THE CHRONIC EFFECTS OF DIETARY LEAD IN FRESHWATER JUVENILE RAINBOW TROUT

THE CHRONIC EFFECTS OF DIETARY LEAD IN THE FRESHWATER JUVENILE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): PHYSIOLOGICAL AND TOXICOLOGICAL APPROACHES

By

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ABSTRACT

The aim of this research was to determine how freshwater fish respond to dietary lead (Pb) in their environment with a respect to both physiological and toxicological endpoints. Previous acute exposure studies have shown that waterborne Pb accumulates in the internal tissues, resulting in hematological alterations and disruptions in Na^+, Cl^- and Ca^{2+} ion-regulation, with waterborne Ca^{2+} being protective against Pb tissue burden. In comparison with other metals such as silver, zinc, cadmium and copper, very little is known about Pb toxicity via the trophic food chain.

Using rainbow trout as the model freshwater fish species, it was discovered that dietary Pb accumulated mainly in the gastrointestinal tract, with the intestine and the stomach being suggested as the primary tissues involved in Pb toxicity. Mild physiological disruptions in terms of plasma Ca^{2+}, Mg^{2+} and Na^+ levels were evident. On a per fish weight basis, the bone accumulated the greatest Pb burden (38%) followed by the anterior intestine (19%) and white muscle (12%). The order of Pb accumulation reflected the exposure pathway of dietary Pb in the internal tissues after 42 days; anterior intestine> stomach> mid intestine> posterior intestine> bone> posterior kidney> anterior kidney> liver> spleen> gill> carcass> brain> white muscle (μg Pb/g tissue wet weight). Despite this accumulation of Pb into the internal tissues, there were no effects on growth and survival rates, indicating that growth is not a sensitive indicator of dietary Pb toxicity in fish. Elevated dietary Ca^{2+} levels had an overall effect in reducing

dietary Pb burdens in the whole body and most of the internal tissues analysed. The red blood cells (RBC) accumulated 99% of the Pb in the blood, with less than 1% in the plasma. The enzyme δ-aminolevulinic acid dehydratase (ALAD), important in hemoglobin synthesis was significantly inhibited by the end of the experiment in fish exposed to high concentrations of dietary Pb. In contrast to waterborne Pb studies, ALAD was not a sensitive biomarker of dietary Pb toxicity.

Overall, this study has shown the mild physiological and toxicological disturbances of dietary Pb exposure to juvenile rainbow trout. It encourages studies on transport mechanisms at the intestine and in the blood of fish exposed to dietary Pb. It is the hope that the work from this thesis will be used in risk assessments and environmental policies that mitigate the toxic effects of dietary Pb in the aquatic environment.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized into four chapters. Chapter 1 provides the general introduction and an outline of the objectives for this project. Chapter 2 and 3 are formatted manuscripts with minor revisions submitted for publication in peer-reviewed journals. Chapter 4 is a summary of the results and conclusions from Chapters 2 and 3.

Chapter 1: **Introduction and Project Objectives**

Chapter 2: **Dietary Pb accumulation in juvenile freshwater rainbow trout (*Oncorhynchus mykiss*).**

Authors: Lara C. Alves, Chris N. Glover, and Chris M. Wood

Comments: This study was conducted by LCA under the supervision of CMW and with the help of CNG. This paper has been submitted to *Archives of Environmental Contamination and Toxicology.*

Chapter 3: **Dietary Pb accumulation in juvenile freshwater rainbow trout (*Oncorhynchus mykiss*) in the presence and absence of elevated dietary Ca^{2+} levels**

Authors: Lara C. Alves and Chris M. Wood

Comments: This study was conducted by LCA under the supervision of CMW. This paper will be submitted for publication to *Aquatic Toxicology.*

Chapter 4: **Summary of Results and Conclusions**

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INTRODUCTION

Lead in the Aquatic Environment

Lead (Pb), which is naturally found in the earth's crust, sediment, soil and water, is mainly introduced into the aquatic environment via anthropogenic activities such as mining, smelting and refining of other metals. There are three basic forms in which Pb can exist: dissolved, colloidal and particulate (Demayo et al., 1982; Stiff, 1971). In theory the dissolved form of Pb is the most toxic to aquatic organisms. Background levels of waterborne Pb in the uncontaminated aquatic environment usually fall between the range of 0.6 to 120 μg Pb/L (Demayo et al., 1982), with close to 900 μg Pb/L being reported at contaminated sites (Research Triangle, 1999). Similarly benthic invertebrates, a source of food for fish, have been found to contain <0.1 μg Pb/g dry weight at uncontaminated Pb sites and up to 792 μg Pb/g at contaminated Pb sites (Woodward et al., 1994, 1995; Farag et al., 1994, 1999).

Routes of Exposure

Aquatic organisms can be exposed to Pb through their water and/or diet. Depending on the route of exposure, and the environmental conditions (temperature, pH, water chemistry, metal speciation and metal distribution) there are essentially two important pathways in which metals may enter and accumulate in fish-the gills and the gastrointestinal tract (Kumada et al., 1973; Dallinger et

al., 1987, Szebedinszky et al., 2001). The skin has also been suggested to play a role in metal uptake, however, less is known about this route of uptake (Varanasi and Markey, 1977; Eddy and Fraser, 1982; Pärt and Lock, 1983; Dallinger et al., 1987). Metal uptake at the gills may occur by transporting dissolved metals from the water into blood via intracellular transport proteins (Thomann et al., 1997). For the gastrointestinal tract, uptake may occur by dissolution of the particulate form of the metal from the food, followed by absorption by the enterocytes, to the blood and transport eventually to other tissues in the fish (Thomann et al., 1997).

Gill Ionoregulation

The gill of teleost fish plays an important role in ion regulation, gas exchange, acid-base balance and the excretion of nitrogenous waste (Wood, 2001). The gill is considered the primary site of waterborne Pb toxicity, since it is able to take up and retain dissolved metals from the water via active and passive processes (Hodson et al., 1976; 1977, 1978a; Rogers et al., 2003, 2005; Rogers and Wood, 2004). In many systems, Pb is considered to be a Ca^{2+} mimic or antagonist (Varanasi and Gmur, 1978; Rogers et al., 2003, 2005; Rogers and Wood, 2004). Ca^{2+} is an essential metal that is transported across the apical membrane of chloride cells (mitochrondrial rich cells) by passive diffusion through voltage-insensitive channels, which are under tight control of the stanniocalcin-mediated pathway. Once in the chloride cells, Ca^{2+}-binding proteins transport Ca^{2+} to the basolateral membrane, where Ca^{2+} is extruded into

circulation via high-affinity Ca^{2+}-ATPase (Flik et al., 1985; Flik et al., 1993;Verbost et al.,1994; Marshall, 2002; Rogers et al., 2003, 2005). Waterborne studies (Rogers and Wood, 2004; Rogers et al., 2005) have shown that Pb is transported from the water and into the gills by the same mechanisms of those of Ca^{2+}, thus disrupting Ca^{2+} ion regulation. Not surprisingly, earlier studies have found that both waterborne Ca^{2+} and dietary Ca^{2+} is protective against waterborne Pb uptake at the gills (Varanasi and Gmur, 1978).

Na^+ is thought to move across the apical membrane of chloride cells via an active apical H^+-ATPase that extrudes protons and generates a favourable electrochemical gradient that enables Na^+ to pass through a coupled Na^+ channel (Perry and Fryer, 1997; Wilson et al., 2000). The protons (H^+) are provided by intracellular carbonic anhydrase which hydrates CO_2 to H^+ and HCO_3^-. The transport of Na^+ across the basolateral membrane to the plasma is via the direction of Na^+, K^+-ATPase, located on the basolateral membrane. Waterborne Pb appears to inhibit this process in a noncompetitive manner, by inhibiting the basolateral Na^+, K^+-ATPase and the intracellular carbonic anhydrase (Rogers et al., 2003, 2005).

It has been suggested that Cl^- is transported across the apical membrane of the chloride cells by a 1:1 exchange with bicarbonate through the action of an apical Cl^-/HCO_3^- exchange mechanism (Wood and Goss, 1990; Perry, 1997). The movement of Cl^- across the apical membrane appears to be powered indirectly by Na^+, K^+-ATPase, and depends on the hydration of intracellular CO_2 to HCO_3^- by

carbonic anhydrase. The mechanism of Cl^-extrusion across the basolateral membrane of chloride cells is presently not known. Decreases in Na^+, K^+-ATPase and carbonic anhydrase activities, and disruptions in Na^+ and Cl^- balance in the presence of waterborne Pb have been shown in both freshwater fish and crustaceans (Morgan et al., 1997; Ahern and Morris, 1998; Ay et al., 1999, Rogers et al., 2003, 2005).

Intestinal Ionoregulation

The intestine is a multifunctional organ in that it is important in the digestion of food, nutrient absorption, elimination of undigested waste, and in marine teleosts it is a tissue of major importance in ion and water balance (Horn, 1998). The intestine is considered the primary site of dietary metal toxicity (Kleinow and James, 2001; Meyer et al., 2005), since it is able to assimilate metals from the diet via both active and passive processes (Meyer et al., 2005). In contrast to the gills, the absorption of Ca^{2+} in the intestine of freshwater fish at the basolateral membrane of enterocytes is primarily via a Na^+/Ca^{2+}-exchanger and to a lesser degree by Ca^{2+}-ATPase (Flik et al., 1993). Ca^{2+} transport in the intestine is dependent on Na^+ concentration and Na^+, K^+-ATPase activity, which maintains the Na^+ gradient and acts as the driving force for the Na^+/Ca^{2+} -exchanger (Flik et al., 1993). However, there is evidence that passive entry of Ca^{2+} into the intestine may be under the control of stanniocalcin, similar to the situation at the gills (Flik et al., 1993).

In comparison to freshwater fish, marine fish have high drinking rates and high rates of intestinal ion and water absorption. The ion absorption osmotically drives the water absorption, which serves to replace the osmotic loss of water to the external environment (Karnaky, 1998). In contrast, freshwater teleosts absorb Na^+ and Cl^- through the diet in order to compensate for the salt that has been lost by diffusion across the gills to external environment. Hence, the bulk of research in terms of NaCl absorption at the intestine in fish has been mainly conducted on marine teleosts. In marine fish, the majority of NaCl absorption takes place in the small intestine. The Na^+, K^+-ATPase enzyme present on the basolateral side of the enterocytes creates a transmembrane electrochemical gradient for Na^+, by which the apical membrane coupled Na^+-K^+-$2Cl^-$ cotransporter brings Na^+ and Cl^- into the enterocytes. Na^+ is then extruded by the basolateral Na^+, K^+ ATPase, while Cl^- diffuses out down an electrochemical gradient or is exported by Cl^- /HCO_3^- exchangers or K^+-Cl^- cotransporters (Loretz, 1995). Baldisserotto and Mimura (1994) found that the posterior intestine of the freshwater-adapted *Anguilla anguilla* possesses a Na^+-K^+-Cl^-- cotransporter, a Na^+, K^+-ATPase pump, and a K^+-Cl^- cotransporter at the basolateral membrane. This observation suggests that the absorption of Na^+ and Cl^- in freshwater fish may be similar to that of marine fish. However, more studies are necessary in order to understand the intestine's role in freshwater osmoregulation.

Little is known about the effects of metals on ionoregulatory and osmoregulatory processes in the gastrointestinal tract of freshwater teleosts. In

terms of dietary Pb exposure, a study by Crespo et al. (1986) found that fish fed 10 μgPb/g fish/day had reduced Na^+, K^+-ATPase activities and reduced Na^+ and Cl^- influx rates at the mid intestine after 15 days. This study suggests that Pb may also be involved in ionoregulatory disruptions at the intestine.

Waterborne Pb studies

Cd is similar to Pb in that it is both a Pb and Ca antagonist. Cd studies have demonstrated that waterborne Cd accumulates to the greatest extent at the gills (Kumada et al., 1973; Handy, 1992, Hollis et al., 2000). This is consistent with studies (Hodson et al., 1977, 1978a; Rogers et al., 2003, 2005; Rogers and Wood, 2004) on waterborne Pb that found that the gills accumulated significantly higher Pb concentrations in comparison to other tissues. Sastry and Gupta (1978) found that when *Channa punctatus* were exposed to sublethal concentrations of 6.8 mg Pb/L for 125 days, there were degenerative changes in the histological structure of the liver, intestine and pyloric cecae. Hepatic damage was in the form of necrosis, inflammation of the portal area, hardening of the vessels and accumulation of lipofusion granules in the cytoplasm of the hepatocytes. In the intestine and pyloric cecae there was a flattening of the villi, inflammation and necrosis. In addition, there was an inhibition of alkaline phosphatase activity at the stomach and increases in acid phosphatase activity at the intestine and pyloric cecae.

Studies from this laboratory (Rogers et al., 2003, 2005; Rogers and Wood, 2004) have shown that acute waterborne Pb exposure to rainbow trout resulted in significant ionoregulatory disruptions in terms of Na^+ and Cl^- balance and Ca^{2+} homeostasis, with no significant effects on respiratory or acid/base regulation. In short, these studies have found that waterborne Pb accumulation inhibits both branchial Na^+, K^+-ATPase and carbonic anhydrase activities by binding to these enzymes, thus influencing Na^+ and Cl^- balance. In addition, as mentioned above, evidence has shown that waterborne Pb follows a similar uptake mechanism to that of Ca^{2+} at the gills.

Somero et al. (1977) found that fish exposed to waterborne Pb and held at higher temperatures accumulated more Pb than at lower temperatures and the rate of Pb accumulation was inversely proportional to the salinity of the environment in the estuarine teleost fish *Gillichthys mirabilis.* This is important since the rates of exchange of water and inorganic ions are temperature-dependent in marine fish and drinking rates are proportional to the salinity of the medium. This indicates that Pb may compete with other divalent ions, Ca^{2+} and Mg^{2+} for uptake at the gills and intestine and, in turn, may disrupt osmoregulation (Maetz and Evans, 1972; Somero, 1977). In addition, Merlini and Pozzi (1977) found that at pH 6, the sunfish, *Lepomis gibbosus* L. accumulated three times more lead than fish held at pH 7.5, when exposed to waterborne Pb. This suggests that the Pb present in the water was converted to its ionic form at low pH, and therefore became more bioavailable. In agreement, Hodson et al. (1978b) found that juvenile rainbow

trout exposed to nominal waterborne concentration of up to 1000 μg Pb/L for 3 days showed increasing Pb concentrations as the pH of the water decreased from 10 to 6. A decrease in pH by 1.0 unit from any reference pH resulted in an increase of blood-Pb by a factor of 2.1. Moreover, Pb uptake has been found to be linearly related to Pb concentrations in the water (Hodson et al., 1978a).

Davies et al. (1976) found that Pb is more toxic to rainbow trout in soft-water than hard-water. It was suggested that in hard-water there is decreased solubility due to the formation of complexes between metals and anions present in such water. For example, the carbonate component of water hardness ($CaCO_3$) is protective since it binds Pb to form insoluble $PbCO_3$ that is not taken up by the gills. Varanasi and Gmur (1978) have also shown that Ca^{2+} in both the water and diet is protective against Pb uptake in the tissue of coho salmon, *Oncorhynchus kisutch*, but that Ca^{2+} in the diet is more effective. Basically, these authors found that the uptake of waterborne Pb into the gills, blood, kidney, and bone was reduced in fish force-fed with dietary Ca^{2+}. Similarly, waterborne Ca^{2+} (6.2 mg/L) also reduced Pb burdens in these tissues.

Dietary Pb studies

In contrast to waterborne Cd studies, dietary Cd accumulates more in the intestine (Handy, 1992; Hollis et al., 1999; Franklin et al., 2005). Studies that have employed isolated gut sacs (Crespo et al., 1986; Ojo, 2005) have found reduced transepithelial Na^+ and Cl^- fluxes and elevated Pb uptake in the mid-

intestine compared to other metals and other sections of the intestine. Varanasi and Gmur (1978) found that two weeks after terminating Pb exposure in their force-feeding study, Pb concentrations in the bone continued to increase, suggesting that the bone may act as a sink for storing Pb, after relocation from soft tissues.

Hodson et al. (1978a) found that fish fed dietary Pb concentrations of 4-118 μg Pb/g dry weight for 32 weeks, did not accumulate Pb into the internal tissues, since > 90% of the Pb was bound to the fecal matter. However, these authors suggested that the beef liver used to make up the dietary Pb treatment may have bound up the majority of the Pb, making it unavailable for fish to take up via the diet.

Mount et al. (1994) exposed brine shrimp (*Artermia* sp.) to individual metals (Cd, Cu, Pb, Zn, Ag) and fed these contaminated brine shrimp to rainbow trout for 60 days, with simultaneous exposure to waterborne metals. Concentrations employed in the diet were much higher (~50, 000 fold more) than in the water, reflecting the situation in the "natural" exposures at contaminated sites. They found that over the range of waterborne and dietary concentrations, the diet accounted for more of total body Pb than the water.

Woodward et al. (1994) found a 40% reduction in growth and survival in adult rainbow trout that fed on contaminated benthic invertebrate diets containing elevated levels of As, Cu and Pb for 91 days from the Clark Fork River in Montana. In a follow-up study, Farag et al. (1994) found that there were no

significant effects on juvenile trout survival and growth and no significant differences were seen in serum Ca^{2+}, K^{+}, Na^{+} in adult rainbow trout that were fed a similar diet from the same river. When cutthroat trout (*Oncorhynchus clarki*) were fed metal-contaminated invertebrate diets collected from different sites along the the Coeur d' Alene River, Idaho there was a 30% reduction in survival and 87% reduction in growth of fish fed the Cataldo diet, which had lower metal concentrations versus the North and South Fork diets which had higher metal concentrations (Farag et al., 1999). These authors suggested that the differences between these diets and studies reflected differing bioavailability of the metals in the diet.

Elevated Ca^{2+} in the diet has been found to not only significantly reduce Cd uptake from the water, but also Cd uptake from the diet when compared to fish fed Cd alone (Baldisserotto et al., 2004a,b, 2005; Franklin et al., 2005). These results suggest that Ca^{2+} and Cd share a common pathway/transport mechanism in the gut, as in the gills (Verbost et al., 1989). In light of these dietary Cd studies and waterborne Pb studies, (Varanasi and Gmur, 1978; Rogers et al., 2003, 2005; Rogers and Wood, 2004) it would be speculated that elevated Ca^{2+} in the diet would also be protective against dietary Pb accumulation. With the exception of the laboratory experiment of Mount et al. (1994) and the mixed metal studies of Woodward et al. (1994, 1995) and Farag et al. (1994, 1999) using field collected diets, both of which found that dietary Pb does accumulate into internal tissues of

fish, in contrast to the work of Hodson et al. (1978a), research on the effects of chronic sublethal dietary Pb exposure in fish remains limited.

Pb and the Hemoglobin Pathway

There is evidence that Pb interferes with several enzymes in the biosynthesis pathway of hemoglobin. Pb readily reacts with sulfhydryl, amine, phosphate and carboxyl groups (Goering, 1993). The increase in protoporphyrin in erythrocytes of mammals exposed to Pb is the result of Pb inhibiting the mitchondrial enzyme ferrochelatase (World Health Organization, 1995). Reductions in δ-aminolevulinic acid dehydratase (ALAD) activity, an enzyme that catalyses the formation of porphobilinogen (PGB) from the substrate aminolevulinic acid (ALA), has been attributed to Pb binding to essential sulfhydryl (SH) groups and displacing Zn^{2+} on ALAD (World Health Organization, 1977; Finelli, 1977; Sassa, 1982). Zn is the only other metal that affects ALAD activity in fish, by ameliorating the inhibition of Pb, through the protection of the SH groups from oxidation and competition with Pb for position on ALAD (Sassa, 1982; Dwyer et al., 1988; Schmitt, 1993). The activity of ALAD has long been used as a sensitive biomarker of Pb toxicity in humans, birds and fish (Hodson et al., 1976, 1977; Dieter, 1979; Schmitt et al., 1984, 1993). Decreased activity in ALAD can be detected at exposures near the "no effect" level (Hodson et al., 1977). After two weeks of exposing both brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss)* to 10 μg/L of

waterborne Pb, Hodson et al. (1977) found there was a marked reduction in ALAD activity. In a follow up study Hodson et al. (1978a) found that waterborne Pb concentrations as low as 13 μg Pb/L caused significant increases in red blood cell (RBC) numbers, decreases in RBC volumes, decrease in RBC cellular iron content and decreases in RBC ALAD activity, but no changes in hematocrit. These authors suggested that the removal of mature red cells from the blood and acceleration of hemopoiesis helped to compensate for the loss in oxygen carrying capacity caused by the toxic action of waterborne Pb in the blood on ALAD.

The sensitivity of ALAD to Pb varies among different taxa. Haux et al. (1986) reported 88% inhibition of ALAD in whitefishes (*Coregonus)* from the field with liver Pb-concentrations of 1.6 μg/g wet weight, while Johansson-Sjobeck and Larsson (1979) found a 70% inhibition in rainbow trout exposed to waterborne Pb concentrations of 80 μg Pb/L for 30 days. In addition, rainbow trout showed a linear response to ALAD inhibition with blood-Pb levels of 30-5400 μg/L, whereas the brook trout accumulated less blood-Pb and was less sensitive to Pb exposure and ALAD inhibition when both species were exposed to waterborne Pb concentrations of 4-92 μg/L (Hodson et al., 1977, 1978a). These authors suggested that species that take up more Pb at a faster rate and lower concentrations are more susceptible to decreased ALAD activity. This is probably because these species are different in terms of their gill permeability to Pb (Holcombe et al., 1976). Hodson et al. (1978a), the only study to my knowledge that examined the effects of dietary Pb exposure on ALAD activity, found that

ALAD activity was not affected by Pb concentrations up to 4-118 μg Pb/g for 32 weeks. Nevertheless this study also reported that dietary Pb was not taken up via the gastrointestinal tract.

Pb and Neurotoxicity

Lead has been found to cause black tail, which eventually leads to lordoscoliosis, a spinal deformity (Davies et al., 1976; Holcombe et al., 1976). Behavioral and neurological abnormalities such as disturbances in predator avoidance, feeding, visuomotor reflexive responses, and reproductive behaviours have been observed in fish exposed to waterborne Pb (Rademacher et al., 2003; Scott and Sloman, 2004). For instance when male-female pairs of fathead minnows (*Pimephales promelas*) were exposed for 30 days to waterborne Pb concentrations of 500 μg/L, there was a longer period between spawning and nest preparations by males when compared to the controls (Weber, 1993). Studies have found that dimercaptosuccinic acid (DMSA), an antidote for toxic metal exposure, is effective in lowering bone and brain-Pb concentrations, without causing relocation of Pb to other tissues, and reversing abnormalities of locomotor activity in humans, monkeys and fish exposed to Pb (Fournier et al., 1988; Tandon et al., 1994; Weber et al., 1997; Smith et al., 2000, Rademacher et al., 2003). Pb disrupts integrative functioning of the medulla, cerebellum and optic tectum, by increasing serotonin (5-HT), a substrate involved in coordinating specific behaviour. However, Pb has also been associated with a dose-dependent

fall in hypothalamic 5-HT levels in fish (Weber et al., 1991; Khan and Thomas, 2000). Sloman et al. (2005) found that when dominant fish were exposed to 325 μg/L of waterborne Pb for 48 h there were significantly higher hypothalamic 5-hydroxy-3-indoleacetic acid (5-HIAA)/5-HT ratios when compared to the subordinates. 5-HIAA is the primary metabolite of 5-HT. Rademacher et al. (2003) found that 5-HT levels decreased in the medulla and cerebellum, whereas 5-HIAA increased when dietary Pb (1500 μg/L) was replaced with DMSA (25 DMSA : 1Pb molar ratio). These researchers suggested that treatment with DMSA may be more effective than removing Pb from the diet in reversing Pb-induced alterations in 5-HT, since DMSA elicits concentration-dependent increases in the renal intracellular Ca^{2+} levels, which are blocked by Pb.

PROJECT OBJECTIVES

Recently, there has been a great interest in using biotic ligand models (BLM) for predicting and evaluating metal toxicity as a function of water chemistry and its effect on the speciation and biological availability in the aquatic environment (Paquin et al., 2002; Macdonald et al., 2002). Pb concentrations in the sediment are usually much higher than that of water and there is always the potential of Pb being transferred from sediment to benthic invertebrates and eventually to fish in the food chain. Many environmental regulations (e.g. Ambient Water Quality Criteria of the U.S.-Environmental Protection Agency) do not take into consideration the possible chronic effects of Pb in the diet alone or in

combination with waterborne Pb. However, over the past few years, the issue of dietary exposure and it's potential harmful effects to aquatic animals has received increasing attention (Szebedinszky et al., 2001; Paquin et al., 2002; Meyer et al., 2005). In light of the limited work on dietary Pb exposure, additional research is not only vital to determine the intestine's role in facilitating and/or preventing Pb toxicity, but in addressing future regulations that are able to mitigate the effects of Pb toxicity via the food chain.

Therefore, the main objective of this thesis was to investigate both the physiology and toxicology of juvenile freshwater rainbow trout (*Oncorhynchus mykiss*) chronically exposed to dietary Pb. In order to accomplish this goal, the following objectives were set forth:

1. To determine if rainbow trout exposed to sublethal chronic concentrations of dietary Pb would accumulate Pb via the diet in internal tissues, and if so to describe the temporal and tissue-specific pattern of accumulation. If Pb accumulation did take place in the internal tissues, a further goal was to determine whether Pb has an effect on growth and survival rates, hematological disruptions in terms of plasma protein and hematocrit, Na^+ and Ca^{2+} influx rates from the water, and plasma Ca^{2+} and Mg^{2+} regulation (Chapter 2).

2. To investigate if elevated Ca^{2+} in the diet is protective against Pb burden. Based on the findings of Chapter 2, a particular goal was to differentiate the distribution of dietary Pb within certain tissues (e.g. anterior and posterior sections of the kidney, and stomach, pyloric caeca, anterior, mid and posterior regions of the gastrointestinal tract), in order to determine any evident regulation and/or depuration of accumulated Pb in these tissues. An additional goal was to establish any hematological disruptions in terms of the RBC enzyme ALAD, hemoglobin levels, total blood protein, whole blood Pb and Zn levels, plasma-Pb levels and RBC-Pb levels. A final goal was to establish any effects of chronic dietary Pb exposure on growth and survival rates, plasma Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+} regulation, and Na^+, K^+-ATPase activity in the gills and different sections of the intestine (Chapter 3).

CHAPTER 2

ABSTRACT

Three different diets amended with lead (Pb) nitrate $Pb(NO_3)_2$ (7, 77 and 520 μg Pb/g dry weight) and a Pb–free control diet (0.06 μg Pb/g dry weight) were fed to juvenile freshwater rainbow trout for 21 days. The patterns of Pb accumulation over time were determined in various tissues (gills, liver, kidney, intestine, carcass), red blood cells (RBC), and plasma, as well as feeding, growth, hematological, and ionoregulatory parameters. Pb accumulation occurred in a dose-dependent manner in all tissues except the plasma, where accumulation was minimal. Overall, when fed the highest Pb diet, the intestine exhibited the greatest Pb burden (17.8 μg Pb/g tissue wet weight), with high concentrations also found in the kidney (2.4 μg Pb/g tissue wet weight) and liver (1.9 μg Pb/g) at the highest dietary Pb treatment by day 21. The RBCs accumulated a substantial amount of Pb (1.5 μg Pb/g) when compared to the plasma (0.012 μg Pb/g) in the high treatment group. Growth, survival, plasma protein and hematocrit were not significantly affected by dietary Pb. Plasma Ca^{2+} levels decreased at the beginning of the experiment, whereas Mg^{2+} levels decreased during the middle of the experiment in both the intermediate and high dietary treatments. Both the Ca^{2+} and Mg^{2+} levels stabilized by day 21. Branchial Ca^{2+} and Na^{+} influx rates were not affected by dietary Pb, except on day 8 where Na^{+} influx rates were significantly elevated. The results of this study show that Pb does accumulate internally from the diet when present at levels within the range reported in

contaminated benthic invertebrates in nature. We further identify the intestine as an important tissue for potential chronic toxicity of Pb via the diet, and RBCs as a reservoir of dietary Pb.

INTRODUCTION

Lead (Pb) is a non-nutrient metal found in the earth's crust, and thus may enter the aquatic environment through natural processes of geological weathering and volcanic emissions (Demayo et al., 1982). The background concentration of Pb in uncontaminated surface water has been estimated to be 0.02 μg Pb/L (Flegal et al., 1987). Pb may also enter the aquatic environment through anthropogenic practices such as the mining, refining and smelting of Pb (Sorensen, 1991; World Health Organization, 1995).

