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The Dynamics of Chronically Bioaccumulated Cd in Rainbow Trout (*Oncorhynchus mykiss*) During Both Moderately Hard and Soft Waterborne Exposures

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by

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Abstract

Aquatic organisms respond to metal exposures by modulating uptake, elimination and detoxification capacity and, therefore, it is difficult to link accumulation within fish to adverse chronic effects. When fish are exposed to sublethal concentrations of metal, physiological responses follow a damage-repair scenario with rapid uptake into the gills followed by transfer via the circulatory system and accumulation into key organs such as the liver and kidney. Anthropogenic sources of cadmium (Cd) arise mainly from mining, smelting, and refining sulphide ores of Zn, Pb and Cu (Norton et al., 1990). The free divalent ion (Cd^{2+}) is the toxic form of Cd in water. Cd^{2+} competes with calcium ions (Ca^{2+}) for binding sites and transport channels across the basolateral membrane of the gill. Ambient Ca is the primary cation responsible for reduced Cd toxicity to species of trout, during both acute and chronic waterborne exposures. The objective of this research was to examine the dynamics of chronically accumulated Cd within tissues of rainbow trout (*Oncorhynchus mykiss*) during chronic (one month) sublethal waterborne exposures to Cd (0.75 and 2.0 μg/L) in both moderately hard (140 mg/L as $CaCO_3$) and moderately soft (50 mg/L as $CaCO_3$) water. Accumulation was assessed on a tissue (gills, liver and kidney) and subcellular levels. Tissues accumulated Cd in a time- and dose- dependent manner. Results indicate that fish exposed to sublethal Cd concentrations in moderately soft water attained higher amounts of Cd in gills, and more Cd bound to metal-sensitive fractions (mitochondria and proteins) in all tissues in comparison to fish exposed to Cd in moderately hard water. Early mortality and ion-loss may link to accumulation in metal-sensitive fractions (particularly within the gills). However, Cd concentrations in metal-sensitive fractions continued to rise throughout both sublethal exposures suggesting damage-repair mechanisms other than metallothionein are induced, or threshold Cd concentrations in tissues and metal-sensitive fractions associated with adverse effects have not been exceeded. The kidney appeared to be most capable organ to offer protection from Cd accumulation in metal-sensitive fractions, as Cd dissipated from metal-sensitive fractions with time and dose. Also kidneys displayed the highest capacity to induce detoxification of Cd by metallothionein.

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Chapter 1

Introduction

1.1 Cd in the Aquatic Environment

Metals in aquatic environments originate from many anthropogenic and natural sources. Metals are found everywhere in the surface environment, usually at trace concentrations. Natural sources of Cd generally arise from volcanic emissions, inputs from erosion and weathering of rocks, specific events such as forest fires as well as cosmic fallout (Thornton 1983; Dias and Edwards 2003; Nordic Council of Ministers, 2003). All of these natural sources have the potential to enter aquatic environments through terrestrial run-off and atmospheric deposition (Thornton 1983; Dias and Edwards 2003) These emissions are involved in the global Cd cycle, but generally do not result in high concentrations within the environment (Nordic Council of Ministers, 2003). Anthropogenic releases of Cd occur as a result of the mining, smelting, and refining of sulphide ores of zinc (Zn), lead (Pb) and copper (Cu) (Norton et al., 1990). The emissions from such processes may enter aquatic environments directly as effluents and/or through atmospheric deposition (Boudou and Ribeyre, 1997; Canadian Council of Ministers of the Environment (CCME), 1999; Dias and Edwards, 2003). Other industrial applications of Cd include nickel Cd batteries, pigments, PVC stabilisers, plating and alloys. Disposal of Cd containing product/material into waste streams accounts for major releases of Cd into the environment (Nordic Council of Ministers, 2003).

The concentration of trace-metals (including Cd) in sediment has been shown to be an important measure of metal contamination, and can be used to assess historic metal contamination over long periods of time (Keller et al., 1992). Metal concentrations in both water and sediment have been shown to be elevated immediately downwind of point sources (for example, downstream from smelter stacks). These concentrations decrease with increasing distance from the source (Keller et al., 1992).

1.2 The Influence of Water Chemistry on Cd Bioavailability

Throughout aquatic environments, Cd may be present in solution, in or on suspended particles and in sediments. Within each of these phases, Cd is found in different chemical forms or species (e.g. $CdCl_2$, $CdCO_3$, CdS, $Cd(NO_3)_2$) that can alter the bioavailability at uptake sites (e.g. gills) of fish (Boudou and Ribeyre, 1997). Water chemistry from site to site will differ as it is strongly influenced by the underlying

geology and watershed characteristics, including the type of sediment, vegetation and industrial developments present. The formation of inorganic and organic Cd complexes alters the proportion of free Cd^{2+} so that it has reduced availability to the uptake sites on fish gills, the Ca^{2+} transporters (Playle 1993a; Niyogi et al. 2008). Certain water chemistry variables (e.g. dissolved organic carbon, particulate matter and sulphur containing compounds) can influence the concentration of freely available Cd ions in the water due to complexation effects, while others (e.g. hardness (Ca^{2+}, Mg^{2+}), Na^{+}) may compete with Cd uptake at the gills (Fig. 1.1).

Water hardness (particularly calcium concentrations) is often regarded as the most influential factor regarding the sensitivity of fish to Cd (Playle et al., 1993b; Hollis et al., 2000b). The bioavailable form of Cd is the free divalent ion Cd^{2+} that resembles the divalent calcium ion (Ca^{2+}) in terms of radius (R-Ca = 0.99Å; R-Cd = 0.97 Å), 2^{+} charge and negative reduction potentials, thus it is thought to compete with Ca-binding sites (i.e. Ca^{2+} channels and ATPase pumps) on the gill (Verbost et al., 1988, 1989; Playle, 1993b; Niyogi and Wood, 2004) (Fig. 1.1). Futhermore, the binding affinity of Cd to Ca-binding sites (e.g. ion channels, transport proteins, ion-exchangers) located in fish gills is greater, which facilitates the displacement of Ca by Cd, competitively inhibiting Ca transport (Verbost et al., 1987; 1988; 1989). Thus free ionic Cd^{2+} induces acute toxicity in freshwater fish by causing hypocalcaemia. During both acute and chronic Cd exposures, branchial ion disruption ensues due to a reduction in the influx of essential elements such as Ca^{2+} and Na^{+}, and inhibition of branchial ATPase activities (Na^{+}/K^{+} and Ca^{2+}) (Verbost et al., 1989; Pratap and Wandelaar Bonga, 1993). ATPases are responsible for transporting essential nutrients across the basolateral membrane of the gill into the fish's internal environment (Verbost et al., 1988; 1989) (Fig 1.1). Ca^{2+} is an essential element in fish, thus increased aqueous Ca^{2+} ions compete with the harmful Cd^{2+} ions to reduce uptake of potentially toxic metals at the gill (Wood et al., 2006; Niyogi and Wood, 2004; Niyogi et al., 2008). For example, Hollis et al. (2000b) have shown that mortality and tissue accumulation increased as Ca^{2+} concentrations decreased throughout a 30 day exposure to 2 μg/L of Cd. Many studies have shown a relationship between Cd^{2+} and Ca^{2+} uptake and

regulation from both diet and aqueous sources (Zahouri et al., 2001; Baldisserotto et al., 2004; Hollis et al., 2000; Wood et al., 2006). The protective effect of increased Ca^{2+} ions and Cd^{2+} toxicity explains the mitigating effects of water hardness on the toxicity of Cd (Allen, 2002).

1.3 Tissue Accumulation of Cd

When fish are exposed to sublethal Cd concentrations, initial damage and accumulation occurs at the primary site of uptake, the gills (McDonald and Wood, 1993; Niyogi et al., 2008). The damage phase is usually followed by a repair phase as essential element balance is restored and ATPase activity is increased (McDonald and Wood, 1993). One mechanism involved during the repair phase is the internal mobilization of metal binding proteins, such as metallothionein, that act to detoxify and store Cd (Roch et al., 1986; Deeds and Klerks, 1999; Rose et al., 2006). However, Cd will continue to be taken up via essential element pathways (e.g. Ca^{2+}) and be subsequently distributed through the internal environment of the organism.

Cd has no essential role in cellular metabolism, thus specific mechanisms for internal regulation do not exist. However, it has been proposed that the main strategies of regulation for essential metals can be utilized for the non-essential metals, including excretion and internal storage without elimination (Vijver et al., 2004). Accumulation of Cd by fish typically increases with increasing exposure concentrations in water (Spry and Wiener, 1991). Accumulation of Cd displays a pattern of loading onto the gills, followed by distribution to internal tissues (McGeer et al., 2000b; McGeer et al., 2007). Once Cd enters the bloodstream, the levels and distribution of the metal will depend on a variety of factors including blood distribution, tissue specific uptake processes and detoxification mechanisms. Usually accumulated Cd will be slowly cleared from tissues (Wicklund Glynn, 1991). After the gills, fish accumulate Cd primarily within the kidney along with substantial concentration increases within the liver. Cd does not appreciably accumulate in muscle tissue of fish (Norey et al., 1990; Harrison and Klaverkamp, 1989; Karaytug et al. 2007). Cd tissue accumulation, particularly in the kidney, has been

proposed as an indicator of chronic waterborne Cd exposure because this organ generally bioaccumulates Cd to elevated levels (McGeer et al., 2000b) and is slowly eliminated (Hogstrand and Haux, 1991). Chronically exposed fish returned to Cd-free water retain elevated Cd concentrations in the kidney, which demonstrates the importance of the kidney as a storage organ (Benoit et al., 1976; Kumada et al., 1980; Wicklund Glynn, 1991).

Cd accumulation within tissues (e.g. gills, liver and kidney) is a dynamic, time- and dose-dependent process characterized by an initial phase of increasing tissue concentration that gradually reaches stabilization, reflecting the pattern of damage, recovery, and acclimation with chronic metal exposure (McGeer et al., 2000b). McGeer et al. (2000b) demonstrated that a 100 day Cd exposure (3 μg Cd/L) with rainbow trout (*Oncorhynchus mykiss*) in moderately hard water showed that the half-time to saturation of Cd within the liver and kidney takes longer in comparison to essential metals like Cu (less than two weeks) which was suggestive of active Cu regulation.

Cd concentrations within tissue of fish have been examined in attempt to link long-term metal exposure to toxic effects (Bergman and Dorward-King, 1997). Studies of invertebrates (Borgmann et al., 2004; Ma, 2005) and fish (Marr et al., 1996; Hansen et al., 2002a) demonstrate links between metal accumulation and chronic toxicity across a range of conditions. These studies suggest that accumulation rate and concentrations of accumulated metal are important determinants associated with the development of a toxic response. This pertains to the basic understanding of bioaccumulation kinetics and physiological responses to metal exposure. For example, a relatively short-term exposure to a high concentration of Cd might result in rapid accumulation of Cd in sensitive tissue compartments allowing for minimal time to initiate an appropriate detoxification response (Meador et al., 2010). This in turn may also lead to a toxic response that will be linked with a relatively low tissue concentration. A long-term exposure to a lower concentration of Cd might result in continuous accumulation of Cd in sensitive compartments but with an induction of a sufficient detoxification response (Meador et al., 2010). This may not lead to the same toxic response despite resulting in tissue concentrations that are significantly higher than those attained in the short term higher concentration exposure. Therefore, associating Cd

concentrations in tissues with adverse effects must be interpreted with caution as there are several factors, such as internal detoxification, influx and efflux rates that may cause uncertainty regarding tissue residues as an indicator of toxicity (Meador et al., 2010). In general, metal accumulation in fish is complex and depends on the specific mechanisms of uptake, regulation and elimination which vary among species, tissues and metals.

1.4 Subcellular Distribution of Cd

Within tissues, Cd can enter cells and bind to physiologically sensitive target molecules (small peptides, enzymes, RNA or DNA) and these can be in organelles (mitochondria, nuclei, endoplasmic reticulum) and some of this binding may induce damage (Giguère et al., 2005). A variety of detoxification and storage mechanisms within fish help to immobilize Cd including; binding to metal binding proteins such as metallothionein and to a lesser extent, granule sequestration (Campbell et al., 2005, 2008; Kamunde and MacPhail, 2008; Kamunde, 2009). Metallothionein has been identified as one of the mechanisms for cellular sequestration of Cd employed by fish in response to increased ambient Cd (Kraemer et al., 2005; Campbell et al., 2008; Roch et al., 1985; Olsson and Hogstrand, 1987). Binding to metallothionein reduces the amount of freely available Cd in cells, thereby decreasing toxicity (Roesijadi, 1996). The induction of metallothionein has been proposed by many studies as a biomarker for metal exposure (Roch et al., 1986; Deeds and Klerks, 1999; Rose et al., 2006)

Subcellular Cd partitioning in fish species has received increasing attention as it provides valuable information on metal toxicity and bioavailability (Campbell et al., 2008). Furthermore, pattern of subcellular Cd distribution during chronic exposure in fish remains largely unexplored. The majority of studies (Wallace et al., 2003; Campbell et al., 2005; Kraemer et al., 2005; Wang and Rainbow, 2006) on the subcellular distribution of metal have divided metal accumulation in organisms into five subcellular compartments; metal-rich granules (MRG), cellular debris, organelles (mitochondria, microsomes and lysosomes), heat-sensitive proteins (HSP), and metallothionein-like proteins (MTLP). Studies have grouped subcellular compartments into metal-sensitive pools (MSP) verses metabolically detoxified

(inactive or insensitive) pools (MDP) (Campbell et al., 2005, 2008; Kamunde and MacPhail, 2008; Kamunde, 2009). The MSP includes mitochondria and HSP, whose functions may be impaired by Cd accumulation, leading to toxicity (Rainbow, 2002; Campbell et al., 2008). The MDP includes MTLP, and MRG (Campbell et al., 2008), and for the purpose of this study, MDP will also include the combined microsomes + lysosomes fraction (ML) since studies examining metal partitioning in aquatic invertebrates have shown that lysosomes can form detoxified concretions (George et al., 1982; 1983; Sterling et al., 2007; 2010) and may be associated with metallothionein-metal degradation in both invertebrates and vertebrates (Cousins, 1979; Bremner, 1991; Nassiri et al., 2001; Amiard et al., 2006). The consequence, if any, of metal accumulation in cellular debris (mostly cell fragments) is poorly understood.

Metal accumulation in tissues of fish is dependent upon the rate of uptake, storage and elimination. Generally, internal storage of metal occurs in two compartments, which include biologically in-active metal (BIM) and biologically active metal (BAM). The BIM is associated with detoxified metal whereas the BAM is associated with metal-sensitive fractions which may link to adverse effects when excessive metal accumulation ensues. The internal distribution and intracellular trafficking of metal is dynamic and changes may occur rapidly which may or may not result in a toxic response. The current working hypothesis for subcellular accumulation into metal-sensitive pools is the spill-over hypothesis (Fig. 1.2), whereby below a certain threshold concentration (in tissues and/or exposure concentration) the combined rate of excretion and detoxification mechanisms such as metallothionein and other metal-binding proteins, can limit uptake into metal-sensitive compartments of the cell. However, an increase in bioavailability of metal will cause an increase in uptake rate of that metal into the tissues. If this uptake rate exceeds the combined detoxification and excretion rate, metal will spill-over into metal-sensitive fractions, concentrations will rise and then exceed the threshold concentration that can be tolerated leading to toxic effects (Rainbow, 2002; Wang and Rainbow, 2006). The concept of spill-over appeared to be validated by earlier studies (Sanders et al., 1983; Sanders and Jenkins, 1984) that examined the relationships between bioavailable Cu in seawater and cytosolic Cu accumulation and distribution in crab

larvae and impacts on growth. Important findings from these studies suggest that larvae could regulate both the accumulation of Cu and the distribution of Cu within the cell, as concentrations in HSP remained unchanged whereas increases in Cu associated with both MTLP were observed over the entire range of Cu concentrations tested. However, studies (e.g. Jenkins and Sanders, 1986) have not confirmed regulation of Cd by other aquatic invertebrates (e.g. polychaetus annelids) and suggest that ability to regulate subcellular metal distribution can depend on differences between the metabolisms of essential and nonessential metals or are species specific (Rainbow, 2002). Therefore, there remains some uncertainty concerning the dynamics of metal accumulation within subcellular pools and linkages to effects.

Recent data using fish as the test organism (Kraemer et al., 2005; Giguère et al., 2006; Campbell et al., 2005, 2008; Kamunde and MacPhail, 2008), suggest that this hypothesis is too simplistic and may not fully explain toxicity. Studies in the field with juvenile yellow perch (*Perca flavescens*) along a Cd gradient, have shown that chronic exposures at low concentrations of Cd accumulated in liver metal-sensitive fractions (particularly HSP), and did not find evidence of a threshold concentration that exists whereby Cd did not accumulate in metal-sensitive compartments (Giguère et al., 2005; Campbell et al., 2008; Kraemer et al., 2005). These studies have concluded that metal detoxification was imperfect and did not confirm to predictions of the spill-over hypothesis. It should be noted that the majority of studies have only examined subcellular partitioning of Cd within the liver, which is not the main storage organ for Cd accumulation in fish during chronic exposures. In addition to field exposures, a recent study by Kamunde (2009) exposed juvenile rainbow trout (*Oncorhynchus mykiss*) for 96 hrs to three Cd exposures (5, 25 and 50 μg/L). The results revealed that the accumulation pattern over time was linear in the liver and biphasic (characterized by initial rapid accumulation followed by saturation and/or decline) in the gill. Generally, the pattern of accumulation within subcellular fractions of the gill and liver was similar in comparison to total tissue accumulation patterns of each respective organ. However, the majority of Cd accumulated in both HSP and MTLP fractions. Since Cd accumulated in potentially metal-sensitive

compartments throughout the duration of the exposure, there was no threshold concentration that prevented spill-over into metal-sensitive compartments.

1.5 Objectives and Hypotheses

Currently, freshwater-quality criteria for aquatic life in water hardnesses ranging from 20 – 120 mg/L as $CaCO_3$ recommends an acute limit of 0.6 – 4.8 μg Cd/L for acute exposures and a chronic limit of 0.3 – 1.3 μg Cd/L for chronic exposures, according to the U.S. Environmental Protection Agency (Thornton, U.S. EPA-822-R-01-001, 2001). These are considerably different to the interim Canadian water quality guideline for Cd for the protection of freshwater life that is established at 0.017 μg Cd/L (CCME, 1999). This study will chronically expose fish to Cd within the salmonid family, particularly *Oncorhynchus mykiss* (rainbow trout) as they are highly sensitive to Cd toxicity in comparison to bull trout (*Salvelinus confluentus*) (Hansen et al., 2002b) and yellow perch (*Perca flavescens*) (Raynal et al., 2005). Nominal concentrations of 0.75 and 2.0 μg Cd/L in combination with water hardness of 50 and 140 mg/L as $CaCO_3$ will be used for our study which is relevant to both U.S and Canadian water quality criteria.

