumulation above the concentration attributable to the respective singular waterborne or dietary exposures. In the kidney (Fig. 12) Zn concentrations ranged between 120 and 250 $\mu g\ g^{-1}$ dry wt with the highest levels occurring in the high Zn diet group on day 14 and the lowest occurring in the controls on day 40. This translated into a significant overall increase with time ($p < 0.001$) and a significant dose-dependent increase ($p < 0.05$ for waterborne and $p < 0.01$

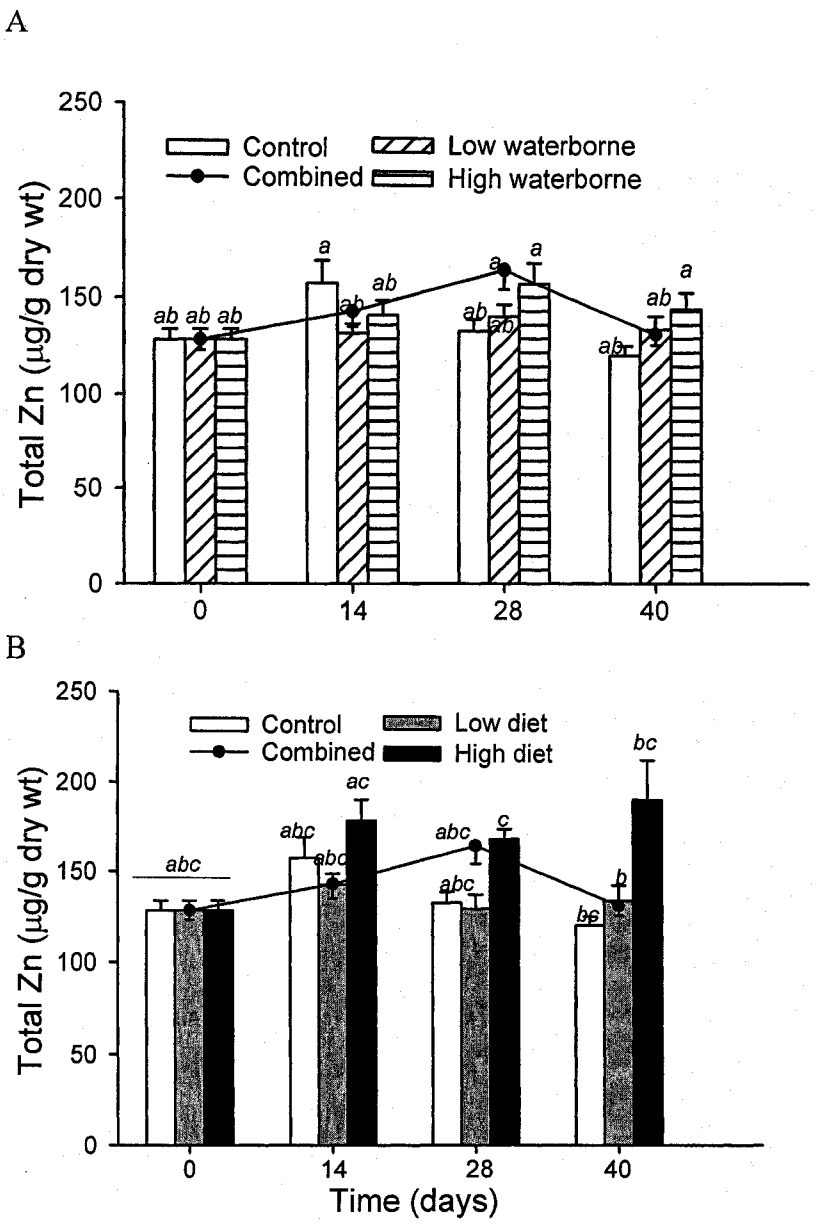
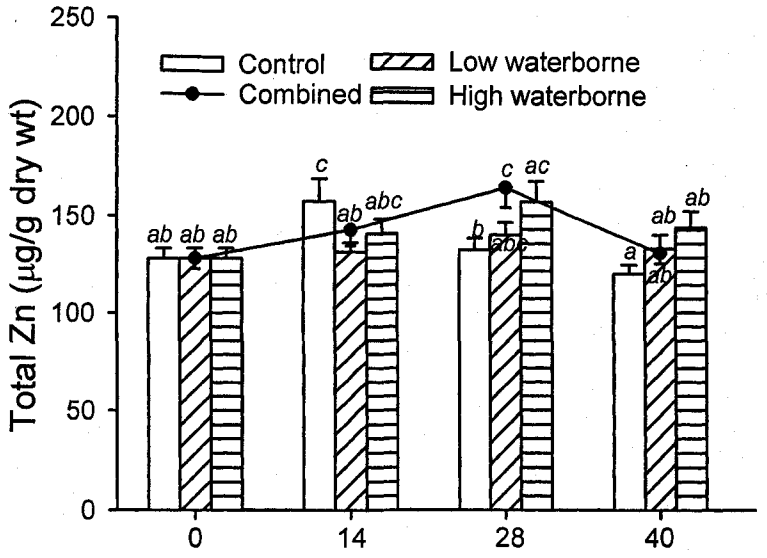


Figure 11. Zinc concentrations in carcass of juvenile rainbow trout. All values are mean ± SEM, n = 12 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

A



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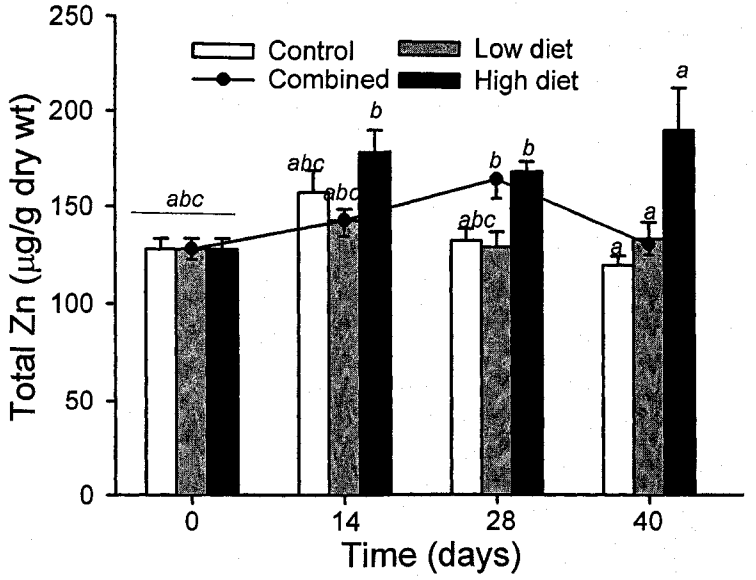


Figure 11. Zinc concentrations in carcass of juvenile rainbow trout. All values are mean ± SEM, n = 12 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

for dietary) of the Zn concentrations. The interaction terms were not significant ($p = 0.33$ for waterborne and $p = 0.08$ for dietary). In the combined exposure no additional accumulation above the concentration attributable entirely to the respective singular waterborne or dietary exposure was observed.

3.3.4 *Overall tissue Zn distribution*

Table 6 compares the Zn concentration ($\mu g\ g^{-1}$ dry wt) and content (μg tissue^{-1}) among rainbow trout tissue. The gill, liver and intestine are included although the detailed results for these organs are reported on a per mg protein basis for comparison with subcellular Zn data. The tissue Zn concentrations were: intestine > gill > kidney ≥ liver > carcass > blood cells > plasma. Proportionally however, the majority of the Zn partitioned in the carcass (84-90%), followed by the intestine (3-7%), gill (4-6%), liver (1%) and kidney 1%.

3.4 Subcellular distribution (speciation) of Zn

3.4.1 *Branchial Zn*

Whole gill Zn concentrations are shown in Figure 13. The Zn concentrations increased significantly over time ($p < 0.001$) from an initial value of 1000 ng mg^{-1} (1 $\mu g\ mg^{-1}$) protein on day 0 to 3000 ng mg^{-1} protein on day 40. The means of the low water Zn, high water Zn, high Zn diet and combined exposure groups were all significantly higher on day 40 compared to the day 0 controls. The treatment effect ($p < 0.01$) was significant among the waterborne exposure groups but not for the dietary groups ($p = 0.09$). The interactions term between time and treatment was also

Table 6: Concentrations (μg g^{-1}) and content (μg $tissue^{-1}$) of Zn in tissues of juvenile rainbow trout. All values are mean ± SEM. The values written in brackets are SEMs. Values on the left side of the columns for both concentrations and contents represent controls whereas those on the right side represent the highest mean concentrations observed in Zn-exposed groups regardless of the intervals and treatments.

Organ/Tissue	Concentrations (μg g^{-1} dry wt)	Contents (μg $tissue^{-1}$)	% of total
Gill	490.80 (20.50) – 1013.37 (120.36)	16.85 (0.40) – 61.94 (4.85)	4 – 6
Liver	112.61 (2.29) – 234.81 (112.70)	4.56 (0.70) – 12.79 (0.47)	1
Intestine	629.82 (73.60) – 1918.32 (830.94)	11.66 (0.99) – 70.87 (3.43)	3 – 7
Kidney	117.74 (11.01) – 241.43 (35.57)	3.27 (0.27) – 9.08 (1.19)	1
Carcass	119.80 (4.88) – 189.54 (22.21)	334.19 (20.80) – 834.15 (52.74)	84 – 90
Plasma	16.18 (0.45) – 23.78 (1.67)	-	-
Blood cells	34.92 (2.49) – 68.16 (16.11)	-	-

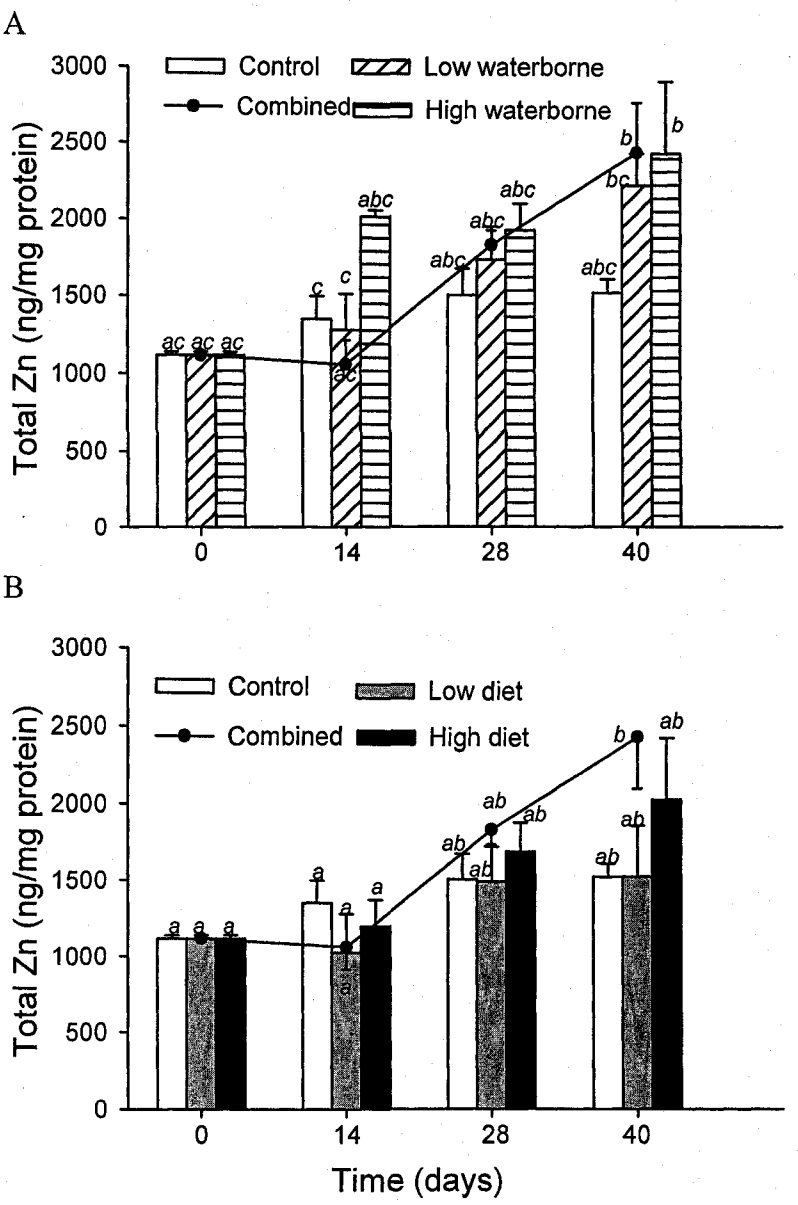


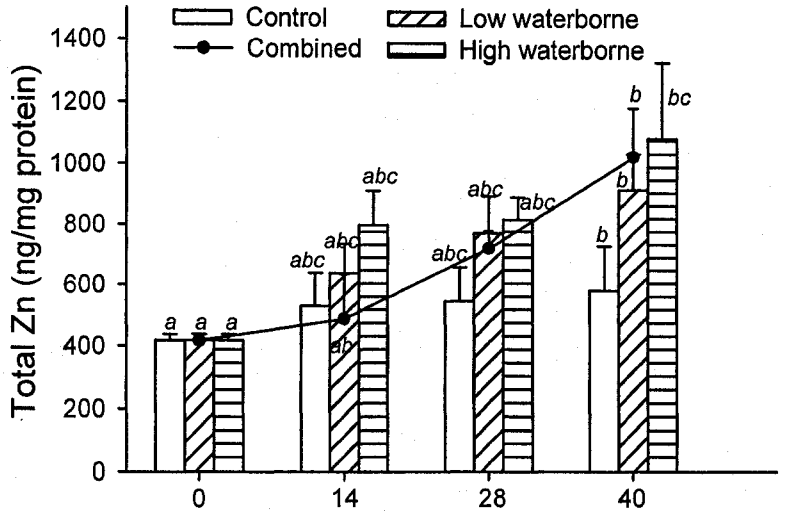
Figure 13. Branchial concentrations of Zn in juvenile rainbow trout. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

insignificant ($p = 0.07$ for waterborne and $p = 0.16$ for dietary). Overall the highest Zn concentrations were observed in combined exposure and were attributable mainly to waterborne uptake.

Zinc concentrations in branchial subcellular fractions are shown in Figures 14 to 19. In the branchial nuclei-cell debris fraction (Fig. 14) Zn concentrations increased with time ($p < 0.001$). The treatment effect ($p < 0.05$) was significant only among the waterborne exposure groups but not the dietary ($p = 0.16$) groups. The interactions term between time and treatment was also not significant ($p = 0.51$ for waterborne and $p = 0.57$ for dietary). Generally Zn concentrations ranged from 417 to 1020 ng mg^{-1} protein and, overall, this fraction contained the highest concentration of Zn (high water Zn group, day 40) among the branchial subcellular fractions. In the combined exposure, Zn accumulation was attributable mainly to waterborne uptake.

Branchial mitochondrial fraction (Fig. 15) Zn concentrations increased significantly with time ($p < 0.01$ for waterborne and $p < 0.01$ for dietary) with the concentrations increasing from approximately 50 to 120 ng mg^{-1} protein. The treatment ($p = 0.07$ for waterborne and $p = 0.20$ for dietary) and interaction ($p = 0.32$ for waterborne and $p = 0.43$ for dietary) terms were, however, not significant. In the combined exposure Zn accumulation was dominated by waterborne uptake and there was no interaction because dietary exposure did not cause additional Zn accumulation.

A



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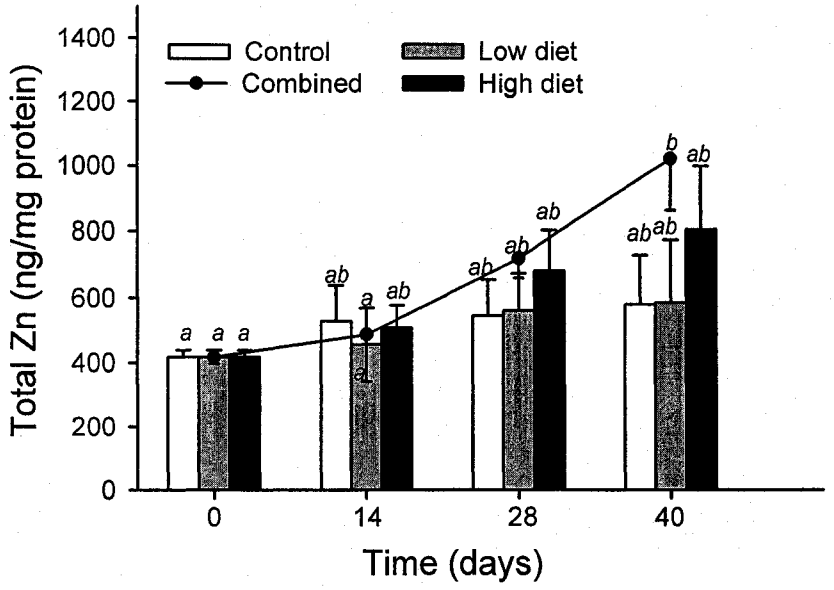

Figure 14. Zinc concentrations in nuclei-cell debris fraction of gills. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

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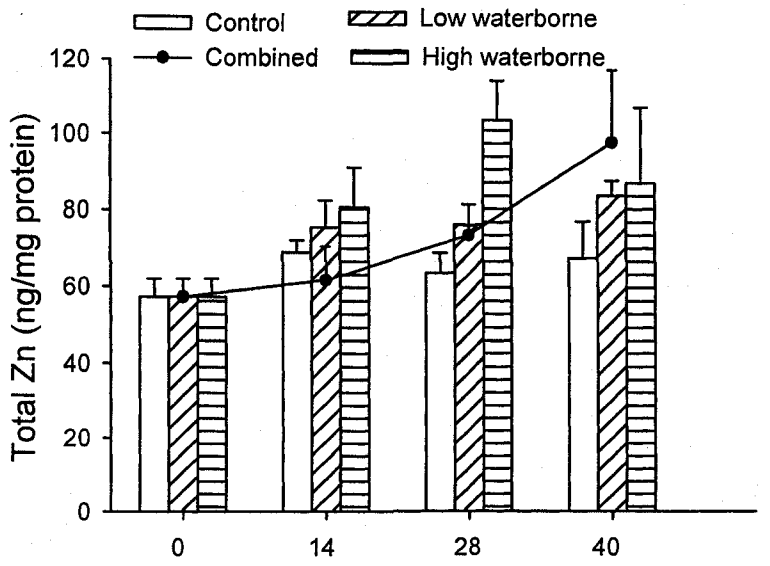

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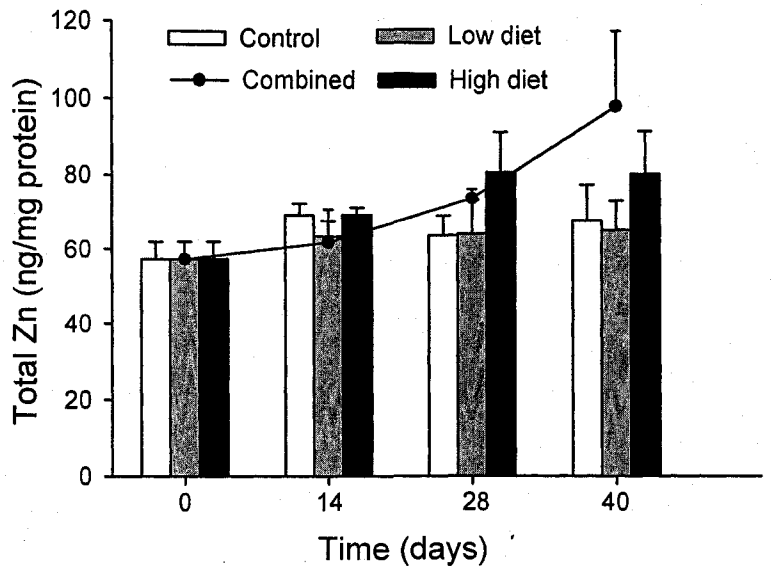


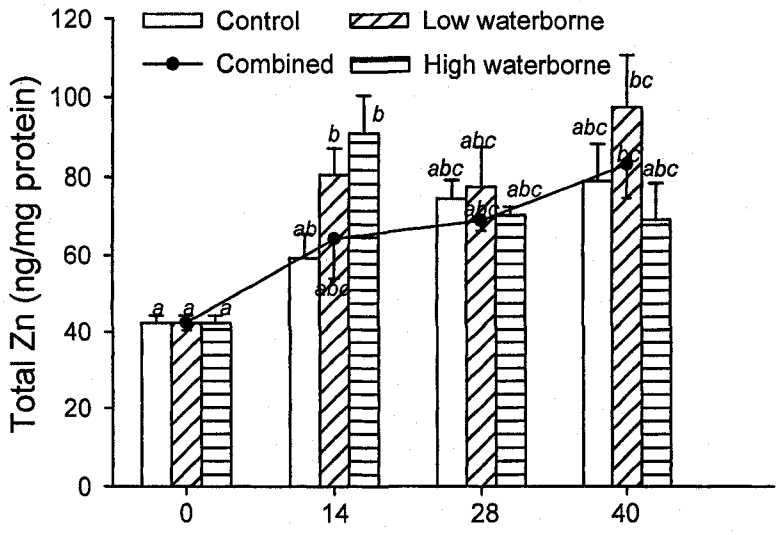
Figure 15. Zinc concentrations in mitochondrial fraction of gills. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure.