In fish, the primary site of Pb toxicity is at the gills (Varanasi and Gmur, 1978), where inhibitory actions of Pb on Ca^{2+}, Na^{+}, and Cl^{-} uptake have recently been documented (Rogers et al., 2003, 2005; Rogers and Wood, 2004). However toxicity may also occur via the gastrointestinal tract (Sorensen, 1991). Crespo et al. (1986) showed that rainbow trout that were orally administered with Pb in the diet (10 μg Pb/g dry weight (dw)/fish/day) had morphological alterations of the intestinal brush border which resulted in an impairment of intestinal absorption. Studies have demonstrated that fish fed diets containing metals have reduced feeding activity, growth, and survival rates (Farag et al., 1994; Woodward et al., 1994, 1995). Farag et al., (1994) reported that cutthroat trout (*Oncorhynchus clarki)* fed benthic macroinvertebrates containing high levels of metals (As, Zn,

Cd, and Pb) from different contaminated sites along the Coeur d' Alene River, Idaho, had a higher concentration of these metals in the stomach and pyloric cecae when compared to the gills and kidney. Normal Pb levels in uncontaminated benthic invertebrates are usually less than 1 μg/g dw, however body burdens up to 792 μg/g dw have been reported in benthic invertebrates in the South Fork of the Coeur d' Alene River (Farag et al., 1994, 1999). While these studies using naturally contaminated diets have displayed evidence of dietary Pb uptake and toxic effects to fish, the laboratory study of Hodson et al. (1978a) reported that rainbow trout fed diets contaminated with up to 118 μg Pb/g did not take up any Pb into internal tissue.

There is evidence for a specific chronic toxic action of waterborne Pb on erythrocyte function in fish. Hodson et al. (1978a) found that a chronic waterborne Pb exposure (13 μg Pb/L; 32 weeks) to rainbow trout caused significant increases in red blood cell (RBC) numbers, decreases in RBC volumes, RBC cellular iron content, and δ- aminolevulinic acid dehydratase (ALAD) activity, an enzyme that catalyses the formation of porphobilinogen from aminolevulinic acid in the heme synthesis pathway. This suggests that fish exposed chronically to Pb may be at risk of anemia.

Although acute waterborne Pb interactions at the gills have been well studied (Davies et al., 1976; Holcombe et al., 1976; Hodson et al., 1978a, Rogers et al., 2003, 2005; Rogers and Wood, 2004), the effects of dietary Pb on toxicological and physiological parameters have received very little attention. The

initial objective of this study was to assess whether three different Pb-contaminated diets (7, 77, 520 μg Pb/g dw) fed to juvenile rainbow trout for 21 days, resulted in Pb accumulation in specific internal tissues - liver, kidney intestine, gills, carcass, and blood. These concentrations were specifically selected to cut across the range used by Hodson et al. (1978a; 4-118 μg Pb/g dw) and the concentrations found in benthic invertebrates (Farag et al., 1994, 1999; 0-792 μg/g dw). If gastrointestinal Pb uptake occurred, a second objective was to evaluate the pattern of tissue-specific accumulation over time, and to look for any evidence of regulation or depuration. In light of the known actions of waterborne Pb on RBC function, a third objective was to test for possible hematological abnormalities in terms of plasma protein and hematocrit. A final objective was to examine if there were any disruptions in growth and survival rates, plasma Ca^{2+} and Mg^{2+} regulation, and Na^{+} and Ca^{2+} influx rates from the water, since many of these parameters are affected by waterborne Pb.

MATERIALS AND METHODS

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss)* (N=368) weighing 3-6 g were obtained from Humber Springs Trout Hatchery (Orangeville, Ontario). Upon arrival at McMaster University, Hamilton, Ontario, fish were randomly selected and placed in eight 200 L flow-through, aerated holding tanks (46 fish per tank). Each tank was supplied with 0.8 L/min of dechlorinated Hamilton water (in mM:

Na^+,0.6 mM; Cl^- 0.7; Ca^{2+}1.0; Mg^{2+} 0.2; K^+ 0.05, and water hardness as $CaCO_3$ =140 mg/L, total Pb =1.4 ± 0.2 μgPb/L) and allowed to acclimate to ambient conditions (11-13°C and pH 7.5-8.0) for at least seven weeks prior to the start of the experiment.

Fish were fed commercial salmon fry pellets (Nelson's Silver Cup fish feed; Murray, Utah, USA; 52% crude protein (min.); 14% crude fat (min.); 3% crude fiber (max.); 12% ash (max.); and 1% sodium (actual)) once daily to satiation from the third day of arrival until the start of the experiment. By this time, fish weighed about 10-13 g. Each tank was assigned to one of four treatment groups, with each treatment replicated. The four treatment groups included a nominally Pb-free diet (control), a low Pb diet (nominally 10 μg Pb/g dw), an intermediate diet (nominally 100 μg Pb/g dw), and a high Pb diet (nominally 500 μg Pb/g dw). The actual measured mean concentrations were 0.06 ± 0.004, 7.2 ± 0.9, 76.5 ± 6.7 and 519.8 ± 50.0 μg Pb/g dw.

Two weeks into the acclimation period, eight fish were randomly selected from each of the eight tanks and given a passive integrative transponder (PIT) tag, placed into the peritoneal cavity under MS-222 (3-aminobenzoic acid ethylester; 0.5 g/L) anesthesia. This was done to establish the growth rate of individual fish over the 21 day experiment.

Diet Preparation

Pb-enriched diets were made by adding lead nitrate ($Pb(NO_3)_2$, Sigma-Aldrich) into the same commercial salmon fry food (Silver Cup Feed, Murray, Utah, USA) as used during the acclimation period. The commercial food was pulverized into a fine powder using a household blender for approximately two minutes. Then, 500 g of the fine powder was hydrated with 40% v/w of double distilled water (NANOpure II; Sybron/Barnsted, Boston, MA, USA) containing different proportions of dissolved $Pb(NO_3)_2$ and blended in a commercial pasta maker for 1.5 h in order to achieve the intended doses of 10, 100 and 500 μg Pb/g dw food. The paste was then passed through a cutter where small strands were broken into small pellets. The control diet was prepared the same way except Pb was not added. The food pellets were first air-dried for 48 h, then dried in a 60°C oven to a constant weight, and frozen until further use. The measured concentration of Pb (Table 1) was determined by heat digesting the food pellets in five volumes of 1N HNO_3 at 60°C for 48 h. The supernatant was then diluted and measured on a graphite furnace atomic absorption spectrophotometer (AAS; 220 SpectrAA; Varian GTA-110; Varian, Australia) with a detection limit of 0.06 μg Pb/L against a certified multi-element Pb standard (Anachemia Inc., Quebec), employing appropriate blank samples and reference samples. Moisture was measured to be 6% by drying food to a constant weight in a 60°C oven.

Feeding, Mean Weight, Growth and Food Consumption

A total of four replicated treatments (0, 7, 77, 520 μg Pb/g dw, Table 2-1) were tested in this study. On the third day of the experiment mechanical failure resulted in an alteration in the exposure conditions in one of the replicated 77 μg Pb/g dw diet treatment tanks. This tank was eliminated from this experiment and thus the n value for this treatment was half that of all others. Each group was fed once daily to satiation, by placing 10-15 pellets on the surface of the water every minute until the fish were no longer striking at the pellets. The pellets not eaten on the surface water were removed and the bottom of each tank was siphoned approximately one hour post feeding to control any leaching of Pb from the food and feces. The amount of all food consumed by the fish was recorded in order to determine the voluntary ration. About 10 mL of water was taken before and after siphoning in order to determine the extent of any waterborne Pb contamination.

Mean fish weights were calculated every week by bulk weighing all fish from each holding tank. The mean fish weight was determined by dividing the number of fish in each tank by the total biomass. Fish that were tagged were individually weighed and their fork lengths were measured. The condition factor was determined for each fish using the formula

$$CF=(W/L^3)*100 \quad (1),$$

where W is the weight in grams and L is the fork length in centimeters.

Specific growth rates (SGR) expressed on a %/day basis (Ricker, 1979) were determined using the least-squares linear regression (SigmaStat, version 3.0)

through the natural logarithm of the individual tagged fish weights versus time. The ration of food consumption per fish at satiation (%/day) was determined using the formula

$$r = (y/n*w)*100 \quad \textbf{(2)},$$

where r is the ration (%/day), y is the total food in grams fed to fish in each tank, n is the number of fish in each tank, and w is the average mean fish wet weight in grams. Food conversion efficiency (FCE) (in %) for each treatment was expressed using the formula

$$FCE = (SGR_{ave}/r) *100 \quad \textbf{(3)},$$

where SGR_{ave} is the mean specific growth rate (%/day), and r is the ration (%/day).

Tissue Sampling

At day 0, two fish from each tank, and on days 7, 14 and 21 six randomly selected fish per tank were sacrificed with 1.0 g/L MS 222. Tagged fish were not sampled. Blood was taken by caudal puncture using an ice-chilled 250 μL gas tight Hamilton syringe pre-rinsed with lithium heparin (50 i.u./mL). Some of the whole blood was used for measurement of hematocrit by capillary tube centrifugation at 13700 g for 2 min. The hematocrit was read directly from the tube, which was then broken in order to extract plasma for total plasma protein (g/100 mL) by refractometry (American Optical, Buffalo, NY) (Alexander and Ingram, 1980). The rest of the whole blood was centrifuged at 10000 g for 2 min

and plasma and RBCs collected, snap-frozen in liquid nitrogen and stored at –70 °C until further analysis for plasma Ca^{2+}, Mg^{2+}, total plasma Pb and total RBC Pb concentrations.

The liver, kidney, intestine, and gill baskets were dissected, rinsed, blotted, weighed and kept at 4°C until analysis for Pb content. The gills were rinsed with double-distilled water and the intestinal tract was flushed out with 0.9% NaCl to remove any source of non-tissue Pb. The pyloric cecae were removed from the anterior intestine and placed with the carcass, which also included the similarly flushed stomach.

For plasma Ca^{2+}, Mg^{2+}, and Pb, samples were diluted with appropriate modifiers and measured against known standards by flame (Ca^{2+}, Mg^{2+}) or furnace (Pb) atomic absorption spectrophotometry (AAS; Varian 220FS or Varian GTA-110, Australia).

Weighed liver, kidney, intestine, gill, carcass and RBC samples were digested in 5 volumes of 1 N HNO_3 in a 60°C oven for 48 h. After 48 h, these samples were centrifuged at 10000 g for 20 min and the supernatant was appropriately diluted and measured by graphite furnace AAS for Pb accumulation, as outlined earlier for analysis of Pb in food.

Na^+ and Ca^{2+} Influx Rates

On day 0, two randomly selected fish from each tank, and on days 8 and 22, six randomly selected fish from each tank were used to measure the

unidirectional Na^+ and Ca^{2+} influx rates from the water. At time 0, 0.1 μCi of ^{22}Na and 10 μCi of ^{45}Ca were added to the water (2 L) and allowed to mix for 10 min. Two 5 mL water samples were taken at 10 min, 2 h, and 4 h for determination of specific activity.

The fish were then removed and placed in a plastic bag containing 200 mL of 10 mM NaCl plus 10 mM $CaCl_2 \cdot 2H_2O$ for 10 min, as a cold displacement to remove any loosely bound ^{22}Na and ^{45}Ca. The fish were blotted dry, weighed and placed in plastic vials to count for ^{22}Na on a γ-counter (Canberra-Packard A5000 Minaxi). The γ-counter did not detect ^{45}Ca radioactivity. Samples were prepared for scintillation counting of ^{45}Ca by acid-digesting the fish in the same manner as for the Pb tissue burden study. The tubes were centrifuged at 5525 g for 10 min (Sigma 4K15C Refrigerated Centrifuge); 2.5 mL of the supernatant was then placed into a glass scintillation vial, with 10 mL of Ultima Gold AB cocktail (Packard Science, Netherlands). For water samples, 5 mL of aqueous counting scintillant (ACS, Amersham, Canada) was added to 2 mL of water. The processed tissue and water were placed in a dark room overnight to reduce chemiluminescence, then counted on a liquid scintillation counter (RackBeta 1217, LKB Wallac, Finland). Since ^{22}Na is a γ/β emitter, the scintillation counter detected both ^{45}Ca and ^{22}Na radioactivity, so it was necessary to distinguish the radioactivity due to ^{45}Ca alone (β emitter) using the protocol of Van Ginneken and Blust (1995).

The ^{45}Ca values from tissue digests were then quench-corrected to the same counting efficiency as ^{45}Ca found in water samples by the method of external standard ratios (ESR), using a ^{45}Ca quench curve that was generated from carcass tissue of varying weights, processed the same as above and counted in the same cocktail.

The unidirectional Na^+ and Ca^{2+} influxes (μmol/kg/h) were calculated using the formula

$$J_{in} = \text{Fish CPM}/(\text{MSA}.W t.) \quad \textbf{(4)},$$

where MSA is the mean specific activity (CPM/umol) of water in the γ counter for ^{22}Na or in the scintillation counter for ^{45}Ca, W is the weight of the fish, and *t* is the time in hours, and Fish CPM is the total cpm of ^{22}Na or ^{45}Ca accumulated by the fish during the flux period.

Statistics

All statistical tests were performed using Sigmastat version 3.0. Data were tested for homogeneity of variances among groups using the Bartlett test. Those that failed were subjected to natural logarithm or square root transformations to obtain homogeneity among groups. Data that could not be normalized were subjected to non-parametric analysis, using the Kruskal-Wallis procedure, where all ranks were corrected for ties. Comparisons between treatments and days were made by one way or two way analysis of variance (ANOVA), as appropriate,

followed by a Tukey (parametric analysis) or Dunn's test (non-parametric analysis).

RESULTS

Waterborne Pb values (Table 2-1) were not significantly above background in the low (7 μg Pb/g) and intermediate (77 μg Pb/g) dietary Pb exposures, but were significantly elevated to about 10 μg/ L in the high (520 μg Pb/g) dietary Pb exposure, presumably due to the leaching from the diet and/or feces. This suggests that Pb accumulation for the 520 μg Pb/g treatment may not be solely based on dietary Pb exposure, but a combination of dietborne and waterborne Pb. No mortality was associated with the dietary Pb treatments throughout the course of the experiment. Specific growth rates over 21 days and voluntary rations consumed each week did not differ among treatment groups (Table 2-1). Total food eaten over 21 days also did not differ between treatments. Condition factors were extremely high and fish in all tanks were considered fat both at the start and end of the experiment (Table 2-1). The food conversion efficiency, calculated based on mean SGR values of tagged fish, did not differ consistently among treatments (Table 2-1).

At all dietary Pb doses, Pb accumulation was seen in the gills (Figure 2-1), intestine (Figure 2-2), liver (Figure 2-3), kidney (Figure 2-4) and carcass (Figure 2-5) during the course of the 21 day experiment. At day 21, the order of Pb concentration in specific tissues was intestine> carcass> kidney > gills> liver. The

intestine showed a substantial accumulation with a burden of 17.8 μg Pb/g tissue wet weight in the 520 μg Pb/g exposure which was 445x greater than the control (0.04 μg Pb/g) on day 21. The intestine (17.8 μg Pb/g tissue wet weight), carcass (2.7 μg Pb/g tissue wet weight), kidney (2.4 μg Pb/g tissue wet weight) and the liver (1.9 μg Pb/g tissue wet weight) all exhibited their highest Pb burdens on day 21 compared to day 0, 7 and 14. In contrast, the gills, at least at the highest dose, had the greatest Pb accumulation on day 7 (8.0 μg Pb/g tissue wet weight) and a much lower Pb burden (2.2 μg Pb/g tissue wet weight) by day 21. Pb burden in the intestine (Figure 2-2) increased with time in all dietary Pb treatments.

When the percentage distribution of Pb burden in the whole fish is considered (Figure 2-8), the carcass accumulated about 80%, the intestine about 10%, while the gills, kidney and liver made up the remaining 10% of the Pb burden. These values are not surprising since the carcass makes up 85-90% of the weight of the fish. Notably, the biggest change from the % Pb distribution in the non-exposed fish was a much larger % contribution from the intestine in all dietary Pb treatments.

The RBCs accumulated a substantial concentration of Pb by day 21 (Figure 2-6), with almost no Pb present in the plasma (Table 2-3). Indeed, only at day 21 at the highest dose level was the plasma Pb significantly elevated. RBC Pb increased from background concentrations of 0.05 ug Pb/g to about 1.5 μg Pb/g in the highest dose level by day 21, about 105 times more than that of the plasma.

There were only a few significant effects of dietary Pb exposure on plasma Ca^{2+} and Mg^{2+} regulation. Plasma Mg^{2+} levels fell significantly in the intermediate and high Pb treatments on day 14, but had stabilized by day 21 (Table 2-3). A similar pattern was seen with the plasma Ca^{2+} of fish exposed to the intermediate and high diets, where there was a significant decrease in Ca^{2+} levels on day 7 when compared to the control. The Ca^{2+} levels had recovered by day 14, and thereafter remained stable. The hematocrit (30-40%) and the plasma protein (5.8-6.4 g/100mL) remained high throughout the experiment and were not affected by the dietary Pb exposure.

Ca^{2+} influx rates from the water were not significantly affected in juvenile rainbow trout and remained stable throughout the experiment (Table 2-4). However, on day 8, Na^{+} influx rates were significantly elevated in all treatments, and this occurred to the greatest extent in the high Pb diet treatment (Table 4). These effects had disappeared by day 22.

DISCUSSION

Dietary Pb concentrations were chosen to mimic environmentally relevant concentrations in terms of those found in benthic invertebrates at both contaminated and uncontaminated sites in the environment (0–792 μg Pb/g dw; Woodward et al., 1994; Farag et al., 1999), as well as to cut across the same range (4-118 μg Pb/g dw) as used by Hodson et al. (1978a) in an earlier dietary study. Dietary exposure to Pb had few apparent adverse effects on rainbow trout during

the 21 day experiment. There were no mortalities and no significant differences were observed in specific growth rates and rations among fish fed either the control diet or the various Pb diets. These results are consistent with Mount et al. (1994) who reported no effects on survival and growth of rainbow trout fed a dietary concentration as high as 170 μg Pb/g.

In the present study, total waterborne Pb was significantly elevated above background levels in the high dietary treatment tanks (from ~1.5 to 10 μg Pb/L; Table 2-2). Therefore it is possible that the Pb accumulation in the tissues, especially in the gill, was attributable to the combination of both dietary and waterborne Pb exposure in this treatment group only. The primary site of acute waterborne Pb uptake in fish is via the gills (Varanasi and Gmur, 1978). Rainbow trout exposed to chronic waterborne Pb (13 μg Pb/L for 32 weeks) also showed elevated gill Pb concentrations (approximately 2.5 μg Pb/g) relative to other tissues such as the kidney and liver (Hodson et al., 1978a). The gills in the present study did accumulate Pb (to about 2.1 μg Pb/g on day 21), mainly in the high dietary Pb treatment. Waterborne Pb levels (Table 2-2) in the low and intermediate dietary treatments were similar to background levels. This suggests any accumulation that was observed in the gills and in the other tissues of fish in the low and intermediate treatments was the result of dietary Pb, and not waterborne Pb. Notably, in the present study, the gill Pb burden substantially dropped from 8.0 μg Pb/g tissue wet weight on day 7 to 2.1 μg Pb/g tissue wet weight on day 21 (Figure 2-1), despite a relatively constant waterborne Pb level

(Table 2-3). This suggests that the gill Pb burden may have been excreted, or redistributed to other tissues. Pb burdens in the low dietary treatment in all the tissues were close to background control levels, which suggests that a dietary concentration >7 μg Pb/g is needed to see an effect on Pb burden in internal tissues.

Tissue accumulation data (Figures 2-1 to 2-5) point to the intestine, which accumulated a much greater Pb burden than any of the other tissues, as a potential site for chronic Pb toxicity via the diet. This is consistent with Farag et al. (1994) who found that adult rainbow trout fed metal-contaminated benthic invertebrates from the Clark Fork River, Montana, exhibited substantial metal accumulation in the gut tissues, with the highest concentration in the pyloric cecae and stomach. In the present study the stomach and pyloric cecae were not analysed individually, but grouped with the carcass. The presence of these tissues in the carcass may explain the high proportion of lead associated with this tissue.

The high Pb burden in the intestine in this study may be due to binding of Pb from the diet by mucus (Powell et al., 1999), whose secretion may be stimulated by dietary metals (Glover and Hogstrand, 2002). This mucus layer can act to sequester high levels of metals, and thus prevent the exposure of the underlying epithelial tissue to potentially toxic metal levels. Such a scenario has previously been observed in fish exposed to intestinally-perfused zinc (Glover and Hogstrand, 2002). The trapped metal burden may subsequently be sloughed off as a result of movement of food through the intestine. As such the high levels of Pb

associated with the intestine, at least in part, may represent Pb that is adsorbed, but not absorbed.

The present results demonstrate that dietary Pb can cross the intestinal epithelium and significantly accumulate in internal soft tissues such as the liver (Figure 2-3), kidney (Figure 2-4), and RBCs (Figure 2-6). When coho salmon (*Oncorhynchus kisutch)* were exposed to 150 μg Pb/L in sea water for 15 days, twice as much Pb was found in the posterior kidney (1.8 μg Pb/g) than in the anterior kidney (0.5 μg Pb/g) (Reichert et al., 1979). These findings of significant renal Pb accumulation were confirmed by the present study, where kidney Pb burden data on day 21 in the high dietary Pb treatment was 2.4 μg Pb/g tissue wet weight (Figure 2-4). These authors suggested that the high renal Pb concentration may be associated with the excretory/ionoregulatory function of the posterior kidney in fish (Smith and Bell, 1976; Reichert et al., 1979). In particular, since Ca^{2+} and Pb are believed to be antagonists of one another, these ions may be competing for transport sites at renal tubule cells. This could lead to Pb becoming trapped in the tubule cells and result in considerable Pb tissue burden in this tissue. Kidney Pb accumulation could contribute to chronic nephrotoxicity, however studies investigating possible nephrotoxic and renal clearance effects have not been performed for either waterborne or dietary Pb in fish. Farag et al. (1999) nevertheless found increased numbers of macrophage aggregates and hyperplasia of cells in the kidney of cutthroat trout fed mixed metal-contaminated

benthic invertebrate diets which included substantial Pb levels of 452 and 792 μg Pb/g dw.

The lower Pb accumulation of liver (Figure 2-3), when compared to the intestine, kidney and gills, suggests that Pb is deposited preferentially into internal soft tissues other than the liver. The low levels of Pb in the liver may be explained by the fact that Pb is not able to induce hepatic detoxifying proteins, namely metallothioneins (MT) (Reichert et al., 1979; Campana et al., 2003). Notably, Reichert et al. (1979) found that Pb was associated in the liver cytosol with much larger proteins (55000 molecular weight) than MT (8900 molecular weight). Nevertheless, if detoxifying proteins were present in the liver, Pb burden would be expected to be higher, since the detoxifying proteins would likely bind, sequester, and prevent the Pb from relocating to other tissues.

When net Pb retention from the diet over 21 days in the whole fish was calculated, the low Pb diet had the highest net Pb retention (4.8%) followed by the high Pb exposure (1.1%) and the intermediate Pb exposure (0.8%). Therefore, the higher the dietary Pb concentration, the lower the proportion of dietary Pb retained, suggesting that Pb levels may be regulated in terms of reduced Pb absorption and/or an increased Pb excretion.

These accumulation results contrast with the study of Hodson et al. (1978a), who found that dietary Pb (up to 118 μg Pb/g for 32 weeks) was not taken up by juvenile rainbow trout, and did not affect the fish at all. The difference may be that the $Pb(NO_3)_2$ in the Hodson et al. (1978a) study was added

to commercial trout chow combined with beef liver protein. It may be that the beef liver reduced Pb bioavailability.

In mammals, under steady state conditions, about 96% of Pb in the whole blood is in the red blood cells (World Health Organization, 1995). In this study 99 % of Pb was in the RBCs on day 21 in fish fed the 520 μg Pb/g diet. Given that there was an increase in RBC Pb (Figure 6), a portion of Pb accumulation in the various tissues may be a function of vascularization. Figure 7 plots the accumulation of Pb in each tissue (gill, intestine, liver and kidney) that may be explained by accounting for the amount of Pb present in trapped RBCs in the tissue, using estimates of ^{51}Cr RBC spaces from Olson (1992). In all three Pb diet treatments, about 9-14% of the Pb accumulated in the kidney is estimated to be the result of trapped RBCs in this tissue, whereas 86-91% of Pb accumulation in the kidney is truly in the renal tissue. Comparable figures for the liver and gill were only 1-3% in the trapped RBCs. In the intestine, 0.3% of Pb accumulated is the result of vascularization in terms of trapped RBC, while the rest has been built up in the intestinal tissues. These data along with the tissue burden data suggest that the intestine may be an important target tissue in chronic Pb poisoning via the diet.

Ca^{2+}, a tightly regulated ion in freshwater fish, is continuously absorbed from the water via the gills (Filk and Verbost, 1993). A significant decrease in plasma Ca^{2+} levels in the intermediate and high Pb diets occurred on day 7 (Table 2-4). However, Ca^{2+} levels recovered thereafter. Notably, this recovery occurred

without any change in Ca^{2+} influx rates from the water (Table 2-4). It may be that Ca was released from the bone or scales, which may serve as a Ca^{2+} reservoir, when the extracellular fluid Ca^{2+} concentrations are low (Persson et al., 1994). Studies have shown decreased plasma Ca^{2+} levels in rainbow trout exposed to waterborne Pb, indicating the presence of a Pb/Ca^{2+} interaction at the gills (Rogers et al., 2003, 2005; Rogers and Wood, 2004). The significant decrease in plasma Ca^{2+} levels in this study may be the result of a Pb/Ca^{2+} interaction at the intestine (see discussion below) which in addition to the gill may act as an important Ca^{2+} uptake route (Flik and Verbost, 1993). In addition, the intermediate and high dietary Pb treatment groups exhibited a significant decrease in Mg^{2+} levels on day 14 with stabilization thereafter. Since most Mg^{2+} normally comes from the diet in fish (Bijvelds et al., 1998), this may indicate an impact of Pb on gastrointestinal Mg^{2+} absorption.

Although there was an increase in Pb accumulation in the liver, the organ where the majority of plasma proteins are formed, there were no significant differences in plasma protein concentrations among the treatments. The majority of Pb was bound to the RBC (Figure 2-6), but there was a small but significant increase by day 21 in the plasma Pb levels in trout on the high Pb diet (Table 2-4). Possibly the RBCs may have become saturated with Pb. Manton and Cook (1984) showed that human RBCs have the capacity to fully bind Pb, at blood Pb levels up to about 2.4 μmol Pb/L (0.5 μg Pb/g). However, when blood Pb concentrations are above this level there is an increase in serum Pb levels. The data in the present

study suggest that the RBC in trout have the capacity to bind about 4.8 μmol Pb/L (1.0 μgPb/g) (Figure 6), as there was a significant increase in plasma Pb beyond this level (after 21 days in trout fed the high dietary Pb treatment). The ability of trout to potentially bind more Pb in the RBCs, may be related to the high oxygen carrying capacity (higher hematocrit), and longer life span of the erythrocytes (150 days), when compared to humans (Bushnell et al., 1985; Fänge, 1992).

Hematocrit was not affected in this study in contrast to the waterborne Pb study of Hodson et al. (1978a). This result suggests, but does not prove, that ALAD activities were not affected by the present dietborne Pb exposure. In future studies, it will be of interest to directly assess possible dietary Pb effects on ALAD.

The physiological mechanism of acute waterborne Pb toxicity has been characterized as an inhibition of Ca^{2+} uptake and an inhibition of the Na^+, K^+-ATPase and carbonic anhydrase enzymes at the gill epithelium, which result in disruptions in Ca^{2+} homeostasis and in Na^+ and Cl^- regulation (Rogers et al., 2003, 2005; Rogers and Wood, 2004). In contrast, dietary Pb did not inhibit Ca^{2+} or Na^+ influx rates from the water. Rogers et al. (2003) found that Ca^{2+} and Na^+ influx rates of juvenile rainbow trout were inhibited by 65% and 50% respectively during waterborne Pb exposures of 890 to 1200 μg/L. The gills accumulated close to 200 μg Pb/g at 96 h LC50 waterborne Pb concentrations of 1004 μg/L in the same study. Based on the above studies, it seems likely that Ca^{2+} or Na^+ influx rates would not be expected to change during the present dietary exposure, since

the gill Pb burden was only about 2.1 μg/g at day 21 in the highest dose treatment. Nevertheless, waterborne Pb studies showing major impacts on Ca^{2+} and Na^{+} influx rates at the gill suggest that future experiments that involve dietary Pb exposure in fish should consider measuring Ca^{2+} and Na^{+} influx rates at the intestine, as well as intestinal Na^{+},K^{+} ATPase activity, since this study points towards the intestine as the principal tissue for Pb accumulation and therefore potential chronic dietary Pb toxicity.

CONCLUSION

In summary, the present study provides evidence that dietary Pb, at levels representative of those in naturally contaminated diets, does accumulate in the internal tissues of rainbow trout and that the intestine has a potential role in sublethal Pb toxicity via the diet. Nevertheless, physiological disturbances were minimal, and feeding, growth, and food conversion efficiency were relatively unaffected over the 21 days of the experiment. Effects of this level of accumulation over a longer timeframe, and the possibility of neurological and reproductive effects have not been evaluated. This present study has provided a basis to explore Pb effects on membrane transport in the intestine, and given the high accumulation in RBCs, its effect on the heme synthesis pathway in future studies.