This objective of this research was to characterize the intracellular bioaccumulation of Cd in rainbow trout (*Oncorhynchus mykiss*) during chronic waterborne exposures. Accumulation was studied at both tissue and subcellular levels of organization in order to establish linkages between exposure, uptake, detoxification and accumulation in toxicologically sensitive "pools" within the cell. Examining patterns of tissue and subcellular distribution when rainbow trout (*Oncorhynchus mykiss*) are chronically exposed to sublethal concentrations of waterborne Cd will help determine if tissues (liver, kidney and gills) differ in their detoxification ability to protect Cd from accumulating within sensitive compartments (mitochondria, HSP) of the cell. This in turn may help determine which tissues or fractions should be focused on in order to link accumulation with Cd toxicity. Furthermore, this study will attempt to determine if there is a threshold concentration within each tissue whereby Cd begins to accumulate in sensitive fractions by testing the spill-over hypothesis. This project will also address the protective effects

of Ca^{2+} (a component of water hardness, measured as $CaCO_3$) on bioavailability and accumulation of Cd at both tissue and subcellular organizational levels. Increased ambient Ca^{2+} could offer stronger competition with Cd^{2+} and influence the number of binding sites for Ca uptake and transport, and indirectly Cd uptake at the gill. For the objectives mentioned above, the four working hypotheses related to the response of rainbow trout (*Oncorhynchus mykiss*) to Cd exposure are as follows:

(1) tissue concentrations of Cd within rainbow trout (*Oncorhynchus mykiss*) will increase in a time- and dose-dependent manner

(2) in comparison to liver and kidney, the gills will accumulate the majority of Cd in MSP and will contain lower amounts of Cd bound to detoxified compartments (e.g. due to metabolically active mitochondrial-rich transport cells that take up Cd)

(3) adverse effects (mortality or ion loss) are linked to spill-over of Cd into metal-sensitive fractions (HSP and mitochondria)

(4) Ca^{2+} concentrations in moderately hard water will dampen increases of Cd in tissues in relation to soft water and therefore "protect" against accumulation in metal sensitive compartments

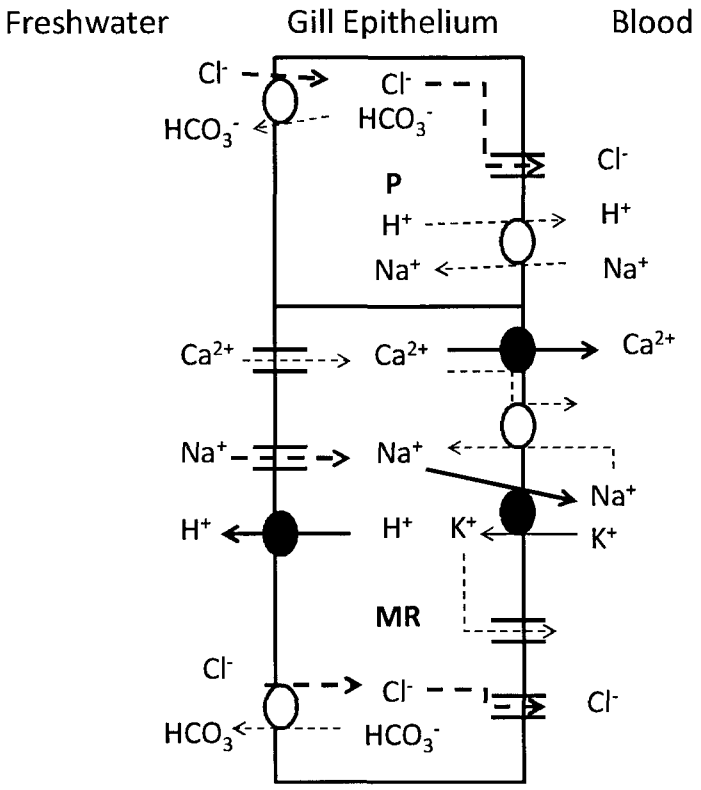

Fig. 1.1 Adapted from Marshall (2002). Model of ion uptake in freshwater teleost fish gills. Uptake involves mitochondrial-rich (MR) and pavement (P) cells. Solid lines indicate active transport whereas dashed lines indicate uptake via diffusion through ion channels or passage across cell membranes. Co-transporters and exchangers are represented by open ovals whereas ATPase pumps are represented by solid circles.

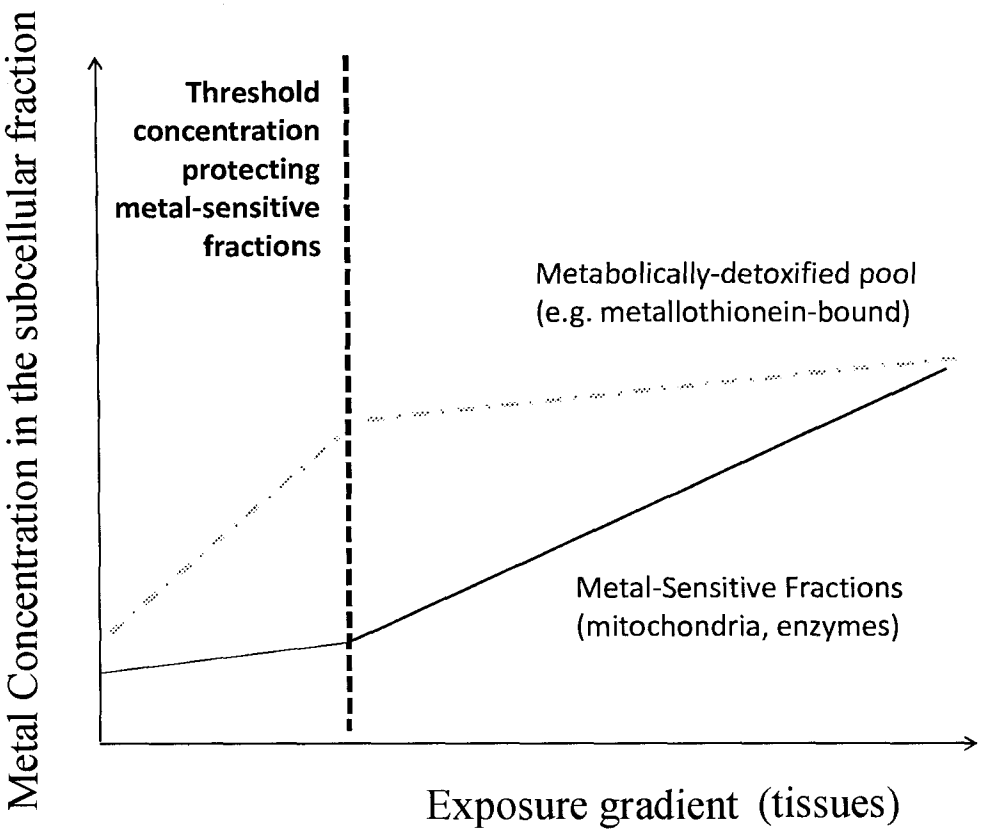

Fig. 1.2 Adapted from Kraemer, 2005. Graphical representation of the spill-over hypothesis that predicts metal detoxification is efficient at low concentrations of metal, and metal-sensitive cellular targets are thus protected from excessive accumulation. Above a threshold exposure concentration (vertical dashed line) the binding capacity of metabolically-detoxified fractions (e.g. metallothionein-bound) becomes saturate and/or overwhelmed (dashed gray line) and metals spill-over into metal-sensitive fractions (solid black line).

Chapter 2

Tissue and subcellular distribution of Cd during chronic sublethal exposure in moderately hard water to rainbow trout (*Oncorhynchus mykiss*)

2.1 Abstract

Aquatic organisms respond to metal exposures by modulating uptake, elimination and detoxification capacity and therefore it is difficult to link accumulation with chronic effects. When fish are exposed to sublethal concentrations of metal, physiological responses follow a damage-repair scenario with rapid uptake into the gills followed by transfer via the circulatory system and accumulation into key organs such as the liver and kidney (McDonald and Wood, 1993). The purpose of this research was to understand the distribution of chronically accumulated Cd within tissues of rainbow trout (*Oncorhynchus mykiss*) in relation to physiological disruption (mortality and plasma ion concentrations). Accumulation was assessed on a tissue (gills, liver and kidney) and subcellular levels. Fish were exposed to two sublethal concentrations of Cd (0.75 and 2.0 μg/L) for 29 days in moderately hard water (140 mg/L as $CaCO_3$, pH 7.2). Acute mortality (20% and 9% for low and high sublethal Cd concentrations) was observed and ceased by day 4. Acute (96 hrs) plasma Na and Ca disruption occurred in both Cd exposure groups and Ca levels in fish exposed to 2.0 μg Cd/L did not recover (1.2-2.2 mM Ca, controls = 2.5-3.2 mM Ca). Cd accumulated in all tissues in a concentration and time dependent manner. At day 29, kidney and gill Cd concentrations were comparable (~1.6-2.5 and 0.8-3.2 μg Cd/g wet tissue for gill and kidney respectively; kidney exceeded gill in fish exposed to 2.0 μg Cd/L) and ~ 2.3 times higher than liver levels for both low and high Cd exposed fish. MTLP and mitochondrial fractions were enriched with Cd and generally contained the highest amount of Cd at day 29, for all tissues. Subcellular partitioning of Cd in all tissues indicated acute spill-over in MSP which coincided with early mortality and reduced plasma ion concentrations. The kidneys appeared to dissipate Cd from excessive accumulation in metal-sensitive fractions (e.g. HSP) with time and exposure. However, accumulation of Cd in MSP was observed in both liver and gills, particularly in the gills, as partitioning of Cd into metal-sensitive fractions (mitochondria and HSP) continued to rise or remained elevated. These results must be interpreted with caution as placing fractions into categories is likely an oversimplification.

2.2 Introduction

Although some metals act as co-factors for many biomolecular processes, in vertebrates, Cd has no essential role in cellular metabolism and can be toxic to aquatic organisms. Effects of chronic exposure to waterborne Cd at sublethal concentrations include tissue accumulation, disturbances in whole-body or plasma ion homeostasis and stress response parameters such as plasma cortisol and glucose (Haux and Larsson, 1984; Giles, 1984; Pratap et al., 1989; McGeer et al., 2000a; Baldisserotto et al., 2004b; Fu et al., 1990; Firat and Kargin, 2010). However, most of the effects are temporary with recovery if exposure is prolonged (Haux and Larsson, 1984; Giles, 1984; Fu et al., 1990; McGeer et al., 2007), suggesting the ability of fish to acclimate to waterborne Cd. The majority of studies (Hollis et al., 1999; Hollis et al., 2000a; McGeer et al., 2007) describe acclimation as physiological changes (i.e. increased biosynthetic processes such as mobilization of metal binding proteins) that help repair earlier homeostatic disruption, which can result in increased tolerance to acute challenges such as 96 h LC_{50} tests (lethal concentration resulting in 50% mortality).

Fish encounter elevated Cd concentrations in their aquatic environment due to both natural and anthropogenic sources, which usually results in elevated Cd bioaccumulation in tissues (Rainbow, 2007; Kamunde, 2009). Studies have shown that Cd accumulates primarily in the kidney, gills, liver and gut, and in general tends not to accumulate in the brain and skeletal muscle tissue of fish (Norey et al., 1990; Harrison and Klaverkamp, 1989; Hollis et al., 2000). However elevated tissue concentrations of Cd are not always associated with adverse effects (Kamunde, 2009). During chronic exposure to Cd and other metals, fish can undergo acclimation by changes in physiological status and an increased tolerance and/or resistance to Cd accumulation can develop (McDonald and Wood, 1993; Hollis et al., 1999; Wood, 2001; McGeer et al., 2007). In addition to internal protection, some aquatic environments such as alkaline lakes, may aid in dampening Cd accumulation due to the competition of Cd^{2+} with Ca^{2+} for binding sites and transport channels across the basolateral membrane of the gill (Verbost et al., 1988; 1989; Spry and Wiener, 1991; Wood et al., 2006).

The ability of fish to develop tolerance to Cd accumulation can be examined when fish are chronically exposed to sublethal waterborne concentrations. Initial damage and accumulation occurs at the primary site of uptake, the gills (Wicklund Glynn et al., 1991; McDonald and Wood, 1993), which results in the reduction in the influx, stimulation of efflux of essential elements (e.g. Ca^{2+}, Na^{+}) related to inhibition of branchial ATPase activities (Na^{+}/K^{+} and Ca^{2+} particularly the Ca^{2+}) and low plasma Ca^{2+} levels develop (Verbost et al., 1987; 1988; 1989). The damage phase is usually followed by a repair phase as essential nutrient influx and efflux rates are restored and re-establishment of homeostasis or a new physiological set point occurs (McDonald and Wood, 1993) while tissue levels of Cd continue to rise. This suggests the existence of mechanisms that internalize metal in forms that are biologically unavailable to interact (metabolically detoxified) with metal-sensitive cellular targets (Kamunde, 2009; Liao et al., 2010).

Metal detoxification is a process of maintaining low cytosolic metal concentrations (Sterling et al., 2010). Mechanisms of detoxification include chelation and sequestration by soluble metallothionein-like proteins in the cytosol and through the formation of insoluble particulate structures also know as metal-rich granules (Lanno et al., 1987; Wallace et al., 2003). In fish, studies (Campbell et al., 2005; Kraemer et al., 2005; Giguère et al., 2006; Kamunde et al., 2009) regarding the subcellular distribution of Cd have shown that detoxification processes depend mainly on Cd binding to soluble thionein ligands in the cytosol, which contains the majority of intracellular metal. Furthermore, metals such as Cd and Cu mainly have affinity to nitrogen- or sulphur- containing ligands based on their specific ionic radius and electronegativity (Nieboer and Richardson, 1980). Detoxification through sequestration and chelation is accomplished either through interception of metal ions as they are taken up and trafficked through cellular compartments or abstraction of them from metal-sensitive biomolecules (enzymes) and organelles (mitochondria) (Amiard et al., 2006). Different regulatory mechanism may exist for essential and non-essential metals (Rainbow, 2002). Essential metals may be subject to regulation including limiting metal uptake and species-specific accumulation strategies with active excretion from the metal excess pool

and/or storage in an inert form and/or excretion of stored (detoxified) metal. Major strategies to cope with accumulation of non-essential metals are excretion from metabolically active pools and internal storage without elimination, thus body concentrations tend to increase with increasing external concentrations (Rainbow, 2002).

The existence of these metal-insensitive vs sensitive systems helped form the spill-over hypothesis which suggests that there is a threshold concentration of metal that exists within tissues whereby if exceeded, metal protective (detoxification) capacities become overwhelmed, and toxicity ensues from excessive metal accumulation in sensitive cellular compartments that play significant roles in cellular physiology (Rainbow 2002; 2007; Wallace et al., 2003). The spill-over concept was first proposed by Winge et al. (1974) and although some studies have appeared to confirm it (Sanders et al., 1983; Sanders and Jenkins, 1984), recent data using fish as the test organism suggest that this hypothesis is too simplistic to fully explain toxicity (Kraemer et al., 2005; Giguère et al., 2006; Campbell et al., 2005, 2008; Kamunde and MacPhail, 2008; Kamunde 2009). Furthermore, the majority of studies testing the validity of the spill-over hypothesis have been conducted in the field along metal gradients (Kraemer et al., 2005; Giguère et al., 2006; Campbell et al., 2005, 2008) or examining acute toxicity of Cd to fish (Kamunde, 2009). The dynamics of subcellular partitioning of Cd during chronic exposures remain largely un-explored.

Although metal toxicity may coincide with accumulation in sensitive compartments, it should not imply that saturation of detoxification systems is a prerequisite to result in adverse affects within the fish (Kamunde and MacPhail, 2008). Examining the pattern of intracellular metal binding will help determine the functional significance of the metal and may reveal potential mechanisms of toxicity and fate of accumulated metal (Sappal and Kamunde, 2009). For example, Cd bound to mitochondria impairs oxidative phosphorylation with reduction in ATP production (Sokolova, 2004; Sokolova et al., 2005) whereas Cd bound to metallothionein-like proteins reduces toxicity and imparts tolerance to Cd accumulation (Hollis et al., 2001; Chowdhury et al., 2005). If Cd accumulates substantially in MSP during the early phase of the exposure, then perhaps accumulation may link to early effects of Cd toxicity

(i.e. hypocalcaemia) during the damage phase of sublethal chronic Cd toxicity. Furthermore, post damage recovery may suggest that detoxification mechanisms experience an initial lag period before they are capable to match up-take of Cd into sensitive compartments and/or changes in the partitioning of Cd from metal-sensitive into detoxified pools may occur. This example of Cd accumulation could correspond to the damage-repair model mentioned previously for prolonged exposures.

The objective of the present study therefore was to characterize the subcellular partitioning of Cd in gill, liver and kidneys of juvenile rainbow trout (*Oncorhynchus mykiss*) during a 29-day sublethal waterborne Cd exposure. It was predicted that Cd would accumulate in tissues and metabolically detoxified pools in a time- and dose- dependent fashion. If mortality and plasma ion loss occur early during the exposure, this will correspond to excessive Cd accumulation in MSP. If fish are able to recover from initial damage, then there will be a shift from Cd partitioning from sensitive to detoxified pools. Furthermore, each tissue will have different patterns of subcellular partitioning of Cd corresponding to different physiological roles (i.e. detoxification (liver); storage or elimination (kidney) ionoregulation (gill), and characterization of Cd bound to sensitive and detoxified compartments will reveal tissue specific strategies of intracellular Cd accumulation.

2.3 Materials and Methods

2.3.1 Fish husbandry and holding conditions

Juvenile rainbow trout; 27.1 ± 7.64 g (mean ± SD, n = 100) were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada). Initially, fish were held in 180-L tanks (2 tanks with 210 fish in each) with water flowing to each tank at 700-mls min^{-1}. Water was a 1:1 mix of well water and soft water produced by reverse osmosis (500 mg/L as $CaCO_3$, 650 µS/cm, pH 7.6, 10.9 °C). Fish were acclimated to moderately hard water by gradually decreasing the flow of the well water over a two-week period. Post two-weeks, fish were randomly distributed among six 200-L polyethylene tanks (70 fish in each). A 60-L polyethylene mixing head tank received 2.8-L/min of soft water plus 1.4-L/min of well water, for a total of 4.2-L/min to achieve the chemistry of moderately hard water used for experimental exposures (140

mg/L as $CaCO_3$, 868 ± 28 Ca, 480 ± 16 Mg, 338 ± 16 Na (all in μM, mean ± 1 SD, n = 37)), with a conductivity, pH and temperature of 220 μS/cm, pH 7.1, 11.0 °C respectively. The mixing head tank delivered water (2-L/min) to three smaller 11.2-L polyethylene head tanks that have equally divided (split) outflows of water (700 mls/min) delivered to two fish tanks each. All water in head tanks and fish tanks were well aerated. Fish were acclimated to their respective tanks for two weeks prior to Cd exposure and fed at 2% of their body weight daily as a single meal (Bio Oregon Protein Inc., Warrenton, OR).

2.3.2 Cd Exposure

Two head tanks were used for Cd exposures at nominal concentrations of 0.75 and 2.0 μg Cd/L (as $CdCl_2$, VWR International, Mississauga, ON, Canada); the remaining head tank was used for control (nominal 0). Dissolved Cd concentrations measured in fish tanks were (means ± 1 SEM (n)): 0.03 ± 0.002 (4) μg Cd/L (control), 0.71 ± 0.101 (6) μg Cd/L, 1.85 ± 0.119 (6) μg Cd/L (see Table 2.1 for all measured exposure concentrations). All three exposure conditions (control (0), 0.75 and 2.0 μg Cd/L) occurred in duplicates, therefore n = 140 fish for controls and n = 140 fish per treatment. Initially, head tanks and fish tanks were spiked with appropriate volumes from a master stock of 1.0 g/L of Cd to achieve exposure concentrations. Additionally, appropriate volumes of the master stock were added to two 10-L carboys, each delivering Cd solution to the head tanks via pumps (FIM lab pump, Fluid Metering Inc., Oyster Bay NY; 1.2-mls min^{-1}) to maintain the desired Cd concentrations in exposure tanks. The Cd solutions in each 10-L carboy were renewed weekly. Water pH, conductivity (μS) and temperature were also measured weekly using a pH meter (Mettler Toledo SevenGo™, Fisher Scientific) and conductivity and temperature using a conductivity meter (YSI 30, Yellow Springs Instruments, Yellow Springs, Ohio).