Zinc concentrations in gill microsomes-lysosomes fraction (Fig. 16) were significantly influenced by the duration of exposure to Zn ($p < 0.001$) with the concentration increasing from approximately 40 to 120 ng mg^{-1} protein. The concentrations were increased with time in all the groups including controls although the treatment ($p = 0.16$ for waterborne and $p = 0.19$ for dietary) and interaction ($p = 0.10$ for waterborne and $p = 0.91$ for dietary) terms were insignificant. In the combined exposure Zn concentrations were comparable to the single route exposures suggesting no interaction between waterborne and dietary Zn uptake.

Branchial HDP fraction (Fig. 17) Zn concentrations significantly increased with time ($p < 0.001$) from approximately 200 ng mg^{-1} protein on day 0 to approximately 450 ng mg^{-1} protein on day 40 in both the waterborne (Fig. 17A) and dietary (Fig. 17B) exposures. However, the treatment ($p = 0.55$) and interaction ($p = 0.12$) terms were not significant for the waterborne exposures. In contrast, the treatment term ($p < 0.05$) was significant for the dietary exposures. For this fraction, the combined exposure showed that there was no interaction between waterborne and dietary Zn uptake.

In the branchial HSP fraction (Fig. 18) Zn concentrations increased from 50–100 ng mg^{-1} protein to 150–250 ng mg^{-1} protein. Both the time ($p < 0.001$) and treatment ($p < 0.05$ for waterborne and $p < 0.01$ for dietary) terms were significant. Generally, the concentrations increased with time from day 0 to day 40 in a dose-dependent manner. In addition the interaction term between the time and treatment was significant ($p <$

A



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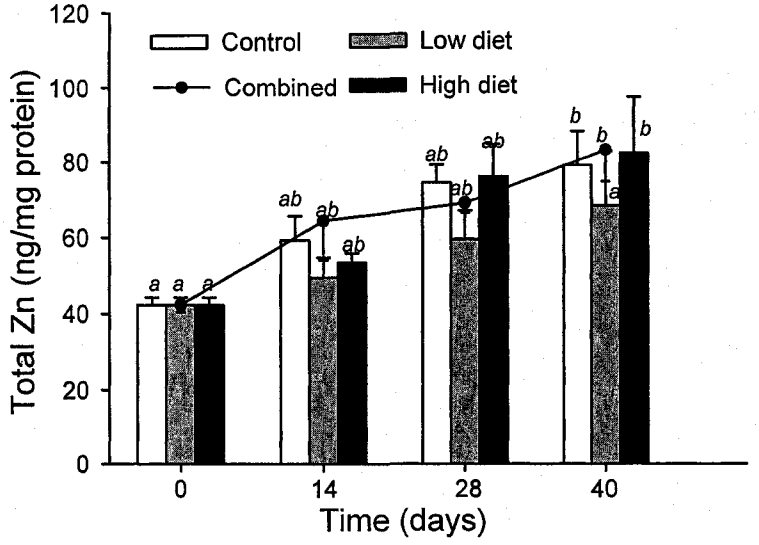

Figure 16. Zinc concentrations in microsomes-lysosomes fraction of gills. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

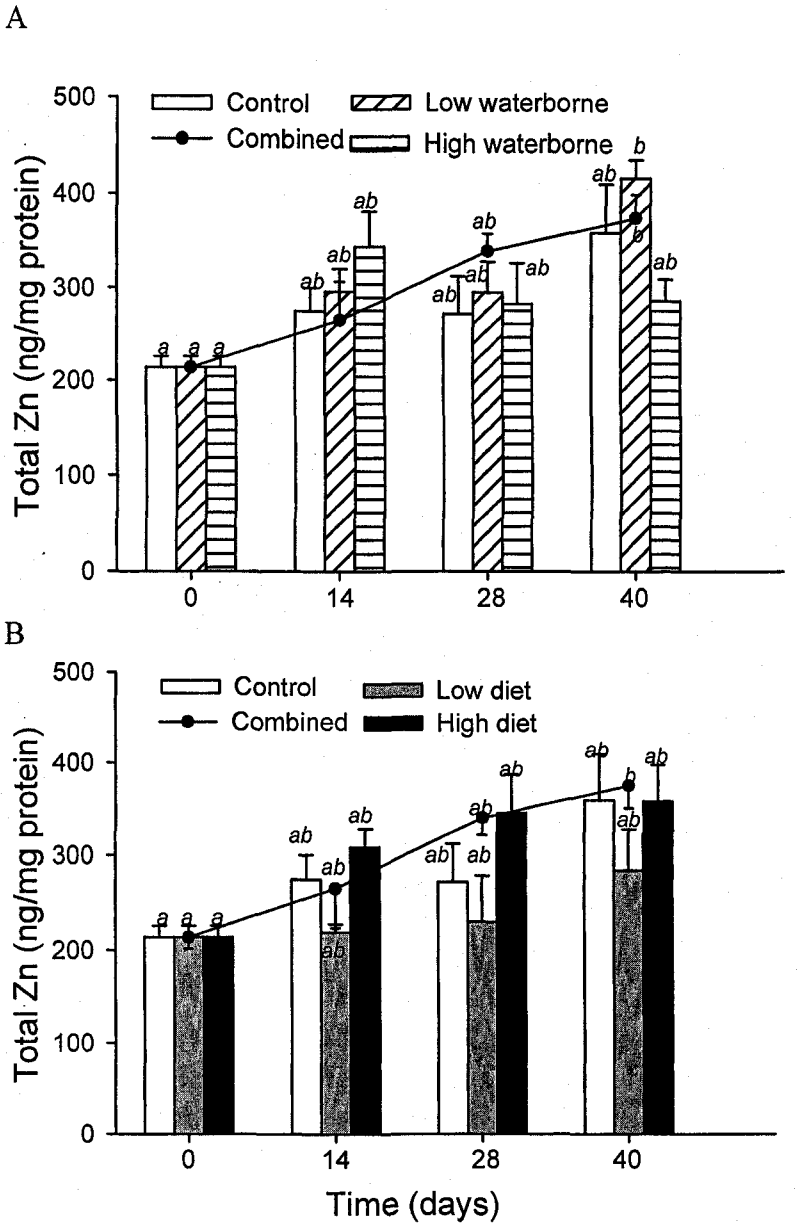

Figure 17. Zinc concentrations in heat-denaturable protein fraction of gills. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

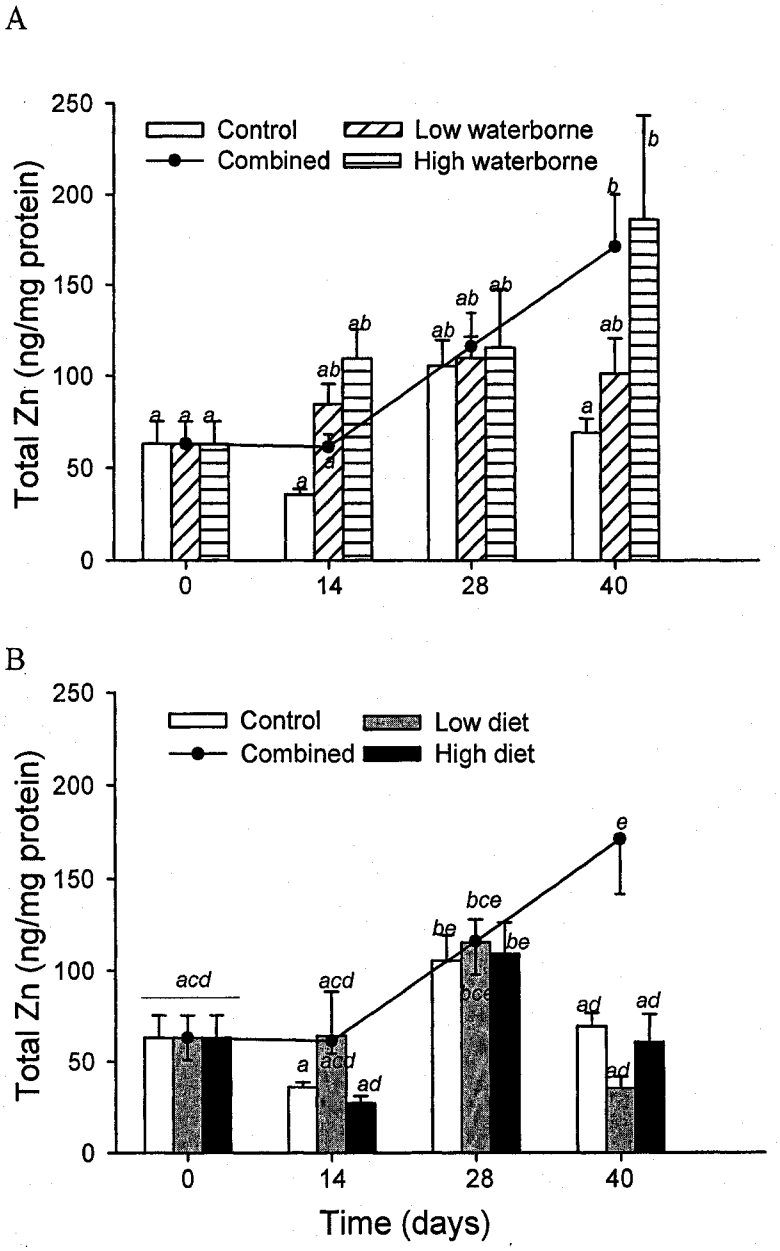

Figure 18. The concentrations of Zn in heat-stable protein fraction of gills. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

0.01) in the dietary but not in the waterborne ($p = 0.11$) exposures. In this fraction there was additional accumulation associated with the combined exposure relative to the comparable singular waterborne or dietary exposures, with a dominance of waterborne uptake.

Gill NaOH-resistant granules Zn concentrations (Fig. 19) increased with time ($p < 0.001$ for waterborne and $p < 0.01$ for dietary). Overall in this fraction the Zn concentration ranged from 60 to 180 ng mg^{-1} protein. The highest concentration was observed in the low waterborne group on day 40. The treatment effect ($p < 0.05$) was significant among the waterborne exposure groups but not the dietary groups ($p = 0.05$). The interactions terms between time and treatment ($p = 0.21$ for waterborne and $p = 0.08$ for dietary) were not significant. Generally dietary Zn did not accumulate (above background) in this fraction and the combined exposure confirmed that Zn accumulated mainly from the water.

3.4.2 Hepatic Zn

Whole liver Zn concentrations are shown in Figure 20. Overall the Zn concentrations increased significantly over time ($p < 0.001$) from an initial value of approximately 170 ng mg^{-1} protein on day 0 to approximately 330 ng mg^{-1} protein on day 40 and appeared to plateau after day 28. The treatment term was insignificant ($p = 0.28$) for the waterborne in contrast to the dietary ($p < 0.05$) exposure. The interaction terms between time and treatment also were not significant ($p = 0.17$ for waterborne and $p = 0.66$ for dietary). The concentrations of Zn were significantly increased on day 14 for

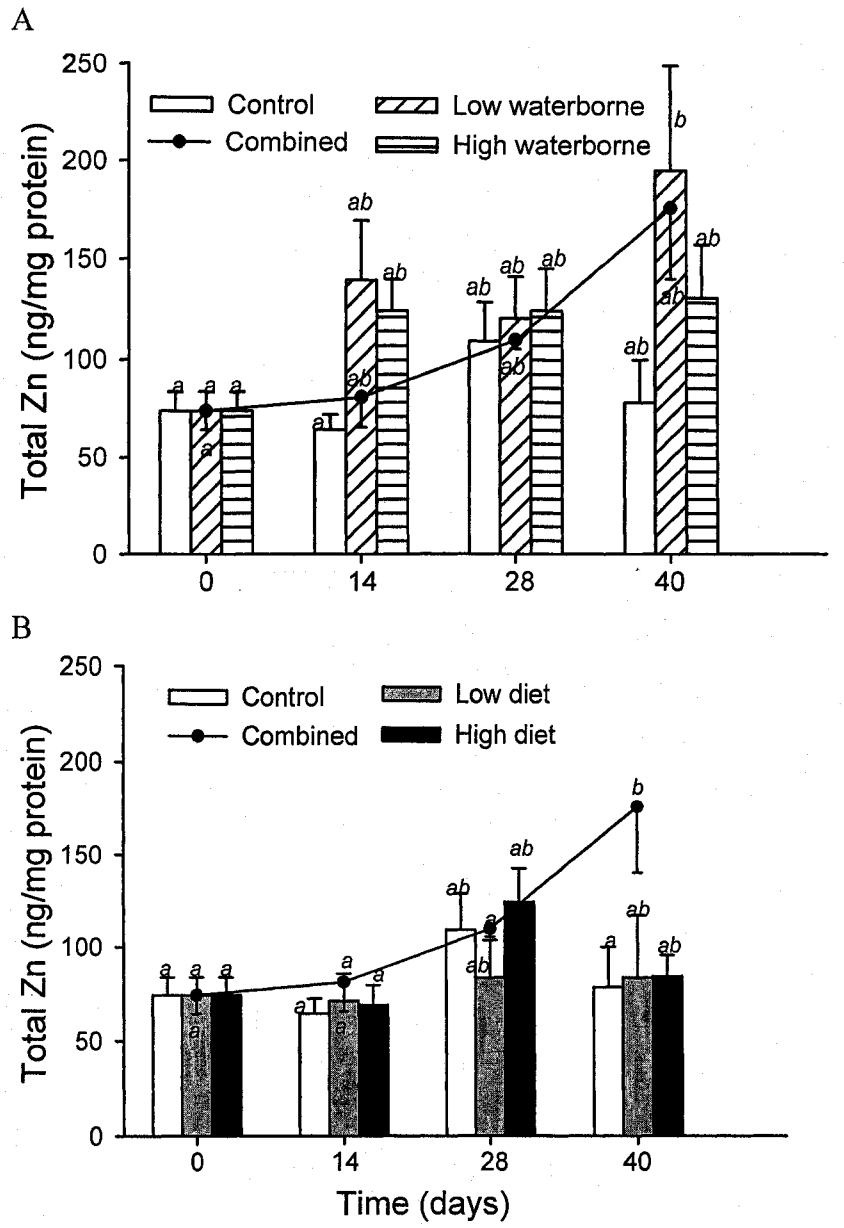

Figure 19. Zinc concentrations in NaOH-resistant granule fraction of gills. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

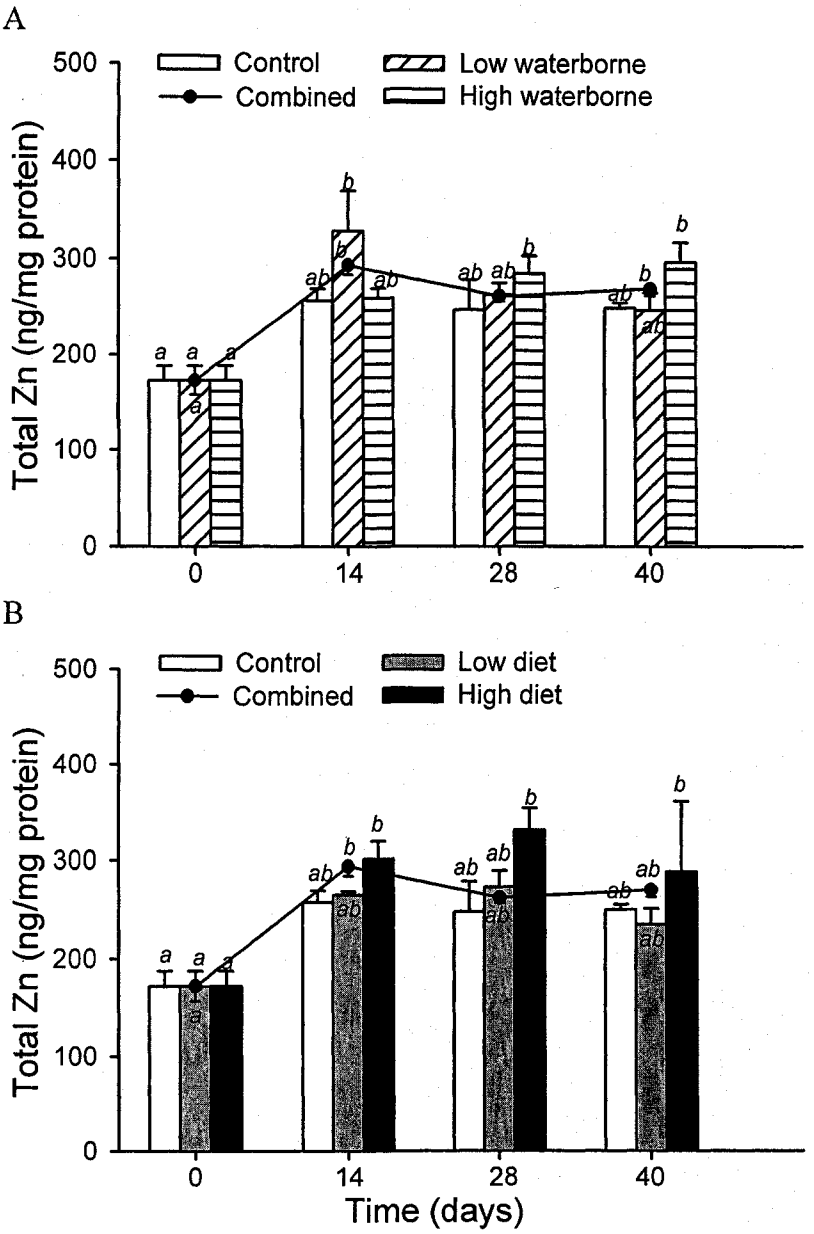

Figure 20. Zinc concentrations in liver of juvenile rainbow trout. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

low water Zn, high Zn diet and combined treatments, day 28 for the high water Zn and high Zn diet treatments, and day 40 for high water Zn, high Zn diet and combined exposure as compared to day 0 controls. In addition, the combined exposure revealed no additional accumulation beyond the concentration attributable to the respective singular waterborne or dietary exposure.

Zinc concentrations in hepatic subcellular fractions are shown in Figures 21 to 26. It was observed that Zn concentrations in hepatic nuclei-cell debris fraction (Fig. 21) were neither altered by time ($p = 0.63$ for waterborne and $p = 0.43$ for dietary) nor by waterborne ($p = 0.41$) or dietary ($p = 0.27$) Zn exposures. The interaction terms between time and treatment were also not significant ($p = 0.57$ for waterborne and $p = 0.71$ for dietary). Generally, Zn concentration in all the groups remained within a narrow range of 30–60 ng mg^{-1} protein with no significant differences among the treatments. In addition, the combined exposure showed no additional accumulation above the concentration attributable to the respective singular waterborne or dietary exposure.

Hepatic mitochondrial Zn concentrations remained relatively stable in all the groups although the day 28 low water and high water Zn groups had significantly lower concentrations than day 0 controls (Fig. 22). The highest concentration was recorded in the high Zn diet group on day 14. The treatment effect ($p < 0.05$) was significant among the dietary exposure groups but not among the waterborne groups ($p = 0.19$).

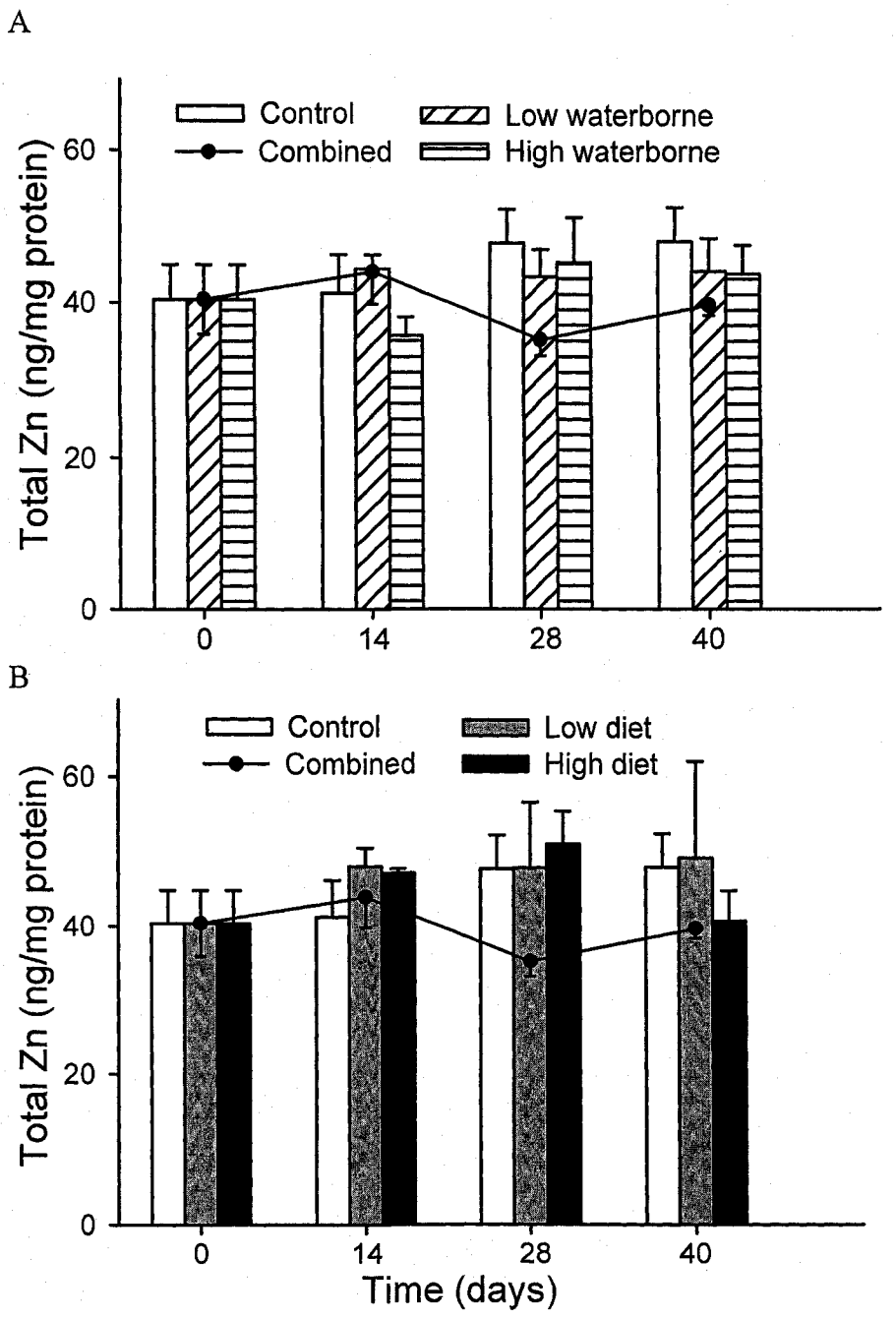

Figure 21. Zinc concentrations in nuclei-cell debris fraction of liver. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure.