Table 2-1: Total waterborne Pb concentrations (μg/L) for different tank diets throughout the 3 week experiment. Data represented as mean ± 1 SEM, N=28 for all treatments, except the 77 μg Pb/g dw treatment, N=14. Values sharing lower case letters are not significantly different from other treatment values within the same week (*P<0.05)*.

Week	Control 0 µg Pb/g	Low 7 µg Pb/g	Intermediate 77 µg Pb/g	High 520 µg Pb/g
0	1.4 ± 0.2 a	1.4 ± 0.2 a	1.4 ± 0.2 a	1.4 ± 0.2 a
1	1.7 ± 0.2 a	1.7 ± 0.2 a	2.8 ± 0.5 a	9.5 ± 1.2 b
2	1.4 ± 0.2 a	1.2 ± 0.1 a	1.4 ± 0.1 a	10.1 ± 1.5 b
3	1.7 ± 0.2 a	1.5 ± 0.1 a	2.4 ± 0.5 a	11.0 ± 2.0 b

Table 2-2: Specific growth rates (SGR), voluntary rations, food conversion efficiency (FCE), and condition factors (CF) of juvenile rainbow trout fed to satiation with diets containing different concentrations of Pb. [a] SGR and rations are mean ± SEM. [b] SGR was calculated using the linear regression (SigmaStat version 3) of the natural logarithm of individual tagged fish weight versus time. The estimates for each fish were then averaged. [c] Condition factor (CF) was measured using the formula $CF=(W/L^3)x100$. [d]FCE was calculated by the formula (SGR/average ration during the week) multiplied by 100 Values sharing lower case letters are not significantly different from other treatment values within the same week ($P<0.05$).

	Week	0 µg Pb/g	7 µg Pb/g	77 µg Pb/g	520 µg Pb/g
SGR (%/day)[a,b]		2.47± 0.08 a	2.47 ± 0.11 a	2.33 ± 0.13 a	2.37 ± 0.18 a
Ration (%/day)[a]	1	2.33 ± 0.23 a	2.56 ± 0.14 a	2.72 ± 0.27 a	2.51 ± 0.21 a
	2	2.69 ± 0.17 a	2.41 ± 0.10 a	2.56 ± 0.13 a	2.40 ± 0.15 a
	3	2.09 ± 0.24 a	2.59 ± 0.17 a	2.20 ± 0.14 a	2.54 ± 0.19 a
CF[c]	0	1.85 ± 0.04 a	1.97 ± 0.04 a	1.94 ± 0.04 a	1.85 ± 0.04 a
	1	1.97 ± 0.05 a	2.01 ± 0.05 a	1.98 ± 0.07 a	1.97 ± 0.06 a
	2	2.04 ± 0.08 a	1.97 ± 0.03 a	2.05 ± 0.08 a	2.34 ± 0.12 b
	3	1.84 ± 0.05 a	1.95 ± 0.03 a	2.01 ± 0.08 a	1.91 ± 0.07 a
FCE (%)[d]	1	106 ± 3 a	97 ± 4 a	86 ± 4 a	94 ± 7 a
	2	92 ± 3 a	102 ± 4 a	91 ± 5 a	99 ± 7 a
	3	118 ± 4 a	95 ± 4 b	106 ± 6 ab	93 ± 7 b

Table 2-3: Plasma protein, hematocrit (%), and plasma Ca^{2+}, Mg^{2+} and Pb concentrations over 21 days in rainbow trout fed different concentrations of Pb in the diet. Values sharing lower case letters are not significantly different from other treatment values within the same week ($P<0.05$).

Day	Treatment (μg Pb/g dw)	Hematocrit (%)	Plasma Protein (g/100mL)	Plasma Ca^{2+} (μM)	Plasma Mg^{2+} (μM)	Plasma Pb (μg/L)
0	0	39.6 ± 2.4	5.8 ± 0.4	2.1 ± 0.3	0.8 ± 0.1	6.3 ± 1.6
7	0	31.2 ± 1.0 a	5.9 ± 0.3 a	4.4 ± 0.5 a	0.9 ± 0.1 a	7.6 ± 0.5 a
	7	34.4 ± 1.9 a	5.8 ± 0.3 a	3.6 ± 0.4 a	0.9 ± 0.1 a	5.5 ± 1.1a
	77	32.9 ± 4.2 a	5.0 ± 0.4 a	2.9 ± 0.2 b	1.0± 0.1 a	4.5 ± 1.0 a
	520	38.2 ± 3.2 a	5.3 ± 0.5 a	2.8 ± 0.2 b	1.1 ± 0.1 a	12.3 ± 3.1a
14	0	31.0 ± 2.4 a	6.3 ± 0.2 a	4.4 ± 0.3 a	1.6 ± 0.2 a	16.5 ± 3.1a
	7	33.1 ± 1.2 a	6.4 ± 0.6 a	4.4 ± 0.3 a	1.2 ± 0.2 a	6.3 ± 0.9 b
	77	30.4 ± 2.0 a	5.4 ± 0.6 a	3.1 ± 0.3 a	0.9 ± 0.2 b	10.1 ± 4.0ab
	520	32.0 ± 1.6 a	6.1 ± 0.3 a	4.5 ± 0.3 a	0.7 ± 0.1 b	14.8 ± 3.3 a
21	0	35.9 ± 1.6 a	6.2 ± 0.2 a	4.6 ± 0.2 a	1.3 ± 0.3 a	4.1 ± 0.7 a
	7	32.8 ± 1.6 a	5.3 ± 0.2 a	3.7 ± 0.3 a	0.9 ± 0.1 a	6.0 ± 0.7a
	77	31.4 ± 1.6 a	5.6 ± 0.2 a	3.8 ± 0.4 a	1.0 ± 0.2 a	7.1 ± 0.9 ab
	520	31.3 ± 1.4 a	6.4 ± 0.3 a	4.1 ± 0.5 a	1.1 ± 0.2 a	11.9 ± 1.8 b

Table 2-4: Na^+ and Ca^{2+} influx rates from the water throughout the course of the experiment. Values sharing lower case letters are not significantly different from other treatment values within the same week ($P<0.05$).

Day	Treatment (µg Pb/g)	Ca^{2+} (µmol/kg/hr)	Na^{+} (µmol/kg/hr)
0	0	128.4 ± 31.8	185.9 ± 18.1
8	0	156.6 ± 13.9 a	344.0 ± 37.1 a
	7	123.1 ± 16.7 a	392.1 ± 55.3 ab
	77	146.0 ± 14.5 a	441.7 ± 30.4 ab
	520	137.7 ± 26.1 a	512.1 ± 45.2 b
22	0	126.2 ± 5.0 a	186.3 ± 22.8 a
	7	145.3 ± 16.0 a	242.2 ± 29.1 a
	77	129.6 ± 11.7 a	167.4 ± 10.6 a
	520	113.4 ± 14.0 a	195.5 ± 29.8 a

Figure 2-1: Lead accumulation in the gills of juvenile rainbow trout exposed to different levels of Pb in the diet over 21 days. Pb tissue burden is expressed in μg Pb/g wet tissue weight. Data represented as mean ± 1 SEM, N=12, except the 77 μg Pb/g treatment, N=6. Lower case letters indicate significant differences ($P<0.05$) between treatment means within a day and upper case letters indicate significant differences between days within treatment means using a one way ANOVA with a Tukey multiple comparison, or Kruskal- Wallis ANOVA on ranks with a Dunn's multiple comparison test.

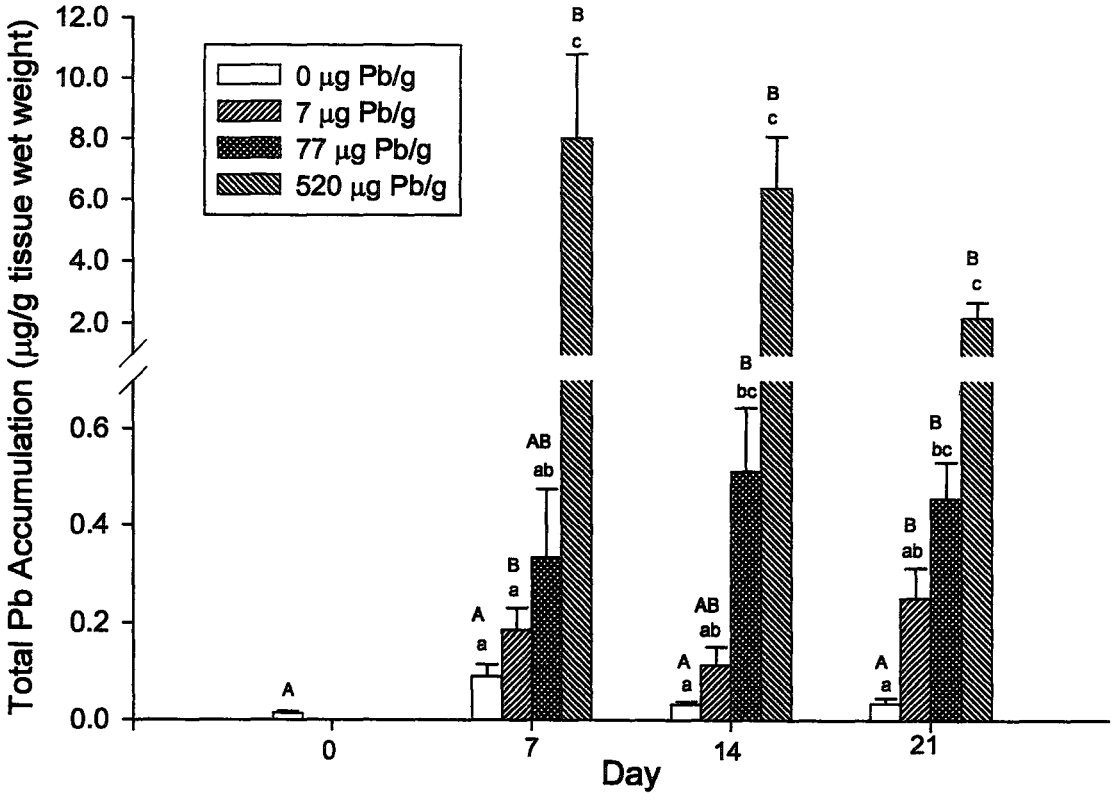

Total Pb Accumulation (µg/g tissue wet weight)
12.0
10.0
8.0
6.0
4.0
2.0
0.6
0.4
0.2
0.0
0 µg Pb/g
7 µg Pb/g
77 µg Pb/g
520 µg Pb/g
A
a
B
a
AB
ab
B
c
A
a
AB
ab
B
bc
B
c
A
a
B
ab
B
bc
B
c
0
7
14
21
Day

Figure 2-2: Lead accumulation in the intestine of juvenile rainbow trout exposed to different levels of Pb in the diet over 21 days. The intestine does not include the pyloric ceca. Other details as in legend of Figure 2-1.

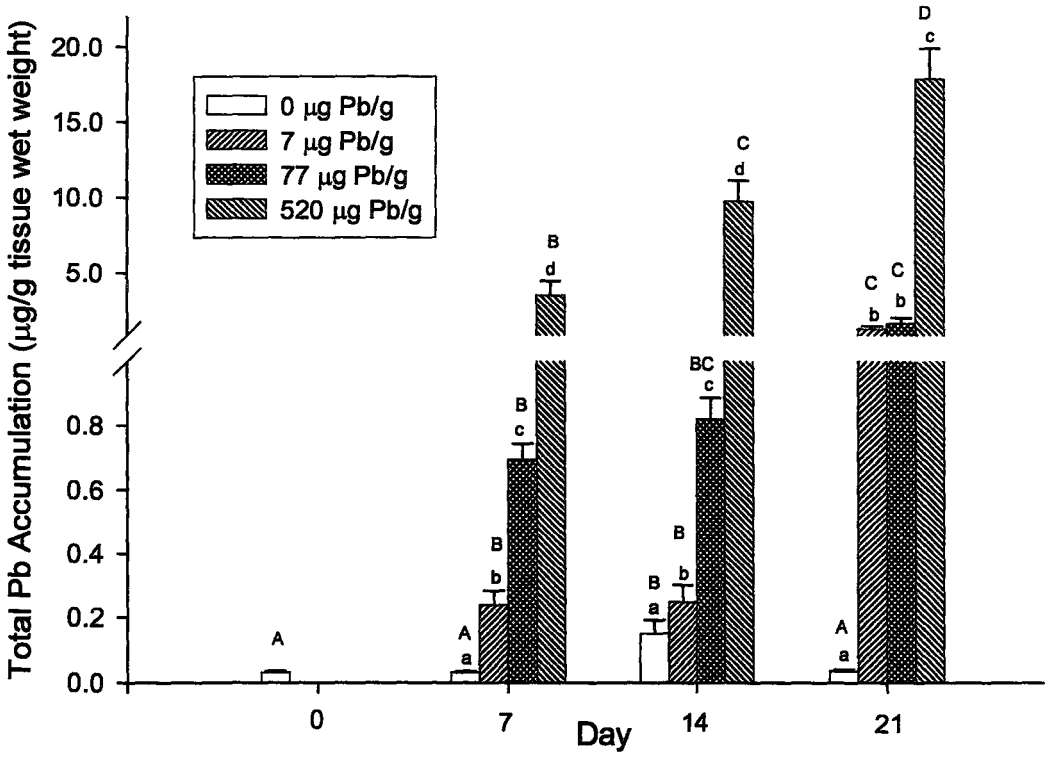

Total Pb Accumulation (µg/g tissue wet weight)
20.0
15.0
10.0
5.0
0.8
0.6
0.4
0.2
0.0
0 µg Pb/g
7 µg Pb/g
77 µg Pb/g
520 µg Pb/g
0
7
14
21
Day

Figure 2-3: Lead accumulation in the liver of juvenile rainbow trout exposed to different levels of Pb in the diet for 21 days. Other details as in legend of Figure 2-1.

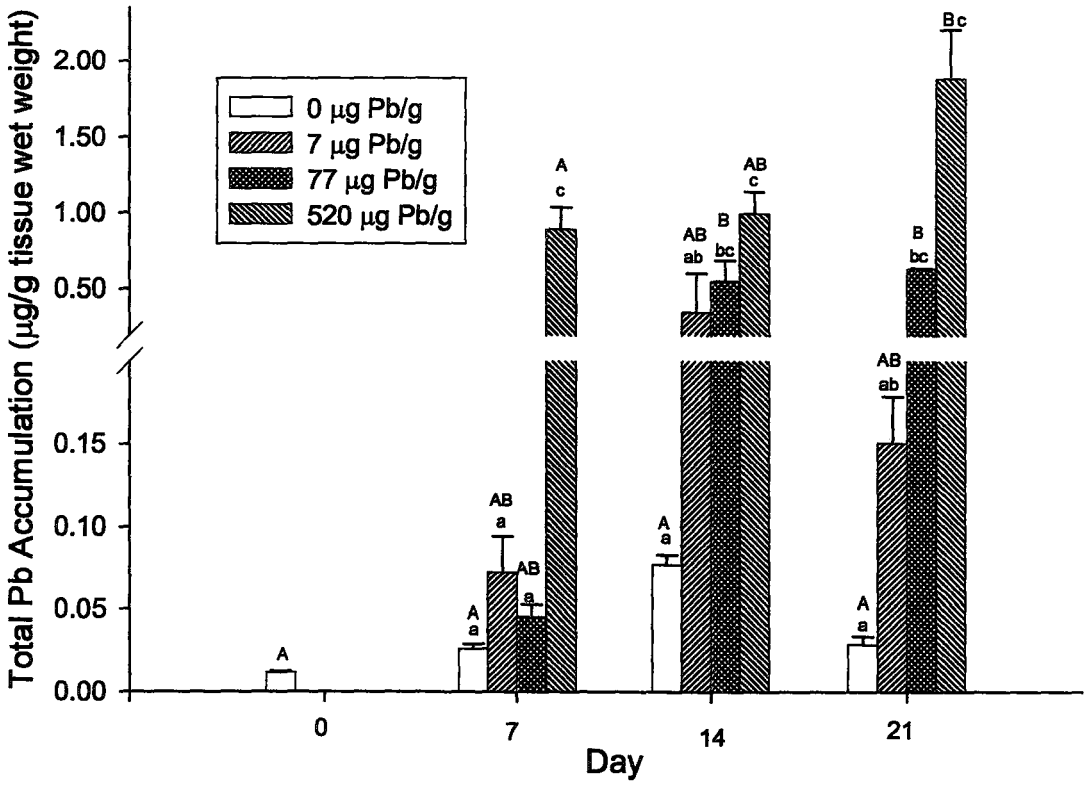

Total Pb Accumulation (µg/g tissue wet weight)
2.00
1.50
1.00
0.50
0.15
0.10
0.05
0.00
0 µg Pb/g
7 µg Pb/g
77 µg Pb/g
520 µg Pb/g
A
AB
B
Bc
a
ab
bc
c
0
7
14
21
Day

Figure 2-4: Lead accumulation in the kidney of juvenile rainbow trout exposed to different levels of Pb in the diet for 21 days. Other details as in legend of Figure 2-1.

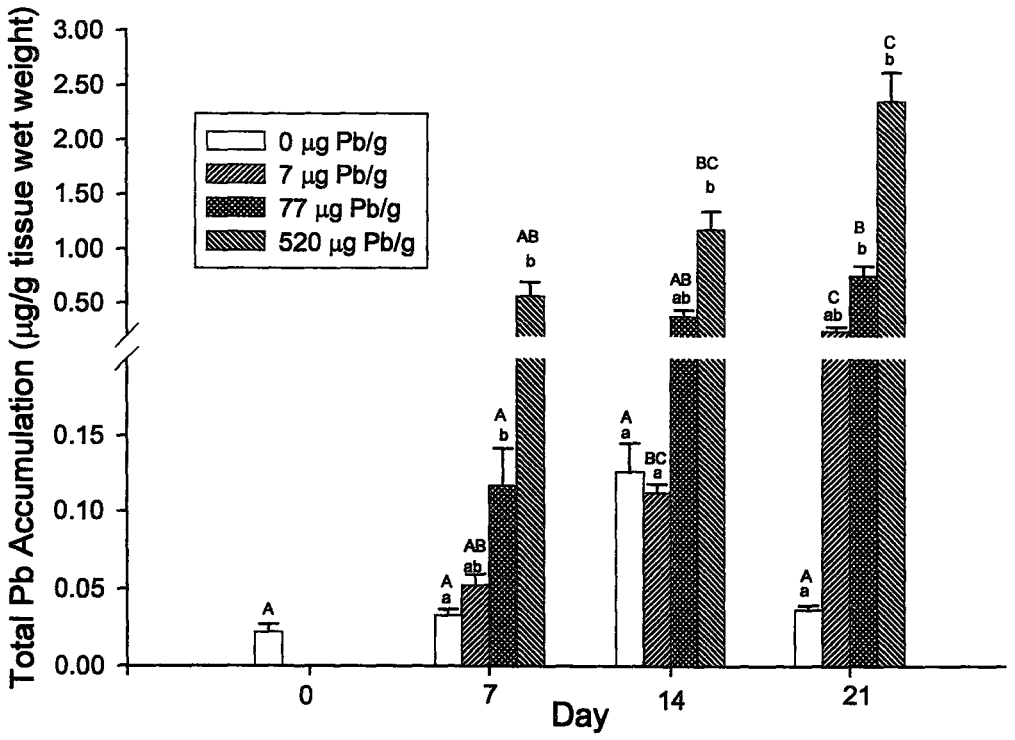

Total Pb Accumulation (µg/g tissue wet weight)
3.00
2.50
2.00
1.50
1.00
0.50
0.15
0.10
0.05
0.00
0 µg Pb/g
7 µg Pb/g
77 µg Pb/g
520 µg Pb/g
A
A a
AB ab
A b
AB b
A a
BC a
AB ab
BC b
A a
C ab
B b
C b
0
7
14
21
Day

Figure 2-5: Lead accumulation in the carcass of juvenile rainbow trout exposed to different levels of Pb in the diet for 21 days. Other details as in legend of Figure 2-1.

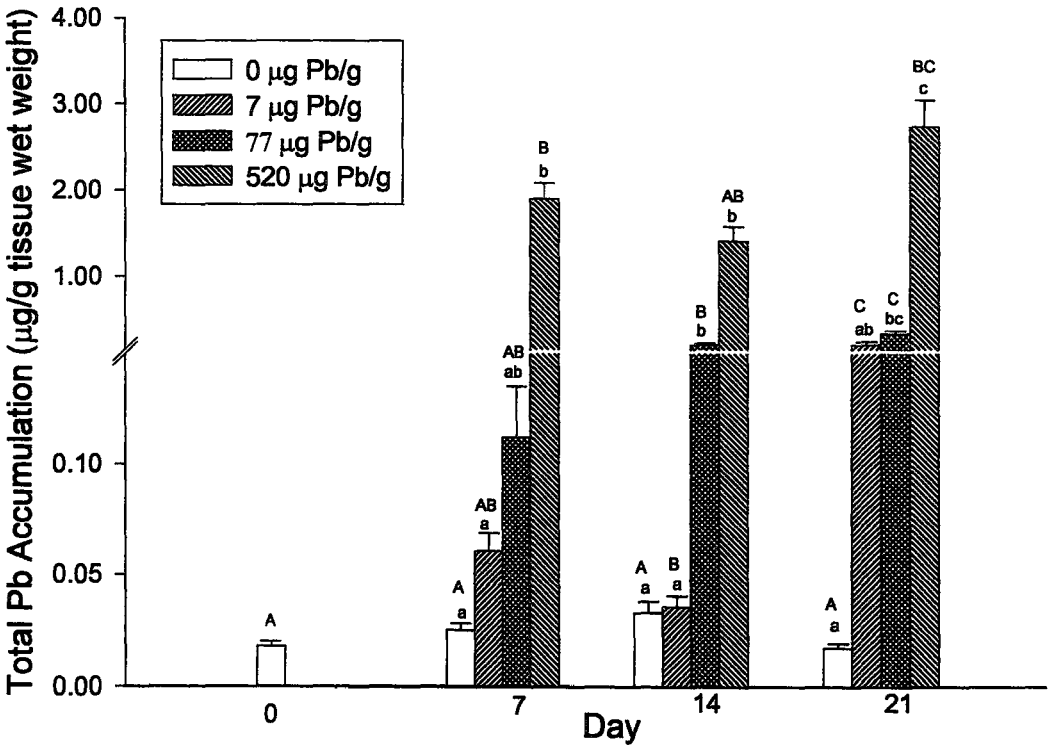

Total Pb Accumulation (µg/g tissue wet weight)
4.00
3.00
2.00
1.00
0.10
0.05
0.00
0 µg Pb/g
7 µg Pb/g
77 µg Pb/g
520 µg Pb/g
A
a
AB
a
AB
ab
B
b
A
a
B
a
B
b
AB
b
A
a
C
ab
C
bc
BC
c
0
7
14
21
Day

Figure 2-6: Lead accumulation in the red blood cells of juvenile rainbow trout exposed to different levels of Pb in the diet for 21 days. Other details as in legend of Figure 2-1.

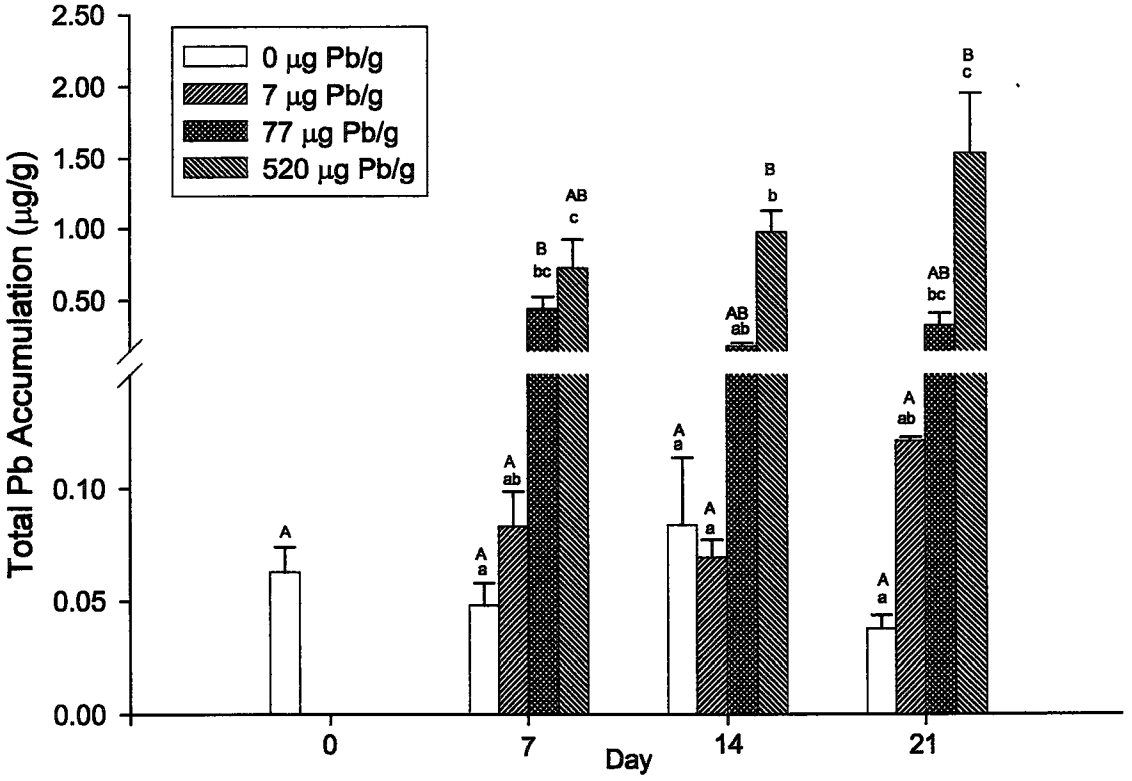

0 µg Pb/g
7 µg Pb/g
77 µg Pb/g
520 µg Pb/g
Total Pb Accumulation (µg/g)
2.50
2.00
1.50
1.00
0.50
0.10
0.05
0.00
0
7
14
21
Day
A
a
A
ab
B
bc
AB
c
A
a
A
a
AB
ab
B
b
A
a
A
ab
AB
bc
B
c

Figure 2-7: Percentage of accumulated Pb burden explained by Pb within the trapped RBC's on day 21. The intestine does not include the pyloric cecae

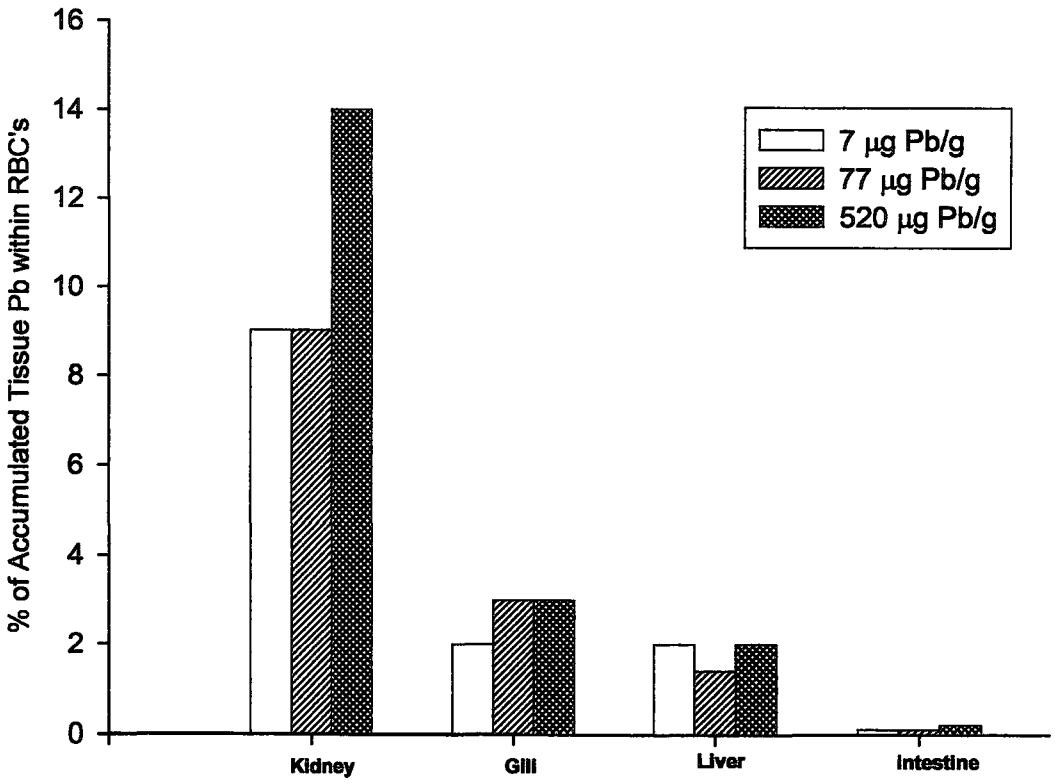
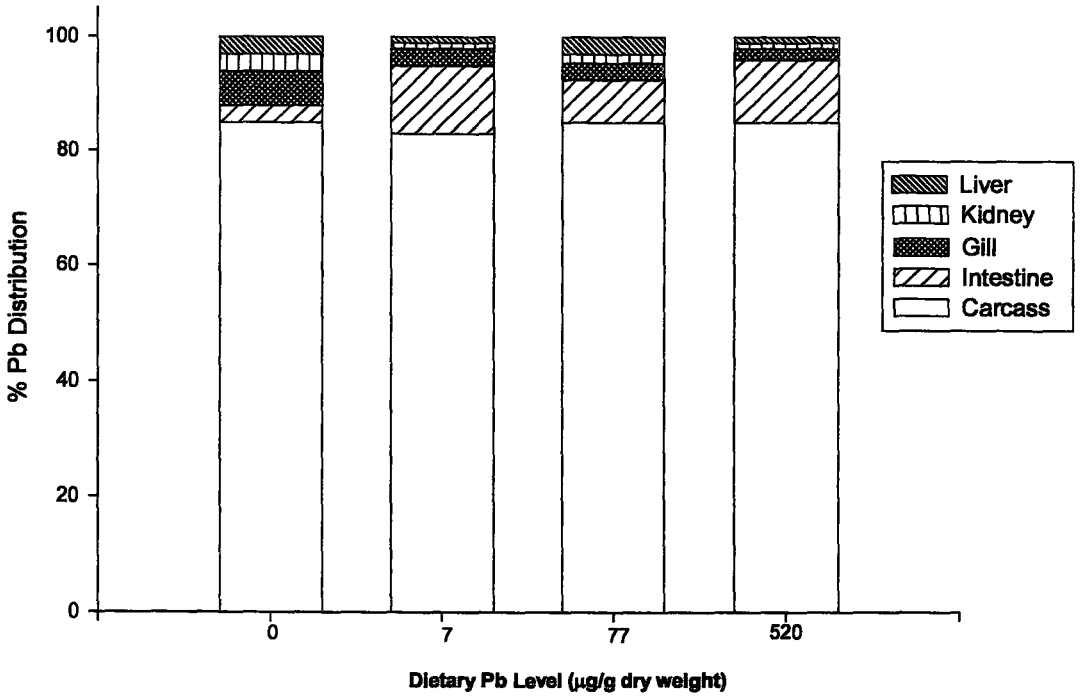

% of Accumulated Tissue Pb within RBC's
16
14
12
10
8
6
4
2
0
7 µg Pb/g
77 µg Pb/g
520 µg Pb/g
Kidney
Gill
Liver
Intestine

Figure 2-8: Per cent distribution of total Pb burden on day 21 in different tissues of juvenile rainbow trout fed different Pb diets.