2.3.3 Sampling

The exposure was carried out for 29 days and samples were collected on days 1, 4, 7, 17 and 29 and fish were fed throuhgout the duration of the exposure except the day prior to sampling. At each sampling time, both filtered (0.45 μm syringe filter; 22 mm Acrodise HT tuffryn membranes, Pall Corporation, Ann Arbor, MI, USA) and un-filtered 10 ml water samples were taken from exposure tanks, acidified to 1% by

adding 100 µl of concentrated HNO_3 (Trace Metal Grade, Fisher Scientic, Mississuaga ON). Additionally, 3 fish per exposure replicate (n = 6 per treatment) were non-selectively netted and euthanized with an overdose of 0.3 g/L tricaine methanesulfonate (Syndel Laboratories Ltd., Vancouver, BC, Canada) buffered with a 0.6 g $NaHCO_3$ /L.

Blood samples were also taken from fish sacrificed for tissue and subcellular Cd accumulation to measure plasma Na^+ and Ca^{2+} concentrations. Blood samples (300-1000 µl) were taken by caudal puncture and collected in 1.5-mL centrifuge tubes containing 10 µL of lithium heparinized saline (100 units) to minimize clotting. Blood samples were immediately centrifuged at 10,000 g for 2 minutes. Plasma was then removed using 1.5 mL insulin syringes, collected in new 1.5-mL centrifuge tubes and stored at -20°C.

Tissue samples of liver, kidney and gills were collected from all sampled fish. All tissues were rinsed for 10 seconds in deionized water, blotted dry then divided into two portions, saved in 1.5 mL centrifuge tubes, frozen in liquid nitrogen then stored at -80°C until analysis of Cd content. One portion of the tissue (liver, kidney, and gill) was used for total Cd concentration, while the remaining portion was used for subcellular Cd accumulation analysis.

2.3.4 Analytical techniques

All liquid and dry chemicals were obtained from Fisher Scientific (Mississauga, ON, Canada) unless otherwise mentioned. Plasma samples were thawed and diluted, 50 times for Ca^{2+} and 1000 times for Na^+ using ultra-pure water. Diluted samples were analysed for concentrations of both ions using atomic absorption spectroscopy (AAS 880, Varian Inc, Palo Alto CA). Water Ca, Mg and Na were similarly measured in flame mode (SpectraAA 880).

Gills, liver and kidney samples (75 - 300 mg) were weighed, thawed and digested in 5-fold of their wet weight with 1N TraceMetal grade HNO_3 then baked for 3 hrs at 80 °C (Playle et al. 1993a, 1993b). After digestion, tissues were vortexed for 5 seconds, then centrifuged for 2 min at 10,000 rpm (Spectrafuge 16M; Labnet International, Edison, NJ, USA), and left to settle. The supernatant was diluted to 10- to 100-fold with 1% acidified (concentrated HNO_3 TraceMetal grade) ultra-pure water to measure

Cd concentrations. Water and tissue samples were measured for Cd using graphite furnace atomic absorption spectrophotometer (GFAAS; SpectraAA 880 GTA 100 atomizer, Varian, Mississauga, Ontario). The concentration of Cd in all measured tissue and subcellular compartments are expressed as μg/g wet weight

2.3.5 Subcellular distribution analyses

The differential centrifugation protocol of Kamunde and MacPhail (2008) was adapted for the isolation of tissue subcellular compartments (1) a fraction comprised of nuclei, cell membranes, intact cells and connective tissue, (2) a granule-like or resistant fraction, (3) mitochondria (heavy and light), (4) a fraction combining microsomes and lysosomes (5) cytosolic heat-stable proteins (HSP) including metallothioneins, and (6) cytosolic heat-denaturable proteins or enzymes (HDP; see Appendix A for protocol).

Individual tissues (gill, liver, kidney; 100 - 500 mg) were homogenized on ice in phosphate-buffered saline [PBS: 137 NaCl; 4.3 KCl; 4.3 Na_2HPO_4; 1.4 NaH_2PO_4 (in mM), pH 7.2] at a dilution of 1:3 of their wet weight by using digital tissue homogenizers set at 20 RPM (Omni THQ, Omni International, Marietta, GA, USA). The resulting tissue homogenates were initially centrifuged at 4°C, 800 g (IEC-CL31R Multispeed; Thermo Electron Corp., Milford, MA, USA) for 15 min. Separation of the granule-like (P1) and supernatant containing the nuclei + debris (S2) fraction from the initial centrifugation pellet was performed by (1) re-suspending the pellet (P1) in 0.5 ml ultrapure water, (2) heating this suspension at 100°C for 2 min, (3) adding 0.5 ml of 1N NaOH (Sigma-Aldrich, Nepean ON, Canada), (4) heating again at 60-70°C for an hour and (5) centrifugation at 10 000 X g for 10 min at 20°C. Separation of mitochondria and lysosomes was performed using a two-step isolation protocol, by centrifuging the S1 supernatant initially at 4°C, 3000 g for 15 min to isolate heavy mitochondria (P3) and subsequently at 4°C, 10,000 g for 30 min to isolate the light mitochondria (P10; both heavy and light mitochondria fractions were combined for analyses). The 10,000 g supernatant (S10) was further ultracentrifuged at 4°C and 100,000 g (Optima MAX; Beckman Instruments, Mississauga, Ontario, Canada) for 60 min, isolating the microsomes and lysosomes fraction (P4) and cytosol (S4). Separation of

the HDP (P5) and HSP (S5 or metallotionein-like proteins, MTLP) was done by (1) heating supernatant S4 at 80°C for 10 min, (2) cooling it on ice for an hour and (3) ultracentrifuging it at 50 000 x g for 10 min at 4°C. To ensure the purity of each pellet, they were rinsed three times in 1 ml of buffer solution and spun for 10 min at 10 000 x g at 4°C. Upon isolation, all of the subcellular fractions were stored at -80°C until analyzed for Cd accumulation.

All pelleted subcellular fractions were thawed and digested in a 5-fold of their wet weight with 1N HNO_3 (TraceMetal grade) for 3 hrs at 80°C, whereas the supernatants were digested in a 3- fold volume of 1N HNO_3 (TraceMetal grade) for 3 hrs, also at 80°C. The digests were diluted accordingly with 1% acidified (concentrated HNO_3, TraceMetal grade) ultra-pure water and concentrations of Cd in pellets and supernatants were measured by GFAAS.

2.3.6 Quality control

For quality control, pre-prepared certified reference material (trace metal fortified Lake Ontario water [TMDA-28.3 and TM 26.3]; National Water Research Institute, Burlington, ON, Canada) and blanks (MilliQ water and solutions used for digestion) were analyzed by GFAAS together with experimental samples.

With respect to subcellular compartmentalization in each tissue, in order to check for fraction enrichment, cross-contamination, and potential rupture of organelles during the isolation process, activities of marker enzymes, citrate synthase (mitochondria) and acid phosphatase (lysosomes) were measured (Figs. 3.3.1 – 3.3.3) in the mitochondria and S10 supernatant fractions or microsome/lysosome and S4 supernatant fractions for the latter (see Appendix A for isolation steps with differential centrifugation protocol). Measurements of citrate synthase and acid phosphatise activity were adapted from Srere (1969) and Bergmeyer (1974) respectively, using commercial kits (CS0720 and CS0740; Sigma-Aldrich, Nepean, ON, Canada; see Appendix B for protocols). The enzyme assays were carried out in 96-well plates and analysed using a microplate spectrophotometer set at 405nm (SPECTRAmax 190; Molecular Devices Inc., Sunnyvale, CA, USA). Also, since one portion of the tissue (gill, liver and kidney) was measured for total Cd content, this enabled the verification of the recovery of Cd in the

various subcellular fractions by calculating the percentage recovery ([sum of Cd burdens in the five fractions/Cd burden in the whole tissue] x 100). Similarly, proportions (%) of Cd distribution in the metal-sensitive pool (MSP; mitochondria (P10) and enzymes (P5)) and metabolically detoxified pool (MDP; metallothionein-like proteins (S5), metal right granules (P2) and microsomes + lysosomes (P4) were calculated in the same manner ([sum of Cd burdens in MSP or MDP/Cd burden in the whole tissue] x 100).

2.3.7 Statistical analyses

Data are expressed as mean ± 1 SEM. All graphs and statistical analysis were performed using the computer software SigmaPlot 11.0 (Systat Software Inc. (SSI), San Jose, CA, USA) or SPSS 18.0 (SPSS Inc. IBM Company Headquarters, Chicago, IL, USA). All data were checked for normality and homogeneity of variances, and subsequently submitted to a two-way analysis of variance (ANOVA). All accumulation data were subjected to Tukey's honest significant difference (HSD) post hoc test and plasma ion measurements were subjected to Dunnet's post hoc test, to detect significant differences among the means using $P < 0.05$.

Initially, to ensure the data agreed with distributional assumptions of the ANOVA and other statistical tools, relationships between exposure variables (i.e., treatments, time) and Cd bioaccumulation (i.e., Cd concentrations in subcellular fractions of liver, gill and kidney, and total Cd concentrations in each tissue) were examined in bivariate scatterplots and tested by simple correlation (Pearson's *r*) and linear regression analyses using non-transformed data. The Kolmogorov–Smirnov test was used to verify that regression standardized residuals were normally distributed and homoscedasticity was checked by examination of the biplots of residuals against predicted values (Bonneris et al., 2005).

The enzyme activity data for the subcellular method quality check were statistically analyzed using a two-tailed unpaired *t*-test, $P < 0.05$. Plasma ion concentrations were statistically compared to control values at each sampling day using one-way analysis of variance (ANOVA; Dunnet's test, $P < 0.05$). Relationships between Cd accumulation in tissue and within the subcellular fractions were

examined by linear regression analyses using Pearson's r^2 value and relationships were reported significant at $p < 0.05$.

Tissue and subcelluar Cd accumulation data were statistically analyzed using two-way ANOVA with time and treatment as independent variables. Statistical comparisons were made between treatments (control (0), 0.75 and 2.0 μg Cd/L) at each time point and within treatments throughout the 29-day exposure (see Appendix D for Anova Tables). Proportional data of Cd distributions in MSP and MDP were initially arcsine-transformed and subsequently submitted to a two-way ANOVA.

2.4 Results

2.4.1 Mortality and plasma ion concentrations

Mortality occurred at both exposure concentrations (075 and 2.0 μg Cd/L) during the early phase of the exposure (days 1-5), resulting in 91% and 80% survival for both the low and high exposure concentrations respectively (Fig. 2.4). There were no changes in plasma Na concentrations in trout exposed to 2.0 μg Cd/L compared to controls; however trout exposed to 0.75 μg Cd/L had significantly lower plasma Na concentrations, only on day 4 (Fig. 2.6), which was also true for plasma Ca concentrations in the low Cd exposure group. Trout exposed to 2.0 μg Cd/L had significantly lower plasma Ca concentrations in comparison to control values during the 30 day exposure (Fig. 2.5), and were unable to recover back to control levels.

2.4.2 Total Cd burdens in tissues

Cd concentrations in all tissues were higher than background concentrations over the 29-day exposure to 2.0 μg Cd/L (Fig.2.7); the increases in all tissues except liver were significant by day 4. Similarly, Cd concentrations were higher than control values in the gills and kidney during the 29-day exposure to 0.75 μg Cd/L (Fig 2.7); the increases in both tissues were significant by day 17 and onwards. At day 29, kidney and gill Cd concentrations were comparable (kidney exceeded gill in fish exposed to 2.0 μg Cd/L) and were 2 times higher than liver levels (Fig. 2.7) for both low and high Cd exposure groups. Kidney Cd

levels increased 28 and 127 times (low and high Cd exposure) from control values (value ± 1SEM (n); 0.025 ± 0.008 (30) μg Cd/g wet weight; Figure 2.7). Liver Cd concentrations increased 13 and 80 times from control values (0.015 ± 0.001 (30) μg Cd/g wet weight) and gill Cd concentrations increased 30 and 34 times from control values (0.011 ± 0.002 (30) μg Cd/g wet weight) for Cd exposed fish after 29 days of exposure (Fig. 2.7).

2.4.3 Bioaccumulation and distribution of Cd in subcellular fractions

2.4.3.1 Marker enzyme assays and recovery of Cd

Citrate synthase and acid phosphatase activities (Figs. 2.1 – 2.3) were measured to examine both the level of enrichment and integrity of the mitochondrial and microsomes + lysosomes fractions, and to determine possible contamination of the cytosol. The citrate synthase activity was 8 times, 7 times and 10 times higher in the mitochondrial fraction relative to the cytosol (30 - 80 μmole/ml/min), for the liver, kidney and gills respectively, indicating significant enrichment with mitochondria. Measured mitochondrial citrate synthase activity ranged between 350 – 500 μmole/ml/min for all tissues. Similarly, the ML fraction was enriched with lysosomes, indicated by 24 times, 6 times and 5 times increase in acid phosphatase activity compared to the cytosol (0 - 0.2 Units/ml), for the liver, kidney and gills respectively. Measured lysosomal acid phosphatase activity ranged between 0.7 – 1.2 Units/ml for all tissues. Cd concentrations in all subcellular fractions were summed and compared to the respective tissue Cd burden. Recovery of Cd ranged between 85-97, 88-111 and 87-114 % for the gill, liver and kidney respectively.

2.4.3.2 Subcellular Cd partitioning

The concentrations of Cd partitioning into subcellular compartments within the gills, liver and kidney of trout exposed to waterborne Cd are shown in Figs. 2.8 – 2.10. Briefly, Cd concentrations in subcellular fractions (cellular debris, metal-rich granules (MRG), mitochondria, microsomes+lysosomes (ML), heat-sensitive proteins (HSP) and metallothionein-like proteins (MTLP)) from fish chronically exposed to sublethal Cd were:

Gill - mitochondria > cellular debris > MTLP > HSP > ML > MRG

Liver - MTLP > mitochondria > HSP > cellular debris > ML > MRG

Kidney - cellular debris > MTLP > MRG > ML > HSP > mitochondria

Cd concentrations in gill mitochondrial fractions (Fig. 2.8) gradually increased in fish exposed to 0.75 μg Cd/L and were significantly higher than control levels on days 17 and 29. Gill Mitochondrial concentrations from fish exposed to 2.0 μg Cd/L significantly increased with time. In exposed fish, gill cellular debris fraction (Fig. 2.8) Cd concentrations were higher than control levels, and concentrations remained unchanged within each exposure group throughout all sampling days. The gill HSP fraction (Fig. 2.8) Cd concentrations from the low Cd exposure group gradually increased during the first week, peaked on day 7 when Cd concentrations were significantly higher than control levels, then declined. Cd concentrations in the gill HSP fraction from the high Cd exposure group significantly increased with time until day 17 and achieved significantly higher Cd concentrations in comparison to both control and low Cd exposure values from days 7 – 29.

Liver metal-sensitive fractions did not accumulate Cd when exposed to 0.75 μg Cd/L, except mitochondrial fractions gradually increased from background levels and were significantly higher by day 30 (Fig. 2.9). However, increases from both control values and concentrations attained by fish exposed to 0.75 μg Cd/L were apparent in all fractions in fish exposed to 2.0 μg Cd/L. The liver cellular debris fraction (Fig. 2.9) significantly accumulated Cd during all sampling days and remained stable from days 4-29. Liver mitochondrial concentrations of Cd (Fig. 2.9) were higher than both control and low Cd exposure levels by day 7 and continued to significantly rise. The liver HSP (Fig 2.9) fraction Cd concentrations fluctuated but remained high in comparison fish livers from control and low Cd exposure.

Cd did not accumulate appreciably in mitochondrial kidney fractions of fish exposed to 0.75 μg Cd/L (Fig. 2.10). However, fish exposed to 2.0 μg Cd/L experienced significant increase in Cd concentrations by day 7 and onwards. The kidney HSP fraction (Fig. 2.10) initially accumulated

significantly higher concentrations of Cd in comparison to control values on day 1 then declined for both low and high Cd exposure groups. Fish exposed to 0.75 μg Cd/L demonstrated a gradual decline in HSP Cd concentrations with levels comparable to control values by day 7 and onwards, whereas fish exposed to 2.0 μg Cd/L decreased to control levels by day 4, and remained relatively unchanged, with significantly higher Cd concentrations on day 17 only. In contrast, kidneys from exposed fish gradually accumulated Cd in cellular debris fractions (Fig. 2.10) and concentrations were elevated from control values after one week of exposure; fish exposed 2.0 μg Cd/L attained higher amounts of Cd by day 4 and onward.

2.4.3.3 Cd partitioning in detoxified metal compartments

Figures 2.8 – 2.10 show the concentrations of Cd in the liver, kidney and gill in detoxified subcellular fractions; MRG, ML, and MTLP. The gill MTLP Cd concentrations (Fig 2.8) gradually increased in comparison to controls, and were significantly higher than control levels on day 29 for both the low and high Cd exposure groups. Gill MRG contained the lowest amount of Cd (Fig. 2.8) however concentrations in exposed fish were higher than control values (only after one week for the low Cd exposure group). Cd concentrations in gill MRG remained unchanged in fish exposed to 0.75 μg Cd/L and fluctuated in fish exposed to 2.0 μg Cd/L. Cd concentrations in gill ML (Fig. 2.8) gradually increased in comparison to controls for fish exposed to 0.75 μg Cd/L and were significantly higher than control levels on days 17 and 29. Fish exposed to 2.0 μg Cd/L attained higher amounts of Cd than both control and low Cd exposed fish by day 7, coinciding with the highest Cd accumulation in ML fraction; however there was a significant decreased between day 7 and 17.

The liver MTLP Cd concentrations (Fig. 2.9) of trout exposed to 0.75 μg Cd/L were significantly higher than control values on days 1 and 7 and did not change appreciably between sampling days. Cd concentrations in the MTLP fraction from trout livers exposed to 2.0 μg Cd/L increased significantly and were higher than fish exposed to 0.75 μg Cd/L after one week. Liver ML fraction (Fig. 2.9) Cd concentrations achieved significantly higher amounts by day 7, and Cd levels continued to increase significantly with time. Liver MRG contained the lowest amount Cd (Fig. 2.9). Cd concentrations in

MRG fluctuated in trout exposed to 0.75 μg Cd/L and attained higher amounts of Cd than control levels on days 7 and 29; however Cd did not accumulate appreciably in MRG of fish exposed to 2.0 μg Cd/L.

The kidney MTLP Cd concentrations (Fig. 2.10) gradually increased. Cd concentrations in trout exposed to 0.75 μg Cd/L were significantly higher than control levels on day 29, whereas concentrations in trout exposed to 2.0 μg Cd/L increased significantly from day 7 and onwards, and attained higher concentrations than both background and low Cd exposure levels. Kidney MRG and ML Cd concentrations (Fig. 2.10) gradually increased in fish exposed to 0.75 μg Cd/L and Cd concentrations in ML were elevated from control values after one week. Significant increases from control values were attained from day 7 and onwards in both ML and MRG fractions from fish exposed to 2.0 μg Cd/L.

2.4.4 Metabolically active vs. metabolically detoxified Cd

Figure 2.11 displays the overall change in proportion of Cd (%) partitioned into MSP which includes mitochondria and HSP; and MDP that is comprised of MTLP, MRG and ML in each tissue throughout the 29-day exposure to waterborne Cd. Cd bound to MSP and MDP in the gill remained comparable to control values, and varied with time and exposure (Fig. 2.11). Initially, there was significantly less Cd bound to MSP on day 1 in 2.0 μg/L exposed fish, however, Cd concentrations appeared elevated from control values after one week. Generally, decreased partitioning of Cd into MDP was observed in fish gills exposed to 2.0 μg Cd/L, whereas Cd bound to MDP appeared to gradually rise, in comparison to controls. The proportions of Cd bound to MSP ranged from 38-57% and 18-57% for low and high Cd exposed fish respectively. The proportions of Cd bound to MDP ranged from 24-53% and 22-42 % for low and high Cd exposed fish respectively. Background proportions of MSP and MDP ranged from 49-53% and 37-42% respectively.