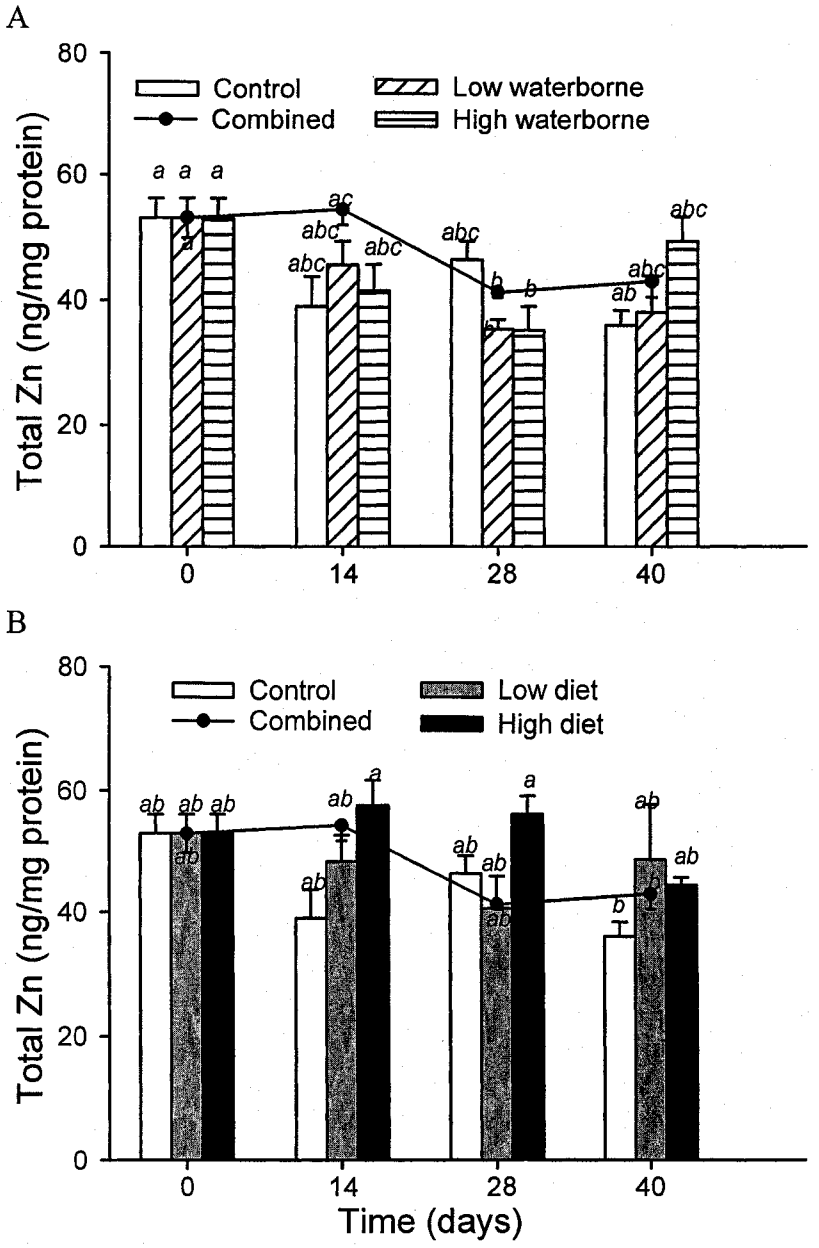

Figure 22. Zinc concentrations in mitochondria of liver. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

A significant interaction between time and treatment ($p < 0.05$) also was observed for both exposure pathways. In the combined exposure there was no additional accumulation beyond that attributable to the respective singular waterborne or dietary exposure.

Zinc concentrations in the microsomes-lysosomes fraction (Fig. 23) were significantly influenced by time ($p < 0.01$) with the concentration increasing from 14 ng mg^{-1} protein to 26 ng mg^{-1} protein among the Zn-exposed fish. The treatment ($p = 0.25$ for waterborne and $p = 0.27$ for dietary) and interaction ($p = 0.89$ for waterborne and $p = 0.85$ for dietary) terms were insignificant. No additional accumulation above that attributable to the respective singular waterborne or dietary exposure was observed in the combined exposure although there was a consistent, albeit insignificant ($0.05 < p < 0.10$), additive trend.

Hepatic HDP Zn concentrations (Fig. 24) changed significantly with time ($p < 0.001$). The Zn concentration increased from 20 ng mg^{-1} protein on day 0 to 40 ng mg^{-1} protein on day 40 in the waterborne exposures and 28 ng mg^{-1} protein in the dietary exposures. The concentrations of Zn in the controls also were increased with time. The treatment ($p = 0.21$) and interaction ($p = 0.12$) terms were not significant for waterborne exposures. In contrast, the treatment term ($p < 0.05$) was significant for the dietary exposures. The highest Zn concentrations were observed in the combined exposures and an additive trend ($p = 0.05$) of waterborne and dietary uptake was observed on day 40.

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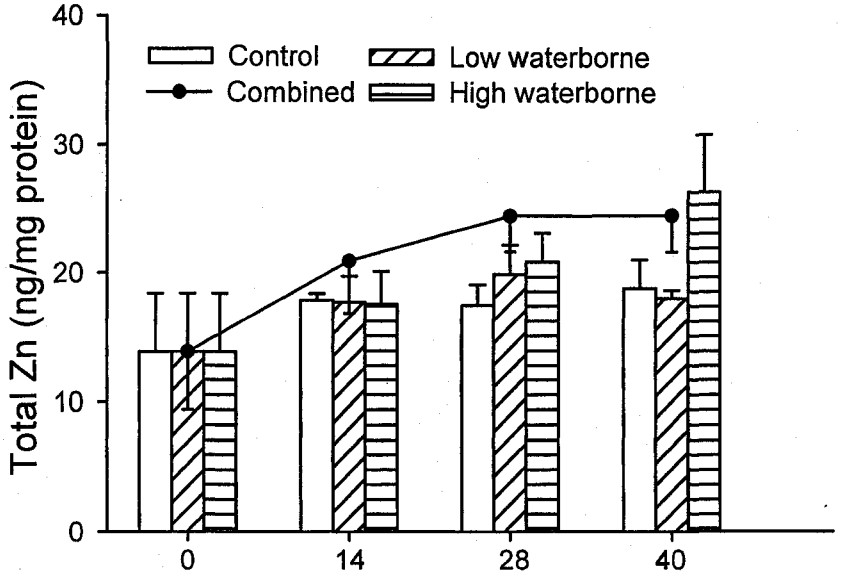

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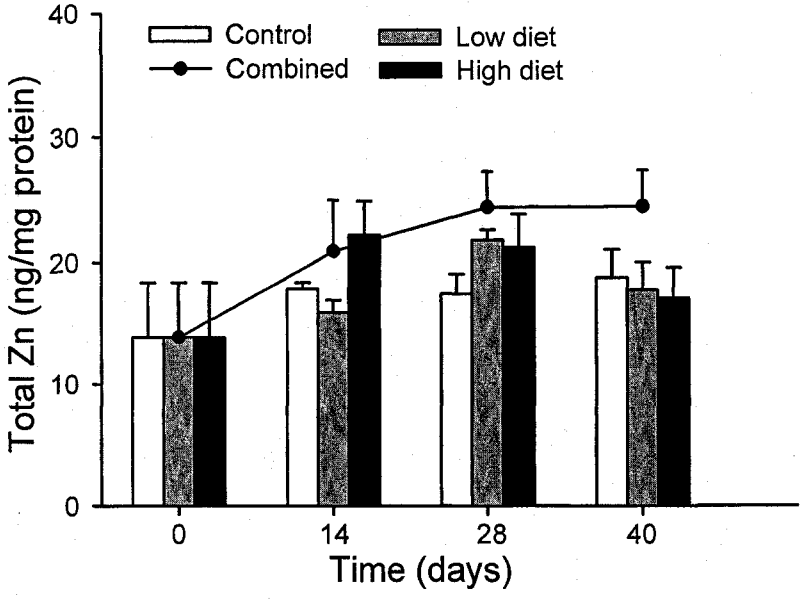

Figure 23. Zinc concentrations in microsomes-lysosomes fraction of liver. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure.

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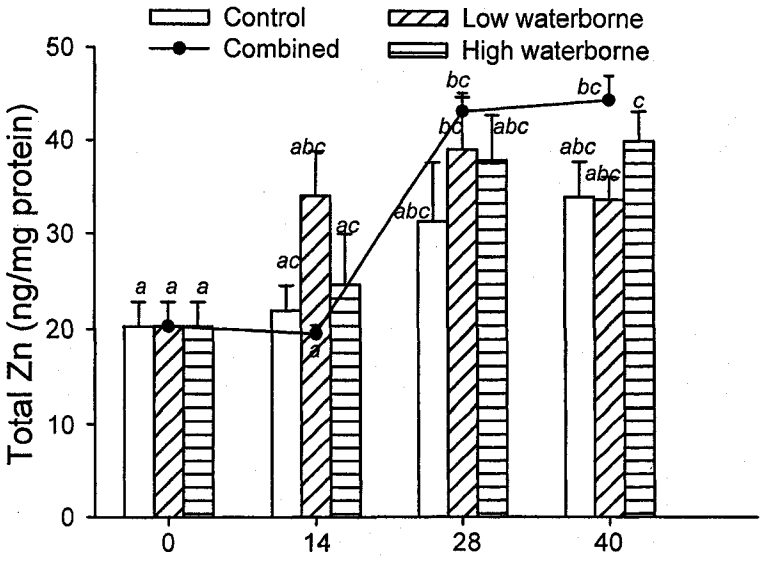

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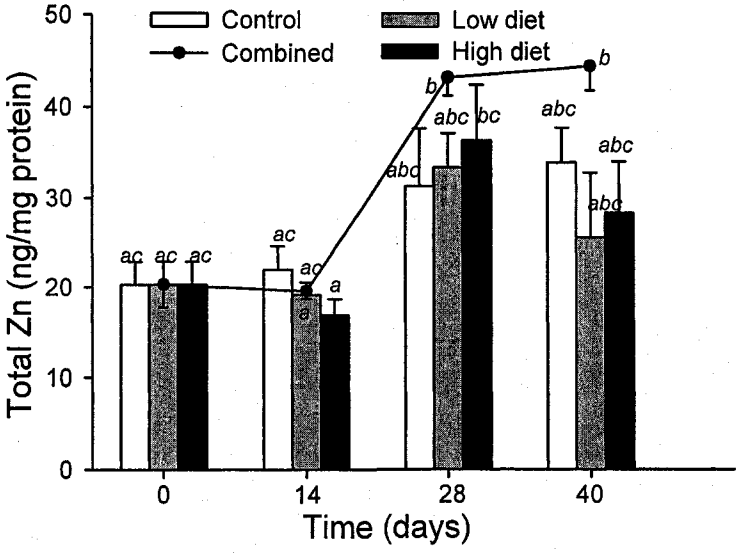


Figure 24. Zinc concentrations in heat-denaturable protein fraction of liver. All values are mean ± SEM, n = 4 per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

Figure 25 shows the Zn concentrations in the hepatic HSP fraction. The concentrations ranged between 27 and 66 ng mg^{-1} protein and both the time ($p < 0.01$) and treatment ($p < 0.05$ for waterborne and $p < 0.001$ for dietary) terms were significant. In addition the interaction term ($p < 0.05$) between the time and treatment for both exposure routes was significant. Overall this fraction recorded the highest concentration of Zn (day 28 high diet group) among all the hepatic fractions isolated. In the combined exposure Zn uptake was biphasic with the concentration peaking on day 14 then declining to control values on days 28 and 40. There was no additional accumulation associated with the combined exposure relative to the comparable singular waterborne or dietary exposure. Relative to the comparable singular dietary exposure, the combined exposure attained consistently lower Zn concentrations in HSP fraction.

In the NaOH-resistant granules a significant time effect ($p < 0.001$) and interaction ($p < 0.001$ for waterborne and $p < 0.05$ for dietary) between time and exposure were observed (Fig. 26). Overall in this fraction the Zn concentrations ranged from 11 ng mg^{-1} to 25 ng mg^{-1} protein and increased with time in all the exposures except for the combined and day 40 high Zn diet groups in which the accumulation patterns were biphasic. Specifically, Zn concentration in the combined exposure peaked on day 14 and gradually decreased to control values by day 40. A similar pattern was evident for the high diet treatment but the peak was attained on day 28. There were no significant differences between the respective singular exposure and the combined exposure.

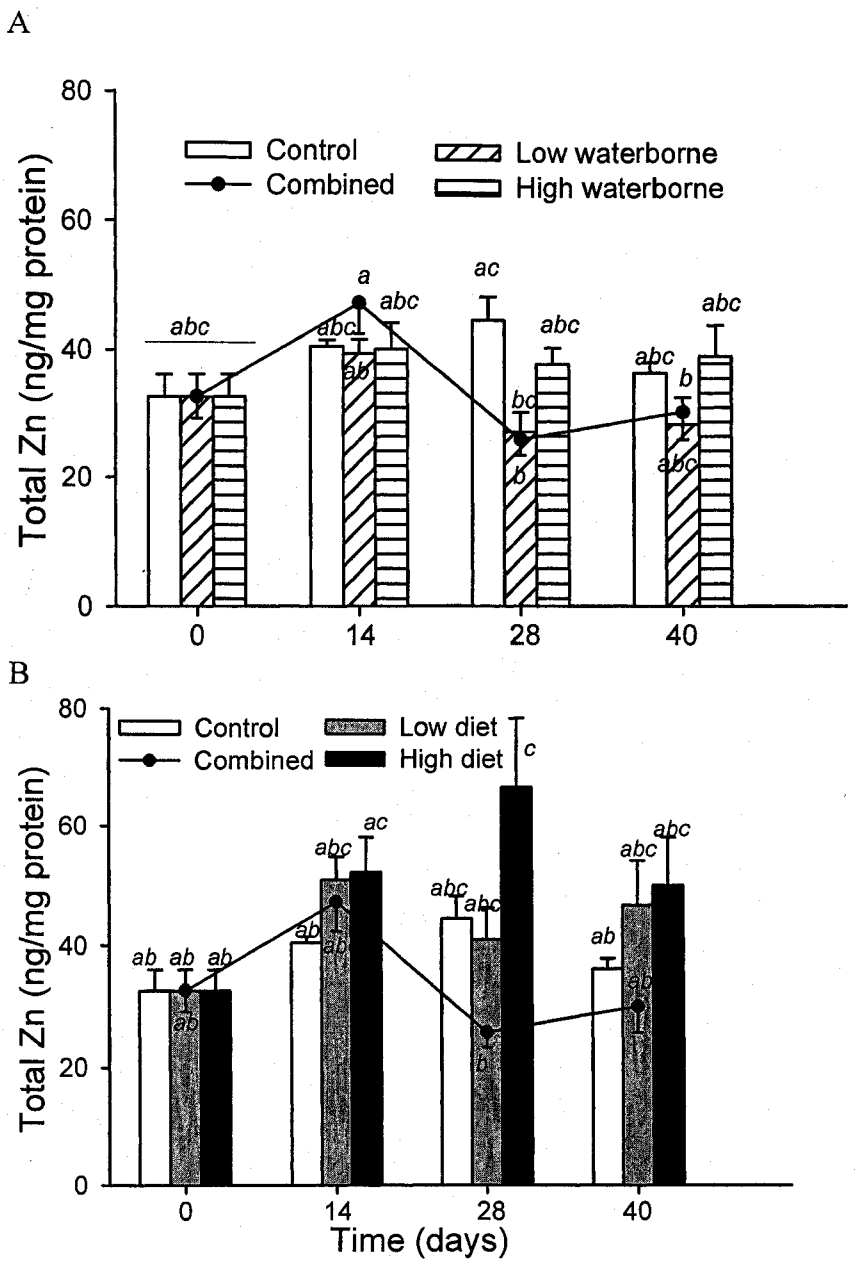


Figure 25. The concentrations of Zn in heat-stable protein fraction of liver. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

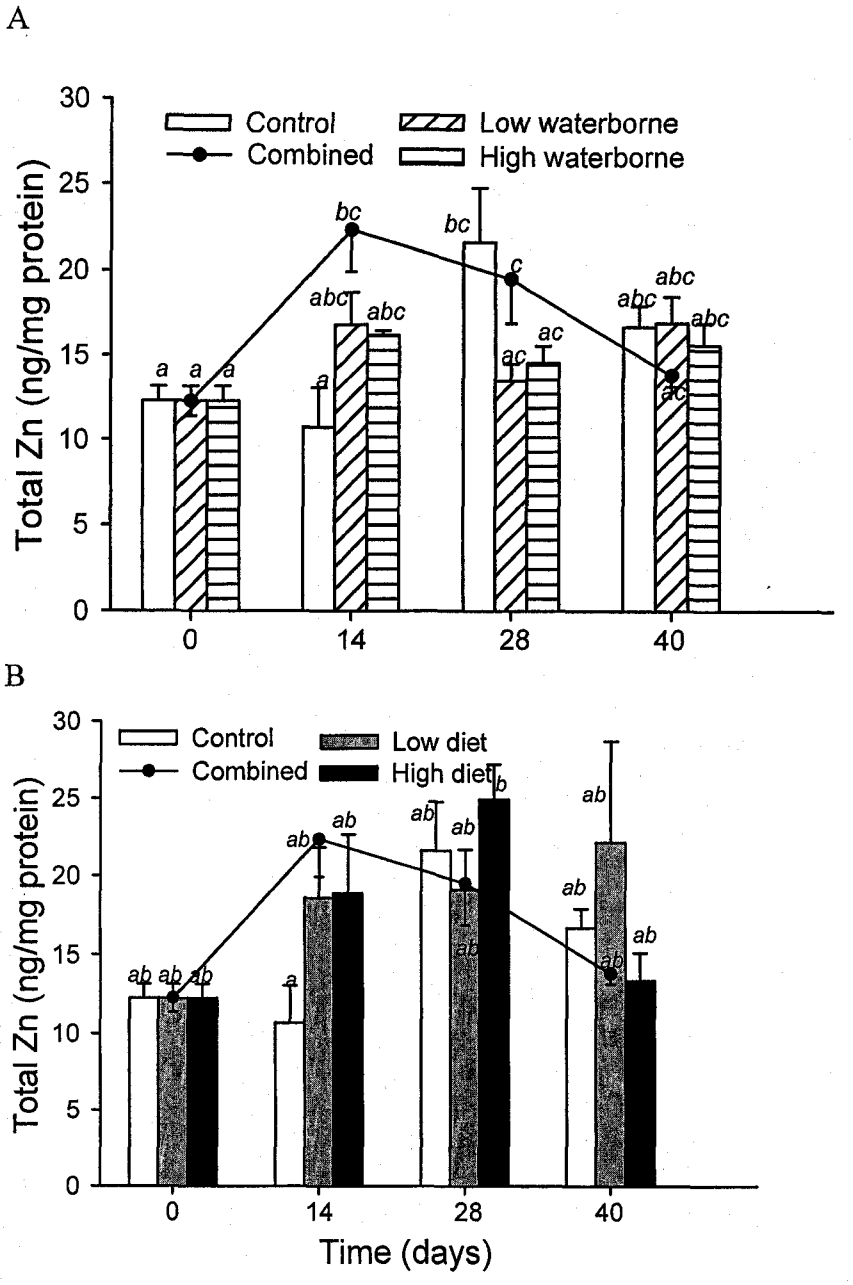


Figure 26. Zinc concentrations in NaOH-resistant granules of liver. All values are mean ± SEM, n = 4 per group. A: open bars, control; medium hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

3.4.3 Intestinal Zn

Whole intestinal Zn concentrations are shown in Figure 27. Overall there were no dramatic changes in the Zn concentrations over time. From an incipient value of approximately 2800 ng mg^{-1} protein on day 0, the concentrations peaked on day 14 with the highest value being approximately 4200 ng mg^{-1} protein in the combined exposure group. Thereafter the concentrations decreased particularly in the low diet group whose concentrations on day 28 were significantly lower than those of the combined exposure group on day 14. The treatment term was significant ($p < 0.05$) for the dietary but not the waterborne ($p = 0.90$). In addition, the interaction terms between time and treatment ($p = 0.98$ for waterborne and $p = 0.61$ for dietary) were insignificant. The combined exposure revealed no additional accumulation beyond the concentration attributable to the respective singular dietary exposure. Figures 28 to 33 show the Zn concentrations in intestinal subcellular fractions.