100
80
60
40
20
0
% Pb Distribution
0
7
77
520
Dietary Pb Level (µg/g dry weight)
Liver
Kidney
Gill
Intestine
Carcass

CHAPTER 3

ABSTRACT

This study examined the impact of elevated dietary Ca^{2+} on the responses to chronic dietary Pb exposure in juvenile rainbow trout, in light of previous reports that dietary Pb is taken up internally, that dietary Ca^{2+} protects against the uptake of several waterborne metals, including Pb, and that dietary Pb causes mild ionoregulatory disturbances. Juvenile rainbow trout were fed reference (0.3 μg Pb/g, ~20 mg Ca^{2+}/g) and Pb-enriched diets (~ 50 or 500 μg Pb/g) in the presence (~ 60 mg Ca^{2+}/g) or absence (~20 mg Ca^{2+}/g) of added Ca^{2+} (as $CaCO_3$) for 42 days. The RBCs accumulated 100 times more Pb when compared to the plasma, while δ-aminolevulinic acid dehydratase was inhibited in the high treatment group without added Ca^{2+}, by the end of the exposure. Neither plasma Cl^-, K^+ and Mg^{2+} nor Na^+, K^+-ATPase activities in the gills, mid and posterior intestine were affected. However, there were mild disruptions in plasma Na^+ and Ca^{2+} levels in the elevated Pb and Ca^{2+} treatment groups, and a significant up-regulation in Na^+, K^+ -ATPase activity at the anterior intestine in fish fed the high Pb diets with and without added Ca^{2+}. The order of Pb accumulation reflected the exposure pathway of Pb via the diet (per tissue wet weight): gut> bone> kidney> liver> spleen > gill > carcass > brain > white muscle. The anterior intestine accumulated the most Pb per tissue wet weight, while the bone accumulated the most Pb per fish weight. Pb concentrations were much higher in the posterior kidney than the anterior kidney.

Simultaneous addition of Ca^{2+} to the diet had an overall protective effect in all the tissues analysed in reducing Pb accumulation.

INTRODUCTION

Lead (Pb) is a non-essential metal. Pb enters the aquatic environment through the mining, smelting, and refining of Pb, and the burning of leaded gasoline (World Health Organization, 1989). In contaminated aquatic environments, fish can take up Pb through the water, diet and to a lesser extent the skin (e.g. Dallinger et al., 1987, Hodson et al., 1978a; Rogers et al., 2003, 2005; Rogers and Wood, 2004; Chapter 2). Naturally occurring levels of waterborne Pb range between 0.6 to 120 μg Pb/L (Demayo et al., 1982). The most important factor that determines waterborne Pb toxicity to fish is the free ionic concentration of Pb, which is the most bioavailable form of the metal and therefore the most toxic (Davies et al., 1976). In the natural fish diet, Pb levels in uncontaminated benthic invertebrates contain <0.1 μg Pb/g dry weight (dw), while up to 792 μg Pb/g dw may be present in contaminated benthic invertebrates (Woodward et al., 1994, 1995; Farag et al., 1994, 1999).

Waterborne Pb causes the disruption of Na^+, Cl^- and Ca^{2+} regulation during acute exposure, the induction of spinal deformities and black tails during chronic exposure, and disruption in hemoglobin synthesis during both types of exposure (Hodson et al., 1978a, Rogers et al., 2003, 2005; Rogers and Wood, 2004). Dietary Pb is less well studied than waterborne Pb. However, reductions in

Na^+, K^+-ATPase activity and morphological damage to the enterocytes at the mid and posterior intestine have been observed in rainbow trout fed 10 μg Pb/g fish/day for 15-30 days (Crespo et al., 1986).

In contrast to the many acute waterborne Pb experiments (Hodson et al., 1976, 1977, 1978; Varanasi and Gmur, 1978; Rogers et al., 2003; 2005, Rogers and Wood, 2004), only four studies have looked at the effects of chronic sublethal dietary Pb exposure to fish, all in rainbow trout. Hodson et al. (1978a) found that Pb was not taken up via the diet, but in contrast, the studies of Chapter 2, Mount et al. (1994), and Crespo et al. (1986) all found that dietary Pb accumulates in the whole body and in a number of internal tissues when present in the diet.

In Chapter 2, fairly mild physiological disturbances in rainbow trout fed diets containing up to 520 μg Pb/g for 21 days were reported. These disturbances included transient decreases in plasma Mg^{2+} and Ca^{2+} levels, and increases in whole body waterborne Na^+ influx rates. Despite no effects on growth and survival rates, rainbow trout accumulated significant Pb burdens in the intestine, kidney, gills, liver and carcass, with the RBCs accumulating 105 times more Pb when compared to the plasma by the end of the experiment. Pb accumulated to the greatest extent in the intestine, suggesting that the intestine may be the primary site of potential Pb toxicity in juvenile freshwater rainbow trout exposed to dietary Pb. Similarly, Mount et al. (1994) found that despite no effects on survival and growth, rainbow trout accumulated whole body dietary Pb burdens when fed brine shrimp contaminated with Pb concentrations as high as 170 μg

Pb/g dw for 60 days. In addition, Crespo et al. (1986) reported morphological changes, decreased Na^+, K^+-ATPase activity, and decreased Na^+ and Cl^- absorption in the intestine of trout fed for 15-30 days with a comparable dietary load of Pb.

Generally, divalent metals such as Pb, Cd and Zn^{2+} are considered Ca^{2+} antagonists. Early studies have shown that Ca^{2+}-deficient diets enhance Pb absorption in rats (Six and Goyer, 1970), while increased levels of dietary Ca^{2+} decrease the absorption and retention of Pb in rat tissues (Quarterman et al., 1975). Moreover, coho salmon (*Oncorhynchus kisutch)* force-fed gelatin capsules containing 8.4 mg of calcium chloride ($CaCl_2$) and then exposed to 1300 μg/L of waterborne Pb for 168 h, had reduced Pb tissue burdens (Varanasi and Gmur, 1978). Similarly, several studies (Zohouri et al., 2001; Baldisserotto et al., 2004 a, b; 2005; Franklin et al., 2005), have shown that dietary Ca^{2+} is protective against the uptake of both waterborne and dietary Cd, as well as against the uptake of waterborne Zn^{2+} (Niyogi and Wood, unpublished results).

δ-aminolevulininc acid dehydratase (ALAD), an enzyme that catalyses the formation of porphobilinogen (PGB) from the substrate aminolevulinic acid (ALA), has long been used as a biomarker for Pb toxicity in humans (Secchi et al., 1974), waterfowl (Dieter, 1979) and fish (Hodson et al., 1977; Schmitt et al., 1984, 1993, 2002; Burden et al., 1998). Pb inhibits ALAD by binding to essential sulfhydryl (SH) groups and displacing the Zn^{2+} cofactors on ALAD (World Health Organization, 1977; Finelli, 1977; Sassa, 1982). Hodson et al. (1976,

1977, 1978a) found that ALAD activity in rainbow trout was inhibited after exposing fish to waterborne Pb concentrations as low as 13 μg/L for four weeks.

Therefore, the objectives of the present investigation were five-fold. The first goal was to investigate the potential role of dietary Ca^{2+} in protecting fish from dietary Pb burden over a 42 day exposure. A second goal was to differentiate the distribution of dietary Pb between the anterior and posterior sections of the kidney, and different compartments of the gastrointestinal tract (stomach, anterior intestine + pyloric ceca, mid intestine and posterior intestine) to determine where dietary Pb might exert the greatest physiological and/or toxicological effects. A third objective was to analyse any Pb accumulation that may have taken place in the gills, liver, bone, white muscle, spleen, and carcass over time, and to look for any evident regulation and depuration of Pb in these tissues during continued exposure to dietary Pb and supplemented Ca^{2+} diets. Chapter 2 showed that 99% of the Pb in the blood was present in the RBCs when compared to the plasma. Based on the known inhibitory effect of waterborne Pb on the enzyme ALAD, a fourth objective was therefore to detect any hematological disruptions in terms of the enzyme ALAD, whole blood Zn levels, hemoglobin levels, total blood protein, whole blood Pb levels, plasma Pb levels and RBC Pb levels. A final objective was to determine if there were any effects on growth and survival rates, plasma Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+} regulation, and Na^+, K^+-ATPase activity, since whole body Na^+ influx rates were increased, while Mg^{2+} and Ca^{2+} plasma levels were decreased in the Chapter 2 study.

MATERIALS AND METHODS

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) (N~ 400) with a mean weight of 25.8 ± 0.5 g were obtained from Humber Springs Trout Hatchery (Orangeville, ON). After arrival, fish were randomly selected and placed into eight, 200 L polypropylene flow-through, aerated tanks that were divided into 2 x 100 L sections (25 fish per section). The division was achieved using a 0.2 mm^2 mesh screen, which allowed free-mixing of water, but not the food or feces between the two sides. Each tank was supplied with 1 L/min of dechlorinated Hamilton water with the composition (mM) of Na^+ = 0.65, Cl^- = 0.8, Ca^{2+} = 1.0, Mg^{2+} = 0.4, K^+ = 0.06 and water hardness as $CaCO_3$ = 140 mg/L, total Pb = 1.3 ± 0.1 μg/L. The pH and temperature were kept at ambient conditions, 7.4-7.7 and 11-13°C, respectively. Photoperiod was maintained at 12 h light and 12 h dark. Fish were acclimated three weeks prior to their use in the 42 day experiment.

Fish were fed commercial salmon fry pellets once daily (Silver Cup feed; Murray, Utah, USA, see below for composition) at a ration of 1.5% body mass/day upon arrival and until the beginning of the experiment. At the start of the experiment, each tank section was assigned to one of six replicated nominal dietary Pb and/or Ca^{2+} treatments: 0 μg Pb/g + 20 mg Ca^{2+}/g dry weight (dw) (A); 0 μg Pb/g + 60 mg Ca^{2+}/g dw (B); 50 μg Pb/g + 20 mg Ca^{2+}/g dw (C); 50 μg Pb/g + 60 mg Ca^{2+}/g dw (D); 500 μg Pb/g + 20 mg Ca^{2+}/g dw (E), and 500 μg Pb/g +

60 mg Ca^{2+}/g dw (Figure 3-1). Additional replicated control tank sections (0 μg Pb/g + 20 mg Ca^{2+}/g and 0 μg Pb/g + 60 mg Ca^{2+}/g, Figure 3-1) in tanks 1 and 5, in comparison to tanks 3 and 7, and 4 and 8, where the same treatments were paired with high dietary treatments, were used to control for possible waterborne Pb contamination as a result of Pb leaching across from the feces and/or food in the neighbouring tank section.

Food Preparation

Pb-enhanced diets were made by adding Pb, in the form of lead nitrate, $Pb(NO_3)_2$ (Sigma-Aldrich) into 0.5 pt. commercial salmon fry food (Silver Cup feed, Murray, Utah, USA). The manufacturer's specifications for the food were of 52% crude protein (min.); 14% crude fat (min); 3% crude fiber (max.); 12% ash (max.), and 1% sodium (actual). The background levels of Pb were 0.3 ± 0.1 μg Pb/g dw, and background levels of Ca^{2+} were 19.2 ± 0.3 mg Ca^{2+}/g dw. The commercial food was powdered, then hydrated with 40% v/w of double distilled water (NANOpure II; Sybron/Barnstead, Boston MA) containing different proportions of dissolved $Pb(NO_3)_2$. For the Ca^{2+}-enhanced diets, the powdered food was mixed with powdered $CaCO_3$ before adding water that contained $Pb(NO_3)_2$. The mixtures were blended in a pasta maker (Popeli, Ronco Inventions, Chastworth, CA, USA) for 1 h, to yield 0, 50 and 500 μg Pb/g dw diet (nominal concentrations), each with 20 (control) or 60 mg Ca^{2+}/g dw (nominal concentrations). The mixed paste was then passed through a cutter where small

strands of approximately 5 mm were broken into small pellets by hand. All food pellets were air-dried to a constant weight for approximately four days and frozen until further use. Moisture in the food was determined by placing 10 g of food from each treatment into a 60°C oven and drying to a constant weight which took about 12 h. The moisture content was 6%.

Concentrations of Pb in food (Table 3-1) were determined by heat digesting the food pellets in five volumes of 6N HNO_3 at 60°C for 48 h. The supernatant was then diluted in 1% HNO_3 and measured against a certified multi-element Pb standard (Anachemia Inc. Quebec), blank samples, reference samples, and spiked samples on the graphite furnace atomic absorption spectrophotometer (AAS; 220 SpectrAA; Varian GTA-110; Varian, Australia) with a detection limit of 0.06 μg Pb/L. Mean spike recovery for the food spiked with a known amount of Pb was 84% ± 1.6%. The data were not corrected for total Pb recovery. Ca^{2+} concentrations (Table 3-1) were measured as above, expect that the samples were diluted in 0.5% $LaCl_3$ and measured against known standards made with 0.5% $LaCl_3$ on a flame AAS (220FS Spectra AA; Varian, Australia).

Feeding, Mean Weight, Specific Growth Rates and Food Conversion Efficiency

Fish from each tank section were bulk-weighed on days 0, 8, 15, 22, 29, and 36, by removing all the fish and placing them into a 4.5 L bucket filled with water. The total biomass was determined from the difference between the weight of the bucket and water with and without fish. The mean fish weight was

calculated by dividing the total biomass by the number of fish in each tank section. Each of the six replicated treatments and the replicated control tanks (i.e A/B, Figure 3-1) were fed a daily ration of 1.5% total body weight per tank section per day determined weekly by bulk weighing. The 1.5% ration was determined using the formula:

$$(0.015^* f^* w = x) \quad (1),$$

where 0.015 represents the 1.5 % ration, f is the total number of fish in each tank section, w is the average mean weight, x is the total amount of food needed to maintain the given ration. The bottom of each tank was siphoned approximately 3 h post-feeding to control for any leaching of Pb and Ca^{2+} from both the food and feces. Water samples were taken twice weekly, 1 h after siphoning (on the sampling day and 3 days post sampling), to detect total and dissolved Pb contamination.

Specific growth rates (SGR) expressed as a % per day were determined using linear regression (SigmaStat, 3.0) of the natural logarithm of mean bulk weight versus time data. The food conversion efficiency (FCE; %) for each treatment was calculated using the formula:

$$FCE = (SGR_{ave}/r)^*100 \quad (2),$$

where SGR_{ave} is the mean specific growth rate (%/day), and r is the ration (%/day).

Tissue Sampling and Analytical Techniques

On day 0, prior to the implementation of the different dietary treatments, two randomly selected fish per tank section were sacrificed with 1.0 g/L MS 222. On days 14, 28, and 42, four randomly selected fish per tank section were similarly sacrificed, 16 h post fish feeding. Blood samples (see below) were taken immediately. Fish wet weights (g) and fork length (cm) were taken in order to measure individual condition factors, using the formula:

$$K = W/L^3 \quad (3),$$

where K is the condition factor, that remains constant if the linear proportional shape of the fish remains constant, W is the weight in grams and L is the fork length (Brett et al., 1979). Vertebral bone, brain, gill baskets, anterior, mid and posterior intestine, anterior and posterior kidney, liver, muscle, spleen, stomach, and remaining carcass were dissected out in order to determine Pb tissue burden.

The gills, once dissected, were rinsed with 0.6% NaCl in order to remove any fine particulate matter. The stomach and the three sections of the intestine were longitudinally cut and rinsed with 0.6% NaCl to remove any undigested food and feces. A section of bone was removed from the posterior neural spine of the vertebrae, while white muscle was removed anterior to the dorsal fin. The bone, brain, gills, intestine sections, kidney sections, liver, muscle, spleen and stomach were blotted dry, placed into pre-weighed plastic tubes, re-weighed and stored in a 4°C cold room until further analyses.

Each tissue was digested in five volumes of the weight of the tissue in 1N HNO_3. The acid digests were placed in an oven for 48 h at 60°C. The tissues were then centrifuged at 14000 g (Eppendof 5145C) for 20 min; the supernatant was diluted in 1% HNO_3 and measured by the graphite furnace AAS for Pb burden. The mean spike recovery for tissue (carcass) spiked with a known amount of Pb was 98% ± 2.4%. The data were not corrected for total Pb recovery.

Net Retention Calculations

Net Pb retention for each of the treatment groups, including the controls, over the 42 day experiment were calculated using the following formulae:

Food ingested (kg) = [Ration (kg/kg fish.day)]* [weight of fish in kg] **(4),**

Total Pb ingested (mg/kg fish) = [Measured Pb in the food (mg/kg)]*

[Food ingested (kg/kg fish)] **(5),**

Total Pb accumulated (mg/kg fish) over time = [∑Total Pb accumulation in each

tissue]/ [fish weight (kg)] **(6),**

Net Pb Retention (%)= [Total Pb accumulated]/[Total Pb ingested]*100. **(7),**

Determination of Whole Body Pb and Per Cent Pb Distribution

Whole body Pb distribution was calculated in order to determine Pb distribution in different tissues of the fish using the formula:

$$WB= [\sum (T_n*Twt_n)]/Fwt \quad \textbf{(8)},$$

where WB is the whole body Pb accumulation (μg/g wet weight), T is the accumulation of Pb in each tissue (μg/g), Twt is the weight of each individual tissues (g), and Fwt is the combined weight of all tissues (g) in the whole fish. The lower case n represents an individual tissue (i.e. WB= [(liver Pb*liver weight) +...+ (carcass Pb* carcass weight)]/ (liver weight +...+ carcass weight).

Since only a small section of the bone and white muscle was removed from the fish, estimates of tissue weight (Twt, g/kg fish) in rainbow trout from Gingerich et al. (1990) were used to determine whole body bone and white muscle weight in fish using the formula:

$$Twt= (Tewt)*(aveFwt) \quad \textbf{(9)},$$

where Tewt represents the estimated tissue weight (g/kg fish) from Gingerich et al. (1990), and aveFwt is the mean fish weight in the study (kg). Once total bone weight and muscle weight were determined, these values were substituted into equation 8, for WB Pb determination. The carcass remnants represents the leftover tissue, after the estimate of total bone and white muscle are considered, by the equation above.

Per cent of total Pb distribution contributed by each tissue in reference to whole body Pb burden was determined using the formula:

$$PD= [(T*Twt)/Fwt]*100 \quad (10),$$

where PD represents per cent of the total distribution (%) contributed by an individual tissue, while T, Twt and Fwt represent the same notation as above.

Blood Analytical Techniques

Blood was taken by caudal puncture using an ice-chilled 1-mL disposable syringe and 21-gauge needle pre-rinsed with lithium heparin (50 i.u./mL). In addition to the blood samples taken at the times of tissue sampling (days 0, 14, 28, and 42), blood samples were also taken on days 0, 7, 21 and 35 from fish sacrificed for the Na^+, K^+-ATPase assay (see below) in order to measure plasma Ca^{2+}, Cl^-, K^+, Mg^{2+}, Na^+ and total Pb. RBCs were analysed for total Pb. Whole blood taken on day 0, 14, 28 and 42 was used to measure ∂-aminolevulinic acid dehydratase (ALAD), total protein (TP), hemoglobin (Hb), total Pb, and total Zn.

Plasma was obtained by centrifuging whole blood at 14000 g for 2 min. The plasma and RBCs were decanted into separate pre-weighed tubes, then placed in liquid nitrogen and stored in a -70°C freezer until further use. For analysis, the plasma was thawed at room temperature (22°C) and sonicated to remove any blood clots. Plasma Ca^{2+} and Mg^{2+} concentrations were determined by diluting the plasma with 0.5% $LaCl_3$ and assaying it against known standards diluted in 0.5% $LaCl_3$ using the flame AAS. Plasma Na^+ and K^+ were handled similarly,

except that the plasma Na^+ and standards were diluted in 1% HNO_3, and plasma K^+ and standards were diluted in 0.1% $CsCl_2$. Total plasma Pb was determined by diluting the plasma with 1% HNO_3 and measuring it against a certified multi-element Pb standard (Anachemia Inc, Quebec) on the graphite furnace AAS. Plasma Cl^- was measured using the coulometric titration method (Radiometer CMT10). The RBCs were acid-digested and assayed for total Pb concentration following the protocol as above for the tissues.

Whole blood was directly transferred into liquid nitrogen and stored at -70°C, until analysis for Hb, TP, ALAD, total Pb, and total Zn. Hb levels were determined in duplicate using the cyanmethemoglobin method. Basically, 10 µL of whole blood homogenized in double-distilled water was mixed with 2 mL of Drabkin's reagent (Ponite Scientific Inc., Michigan) and measured against known standards (Pointe Scientific Inc, Michigan) at 540 nm (UltraSpec Plus, LKB BIOCHROM).

TP was assayed in duplicate using the Bradford (1976) method with bovine serum albumin standards (Sigma-Aldrich) and measured on a microplate reader (SpectraMax 340 PC, Molecular Devices, CA) at 540 nm.

The whole blood was acid-digested and assayed for total Pb concentration following the same protocol as above for the tissues and RBCs. The mean recovery for whole Pb spiked with a known amount of Pb was 68% ± 3.7%. Data were not corrected for total Pb recovery.

Total Zn concentrations in the whole blood were determined by diluting the acid-digested whole blood with 1% HNO_3 and assaying it against known Zn standards (Sigma-Aldrich) diluted in 1% HNO_3 using the flame AAS.

∂-Aminolevulinic Acid Dehydratase (ALAD) Assay

On day 0, two fish, while on days 14, 28 and 42, four randomly selected fish per tank section were used to determine ALAD in the blood, using the Ehrlich color reaction. The second step of the heme synthesis pathway involves the enzyme ∂ aminolevulinic acid dehydratase to catalyze the conversion of two molecules of aminolevulinic acid to one molecule of porphobilinogen (PBG). The Ehrlich colour reaction (1.0 g of p-dimethylamino benzaldehyde (PAB) in 55 mL of water/acetic acid/perchloric acid) was used to measure the conversion of these substrates in the blood homogenate. Blood was collected as above and ALAD was analysed using a procedure modified from Schmitt et al. (1993, 2005) and Whyte (2002). In short, frozen blood was thawed and homogenized in an equal volume of ice cold double-distilled water, and 15 µL of this homogenate was placed into four tubes, two tubes which contained 75 µL of 0.2% Triton X-100 in 0.1 M phosphate buffer, used as the blank, and two other tubes containing 75 µL of 0.2% Triton X-100 in 0.1 M phosphate buffer plus 670 µg/mL of ∂ aminolevulinic acid hydrochloride (ALA-HCL), used for ALAD determination. Each tube was vortexed and incubated in a 37°C water bath for 1 h. The reaction was terminated with 600 µL of TCA/n-ethylmaleimide solution (4.0 g

trichloroacetic acid and 2.7 g N-ethylmaleimide in 100 mL of double-distilled water). The tubes were then centrifuged at 1000 g for five minutes. 100 μL of the supernatant plus 100 μL of Ehrlich-PAB (3 mL of double distilled water, 42 mL glacial acetic acid and 10 mL 70% perchloric acid and 1.0 g p-dimethylamino benzaldehyde) solution were mixed and measured against PBG standards at 540 nm on the microplate reader.

Determination of Tissue Pb Burdens Due to Trapped Red Blood Cells (RBC)

It was found that the whole blood accumulated a substantial amount of Pb, virtually all of which was in the RBCs, with negligible amounts in the plasma. Therefore, there was a need to estimate how much of an observed Pb burden in the specific internal tissues was actually due to trapped whole blood content. Percentage of Pb in each tissue as a function of vascularization in terms of blood Pb concentrations was accounted for using the estimate of tissue blood volume reported by Olson (1992) for each tissue of freshwater rainbow trout (brain, bone, gill, liver, large intestine (posterior intestine), small intestine (mid intestine), pyloric cecae, anterior, and posterior kidney, stomach, spleen, and white muscle), and the formula;

$$\% T_{wb} = [(V_{ave})*(Pb_{wb}-C_{wb})/(Pb_T-C_T)]*100 \quad (11),$$

where % T_{wb} is the percentage of total accumulated Pb in the tissue explained by trapped whole blood content, V_{ave} is the estimated tissue blood volume for rainbow trout from Olson (1992) in g/kg tissue weight, Pb_{wb} is the Pb burden in

blood in g/kg, C_{wb} is the background Pb burden in blood in g/kg, Pb_T is the Pb burden in the tissue of interest in g/kg, and C_T is the background Pb burden in the tissue of interest in g/kg.

Na^+, K^+-ATPase Assay

On day 0, two fish were randomly selected per tank section, while on days 7, 21, and 35, three randomly selected fish per tank section were used to determine Na^+, K^+-ATPase activity in the gill and intestinal segments. Each fish was anaesthetized with 1.0 g/L MS 222 and dissected on ice. Each gill and intestinal segment was placed into a pre-weighed plastic tube, frozen in liquid nitrogen, and stored at -70°C, until further use. Na^+, K^+-ATPase activity was measured using the UV detection microplate method of McCormick (1993). In short, an enzyme-coupling reaction in which each mole of ATP was hydrolyzed by ATPase enzymes to convert NADH to NAD^+, was used to determine absorbance changes due to NADH oxidation measured at 340 nm at 20 s intervals for 10 min, or until the reaction substrates were depleted. Na^+, K^+-ATPase activity was determined by calculating the difference in ATP hydrolysis in the absence and presence of oubain. Gill and intestinal Na^+, K^+-ATPase activity was then normalized against total gill or intestinal protein using the Bradford (1976) method.

Water Samples

Two 10 mL water samples were taken twice weekly (on the sampling day and 3 days post sampling) for each tank section in order to determine total Pb and dissolved Pb. Samples were acidified to 1% HNO_3 (trace-metal grade acid, Fisher Scientific) and analysed on the furnace AAS. Dissolved Pb was measured similarly to total Pb, except that the water was first passed through a 0.45 μm Supor® low protein binding non-pyrogenic membrane acrodisc syringe filter (Pall Corporation, MI, USA) and then acidified with 1% HNO_3.

Statistics

All statistical tests were performed using SigmaStat version 3.0. Prior to analysis of variance, all data were tested for homogeneity of variances among groups using the Bartlett test. Those that failed were subjected to various transformations to obtain homogeneity among groups. Data that could not be normalized were subjected to the Kruskal-Wallis rank test, where all ranks were corrected for ties and a Dunn multiple comparison was used to determine differences between treatments and groups ($P<0.05$). Comparisons in normalized data between treatments and groups were made by one-way, two-way or three-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for differences among treatments and groups ($P<0.05$). In some cases residuals were plotted against non-normalized data to detect for any outliers. Outliers that were present were removed. Data were again tested for homogeneity

of variances, as indicated above. In many instances, the removal of outliers resulted in normalized data. All data have been reported as means ± SEM, N= sample size.