In the liver of Cd exposed fish (Fig. 2.11), it was observed that the proportion of Cd bound to MSP was low during the first week of exposure (particularly in fish exposed to 0.75 μg Cd/L) and gradually rised, whereas Cd bound to MDP remained elevated (particularly in fish exposed to 0.75 μg Cd/L), in comparison to control values. The proportions of Cd bound to MSP ranged 21-51% and 29-44% for low

and high Cd exposed fish respectively. The proportions of Cd bound to MDP ranged from 38-65% and 41-50% for low and high Cd exposed fish respectively. These values were comparable to control MSP and MDP values ranging from 39-43% and 35-42% respectively.

The kidney partitioned Cd from MSP throughout the exposure (Fig. 2.11), as Cd bound to MSP declined linearly in both low and Cd exposed fish. The proportion of Cd bound to MSP ranged from 11-47% and 4-30% for low and high Cd exposures respectively, whereas Cd bound to MDP in kidneys ranged from 38-65% and 40-60% respectively. Cd bound to MSP was significantly lower from control values (26-30%) after one week of exposure to both Cd concentrations, whereas Cd bound to MDF remained comparable to control values (49-53%) and appeared elevated in comparison to control levels on day 29.

2.4.5 Correlation between fraction Cd and total tissue Cd burdens

In order to indentify which subcellular fractions responded to the increases in Cd accumulation, concentrations in each fraction (μg/g; y-axis) were regressed against total tissue burdens (μg/g; x-axis). Tables 2.2 – 2.4 show the coefficients of determination for the relationships between Cd concentrations in cell fractions and the respective whole liver, kidney and gill concentrations. Correlations were determined throughout the 29-day exposure, during the first week (days 1, 4 and 7) and after one week (days 7, 17 and 29) of exposure to observe potential differences in fractions that become particularly enriched with Cd early in the exposure and vice versa. In the liver, only the MRG fraction Cd concentrations were not correlated with hepatic Cd, which was also true for the kidney HSP fraction and both cellular debris and MRG fractions in the gill throughout the 29-day exposure. However, coefficients of determination (r^2) changed between the first week of exposure and after one week of exposure within each subcellular fraction from all tissues. Correlations between each fraction and the total hepatic Cd concentration demonstrated a stronger relationship within ML, mitochondria and HSP subcellular fractions during the first week of exposure; in contrast the kidney displayed stronger correlations among all subcellular fractions after one week of exposure. Gill correlations between each fraction and total Cd burden were not

significant during the first week of exposure, and were only significant within the MTLP fraction post one week exposure and r^2 values were less than 0.26 for all fractions compared to total gill Cd burdens.

2.5 Discussion

2.5.1 Plasma ion concentrations

Disturbances in plasma ion concentrations have been previously linked with metal exposure (Wicklund and Runn, 1988; Richards and Playle, 1999; Reynders et al., 2008). In this study, plasma Ca and Na concentrations in rainbow trout (*Oncorhynchus mykiss*) were affected by Cd exposure (Fig. 2.5-2.6). Trout exposed to 0.75 μg Cd/L had reduced levels of plasma Na and Ca on day 4. For trout exposed to 2.0 μg Cd/L no changes in plasma Na levels occurred, however plasma Ca levels remained lower than control values throughout the duration of the exposure, suggesting that detoxification of Cd was imperfect, most probably within gills since they play a significant role in serum Ca homeostasis (McDonald and Wood, 1993; Richards and Playle, 1999).

The survival of fish chronically exposed to Cd with low plasma Ca concentrations is consistent with results from Van Campenhout et al. (2010) which found significantly lower Ca concentrations at the sampling site associated with the highest environmental Cd pollution in gibel carp (*Carassius auratus gibelio*). Similar results of reduced plasma Ca levels in rainbow trout (*Oncorhynchus mykiss*) after 24 hr of Cd exposure (10 μg/L) in moderately hard water were obtained by Chowdhury et al. (2004). However, a study done by Hollis et al. (2000b) reported no significant changes in plasma Ca concentrations to rainbow trout (*Oncorhynchus mykiss*) exposed chronically to 2.0 μg Cd/L in four different Ca concentrations (260 – 1200 μM Ca) in synthetic soft water. This study also demonstrated that trout acclimated to increased ambient Ca concentrations resulted in a decrease in influx and binding availability of Ca at the gills, which may contribute to reduced plasma Ca in fish within the present study. Waterborne Cd causes toxicity in fish by decreasing plasma Ca concentrations due to Cd interfering with Ca regulation at the gills (Verbost et al., 1989; McDonald and Wood, 1993; Richards and Playle, 1999; Hollis et al., 1999). The work carried out by Verbost et al. (1988; 1989) demonstrated that there is a

strong inhibition of basolateral Ca^{2+}ATPase activity which indirectly results in a blockade of apical Ca channels. Due to the strong inhibition of Ca transport by Cd, plasma Ca levels may have been unable to recover in fish exposed to 2.0 μg Cd/L, and Ca concentrations (868 ± 28 μM) present in moderately hard water (140 mg/L as $CaCO_3$) used in this study were too low for Ca to effectively compete with Cd binding sites on the gill. Additionally, Cd exposure has been shown to disrupt gill epithelium (Oronsaye, 1989) which may lead to Ca efflux resulting in an increase of diffusive ion losses.

Consistent with results obtained in this study for trout exposed to 0.75 μg Cd/L, a study carried out by Fu et al. (1990) also demonstrated a small but significant decrease in plasma Na at day 4 in tilapia (*Oreochromis niloticus*) exposed to 10 μg Cd/L. However effects of Cd on Na balance in teleost fish are variable. Reader and Morris (1988) observed an increase in plasma Na in brown trout (*Salmo trutta*) exposed to elevated waterborne Cd, whereas studies (Pratap et al., 1989; Verbost et al., 1989; Baldisserotto et al., 2004; Chowdhury et al., 2004) with brown trout (*Salmo trutta*), tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*) have demonstrated unperturbed plasma Na levels in exposed fish. Although studies (Chowdhury et al., 2004; Garcia-Santos et al., 2005) have not reported changes in plasma Na with elevated waterborne Cd concentrations, it has been shown that Cd can inhibit the basolateral Na^+/K^+ATPase activity in gill and intestinal epithelium of many fish species (Schoenmakers et al., 1992; Pratap and Wendelaar-Bonga, 1993; Atli and Canli, 2006). Another possibility is that Cd can inhibit apical Na transport by binding to sensitive exchangers such as inracelllular carbonic anhydrase, similar to mechanism of other metals (i.e. silver and lead) described by other studies with rainbow trout (*Oncorhynchus mykiss*) (Rogers et al., 2005). Briefly, carbonic anhydrase provides H^+ and HCO_3^- ions for apical Na^+ and Cl^- uptake/exchangers sites of Cl^-/HCO_3^- (Rogers et al., 2005). However, short term (3h) unidirectional Na influx in ranbow trout was not inhibited by 5 μg Cd/L in soft water, but was exacerbated in combined Cd and Pb exposures in comparison to Pb exposure alone (Birceanu et al. 2008). Potentially, other mechanisms for Cd disruption of plasma Na levels exist and interactions with intracellular Cd and Na transport should continue to be explored.

2.5.2 Tissue Cd burdens

Overall, the 29-day uptake of Cd into tissues showed dose-, duration- and organ dependent differences in which Cd concentrations in the gill were approximately 2-3 times those attained in the liver and kidney during the first week of exposure (Fig. 2.7). This is not surprising since gills are in direct contact with the environment. Cd accumulation and retention in gills during the early phase of exposure coinciding with low Cd concentrations in internal organs (liver and kidney) is consistent with previous studies (McGeer at al., 2000b, McGeer et al., 2007; Hollis et al., 2001; Szebedinzky et al., 2001) and may indicate that the gill acts as a barrier in transfer of Cd thus limiting internalization and uptake of Cd in internal tissues. In contrast, liver and kidney showed an early lag phase followed by a linear increase in Cd concentrations in the kidney. Lag periods prior to Cd accumulation during chronic sublethal exposures have previously been reported in rainbow trout (*Oncorhynchus mykiss*) (McGeer et al., 2007; Hollis et al., 2001; Szebedinszky et al., 2001) and suggest that time is required to deliver Cd to internal target sites and is possibly delayed by intracellular binding of Cd to ligands in the gill (Olsson and Hogstrand, 1987; Wicklund Glynn, 1991). However, by day 29 the kidney accumulated comparable amounts of Cd to gills, and exceeded concentrations in the gills for fish exposed to 2.0 μg Cd/L.

Both gills and kidney attained approximately 2-3 times higher concentrations of Cd compared to liver at day 30 (Fig. 2.7). Additionally, absolute Cd accumulation was highest in kidneys, suggesting that the kidney is an important organ for Cd accumulation. Previous studies (McGeer et al., 2000b; Hollis et al., 2001; Szebedinszky et al., 2001) have demonstrated equal or higher concentrations of Cd (relative to gills or liver) in kidneys of trout chronically exposed to sublethal waterborne Cd in moderately hard water. Harrison and Klaverkamp (1989) found that kidney Cd burdens were retained even after the fish were returned to Cd-free water, highlighting the significance of the kidney as a bioaccumulator of Cd. The kidney Cd burden typically exceeds that of the liver (McGeer et al., 2000b; Hollis et al., 2001; Szebedinszky et al., 2001). Cd has previously been described by Kumada et al. (1980) as a nephrotoxicant due to selective internal accumulation of Cd in kidneys.

2.5.3 Subcellular partitioning of Cd

Marker enzyme activity of citrate synthase and acid phosphatase were mearsured in order to check for fraction enrichment, cross-contamination, and potential rupture of organelles during the isolation process (Figs. 2.1 – 2.3) in the mitochondria and S10 supernatant fractions or ML and S4 supernatant fractions for the latter (see Appendix A for isolation steps with differential centrifugation protocol). The liver, kidney and gill enzyme marker activities varied between tissues. Citrate synthase and acid phosphatase activity ranged from 7-10 times greater in mitochondrial fractions and ranged from 5-24 times greater in ML fractions, in comparison to the associated cytosol, indicating significant enrichment of both fractions. Measured mitochondrial citrate synthase activity ranged between 350 – 500 μmole/ml/min for all tissues which was comparable to pooled liver citrate synthase activity measured by Kamunde and MacPhail (2008) which ranged between 550-700 μmole/ml/min .Similarly, measured lysosomal acid phosphatase activity ranged between 0.7 – 1.2 Units/mL and were comparable to pooled liver acid phosphatase activity measured by Kamunde and MacPhail (2008) that ranged between 0.7-0.9Units/mL.

Intracellular metal-sensitive compartments including mitochondria and HSP accumulated Cd early (on day 1), especially in the gill (Fig. 2.8-2.10). This could potentially indicate temporary saturation of the capacity for Cd binding before induction of metal-binding proteins. Generally, gill metal-sensitive compartments accumulated more Cd than in the respective liver and kidney compartments in Cd exposed fish, which is undoubtedly due to the proximity and accessibility of waterborne Cd to the gill.

In both the liver and kidney, mitochondria appear to have a low capacity for Cd accumulation and ranked low in terms of concentrations attained compared to the gills (Fig. 2.8; 2.10). Throughout the duration of the exposure, trout exposed to 0.75 and 2.0 μg Cd/L accumulated approximately 2-6x and 8-11x more Cd in gill mitochondrial fractions compared to the liver and kidney respectively. During the first week of exposure, gill mitochondrial Cd concentrations increased, whereas liver and kidney Cd experienced a lag period before substantial increases were attained, similar to tissue burdens of Cd (Fig. 2.8-2.10). These findings are consistent with the work of Kamunde et al., (2009) which demonstrated that

Cd accumulated in the gill in a dose (5-50 μg Cd/L) and time (96 hrs) dependent pattern similar to whole Cd gill burdens, with minimal accumulation in the liver between 0 and 48 hrs. A study done by Kraemer et al., (2005) with yellow perch (*Perca flavescens*) transplanted to a metal-impacted lake with Cd concentrations of 1.7 nM for 70 days also confirmed low levels of Cd bound to organelles (mitochondria and ML) in the liver. Mitochondria are important metal-sensitive organelles in which Cd accumulation can impair oxidative phosphorylation resulting in ATP deficits, generate reactive oxygen species and cell death (Belyaeva et al., 2001; Sokolova, 2004).

Elevated concentrations of Cd partitioning to HSP occurred on day 1 in all tissues of Cd exposed trout (Fig. 2.8-2.10). However, after day 1 kidney HSP concentrations gradually declined in contrast to the gill which continued to increase (Fig. 2.10), the liver pattern of Cd partitioning displayed a median response with fluctuating Cd concentrations (Fig. 2.9). These results may indicate that the detoxification mechanisms within the kidney were able to dissipate Cd from this sensitive fraction with time. Discrepancies of Cd accumulation in liver HSP exist within the literature; numerous authors (Brown et al., 1990; De Smet at al., 2001; Kamunde et al., 2009) reported increased Cd partitioning into HSP, however lack of effect (Kraemer et al., 2005) and decreased partitioning (Giguère et al., 2006) have also been observed. This is most likely due variable experimental parameters such as exposure concentrations, water chemistry and fish species. Consistent with this study, Kamunde (2009) also observed early partitioning of Cd into HSP of both liver and gills. HSP are considered a metal-sensitive fractions since binding of metal can result in structural damage and perturb protein function, resulting in toxicity to fish when Cd concentrations become elevated (Pruell and Engelnardt, 1980).

Initially the cellular debris fraction contained approximately 2x and 4x as much Cd in gills compared to the kidney and liver respectively, while its contribution to the total Cd burden did not change in gill and liver, but gradually increased with time and dose in the kidney (Fig. 2.8-2.10). High concentrations of Cd in cellular debris fraction in the gill compared to that of the liver were also demonstrated by Kamunde (2009). Cd accumation in cellular debris fractions is difficult to interpret since

it is comprised of a heterogenous mixture of membranes, intact cells, and tissue fragments (Vijver et al., 2004).

2.5.4 Subcellular partitioning of Cd into metabolically detoxified compartments

In trout, the metal-rich granule fraction appears to play a minor role in Cd sequestration and detoxification in gills and liver (Fig. 2.8; 2.9) which attained the lowest concentrations of Cd in this study and previous studies (Giguère et al., 2006; Kamunde, 2009). In contrast, this detoxification mechanism is important in invertebrate species in which extremely high amounts of Cd are sequestered in granules (Rainbow, 2002 and 2007). Similarly, this study demonstrated that fish kidneys may also employ granule sequestration to detoxify Cd, as indicated by increases of Cd partitioning into MRG from exposed fish with time (Fig. 2.10). Interestingly, a study done by Oronsaye (1989) examined histological changes in kidneys of stickleback chronically exposed to dissolved Cd in hard water and observed excessive formation of granular masses, particularly in the tubule lumina, suggesting that the kidneys were excreting detoxified Cd.

The role of metallothionein in the sequestration and detoxification of Cd has been well documented in fish (Olsson and Hogstrand, 1987; De Smet et al., 2001; Hollis et al., 2001; Chowdhury et al., 2005). The MTLP fraction accumulated the highest concentration and % of the Cd burden which is consistent with many previous studies (Kraemer et al., 2005; Giguère et al., 2006; Campbell et al., 2006; 2008; Kamunde et al., 2009). However, there appeared to be a lag period during the first week of exposure in all tissues, whereby Cd gradually begins to accumulate in MTLP (Fig. 2.8-2.10).

In gills, lag periods were also observed by Wicklund Glynn (1988) and Kamunde (2009) in fish exposed to elevated levels of Cd, suggestive of temporary saturation of metal-binding capacity followed by the gradual synthesis of new metal-binding proteins. Delay of Cd accumulation into MTLP with concurrent rapid accumulation of Cd in metal-sensitive compartments in gills may be tempting to interpret as acute spill-over. Gills contain three main cell types; pavement, mucous and mitochondrial-rich cells (Laurentt, 1984) that could be involved in Cd uptake, however much attention has focused on

mitochondrial-rich cells due to their morphological appearance to that of transport cells. Waterborne Cd enters branchial mitochondrial-rich cells by passing through apical Ca channels, whereas the transport of Cd into the blood stream across the basolateral membrane occurs by an unknown mechanism, for Cd itself tends to inhibit the high-affinity Ca^{2+} -ATPase (Verbost et al., 1989; Playle et al., 1993a,b; Wood, 2001). Mitochondrial-rich cells are highly packed with mitochondria and rich in tubular endoplasmic reticulum (Laurent, 1984), making these cells particularly sensitive to Cd accumulation. Additionally, synthesis of metallothionein-like proteins occurs primarily in the mitochondrial-rich cells which comprises the smallest proportion (<10%) of the branchial epithelial surface area (Potts, 1977) thus the amount of metal-binding proteins produced is likely low. Initial rapid accumulation of Cd may overwhelm the detoxification capacity of mitochondrial-rich cells, rendering Cd accumulation into highly available sensitive fractions inevitable. Cd concentrations continued to significantly increase in both sensitive and MTLP fractions after one week of exposure, suggesting that the gills may be using additional mechanisms to help cope with potential consequences of accumulation. For example heat-shock proteins help repair structural damage to proteins and are synthesized during Cd exposure in gills of bivalves (Piano et al., 2004; Ivanina et al., 2008). Additionally, it could be that the gill is tolerant to accumulation of Cd in sensitive fractions and has not exceeded a threshold concentration that may be linked to adverse effects.

Slow rates of accumulation in the early stages of the exposure in the liver and kidneys (Fig. 2.9; 2.10) may be expected due to the same rationale of delayed tissue Cd accumulation mentioned previously. Ultimately, Cd partitioning into MTLP in both liver and kidney contributed to the highest proportion of total Cd. However, similar to gills, early Cd partitioning into metal-sensitive compartments (mitochondria, HSP) was observed potentially indicating acute spill-over in both liver and kidney. In contrast to gills, concentrations of Cd in metal-sensitive fractions declined or remained unchanged after one week of exposure due to possible changes in partitioning of Cd or incoming Cd partitioned to newly synthesized MTLP. Although previous studies have not examined subcellular distribution of Cd in

kidneys, studies done by Hollis et al, (2001) and Chowdhury et al., (2005) observed the highest relative increases in metallothionein concentrations in the kidney, followed by gills then liver, in fish chronically exposed to waterborne Cd (3 μg Cd/L) in moderately hard water (140 mg as $CaCO_3$/L). These findings are consistent with the present study; Cd exposed fish sequestered the highest concentrations of Cd in MTLP in the kidneys, followed by gills, then liver, similar to total tissue burdens of Cd (Fig 2.8-2.10), indicating that accumulators of Cd rely heavily on the binding of Cd to intracellular thionein-rich ligands.

Partitioning of Cd into the ML fraction was minimal early in the exposure within all tissues (Fig. 2.8-2.10). Increases of Cd partitioning into ML were after one week of exposure, however concentrations did not continue to increase in the gill or liver; only Cd concentrations in the kidney increased. These results are consistent with Kamunde (2009) which demonstrated that Cd partitioning to ML fraction was complex in the gills (some exposure doses (25 and 50 mg Cd/L) displayed similar plateaus and declines in Cd concentrations) and liver absolute accumulation was minimal until 96 hrs of exposure. It has been suggested that ML plays a role in detoxification (Kraemer at al., 2005) since it has been shown that metal-bound to metallothionein and other metal-thiol conjugates are ultimately degraded in microsomes and lysosomes in aquatic invertebrates (Viarengo et al, 1985; Barka et al., 2001; Nassiri et al., 2000) and vertebrates (Cousin, 1979; Mehra and Bremner, 1985, cited in Bremner, 1991). Additionally, bound metals have been shown to have different capacities to resist lysosomal degradation in vertebrates (Cousins, 1979; Mehra and Bremner, 1985, cited in Bremner, 1991), possibly in order of increasing resistance to lysosomal degradation: Cu > Cd > Zn, based on the stability of the disulphide links in their molecular conformation. Due to complex role of ML to potentially detoxify, sequester, and release Cd into other cellular components possibly by mechanisms similar to molluscs, crustaceans and annelids (Langston et al., 1998; Ahearn et al., 2004), future research should focus on partitioning of Cd within this fraction in fish to delineate the consequence, if any, of Cd accumulation.