Zinc concentrations in intestinal nuclei-cell debris fraction (Fig. 28) were variable among the exposure groups. Generally the Zn concentration; ranged from 590 to 1860 ng mg^{-1} protein with no significant treatment terms ($p = 0.64$ for waterborne and $p = 0.43$ for dietary). Peak accumulation occurred on day 14 in the combined exposure group and the lowest accumulation was associated with the low Zn diet group on day 28. This fraction contained the highest concentration of Zn among all the intestinal fractions isolated, and also more than the concentrations seen in the same fraction for liver and gill. In the combined exposure Zn accumulation was biphasic with the concentration peaking on day 14 then declining on day 28. There was no additional

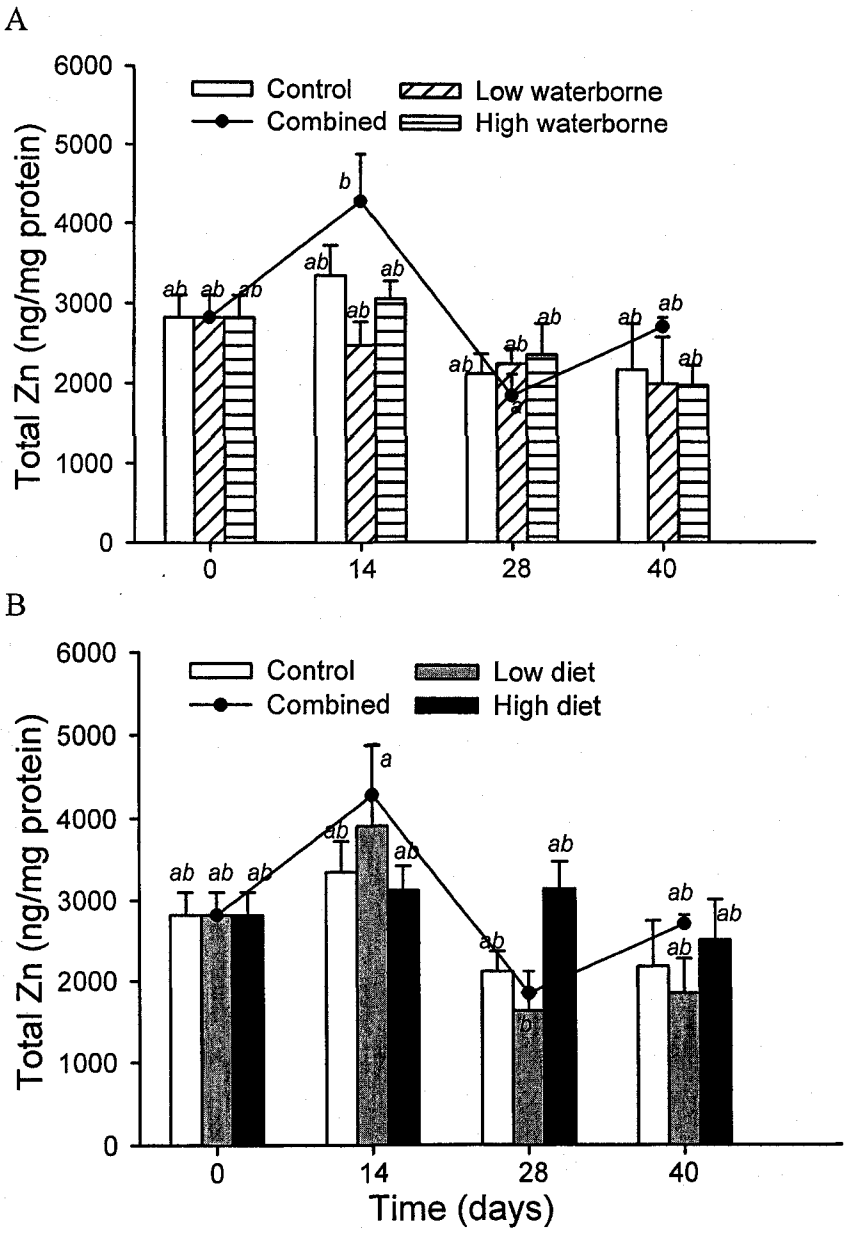


Figure 27. Zinc concentrations in juvenile rainbow trout intestine. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

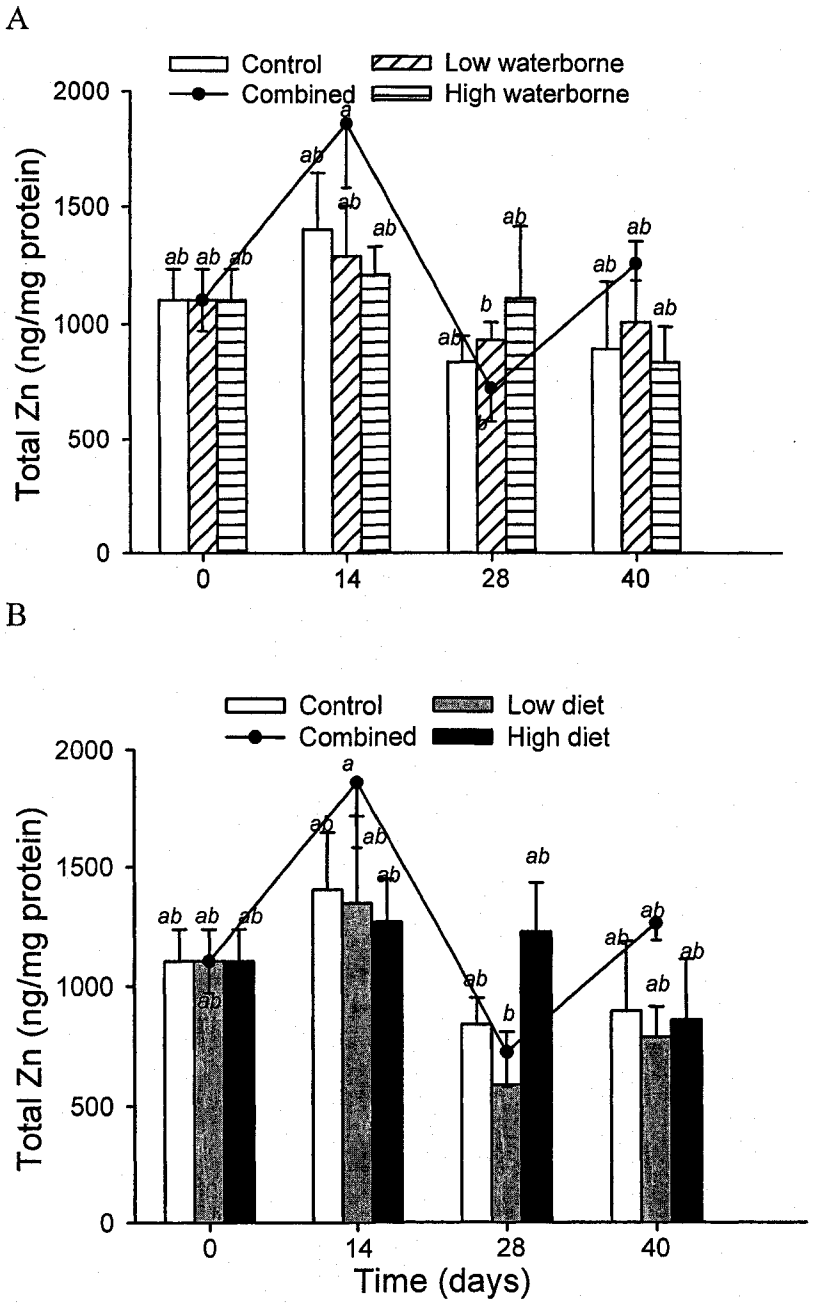


Figure 28. Zinc concentrations in nuclei-cell debris fraction of intestine. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

accumulation associated with the combined exposure relative to the comparable singular waterborne or dietary exposure.

Intestinal mitochondrial Zn concentrations remained relatively stable throughout the exposure (Fig. 29). The only significant change was seen in the combined exposure group in which the concentrations declined significantly on day 28. Overall the Zn concentrations ranged from 200 to 330 ng mg^{-1} protein in all the exposures. The treatment effect ($p < 0.05$) was significant among the dietary exposure groups but not in the waterborne groups ($p = 0.81$). In addition, the interaction terms between the time and treatment ($p = 0.21$ for waterborne and $p = 0.20$ for dietary) were insignificant. In the combined exposure, there was no additional accumulation relative to the comparable singular waterborne or dietary exposures.

Zinc concentrations in the intestinal microsomes-lysosomes fraction significantly increased with time ($p < 0.05$) only in the waterborne exposures (Fig. 30). The concentration of Zn in this fraction ranged from 80 to 230 ng mg^{-1} protein. The treatment effect ($p < 0.05$) was significant among the dietary exposure groups but not in the waterborne groups ($p = 0.57$) but it was observed that in day 14 high water Zn group the Zn concentration was significantly higher than in the same group on day 28. The interaction terms between the time and treatment ($p = 0.20$ for waterborne and $p = 0.86$ for dietary) were insignificant. In the combined exposure the Zn concentrations observed were consistently higher than those attained in the singular dietary exposure.

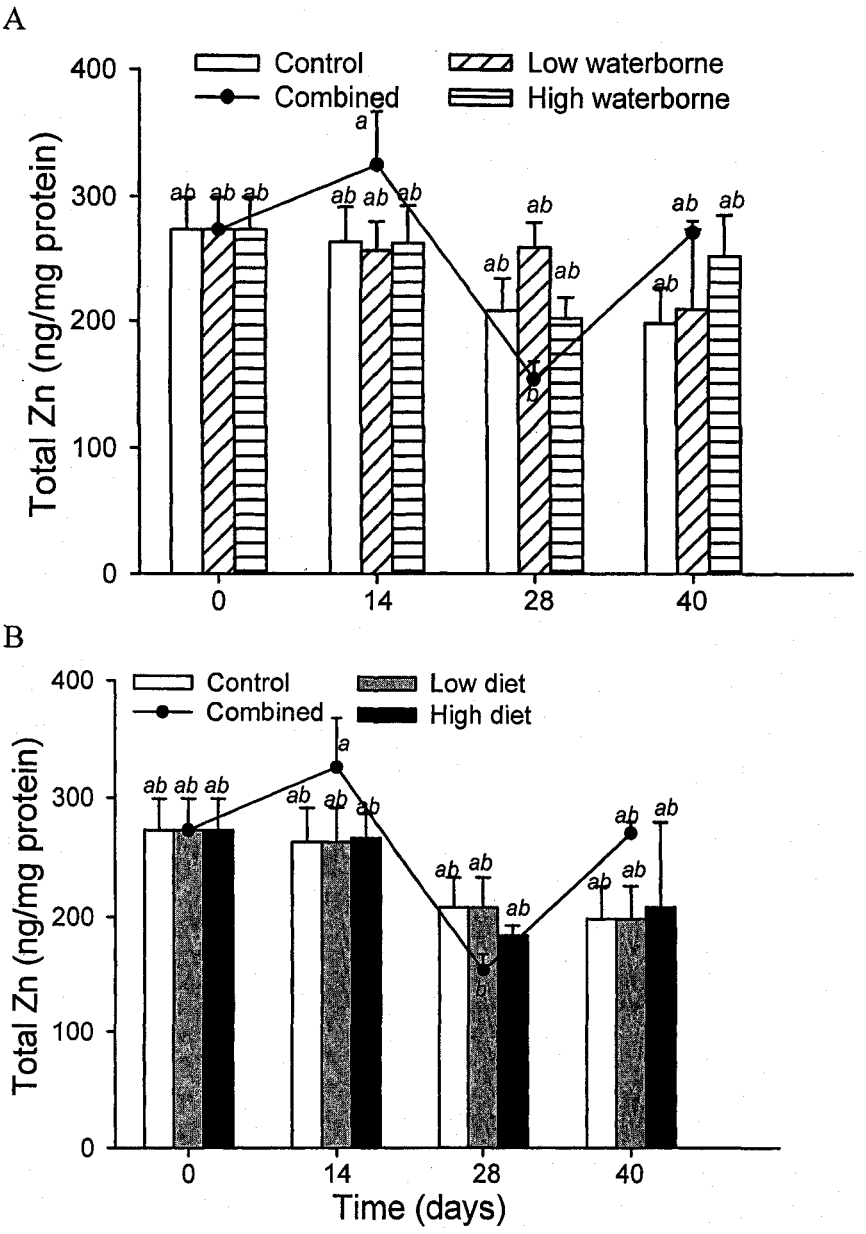
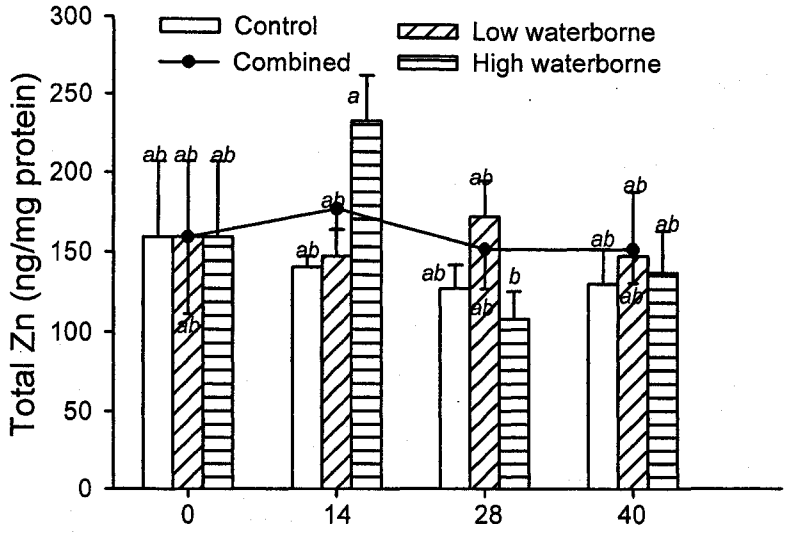


Figure 29. Zinc concentrations in mitochondrial fraction of intestine. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

A



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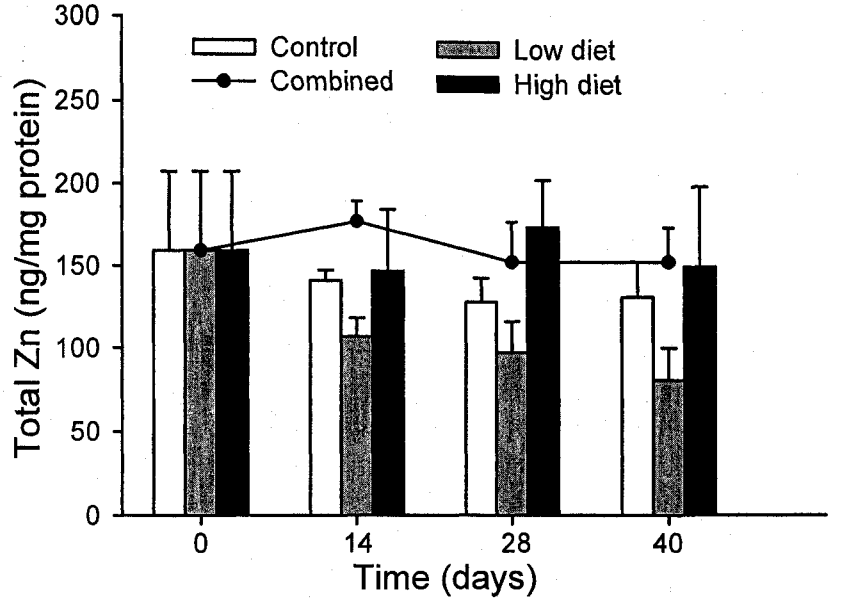


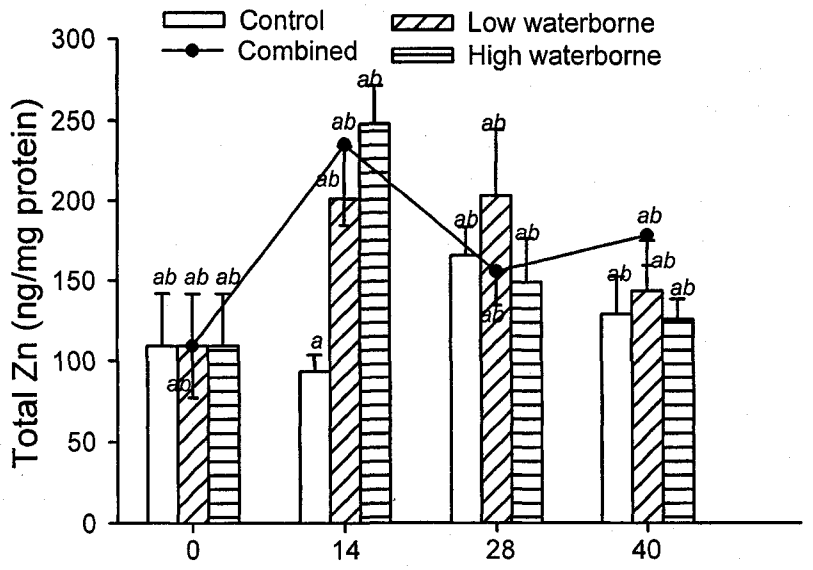
Figure 30. Zinc concentration in intestinal microsomes-lysosomes fraction. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

Intestinal HDP (Fig. 31) Zn concentrations ranged from 100 to 250 ng mg^{-1} protein and significantly increased with time ($p < 0.01$) in the waterborne exposures. The treatment ($p = 0.14$ for waterborne and $p = 0.12$ for dietary) and interaction ($p = 0.11$ for waterborne and $p = 0.17$ for dietary) terms were not significant. There was, however, a significant increase in the concentration of Zn relative to the controls early in the exposure (day 14) in the high water Zn group followed by a decline. Zinc concentrations in the dietary Zn-exposed groups did not change. In the combined exposure, Zn concentration peaked on day 14 and generally there was no difference relative to respective singular exposures.

Zinc concentrations in intestinal HSP fraction are shown in Figure 32. In this fraction the concentrations ranged between 420 and 1050 ng mg^{-1} protein and both the time ($p < 0.001$) and treatment ($p < 0.05$ for waterborne and $p < 0.01$ for dietary) terms were significant. Generally the Zn concentrations declined with time and increased with the level of dietary Zn exposure. The interaction terms ($p = 0.38$ for waterborne and $p = 0.58$ for dietary) were not significant. In the combined exposure Zn uptake was biphasic with the concentration peaking on day 14 then declining on days 28 and 40. In the combined exposure, Zn accumulation was attributable to either waterborne or dietary uptake because there was no additional accumulation above that attributable to the respective singular exposures.

In the NaOH-resistant granules (Fig. 33) a significant time effect ($p < 0.001$ for waterborne and $p < 0.01$ for dietary) was observed, in which the Zn concentrations

A



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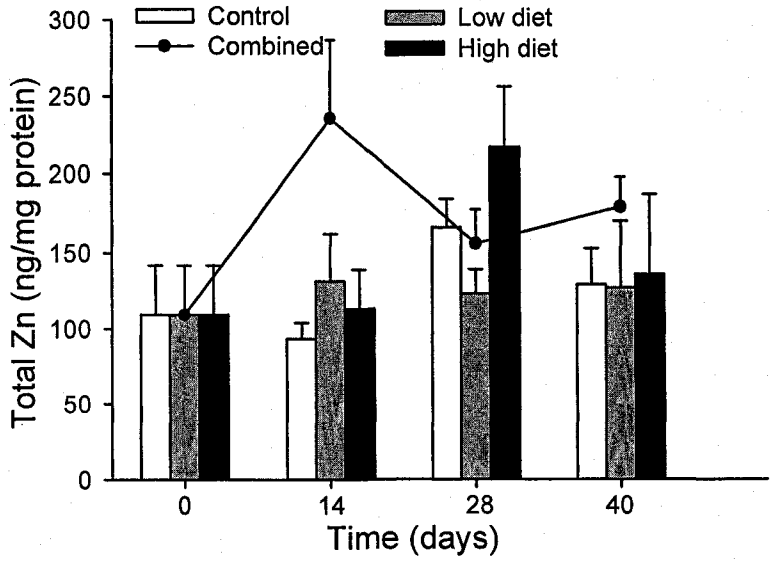
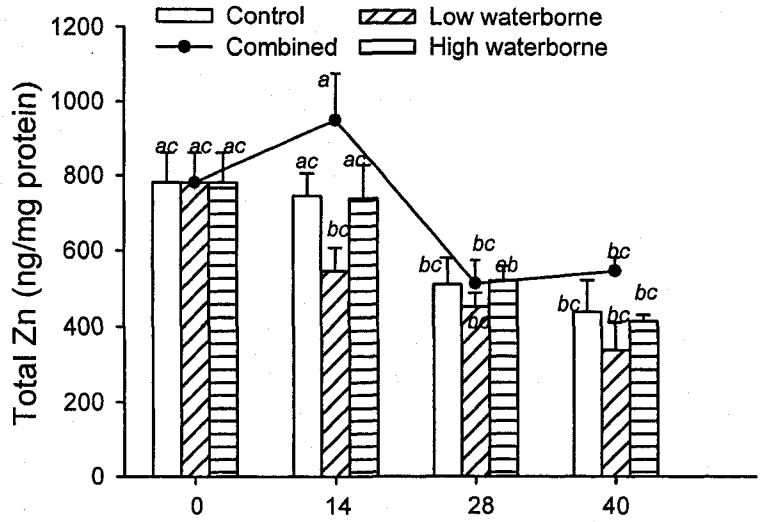


Figure 31. Zinc concentrations in heat-denaturable protein fraction of intestine. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

A



B

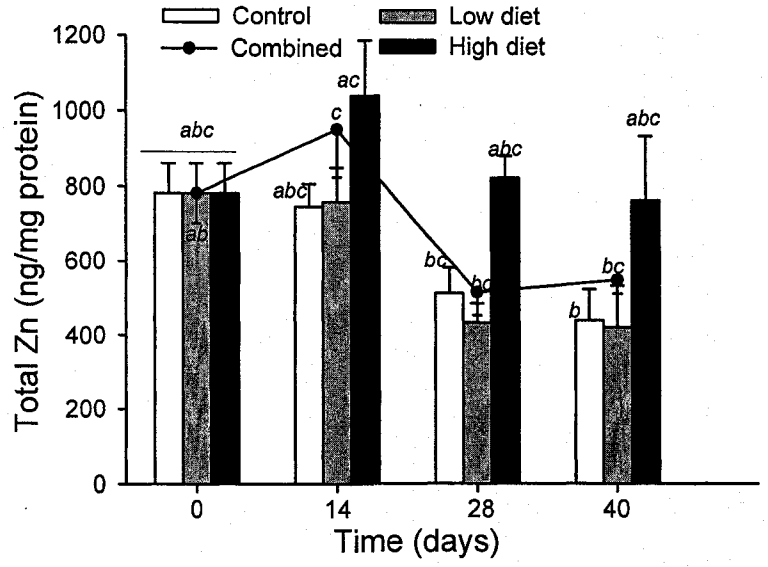


Figure 32. Zinc concentrations in heat-stable protein fraction of intestine. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure. Bars or points with different letters are significantly different (ANOVA, $p < 0.05$).