RESULTS

Water Samples

Pb concentrations in both filtered and unfiltered water samples were not significantly different between treatments (Table 3-2). Waterborne Pb concentrations were not significantly greater than waterborne Pb background levels of 1.3 ± 0.1 μg Pb/L measured during the pre-exposure acclimation period. When the additional control tanks were compared to the other control treatment tanks (Figure 3-1) that were sectioned with high dietary Pb levels, there were no significant differences in waterborne Pb concentrations, Pb accumulation in the different tissues, hematological, or ionoregulatory parameters, indicating that leaching of dietary Pb and transport of waterborne Pb between the tank sections was not a problem.

Growth and Survival

Chronic dietary exposure in the range of 50-500 μg Pb/g dw resulted in no significant effects on the growth and survival of juvenile rainbow trout over 42 days. Enhanced-Ca^{2+} diets (60 mg Ca^{2+}/g dw) had no effects on survival, despite a tendency for SGR to be lower in fish fed these diets (Table 3-3). The mean fish weight (g) remained constant across all treatments during the course of the

experiment. FCE was significantly lower in the 0 μg Pb + 60 mg Ca^{2+}/g, 500 μg Pb/g + 20 mg Ca^{2+}/g and 500 μg Pb/g + 60 mg Ca^{2+}/g, compared to the 0 μg Pb/g + 20 mg Ca^{2+}/g and 50 μg Pb/g + 20 mg Ca^{2+}/g treatments. Fish started and remained fat throughout the experiment as shown by the condition factor (CF) in Table 3-3, where values were uniformly above a value of 1 (normal, healthy weight).

Pattern of Pb Accumulation in Tissues

In terms of accumulated whole body Pb burden (μg/kg fish; Figure 3-2) throughout the duration of the experiment, the fish fed the 500 μg Pb/g + 20 mg Ca^{2+}/g diet accumulated 18-34 times more Pb when compared to the control (500 μg Pb/g + 20 mg Ca^{2+}/g), and 6-9 times more when compared to the 50 μg + 20 mg Ca^{2+}/g treatment. The fish receiving the 50 μg Pb/g + 20 mg Ca^{2+}/g diet accumulated 2-5 times more Pb when compared to the controls.

Figure 3-3 shows the per cent of total Pb distribution (per fish weight) in the different tissues of juvenile rainbow trout fed the 500 μg Pb/g + 20 mg Ca^{2+}/g diet for 42 days. In short, the bone accumulated the highest percentage of Pb (37.7%), while the brain accumulated the least burden of Pb (0.1%). In terms of Pb distribution in the gastrointestinal tract, the anterior intestine (18.6%) had the highest Pb burden followed by the stomach (9.9%), the mid intestine (1.0%), and the posterior intestine (0.9%). The posterior kidney, however, had a much higher Pb load (2.4%) when compared to the anterior kidney (0.3%). Despite the

spleen's relatively small weight when compared to the posterior kidney, posterior intestine, and mid intestine, it accumulated a substantial percentage of Pb burden (2.7%). Overall, the three tissues with the highest percentage of Pb burden were the bone (37.7%), anterior intestine (18.6%), and the white muscle (11.5%), whereas, the three tissues with the least percentage of Pb burden were the brain (0.1%), the anterior kidney (0.3%) and the posterior intestine (0.9%).

Pb concentrations in the control treatment (0 μg Pb/g + 20 mg Ca^{2+}/g) of all tissues were low (<0.05 μg/g tissue wet weight), but were significantly elevated on days 14 and 28, and occasionally on day 42, when compared to day 0. In the high dietary Pb treatments without enhanced Ca^{2+}, Pb accumulated in all the tissues analysed (brain, bone, gill, anterior intestine, mid intestine, posterior intestine, liver, anterior kidney, posterior kidney, spleen, stomach, white muscle and carcass) when compared to the controls (Figures 3-4 to 3-9; Table 3-4). The order of Pb accumulation reflected the exposure pathway of fish fed the high Pb diet (500 μg Pb/g + 20 mg Ca^{2+}/g) after 42 days: gut> bone> kidney > liver > spleen> gills > carcass> brain> white muscle. In the gastrointestinal tract, Pb levels were the highest in anterior intestine (Figure 3-5b)>mid intestine (Figure 3-5c) > stomach (Figure 3-5a)> posterior intestine (Figure 3-5d). Within the internal tissues the following order of Pb accumulation was observed: bone (Figure 3-8)> posterior kidney (Figure 3-6b)> anterior kidney (Figure 3-6a)> liver (Figure 3-7)> spleen (Table 3-4)> gills (Figure 3-4)> carcass (Figure 3-9)> brain (Table 3-4)> white muscle (Table 3-4) in fish exposed to the high Pb diet.

Similarly, for the low Pb diet (50 μg Pb/g + 20 mg Ca^{2+}/g), the order reflected the exposure pathway after 42 days: anterior intestine>mid intestine>bone>posterior kidney> posterior intestine>stomach>anterior kidney>liver>spleen>gills>white muscle>brain> carcass. In the gastrointestinal tract in the low dietary Pb treatment (50 μg Pb/g + 20 mg Ca^{2+}/g), anterior intestine accumulated the most Pb >mid intestine>posterior intestine> stomach. Within the internal tissues, the bone accumulated the highest Pb burden followed by >posterior kidney>anterior kidney>liver>spleen>gills>white muscle>brain> carcass.

The gills significantly accumulated Pb from days 0 to 28 (2.2 μg Pb/g tissue wet weight) in the 500 μg Pb/g + 20 mg Ca^{2+}/g exposed fish, followed by a significant depuration by day 42 (0.6 μg Pb/g tissue wet weight; Figure 3-4). A similar pattern was observed in the mid intestine (Figure 3-5c), and the anterior kidney (Figure 3-6a), whereas Pb burdens in most other tissues (stomach, anterior intestine, posterior intestine, posterior kidney, spleen, and carcass) were more or less stable from days 14 through 42. Taking these data together suggests that Pb regulation may have taken place in the high dietary Pb treatments. Similarly, fish exposed to the 50 μg Pb/g + 20 mg Ca^{2+}/g exhibited approximately stable Pb burden from days 14 to 42 in many tissues, suggesting homeostatic regulation.

The bone (Figure 3-8) had a distinct pattern of Pb accumulation in fish fed the 500 μg Pb/g + 20 mg Ca^{2+}/g diet. In short, Pb accumulation was significantly increased on day 14 with a significant reduction on day 28. No significant

differences were observed in Pb accumulation between days 28 and 42, and days 14 and 42. In fish fed the low dietary Pb, bone Pb levels remained fairly close to control background levels.

The two tissues with the lowest Pb accumulation, the white muscle (Table 3-4) and brain (Table 3-4) had approximately 3 times greater Pb accumulations than background control levels in fish fed the high Pb diets over the duration of the experiment. In the low dietary Pb treatments without supplemented dietary Ca^{2+}, Pb accumulations were close to control background levels. The carcass (Figure 3-9) had an initially high Pb accumulation by day 14 (0.55 μg Pb/g), with Pb levels remaining stable around 0.43 μg Pb/g over the duration of the experiment in fish fed a 500 μg Pb/g + 20 mg Ca^{2+}/g diet. In fish fed the low dietary Pb, Pb accumulations in these compartments were close to control background levels.

Effects of Elevated Dietary Ca^{2+} *on Patterns of Pb Accumulation in Tissue*

Elevated dietary Ca^{2+} levels had a protective effect in reducing whole body Pb burden (Figure 3-2) on all days in fish fed the high dietary Pb diet. Whole body Pb burden were significantly reduced by 61-78% on all days in fish fed the 500 μg Pb/g + 60 mg Ca^{2+}/g treatment when compared to fish fed the 500 μg Pb/g + 20 mg Ca^{2+}/g diet. However, elevated dietary Ca^{2+} levels had a significant effect in reducing whole body Pb burden by 66% only on day 14 in

fish fed the 50 μg Pb/g + 60 mg Ca^{2+}/g diet when compared to fish fed the 50 μg Pb/g + 20 mg Ca^{2+}/g diet.

A three way ANOVA was done to factor in time, dietary Ca^{2+} and Pb levels on the overall effect of Ca^{2+} on each tissue. Overall, elevated Ca^{2+} levels had a protective effect in reducing tissue Pb burden in all tissues, except the brain of trout fed the high dietary Pb levels. The same was true for fish fed the low dietary Pb levels with elevated Ca^{2+} but only in the anterior intestine, anterior kidney, bone and liver. Despite, the overall effect of Ca^{2+}, the individual time points were not significantly different in many of the tissues between the 500 μg Pb/g + 20 mg Ca^{2+}/g and 500 μg Pb/g + 60 mg Ca^{2+}/g treatments and the 50 μg Pb/g + 20 mg Ca^{2+}/g and 50 μg Pb/g + 60 mg Ca^{2+}/g treatments.

The following effects of elevated dietary Ca^{2+} are particularly notable.

Fish fed the elevated Ca^{2+} diets (500 μg Pb/g + 60 mg Ca^{2+}/g) exhibited a 90% reduction in gill-Pb concentrations when compared to fish fed elevated Pb alone (500 μg Pb/g + 20 mg Ca^{2+}/g) on day 28, with lesser reductions also present on other days (Figure 3-4).

The stomach had an equally high Pb accumulation on day 14 in fish fed control and elevated Ca^{2+} diets, but thereafter, there was a continuous decrease in the latter (Figure 3-5A). A significant 6 fold lower Pb burden in fish fed the 500 μg Pb/g + 60 mg Ca^{2+}/g was observed on day 42 in the stomach relative to the 500 μg Pb/g + 20 mg Ca^{2+}/g treatment.

Pb accumulations in the anterior intestine (Figure 3-5b) of fish fed the elevated dietary Ca^{2+} levels were stable around 2.7 μg Pb/g tissue wet weight over the duration of the experiment. Elevated dietary Ca^{2+} significantly reduced Pb burden in the anterior intestine by 67% on day 28 between 500 μg Pb/g + 20 mg Ca^{2+}/g and 500 μg Pb/g + 60 mg Ca^{2+}/g treatments. In the mid intestine (Figure 3-5c), fish fed elevated dietary Ca^{2+} had a similar pattern of Pb uptake as that of fish fed the high Pb diet alone. Despite elevated dietary Ca^{2+} having an overall effect on Pb burden in the mid intestine, elevated Ca^{2+} did not have a significant effect in reducing Pb burden on individual sampling days in this tissue. Elevated Ca^{2+} was effective in reducing Pb burden in the posterior intestine (Figure 3-5d) by 65-80% on days 28 and 42 when compared to high dietary Pb alone.

Dietary Ca^{2+} was significant in reducing Pb burden by 70-90% over the duration of the experiment in the anterior kidney (Figure 3-6a). Similarly, dietary Ca^{2+} was effective in reducing Pb burden in the posterior kidney (Figure 3-6b) by 70% on day 28 when compared to the high dietary Pb alone.

Elevated dietary Ca^{2+} significantly reduced Pb burden in the liver by 41 % on day 42 (Figure 3-7). Dietary Ca^{2+} (500 μg Pb/g + 60 mg Ca^{2+}/g) resulted in a significant 3 fold and 7 fold decrease in Pb accumulation in the bone on day 28 and 42, respectively.

Notably, the Pb burdens in the elevated Ca^{2+} treatment were identical to those in fish fed the high Pb diet alone in the brain (Table 3-4). Pb burdens in the white muscle (Table 4) were significantly reduced by 66% on day 14 in fish fed

the high Pb, high Ca^{2+} diet, when compared to fish fed the high Pb, low Ca^{2+} diet. Compared to the high Pb diet alone, the elevated Ca^{2+} diet significantly reduced Pb burden in the carcass by approximately 70% on days 14 and 28 (Figure 3-9).

Pb Retention

Table 3-8 shows the net retention of Pb after 42 days at a ration of 1.5% body weight per day. The controls (0 μg Pb/g + 20 mg Ca^{2+}/g; 0 μg Pb/g + 60 mg Ca^{2+}/g) had much higher % Pb retention (10.84 %; 32.52 %, respectively) than any of the elevated Pb treatments (all were < 0.35 %, Table 3-8). Nevertheless fish fed the elevated Pb and Ca^{2+} diets, on average had lower Pb retention than those fed elevated dietary Pb alone.

Plasma and RBC Pb Burden, Whole Blood Burden and ALAD Activity

When the blood plasma (Table 3-5) and RBC (Figure 3-10) were compared in terms of Pb burden on day 35 in all dietary treatments, it was found that virtually all the Pb (~98-99%) was present in the RBCs, with almost no Pb (1-2%) being present in the plasma. Effects of elevated dietary Pb and/or Ca^{2+} on plasma Pb concentrations could not be detected, though there was a general tendency for plasma Pb levels to decrease with time (Table 3-5). Pb burden in the RBCs increased gradually over time from day 7 (0.6 μg Pb/g RBC wet weight) to day 35 (1.0 μg Pb/g RBC wet weight) in the 500 μg Pb/g + 20 mg Ca^{2+}/g treatment. RBC Pb burden in the 500 μg Pb/g + 60 mg Ca^{2+}/g treatment increased

from 0.4 ug Pb/g RBC wet weight on day 7 to 0.6 μg Pb/g RBC wet weight on day 35. Despite there being no significant differences on individual days, elevated Ca^{2+} did have a significant overall effect in reducing Pb burdens in the RBC (Figure 3-10) and whole blood (Table 3-5).

Whole blood Pb burden was significantly elevated in the high Pb treatments groups with and without Ca^{2+} when compared to the controls on days 14 to 42 (Table 3-5). Elevated dietary Ca^{2+} levels were significant in reducing whole blood Pb burden between the 500 μg Pb/g + 20 mg Ca^{2+}/g (0.30 μg Pb/g blood wet weight) and the 500 μg Pb/g + 60 mg Ca^{2+}/g (0.15 μg Pb/g blood wet weight) treatments on day 28. Similarly, a significant reduction in Pb accumulation was also present between the 50 μg Pb/g + 20 mg Ca^{2+}/g diet (0.14 μg Pb/g blood wet weight) and the 50 μg Pb/g + 60 mg Ca^{2+}/g diet (0.07 μg Pb/g blood wet weight) on day 42.

When ALAD activity (nMol PBG/g/h) was plotted against the natural logarithm of whole blood Pb concentration (Figure 3-11), it was found that only 9% of variability in ALAD activity was explained by dietary Pb in the blood in all treatments tested; this relationship was not significant (*P=0.45*). However, a significant reduction in the ALAD activity between the 0 μg Pb/g + 20 mg Ca^{2+}/g treatment and 500 μg Pb/g + 20 mg Ca^{2+}/g treatment on day 42 was observed, with whole blood-Pb concentrations of 0.07 μg Pb/g and 0.47 μg Pb/g, respectively (Table 3-7). Despite this significant decrease, there were no

significant effects on Hb levels, total blood protein, and whole blood Zn levels (Table 3-7) over the duration of the experiment in all treatments.

Ion Regulation and Na^+, K^+-ATPase Activity

There were no significant differences observed in plasma Cl^- and K^+ (Table 3-6) in all treatments when compared to the controls over the duration of the experiment. However, significant differences did exist in plasma Na^+ levels on day 21 and Ca^{2+} levels on days 7 and 21 (Table 3-6). In short, plasma Na^+ levels were significantly increased in the 50 μg Pb/g + 20 mg Ca^{2+}/g and 500 μg Pb + 20 mg Ca^{2+}/g treatments when compared to both of the controls. Plasma Na^+ concentrations in the 500 μg Pb/g + 60 mg Ca^{2+}/g treatment were significantly elevated when compared to the 0 + 60 mg Ca^{2+}/g diet. Plasma Ca^{2+} levels were significantly decreased on day 7 in the 500 μg Pb/g + 60 mg Ca^{2+}/g treatment when compared to the 0 μg Pb/g + 20 mg Ca^{2+}/g and 50 μg Pb/g + 20 mg Ca^{2+}/g treatments. However, Ca^{2+} levels were significantly elevated on day 21 in the above treatments when compared to the controls. Furthermore, Mg^{2+} and Ca^{2+} levels were significantly lower on days 21 and 35 within the same treatment when compared to day 0 and 7.

There were no significant effects of dietary Pb on Na^+, K^+-ATPase activity in the gills, mid intestine, and posterior intestine on day 35 (Figure 3-12). However, there was a significant up-regulation in Na^+, K^+-ATPase activity in the

500 μg Pb/g + 20 mg Ca^{2+}/g and 500 μg Pb/g + 60 mg Ca^{2+}/g when compared to the 0 μg Pb/g + 20 mg Ca^{2+}/g treatment on day 35 in the anterior intestine

DISCUSSION

Diet

Dietary Pb concentrations (Table 3-1) were chosen to duplicate reported Pb concentrations found in contaminated benthic invertebrates in the environment (Woodward et al., 1994, 1995; Farag et al., 1994, 1999), and concentrations used in Chapter 2. Previous studies have found significant reductions in whole body Cd uptake from the water and diet when rainbow trout were fed a diet supplemented with 60 mg $CaCO_3$/g (Baldisserotto et al., 2004b, 2005; Franklin et al., 2005), or 60 mg $CaCl_2$ (Zohouri et al., 2001; Baldisserotto et al., 2004a), though some negative physiological effects associated with the Cl^- anion were found in the latter studies. Since Cd is a Pb antagonist, a dietary Ca^{2+} concentration of 60 mg $CaCO_3$/g was chosen for this study.

Growth and Survival

There were no mortalities (Table 3-3), no significant effects on fish weight or CF (Table 3-3) and only minor differences in FCE in this experiment. These results are consistent with Chapter 2 that found no effects on survival and growth when juvenile rainbow trout were fed commercial trout pellets amended with various levels of Pb (7-520 μg Pb/g dw) for 21 days. They are also consistent with

the results of Hodson et al. (1978a) who found no effects on survival of rainbow trout fed beef liver diets contaminated with Pb levels up to 118 μg Pb/g dw for 32 weeks, and Mount et al. (1994) who found no effects on survival and growth of rainbow trout fed brine shrimp contaminated with 170 μg Pb/g dw for 60 days.

Fish fed the high Ca^{2+} and Pb diets, nevertheless, did have non-significantly lower SGR, weight gains, and FCE than those fed the low dietary Ca^{2+} and Pb diets. Andrews et al. (1973) noted that when channel catfish (*Ictalurus punctatus*) fingerling were fed diets containing 2% Ca^{2+} in the form of $CaCO_3$ and reared at waterborne Ca^{2+} levels of 56 mg Ca^{2+}/L (1.4 mM Ca^{2+}), there were decreases in growth and FCE in these fish compared to fish fed diets containing 0.5, 1, and 1.5% Ca^{2+}. In this study the background and enhanced Ca^{2+} diets contained 2% and 6% Ca^{2+}, respectively. In contrast to channel catfish study, juvenile rainbow trout may need a higher Ca^{2+} diet thershold (>6%) in order for there to be a significant decrease on growth and FCE. The authors suggested that these reductions, may be the result of Ca^{2+} competing with the metabolism of Zn and Mg^{2+} from the diet, which are essential nutrients necessary for optimal growth and maintenance. Overall, growth rates do not appear to be sensitive indicators of dietary Pb toxicity.

Tissue Specific Accumulation

In contrast to Chapter 2, the present exposures were completed without significant elevations in waterborne Pb levels (Table 3-2). Therefore, it can be

concluded unequivocally that Pb of dietary origin does accumulate in the internal tissues and whole body in agreement with Crespo et al. (1986) and Mount et al. (1994) respectively, but in contrast to the data of Hodson et al. (1978a) who found that dietary Pb (4-118 μg Pb/g dw; 32 weeks) did not accumulate in the internal tissues. However, the latter study used beef liver as the diet, and reported Pb bound to beef liver was not leached from the food when placed in water and acid solutions, suggesting that Pb was not bioavailable for uptake by the fish in this diet.

The order of Pb accumulation in this study reflects the exposure pathway (μg/g tissue wet weight) after 42 days: gut>bone>kidney>liver>gill>carcass> brain. Ten out of the thirteen tissues (stomach, mid intestine, liver, posterior kidney, gills, bone, carcass, brain, spleen, and white muscle) analysed in the low dietary Pb treatments with and without supplemented Ca^{2+} were within a factor of 3 of background tissue levels (0.07 μg Pb/g). This suggests that concentrations greater than 50 μg Pb/g are necessary to result in clear Pb accumulation in these tissues. This is consistent with the Chapter 2 study that found dietary Pb concentrations >7 μg Pb/g dw and <77 μg Pb/g dw are necessary for Pb accumulation in the internal tissues via the diet. Moreover, whole body net Pb retention of fish fed any of the diets elevated in Pb were relatively low (all <0.35%; Table 3-8) both in this study, and Chapter 2, suggesting that the gastrointestinal tract may provide a protective barrier against dietary Pb accumulation.

In animals the absorption of Pb in the gastrointestinal tract depends on the biochemical and physical nature of the food, nutritional status of the animal, and the environmental milieu of the gastrointestinal tract (World Health Organization, 1995). This study and Chapter 2 both point towards the intestine as a primary target tissue for sublethal chronic dietary Pb toxicity. When the whole body tissue burdens are considered in μg of Pb in the total tissue (not shown), the anterior intestine + pyloric cecae, mid intestine (Figure 3-5c) and posterior intestine (Figure 3-5d) accumulated 2.9 μg Pb (total tissue), 0.32 μg Pb (total tissue) and 0.18 μg Pb (total tissue), respectively in fish fed the high Pb, low Ca^{2+} diets. This suggests that the anterior intestine + pyloric cecae acts as a sink for Pb, when compared to the mid intestine and posterior intestine.

The importance of the anterior intestine + pyloric cecae in Pb uptake is probably due to it's large surface area, and ability to digest proteins and lipids (Fänge and Grove, 1979; Kleinow and James, 2001). Pb's ability to inhibit protein/enzyme activity by either directly binding to the enzymes, or by reacting with sulfhydryl (SH), amine, phosphate and carboxyl groups (Goering, 1993) on other compounds may help explain the high concentration of Pb in the anterior intestine + pyloric cecae.

In contrast to this *in vivo* study which found that the anterior intestine + pyloric cecae to have accumulated the most Pb, Ojo (2005) found that the mid intestine accumulated the most Pb when isolated intestinal sacs were incubated with 50 μM Pb (~10 μg Pb/mL) on the lumenal surface for 4 h. Nevertheless, the

mid intestine (Figure 3-5c) did accumulate a significant amount of Pb (3 μg Pb/g wet weight) in the 500 μg Pb/g + 20 mg Ca^{2+}/g treatment on day 42 in the present study. The form in which Pb was presented to the intestine (i.e. partial digested food versus dissolved Pb in saline) may help explain the different Pb concentrations between the anterior and mid intestine in these studies. For instance, the food used to make up the various amended levels of Pb and Ca^{2+} diets in this study had a manufacturer's specification of 52% crude protein, while Ojo (2005) used Pb dissolved in saline, which probably contained only low concentrations of protein. Since, the anterior intestine functions in protein digestion when compared to the mid-intestine, Pb's ability to bind to proteins and enzymes, especially during protein digestion, may help explain the different Pb burdens between these two tissues in these different studies.

Pb's ability to bind to the intestinal mucus may serve to sequester Pb that can be absorbed by the gut or excreted or sloughed off during the renewal of the epithelial cells in freshwater fish (Stroband and Debets, 1978), therefore, preventing Pb accumulation and toxicity, and explaining the high Pb concentrations along the gastrointestinal tract. For instance, Glover and Hogstrand (2002) found that mucus and epithelial cells accumulated up to 74% of the retained Zn, whereas Chowdhury et al. (2004) observed that <7% of the Cd infused into the gastrointestinal tract was absorbed by the intestine, whereas, 10-24% remained bound to the gut wall and 16-33% was present in the lumen.

The stomach (Figure 3-5a) accumulated a significant concentration of Pb throughout the whole study (3.7 –2.5 μg Pb/g tissue wet weight, day 14 and 42) when compared to the controls. This is consistent with Farag et al. (1994) that found that rainbow trout fed a contaminated mixed metal (Al, As, Cd, Cu, Cr, Pb, Hg, Zn) benthic invertebrate diet for 21 days exhibited higher Pb burden in the stomach and pyloric cecae when compared to the gills and kidney.

The acidic environment of the stomach (pH 2-4) (Kleinow and James, 2001) may permit Pb to exist in the dissolved form. However, mucus, which is secreted copiously in the stomach, and which acts as a pH buffer, may prevent Pb absorption (Whitehead et al., 1996). Nevertheless, this mucus may bind and sequester the Pb, as in the intestine, thus explaining the high Pb concentrations in the stomach. In addition, since the partial digestion of proteins begins in the stomach, Pb's ability to inhibit enzyme activity by binding directly to the enzymes that contain SH groups, for example, may also explain the high Pb burden at the stomach.

The posterior kidney (Figure 3-4b) accumulated more dietary Pb on average than the anterior kidney (Figure 3-6A) in fish fed the 500 ug Pb/g + 20 mg Ca^{2+}/g treatment on days 14 and 42. These results are similar to Reichert et al. (1979) who found that coho salmon exposed to 150 μg/L of waterborne Pb for 15 days accumulated Pb primarily in the posterior kidney when compared to the anterior kidney. The posterior kidney mainly functions in renal ion transport, such as Ca^{2+} re-absorption (Larsen and Perkins, 2001). Since Pb is a Ca^{2+} antagonist,

Pb may compete with Ca^{2+} for uptake at the posterior kidney explaining the high Pb burden present in this tissue. Furthermore, the peptide glutathione, which is involved in the renal transport of metals, contains SH groups, which makes it a good peptide to complex with Pb (Larsen and Perkins, 2001). Therefore, the direct binding of Pb to glutathione may also help explain the high Pb concentrations in the posterior kidney. Moreover, Larsen and Perkins (2001) suggested that the binding of metals to SH groups in the kidney may result in cellular injury, disrupt enzyme function, cause alterations in membrane integrity, and reduced cytoprotective function. The same disturbances may be present in this study, and could explain the observed disturbances in plasma Ca^{2+} regulation seen in fish on high Pb diets (Table 3-6).

In light of the significant amount of Pb accumulated by the whole blood (0.47 μg Pb/g on day 42 in the 500 μg Pb/g + 20 mg Ca^{2+}/g treatment; Table 3-5), Figure 3-13 shows the percentage of accumulated Pb that can be explained purely by trapped blood within each tissue using the estimated mean blood volumes in different tissues from Olson (1992). Thus, high blood content and vascularization may also help explain the high Pb burden in the renal tissue, accounting for about 20% of the Pb burden (Figure 3-13).

The liver (Figure 3-7) continuously accumulated Pb from 0.1 to 0.7 μg Pb/g tissue wet weight in fish fed the high Pb, low Ca^{2+} diet throughout this study, and about 10% of this Pb in the liver was explained by blood trapped in this tissue (Figure 3-13). Pb levels in the liver were lower than those of the intestine, kidney

and bone. Studies with Pb (Reichert et al., 1979; Campana et al., 2003) have found fish livers to lack Pb-binding proteins, such as metallothioneins, that help in the detoxification of other metals. This may explain why the liver on average had a relatively low Pb burden.

The bone (Figure 3-8) accumulated a substantial concentration of Pb (2.6 μg Pb/g tissue wet weight) when compared to many other tissues. Indeed, 38% of whole body Pb burden (Figure 3-3) was found in the bone. Hodson et al. (1978a) found that the opercular bone had the greatest accumulation of Pb in rainbow trout exposed to waterborne Pb when compared to the gills and kidney. These authors suggested that the bone provides a site for Pb storage and detoxification. Since Pb is a Ca^{2+} antagonist, the high concentrations of Pb in the bone may be explained by Pb using similar uptake pathways to that of Ca^{2+} (see discussion below). In the long term, this detoxification mechanism may have negative consequences. For instance, when dietary and/or waterborne Ca^{2+} levels are low, Ca^{2+} may be relocated from the bone (Persson et al., 1994), and this could simultaneously release Pb into circulation, and cause disturbances in Ca^{2+} ion regulation.

The spleen (Table 3-4) accumulated a substantial amount of Pb (0.52 μg Pb/g tissue wet weight) by day 42 in fish exposed to 500 μg Pb/g + 20 mg Ca^{2+}/g dw. When these results are compared to the Olson (1992) estimates, it was found that all of the Pb accumulation in the spleen was explained by blood being trapped

in this tissue. These results are not surprising, given that the spleen functions to manufacture, store, and recycle RBCs and blood components in fish.

The brain accumulated only minor amount of Pb (Table 3-4), and 11.5% of the Pb accumulation in the brain was explained by the trapped blood in this tissue. Similarly, the white muscle (Table 3-4) also exhibited low, but significant levels of Pb accumulation. When Pb distribution (Figure 3-3) in terms of whole body is considered, the white muscle accounted for 12% of the Pb accumulation. The same was true for the carcass remnants, which made up 10% of whole body Pb accumulation. These results are not surprising since the carcass and white muscle combined make up 80-85% of the fish mass.