In order to examine the relationship between subcellular fraction enrichment and tissue Cd accumulation, simple linear regression analysis was performed (Tables 2.2-2.4). Correlations were

determined throughout the 29-day exposure, during the first week (days 1, 4 and 7) and after the first week (days 7, 17 and 29) of exposure to observe potential differences in fractions that become particularly rich early in the exposure and vice versa. This will help reveal accumulation patterns at the subcellular level that do not match whole tissue patterns. All fractions in the liver except MRG were enriched with Cd, and slightly stronger correlations were observed during the first week of exposure. Similarly, all fractions in the kidney except HSP were enriched with Cd, however slightly stronger correlations were observed after one week of exposure. Cd accumulation in subcellular fractions did not display a strong relationship to total gill burden. Fractions that were particularly enriched with Cd in all tissues include MTLP and mitochondria. Thus, consequences of Cd accumulation, if any, could be related to partitioning of Cd to MTLP and mitochondria.

2.5.4Partitioning of Cd into metal-sensitive and detoxified pools

In fish gills exposed to 2.0 μg Cd/L, Cd concentrations partitioned into the MSP (mitochondria and HSP) varied with exposure and time, and values were comparable to controls after day one (Fig. 2.11). The gills partitioned less Cd into MDP during the first week of exposure. Similarly, partitioning of Cd in the liver into MSF also varied with time and exposure, however, in contrast to the gill, Cd bound to MDP appeared to remain elevated in both high and low Cd exposed fish and was higher than control values on day 7 in fish exposed to 0.75 μg Cd/L. In contrast to both liver and gill, Cd binding to MSP in the kiney declined in both low and high Cd exposed fish, however a shift in Cd partitioning into MDP was not observed, although levels appeared to be elevated in comparison to control values after one week (Fig. 2.11). The lack of significant partitioning into MDF was most-likely due to substantial amounts of Cd bound to cellular debris in the kidneys (Fig. 2.10). These results may indicate that the detoxification capacity of the liver and particularly the kidney has not been exceeded during the 29-day exposure. Similar observations have been made in juvenile rainbow trout (*Oncorhynchus mykiss*) liver and gills (Kamunde, 2009) and yellow perch liver (Giguère et al., 2006). These results indicate that fish gills are most sensitive to waterborne Cd accumulation due to lower amount of Cd bound to MDP.

The objective of the present study was to characterize the subcellular partitioning of Cd in gill, liver and kidneys of juvenile rainbow trout (*Oncorhynchus mykiss*) during a 29-day sublethal waterborne Cd exposure. The results indicated that all tissues did accumulate Cd in a time- and concentration-dependent manner. It was predicted that acute toxic effects (mortality and ion loss) will correspond to Cd accumulation in MSP, which was supported by these results in some tissues. The kidneys appeared to dissipate Cd from excessive accumulation in metal-sensitive fractions (e.g. HSP) with time and exposure. However, accumulation of Cd in MSP was observed in both liver and gills, particularly in the gills, as partitioning of Cd into metal-sensitive fractions (mitochondria and HSP) continued to rise or remained elevated. Additionally, it was predicted that there will be a shift from Cd partitioning from metal-sensitive to detoxified pools which could be related to the recovery phase of the damage-repair-acclimation scenario (McDonald and Wood, 1993). No significant shifts in Cd partitioning were observed during the first week of exposure; however increased partitioning of Cd was observed in the kidney at day 29 in fish exposed to 2.0 μg Cd/L.

Lastly, it was predicted that each tissue will have different patterns of subcellular Cd partitioning corresponding to different physiological roles. Characterization of Cd bound to metal-sensitive and detoxified compartments did reveal that the kidney is most efficient at detoxification as indicated by decreased partitioning of Cd into MSP and contained the highest amounts of Cd bound to MTLP and MRG in comparison to the liver and gills. The liver displayed intermediate detoxification capacity, whereas the gills partitioned the lowest amount of Cd into MDP. With regard to determining which particular fractions are enriched with Cd, the present study shows that: (a) the relative contribution to of each fraction to total liver or kidney burden was reasonably constant with few exceptions (MRG in liver; HSP in kidney) (b) complications arise when evaluating fraction enrichment of Cd in gills (c) MTLP and mitochondria appear to be the most important metal binding ligands in gills, liver and kidney, thus both should continue to be examined in future studies concerning the fate and potential consequences of

chronic sublethal Cd exposure in fish. Overall, more knowledge and studies concerning subcellular partitioning of Cd in tissues (particularly the kidney) of fish are strongly encouraged.

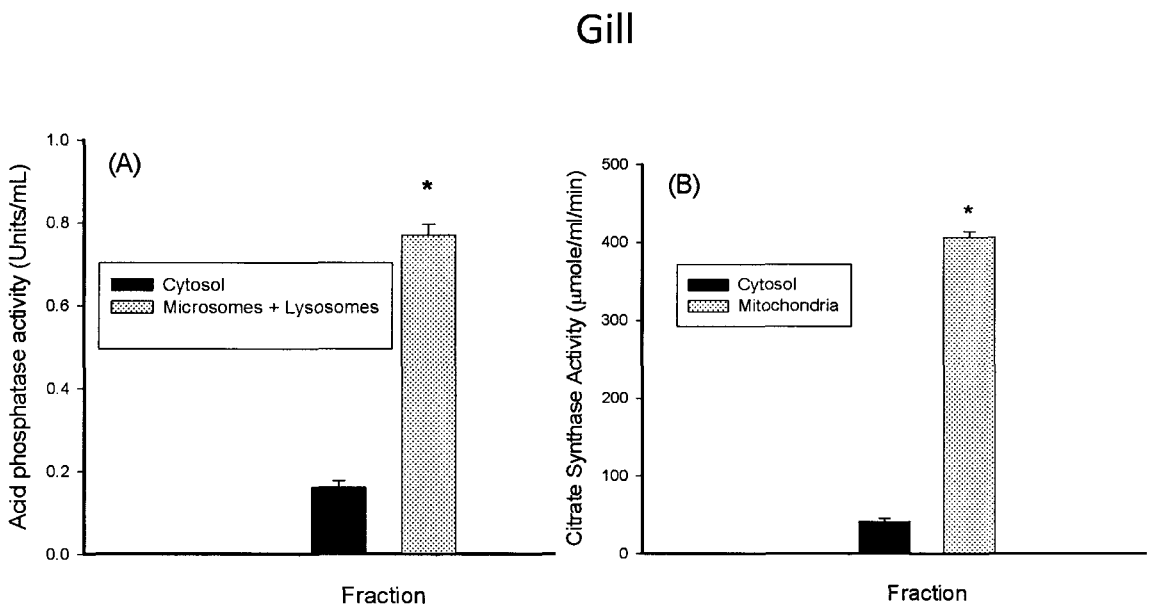

Fig. 2.1 Acid phosphatase (A) and citrate synthase (B) activities in microsomes + lysosomes and mitochondrial fractions (gray bars) and cytosol (black bars) of gills from control fish. Values are mean ± 1 SEM, n = 6 fish. * Indicate significant difference from cytosolic fractions (unpaired t-test, $p < 0.05$)

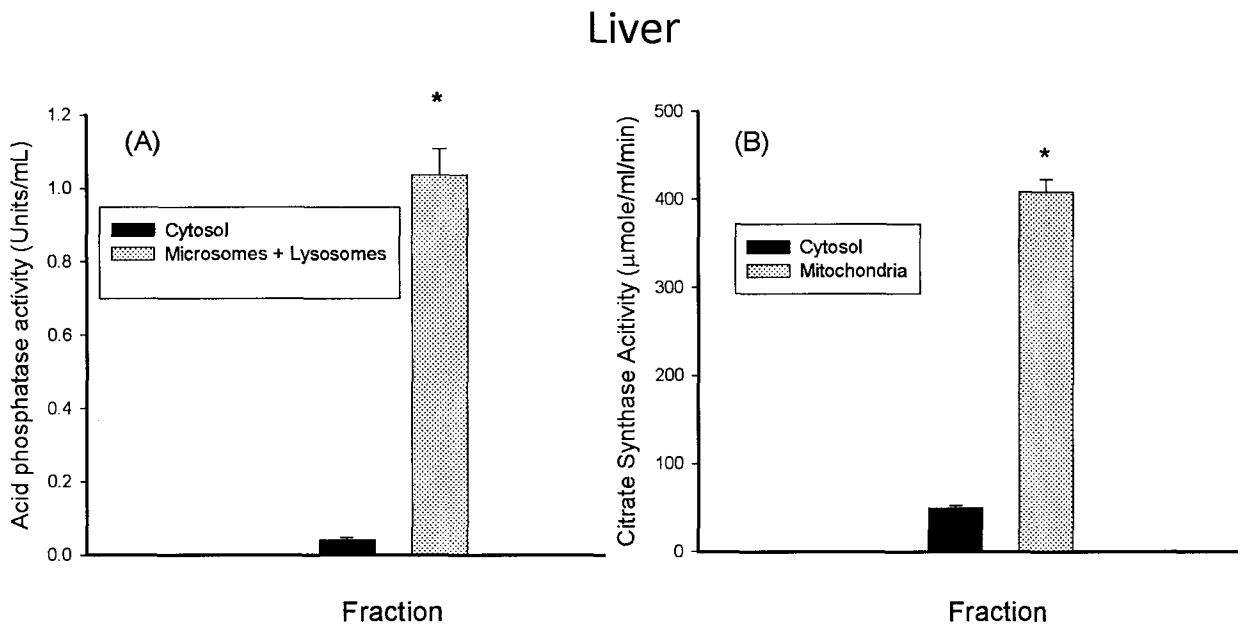

Fig. 2.2 Acid phosphatase (A) and citrate synthase (B) activities in microsomes + lysosomes and mitochondrial fractions (gray bars) and cytosol (black bars) of livers from control fish. Values are mean ± 1 SEM, n = 6 fish. * Indicate significant difference from cytosolic fractions (unpaired t-test, $p < 0.05$)

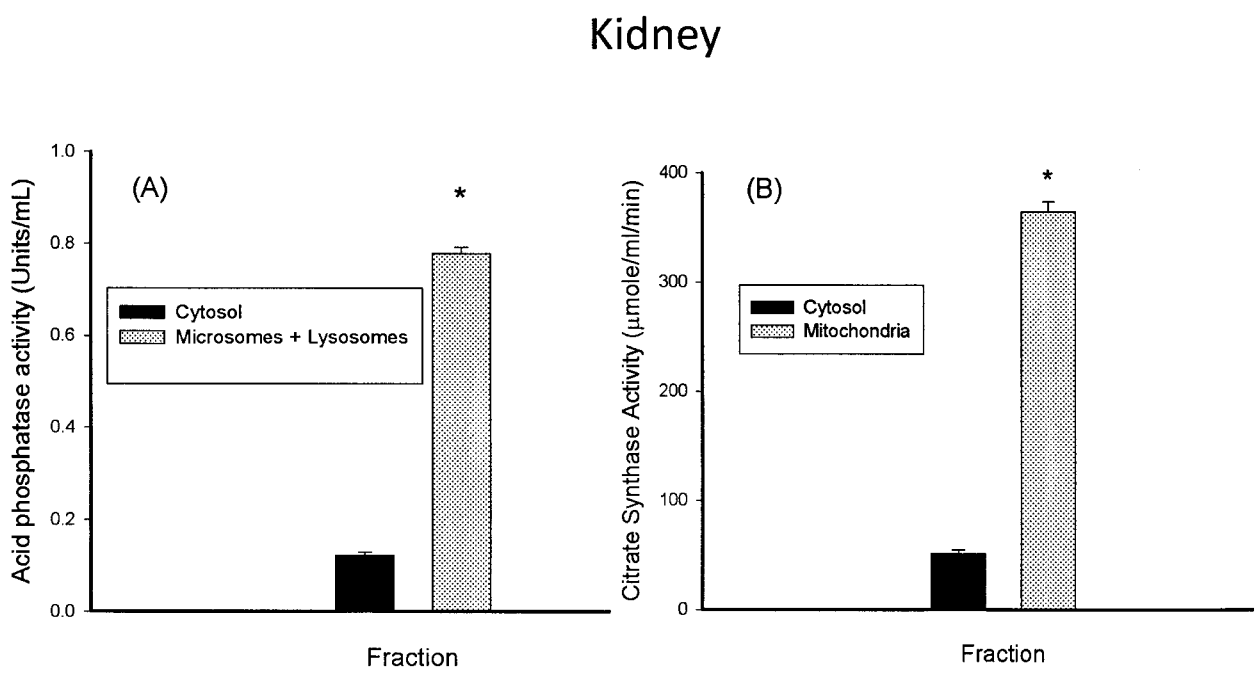

Fig. 2.3 Acid phosphatase (A) and citrate synthase (B) activities in microsomes + lysosomes and mitochondrial fractions (gray bars) and cytosol (black bars) of kidneys from control fish. Values are mean ± 1 SEM, n = 6 fish. * Indicate significant difference from cytosolic fractions (unpaired t-test, $p < 0.05$)

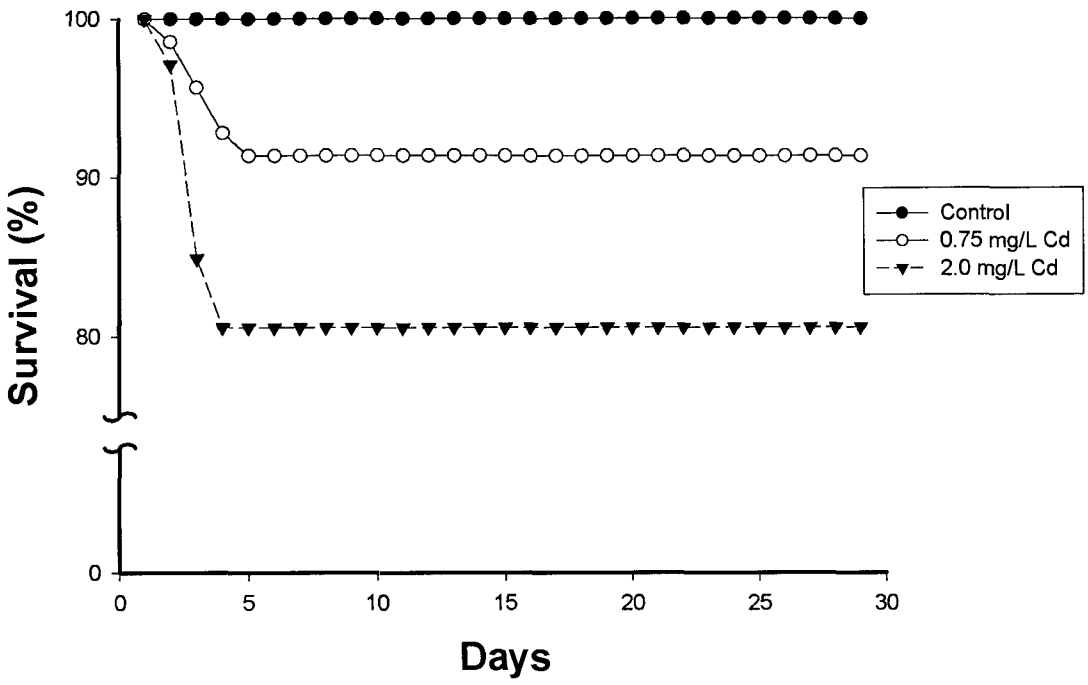

Fig. 2.4 The effect of Cd exposure to rainbow trout (*Oncorhynchus mykiss*) 27.1 g ± 7.64 SD, n = 117) survival (%) throughout 29-day exposure to control (nominal 0), 0.75 and 2.0 μg/L of waterborne Cd in moderately hard water. Initially, n = 140 fish per treatment.

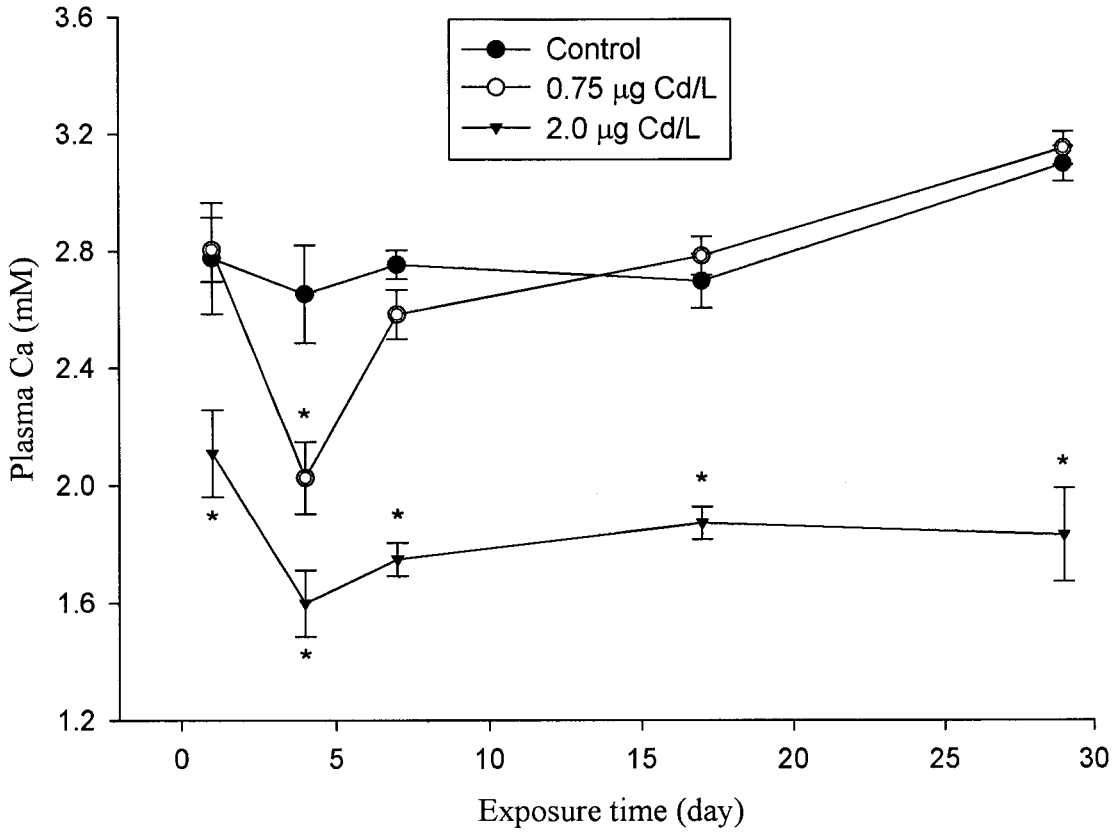

Fig. 2.5 The effect of waterborne Cd exposure to plasma Ca^{2+} concentrations in rainbow trout (*Oncorhynchus mykiss*) during 29-day exposure to control (nominal 0), 0.75 and 2.0 µg/L in moderately hard water. Values are means ± 1 SEM, n = 6. * indicates a significant difference compared to controls (ANOVA; Dunnet's test, $p < 0.05$)