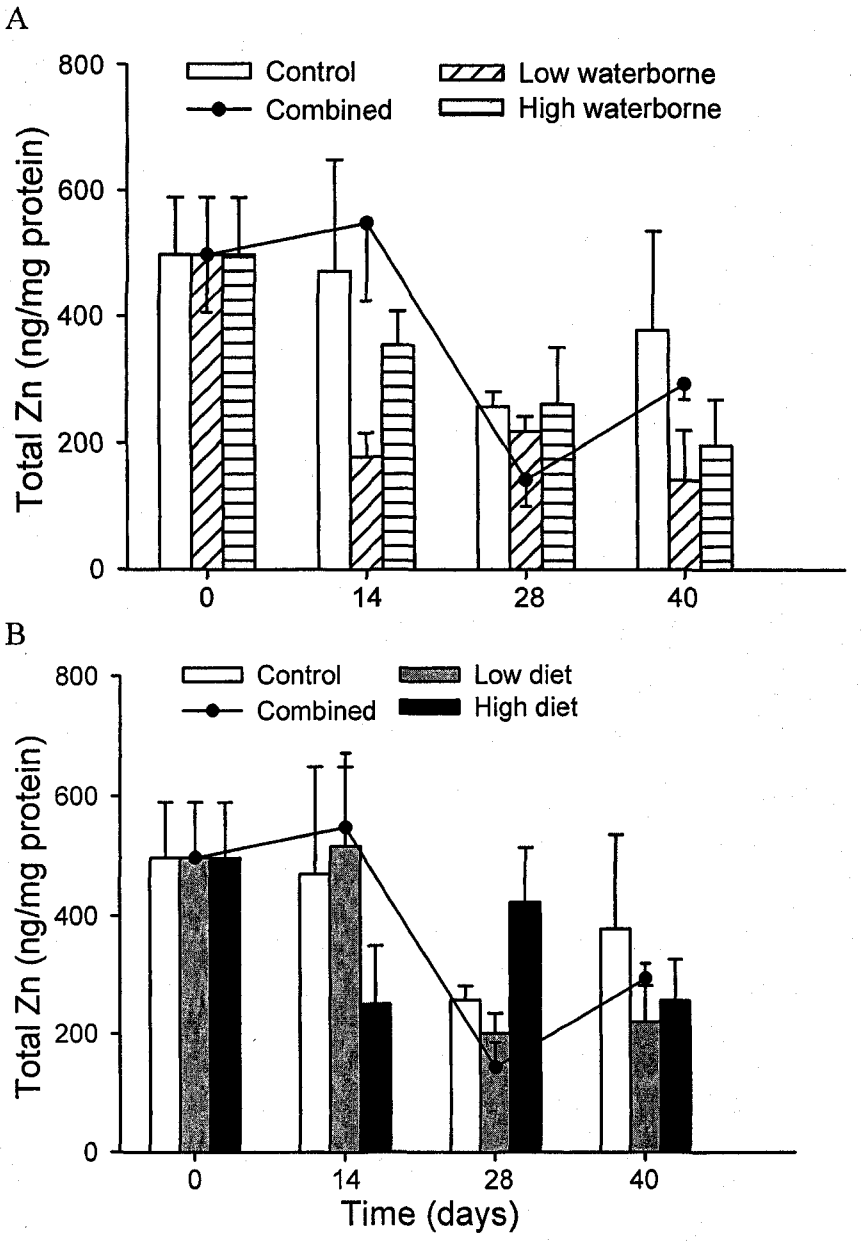


Figure 33. Zinc concentration in intestinal NaOH-resistant granule fraction. All values are mean ± SEM, $n = 4$ per group. A: open bars, control; diagonally hatched bars, low waterborne; cross-hatched bars, high waterborne; line, combined exposure. B: Open bars, control; grey bars, low diet; black bars, high diet; line, combined exposure.

declined with time. The treatment ($p = 0.16$ for waterborne and $p = 91$ for dietary) and interaction ($p = 0.47$ for waterborne and $p = 0.26$ for dietary) terms were insignificant. Overall in this fraction the Zn concentration ranged from 140 ng mg^{-1} to 550 ng mg^{-1} protein. There was no additional accumulation associated with the combined exposure relative to that attributable to the comparable singular waterborne or dietary exposure.

3.5 Correlation between fraction Zn and whole tissue Zn concentration

Table 7 shows the coefficients of determination for the relationships between fraction Zn and the respective tissue Zn concentrations. The correlation between HSP and MT concentrations also is shown. In the gill, fraction Zn concentrations in all the fractions were positively significantly correlated with the total gill Zn concentration for both the waterborne and dietary exposures. The nuclei-cellular debris Zn showed the highest correlation coefficients (0.90 for waterborne and 0.89 for dietary). The gill HSP was very highly correlated to the gill MT with $r = 0.95$ (waterborne) and 0.97 (dietary). In the intestine, although there were significant correlations with all the fractions except HDP, they were generally medium to weak ($r = 0.28$–0.77). However HSP and MT were highly correlated ($r = 0.97$) in the dietary exposure. In the liver only the mitochondrial Zn was correlated with hepatic Zn concentration, albeit weakly. Hepatic HSP Zn and MT also registered a significant correlation. Interestingly among the three tissues only mitochondria Zn concentration correlated with tissue Zn for both routes of exposure.

Table 7: Linear regression analyses of Zn concentrations in rainbow trout subcellular fractions of gill, intestine and liver against the respective tissue total Zn concentrations. The raw data (n = 4 for each tissue and sampling interval) were used in the analyses. +: The Zn concentrations in HSP fractions were also regressed against the respective MT concentrations. Values are correlation coefficients (*r*) and asterisks indicate significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NS: not significant; MT: metallothionein; HDP: heat-denaturable proteins; HSP: heat-stable proteins.

Fraction	**Gill**		**Intestine**		**Liver**	
	Waterborne	Dietary	Waterborne	Dietary	Waterborne	Dietary
Nuclei-cellular debris	0.90^{***}	0.89^{***}	0.41^{**}	0.44^{**}	NS	NS
Mitochondria	0.77^{***}	0.78^{***}	0.56^{***}	0.69^{***}	0.52^{***}	0.42^{**}
Lysosomes-microsomes	0.64^{***}	0.76^{***}	0.28^{*}	0.33^{*}	NS	NS
HDP	0.66^{***}	0.74^{***}	NS	NS	NS	NS
HSP	0.77^{***}	0.46^{***}	0.71^{***}	0.77^{***}	NS	NS
Granules	0.69^{***}	0.75^{***}	0.28^{*}	0.28^{*}	NS	NS
$^{+}$HSP vs. MT	0.95^{***}	0.97^{***}	0.74^{***}	0.97^{***}	0.58^{***}	0.72^{***}

3.6 Distribution of Zn between metabolically active and detoxified pools

In order to understand the overall subcellular dynamics of Zn, relative changes in the concentration and proportion (%) of Zn accumulated in the MAP and MDP were evaluated. The MAP comprised of Zn accumulated in the nuclei-cell debris, mitochondria, microsomal-lysosomal and the HDP fractions, whereas the MDP comprised of Zn associated with the HSP and NaOH-resistant granules. In terms of proportions, the majority of the Zn was clearly in MAP: 81 – 90% in gill (Table 8), 67 – 78% in the liver (Table 9) and 59 – 75% in the intestine (Table 10). In contrast the MDP ranged from 10 – 19 % in the gill, 23 – 33% in the liver and 25 – 41% in the intestine. It was also seen that the level of Zn exposure and time did not induce shifts in the proportional distribution of Zn between the two pools in the gill and the liver. However in the intestine there was an increase in the Zn partitioning in the MDP in fish fed elevated levels of Zn in the food.

3.7 Metallothionein

Using the values of cytosolic Zn, Cu and Cd measured in HSP fractions, the theoretical MT concentrations were calculated in the gills, liver and intestine, taking into account the specific capacity of each metal to bind to MT by metal thiolate linkages (7 for Zn and Cd, and 12 for Cu). The calculated concentrations of MT in gills, liver and intestine are shown in Tables 11, 12 and 13, respectively. The MT concentrations in the gills ranged from 2 to 11 $\mu g\ g^{-1}$ and increased significantly with time ($p < 0.001$ in waterborne and $p < 0.01$ for dietary). The treatment effect ($p < 0.05$) was significant for the waterborne but not the dietary ($p = 0.12$) exposures. The

Table 8: Distribution of Zn between metabolically active and detoxified metal pools in trout gill. All values are mean ± SEM, n = 4 per group. Different letters indicate significance at $p < 0.05$.

Day	Treatment	Waterborne		Dietary		Combined	
		Metabolically Active	*Detoxified*	*Metabolically Active*	*Detoxified*	*Metabolically Active*	*Detoxified*
0	**Control**	84.16^{abc} ±1.78	15.84^{xyz} ±1.78	84.16^{abc} ±1.78	15.84^{xyz} ±1.78	84.16^{abc} ±1.78	15.84^{xyz} ±1.78
14	**Control**	90.12^{ac} ±0.93	9.88^{xz} ±0.93	90.12^{ac} ±0.93	9.88^{xz} ±0.93	90.12^{ac} ±0.93	9.88^{xz} ±0.93
	Low	82.86^{abc} ±2.64	17.14^{xyz} ±2.64	85.33^{abc} ±2.30	14.67^{xyz} ±2.30	85.28^{abc} ±1.27	14.72^{xyz} ±1.27
	High	84.85^{abc} ±0.61	15.15^{xyz} ±0.61	90.75^{c} ±0.30	9.25^{z} ±0.30	85.28^{abc} ±1.27	14.72^{xyz} ±1.27
28	**Control**	81.37^{b} ±1.21	18.63^{y} ±1.21	81.37^{b} ±1.21	18.63^{y} ±1.21	81.37^{b} ±1.21	18.63^{y} ±1.21
	Low	84.02^{abc} ±0.48	15.98^{xyz} ±0.48	81.47^{b} ±2.70	18.53^{y} ±2.70	84.26^{abc} ±0.93	15.74^{xyz} ±0.93
	High	84.64^{abc} ±1.70	15.36^{xyz} ±1.70	83.27^{ab} ±1.23	16.73^{xy} ±1.23	84.26^{abc} ±0.93	15.74^{xyz} ±0.93
40	**Control**	88.07^{abc} ±0.47	11.94^{xyz} ±0.47	88.07^{abc} ±0.47	11.94^{xyz} ±0.47	88.07^{abc} ±0.47	11.94^{xyz} ±0.47
	Low	84.15^{abc} ±2.06	15.85^{xyz} ±2.06	89.54^{abc} ±0.28	10.46^{xyz} ±0.28	82.11^{b} ±1.10	17.89^{y} ±1.10
	High	83.41^{abc} ±1.67	16.59^{xyz} ±1.67	89.33^{abc} ±2.51	10.67^{xyz} ±2.51	82.11^{b} ±1.10	17.89^{y} ±1.10

Table 9: Distribution of Zn between metabolically active and detoxified metal pools in trout liver. All values are mean ± SEM, n = 4 per group. Different letters indicate significance at $p < 0.05$.

Day	Treatment	Waterborne		Dietary		Combined	
		Metabolically Active	*Detoxified*	*Metabolically Active*	*Detoxified*	*Metabolically Active*	*Detoxified*
0	**Control**	73.99^{abc} ±0.29	26.01^{xyz} ±0.29	73.99^{abc} ±0.29	26.01^{xyz} ±0.29	73.99^{abc} ±0.29	26.01^{xyz} ±0.29
14	**Control**	69.98^{abc} ±1.45	30.02^{xyz} ±1.45	69.98^{abc} ±1.45	30.02^{xyz} ±1.45	69.98^{abc} ±1.45	30.02^{xyz} ±1.45
	Low	71.59^{abc} ±1.37	28.41^{xyz} ±1.37	65.66^{a} ±2.10	34.35^{x} ±2.10	66.61^{ac} ±0.67	33.39^{xz} ±0.67
	High	67.63^{a} ±2.50	32.37^{x} ±2.50	67.20^{a} ±2.82	32.80^{x} ±2.82	66.61^{ac} ±0.67	33.39^{xz} ±0.67
28	**Control**	68.19^{a} ±1.90	31.81^{x} ±1.90	68.19^{a} ±1.90	31.81^{x} ±1.90	68.19^{a} ±1.90	31.81^{x} ±1.90
	Low	77.27^{b} ±1.49	22.73^{y} ±1.49	70.70^{abc} ±2.11	29.30^{xyz} ±2.11	72.71^{abc} ±3.39	27.29^{xyz} ±3.39
	High	72.57^{abc} ±1.27	27.44^{xyz} ±1.27	64.79^{a} ±2.59	35.21^{x} ±2.59	72.71^{abc} ±3.39	27.29^{xyz} ±3.39
40	**Control**	72.04^{abc} ±1.05	27.96^{xyz} ±1.05	72.04^{abc} ±1.05	27.96^{xyz} ±1.05	72.04^{abc} ±1.05	27.96^{xyz} ±1.05
	Low	74.97^{ab} ±1.80	25.03^{xy} ±1.80	67.60^{abc} ±2.10	32.40^{xyz} ±2.10	77.55^{b} ±1.93	22.45^{y} ±1.93
	High	74.61^{abc} ±1.43	25.39^{xyz} ±1.43	67.24^{a} ±3.73	32.76^{x} ±3.73	77.55^{b} ±1.93	22.45^{y} ±1.93

Table 10: Distribution of Zn between metabolically active and detoxified metal pools in trout intestine. All values are mean ± SEM, $n = 4$ per group. Different letters indicate significance at $p < 0.05$.

Day	Treatment	Waterborne		Dietary		Combined	
		Metabolically Active	*Detoxified*	*Metabolically Active*	*Detoxified*	*Metabolically Active*	*Detoxified*
0	**Control**	68.57^{a} ±3.89	31.43^{x} ±3.89	68.57^{a} ±3.89	31.43^{x} ±3.89	68.57^{a} ±3.89	31.43^{x} ±3.89
14	**Control**	63.55^{a} ±1.15	36.45^{x} ±1.15	63.55^{a} ±1.15	36.45^{x} ±1.15	63.55^{a} ±1.15	36.45^{x} ±1.15
	Low	70.59^{a} ±0.48	29.40^{x} ±0.48	65.14^{a} ±3.84	34.86^{x} ±3.84	61.33^{a} ±1.73	38.67^{x} ±1.73
	High	64.32^{a} ±0.96	35.68^{x} ±0.96	58.78^{a} ±2.98	41.22^{x} ±2.98	61.33^{a} ±1.73	38.67^{x} ±1.73
28	**Control**	63.52^{a} ±0.69	36.48^{x} ±0.69	63.52^{a} ±0.69	36.48^{x} ±0.69	63.52^{a} ±0.69	36.48^{x} ±0.69
	Low	70.00^{a} ±0.43	29.99^{x} ±0.43	65.84^{a} ±2.49	34.16^{x} ±2.49	64.62^{a} ±0.79	35.39^{x} ±0.79
	High	66.41^{a} ±1.86	33.59^{x} ±1.86	60.37^{a} ±0.62	39.63^{x} ±0.62	64.62^{a} ±0.79	35.39^{x} ±0.79
40	**Control**	63.08^{a} ±1.18	36.93^{x} ±1.18	63.08^{a} ±1.18	36.93^{x} ±1.18	63.08^{a} ±1.18	36.93^{x} ±1.18
	Low	74.84^{a} ±1.92	25.16^{x} ±1.92	66.07^{a} ±1.91	33.93^{x} ±1.91	68.96^{a} ±1.42	31.04^{x} ±1.42
	High	68.71^{a} ±1.75	31.29^{x} ±1.75	59.84^{a} ±2.62	40.16^{x} ±2.62	68.96^{a} ±1.42	31.04^{x} ±1.42

Table 11. Metallothionein concentrations ($\mu g\ g^{-1}$) in the heat-stable protein fraction of trout gill. All values are mean ± SEM, $n = 4$ per group. Values with different letters are significantly different (ANOVA, $p < 0.05$).

DAY	TREATMENT					
	Control	Low Waterborne	High Waterborne	Low Dietary	High Dietary	Combined
0	4.20^{ac} ± 0.94	4.20^{ac} ± 0.94	4.20^{ac} ± 0.94	4.20^{ac} ± 0.94	4.20^{ac} ± 0.94	4.20^{ac} ± 0.94
14	2.43^{ac} ± 0.16	5.58^{abc} ± 1.55	4.89^{abc} ± 1.14	6.05^{abc} ± 3.15	2.19^{acd} ± 0.57	4.06^{c} ± 0.48
28	6.45^{abc} ± 0.72	6.12^{abc} ± 0.39	6.96^{abc} ± 1.63	7.02^{abc} ± 0.50	7.42^{abc} ± 1.42	6.99^{abc} ± 1.10
40	4.84^{abc} ± 0.75	5.97^{abc} ± 1.17	11.02^{b} ± 2.63	2.40^{acd} ± 0.52	3.93^{abc} ± 1.27	9.85^{bc} ± 1.58

Table 12: Metallothionein concentrations ($\mu g\ g^{-1}$) in the heat-stable protein fraction of trout liver. All values are mean ± SEM, $n = 4$ per group. Values with different letters are significantly different (ANOVA, $p < 0.05$).

DAY	TREATMENT					
	Control	**Low Waterborne**	**High Waterborne**	**Low Dietary**	**High Dietary**	**Combined**
0	3.01^{a} ± 0.20	3.01^{a} ± 0.20	3.01^{a} ± 0.20	3.01^{a} ± 0.20	3.01^{a} ± 0.20	3.01^{a} ± 0.20
14	4.37^{abc} ± 0.29	3.71^{abc} ± 0.16	4.34^{abc} ± 0.41	4.58^{abc} ± 0.36	4.92^{abc} ±0.59	4.25^{abc} ± 0.24
28	5.06^{b} ± 0.43	4.08^{c} ± 0.28	4.57^{bc} ± 0.17	3.42^{a} ±0.36	3.99^{bc} ± 0.34	5.55^{abc} ± 0.50
40	3.91^{abc} ± 0.22	3.47^{ac} ± 0.27	4.26^{abc} ± 0.36	5.19^{abc} ± 0.42	6.27^{bcd} ± 1.16	3.97^{abc} ± 0.35

Table 13: Metallothionein concentrations ($\mu g\ g^{-1}$) in the heat stable protein fraction of trout intestine. All values are mean ± SEM, n = 4 per group. Values with different letters are significantly different (ANOVA, $p < 0.05$).