Is Dietary Pb Accumulation Regulated?

When accumulated whole body Pb in μg/kg fish was considered (Figure 3-2), Pb burden in all the Pb treatments with and without elevated dietary Ca^{2+} levels remained more or less stable from days 14 through 42. Taken together, this suggests that fish are able to regulate Pb.

Gill burden (Figure 3-4) in this study was maintained around 2 μg Pb/g on days 14 and 28, with a significant reduction to 0.6 μg Pb/g by day 42 when rainbow trout were exposed to the 500 μg Pb/g + 20 mg Ca^{2+}/g treatment. In Chapter 2, gill Pb burdens were more elevated on day 14 (6.4 μg Pb/g tissue wet weight) with a significant reduction to 2.1 μg Pb/g by day 21 in fish exposed to 520 μg Pb/g, though the results were possibly confounded by elevated and

changing waterborne Pb levels in that study. However, taken together, these two studies suggest that the gill is able to adjust, regulate and redistribute Pb to other tissues for detoxification or excretion of Pb.

Both Pb concentrations in the posterior intestine (2.3 μg Pb/g tissue wet weight; Figure 3-5d) and posterior kidney (2.5 μg Pb/g tissue wet weight, Figure 3-6B) remained stable on all days analysed in fish exposed to 500 μg Pb/g + 20 mg Ca^{2+} /g treatment. This suggests that the posterior intestine and posterior kidney are both able to regulate and excrete Pb, perhaps via the feces and urine respectively. In addition, in most of the other tissues (stomach, anterior intestine, spleen, and carcass) Pb burden was stable from day 14 to 42, reinforcing that Pb may be regulated.

As mentioned above, the bone acts as a storage site for Pb. For instance, Varanasi and Gmur (1978) found that 2 weeks after the termination of their waterborne Pb exposure (0.13 and 0.21 mg Pb/L) to the coho salmon, the bone continued to accumulate Pb. Their results suggested that Pb is able to relocate from soft tissues (liver and gills) in order to be stored in the bone. Similarly, the same process may be taking place in the present study. Notably, on day 28 the anterior intestine, mid intestine, anterior kidney and gills had the highest Pb accumulation in fish fed the 500 μg Pb/g + 20 mg Ca^{2+}/g diet compared to days 14 and 42, while the opposite was true for the bone.

Another piece of evidence in favour of some sort of homeostatic regulation of Pb was the relationship between Pb accumulation and Pb dose in the

diet. Despite a 10-fold difference between the two elevated Pb levels (500 versus 50 μg Pb/g), there was a much less than a 10-fold difference in Pb accumulation in the whole body and most tissues. For instance, whole body Pb burden (Figure 3-2) was 6-9 times higher in fish fed the high Pb diets when compared to the low diets. Similarly, a 2-8 fold difference between the two elevated dietary Pb levels (500 versus 50 μg Pb/g) was also evident in most individual tissues (Figure 3-14a-b).

Is Elevated Dietary Ca^{2+} Protective?

Elevated dietary Ca^{2+} had the greatest protective effect in the stomach, bone, posterior intestine, anterior kidney, and liver, and the least effect at the anterior intestine, mid intestine, posterior kidney, spleen, gill, carcass, brain and white muscle at the end of the experiment in fish exposed to the 500 μg Pb/g + 60 mg Ca^{2+}/g diet when compared to 500 μg Pb/g + 20 mg Ca^{2+}/g diet (Figure 3-14a). Despite no effects of elevated dietary Ca^{2+} on different days in the mid intestine, and spleen in fish fed the high Pb, high Ca^{2+} diet, elevated dietary Ca^{2+} had an overall significant effect in reducing Pb burden in all tissues, except the brain. The low Pb, elevated Ca^{2+} diet had an overall effect in reducing Pb burdens only in the anterior intestine, anterior kidney, bone, and liver when compared to the low Pb diet alone. There was not a significant effect of elevated dietary Ca^{2+} on individual tissues at the end of the experiment in fish fed the low Pb, high Ca^{2+} diet when compared to the low Pb, low Ca^{2+} diet (Figure 3-14a).

Elevated dietary Ca^{2+} levels resulted in significant reductions in whole body Pb burdens (μg/kg fish, Figure 3-2) compared to fish fed the high dietary Pb alone, on all days. This is consistent with Franklin et al. (2005) that found that rainbow trout fed elevated dietary Ca^{2+} levels exhibited a reduced whole body dietary Cd burden. In addition, Varanasi and Gmur, (1978) found that the accumulation of waterborne Pb was greatly reduced in the gills, blood, liver, brain, bone and kidney of the coho salmon force-fed 8.4 mg Ca^{2+} gelatin capsules.

When net retention of Pb (Table 3-8) was calculated, it was found that the Pb diets supplemented with Ca^{2+} had a lower whole body Pb net retention than those diets without supplemented Ca^{2+}. This calculation reinforces the conclusion that elevated dietary Ca^{2+} is protective against dietary Pb burdens.

The protective effect of elevated dietary Ca^{2+} was observed on day 28 at the gills in fish fed the high Pb, high Ca^{2+} diet when compared to the high Pb, low Ca^{2+} diet. Since plasma Ca^{2+} levels were significantly elevated on day 21 in fish fed the 500 μg Pb/g + 60 mg Ca^{2+}/g diet, it is suggested that elevated dietary Ca^{2+} and increased Ca^{2+} absorption at the intestine may trigger regulatory mechanisms such as a decrease in Ca^{2+} influx rates and an increase Ca^{2+} efflux rates at the gills in order to maintain Ca^{2+} balance. Since Pb is a Ca^{2+} antagonist and mimic, an increase in Ca^{2+} efflux rates at the gills may also increase Pb efflux rates, thus explaining the protective effect of elevated dietary Ca^{2+} against dietary Pb burden at the gills.

Franklin et al. (2005) found that dietary Ca^{2+} reduced dietary Cd burden in the stomach by at least 2 fold. Similarly in this study, Ca^{2+} significantly, reduced stomach burden by 6 fold on day 42. Despite Pb having a higher affinity for gastrointestinal mucin than Ca^{2+} (Crowther and Marriott, 1984; Conrad et al., 1991; Powell et al., 1999), elevated dietary Ca^{2+} concentrations may out-compete dietary Pb for mucin binding sites, thus explaining the low Pb burdens in the high Pb, high Ca^{2+} diets when compared to the high Pb, low Ca^{2+} diet, along the gastrointestinal tract (Figures 3-5a-d).

The protective effects of elevated Ca^{2+} at the posterior kidney and anterior kidney when compared to the 500 μg Pb/g + 20 mg Ca^{2+}/g treatment may be explained by kidney's role in the re-absorption of Ca^{2+} (Larsen and Perkins, 2001). Elevated Ca^{2+} levels in the glomerular filtration may compete with Pb at the kidney during Ca^{2+} re-absorption, explaining the protective effective of Ca^{2+} against Pb burden in the renal tissues. Since, elevated dietary Ca^{2+} was significant in reducing Pb burdens by 70-90% over the duration of the experiment in the anterior kidney (Figure 3-14b) in fed the high Pb, high Ca^{2+} diet when compared to the high Pb, low Ca^{2+} diet, this suggests that Ca^{2+} and Pb may share a similar transport mechanism at the kidney.

Pb burden in the bone was 72-86% lower in fish fed elevated Ca^{2+} diets. This is consistent with Varansai and Gmur (1978) who found the coho salmon had reduced waterborne Pb burdens in the bone when exposed to increased levels

of dietary Ca^{2+}. These authors suggested that Pb competes with Ca^{2+} for a common pathway and/or transport mechanism at the bone.

Overall, dietary Ca^{2+} was protective in reducing dietary Pb burdens in the whole body and individual tissues in this study. Internal Ca^{2+} status becomes very important to fish that live in Pb-contaminated soft water environments, since Pb in this environment is more likely to exist in the free ionic form, which is the most toxic to fish (Davies et al., 1976). Fish in these environments may need to choose diets that are enriched with Ca^{2+}, such as mollusks and crustaceans, not only to maintain Ca^{2+} ion regulation, but also to protect against sublethal Pb toxicity. Elevated Ca^{2+} diets may not only support internal Ca^{2+} homeostasis, but may also protect fish against waterborne Pb and dietary Pb concentrations. For example, increases in dietary Ca^{2+} may reduce Pb uptake at the intestine, may down-regulate branchial Ca^{2+} uptake pathway, (and therefore Pb uptake pathways), and may increase Ca^{2+} efflux rates, (and therefore Pb efflux rates) so as to maintain Ca^{2+} ion-regulation. For instance, Varansai and Gmur (1978) found that elevated Ca^{2+} levels in the diet were more protective against waterborne Pb burden than elevated Ca^{2+} levels in the water, stressing the important of dietary Ca^{2+} in protecting against Pb accumulation and toxicity in aquatic environments. In aquaculture, the negative impacts of low level Pb contamination in the aquatic environment can be averted by feeding fish diets elevated in Ca^{2+}. However, future studies should investigate at exactly what dietary Ca^{2+} threshold the protective effect of Ca^{2+} is seen against waterborne and dietary Pb in the

environment, while maintaining growth, since too much dietary Ca^{2+} can be detrimental (Andrews et al., 1973). In addition, the protective effects of elevated dietary Ca^{2+} may become important in new environmental regulations and approaches to Pb toxicity (e.g. Biotic Ligand Model; Macdonald et al., 2002; Paquin et al., 2002) that require the understanding of dietary status on Pb accumulation, in order to alleviate the toxic effects of dietary Pb on fish

Plasma Ions and Ionoregulatory Effects

In freshwater fish the absorption of Ca^{2+} via the intestine to the blood is primarily by way of a Na^+/Ca^{2+} exchanger and to a lesser degree by Ca^{2+}-ATPase. Therefore Ca^{2+} uptake at the intestine is dependent on Na^+ concentration at the epithelium and Na^+, K^+ ATPase activity (Flik et al., 1993).

Plasma Ca^{2+} levels were found to be significantly decreased on day 7 in the 500 μg Pb/g + 60 mg Ca^{2+} /g treatment when compared to the controls (Table 3-6). Similarly, Chapter 2 found plasma Ca^{2+} levels to be decreased on day 7 in fish fed a 520 μg Pb/g diet for 21 days, suggesting that dietary Pb interferes with Ca^{2+} ion regulation.

However, plasma Ca^{2+} and Na^+ levels were significantly increased by day 21 in the 500 μg Pb/g + 60 mg Ca^{2+}/g treatment, and the 500 μg Pb/g + 60 mg Ca^{2+}/g and 500 μg Pb/g + 20 mg Ca^{2+}/g, respectively, when compared to the controls (Table 3-6). Na^+, K^+-ATPase activity was also up-regulated at the anterior intestine on day 35 in the 500 μg Pb/g + 20 mg Ca^{2+}/g and 500 μg Pb/g +

60 mg Ca^{2+}/g treatments (Figure 3-12). These results taken together suggest that dietary Pb indirectly disrupts plasma Na^{+} levels. Since, Ca^{2+} absorption at the intestine is dependent on Na^{+} status at the intestine, declines in plasma Ca^{2+} levels, as a result of Pb competing with Ca^{2+} at the intestine, may trigger regulatory mechanisms (such as the up-regulation of Na^{+}, K^{+}-ATPase) to increase Na^{+} concentrations at the intestine to aid in Ca^{2+} absorption at the intestine and thus maintain Ca^{2+} ion balance.

Mg^{2+} and Ca^{2+} are considered to be complementary ions (Bijvelds et al., 1998). In Chapter 2 it was found that Mg^{2+} levels were decreased on day 14 when compared to the control. In this study no significant effects on Mg^{2+} levels (Table 3-6) when compared to the controls were observed. These differences may perhaps be explained by the fact that waterborne Mg^{2+} levels were 2 times higher (0.4 mM) in this study than those of Chapter 2 (0.2 mM).

Na^{+}, K^{+}-ATPase activity (Figure 3-12) in the gills was not affected in this study. This is not surprising since gill burden was 100 x less than that of the Rogers et al. (2003) study that found a 40% inhibition of Na^{+}, K^{+}-ATPase at waterborne Pb concentrations of 1.6 mg/L, associated with gill tissue burdens of 200 μg Pb/g, when trout were exposed to a 96 h LC 50 concentration of 1.0 mg Pb/L. Thus, this study demonstrates that the gills are not a primary site of dietary Pb toxicity, in contrast to the situation with waterborne Pb (Hodson et al., 1978a, Holcombe et al., 1976; Rogers et al., 2003).

There were no significant effects in Na^+, K^+-ATPase activity at the mid and posterior intestine in this study, in contrast to Crespo et al. (1986) who found that Na^+, K^+-ATPase activity was inhibited at the mid-intestine in adult fresh water rainbow trout fed 10 μg Pb/g fish/day for 15-30 days. Despite Crespo et al. (1986) not measuring Pb burden in the mid intestine, they did measure Pb concentrations in the kidney (21.82 μg Pb/g tissue dw, day 15). Assuming that wet weight weighs 5 x more than dry weight, the kidney (~4 μg/g tissue wet weight) in the Crespo et al. (1986) accumulated ~2 fold higher Pb concentrations than in this study (~2.4 μg/g wet weight, Figure 3-6a-b) on day 42. Taken together this suggests that a higher Pb burden threshold may be needed to inhibit Na^+, K^+-ATPase activity at the intestine.

RBC and ALAD Activity

When plasma-Pb (Table 3-5) was compared to RBC-Pb, it was found that 99% of the Pb was bound on or in the RBCs. This is consistent with Chapter 2 that found that 98-99% of dietary Pb was present in the RBCs. The constant Pb accumulation in the whole blood and RBC suggests that besides transporting Pb to various tissues, blood may act as a reservoir of excess Pb in the fish system. In humans about 90% of radiolabelled Pb found within red cells was associated with cytoplasm, while <10% was bound to the membrane (Bruenger et al., 1973). In humans the free ionic form of Pb crosses the erythrocyte membrane by an anion exchanger and is actively extruded by a vanadate-sensitive Ca^{2+} pump in the red

cell membranes (Simons, 1984, 1986, 1988, 1993). A similar transport mechanism may also take place in the erythrocytes of freshwater fish. This type of transport may explain why there was an overall significant effect of Ca^{2+} on Pb burdens in both the RBC and whole blood, despite no significant differences being present on individual sampling days. Since Pb mimics Ca^{2+}, Pb may out-compete Ca^{2+} for uptake by the blood using the vanadate-sensitive pathway or similar transport pathways that involve Ca^{2+}. Future studies should investigate this type of Pb transport in fish.

There were no significant effects of dietary exposure of Pb on Hb levels and ALAD activity in the present study. ALAD activity has been found to be negatively correlated with blood-Pb concentrations (over the range 30-5400 μg/L) and waterborne Pb concentrations up to 100 μg Pb/L in fish (Hodson et al., 1977, 1978a; Schmitt et al., 1984, 1993). In this study a linear response of blood-Pb levels and ALAD activity was evident, however only 9% (non-significant) of the variation of ALAD activity was explained by Pb in the blood after 42 days. This suggests that a longer exposure time may be necessary in order to see an effect. Nakagawa et al. (1995) found that a blood-Pb concentration of 0.1 μg Pb/g of blood resulted in a 50% inhibition of ALAD activity in the blood of the carp, *Cyprinus Carpio*. In this study whole blood-Pb (Table 3-5) ranged from 0.26 to 0.47 μg Pb/g from day 14 to 42 in the 500 μg Pb/g + 20 mg Ca^{2+}/g treatment, and a significant 28% inhibition of ALAD activity was observed between the 0 μg

Pb/g + 20 mg Ca^{2+}/g treatment and the 500 μg + 20 mg Ca^{2+}/g treatment on day 42.

ALAD requires Zn^{2+} cofactors and SH groups in order to function (Finelli, 1977; Sassa, 1982). It has been suggested that Zn alleviates the effects of Pb inhibition on ALAD activity by protecting SH groups from oxidation and the displacement of Zn by Pb on ALAD (Schmitt, 1984, 1993, 2002; Dwyer et al., 1988). Despite blood Zn levels being about 100 times greater than that of blood Pb levels, blood-Zn levels in this study remained constant on all days in all treatments and did not differ significantly between treatments, negating the idea that Zn may exert a protective effect against Pb toxicity to ALAD activity in this study. Taking all these observations together, ALAD is not a sensitive biomarker of Pb toxicity in fish exposed to dietary Pb.

CONCLUSION

All segments of gastrointestinal tract are capable of Pb accumulation. The proximal intestine seems to be the most important site for Pb uptake in the gastrointestinal tract, due its higher absorptive surface area than that of the of the distal intestine. Furthermore, it is the region where the first contact of dietary Pb, digestion, and absorption of nutrients may take place. This study suggests that Pb and Ca^{2+} follow similar uptake pathways in the intestine, bone and possibly the kidney. Future studies should investigate the transport mechanism of Pb in the RBC's, since 99% of the dietary Pb burden was present in the RBCs when

compared to the plasma. In light of Pb having a strong affinity for SH groups, enzyme activities along the gastrointestinal tract should also be investigated. Overall, this study shows that elevated dietary Ca^{2+} levels were effective in reducing Pb burdens in individual tissues and in the whole body of fish. This study shows that the Ca^{2+} status is important to fish, especially in environments where Ca^{2+} levels are low, and fish may need to choose or rely on other diets (i.e mollusks and crustaceans) in order to maintain internal Ca^{2+} levels. In addition, this study points towards the intestine as being the primary tissue potentially involved in Pb toxicity.

Table 3-1: The nominal and achieved concentrations of Pb and Ca^{2+}, in custom made diets. [a] (N=15) Data are expressed as mean ± 1 SEM dw.

Treatment	[a][Pb] (μg/g dw)	[a][Ca] (mg/g dw)
0 μg Pb/g + 20 mg Ca^{2+}/g	0.3 ± 0.1	19.2 ± 0.3
0 μg Pb/g + 60 mg Ca^{2+}/g	0.1 ± 0.0	61.0 ± 0.9
50 μg Pb/g + 20 mg Ca^{2+}/g	45.2 ± 2.1	18.5 ± 0.2
50 μg Pb/g + 60 mg Ca^{2+}/g	51.6 ± 3.6	60.4 ± 1.2
500 μg Pb/g + 20 mg Ca^{2+}/g	480.2 ± 30	18.2 ± 0.4
500 μg Pb/g + 60 mg Ca^{2+}/g	495.2 ± 12.9	59.3 ± 1.6

Table 3-2: Mean total Pb concentrations in filtered versus unfiltered water for each treatment over 42 days. Data represented as mean ± 1 SEM, N=28. Lower case letters indicate significant differences ($P < 0.05$) between filtered and unfiltered water within a treatment.Upper case letters indicate significant differences ($P < 0.05$) between treatments within the same water Pb column. Values sharing the same letter indicates no significant differences.Values not sharing the same letter indicates significant differences.

Treatment	[a] Unfiltered Water (µg/L)	[b] Filtered Water (µg/L)
0 µg Pb/g + 20 mg Ca^{2+}/g	1.55 ± 0.14 AB a	1.60 ± 0.15 A a
0 µg Pb/g + 60 mg Ca^{2+}/g	1.69 ± 0.13 AB a	1.57 ± 0.14 A a
50 µg Pb/g + 20 mg Ca^{2+}/g	1.30 ± 0.10 A a	1.64 ±0.23 A a
50 µg Pb/g + 60 mg Ca^{2+}/g	1.59 ± 0.29 AB a	1.27 ± 0.09 A a
500 µg Pb/g + 20 mg Ca^{2+}/g	1.88 ± 0.15 AB a	1.62 ± 0.20 A a
500 µg Pb/g + 60 mg Ca^{2+}/g	2.94 ± 0.67 B a	1.62 ± 0.17 A a

Table 3-3: Mean fish weight, specific growth rates (SGR), rations, food conversion efficiency (FCE) and condition factors (CF) of juvenile rainbow trout fed different Pb and/or Ca^{2+} diets for 42 days. [a] value is in mean ± 1 SEM. [b]SGR was calculated using the linear regression (SigmaPlot version 8.0) of the natural logarithm of mean bulk weight versus time. Mean ± 1 SEM is based on the regression, not on the tank divisions. [c]FCE was calculated by SGR/ration (1.5) multiplied by 100. [a,d] CF was calculated using the formula K= weight (g)/ (fork length (cm))^{3}x100 (Brett et al., 1979). Lower case letters indicate significant differences ($P < 0.05$) between treatments. Values sharing the same letter indicates no significant differences. Values not sharing the same letter indicates significant differences.

Treatment	[a] Mean Fish wt. (g)	Mortality (%)	[a,b] SGR (%/day)	[c] FCE (%)	[d] CF
0 μg Pb/g + 20 mg Ca^{2+}/g	48 ± 4 a	0	1.46 ± 0.12 ab	97 ± 2 ac	1.60 ± 0.06 a
0 μg Pb/g + 60 mg Ca^{2+}/g	40 ± 4 a	0	1.03 ± 0.07 a	69 ± 1 b	1.51 ± 0.08 a
50 μg Pb/g + 20 mg Ca^{2+}/g	48 ± 4 a	0	1.66 ± 0.09 b	104 ± 1 c	1.54 ± 0.05 a
50 μg Pb/g + 60 mg Ca^{2+}/g	40 ± 2 a	0	1.30 ± 0.13 ab	87 ± 3 a	1.53 ± 0.03 a
500 μg Pb/g + 20 mg Ca^{2+}/g	37 ± 4 a	0	1.16 ± 0.14 a	77 ± 4 b	1.45 ± 0.06 a
500 μg Pb/g + 60 mg Ca^{2+}/g	41 ± 2 a	0	1.19 ± 0.12 a	79 ± 2 b	1.59 ± 0.03 a

Table 3-4: Pb tissue burden in the brain, spleen and white muscle (μg Pb/g tissue wet wt.) over 42 days. Data are expressed as mean ± 1 SEM. Upper case letters indicate significant differences between days within the same treatment ($P<0.05$). Lower case letters represent significant differences between treatments within the same day; values sharing the same letter are not significantly different ($P<0.05$).

Tissue	Day	0 µg Pb/g + 20 mg Ca^{2+}/g	0 µg Pb/g + 60 mg Ca^{2+}/g	50 µg Pb/g + 20 mg Ca^{2+}/g	50 µg Pb/g + 60 mg Ca^{2+}/g	500 µg Pb/g + 20 mg Ca^{2+}/g	500 µg Pb/g + 60 mg Ca^{2+}/g
	0	0.07± 0.02	0.07± 0.02	0.07± 0.02	0.07± 0.02	0.07± 0.02	0.07± 0.02
Brain		A	A	A	AB	A	A
µg Pb/g	14	0.06 ± 0.01	0.06 ± 0.04	0.13 ± 0.02	0.04 ± 0.01	0.27 ± 0.08	0.26 ± 0.1
ssue wet		A a	A a	A b	A a	B b	A b
wt.)	28	0.12 ±0.02	0.10 ± 0.02	0.12 ± 0.03	0.20 ± 0.07	0.31 ± 0.09	0.26 ± 0.1
		A a	A a	A a	B a	B a	A a
	42	0.08 ± 0.03	0.04 ± 0.01	0.08 ±0.02	0.07 ± 0.02	0.24 ± 0.09	0.18 ± 0.04
		A ac	A a	A ac	AB a	AB bc	A bc
	0	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
Spleen		A	A	A	A	A	A
µg Pb/g	14	0.07 ± 0.01	0.04 ± 0.01	0.21 ± 0.05	0.09 ± 0.02	0.69 ± 0.21	0.41 ± 0.17
ssue wet		A a	A a	B cd	B ac	B b	B bd
wt.)	28	0.07 ± 0.02	0.08 ± 0.02	0.09 ± 0.03	0.10 ± 0.02	0.29 ± 0.03	0.42 ± 0.28
		A a	A a	B a	B a	B b	B ab
	42	0.06 ± 0.01	0.09 ± 0.02	0.16 ± 0.03	0.15 ± 0.02	0.52 ± 0.11	0.24 ± 0.04
		A a	A ac	B bc	B bc	B d	B bd
	0	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
		A	A	A	A	A	A
White	14	0.08 ± 0.02	0.12 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.29 ± 0.07	0.10 ± 0.02
Muscle		B ac	B bc	B ac	B a	B b	B ac
µg Pb/g	28	0.05 ± 0.02	0.09 ± 0.04	0.09 ± 0.01	0.05 ± 0.01	0.14 ± 0.02	0.07 ± 0.01
ssue wet		AB a	AB ab	B ab	B a	B b	B ab
wt.)	42	0.07 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.09 ± 0.03	0.17 ± 0.04	0.11 ± 0.02
		B ac	AB a	AB a	B ab	B b	B bc

Table 3-5 Mean plasma-Pb (μg/L) and whole blood (μg/g) concentration over 42 days. Data are expressed as mean ± 1 SEM. Lower case letters indicate significant differences between treatments within the same day. Upper case letters indicate a significant differences between days within the same treatment.

	Day	0 µg Pb/g + 20 mg Ca^{2+}/g	0 µg Pb/g + 60 mg Ca^{2+}/g	50 µg Pb/g + 20 mg Ca^{2+}/g	50 µg Pb/g + 60 mg Ca^{2+}/g	500 µg Pb/g + 20 mg Ca^{2+}/g	500 µg Pb/g + 60 mg Ca^{2+}/g
Plasma Pb (µg/L)	0	9.7 ± 1.8 A	9.7 ± 1.8 A	9.7 ± 1.8 A	9.7 ± 1.8 A	9.7 ± 1.8 A	9.7 ± 1.8 A
	7	7.5 ± 0.9 A ab	9.9 ± 1.6 A ab	8.9 ± 1.3 A a	6.1 ± 0.3 AB a	11.4 ± 2.4 A ab	13.4 ± 1.5 A ab
	21	9.1 ± 1.5 A a	8.2 ± 0.5 A a	8.0 ± 0.3 A a	9.4 ± 1.7 A a	7.7 ± 1.0 AB a	7.2 ± 0.9 A a
	35	7.1 ± 0.4 A a	5.1 ± 1.6 A ab	2.8 ± 0.4 B b	3.7 ±0.4 B ab	3.7 ± 0.4 B ab	4.5 ± 0.6 B ab
Whole Blood Pb (µg/g)	0	0.04 ± 0.00 A	0.04 ± 0.00 A	0.04 ± 0.00 A	0.04 ± 0.00 A	0.04 ± 0.00 A	0.04 ± 0.00 A
	14	0.03 ± 0.01 A ac	0.05 ± 0.01 A ac	0.08 ± 0.01 B bc	0.06 ± 0.01 AB bc	0.26 ± 0.04 B b	0.20 ± 0.04 BC b
	28	0.04 ± 0.01 A a	0.05 ± 0.01 A a	0.07 ± 0.01 AB a	0.07 ± 0.01 B a	0.30 ± 0.03 B b	0.15 ± 0.01 B c
	42	0.07 ± 0.01 B a	0.07 ± 0.01 A a	0.14 ± 0.02 C b	0.07 ± 0.01 B a	0.47 ± 0.13 B c	0.24 ± 0.03 C c

Table 3-6: Plasma Na^-, Cl^-, Ca^{2+}, Mg^{2+} and K^+ concentrations (mM). Data are expressed as mean ± 1 SEM. Upper case letters indicate significant differences between days within the same treatment ($P<0.05$). Lower case letters represent significant differences between treatments within the same day ($P<0.05$). Values sharing the same letter are not significantly different.