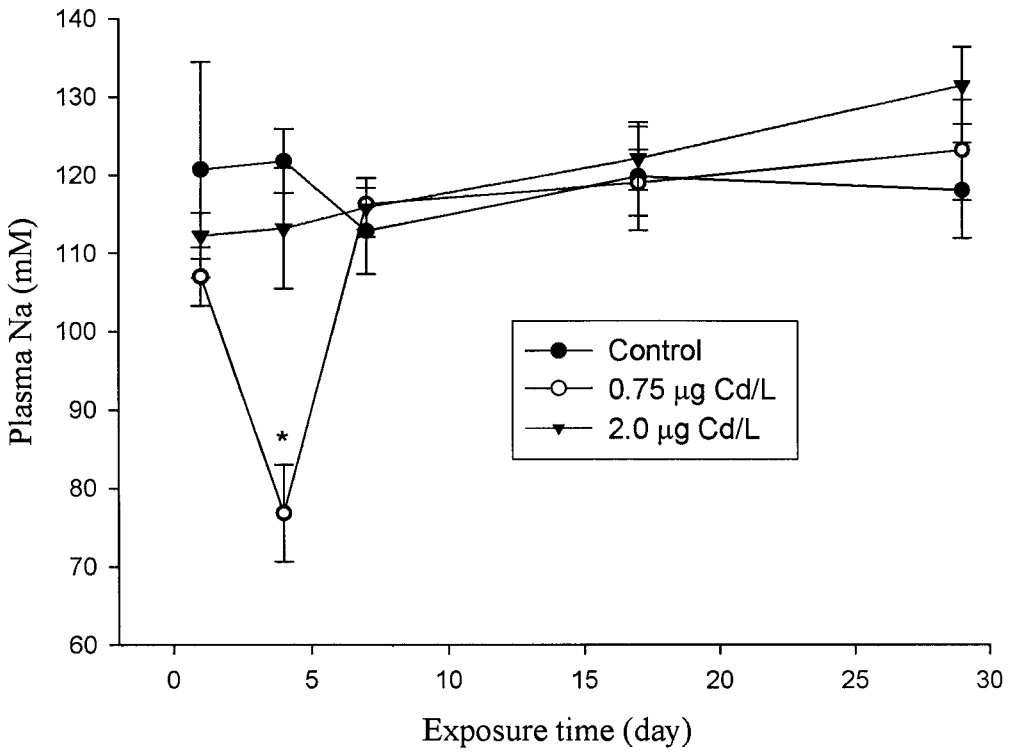

Fig. 2.6 The effect of waterborne Cd exposure to plasma Na^+ concentrations in rainbow trout (*Oncorhynchus mykiss*) during 29-day exposure to control (nominal 0), 0.75 and 2.0 µg/L in moderately hard water. Values are means ± 1 SEM, n = 6. * indicates a significant difference compared to controls (ANOVA; Dunnet's test, $p < 0.05$)

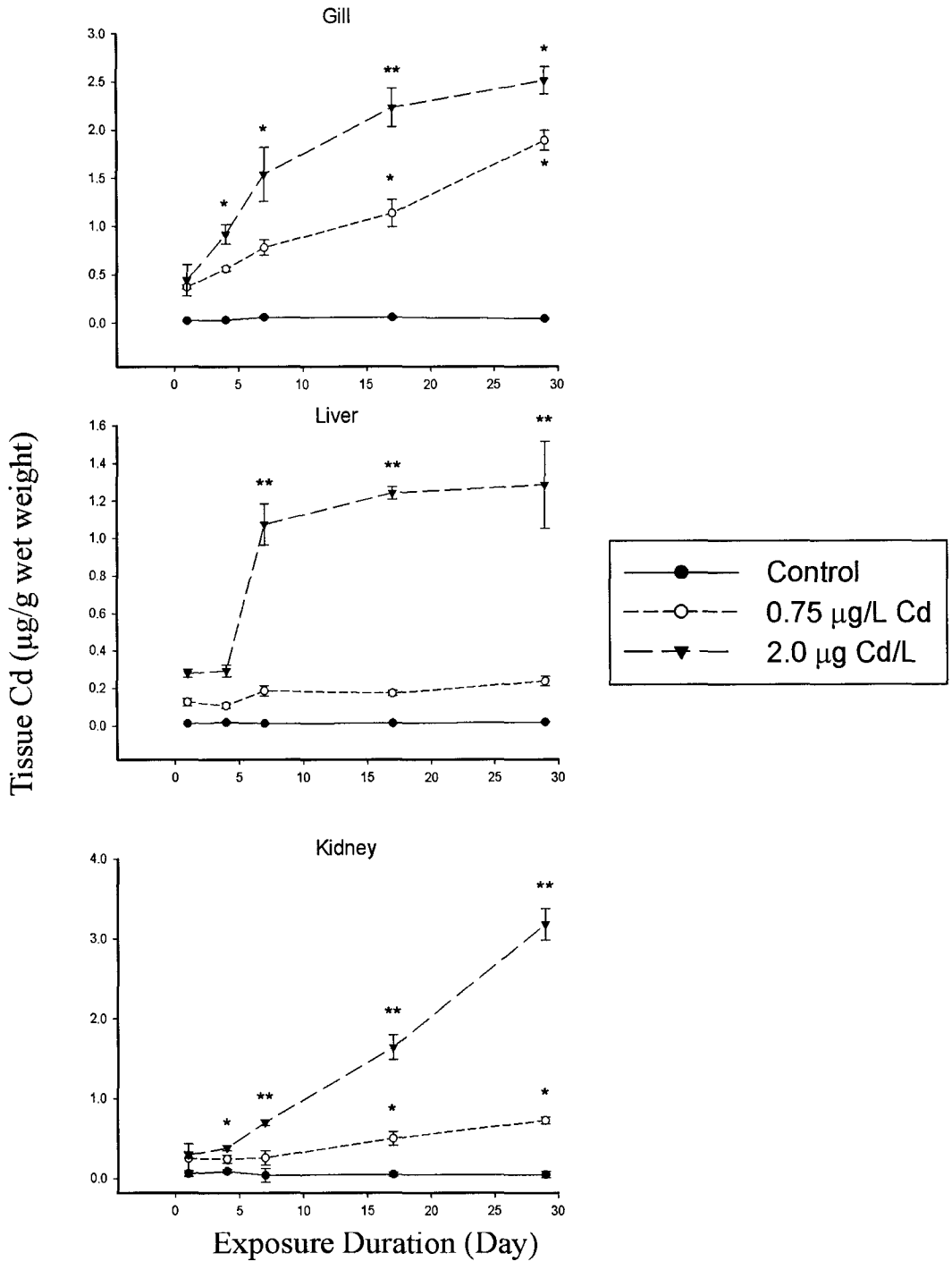

Fig. 2.7 Dose- and time-course of Cd accumulation in rainbow trout (*Oncorhynchus mykiss*) gill, liver and kidney during 29-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd. Values are means ± 1 SEM, n = 6 fish. * indicate significance compared to control values whereas ** indicate significance from both control values and between treatment groups (Anova; Tukeys HSD, $p < 0.05$)

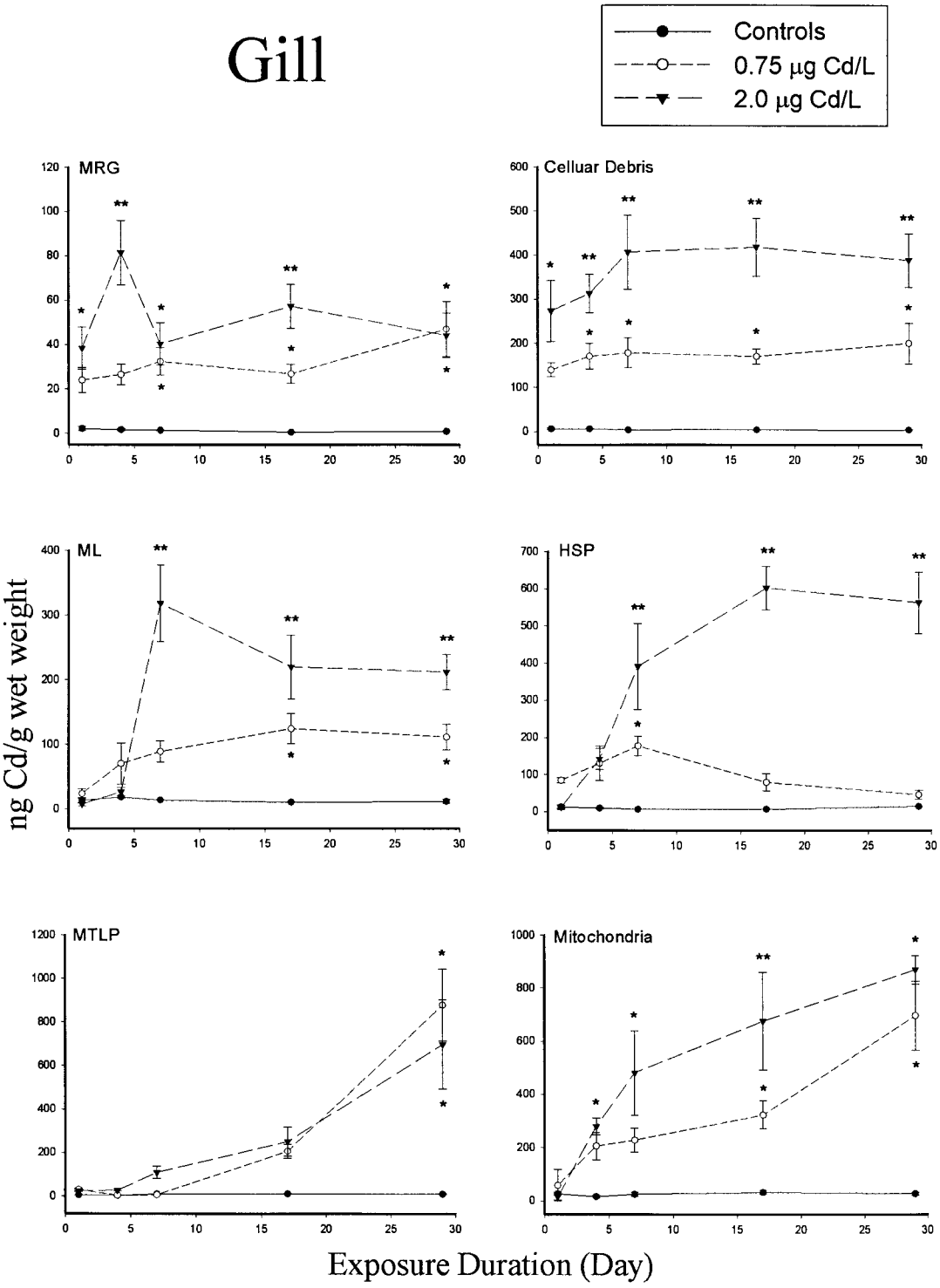

Fig. 2.8 Dose- and time-course of Cd accumulation in gill subcellular fractions of rainbow trout (*Oncorhynchus mykiss*) during 29-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately hard water. Values are means ± 1 SEM, n = 6 fish. * indicate significance compared to control values whereas ** indicate significance from both control values and between treatment groups (Anova; Tukeys HSD, $p < 0.05$)

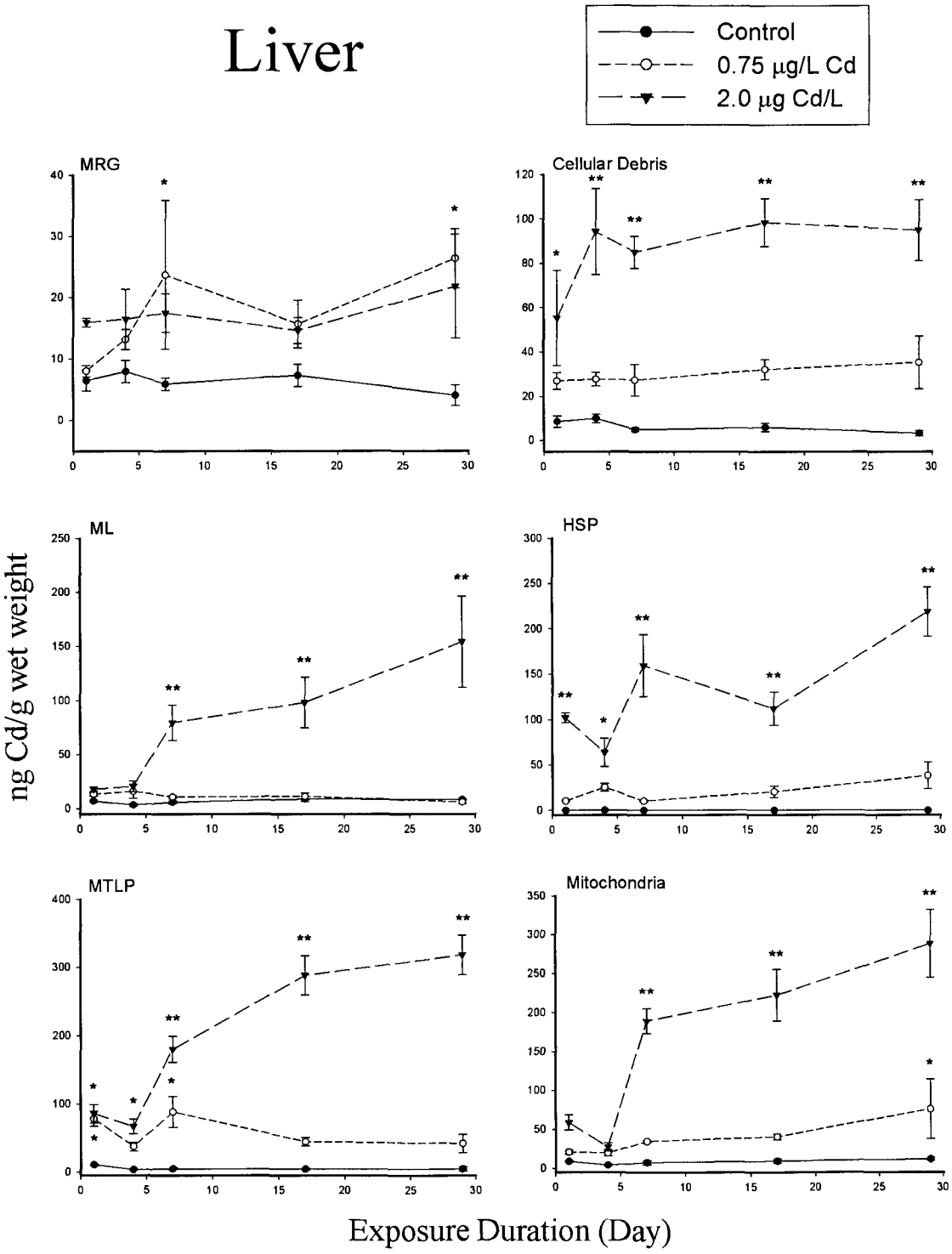

Fig. 2.9 Dose- and time-course of Cd accumulation in liver subcellular fractions of rainbow trout (*Oncorhynchus mykiss*) during 29-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately hard water. Values are means ± 1 SEM, n = 6 fish. * indicate significance compared to control values whereas ** indicate significance from both control values and between treatment groups (Anova; Tukeys HSD, $p < 0.05$)

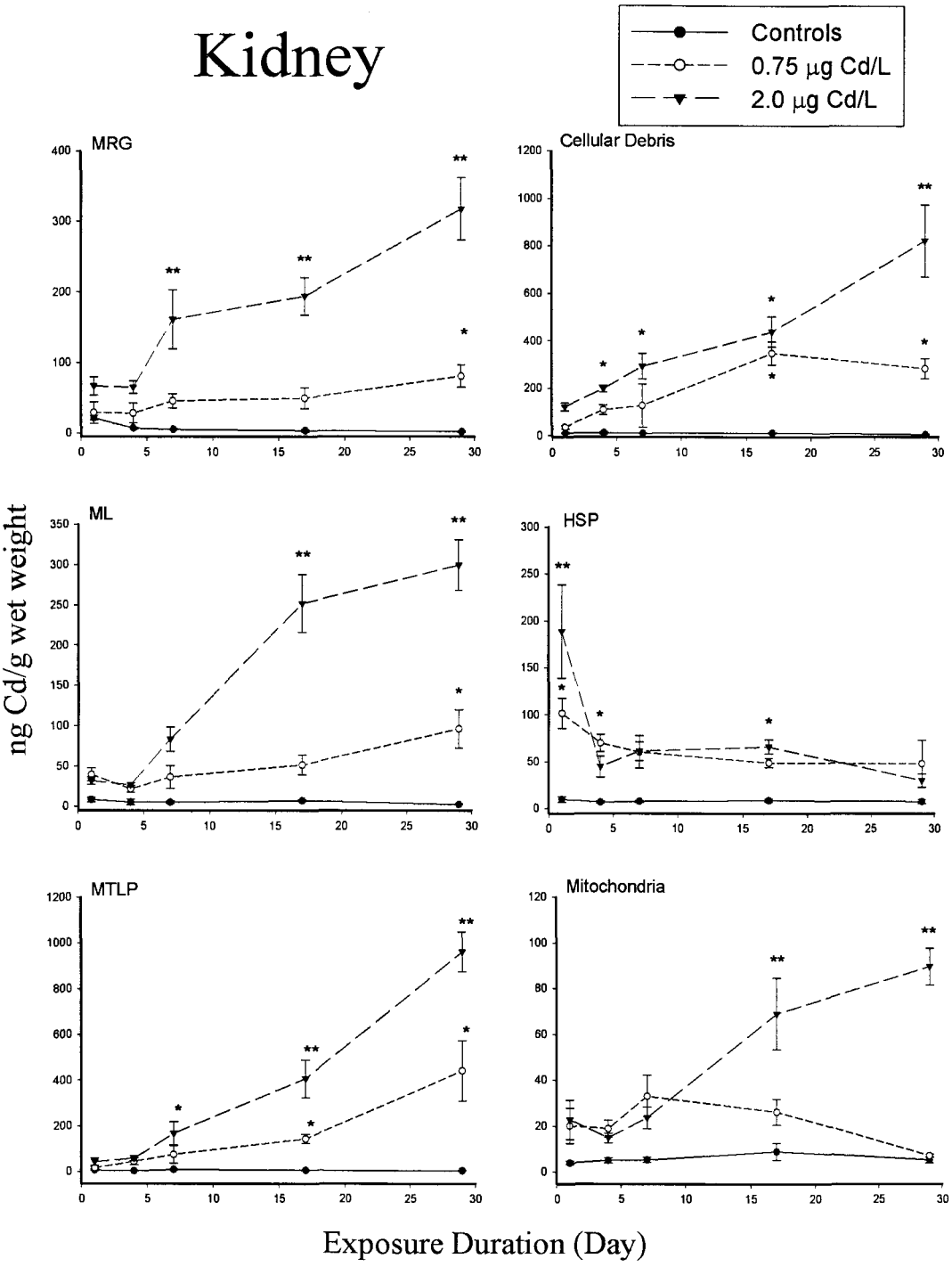

Fig. 2.10 Dose- and time-course of Cd accumulation in kidney subcellular fractions of rainbow trout (*Oncorhynchus mykiss*) during 29-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately hard water. Values are means ± 1 SEM, n = 6 fish. * indicate significance compared to control values whereas ** indicate significance from both control values and between treatment groups (Anova; Tukeys HSD, $p < 0.05$)