DAY	TREATMENT					
	Control	**Low Waterborne**	**High Waterborne**	**Low Dietary**	**High Dietary**	**Combined**
0	24.25^{ad} ± 1.30	24.25^{ad} ± 1.30	24.25^{ad} ± 1.30	24.25^{ad} ± 1.30	24.25^{ad} ± 1.30	24.25^{ad} ± 1.30
14	20.17^{abd} ±0.94	15.08^{bd} ± 2.33	18.32^{abcd} ± 2.41	20.91^{abd} ± 2.19	31.62^{c} ±3.25	23.47^{ad} ± 2.42
28	19.23^{abd} ± 0.56	13.21^{bcd} ± 0.83	17.94^{abcd} ± 1.59	17.36^{ab} ± 2.15	28.71^{abcd} ± 2.05	17.94^{abd} ±1.05
40	14.11^{bc} ± 2.32	13.67^{bcd} ± 1.18	17.18^{a} ±1.19	16.60^{ab} ± 2.90	20.11^{ab} ± 2.27	18.34^{abc} ± 1.59

interaction term between the time and treatment was significant ($p < 0.05$) for the dietary but not waterborne ($p = 0.15$) exposures. Metallothionein concentrations in the liver ranged between 3 and 6 $\mu g\ g^{-1}$ and both the time ($p < 0.001$) and treatment ($p < 0.01$) terms were significant. There was a significant interaction ($p = 0.05$) between time and treatment for the dietary exposures, but not for the waterborne exposure ($p = 0.26$). Similarly, the MT concentrations calculated for the intestine revealed significant time ($p < 0.001$) and treatment ($p < 0.01$) effects. Overall, the highest concentrations of MT were observed in the intestine and were in the range of 13 to 33 $\mu g\ g^{-1}$. In the intestine, the interaction term between the time and treatment was not significant ($p = 0.19$) for the waterborne exposures in contrast to dietary exposures, for which it was significant ($p < 0.05$). A comparison of MT concentrations across the three tissues (gills, liver and intestine) revealed that MT concentrations in the intestine were significantly higher ($p < 0.001$) than in the gill and liver.

4.0 DISCUSSION

Zinc is an important structural and catalytic component of numerous proteins that play essential roles in fish growth, reproduction, development, vision and immune function (Watanabe *et al.* 1997), but is toxic when accumulated in excess of cellular requirements (Wood 2001). Because of these opposing roles, there are extant biochemical and physiological mechanisms in fish that limit assimilation and/or toxicity (Hogstrand and Wood 1996; Glover and Hogstrand 2003). At the cellular level Zn partitions into two tightly regulated pools: a MAP responsible for essentiality (and toxicity), and a MDP comprising detoxified Zn (Wallace *et al.* 2003). The primary objective of this thesis research was to unravel the intracellular compartmentalization of Zn geared toward increasing our understanding of Zn homeostasis and toxicity in fish. Because Zn uptake in fish occurs via both branchial and GI pathways, the secondary objective was to investigate the potential crosstalk between these two pathways. The results obtained revealed significant tissue-specific differences in the handling of Zn, subtle cross-talk between the two pathways, and tight regulation of intracellular Zn with the majority being held in a MAP. Moreover, in order to provide a complete picture of Zn homeostasis and a clearer understanding of chronic toxicity, this thesis in addition investigated the effect of Zn exposure on growth, food intake (visual observation), protein synthesis and Zn distribution among all body tissues.

4.1 Toxicity and growth

The waterborne and dietary Zn exposure regimes employed in the present study were well tolerated by rainbow trout as portrayed by the low overall mortality (2%) unrelated to the level of Zn exposure, and the absence of growth impairment. These observations on mortality and growth are largely consistent with several earlier studies entailing exposure of fish to elevated waterborne and dietary Zn concentrations. For example during waterborne exposure of rainbow trout to 150 and 450 $\mu g\ l^{-1}$ (Alsop *et al.* 1999), 250 $\mu g\ l^{-1}$ (McGeer *et al.* 2002), and 150 $\mu g\ l^{-1}$ (Chowdhury *et al.* 2003), growth was not impaired. Interestingly, at the 450 $\mu g\ l^{-1}$ Zn exposure (Alsop *et al.* 1999) significant mortality occurred in the absence of growth, suggesting that survival is in fact a more sensitive endpoint for assessing effects of Zn exposure than growth. Similarly, in a detailed study geared toward the development of a chronic biotic ligand model, De Schamphelare and Janssen (2004) showed that growth was an insensitive endpoint of chronic Zn toxicity in rainbow trout. Although the absence of significant mortality, even at the high exposure (600 $\mu g\ l^{-1}$) in the present study, appears to contradict the results obtained by Alsop *et al.* (1999), the discrepancy can be explained on the basis of differences in water chemistry. The water in our study was much harder (260 vs. 120 mg l^{-1} $CaCO_3$ in Alsop *et al.* 1999) and contained more calcium than the water used in the study by Alsop *et al.* (1999). Both water hardness and calcium are the major modulators of waterborne Zn toxicity in fish (Wood 2001). In addition the strains of rainbow trout used were likely different; fish strain is known to influence the sensitivity of fish to environmental stressors (McGeer *et al.* 1991).

With regard to dietary Zn exposure, Knox *et al* (1984) found no growth effects when rainbow trout were fed a diet containing 500 mg Zn kg^{-1} bwt for 20 weeks, as did Wekell *et al.* (1983) in fingerling rainbow trout exposed to 1700 mg Zn kg^{-1} dietary Zn. Similarly, Spry *et al.* (1988) found no effect on growth after feeding juvenile rainbow trout diet dry pellets containing 529 mg Zn kg^{-1} for 16 weeks. Overall, a review of dietary Zn exposure in fish suggested that 60-96 mg Zn kg^{-1} body weight d^{-1} is non-toxic in rainbow trout (Clearwater *et al.* 2002). In the present study, fish received 43 (low diet) and 145 (high diet) mg Zn kg^{-1} body weight d^{-1} without exposure-related mortality suggesting that rainbow trout can tolerate a much wider range of dietary Zn exposure. Moreover, our results are both interesting and unique in the sense that waterborne Zn exposure had stimulatory, whereas dietary Zn had inhibitory, effects on early growth. Although this disparate effect did not result in significant treatment effects when comparisons were done against the controls, it culminated in significant differences between the waterborne and dietary Zn-exposed groups. Surprisingly, these effects occurred in the absence of observable reductions in food intake, suggesting that differences existed at the level of energy metabolism and allocation, rather than in the energy intake.

Clearly absence of growth impairment is a common observation upon chronic sublethal exposure of fish to both waterborne and dietary Zn. This, however, does not preclude impacts at the lower levels of biological organization because growth is an apical and relatively insensitive measure of toxicity (Kamunde and MacPhail 2007). This is supported by the fact that studies that measured biochemical, physiological or

morphological endpoints generally indicate that elevated Zn is toxic in fish. In this regard, we measured lower protein concentrations in gills, liver and intestines of fish exposed to the high waterborne and dietary Zn concentration (Fig. 6 – 8). Similarly, Kock and Buchner (1997) reported impaired swimming ability and disorientation following exposure of rainbow trout to 1.6 mg Zn l^{-1} waterborne Zn for 43 days. Microscopic analysis of tissues in the same study showed severe histopathological changes including fusion of secondary gill lamellae, proliferation of chloride and mucous cells, hyperplasia of gill epithelia cells, and increased hyaline droplet degeneration of the first proximal segment of the kidney tubules.

4.2 Bioaccumulation and distribution of Zn in tissues

Bioaccumulation of metals and other toxic substances is routinely used and widely accepted as a potential indicator of adverse effects since accumulation and its correlation with the exposure dose define the dose-response relationship. A fundamental principle of toxicology is that adverse effects occur when toxic agents accumulate at target sites in adequate concentrations and for adequate time. The primary sites of Zn uptake in fish are the gills for waterborne metal and the GI tract for dietary metal (Spry *et al.* 1988; Wood 2001; Clearwater *et al.* 2002; Bury *et al.* 2003). The branchial pathway is considered of high importance toxicologically whereas the GI route has greater significance in meeting the physiological requirement for this essential trace metal.

Zinc is transported from the primary uptake sites in blood, bound to plasma proteins and distributed to various parts of the body. Depending on the exposure concentrations and duration, together with regulatory modulation by the fish, Zn may accumulate at the primary uptake sites and or in internal organs. This accumulation may or may not be associated with toxicity (Hogstrand *et al.* 1996; Sun and Jeng 1999; Wood 2001; Thompson *et al.* 2001, 2002; Clearwater *et al.* 2002). In the present study we measured accumulation of Zn in rainbow trout tissues/organs including the gill, liver, gut, plasma, blood cells, kidney, and carcass following exposure to waterborne and dietary Zn, singly and in combination. Overall, Zn accumulation was tissue-specific and depended on the dose, duration, and route of the exposure. In the ensuing sections we discuss the homeostatic and toxicological implications of this accumulation and the interaction between the two routes of uptake. Note that in addition to measuring organ level concentrations, the accumulation of Zn in the gill, liver and intestinal tissue was subjected to a finer dissection at the subcellular level and is discussed separately.

4.2.1 Plasma and blood cells Zn concentrations

Blood plays a vital role in Zn homeostasis by providing a transport medium for delivery of Zn to internal organs where it serves important physiological functions. This transport is facilitated by several proteins including albumin which are able to bind and release Zn as required (Golaz *et al.* 1993). The transport of Zn from the primary organs of uptake into the blood is a passive process (Shears and Fletcher, 1983). For dietary uptake, Zn transporter proteins (e.g. the ZnT and ZIP gene families

of transporters), which are localized to the apical (ZIP) and basolateral (ZnT) membranes of enterocytes, import and export Zn to and from the intestine into the blood stream, respectively (Cousins and McMahon 2000; Bury *et al.* 2003; Liuzzi and Cousins 2004). For waterborne exposure, Zn uptake occurs through the epithelial calcium channel as well as via the ZIP family of metal transporters (Hogstrand and Wood 1994, 1996; Bury *et al.* 2003; Qui *et al.* 2007). Once in the blood, up to 99.8% of the total Zn content is loosely bound to plasma proteins especially albumin (Fletcher and Fletcher 1978; Bettger *et al.* 1987; Bentley 1991).

In the present study, the measured plasma Zn concentrations ranged from 14 to 24 μg ml^{-1} with the waterborne Zn-exposed fish achieving higher concentrations than the dietary Zn-exposed fish. Note that because blood sample volumes collected on days 0 and 14 were inadequate, only days 28 and 40 plasma Zn data are reported (Fig. 9). Significant accumulation occurred only in the plasma of waterborne and in the combined Zn-exposure trout suggesting that rainbow trout regulate dietary Zn better than waterborne Zn. This is consistent with the notion that waterborne Zn uptake is toxicologically more significant possibly because Zn easily permeates the gill into blood for onward transport to internal organs. Several authors including Spry *et al.* (1988) and Zia and McDonald (1994) have reported that exposure to elevated waterborne Zn increases plasma Zn concentrations; thus plasma Zn is responsive to changes in the plane of Zn exposure. Worthy of note for comparative purposes is that the fish plasma Zn levels reported here are 14-24 times higher than in mammals; human plasma contains about 1 μg ml^{-1} Zn (Underwood 1977).

Signs of toxicity or deficiency of essential metals like Zn and Cu result when their homeostatic mechanisms are impaired. A common indicator of impaired homeostasis is alteration in the levels of these essential metals in plasma. For example Spry *et al.* (1988) reported that homeostasis of Zn in fish is characterized by regulation of plasma Zn levels within narrow margins regardless of the input and that the concentrations are depressed only in case of extreme Zn deprivation and elevated only at very high dietary and waterborne input.

Although we report changes in plasma Zn concentrations in the present study, our data do not allow a definitive conclusion regarding the link between increased plasma Zn concentration and onset of toxicity since no toxicity was observed, at least with regard to survival and growth effects. Furthermore, although it is tempting to conclude from our observations that waterborne Zn exposure levels were high enough to cause perturbations of plasma Zn, whereas the dietary exposures were not, a cautionary remark is warranted. In short-term studies where plasma Zn concentrations were measured soon after dietary Zn boluses, plasma exhibited a linear increase in Zn accumulation with increasing Zn concentration (Glover and Hogstrand 2002). This suggests that elevation of plasma Zn concentrations during dietary exposure may be transient and that a possible reason why we did not observe an increase in plasma Zn concentrations despite the relatively high dietary Zn exposure doses may be due to the fact fish were sampled after 48 h of starvation. In contrast there was no interruption of dosing in the waterborne Zn-exposed fish.

The combined waterborne and dietary Zn exposure provides, to the best of our knowledge, the first conclusive evidence that accumulation of Zn in plasma in rainbow trout is dominated by waterborne uptake, confirming the observation by Kock and Buchner (1997) of greater sensitivity of rainbow trout to waterborne Zn. Thus while the concentration of Zn accumulated in plasma in the combined treatment was higher than that accumulated in the respective dietary exposure alone, it was similar to that accumulated in the respective singular waterborne exposure (Fig. 9). This lends further support to the notion that waterborne Zn uptake is more important toxicologically, because elevation of plasma Zn concentration signifies a disturbance in Zn homeostasis, a probable prelude to toxicity.

Similar to plasma, Zn accumulation in the blood cells was dominated by waterborne Zn uptake, with the accumulation from waterborne exposure being almost double that from the dietary exposure. The blood cells Zn concentrations expressed on a dry wt basis were higher than the concentrations obtained in plasma ($\mu g\ ml^{-1}$ directly compared with $\mu g\ g^{-1}$). This may be due to the high expression of carbonic anhydrase and superoxide dismutase, two Zn metalloenzymes, in red blood cells which comprise the majority of the blood cells. Indeed 90% of the Zn in human red blood cells is found in the two enzymes (Ohno *et al.* 1985). Although we observed significant dose-dependent (especially on day 40, Fig. 10A) a previous exposure of rainbow trout to waterborne Zn (150 mg l^{-1}) for 30 days did not cause changes in blood cells Zn concentrations (Chowdhury *et al.* 2003). This discrepancy with our study could be

attributed to the size/age of the fish used in the two studies (14-30 g vs. 275-455 g): early life stages of fish including larvae and juveniles are generally more sensitive to metals (Hutchinson *et al.* 1998) and are therefore more likely to exhibit impaired Zn homeostasis.

A unique observation in the present study was the consistent dose-dependent decline in blood cell Zn concentrations in the dietary exposures which is the reverse of the blood cell Zn concentration in the waterborne exposures. The actual reason for this is unknown but it does suggest that multiple mechanisms are involved in regulating red blood cell Zn concentrations. It is possible that under some circumstances, Zn may be mobilized from the blood cells to the plasma and transported to organs such as the gill, liver, gut and kidney for excretion to protect red blood cells from Zn overload and toxicity. Furthermore, although the Zn concentration in red blood cells of dietary Zn-exposed fish declined, it is feasible that maintenance of whole-body Zn homeostasis may inevitably involve maintenance of increased concentrations in some tissues and reduced concentrations in others. Indeed McGeer *et al.* (2000) observed increased whole-body Zn concentration concurrent with decreased Zn concentration in internal tissues. It is also worthy of note that accumulation of new Zn (as measured with ^{65}Zn) in rainbow trout was significantly lower in red blood cells of rainbow trout pre-exposed to 150 mg l^{-1} waterborne Zn for one month (Chowdhury *et al.* 2003). Clearly, it is important to simultaneously inspect Zn accumulation patterns in all tissues to make correct inferences on Zn homeostasis.

4.2.2 *Zn accumulation in carcass and kidney*

The accumulation of Zn in the carcass increased with time in the waterborne exposures, and with both time and Zn concentration in the dietary exposures. Generally the concentrations above background were low (120 to 190 μg g^{-1} dry wt) and quite variable. This limited accumulation implies that the carcass is not an important site for Zn accumulation in rainbow trout and is in agreement with previous studies (Alsop *et al.* 1999; McGeer *et al.* 2000). Despite the relatively low Zn concentrations in the carcass, it should be noted that in absolute terms the carcass comprised the largest pool of Zn because it makes up the greatest proportion (80%) of the fish body mass. Our analysis (Table 6) indicates that 84-90 % of the whole-body Zn was contained in the carcass. This suggests significant requirement of Zn in muscle, which is the main constituent of the carcass. Moreover some authors suggest that the skin and skeletal tissue, the other key components of the carcass, are also major sites of Zn accumulation in fish (Ogino and Yang 1978; Maage and Julshamn 1993; Shim and Lee 1993).

The concentrations of Zn attained in the kidney (up to 250 μg g^{-1} dry wt) were slightly higher than those measured in the carcass. Although significant accumulation occurred only in the high dietary Zn exposure, the combined exposure suggests that neither route was dominant over the other. This is because the concentrations attained in the combined exposure were similar to those measured in the respective singular route exposures. Several authors have measured Zn concentrations in the kidney following both waterborne and dietary Zn exposures and no consistent trend is

evident since the results varied from study to study. For example in a study done by Kock and Bucher (1997), rainbow trout exposed to 1.6 mg l^{-1} waterborne Zn had elevated Zn concentrations in the kidney from day 6 to the end of the 43-day experiment. The same authors showed that in fish exposed to 3 g Zn kg^{-1} dry wt in the diet for 70 days, accumulation of Zn in kidney was not consistent; significant elevation occurred only early (day 9) and at the end. Moreover accumulation of Zn in the kidney was higher from the waterborne as compared to dietary exposure. In a study done by Holcombe *et al.* (1979), exposure of brook trout (*Salvelinus fontinalis*) to 534 μg l^{-1} waterborne Zn for 24 weeks resulted in a 3-fold increase of Zn concentration above the controls in the kidney. Other studies where fish were fed Zn-enriched diets revealed no change, decline or only minor accumulation of the metal in kidney (Jeng and Sun 1981; Knox *et al.* 1984; Overnell *et al.* 1988; McGeer *et al.* 2000). Clearly there is no unifying theme regarding changes in Zn concentration in fish kidney following exposure to elevated Zn in water or food.

4.2.3 Zn accumulation in gill, liver and intestine

Because the gill, liver and intestine were submitted to subcellular fractionation, Zn concentrations in these tissues were normalized to the protein concentration of the respective tissue, similar to the subcellular fractions. This allowed direct comparison of the contribution of each fraction to the total tissue Zn burden. A recent study from our laboratory employed the same style of presenting subcellular distribution data for Cu (Kamunde and MacPhail 2007). Nonetheless this section briefly highlights the key

findings regarding Zn accumulation in these tissues on a dry weight basis for purposes of comparison with the data from other tissues (Table 6).

In the gill the Zn concentrations increased linearly in a dose-dependent manner with significant accumulation relative to same sampling point controls on days 14 and 40. There was an overall 2-fold increase in gill Zn concentration at day 40 relative to day 0 controls and no indication that a steady state had been achieved. In a comparable study in rainbow trout, branchial Zn was reported to saturate at 34% above background after exposure to 250 $\mu g\ l^{-1}$ Zn (McGeer *et al.* 2000). Other authors reported no or inconsistent accumulation of Zn in the gill following comparable waterborne Zn exposure regime (Farag *et al.* 1994; Hogstrand *et al.* 1994; Alsop *et al.* 1999; Dethloff 1999).

In the present study dietary Zn did not accumulate in the gill in contrast to a study by Wekell *et al.* (1986) in which increased accumulation of Zn in the gill was found following dietary Zn exposure. Assessment of leaching was not done by Wekell *et al* (1986); thus the possibility of concurrent waterborne exposure from leached Zn cannot be ruled out. It is important when studying accumulation of dietary metals in the gill to document whether or not leaching of the dietary metal occurred leading to elevated waterborne metal concentrations and subsequent branchial uptake. In our study measurements of Zn levels in the water 1 h after feeding revealed no contamination possibly because all the food was consumed by the fish within a short time.

Compared to gill, the liver exhibited low levels of Zn which changed very minimally but significantly with Zn exposure with steady state at about 1.5-fold the day 0 control values attained by day 28. Similar to these observations, other authors reported inconsistent or minimal changes in hepatic Zn concentrations following exposure to elevated Zn in water or food (Jeng and Sun 1981; Spry *et al.* 1988; Kock and Bucher 1997; Alsop *et al.* 1999; McGeer *et al.* 2000; Clearwater *et al.* 2002; Chowdhury *et al.* 2003) and concluded that hepatic Zn was under tight homeostatic regulation in rainbow trout. Further, Chowdhury *et al.* (2003) measured bile Zn concentration and found it elevated in waterborne Zn-exposed trout indicating that hepatobiliary excretion is likely part of the mechanisms involved in Zn homeostasis. Although Kock and Bucker (1997) showed a more persistent elevation of hepatic Zn with waterborne exposure, this study indicates that waterborne and dietary Zn exposure have a similar effect on hepatic Zn concentrations.

Zinc concentrations have not been extensively measured in GI tissue of fish. In the present study we show that among all the tissue, the intestinal tissue had the highest Zn concentration irrespective of the plane and route of Zn exposure. Several other authors found high concentrations of Zn in gut of control and Zn-exposed fish. Following both waterborne and dietary Zn exposures (Kock and Bucher 1997), gut Zn concentration increased only in the dietary Zn-exposed rainbow trout. Chowdhury *et al.* (2003) similarly showed that the intestine had the highest Zn concentration among rainbow trout tissues following a 30-day exposure to waterborne Zn. As well, Sun and Jeng (1999) reported high concentrations of Zn in common carp intestinal

tissue. The high intestinal levels of Zn possibly suggest a central role of the gastrointestinal tissue in Zn absorption for normal physiology as well as a role in excretion of both dietary and waterborne Zn. Zinc excreted via bile ends up in the GI tract, thus contributing to the high levels of Zn in the intestinal tissue, irrespective of the route of exposure.