Plasma Ion	Day	0 μg Pb/g + 20 mg Ca^{2+}/g	0 μg Pb/g + 60 mg Ca^{2+}/g	50 μg Pb/g + 20 mg Ca^{2+}/g	50 μg Pb/g + 60 mg Ca^{2+}/g	500 μg Pb/g + 20 mg Ca^{2+}/g	500 μg Pb/g + 60 mg Ca^{2+}/g
Na^+ (mM)	0	158 ± 5 A	158 ± 5 A	158 ± 5 AC	158 ± 5 A	158 ± 5 A	158 ± 5 A
	7	155 ± 5 A ab	143 ± 6 A a	173 ± 7 AB b	149 ± 3 A ab	155 ± 5 A ab	157 ± 10 A ab
	21	145 ± 3 A ac	143 ± 5 A a	184 ± 2 B b	158 ± 4 A ab	179 ± 7 A b	168 ± 8 AB bc
	35	155 ± 12 A ab	158 ± 3 A ab	151 ± 4 C b	149 ± 7 A b	158 ± 12 A ab	189 ± 9 B a
Cl^- (mM)	0	124 ± 2 A	124 ± 2 AC	124 ± 2 A	124 ± 2 A	124 ± 2 A	124 ± 2 A
	7	121 ± 4 A a	123 ± 2 A a	119 ± 2 A a	127 ± 3 AB a	122 ± 4 A a	129 ± 3 A a
	21	129 ± 2 A a	133 ± 1 BC a	129 ± 3 A a	129 ± 4 AB a	130 ± 2 A a	134 ± 3 A a
	35	131 ± 2 A a	136 ± 1 B a	132 ± 2 A a	135 ± 3 B a	132 ± 1 A a	130 ± 4 A a
Ca^{2+} (mM)	0	2.9 ± 0.1 A	2.9 ± 0.1 AB	2.9 ± 0.1 A	2.9 ± 0.1 AB	2.9 ± 0.1 A	2.9 ± 0.1 A
	7	2.9 ± 0.1 A a	2.5 ± 0.1 A ab	2.8 ± 0.1 A a	2.6 ± 0.1 A ab	2.4 ± 0.2 B ab	2.1 ± 0.2 B b
	21	2.6 ± 0.1 A a	2.5 ± 0.1 A a	2.7 ± 0.1 A ab	2.5 ± 0.1 A a	2.7 ±0.1 AB ab	3.0 ± 0.1 A b
	35	2.5 ± 0.3 A a	3.1 ± 0.3 B a	3.0 ± 0.2 A a	3.1 ± 0.1 B a	3.2 ± 0.2 Aa	2.7 ± 0.2 A a
Mg^{2+} (mM)	0	1.1 ± 0.1 A	1.1 ± 0.1 A	1.1 ± 0.1 A	1.1 ± 0.1 A	1.1 ± 0.1 A	1.1 ± 0.1 AC
	7	0.9 ± 0.1 AB a	0.9 ± 0.1 AB a	0.9 ± 0.1 AB a	0.8 ± 0.1 AB a	0.9 ± 0.1 AB a	0.9 ± 0.1 A a
	21	0.9 ± 0.0 B a	0.8 ± 0.0 B a	0.8 ± 0.0 B a	0.7 ± 0.1 B a	0.8 ± 0.0 B a	0.7 ± 0.1 BC a
	35	0.9 ± 0.0 B a	0.8 ± 0.1 AB a	0.9 ± 0.0 B a	0.7 ± 0.1 B a	0.8 ± 0.0 B a	0.6 ± 0.1 B a
K^+ (mM)	0	4.8 ± 0.8 A a	4.8 ± 0.8 A a	4.8 ± 0.8 A a	4.8 ± 0.8 A a	4.8 ± 0.8 A a	4.8 ± 0.8 A a
	7	5.2 ± 0.5 A a	5.9 ± 0.7 A a	4.8 ± 0.3 A a	4.4 ± 0.6 A a	5.3 ± 0.3 A a	4.2 ± 0.4 A a
	21	5.7 ± 0.5 A a	4.9 ± 0.3 A a	5.0 ± 0.3 A a	5.8 ± 0.2 A a	4.5 ± 0.5 A a	5.3 ± 0.2 A a
	35	4.7 ± 0.4 A ab	6.0 ± 0.5 A a	5.8 ± 0.4 A ab	5.0 ± 0.4 A ab	5.4 ± 0.3 A ab	4.2 ± 0.3 A b

Table 3-7: Mean hemoglobin, total blood protein, whole blood-Pb, ALAD concentrations and whole blood Zn concentrations following 42 days exposure to different dietary Pb diets. Data are expressed as mean ± 1 SEM. Lower case letters indicate significant differences between treatment within a day ($P<0.05$, using a one way ANOVA, with Tukey's multiple comparison test).

Treatment	Hemoglobin (g/L)	Total blood Protein (g/L)	Blood-Pb (µg/L)	ALAD (nMol PBG/g RBC/hr)	Blood-Zn (µg/L)
0 µg Pb/g + 20 mg Ca^{2+}/g	132 ± 6 a	167 ± 6 a	68 ± 7 a	447 ± 33 a	7500 ± 1500 a
0 µg Pb/g + 60 mg Ca^{2+}/g	120 ± 7 a	159 ± 9 a	67 ± 10 a	336 ± 27 ab	10700 ± 1000 a
0 µg Pb/g + 20 mg Ca^{2+}/g	130 ± 5 a	153 ± 7 a	137 ± 18 b	337 ±11 ab	10300 ± 700 a
0 µg Pb/g + 60 mg Ca^{2+}/g	128 ± 4 a	161 ± 4 a	70 ± 7 a	428 ± 54 ab	10400 ± 1900 a
00 µg Pb/g + 20 mg Ca^{2+}/g	127 ± 5 a	153 ± 8 a	473 ± 127 c	322 ± 31 b	10100 ± 500 a
00 µg Pb/g + 60 mg Ca^{2+}/g	124 ± 6 a	164 ± 8 a	242 ± 27 c	439 ± 68 ab	11300 ± 800 a

Table 3-8: Net retention of Pb in trout fed different Pb diets with and without dietary Ca^{2+}. Note that fish were not fed on Day 28, since sampling time was longer than expected, resulting in it being too late to feed fish.

Treatment	Measured Dietary Pb (mg/kg)	Pb ingested Over 42 Days (mg/kg fish)	Total Pb accumulated Over 42 Days (mg/kg fish)	Retention (%)
0 μg Pb/g + 20 mg Ca^{2+}/g	0.3	0.18	0.02	10.84
0 μg Pb/g + 60 mg Ca^{2+}/g	0.1	0.06	0.02	32.52
50 μg Pb/g + 20 mg Ca^{2+}/g	45.2	28	0.09	0.32
50 μg Pb/g + 60 mg Ca^{2+}/g	51.6	32	0.07	0.22
500 μg Pb/g + 20 mg Ca^{2+}/g	480.2	295	0.59	0.20
500 μg Pb/g + 60 mg Ca^{2+}/g	495.1	304	0.27	0.09

Figure 3-1: Tank set-up. Tanks were set up by sectioning 200 L tanks into 2 x 100L sections using a 0.2mm^2 mesh screen. The letter in each section corresponds to the diet given in each section: A: 0 μg Pb/g + 20 mg Ca^{2+}/g; B: 0 μg Pb/g + 60 mg Ca^{2+}/g; C: 50 μg Pb/g + 20 mg Ca^{2+}/g; D: 500 μg Pb/g + 20 mg Ca^{2+}/g; E: 50 μg Pb/g + 60 mg Ca^{2+}/g; F: 500 μg Pb/g + 20 mg Ca^{2+}/g. Each of the above tanks were replicated. Tanks 1 and 5 were used as a control for possible waterborne Pb as a result of Pb leaching from the feces and food into neighbouring tank sections. (i.e. treatment A was replicated in Tank 3 and 7, and treatment B was replicated in Tank 4 and 8, both of which had the highest dietary Pb levels on the opposite side.

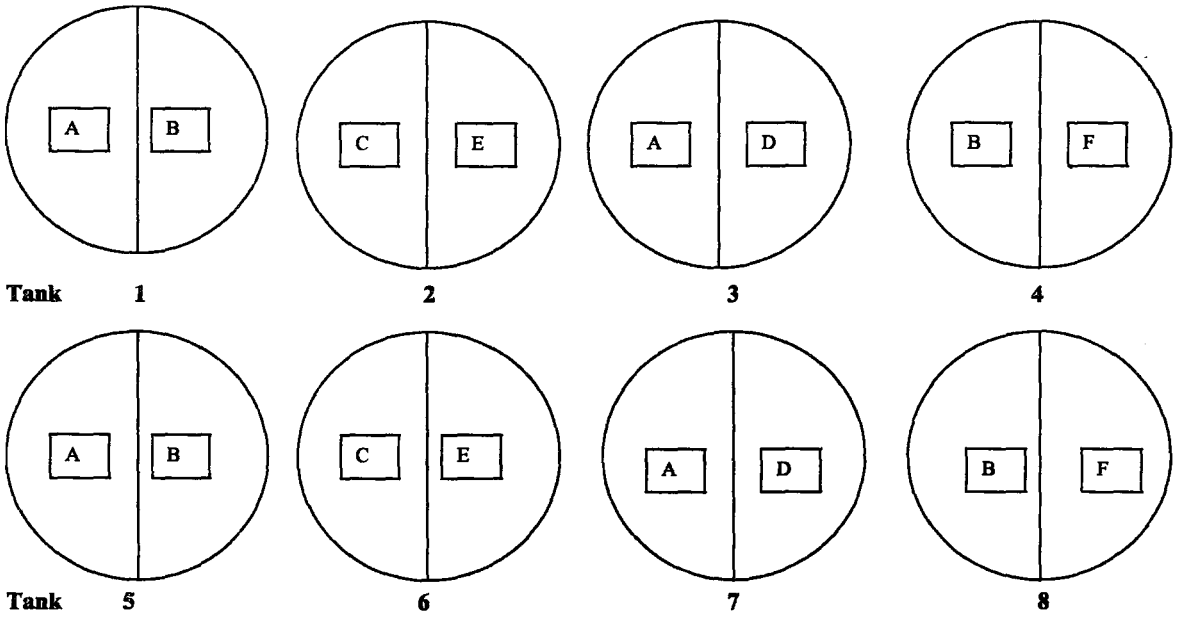

A
B
C
E
A
D
B
F
Tank 1
2
3
4
A
B
C
E
A
D
B
F
Tank 5
6
7
8

Figure 3-2: Accumulated whole body Pb burden in juvenile rainbow trout fed contaminated Pb and/or Ca^{2+} supplemented diets. Data reported as mean ± 1 SEM; N=8. Upper case letters represent significant differences ($P<0.05$) between days within the same treatment. Lower case letters represent significant differences ($P<0.05$) between treatments within the same day. Values sharing the same letters indicates no significant differences. Values not sharing the same letters indicates significant differences. An overall significant effect of Ca^{2+} ($P<0.05$) was determined using a 3-way ANOVA with a Tukey's multiple comparison test.

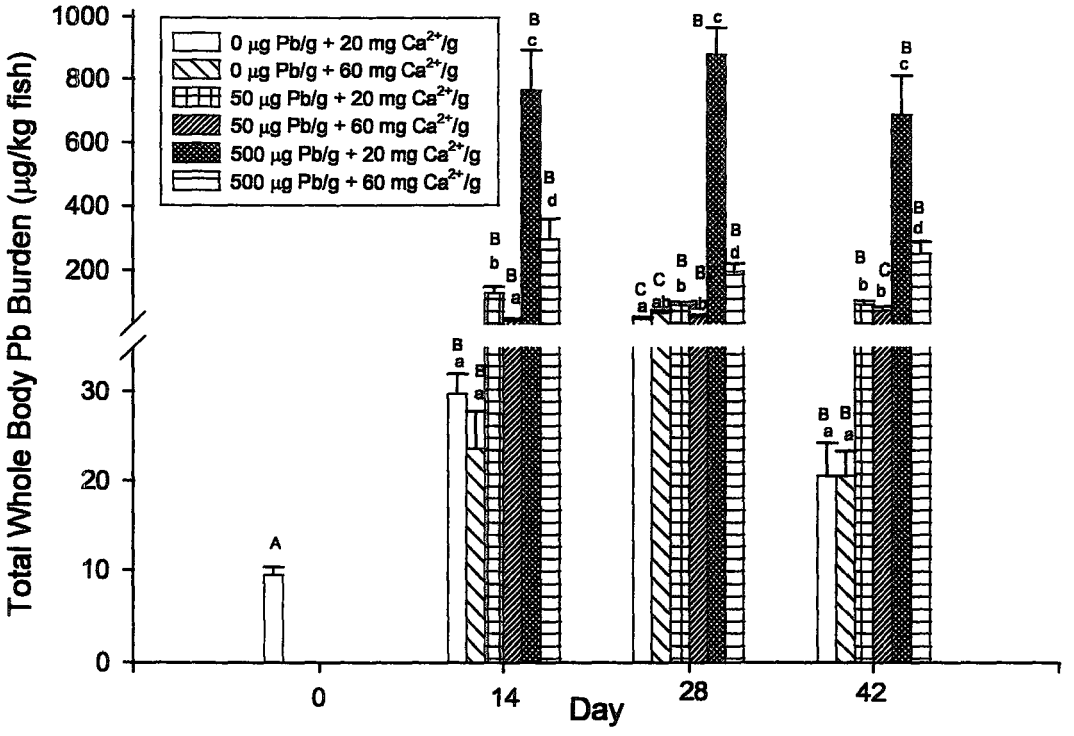

0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
Total Whole Body Pb Burden (µg/kg fish)
1000
800
600
400
200
30
20
10
0
0
14
28
42
Day

Figure 3-3: Average per cent distribution of total Pb burden in different tissues of the juvenile rainbow trout fed the 500 μg Pb/g + 20 mg Ca^{2+}/g diet for 42 days.The bone accumulated the highest Pb burden, while the brain accumulated the least Pb burden in fish fed the 500 μg Pb/g + 20 mg Ca^{2+}/g diet. Calculations to determine per cent Pb burden in each tissue are explained in the section: *Determination of Whole Body Pb and Per Cent Pb Distribution.*

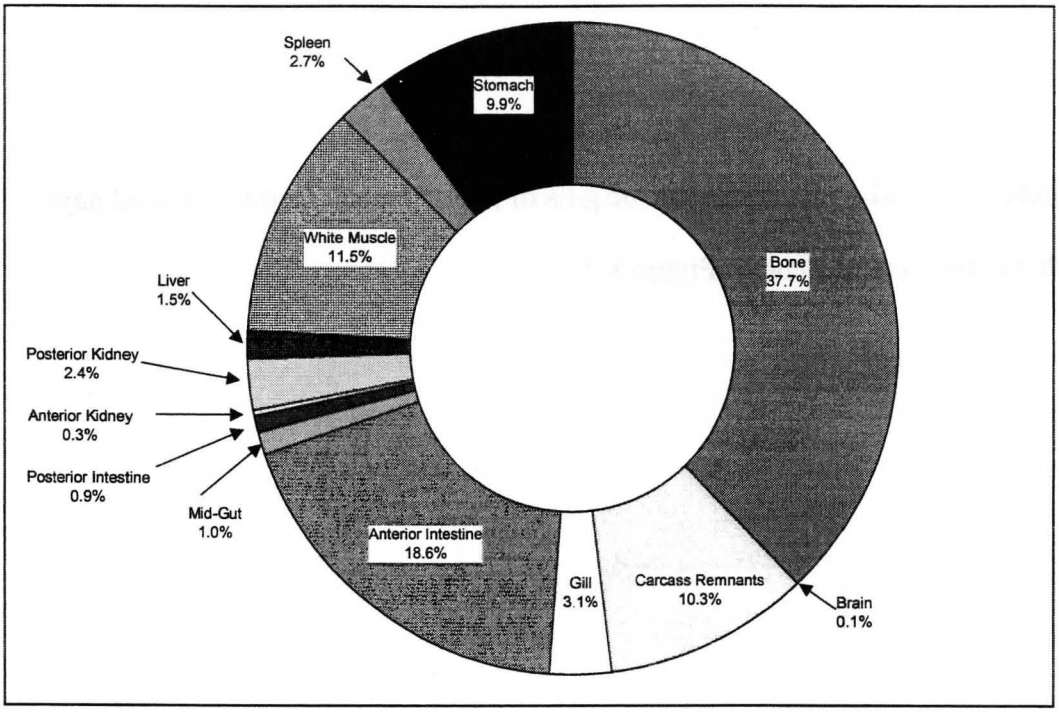

Spleen
2.7%
Stomach
9.9%
White Muscle
11.5%
Liver
1.5%
Posterior Kidney
2.4%
Anterior Kidney
0.3%
Posterior Intestine
0.9%
Mid-Gut
1.0%
Anterior Intestine
18.6%
Gill
3.1%
Carcass Remnants
10.3%
Brain
0.1%
Bone
37.7%

Figure 3-4: Lead accumulation in the gills of juvenile rainbow trout over 42 days. Other details as in legend of Figure 3-2.

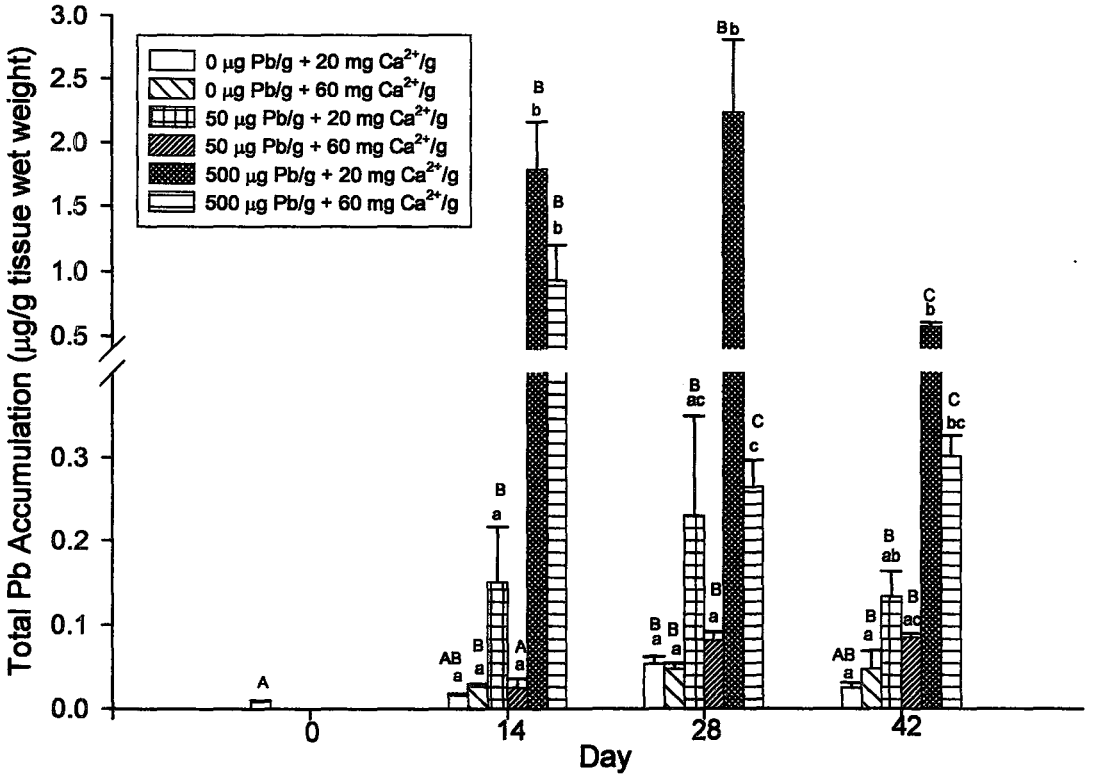

0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
Total Pb Accumulation (µg/g tissue wet weight)
3.0
2.5
2.0
1.5
1.0
0.5
0.3
0.2
0.1
0.0
0
14
28
42
Day

Figure 3-5: Lead accumulation along the gastrointestinal tract of juvenile rainbow trout over 42 days. Note different side used for different sections (a) Stomach. (b) Anterior intestine + pyloric cecae. (c) Mid intestine. (d) Posterior intestine. Other details as in legend of Figure 3-2.

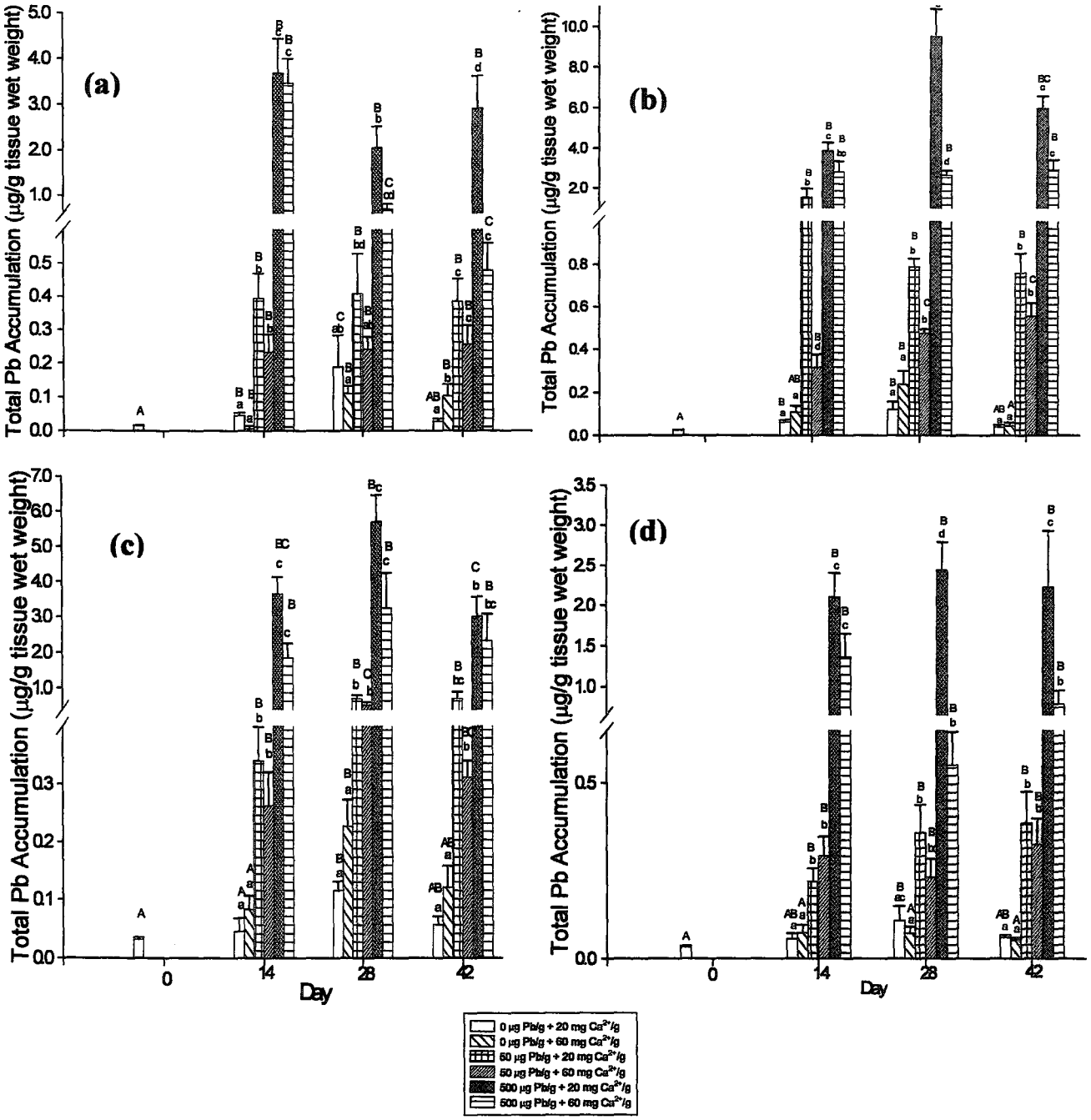

(a)
(b)
(c)
(d)
Total Pb Accumulation (µg/g tissue wet weight)
Day
0
14
28
42
0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g

Figure 3-6: Lead accumulation in the anterior and posterior kidney of juvenile rainbow trout over 42 days. (a) Anterior kidney. (b) Posterior kidney. Other details as in legend of Figure 3-2.

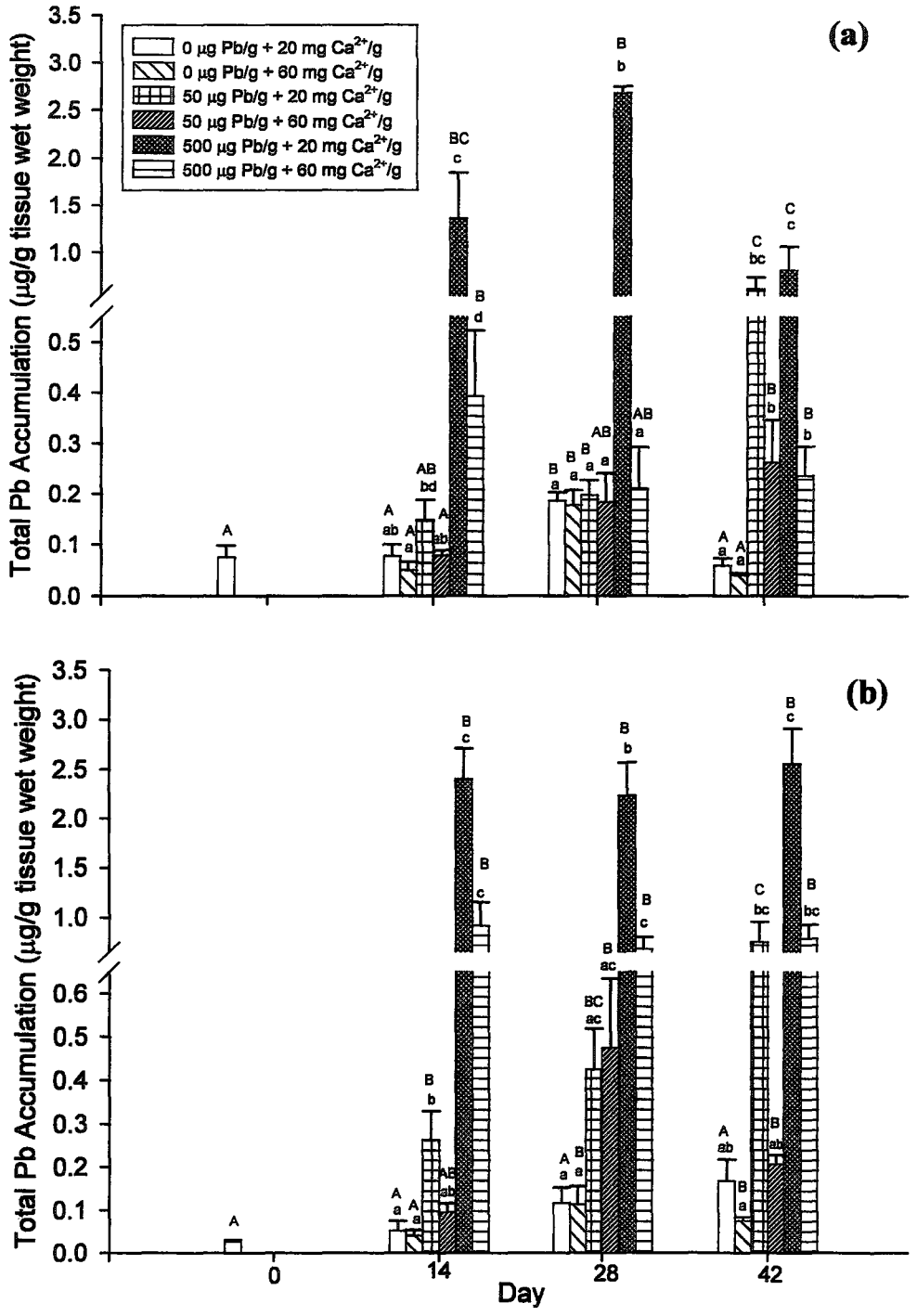

(a)
(b)
0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
Total Pb Accumulation (µg/g tissue wet weight)
Day
0
14
28
42

Figure 3-7: Lead accumulation in the liver of juvenile rainbow trout over 42 days. Other details as in legend of Figure 3-2.

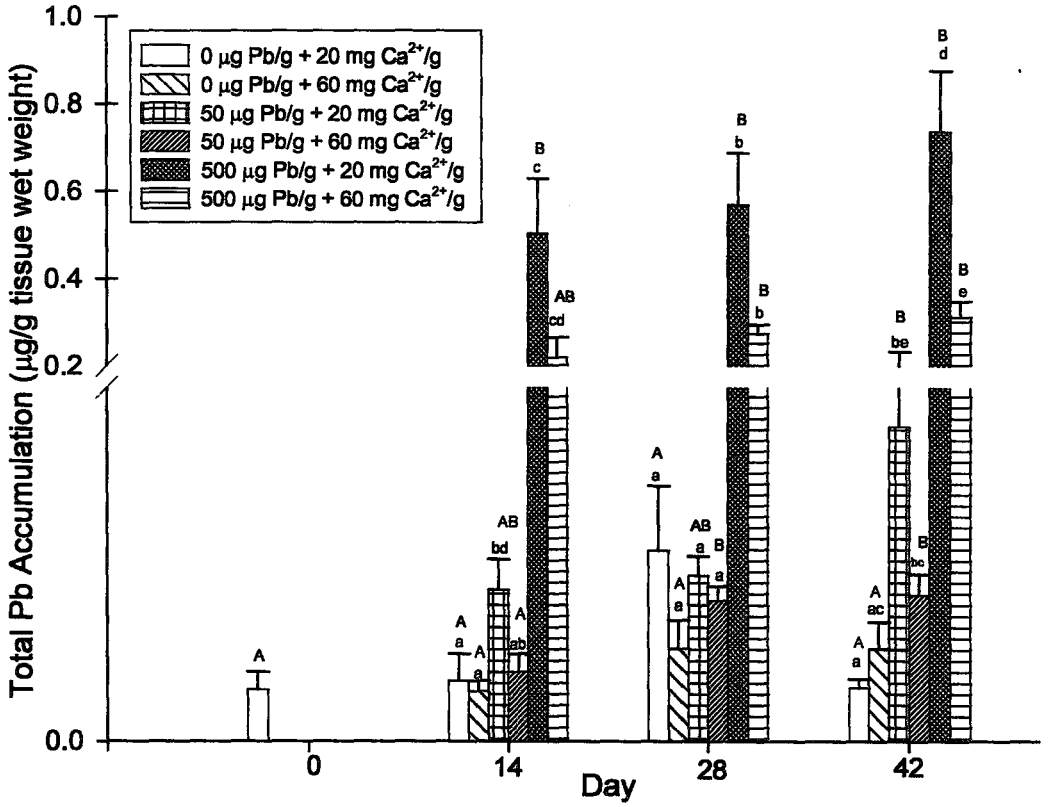

0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
Total Pb Accumulation (µg/g tissue wet weight)
1.0
0.8
0.6
0.4
0.2
0.0
0
14
28
42
Day

Figure 3-8: Lead accumulation in the bone of juvenile rainbow trout over 42 days. Other details as in legend of Figure 3-2.