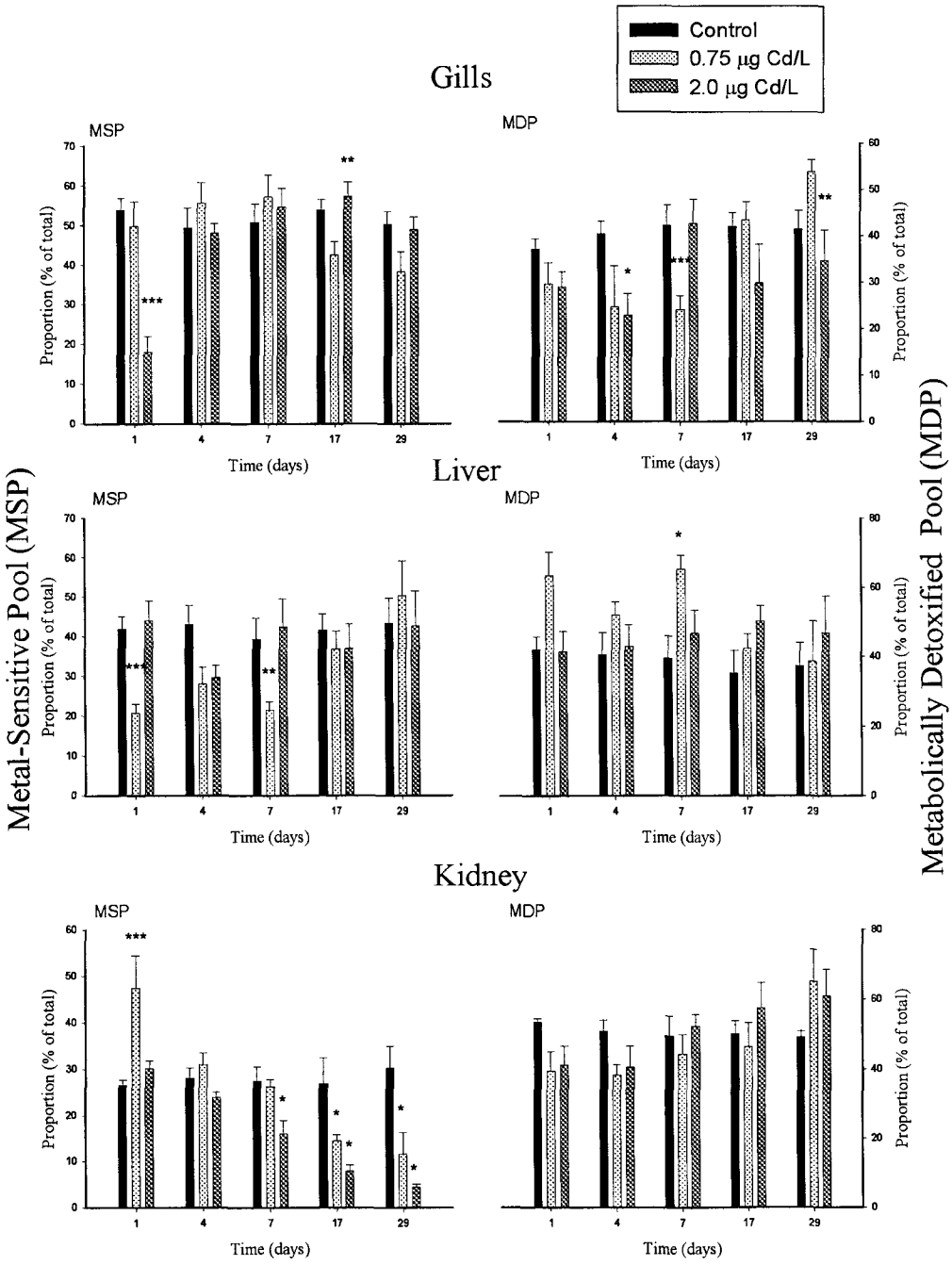

Fig. 2.11 Relative proportions (%) of Cd distributed between metal-sensitive and detoxified pools (MSP and MDP) in rainbow trout (*Oncorhynchus mykiss*) during 29-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately hard water. Values are means ± 1SEM, n = 6 fish. * indicate significance against control values whereas ** indicate significance between treatments and *** indicate significance from both control values and between treatment groups at each time point (day) (Anova; Tukey's HSD, $p < 0.05$)

Table 2.1 Measured exposure concentrations of Cd from head tanks and fish tanks taken during the 29-day exposure to juvenile rainbow trout (*Oncorhynchus mykiss*). Temperature of exposure water ranged from 9.9 – 11.6 °C. Values are expressed as means ± 1SEM (n); HT-F: filtered water samples from head tanks; HT-UF: un-filtered water samples from head tanks; FT-F: filtered water samples from fish tanks; FT-UF: un-filtered water samples from fish tanks.

Exposure	HT-F	HT-UF	FT-F	FT-UF
Control (0)	0.02 ± 0.004 (4)	0.06 ± 0.023 (4)	0.03 ± 0.002 (4)	0.08 ± 0.026 (6)
0.75 µg Cd/L	0.66 ± 0.137 (5)	0.79 ± 0.113 (3)	0.71 ± 0.101 (6)	0.78 ± 0.069 (6)
2.0 µg Cd/L	1.87 ± 0.252 (4)	2.05 ± 0.565 (2)	1.85 ± 0.119 (6)	2.02 ± 0.106 (6)

Table 2.2 Linear regression analyses of Cd concentrations in rainbow trout (*Oncorhynchus mykiss*) gill subcellular fractions against the total gill Cd concentrations throughout all sampling days (1-29), within the first week (1-7) and after one week (7-29) of Cd exposure (0.75 and 2.0 μg Cd/L) in moderately hard water. Values are coefficients of determination (r^2) and asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS: not significant; MT: metallothionein; M + L: microsomes + lysosomes; MTLP: metallothionein-like proteins; Mito: mitochondria; MRG: metal-rich granules.

Subcellular Fraction	Days 1-29	Days 1-7	Days 7-29
M + L	0.0684*	NS	NS
MTLP	0.255***	NS	0.125*
Mito	0.254***	NS	NS
Enzymes	0.138**	NS	NS
Nuclei-debris	NS	NS	NS
MRG	NS	NS	NS

Table 2.3 Linear regression analyses of Cd concentrations in rainbow trout (*Oncorhynchus mykiss*) liver subcellular fractions against the total liver Cd concentrations throughout all sampling days (1-29), within the first week (1-7) and after one week (7-29) of Cd exposure (0.75 and 2.0 µg Cd/L) in moderately hard water. Values are coefficients of determination (r^2) and asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS: not significant; MT: metallothionein; M + L: microsomes + lysosomes; MTLP: metallothionein-like proteins; Mito: mitochondria; MRG: metal-rich granules.

Subcellular Fraction	Days 1-29	Days 1-7	Days 7-29
M + L	0.457***	0.752***	0.391***
MTLP	0.558***	0.544**	0.486***
Mito	0.599***	0.687***	0.509***
Enzymes	0.451***	0.505***	0.434***
Nuclei-debris	0.303***	0.164*	0.477***
MRG	NS	NS	NS

Table 2.4 Linear regression analyses of Cd concentrations in rainbow trout (*Oncorhynchus mykiss*) kidney subcellular fractions against the total kidney Cd concentrations throughout all sampling days (1-29), within the first week (1-7) and after one week (7-29) of Cd exposure (0.75 and 2.0 µg Cd/L) in moderately hard water. Values are coefficients of determination (r^2) and asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS: not significant; MT: metallothionein; M + L: microsomes + lysosomes; MTLP: metallothionein-like proteins; Mito: mitochondria; MRG: metal-rich granules.

Subcellular Fraction	Days 1-29	Days 1-7	Days 7-29
M + L	0.669***	0.182**	0.615***
MTLP	0.657***	0.128*	0.591***
Mito	0.595***	0.112*	0.583***
Enzymes	NS	NS	NS
Nuclei-debris	0.687***	0.210*	0.645***
MRG	0.702***	0.315***	0.655***

Chapter 3

Tissue and Subcellular Distribution of Cd during a chronic sublethal exposure to rainbow trout (*Oncorhynchus mykiss*) in moderately soft water

3.1 Abstract

In aquatic ecosystems, Cd concentrations are elevated over natural background levels due to a continuous release from industrial and agricultural sources. During conditions of acute, high Cd exposure, the ability of the fish to maintain branchial ionregulation and gas exchange is important for the survival of the fish. Ionoregulation may also be affected during chronic sublethal Cd exposure however accumulation and potential effects of Cd within internal organs such as the liver and kidney may gain importance (McDonald and Wood, 1993). During chronic Cd toxicity, a damage-repair-acclimation scenario can be observed in some physiological responses such as ionoregulation, however it is unclear if this scenario pertains to other responses. The objective of this project was to characterize the bioaccumulation of Cd in rainbow trout (*Oncorhynchus mykiss*) during sublethal (0.75 and 2.0 μg Cd/L) chronic waterborne exposure in moderately soft water (50 mg/L as $CaCO_3$) in comparison to a previous study conducted in moderately hard water (140 mg/L as $CaCO_3$). Accumulation was studied at both tissue and subcellular levels of organization in order to establish linkages between exposure, uptake, detoxification and accumulation in toxicologically sensitive pools within the cell. This study also examined metallothionein induction in relation to tissue specific accumulation and subcellular partitioning of Cd. Induction of metallothionein was greatest in kidney, followed by liver and gills as indicated by metallothionein concentrations and Cd levels in MTLP. However, detoxification by metallothionein and other mechanisms (granule and lysosomal sequestration) was imperfect, as Cd partitioned into metal-sensitive fractions (mitochondria and HSP) throughout the course of the exposure. Similar to the previous study, the kidneys appeared to dissipate Cd from MSP with time and exposure. Cd bound to MSP throughout the duration of the exposure and thus elevated Cd concentrations were not linked to mortality and plasma ion loss which were transient. Lastly, the protective effects of Ca^{2+} were observed on both a tissue and subcellular level, as lower amounts of Cd accumulated in tissues (particularly gills) and sensitive fractions during chronic sublethal exposures in moderately hard water.

3.2 Introduction

Cd is a highly toxic metal that is released into aquatic and terrestrial ecosystems by industrial activities which poses a significant health risk to both human and wildlife when concentrations become elevated from background values. In aquatic ecosystems, Cd concentrations are elevated over natural background levels due to a continuous release from industrial and agricultural sources. Numerous physiological impacts result from Cd exposure including disruption of ionoregulation, energy metabolism and stress response elements (McGeer et al., 2010). During conditions of acute, high Cd exposure, the ability of the fish to maintain branchial ionregulation and gas exchange is important for the survival of the fish. Ionoregulation may also be affected during chronic sublethal Cd exposure however accumulation and potential effects of Cd within internal organs such as the liver and kidney may gain importance (McDonald and Wood, 1993). During chronic Cd toxicity, a damage-respair-acclimation scenario can be observed in some physiological responses such as ionoregulation, however it is unclear if this scenario pertains to other responses. Key factors that will most likely determine metal toxicity are tissue distribution of accumulated Cd, intracellular Cd partitioning and the relationship between tissue burden and toxic response. For example, Hollis et al. (2000a) demonstrated that fish previously exposed to Cd (0.07 or 0.11 μg Cd/L) accumulated significantly less "new" Cd in their gills compared to controls when exposed acutely to Cd concentrations below 25 μg/L in soft water. These findings suggest that fish chronically exposed to Cd appear to develop a protective mechanism for reduced uptake and/or increased detoxification of Cd (Hollis et al., 1999; Hollis et al, 2000a; Hollis et al., 2001).

The uptake of Cd in fish exposed to ambient waterborne Cd is primarily through the gills. Numerous studies have characterized branchial Cd uptake and conclude that Cd^{2+} is an analogue to Ca^{2+}, thus Cd competes for Ca uptake pathways that exist at both the apical and basolateral membrane of chloride cells (Verbost et al., 1988, 1989; Niyogi and Wood, 2004; Niyogi, 2008). Acute toxicity of Cd occurs due to a decrease Ca uptake into the body of the fish which has been associated with the inhibition of Ca^{2+}-ATPase and/or resulting blockade of Cd at apical voltage gated Ca ion chanels (Verbost et al.,

1988, 1989). However, these effects can change depending on water hardness (Pärt et al., 1985; Carrol et al., 1979). Numerous studies (Carrol et al., 1979; Calamari et al., 1980; Pärt et al., 1985; Davies et al., 1993) have shown that protection against acute Cd toxicity is related to Ca concentrations in the water (rather than Mg) thus Ca is the primary cation responsible for protective action in hard water. The protective effects of Cd have been attributed to changes in gill permeability and/or competition between Cd and Ca for binding sites at the gills (Calamari et al., 1980; Pärt et al., 1985). In soft water, lower concentrations of Cd can generate toxic effects in fish compared to hard water.

Cadmium has been shown to elicit protective mechanisms against toxicity in fish during both acute and chronic exposures (McDonald and Wood 1993; Hollis et al., 2001). A variety of detoxification and storage mechanisms within fish help to regulate and immobilize Cd; these include, metal binding proteins such as metallothionein, sequestration and storage in lysosomes (Amiard et al., 2006) and to a lesser extent granules (Campbell et al., 2005, 2008; Kamunde and MacPhail, 2008; Kamunde, 2009). Metallothionein has been identified as one of the mechanisms for cellular sequestration of Cd employed by fish in response to increased ambient Cd (Roch et al., 1986; Olsson and Hogstrand, 1987; Kraemer et al., 2005; Campbell et al., 2008), reducing the amount of freely available Cd in cells to interact with metal-sensitive compartments, thereby decreasing toxicity (Kraemer et al., 2005). The induction and metal binding abilities of metallothionein has been proposed in many studies as a biomarker for metal exposure (Roch et al., 1986; Deeds and Klerks, 1999) and metallothionein biosynthesis may be a key step in the transition from damage, to repair and acclimation phases during chronic Cd exposure (McDonald and Wood, 1993).

Metallothionein is a low-molecular weight (6, 000 g/mol; Olsson and Haux, 1985), cysteine rich protein that is important in the homeostatic control of essential metals (Cu, Zn) as they bind and store metal for enzymatic and metabolic processes (Amiard et al., 2006). Non-essential metals such as Cd are able to displace essential metals bound by metallothionein. The binding behaviour of metallothionein is dominated by the chemistry of the thiol group, such that metals resembling the stoichiometric properties

of Cu or Zn can also be bound by metallothionein (Templeton and Cherian, 1991). Synthesis of MTLP for the detoxification (maintaining low cytosolic metal concentrations; Sterling et al., 2010) of Cd in target tissues is known to play an important role in acquired tolerance during acclimation (Hollis et al., 2001; Chowdhury et al., 2004).

Previous studies (Hollis et al., 2001; Chowdhury et al., 2005) examining metallothionein induction during chronic waterborne exposures of Cd to rainbow trout (*Oncorhynchus mykiss*) have shown that the kidney attained the highest increase in metallothionein levels in comparison to other tissues, followed by the gill, then liver. These studies investigated the relationship between tissue-specific Cd accumulation and metallothionein induction to determine whether metallothionein buildup quantitatively matched Cd buildup in tissues (gills, liver and kidney). Although metallothionein concentrations in all tissues increased throughout the 30 days of exposure, the increases were much less than those of Cd based on a molar binding site basis (Hollis et al., 2001; Chowdhury et al., 2004). Therefore, in order to assess the induction of metallothionein and metal-binding abilities, the subcellular accumulation of Cd should be examined to quantify Cd in MTLP fractions, and other detoxified and sensitive compartments to reveal how much protection metallothionein can offer against Cd toxicity at a cellular level.

Generally, studies have confirmed that the increases in Cd accumulation during chronic waterborne exposure occur in the order of kidneys > gills > liver (Szebedinszky et al. 2001; Chowdhury et al. 2005; McGeer et al., 2010). However, partitioning of Cd between intracellular compartments within tissues that accumulate Cd is poorly understood, and subcellular partitioning may be used as a potential indicator of toxicity in fish during chronic sublethal exposures. Generally, studies (Wallace et al., 2003; Kraemer et al., 2005; Kamunde, 2009) have examined five subcellular fractions; MRG, MTLP, cellular debris, organelles (mitochondria, ML) and HSP. Different studies have further categorized fractions into metal-sensitive (mitochondria and HSP) and metabolically detoxified (MRG, MTLP) pools. Contrary to the spill-over hypothesis which claims that low ambient or tissue concentrations of metal will bind entirely to MDP prior to spilling over into MSP, recent studies on fish collected in the field (Campbell et

al., 2005; 2008; Giguère et al., 2005; 2006; Kraemer et al., 2005) and in the laboratory (Kamunde, 2009) revealed that Cd was associated with MSP along metal contamination gradients, independent of time and tissue burdens. Potentially, differences in Cd concentrations may solely be explained by differences in the number and affinity of Cd-binding sites within each tissue and fraction. Additionally, no redistribution of Cd between subcellular pools was observed even at Cd concentrations that are indicative of stressful conditions, thus aquatic organism tested to date may not be able to regulate Cd distribution between subcellular fractions

In the previous study (Chapter 2), tissue accumulation and subcellular distribution of Cd were examined in juvenile rainbow trout (*Oncorhynchus mykiss*) chronically exposed to sublethal concentrations (0.75 and 2.0 μg Cd/L) of waterborne Cd in moderately hard water. This study revealed that acute toxic effects (i.e. mortality and ion loss) may link to Cd partitioning into metal-sensitive compartments, particularly in the gill, however partitioning alone could not link to adverse effects as Cd continuously spilled-over into sensitive fractions after one week of exposure. The kidneys contained the highest amount of Cd bound to MTLP and the least amount of Cd bound to metal-sensitive compartments by day 30 in fish exposed to 2.0 μg Cd/L. However, the pattern of subcellular partitioning of Cd may be different in lower Ca (moderately soft; 50 mg/L as $CaCO_3$) water due to changes that occur at the gills in response to soft water acclimation (i.e. increased binding sites) to compensate for low ambient Ca (Hollis et al., 2000b), which could also result in increased uptake of Cd into tissues.

The objective of this research was to characterize the bioaccumulation of Cd in rainbow trout (*Oncorhynchus mykiss*) during sublethal (0.75 and 2.0 μg Cd/L) chronic waterborne exposure to juvenile rainbow trout (*Oncorhynchus mykiss*) in moderatlely soft water (50 mg/L as $CaCO_3$) in comparison to a previous study conducted in moderately hard water (140 mg/L as $CaCO_3$). Accumulation was studied at both tissue and subcellular levels of organization in order to establish linkages between exposure, uptake, detoxification and accumulation in toxicologically MSP within the cell. This study also examined metallothionein induction in relation to tissue specific accumulation and subcellular partitioning of Cd.

Examining patterns of tissue and subcellular distribution when rainbow trout (*Oncorhynchus mykiss*) are chronically exposed to sublethal concentrations of waterborne Cd will help determine if tissues (liver, kidney and gills) differ in their detoxification ability to protect Cd from accumulating within metal-sensitive fractions (mitochondria, HSP) of the cell. Furthermore, this study tested the spill-over hypothesis to determine if there is a threshold concentration within each tissue whereby Cd begins to accumulate in metal-sensitive fractions. This research also examined the protective effects of waterborne Ca^{2+} (a component of water hardness, measured as $CaCO_3$) on accumulation of Cd at both tissue and subcellular organizational levels. For the objectives mentioned above, the four working hypotheses related to the response of rainbow trout (*Oncorhynchus mykiss*) to Cd exposure are as follows: (1) tissue concentrations of Cd within rainbow trout (*Oncorhynchus mykiss*) will increase in a time- and dose-dependent manner, (2) in comparison to liver and kidney, the gills will be most sensitive to Cd accumulation and will contain lower amounts of Cd bound to detoxified compartments (e.g. due to metabolically active mitochondrial-rich transport cells that take up Cd)) (3) adverse effects (mortality or ion loss) are linked to spill-over of Cd into metal-sensitive fractions (HSP and mitochondria) and (4) Ca^{2+} concentrations in moderately hard water will dampen increases of Cd in tissues in relation to soft water and therefore "protect" against accumulation in metal sensitive compartments.