4.2.4 Tissue Zn accumulation: the overall picture

Taken together, the pattern of Zn bioaccumulation in rainbow trout was characterized by relatively high concentrations in all the tissues including those of naïve (control) fish. This reflects the essentiality of Zn and its role in over 1000 proteins (Berg and Shi 1996, Maret 2000). Unlike other metals (e.g. Cd and Cu) which preferentially accumulate in specific tissues (e.g. liver for Cu and kidney for Cd), no tissue appeared to preferentially accumulate Zn. The tissue Zn concentrations were of the order: intestine > gill > kidney ≥ liver > carcass > blood cells > plasma (Table 6). However, in terms of quantity (μg per tissue) the majority of the Zn occurred in the carcass (84-90%), followed by the intestine (3-7%), gill (4-6%), liver (1%) and kidney 1%. Generally the highest concentrations attained in Zn-exposed fish were about 2.5× the control values in all the tissue, and the increases in concentrations were not consistent throughout the exposure period. Our results are in agreement with several previous studies in rainbow trout chronically exposed to waterborne and/or dietary Zn (Bradley and Sprague 1985; Spry *et al.* 1988; Hogstrand *et al.* 1994, 1996; Alsop *et al.* 1999; McGeer *et al.* 2000). There are several potential reasons for the variability in tissue Zn accumulation data among fishes. First, the low

bioaccumulation factors can be attributed to tight homeostatic regulation of Zn in fish. This tight homeostasis is presumed to occur primarily at the level of uptake (Hogstrand *et al.* 1994, 1995; 1996, 1998; Alsop *et al.* 1999) and excretion via hepato-biliary-fecal and branchial pathways (Pierson 1981; Shears and Fletcher 1983; Spry and Wood 1985; Hardy *et al.* 1987; Handy 1996; Chowdhury *et al.* 2003) with minimal contribution of internal sequestration (Bradley *et al.* 1985; Alsop *et al.* 1999). Second, the not uncommonly observed decreases in tissue Zn concentrations with time during chronic exposures could in part be due to growth dilution (Farmer *et al.* 1979), particularly because so much of the residual Zn resides in the muscle i.e. carcass, or as part of whole-body homeostasis whereby transiently lower Zn concentrations in some tissues can occur due to preferential mobilization.

Overall the tissue bioaccumulation data obtained in this study and other published data to date suggest that a correlation between whole-organ accumulated Zn, exposure, and toxicity is unlikely to exist in rainbow trout. Thus for this essential metal, we submit that use of whole tissue metal residues as a biomarker of exposure (Bergman and Doward-King 1997) has little value. Rather, we hypothesized that dissecting the subcellular localization would isolate the subcellular fraction and pool of Zn directly related to bioaccumulation and by extension to toxicity. This hypothesis was explored with the gill, liver and intestine.

4.3 Internal bioavailability of Zn in rainbow trout

It is now widely accepted that toxicity of metals in aquatic organisms cannot be predicted from total or dissolved metal concentrations in the external milieu (Campbell 1995; Di Toro *et al.* 2001). Instead, it is the bioavailable metal species, primarily the free metal ion, which correlates with toxicity. It is therefore highly recommended to incorporate speciation analysis during measurement and prediction of metal toxicity for ecological risk assessment. An alternative approach to speciation is the use of CBR (McCarty and Mackay 1993). The CBR negates the need for the often tedious determination of chemical species because it deals with accumulated toxicants. Although CBR does appear to be effective in predicting toxicity of narcotic organic pollutants it does not work effectively for metals because of the diverse and complex strategies employed by aquatic organisms to handle accumulated metals (Rainbow 2002; Ahearn *et al.* 2004; Campbell *et al.* 2005). These metal handling strategies render some pools of accumulated metal unavailable to cause toxicity, a scenario similar to the external environment. Thus analogous to the utility of speciation analysis in illuminating bioavailability of metals in the external medium, subcellular fractionation (e.g. by differential centrifugation or chromatography) is used to study bioavailability of accumulated metals in the internal environment (Wallace *et al.* 2003; Campbell *et al.* 2005). Using this approach metals accumulated in tissues can be isolated and broadly categorized into two pools namely, MAP and MDP. These pools can be further broken down into specific constituents. The MAP, comprising metals bound to metal-sensitive cellular components including organelles and HDP, represents bioavailable metal responsible for essentiality and/or toxicity. In

contrast the MDP consisting of metal bound to HSP (MT and other MT-like proteins) and insoluble metal-rich granules, is considered unavailable for physiological roles or to cause toxicity. In this context, toxicity occurs when the detoxification capacity is overwhelmed and an excess of metal spills over to metal-sensitive compartments. Additionally, subcellular fractionation allows inference about potential mechanisms of toxicity based on changes in metal concentrations associated with subcellular compartments coupled with measurement of biomarkers of effects.

The majority of the studies on internal bioavailability of metals involve invertebrates [see reviews by Rainbow (2002); Wallace *et al.* (2003); Ahearn *et al.* (2004)]. The present study presents the first comprehensive analysis of internal bioavailability of Zn in fish gill, intestine and liver following exposure to waterborne and dietary Zn, singly and in combination. Briefly we demonstrate: (i), tissue-specific differences in the pattern of Zn accumulation in subcellular compartments (ii), distribution patterns with the majority of the accumulated Zn being metabolically active (iii), a shift in the distribution of Zn between MAP and MDP in dietary Zn exposure (iv), minimal tissue- and fraction-specific cross-talk between waterborne and gastrointestinal pathways of Zn uptake and (v), tissue-specific dominance of waterborne and dietary uptake pathway in determining subcellular Zn accumulation. These findings are discussed in detail in the following sections.

4.3.1 Internal bioavailability of branchial Zn

Very few studies have investigated subcellular distribution of metals in gills of aquatic species possibly because gentle homogenization, a prerequisite for successful subcellular fractionation, is difficult. Specifically, the bony and cartilaginous parts of the gill are not amenable to gentle homogenization. We overcame this problem by carefully cutting or scrapping the gill filaments/epithelium from the cartilaginous septum. The isolated epithelia/filaments were then easily homogenized with 8 strokes of the pestle without disrupting organelle membranes.

The pattern of Zn accumulation in the gill was of the order: nuclei-cellular debris > HDP > HSP ≥ NaOH-resistant granules > mitochondria ≥ microsomes-lysosomes irrespective of the route of exposure. The nuclei-cellular debris fraction was by far the dominant Zn binding compartment in rainbow trout gill accounting for more than 40% of the branchial Zn. In addition regression analysis (Table 7) indicated that 90-94% of the variation in gill Zn accumulation could be explained by Zn accumulation in the nuclei-cellular debris fraction. We speculate that the presence of high levels of Zn in nuclei-cellular debris reflects both essentiality and storage. The essential nature of Zn in this fraction is highlighted by the high background levels and the fact that Zn is involved in nucleic acid synthesis as many of the nucleoproteins required during replication and transcription of DNA contain Zn atoms (Vallee and Falchuk 1993). We are not aware of another study that has reported subcellular concentrations of Zn in the fish gill; hence we cannot tell whether or not the nuclei-cellular fraction is consistently the main subcellular compartment for Zn accumulation. However,

previous studies have reported high levels of Cu in this fraction in gills and attributed it to the presence of secondary/tertiary lysosomes (Bunton *et al.* 1987; Lanno *et al.* 1987) acting as storage depots. In our study, binding of Zn to secondary or tertiary lysosomes can be ruled out because these structures were isolated in the particulate fraction following heat treatment and NaOH digestion of the 800 × g pellet (see fractionation protocol, Fig. 3). The high levels of Zn possibly suggest the presence of a vast number of Zn binding sites in this fraction. The nature of the Zn binding substances in fish gill nuclei-cellular debris fraction is presently not known. However, MTs have been demonstrated in the nucleus (Panemangalore *et al.* 1983) and it is possible that chronic Zn exposure resulted in their induction and increased Zn binding. Secondly, a possible preponderance of Zn finger proteins in the nucleus or their movement into the nucleus on binding to steroids may account for the high levels of Zn in this fraction. In humans for example, 3% of the genome encode Zn finger proteins (Maret 2003).

The second key finding on gill subcellular Zn distribution was the very high MAP (81-91% of the accumulated Zn, Table 8) while only 9-19% partitioned in the MDP. This indicates high Zn binding to nuclei-cellular debris, mitochondria, microsomes-lysosomes and HDP. Moreover there were no changes in distribution following chronic Zn exposure. While this points to the essentiality of Zn, it also suggests that gill tissue is likely vulnerable to Zn because induction of protective proteins, such as MT, appears to be minimal. We are not aware of previous reports of a comparably high MAP of Zn in any fish tissue. The only gill subcellular Zn distribution data

available are those of the freshwater bivalve, *Pyganodon grandis,* exposed to a natural polymetallic gradient including Zn (Bonneris *et al.* 2005a, b). In this species 73% of the Zn was bound by NaOH resistant granules (detoxified pool) and only 23% was metabolically active, a complete reversal of our observations. Additional fractionation studies of gills are required to better understand the patterns of subcellular metal distribution in aquatic animals and whether there are differences between invertebrates and vertebrates or even between species.

Generally the net (above background) Zn accumulation in the gill subcellular fractions was fairly low relative to the exposure dosages. This suggests that rainbow trout were able to regulate cellular levels of Zn. Tight regulation of subcellular Zn levels has previously been implied for the freshwater bivalve gill (Giguere *et al.* 2003; Bonneris *et. al.* 2005a, b). Among the gill fractions, the HSP fraction was the most responsive and achieved a 4-fold increase in concentration over the duration of the experiment in the waterborne exposure. This observation is consistent with the MT data and the close to 1:1 relationship between HSP and MT in gill tissue (Table 7). Nevertheless, the overall contribution of HSP was low suggesting a minor role of this fraction in Zn homeostasis and toxicity in the gill. A unique observation was that HSP was the only gill fraction that displayed cross-talk between branchial and GI Zn uptake. We report for the first time that Zn accumulation in rainbow trout HSP following waterborne and dietary exposures is additive.

Although there was a linear increase in the concentration of Zn in the NaOH-resistant granules (Fig. 19) the overall contribution of this fraction to total gill Zn did not change with chronic Zn exposure. We concluded that the role of NaOH-resistant granules in branchial Zn homeostasis is minor.

Overall for the gill, there were significant linear correlations between total branchial Zn and all subcellular fraction Zn concentrations suggesting indiscriminate cellular Zn binding. This negates the spillover hypothesis of metal toxicity (Hamilton and Merhle 1986; Rainbow 2002; Campbell *et al.* 2005). The spillover hypothesis contends that metal binding to metal-sensitive compartments occurs when detoxification (e.g. binding to HSP fraction) is exceeded. Zn binding to HSP did indeed increase with time but so did concurrent binding to other fractions indicating that HSP (MTs) afforded little or no protection to potentially metal-sensitive fractions. Moreover the ubiquitous distribution of Zn among the subcellular fractions is consistent with the chemistry of Zn, a borderline metal (Nieboer and Richardson 1980) that binds non-preferentially with many types of functional groups within animal cells.

4.3.2 Internal bioavailability of hepatic Zn

Probably because of its parenchymatous nature and ease of homogenization, the majority of metal fractionation data in aquatic organisms relate to the liver. In the present study, the liver exhibited the lowest Zn accumulation in all the fractions among the three tissues submitted to subcellular fractionation (Table 6). This is not

surprising since Zn is not known to preferentially accumulate in the liver of rainbow trout (Roch *et al.* 1982; Bradley *et al.* 1985; Sorensen 1991). However some fish, such as the female squirrelfish (*Holocentrus adscensionis*), do accumulate remarkable amounts of Zn in the liver (Hogstrand *et al.* 1996; Thompson *et al.* 2001, 2002, 2003). Clearly making general statements about the role of the liver in fish Zn homeostasis is likely to draw erroneous conclusions.

In the present study Zn concentrations among the liver subcellular fractions were in the range of 15-65 ng mg^{-1}. Zinc accumulation was almost uniform across the subcellular fractions with a mild declining gradient of: HSP > HDP ≥ mitochondria ≥ nuclei-cellular debris > microsomes-lysosomes = NaOH resistant granules. Other than for slight increases with time in the HDP, HSP and NaOH-resistant granules, Zn concentrations remained largely unchanged in hepatic subcellular fractions throughout the experiment. However, previous studies found different fractions to dominate the hepatic Zn accumulation. Giguere *et al.* (2006) and Roch *et al.* (1982) found the highest concentration in HDP fraction while Hogstrand *et al.* (1989) reported the highest concentration in the mitochondrial fraction. Fractionation of yellow perch (*Perca flavescens)* liver exposed to a natural polymetallic gradient of contaminated lakes found that the majority of the Zn was bound by organelles (Kraemer *et al.* 2005).

Taken together this study shows that 65-78% of hepatocellular Zn was held in MAP while 22-35% was within a MDP (Table 9). This translates into high Zn

concentrations in the organelle and HDP, where it is required in various Zn-metalloproteins important for normal physiology. In yellow perch, little Zn was within the detoxified compartment pool (Kraemer *et al.* 2005), suggesting internal sequestration and detoxification are not the key strategies of handling Zn in yellow perch liver. This contrasts with our data which suggests significant hepatic detoxification of Zn in rainbow trout liver.

Overall it is apparent from the subcellular Zn concentration and distribution data that hepatic Zn is highly regulated by rainbow trout. Although the fish were exposed to 30-40 fold higher concentrations of waterborne or dietary Zn, minimal increases in fraction Zn concentrations occurred. This homeostasis appears to involve in part increased partitioning of Zn into MDP. Our data are also consistent with observations and conclusions made by several other authors (Andres *et al.* 2000; McGeer *et al.* 2000; Chowdhury *et al.* 2003; Kraemer *et al.* 2005) that the liver is not a primary organ for Zn accumulation in rainbow trout and yellow perch. Interestingly, other than HSP vs. MT, only the mitochondria fraction Zn concentrations were positively correlated with total liver Zn, albeit weakly. This may suggest that hepatic Zn may have been lost via other pathways such as biliary excretion (Chowdhury *et al.* 2003).

4.3.3 Internal bioavailability of intestinal Zn

Similar to the gill, the fish intestine is rarely submitted to fractionation to investigate subcellular distribution of metals. This is the first fractionation of rainbow trout intestine following Zn exposure via both water and food. Starting from the highest to

the lowest the subcellular Zn concentration gradient was in the order: nuclei-cellular debris > HSP > NaOH-resistant granules > mitochondria > microsome-lysosomes = HDP. Thus, as with the gill, nuclei-cellular debris fraction was the main compartment for Zn accumulation. These findings are similar to the only other study Jeng *et al.* (1999) of which we are aware that investigated subcellular distribution of Zn. These authors reported that not only does the common carp intestine accumulate high levels of Zn, but the majority of the Zn partitioned in the nuclei-cellular debris fraction. Jeng *et al.* (1999) performed partial characterization of the Zn binding substances in this fraction and concluded that they were likely to be membrane bound proteins and not MT.

The two compartments associated with detoxified metal (HSP and NaOH-resistant granules) together contained a high amount of Zn as well, suggesting significant detoxification was occurring in the intestine. In line with this observation, the MAP contained 59-75% while the MDP contained 25-41% of the intestinal Zn (Table 10). Thus, as with gill and the liver, there appears to be a requirement for the presence of metabolically available Zn in the intestine to support physiological function. However in the intestine, there was a clear shift in the Zn distribution; a significantly greater proportion of the Zn partitioned into the MDP in the dietary Zn-exposed fish with time, a persuasive indication that intestinal detoxification capacity was increased. The significant induction of intestinal MT was highly correlated to HSP Zn concentration ($r = 0.97$, Table 7) which lends further support to this conclusion.

4.4 Cross-talk between waterborne and dietary Zn uptake

Although metal uptake by fish may be influenced by the route of uptake, a unifying theme has been elusive. In a comprehensive study at the organ and whole-body levels in rainbow trout, Spry *et al.* (1988) found that uptake of Zn from the two pathways depended on the concentration of Zn in water and food. These authors found dominant branchial uptake when waterborne Zn concentrations were high and dominant gastrointestinal uptake when dietary Zn was high. In terms of interactions, Spry *et al.* (1988) concluded Zn uptake via the two routes was independent because increasing waterborne Zn concentration caused additional Zn uptake above that resulting from elevated dietary Zn. Other studies that investigated waterborne-dietary Zn interactions (Pentreath 1976; Milner 1982; Willis and Sunda 1984; Kock and Bucher 1997) were all consistent with the conclusions drawn by Spry *et al.* (1988). However for Cu (Kamunde *et al.* 2001) and Cd (Szebedinsky *et al.* 2001) dietary uptake not only resulted in greater internal accumulation and different tissue distribution patterns but also influenced the subsequent uptake of the respective metal from water. Furthermore, the finding by Miller *et al.* (1993) that waterborne Cu pre-exposure resulted in greater tolerance to subsequent waterborne Cu challenge than did dietary Cu exposure suggests that metals may partition in different subcellular compartments (resulting in different physiological responses) depending on the route of uptake.

The present study did not find any interaction between the two routes at the organ level. The detailed analysis revealed three distinct tissue-specific hitherto unreported

accumulation patterns when rainbow trout were exposed to waterborne and dietary Zn simultaneously. In the plasma, blood cells, and gill, Zn uptake from the combined waterborne and dietary exposure were similar to the uptake attributable to branchial uptake (Figs. 9, 10, and 13) indicating that accumulation in these tissues was driven largely by branchial uptake. The second pattern was exhibited by the intestine where uptake from the combined exposure was similar to the uptake attributable to dietary uptake (Fig. 27) indicating that GI uptake was dominant. The third pattern describes the accumulation in the kidney, carcass and liver (Figs. 11, 12 and 20). Here neither pathway was dominant because the accumulation from the combined exposure was comparable to the accumulation from either pathway singly. Note that these conclusions are based on data from simultaneous exposure of rainbow trout to 150 μg l^{-1} waterborne Zn and 1000 mg kg^{-1} dietary Zn. Whether or not these patterns occur at other combinations of waterborne and dietary Zn exposures remain to be determined. Alternatively if we were to assume that gastrointestinal uptake is the normal route of Zn acquisition and that Zn present in naïve fish emanates from the diet, we would conclude that branchial uptake was additive in plasma, blood cells and gill.

The cross-talk between waterborne and dietary metal uptake was further dissected at lower levels of biological organization. This is the first study to attempt answering the question of whether or not an interaction between branchial and gastrointestinal uptake of Zn exists in fish at the subcellular level. Specifically, we sought to find out whether subcellular distribution of Zn would depend on the route of uptake and if specific fractions responded to both waterborne and dietary Zn exposure. In the gill,

although the waterborne exposure generally resulted in greater accumulation, the pattern of Zn accumulation was the same irrespective of the route of exposure. The greatest difference between the two pathways occurred in the HSP fraction where there was an almost 4-fold difference in the amount of Zn accumulated in the waterborne relative to the dietary exposure (Fig. 32). Moreover, Zn accumulation from the water and food was additive in HSP. In the liver Zn accumulation was almost uniform across the subcellular fractions independent of the route of exposure and Zn accumulation in hepatic HDP fraction was additive. Similarly in the intestine, the pattern of subcellular distribution was not affected by the route of exposure although dietary Zn exposure resulted in higher Zn concentrations in the subcellular fractions. Thus measurement of Zn concentration at the subcellular level in intestine did not reveal new information about the nature of cross-talk between branchial and GI pathways of Zn uptake, except perhaps in the microsome-lysosome fraction.

With regard to identifying a subcellular fraction that is responsive to both waterborne and dietary Zn in all the tissues, the mitochondrial fraction stood out as a possible candidate. This is based on the regression analysis (Table 7) which showed that only the mitochondrial fraction Zn concentrations were correlated with whole tissue Zn among the three organs analyzed. The significance and utility of mitochondrial parameters as sensitive and unifying biomarkers of Zn exposure and toxicity in fish therefore requires further investigation.

4.5 Metallothionein

The utility of MTs as a biomarker of metal exposure and metal-induced stress involves the examination of the intracellular distribution of metals among cytosolic ligand pools (Wang *et al.* 1999). In the present study, MT concentrations were back-calculated from the concentrations of Zn, Cu and Cd in the HSP fraction. This calculation assumes that the entire MT pool is bound to metals (i.e. there is no free cytosolic MT). Moreover, because metal concentrations are in excess of the MT binding sites and MT has very high affinity for metals (stability constants: Cu 10^{19} – 10^{17}; Cd 10^{17} – 10^{15}; Zn 10^{14} – 10^{11}), concentrations of free MT are considered insignificant (Coyle *et al.* 2002). We found that the MTs were dominated by Zn which is consistent with the role of MT as a high-affinity binding protein in cellular Zn homeostasis. That Zn was the principal metal in the MT is attested by the fact that in tissues where there was significant MT induction (gill and intestine) HSP Zn concentrations were highly correlated ($r \geq 0.95$) with the MT concentrations. Other studies have shown that elevation of hepatic Zn is associated with increased levels of MT (Thompson *et al.* 1999). This suggests that MT functions in trafficking or processing of newly acquired cellular Zn. In addition, it appears that chronic exposure of fish to elevated Zn levels ensured that Zn was in excess and could not be effectively displaced by the Cu and Cd despite their higher affinities for MT. Note that the latter two metals were present at low (background) concentrations. Displacement and dominant accumulation of Cu or Cd in MT has indeed been demonstrated in yellow perch (Hogstrand *et al.* 1991; Kraemer *et al.* 2005; Giguere *et*

al. 2006) and rainbow trout (Roch *et al.* 1982) exposed to a metals mixture in naturally contaminated waters.