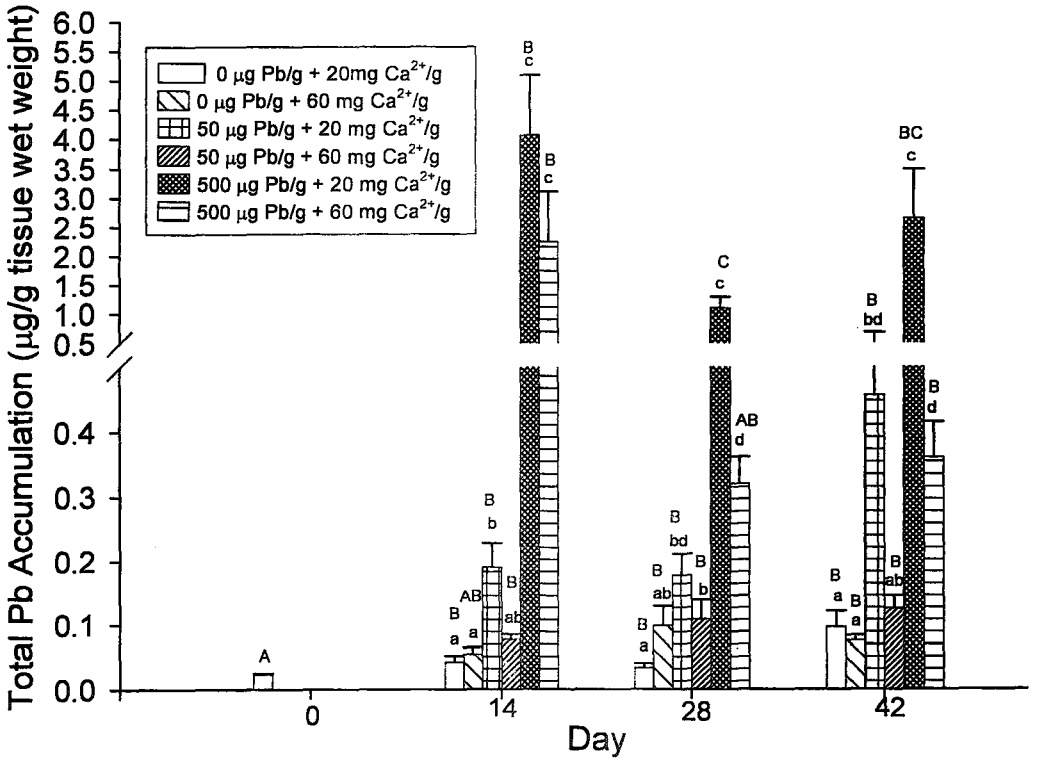

Total Pb Accumulation (µg/g tissue wet weight)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.4
0.3
0.2
0.1
0.0
0 µg Pb/g + 20mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
0
14
28
42
Day

Figure 3-9: Lead accumulation in the carcass of juvenile rainbow trout over 42 days. Other details as in legend of Figure 3-2.

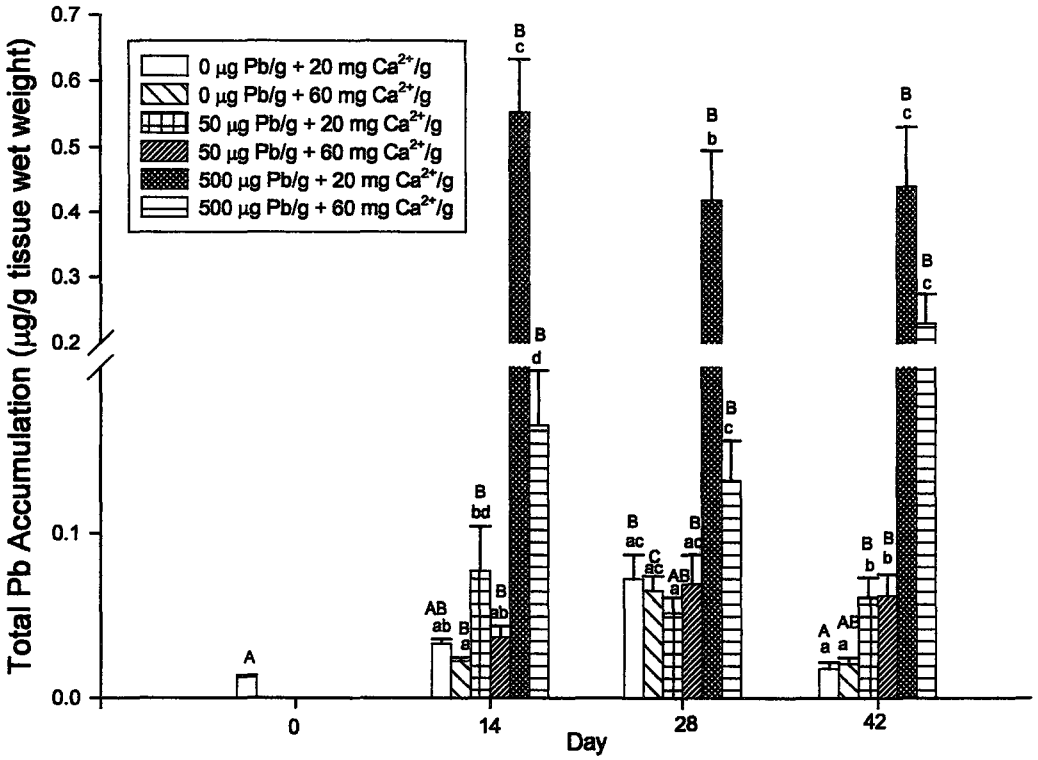

Total Pb Accumulation (µg/g tissue wet weight)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
0
14
28
42
Day

Figure 3-10: Lead accumulation in the red blood cells of juvenile rainbow trout over 35 days. Data reported as mean ± 1; N=6. Other details as in legend of Figure 3-2.

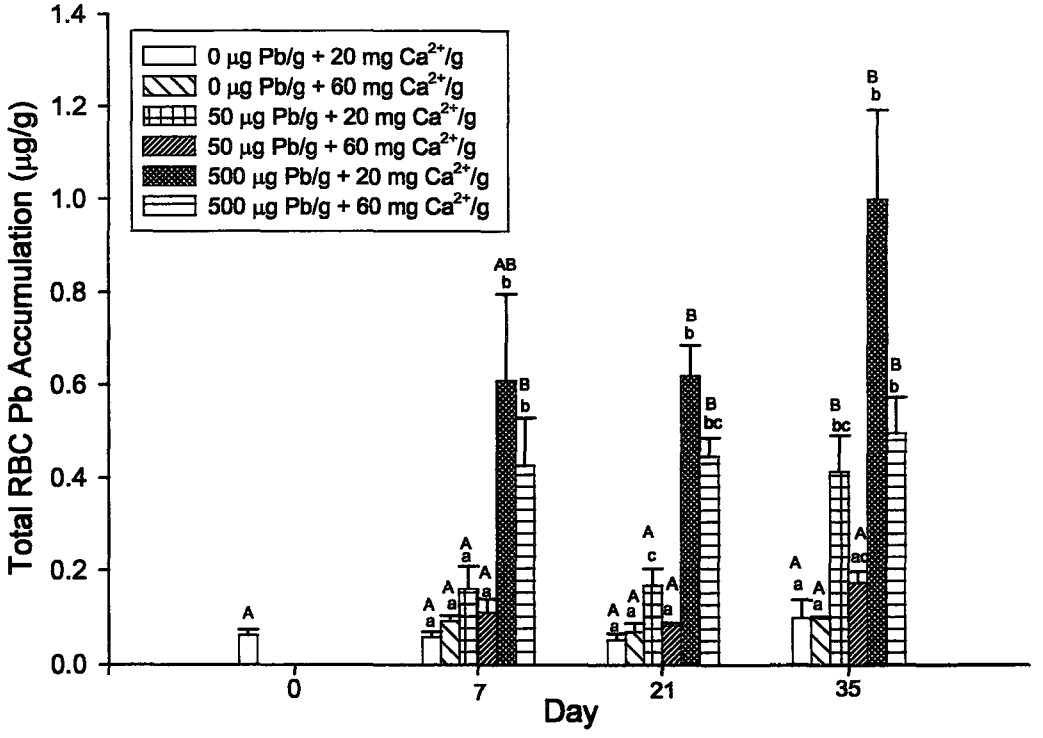

0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
Total RBC Pb Accumulation (µg/g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
21
35
Day

Figure 3-11: Regression between linear ALAD activity (on a linear scale) and log blood Pb concentration on day 42. Each symbol represents a fish in an individual treatment (N=5-8 per treatment). Not enough blood was available for some of the ALAD and Pb burden assays, thus explaining the low N value in some treatments. Solid line represents the linear regression, while the dashed lines represents the upper and lower 95% confidence intervals.

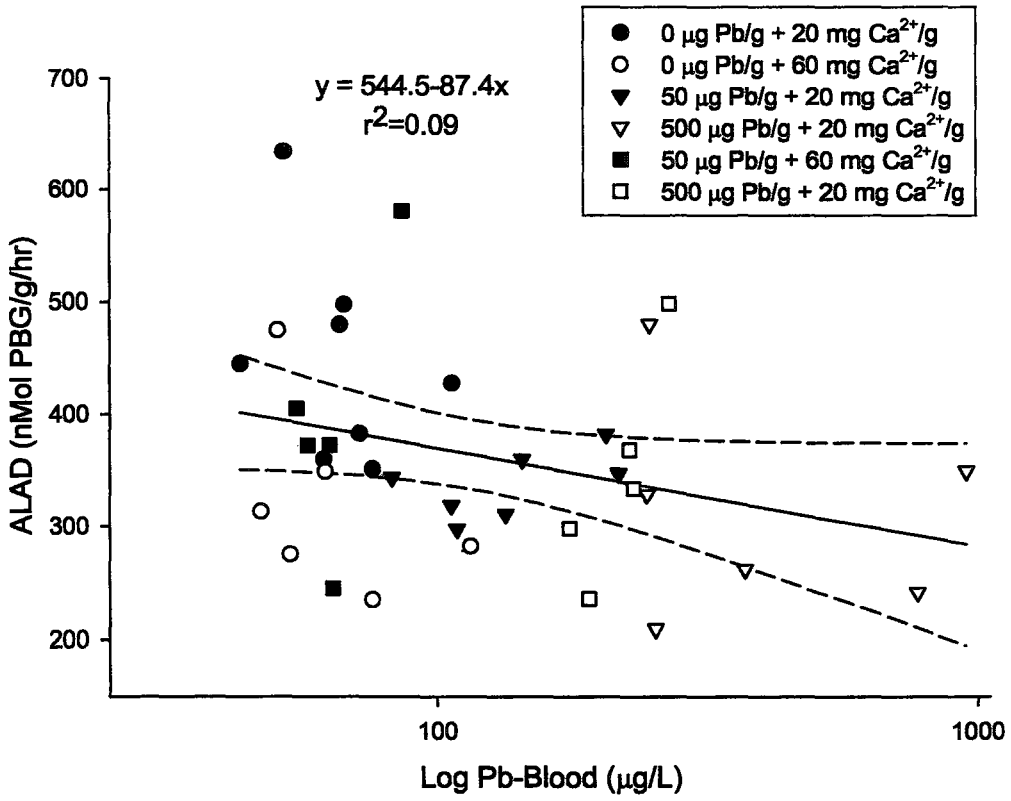

y = 544.5-87.4x
r2=0.09
0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
50 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
ALAD (nMol PBG/g/hr)
700
600
500
400
300
200
100
1000
Log Pb-Blood (µg/L)

Figure 3-12: Na^+, K^+- ATPase activity in the gills and intestine on day 35.

Data represented as mean ± 1 SEM; N=6. Lower case letters represent significant differences between treatments within the same tissue. Values sharing the same letters indicates no significant differences. Values not sharing the same letters indicates significant differences.

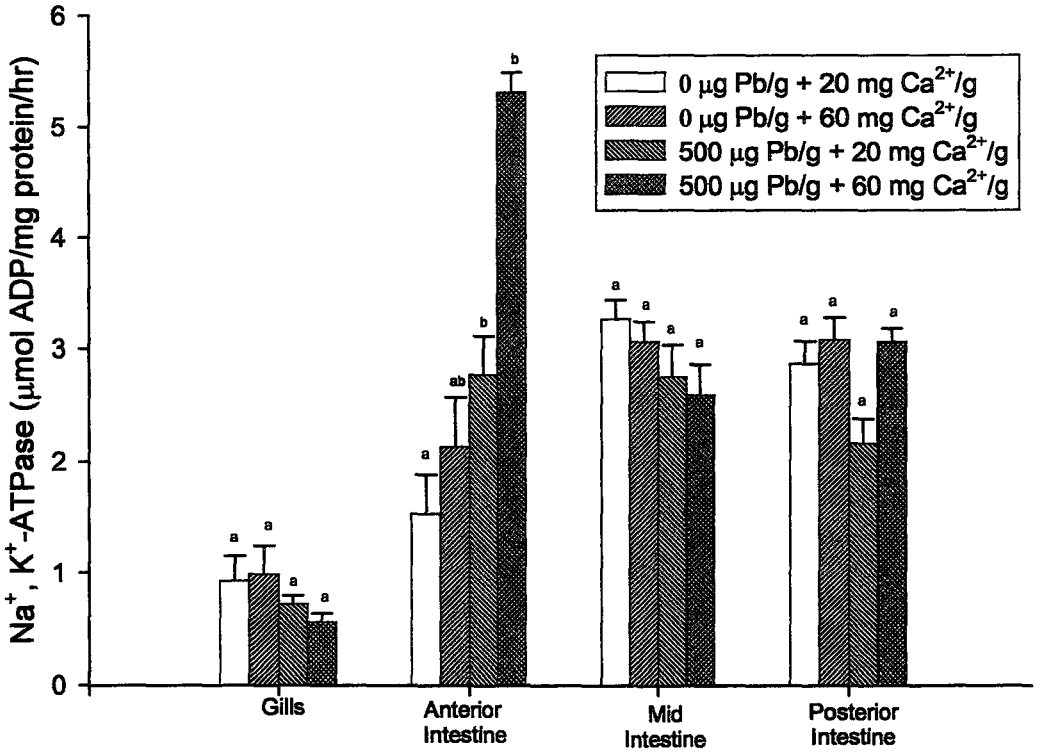

Na+, K+-ATPase (µmol ADP/mg protein/hr)
0
1
2
3
4
5
6
0 µg Pb/g + 20 mg Ca2+/g
0 µg Pb/g + 60 mg Ca2+/g
500 µg Pb/g + 20 mg Ca2+/g
500 µg Pb/g + 60 mg Ca2+/g
a
a
a
a
a
ab
b
b
a
a
a
a
a
a
a
a
Gills
Anterior Intestine
Mid Intestine
Posterior Intestine

Figure 3-13: Percentage of accumulated tissue Pb burden explained by Pb within the blood of juvenile rainbow trout following 42 days. Calculations for the determination of trapped blood within each tissue are explained in the section: *Determination of Tissue Pb Burdens Due to Trapped Red Blood Cells (RBC).* The anterior intestine, mid intestine, posterior intestine, bone, stomach, and muscle had <1% of their Pb burdens explained by trapped blood within the tissue.

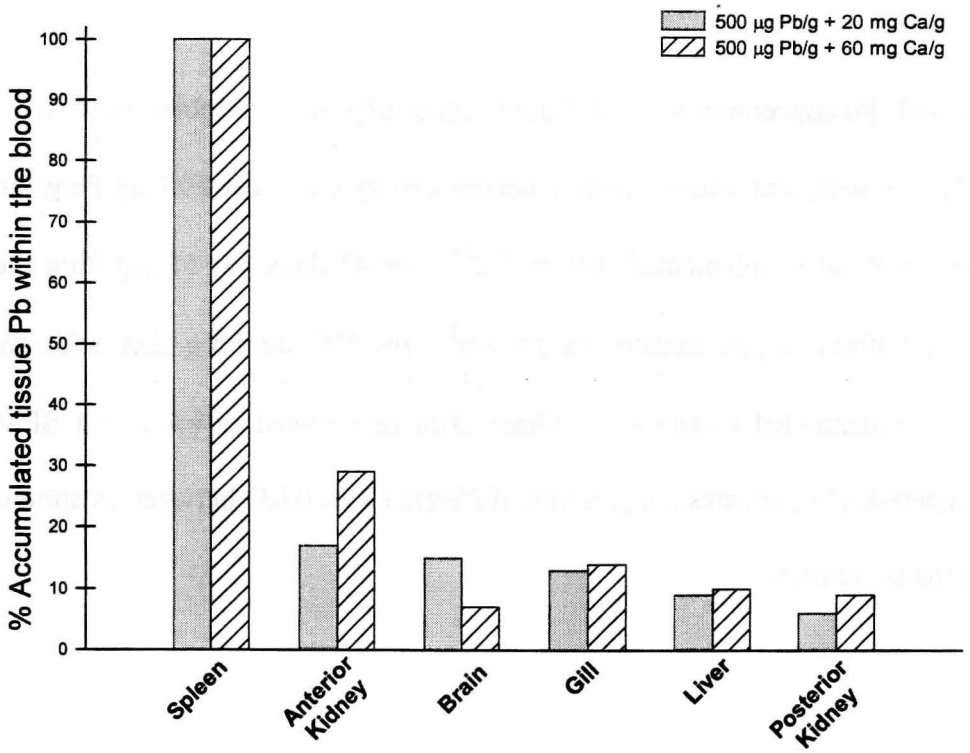
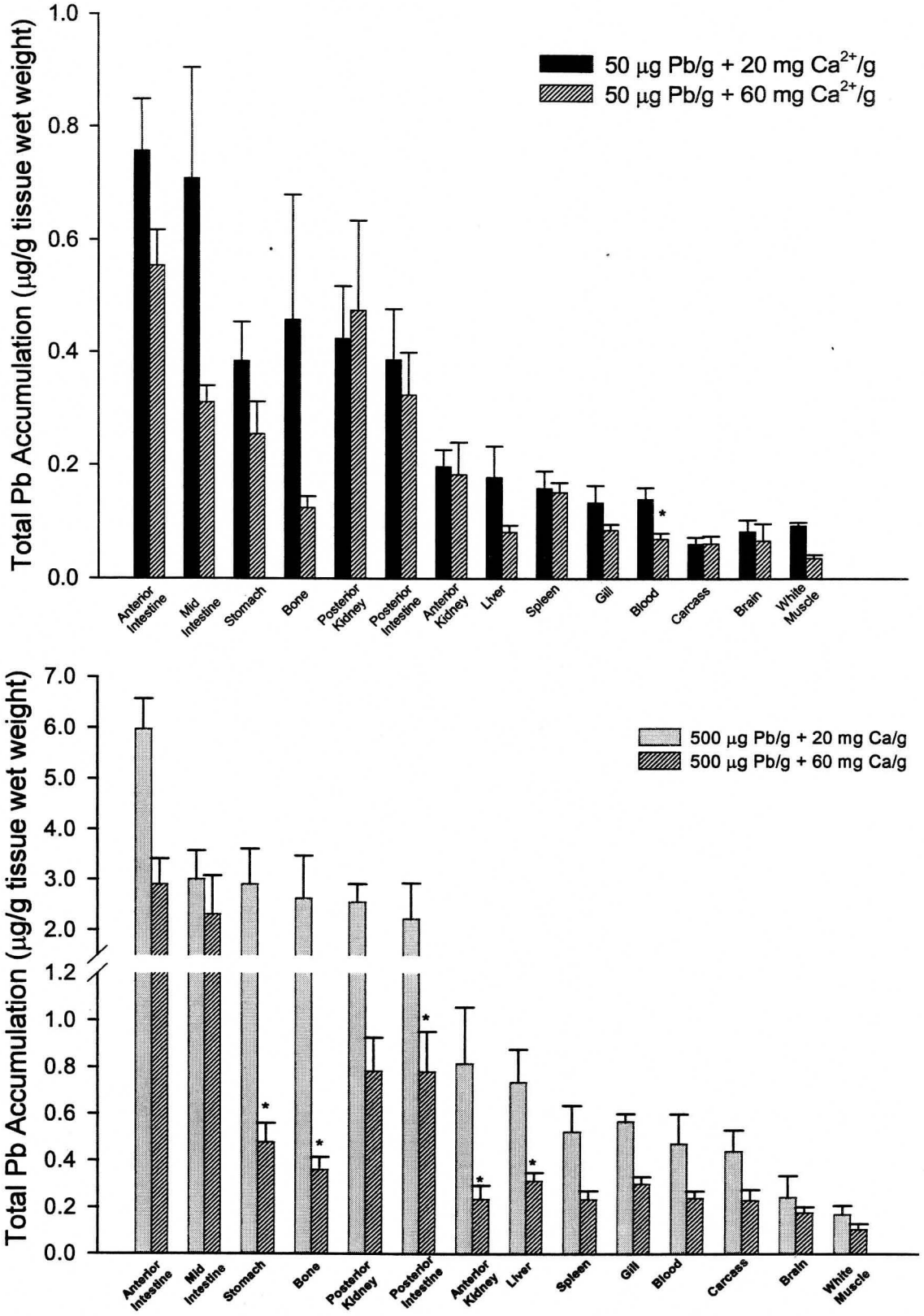

500 µg Pb/g + 20 mg Ca/g
500 µg Pb/g + 60 mg Ca/g
% Accumulated tissue Pb within the blood
100
90
80
70
60
50
40
30
20
10
0
Spleen
Anterior Kidney
Brain
Gill
Liver
Posterior Kidney

Figure 3-14: Pb concentrations in different tissues of juvenile rainbow trout fed 50 μg Pb/g diet with and without supplemented dietary Ca^{2+} and 500 μg Pb/g diet with and without supplemented dietary Ca^{2+} over 42 days. (a) 50 μg Pb/g diet with and without supplemented dietary Ca^{2+}. (b) 500 μg Pb/g diet with and without supplemented dietary Ca^{2+}. Other Data represented as mean ± 1 SEM; N=8. Asterisk (*) indicates a significant difference ($P< 0.05$) between treatments within the same tissue.


Total Pb Accumulation (µg/g tissue wet weight)
1.0
0.8
0.6
0.4
0.2
0.0
50 µg Pb/g + 20 mg Ca2+/g
50 µg Pb/g + 60 mg Ca2+/g
*
Anterior Intestine
Mid Intestine
Stomach
Bone
Posterior Kidney
Posterior Intestine
Anterior Kidney
Liver
Spleen
Gill
Blood
Carcass
Brain
White Muscle
Total Pb Accumulation (µg/g tissue wet weight)
7.0
6.0
5.0
4.0
3.0
2.0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
500 µg Pb/g + 20 mg Ca/g
500 µg Pb/g + 60 mg Ca/g
*
Anterior Intestine
Mid Intestine
Stomach
Bone
Posterior Kidney
Posterior Intestine
Anterior Kidney
Liver
Spleen
Gill
Blood
Carcass
Brain
White Muscle

CHAPTER 4

SUMMARY OF RESULTS AND CONCLUSIONS

As indicated in Chapter 1, the intestine of the teleost serves a multifunctional purpose in terms of the digestion of food, absorption of nutrients, elimination of undigested food, and in marine teleosts, water balance and osmoregulation. The intestine is vulnerable to chemicals and toxicants such as Pb via the diet. In recent years there has been an emphasis on understanding the mechanisms of uptake and physiological disruptions in fish exposed to acute waterborne Pb at the gills, with little research on the effects of sublethal chronic exposure to Pb via the diet. Present studies in this thesis have shown that the intestine is likely the primary target tissue for potential sublethal chronic dietary Pb toxicity of freshwater juvenile rainbow trout exposed to dietary Pb. The high Pb accumulation in many of the internal tissues reflected the exposure pathway in fish fed elevated dietary Pb (500 μg Pb + 20 mg Ca^{2+}/g) after 42 days: anterior intestine > mid intestine > stomach> bone > posterior kidney > posterior intestine > anterior kidney > liver > spleen >gill > carcass > brain > white muscle (per μg Pb/g tissue wet weight). The anterior intestine had the highest Pb accumulation on a per tissue wet weight basis, while the bone had the greatest Pb accumulation on a per fish wet weight basis when compared to other tissues indicating that the bone may be involved in the detoxifying or storage of excess Pb in fish. It was shown that the stomach accumulated dietary Pb, despite the popular belief that hydrolytic metal ions such as Pb are not able to be absorbed by the stomach, due

to the stomach's thick layer of pH-buffered mucus. The posterior kidney accumulated much more Pb than the anterior kidney, probably due to the posterior kidney's function in excretion and Ca^{2+}-reabsorption. In addition, in most of the tissues (stomach, anterior intestine, posterior intestine, posterior kidney, spleen, and carcass), Pb burdens remained stable during the duration of the experiment, suggesting that some sort of homeostatic regulation of Pb had taken place. This was evident in the relationship between Pb accumulation and Pb dose in the diet of Chapter 3. Despite there being a 10-fold difference between the two elevated Pb levels (500 versus 50 μg Pb/g), there was a generally less than 10-fold difference between Pb accumulations in the individual tissues and whole body.

Although dietary Pb exposure did not affect growth rates, or alter hematological parameters in terms of total blood protein, plasma protein, hematocrit, Zn or hemoglobin levels, ALAD activity was inhibited after 42 days in fish fed high dietary Pb diets (500 ug Pb/g + 20 mg Ca^{2+}/g) when compared to the controls. In addition, the longer the exposure and the higher dietary Pb concentrations, the more elevated were RBC Pb burdens when compared to the controls. Virtually all (98-99%) of the Pb blood burden was found in the RBCs with insignificant amounts in the plasma (1-2%). These results suggest that the RBCs serve as a reservoir and transport mechanism of Pb in the trout. In contrast to waterborne Pb studies, the present studies show that chronic growth rates and ALAD activity are not sensitive indicators of Pb toxicity in fish exposed to chronic sublethal concentrations of dietary Pb.

Mild physiological disturbances were evident in terms of increased plasma Na^+ and Na^+ influx rates from the water, and decreases in plasma Ca^{2+}, and Mg^{2+}. The increase in plasma Na^+ were consistent with the up-regulation of Na^+, K^+-ATPase activity at the anterior intestine observed in the presence of high dietary Pb levels. Overall, these results suggest that a mechanism of dietary Pb toxicity in the rainbow tout is a mild ionoregulatory disruption.

Research in Chapter 3 demonstrated that elevated dietary Ca^{2+} (60 mg Ca^{2+}/g, a 3 fold increase from background dietary Ca^{2+} levels) had an overall protective effect in reducing tissue whole body Pb burden by 61-78 % of fish fed high dietary Pb (500 μg Pb/g). Elevated dietary Ca^{2+} had the greatest protective effect on the stomach, bone, anterior kidney, posterior intestine and liver, and the least effect on the gills, anterior intestine, mid intestine, posterior kidney, carcass, spleen, white muscle, and brain in fish fed the high Pb, high Ca^{2+} diets. Elevated dietary Ca^{2+} was also protective in reducing whole body Pb burdens on all days in fish fed the high Pb, high Ca^{2+} diets when compared to the high Pb, low Ca^{2+} diet.

Although this research has not proven that Pb shares a similar pathway to that of Ca^{2+} in certain tissues, it does however, suggest that a similar pathway may be present, based on the inhibitory effects of elevated dietary Ca^{2+} on dietary Pb burdens in the intestine, kidney, and bone. This research has also focused attention on the potential ecological importance of elevated dietary Ca^{2+} levels in fish living in soft water and/or contaminated Pb environments. Fish in these environments may need to choose diets that are enriched with Ca^{2+} such as

mollusks and crustaceans not only to maintain Ca^{2+} ion regulation that may be disturbed by waterborne and dietary Ca^{2+}, but to protect against sublethal chronic Pb toxicity. The same is true in aquaculture where the negative impacts of contaminated Pb in the aquatic environment can be prevented and avoided by feeding fish diets elevated in Ca^{2+}. The protective effects of elevated dietary Ca^{2+} becomes important in new environmental regulations and approaches to Pb toxicity (e.g. Biotic Ligand Model) that require the understanding of the influence of dietary status on Pb accumulation in order to mitigate the toxic effects of dietary Pb along the food chain.

This thesis has set a foundation for further research in terms of investigating the mechanisms of Pb transport via the intestine and Pb transport into the RBCs. Further research should also address the role of gut pH and digestive enzymes in Pb uptake and in Pb toxicity and bioavailability. It is the hope that the findings of this thesis will be incorporated into future regulations that take into consideration not only the effects of metals in the water, but metals present in the diet.

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