According to Hamilton and Mehrle (1986), exposure to sublethal concentrations of metals induces thionein (apoprotein) synthesis and the binding of the apoprotein to the metal, thus forming MT. It has been shown that Cu- and Zn-MTs occur naturally in tissues of fish and that Cd-MTs are naturally absent in control animals (Olsson and Hogstrand 1987; Wu *et al.* 1999). Threshold levels of both essential and non-essential metals are necessary to trigger MT induction in the aquatic environment (Eroglu *et al.* 2005). In the present study, because MTs were present in significant concentrations in naïve fish, we postulate that background levels of metals (Cu, Zn and Cd) in our water and food were adequate to induce MT synthesis. The presence of background MT levels is necessary for the homeostasis of essential trace metals because MT serves as an acceptor or donor of metal ions (Cu and Zn) depending on the physiological requirements (Jacob *et al.* 1998; McMahon and Cousins 1998). It is significant that the highest background MT levels occurred in the intestine; MT is known to play a role in physiological uptake of Zn at this locale by binding the excess Zn in enterocyte cytoplasm (Bury *et al.* 2003, Fig. 1).

In fish, the expression and role of MT have mostly been studied in organs that play central roles in metal uptake and accumulation (Van Campenhout *et al.* 2004). In the present study, MT levels in the gill, liver and intestines of rainbow trout exhibited tissue-specific differences with the highest concentrations occurring in the intestine,

followed by the gill and the liver in that order. This is consistent with previous studies that MT levels vary among tissues within the same fish species (Olsson 1993; Hogstrand and Haux 1996; Chowdhury *et al.* 2005). In addition, significant MT induction occurred in the gill (high waterborne and combined groups) and in the intestine (high dietborne group), but not in the liver. These results are similar to those obtained by Chowdhury *et al.* (2005) in rainbow trout acclimated to waterborne and dietary Cd, a non-essential metal known to induce MT. It also is apparent from our results that the sites of environmental Zn exposure were also the primary sites of MT induction. However, only at high exposure concentrations of Zn in both the water and food was MT induced above the background levels. This suggests that at the higher levels of exposure, additional MT was likely required to sequester and immobilize excess Zn (Hogstrand and Haux 1991), thus reducing both its local effects, as well as transport to potentially metal sensitive sites elsewhere in the fish. Local sequestration not only facilitates excretion through sloughing of mucus and epithelial cells (Sorensen 1991; Handy 1996; Chowdhury *et al.* 2005), but also reduces absorption and favors back-transfer of Zn into intestinal lumen (Cousins 1985).

Overall the MT induction following Zn exposure observed in the present study was low compared to the induction observed with other metals (Cu and Cd) during waterborne and dietary metal exposures (Dang *et al.* 2001; DeSmet 2001; Hollis *et al.* 2001; Wu and Wang 2003; Cheung *et al.* 2004; Chowdhury 2005). This suggests that MT may not be as important in detoxifying/sequestering excess Zn as it is for Cu and

Cd. Indeed only slight accumulation of Zn was associated with elevated branchial MT levels in yellow perch (Hogstrand *et al.* 1995).

5.0 PERSPECTIVES

While the value of metal accumulation data at the whole-body and organ levels for toxicological inference appears to be limited, the significance of measuring internal bioavailability in understanding the homeostasis and toxicology of metals is becoming increasingly apparent. By employing a methodology that permitted measurement of Zn concentration at the subcellular level, this thesis research demonstrated that Zn is tightly regulated in rainbow trout because subcellular accumulation was minimal in the face of elevated dietary and waterborne Zn exposures. It has been hypothesized that Zn is regulated at the level of uptake and excretion but comprehensive studies designed specifically to test this hypothesis in fish have not yet been carried out. Future studies should aim at a better understanding of the mechanisms of regulated Zn uptake at the gill and GI tract, and the physiological and abiotic factors that modulate it. Similarly, the mechanisms of endogenous Zn excretion possibly operating at the gill, intestine and liver should be investigated.

We investigated subcellular localization of Zn not only geared toward improving our understanding of Zn homeostasis, but also isolating a compartment-specific concentration that best responded to elevated Zn exposure irrespective of the route of uptake. Internal toxicant concentrations and, more specifically, the target site concentrations, provide a more direct measure of toxicity since factors related to external bioavailability and toxicokinetics (absorption, distribution, metabolism and excretion) are ruled out. Our hypothesis was that only a fraction of the Zn taken up by

fish reaches target sites to trigger a toxic response and investigating subcellular localization would provide insights on the potential site of toxic action of Zn in fish. Although Zn occurred ubiquitously in all subcellular compartments irrespective of the tissue and plane of Zn exposure, regression analysis revealed mitochondria as a possible common subcellular site of Zn accumulation. The recent development of ion-specific sensing technologies e.g. intracellular Zn sensing using ratiometric fluorescence resonance energy transfer (FRET) (Bozym *et. al.* 2006) offers an exciting avenue for more accurate measurement of intracellular Zn concentrations with a great potential of identifying the toxicologically relevant freely exchangeable Zn concentrations.

An additional aspect of Zn toxicology that requires more research is the identification of relevant, sensitive and specific biomarkers of Zn exposure and toxicity. Such a biomarker would provide a link between exposure, internal dose, and effects, an important step in toxicological inferences and risk assessment. Mitochondrial biochemical and physiological parameters need to be assessed in this context because of the apparent responsiveness of this organelle to Zn exposure. Other preliminary work along this line identified several cytosolic proteins that respond to Zn exposure by gel filtration (Fig. 34). Future studies should indentify and characterize the responses of these proteins to Zn exposure.

The question of whether or not there is cross-talk between branchial and gastrointestinal uptake of Zn also requires further research. Teasing out the

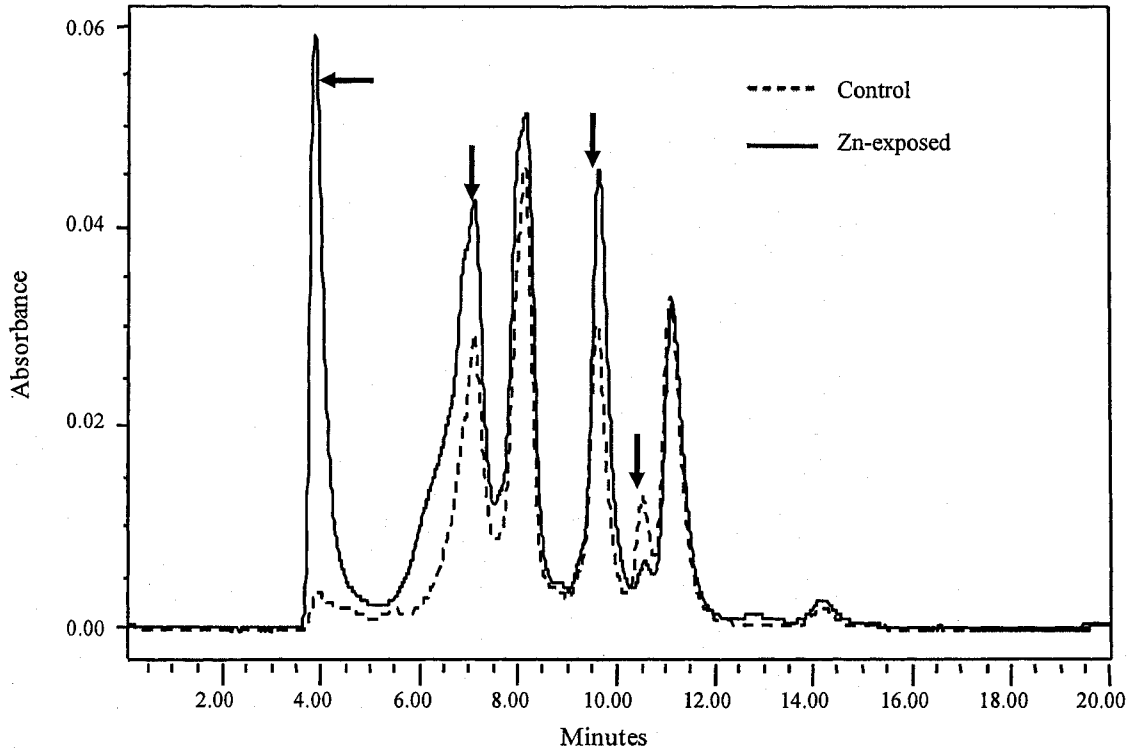


Figure 34: Gel filtration of cytosolic HSP fraction of rainbow trout. Running conditions were: buffer, 50 mM KCl; elution 0.4 ml min^{-1}; detection photo diode array @ 280 nm. Dashed lines: control; solid line: fish exposed to 4000 mg kg^{-1} dietary Zn. Arrows indicate unidentified proteins that were influenced by Zn exposure.

intracellular free Zn from bound Zn, for example with the more sensitive FRET methodology, may reveal differences and interactions that the research in this thesis was not able to detect. Overall we envisage that future studies will identify and accurately measure a single toxicologically relevant internal Zn pool which is independent of the exposure route and link it directly and specifically to toxicological effects.

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APPENDIX: TYPICAL ANALYSIS OF VARIANCE OUTPUT

```
   DESIGN: 2 - way ANOVA        , fixed effects
DEPENDENT: 1 variable:     FISH_WT
  BETWEEN: 1-TIME     ( 4): Day0 Day14 Day28 Day40
           2-TX       ( 6): LW HW LD HD CONTROL COMBINED
   WITHIN: none
```

STAT. GENERAL MANOVA	Summary of all Effects; design: (growth.sta) 1-TIME, 2-TX					
Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
1	3*	1306.626*	264*	38.50855*	33.93081*	.000000*
2	5*	239.939*	264*	38.50855*	6.23079*	.000018*
12	15*	136.756*	264*	38.50855*	3.55132*	.000014*

```
   DESIGN: 2 - way ANOVA          , fixed effects
DEPENDENT: 1 variable:      FISH_WT
  BETWEEN: 1-TIME      ( 4): Day0 Day14 Day28 Day40
           2-TX        ( 6): LW HW LD HD CONTROL COMBINED
   WITHIN: none
```

STAT. GENERAL MANOVA			Tukey HSD test; variable FISH_WT (growth.sta) Probabilities for Post Hoc Tests INTERACTION: 1 x 2				
TIME	TX		{1} 14.19000	{2} 14.19000	{3} 14.19000	{4} 14.19000	{5} 14.19000
Day 0	LW	{1}		1.000000	1.000000	1.000000	1.000000
Day 0	HW	{2}	1.000000		1.000000	1.000000	1.000000
Day 0	LD	{3}	1.000000	1.000000		1.000000	1.000000
Day 0	HD	{4}	1.000000	1.000000	1.000000		1.000000
Day 0	CONTROL	{5}	1.000000	1.000000	1.000000	1.000000	
Day 0	COMBINED	{6}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 14	LW	{7}	.999313	.999313	.999313	.999313	.999313
Day 14	HW	{8}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 14	LD	{9}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 14	HD	{10}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 14	CONTROL	{11}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 14	COMBINED	{12}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 28	LW	{13}	.114550	.114550	.114550	.114550	.114550
Day 28	HW	{14}	.551053	.551053	.551053	.551053	.551053
Day 28	LD	{15}	.245480	.245480	.245480	.245480	.245480
Day 28	HD	{16}	.798929	.798929	.798929	.798929	.798929
Day 28	CONTROL	{17}	.056677	.056677	.056677	.056677	.056677
Day 28	COMBINED	{18}	.432332	.432332	.432332	.432332	.432332
Day 40	LW	{19}	.000018*	.000018*	.000018*	.000018*	.000018*
Day 40	HW	{20}	.019554*	.019554*	.019554*	.019554*	.019554*
Day 40	LD	{21}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 40	HD	{22}	1.000000	1.000000	1.000000	1.000000	1.000000
Day 40	CONTROL	{23}	.000201*	.000201*	.000201*	.000201*	.000201*
Day 40	COMBINED	{24}	.000027*	.000027*	.000027*	.000027*	.000027*

STAT. GENERAL MANOVA			Tukey HSD test; variable FISH_WT (growth.sta) Probabilities for Post Hoc Tests INTERACTION: 1 x 2				
TIME	TX		{6} 14.19000	{7} 17.82250	{8} 16.02917	{9} 15.35250	{10} 13.44333
Day 0	LW	{1}	1.000000	.999313	1.000000	1.000000	1.000000
Day 0	HW	{2}	1.000000	.999313	1.000000	1.000000	1.000000
Day 0	LD	{3}	1.000000	.999313	1.000000	1.000000	1.000000
Day 0	HD	{4}	1.000000	.999313	1.000000	1.000000	1.000000
Day 0	CONTROL	{5}	1.000000	.999313	1.000000	1.000000	1.000000
Day 0	COMBINED	{6}		.999313	1.000000	1.000000	1.000000
Day 14	LW	{7}	.999313		1.000000	.999999	.990193
Day 14	HW	{8}	1.000000	1.000000		1.000000	.999998
Day 14	LD	{9}	1.000000	.999999	1.000000		1.000000
Day 14	HD	{10}	1.000000	.990193	.999998	1.000000	
Day 14	CONTROL	{11}	1.000000	.999996	1.000000	1.000000	1.000000
Day 14	COMBINED	{12}	1.000000	1.000000	1.000000	1.000000	.999963
Day 28	LW	{13}	.114550	.962281	.560912	.353654	.045511*
Day 28	HW	{14}	.551053	.999950	.963517	.871868	.325976
Day 28	LD	{15}	.245480	.994873	.781666	.580349	.113360
Day 28	HD	{16}	.798929	1.000000	.996495	.974810	.579552
Day 28	CONTROL	{17}	.056677	.887313	.381459	.212094	.020235*
Day 28	COMBINED	{18}	.432332	.999658	.922023	.785992	.233563
Day 40	LW	{19}	.000018*	.000763*	.000035*	.000021*	.000018*

STAT. GENERAL MANOVA			Tukey HSD test; variable FISH_WT (growth.sta) Probabilities for Post Hoc Tests INTERACTION: 1 x 2				
TIME	TX		{6} 14.19000	{7} 17.82250	{8} 16.02917	{9} 15.35250	{10} 13.44333
Day 40	HW	{20}	.019554*	.699468	.193461	.092194	.006136*
Day 40	LD	{21}	1.000000	.984288	.999994	1.000000	1.000000
Day 40	HD	{22}	1.000000	.999767	1.000000	1.000000	1.000000
Day 40	CONTROL	{23}	.000201*	.073357	.005449*	.001723*	.000056*
Day 40	COMBINED	{24}	.000027*	.010997*	.000521*	.000141*	.000019*

STAT. GENERAL MANOVA			Tukey HSD test; variable FISH_WT (growth.sta) Probabilities for Post Hoc Tests INTERACTION: 1 x 2				
TIME	TX		{11} 15.14583	{12} 16.48833	{13} 22.72917	{14} 20.92083	{15} 21.99167
Day 0	LW	{1}	1.000000	1.000000	.114550	.551053	.245480
Day 0	HW	{2}	1.000000	1.000000	.114550	.551053	.245480
Day 0	LD	{3}	1.000000	1.000000	.114550	.551053	.245480
Day 0	HD	{4}	1.000000	1.000000	.114550	.551053	.245480
Day 0	CONTROL	{5}	1.000000	1.000000	.114550	.551053	.245480
Day 0	COMBINED	{6}	1.000000	1.000000	.114550	.551053	.245480
Day 14	LW	{7}	.999996	1.000000	.962281	.999950	.994873
Day 14	HW	{8}	1.000000	1.000000	.560912	.963517	.781666
Day 14	LD	{9}	1.000000	1.000000	.353654	.871868	.580349
Day 14	HD	{10}	1.000000	.999963	.045511*	.325976	.113360
Day 14	CONTROL	{11}		1.000000	.298221	.827839	.514354
Day 14	COMBINED	{12}	1.000000		.703914	.988562	.884140
Day 28	LW	{13}	.298221	.703914		1.000000	1.000000
Day 28	HW	{14}	.827839	.988562	1.000000		1.000000
Day 28	LD	{15}	.514354	.884140	1.000000	1.000000	
Day 28	HD	{16}	.959498	.999412	.999997	1.000000	1.000000
Day 28	CONTROL	{17}	.172420	.522578	1.000000	1.000000	1.000000
Day 28	COMBINED	{18}	.729618	.969711	1.000000	1.000000	1.000000
Day 40	LW	{19}	.000020*	.000066*	.485066	.089935	.275113
Day 40	HW	{20}	.071709	.296519	1.000000	.999931	1.000000
Day 40	LD	{21}	1.000000	.999907	.036258*	.282486	.093203
Day 40	HD	{22}	1.000000	1.000000	.149067	.626868	.303360
Day 40	CONTROL	{23}	.001198*	.011357*	.997039	.818266	.974036
Day 40	COMBINED	{24}	.000098*	.001171*	.908103	.413433	.744471

STAT. GENERAL MANOVA			Tukey HSD test; variable FISH_WT (growth.sta) Probabilities for Post Hoc Tests INTERACTION: 1 x 2				
TIME	TX		{16} 20.08500	{17} 23.30833	{18} 21.29833	{19} 29.66750	{20} 24.07833
Day 0	LW	{1}	.798929	.056677	.432332	.000018*	.019554*
Day 0	HW	{2}	.798929	.056677	.432332	.000018*	.019554*
Day 0	LD	{3}	.798929	.056677	.432332	.000018*	.019554*
Day 0	HD	{4}	.798929	.056677	.432332	.000018*	.019554*
Day 0	CONTROL	{5}	.798929	.056677	.432332	.000018*	.019554*
Day 0	COMBINED	{6}	.798929	.056677	.432332	.000018*	.019554*
Day 14	LW	{7}	1.000000	.887313	.999658	.000763*	.699468
Day 14	HW	{8}	.996495	.381459	.922023	.000035*	.193461
Day 14	LD	{9}	.974810	.212094	.785992	.000021*	.092194

STAT. GENERAL MANOVA			Tukey HSD test; variable FISH_WT (growth.sta) Probabilities for Post Hoc Tests INTERACTION: 1 x 2				
TIME	TX		{16} 20.08500	{17} 23.30833	{18} 21.29833	{19} 29.66750	{20} 24.07833
Day 14	HD	{10}	.579552	.020235*	.233563	.000018*	.006136*
Day 14	CONTROL	{11}	.959498	.172420	.729618	.000020*	.071709
Day 14	COMBINED	{12}	.999412	.522578	.969711	.000066*	.296519
Day 28	LW	{13}	.999997	1.000000	1.000000	.485066	1.000000
Day 28	HW	{14}	1.000000	1.000000	1.000000	.089935	.999931
Day 28	LD	{15}	1.000000	1.000000	1.000000	.275113	1.000000
Day 28	HD	{16}		.999902	1.000000	.030340*	.997185
Day 28	CONTROL	{17}	.999902		1.000000	.668281	1.000000
Day 28	COMBINED	{18}	1.000000	1.000000		.138449	.999993
Day 40	LW	{19}	.030340*	.668281	.138449		.867774
Day 40	HW	{20}	.997185	1.000000	.999993	.867774	
Day 40	LD	{21}	.525766	.015762*	.198499	.000018*	.004652*
Day 40	HD	{22}	.853971	.076515	.506671	.000018*	.027587*
Day 40	CONTROL	{23}	.576359	.999726	.895646	.999981	.999997
Day 40	COMBINED	{24}	.201798	.971548	.531083	1.000000	.996978

STAT. GENERAL MANOVA			Tukey HSD test; variable FISH_WT (growth.sta) Probabilities for Post Hoc Tests INTERACTION: 1 x 2			
TIME	TX		{21} 13.27500	{22} 14.42833	{23} 26.73667	{24} 28.09167
Day 0	LW	{1}	1.000000	1.000000	.000201*	.000027*
Day 0	HW	{2}	1.000000	1.000000	.000201*	.000027*
Day 0	LD	{3}	1.000000	1.000000	.000201*	.000027*
Day 0	HD	{4}	1.000000	1.000000	.000201*	.000027*
Day 0	CONTROL	{5}	1.000000	1.000000	.000201*	.000027*
Day 0	COMBINED	{6}	1.000000	1.000000	.000201*	.000027*
Day 14	LW	{7}	.984288	.999767	.073357	.010997*
Day 14	HW	{8}	.999994	1.000000	.005449*	.000521*
Day 14	LD	{9}	1.000000	1.000000	.001723*	.000141*
Day 14	HD	{10}	1.000000	1.000000	.000056*	.000019*
Day 14	CONTROL	{11}	1.000000	1.000000	.001198*	.000098*
Day 14	COMBINED	{12}	.999907	1.000000	.011357*	.001171*
Day 28	LW	{13}	.036258*	.149067	.997039	.908103
Day 28	HW	{14}	.282486	.626868	.818266	.413433
Day 28	LD	{15}	.093203	.303360	.974036	.744471
Day 28	HD	{16}	.525766	.853971	.576359	.201798
Day 28	CONTROL	{17}	.015762*	.076515	.999726	.971548
Day 28	COMBINED	{18}	.198499	.506671	.895646	.531083
Day 40	LW	{19}	.000018*	.000018*	.999981	1.000000
Day 40	HW	{20}	.004652*	.027587*	.999997	.996978
Day 40	LD	{21}		1.000000	.000044*	.000019*
Day 40	HD	{22}	1.000000		.000341*	.000034*
Day 40	CONTROL	{23}	.000044*	.000341*		1.000000
Day 40	COMBINED	{24}	.000019*	.000034*	1.000000	