3.3 Materials and Methods

3.3.1 Fish husbandry and acclimation

Juvenile rainbow trout; 32.4 ± 12.2 g (mean ± SD, n = 100) were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada). Initially, fish were held in 180-L tanks (2 tanks with 170 fish in each) with water flowing to each tank at 700 mls/min. Water was a 1:1 mix of well water and soft water produced by reverse osmosis (500 mg/L as $CaCO_3$, 650 μScm^{-1}, pH 7.6, 10.9 °C). Fish were acclimated to moderately soft water by gradually decreasing the flow of the well water over a three-week period. After acclimation to moderately soft water for two-weeks, fish were randomly distributed among six 200-L

polyethylene tanks (50 fish per control tanks (2); 60 fish per exposure tanks (4)). A 60-L polyethylene mixing head tank received 3.6-L/min of soft water plus 600 mls/min of well water, for a total of 4.2-L/min to achieve the chemistry of moderately soft water used for experimental exposures (53.7 mg/L as $CaCO_3$: 360 ± 22 Ca, 178 ± 13 Mg, 368 ± 20 Na (all in μM, mean ± 1 SD, n = 37)), with a conductivity, pH and temperature of 90 μS/cm, pH 6.8, 9.3 °C respectively. The mixing head tank delivered water (1.4-L/min) to three smaller 11.2-L polyethylene head tanks that have equally divided (split) outflows of water (700 mls/min) delivered to two fish tanks each. All water in head tanks and fish tanks were well aerated. Fish were acclimated to their respective tanks for two weeks prior to Cd exposure and fed at 2% of their body weight daily as a single meal (Bio Oregon Protein Inc., Warrenton, OR).

3.3.2 Cd Exposure

Two head tanks were used for Cd exposures at nominal concentrations of 0.75 and 2.0 μg Cd/L (as $CdCl_2$, VWR International, Mississauga, ON, Canada); the remaining head tank was used for control (nominal 0). The measured dissolved concentrations in fish tanks were (means ± 1 SEM. (n)): 0.01 ± 0.033 (4) μg Cd/L (control), 0.73 ± 0.063 (6) μg Cd/L, 1.82 ± 0.212 (6) μg Cd/L (see Table 3.1 for all measured exposure concentrations). The three exposure conditions (control (0), 0.75 and 2.0 μg Cd/L) occurred in duplicates, therefore n = 100 fish for controls and n = 120 fish per treatment. Initially, head tanks and fish tanks were spiked with appropriate volumes from a master stock of 1.0 g/L of Cd to achieve exposure concentrations. Appropriate volumes of the master stock were added to two 10-L carboys, each delivering Cd solution to the head tanks via rotator pumps (FIM lab pump, Fluid Metering Inc., Oyster Bay NY; 1.2-mls/min) to maintain the desired Cd concentrations in exposure tanks. The Cd solutions in each 10-L carboy were renewed weekly. Water pH, conductivity (μS) and temperature were also measured weekly using a pH meter (Mettler Toledo SevenGo™, Fisher Scientific) and conductivity and temperature using a conductivity meter (YSI 30, Yellow Springs Instruments, Yellow Springs, Ohio).

3.3.3 Sampling

The exposure was carried out for 30 days and samples were collected on days 1, 4, 7, 16 and 30, fish were fed throughout the duration of the exposure except the day prior to sampling. At each sampling time, both

filtered (0.45 μm syringe filter; 22 mm Acrodise HT tuffryn membranes, Pall Corporation, Ann Arbor, MI, USA) and un-filtered 10 ml water samples were taken from exposure tanks, acidified to 1% by adding 100 μl of concentrated HNO_3 (Trace Metal Grade, Fisher Scientific, Mississuaga ON). Additionally, 6 fish per exposure replicate (n = 12 per treatment) were non-selectively netted and euthanized with an overdose of 0.3 g/L tricaine methanesulfonate (Syndel Laboratories Ltd., Vancouver, BC, Canada) buffered with a 0.6 g $NaHCO_3$ /L.

Blood samples were also taken from fish sacrificed for tissue and subcellular Cd accumulation to measure plasma Na^+ and Ca^{2+} concentrations. Blood samples (300-1000 μl) were taken by caudal puncture and collected in 1.5-mL centrifuge tubes containing 10 μL of lithium heparinised saline (100 units) to minimize clotting. Blood samples were immediately centrifuged at 10,000 g for 2 minutes. Plasma was then removed using 1.5 mL insulin syringes, saved in new 1.5-mL centrifuge tubes and stored at -20°C.

Tissue samples of liver, kidney and gills were collected from all sampled fish. All tissues were rinsed for 10 seconds in deionized water, blotted dry then divided into two portions, saved in 1.5 mL centrifuge tubes, frozen in liquid nitrogen then stored at -80°C until analysis of Cd content. One portion of the tissue (liver, kidney, and gill) was used for total Cd concentration, while the remaining portion was used for subcellular Cd distribution analysis. Tissue samples were not large enough to perform both tissue and subcellular Cd accumulation, and metallothionein analyses on the same fish. Separate tissue samples were collected, immediately frozen in liquid nitrogen and stored at -80 °C until analysis for metallothionein concentrations.

3.3.4 Analytical techniques

All liquid and dry chemicals were obtained from Fisher Scientific (Mississauga, ON, Canada) unless otherwise mentioned. Plasma samples were thawed and diluted, 50 times for Ca^{2+} and 1000 times for Na^+ using ultra-pure water. Diluted samples were analysed for concentrations of both ions using atomic absorption spectroscopy (AAS 880, Varian Inc, Palo Alto CA). Water Ca, Mg and Na were similarly measured in flame mode (SpectraAA 880). For quality control, pre-prepared certified reference material

(trace metal fortified Lake Ontario water [TMDA-28.3 and TM 26.3]; National Water Research Institute, Burlington, ON, Canada) and blanks (MilliQ water and solutions used for digestion) were analyzed by GFAAS together with experimental samples.

Gills, liver and kidney portions (75 - 300 mg) were weighed, thawed and digested in 5-fold of their wet weight with 1N TraceMetal grade HNO_3 then baked for 3 hrs at 80 °C (Playle et al. 1993a, 1993b). After digestion, tissues were vortexed for 5 seconds, then centrifuged for 2 min at 10,000 rpm (Spectrafuge 16M; Labnet International, Edison, NJ, USA), and left to settle. The supernatant was diluted to 10- to 100-fold with 1% acidified (concentrated HNO_3, TraceMetal grade) ultra-pure water to measure Cd concentrations. Water and tissue samples were measured for Cd using graphite furnace atomic absorption spectrophotometer (GFAAS; SpectraAA 880 GTA 100 atomizer, Varian, Mississauga, Ontario). The concentration of Cd in all measured tissue and subcellular compartments are expressed as μg/g wet weight

3.3.5 Subcellular distribution analyses

The differential centrifugation protocol of Kamunde and MacPhail (2008) was adapted for the isolation of tissue subcellular compartments (see fig.5.): (1) a fraction comprised of nuclei, cell membranes, intact cells and connective tissue, (2) a granule-like or resistant fraction, (3) mitochondria (heavy and light), (4) a fraction combining microsomes and lysosomes (5) cytosolic heat-stable proteins (HSP) including metallothioneins, and (6) cytosolic heat-denaturable proteins or enzymes (HDP; see Appendix A for protocol).

Individual tissues (gill, liver, kidney; 100 - 500 mg) were homogenized on ice in phosphate-buffered saline [PBS: 137 NaCl; 4.3 KCl; 4.3 Na_2HPO_4; 1.4 NaH_2PO_4 (in mM), pH 7.2] at a dilution of 1:3 of their wet weight by using digital tissue homogenizers set at 20 RPM (Omni THQ; Omni International, Marietta, GA, USA). The resulting tissue homogenates were initially centrifuged at 4°C, 800 g (IEC-CL31R Multispeed; Thermo Electron Corp., Milford, MA, USA) for 15 min. Separation of the granule-like (P1) and supernatant containing nuclei + debris (S2) fraction from the initial centrifugation pellet was performed by (1) re-suspending the pellet (P1) in 0.5 ml ultrapure water, (2)

heating this suspension at 100°C for 2 min, (3) adding 0.5 ml of 1N NaOH (Sigma-Aldrich, Nepean ON, Canada), (4) heating again at 60-70°C for an hour and (5) centrifugation at 10 000 X g for 10 min at 20°C. Separation of mitochondria and lysosomes was performed using a two-step isolation protocol, by centrifuging the S1 supernatant initially at 4°C, 3000 g for 15 min to isolate heavy mitochondria (P3) and subsequently at 4°C, 10,000 g for 30 min to isolate the light mitochondria (P10; both heavy and light mitochondria fractions were combined for analyses). The 10,000 g supernatant (S10) was further ultracentrifuged at 4°C and 100,000 g (Optima MAX; Beckman Instruments, Mississauga, Ontario, Canada) for 60 min, isolating the microsomes and lysosomes fraction (P4) and cytosol (S4). Separation of the HDP (P5) and HSP (S5 or metallotionein-like proteins, MTLP) was done by (1) heating supernatant S4 at 80°C for 10 min, (2) cooling it on ice for an hour and (3) ultracentrifuging it at 50,000 g for 10 min at 4°C. To ensure the purity of each pellet, they were rinsed three times in 1 ml of buffer solution and spun for 10 min at 10 000 x g at 4°C. Upon isolation, all of the subcellular fractions were stored at -80°C until analyzed for Cd accumulation.

All pelleted subcellular fractions were thawed and digested in a 1-to 5-fold of their wet weight with 1N HNO_3 (TraceMetal grade) for 3 hrs at 80°C, whereas the supernatants were digested in a 3- fold volume of 1N HNO_3 (TraceMetal grade) for 3 hrs, also at 80°C. The digests were diluted accordingly with 1% acidified (concentrated HNO_3, TraceMetal grade) ultra-pure water and concentrations of Cd in pellets and supernatants were measured by GFAAS.

3.3.6 Metallothionein analysis

Metallothionein concentrations in the tissues (gill, liver and kidney) of rainbow trout were determined using a spectrophotometric method developed for marine organisms by Viarengo et al. (1997). In this assay, a partially purified metallo-protein fraction is obtained by acidic ethanol/chloroform fractionation of the tissue homogenate. In the extracts, metallothioneins are denatured by low pH and high ionic strength, and quantified spectrophotometrically using the Ellman's SH reagent (see Appendix C for protocol).

Tissue samples (200 - 700 mg) were homogenized on ice in homogenization buffer [0.5 M sucrose, 20 mM Tris-HCl buffer, 0.5 mM PMSF (phenylmethylsulphonylfluoride) and 0.01 % β-mercaptoethanol, pH 8.6] at a dilution 3 to 5 volumes of their wet weight by using an Omni THQ digital tissue homogenizer set at 20 RPM (Omni International, Marietta, GA, USA). The homogenate was then centrifuged at 30,000 x g for 45 min at 4 °C to obtain a supernatant containing metallothionein-like proteins. The supernatant (250 µL) was then treated with ethanol/chloroform as described by Kimura et al. (1979). Briefly, 265 µl of cold (- 20 °C) absolute ethanol and 20 µl of chloroform were added to aliquots of supernatant, then centrifuged at 6000 x g for 10 at 4 °C. The supernatant was collected (300 µL) and combined with 20 µl of HCL (37%) and 900 µl of cold ethanol (100%). The sample was maintained at -20 °C for one hour followed by centrifugation at 6000 x g for 10 min at 4 °C. The metallothionein- containing pellet was then washed with 87 % ethanol and 1 % chloroform solution in homogenizing buffer, then centrifuged at 600 x g for 10 min at 4 C. The pellet was resuspended in 150 µl 0.25 M NaCl and subsequently 150 µl of 1N HCL containing EDTA 4 mM. Additionally, a volume of 1.2 ml 2 M NaCl containing 0.43 mM DTNB (5, 5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8 (Ellmann, 1958) was then added to the sample at room temperature and vortexed. The sample absorbance was evaluated at 412 nm and metallothionein concentration was estimated using reduced glutathione (GSH) as a reference standard.

3.3.7 Statistical analyses

Data are expressed as mean ± 1 SEM. All graphs and statistical analysis were performed using the computer software SigmaPlot 11.0 (Systat Software Inc. (SSI), San Jose, CA, USA) or SPSS 18.0 (SPSS Inc. IBM Company Headquarters, Chicago, IL, USA). All data were checked for normality and homogeneity of variances, and subsequently submitted to a two-way analysis of variance (ANOVA). Tukey's honest significant difference (HSD) post hoc test was used to detect significant differences among the means at a limit of $p < 0.05$, unless mentioned otherwise.

Initially, to ensure the data agreed with distributional assumptions of the ANOVA and other statistical tools, relationships between exposure variables (i.e., treatments, time) and Cd bioaccumulation

(i.e., Cd concentrations in subcellular fractions of liver, gill and kidney, and total Cd concentrations in each tissue) or metallothionein concentration were examined in bivariate scatterplots and tested by simple correlation (Pearson's *r*) and linear regression analyses using non-transformed data. The Kolmogorov–Smirnov test was used to verify that regression standardized residuals were normally distributed and homoscedasticity was checked by examination of the biplots of residuals against predicted values (Bonneris et al., 2005).

Plasma ion concentrations were statistically compared to control values at each sampling day using one-way analysis of variance (ANOVA; Dunnet's test, $p < 0.05$). Relationships between total tissue Cd accumulation and Cd accumulation within the subcellular fractions were examined by linear regression analyses using Pearson's r^2 value and relationships were reported significant at $p < 0.05$. Additionally, Cd concentrations within the MTLP fraction and tissue metallothionein concentration were also submitted to linear regression analyses.

Tissue Cd burdens, subcelluar Cd distribution and metallothionein concentration data were statistically analyzed using two-way ANOVA with time and treatment as independent variables. Statistical comparisons were made between treatments (control (0), 0.75 and 2.0 μg Cd/L) at each time point and within treatments throughout the 29-day exposure (see Appendix D for Anova tables). Proportional data of Cd distributions in MSP and MDP were initially arcsine-transformed and subsequently submitted to a two-way ANOVA.

3.4 Results

3.4.1 Effects of Cd exposure

Mortality was minimal during the 30 day exposure with 0, 3 and 15% mortality for controls, 0.75 μg Cd/L and 2.0 μg Cd/L exposures respectively; mortality ceased after one week (Fig. 3.1). Plasma Ca concentrations (Fig. 3.2) significantly decreased in both treatment groups compared to controls within the first 96 hrs of exposure. However, Ca ions recovered around day 7 in both treatment groups. By the end

of the exposure Ca^{2+} concentrations had returned to baseline levels. Plasma Na concentrations (Fig. 3.3) displayed a similar acute ion loss-recovery pattern as seen with Ca although not statistically significant.

Metal accumulated in all tissues with time and exposure. In both sublethal Cd exposed fish, gradual linear accumulation of Cd was observed in the kidney and liver, whereas accumulation of Cd in the gills was biphasic and characterized by rapid Cd accumulation within the first week followed by saturation and significant decline by day 30 (Fig. 3.4). Gills accumulated the most Cd throughout the exposure and at day 30 contained 7-5x and 5-2x more Cd than liver and kidney, for fish exposed to 0.75 and 2.0 μg Cd/L respectively. In both sublethal Cd exposed fish, concentrations in gills and liver were higher than background values by day 7 and onwards, whereas the kidney did not appreciably accumulate Cd until day 30.

3.4.2 Bioaccumulation and distribution of Cd in subcellular fractions

The concentrations of Cd partitioning into subcellular compartments within the liver, kidney and gills of trout exposed to waterborne Cd are shown in Figs. 3.5 – 3.7. Briefly, Cd concentrations in subcellular fractions; cellular debris, metal-rich granules (MRG), mitochondria, microsomes+lysosomes (ML), heat-sensitive proteins (HSP) and metallothionein-like proteins (MTLP), from fish chronically exposed to sublethal Cd were:

Liver - mitochondria > ML > HSP > MTLP > cellular debris > MRG

Kidney - MTLP > cellular debris > HSP > MRG > mitochondria > ML

Gill – cellular debris > mitochondria > MRG = ML > MTLP > HSP

The recovery of Cd ranged from 91-105, 85-114 and 82-110 % for gill, liver and kidney subcellular fractions in comparison to tissue Cd burdens.

Cd concentrations in gill HSP fraction peaked on day 4 and day 7 for low and high Cd exposed fish respectively, and then significantly declined to levels above control values (Fig. 3.5). Gill cellular

debris fraction Cd concentrations increased significantly with time and fish exposed to both sublethal Cd concentrations attained comparable amounts (Fig. 3.5). Similarly, gill mitochondrial fraction Cd concentrations gradually increased to levels higher than control values by day 7 and onwards, however Cd concentrations in fish exposed to 2.0 μg Cd/L increased higher than both control and low Cd exposed fish (Fig. 3.5).

Liver subcellular fractions gradually accumulated Cd when exposed to 0.75 μg Cd/L and attained higher concentrations from control values at day 30; except Cd partitioning into cellular debris fluctuated in fish exposed to both sublethal Cd concentrations (Fig 3.6). Mitochondrial Cd concentrations were the highest amongst all fractions and significantly increased from control levels after one week of exposure (Fig.3.6). Similarly, HSP Cd concentrations gradually increased and were significant at day 30 (Fig. 3.6).

Kidneys from fish exposed to 0.75 μg Cd/L did not accumulate Cd in cellular debris fractions (Fig. 3.7). Kidney HSP fraction Cd concentrations in low Cd exposed fish gradually increased to levels higher than control values on day 16 then remained unchanged; whereas fish exposed to 2.0 μg Cd/L concentrations significantly increased with time (Fig. 3.7). Similarly, fish exposed to 2.0 μg Cd/L significantly accumulated Cd with time in cellular debris fraction (Fig. 3.7). Kidneys accumulated the lowest amount of Cd in mitochondria, however partitioning of Cd into mitochondrial fractions in fish exposed to 2.0 μg Cd/L was observed (Fig. 3.7).

3.4.2.1 Cd partitioning into detoxified metal compartments

Figures 3.5 – 3.7 show the concentrations of Cd in the liver, kidney and gill detoxified subcellular fractions; MRG, ML and MTLP. The gill MTLP Cd concentrations increased linearly in comparison to controls, and were significantly higher than control levels after one week of exposure (Fig 3.5). Fish exposed to both sublethal Cd concentrations partitioned comparable amounts of Cd into gill MTLP. Gill MRG and ML Cd concentrations attained higher amounts in comparison control levels by day 7 and onwards, however concentrations remained unchanged in fish exposed to 0.75 μg Cd/L whereas levels continued to rise in fish exposed to 2.0 μg Cd/L (Fig. 3.5).

The liver MTLP Cd concentrations (Fig. 3.6) of trout exposed to 0.75 μg Cd/L peaked on day 1 and were significantly higher than control values on days 1 and 30, however concentrations did not change appreciably between sampling days. In fish exposed to 2.0 μg Cd/L, MTLP Cd concentrations gradually increased and were higher than both control and low Cd exposed fish by day 16 and onwards. Liver MRG contained the lowest amount Cd (Fig. 3.6). Cd did not partition into MRG in trout exposed to 0.75 μg Cd/L; however Cd concentrations significantly increased in MRG of fish exposed to 2.0 μg Cd/L. Similarly, fish exposed to 2.0 μg Cd/L attained liver ML fraction Cd concentrations significantly higher than control values by day 16 and onwards (Fig. 3.6).

The kidney MTLP Cd concentrations (Fig. 3.7) gradually increased. Cd concentrations in trout exposed to 0.75 μg Cd/L were significantly higher on day 30, whereas concentrations in trout exposed to 2.0 μg Cd/L increased significantly after one week, attaining higher concentrations than both control and low Cd exposure fish. Kidney MRG Cd concentrations (Fig. 3.7) gradually accumulated Cd in fish exposed to 0.75 μg Cd/L whereas significant increases from control values and low Cd exposed fish were attained by day 7 and onwards in fish exposed to 2.0 μg Cd/L. ML fraction Cd concentrations gradually increased and were higher than control values by day 30, and fish exposed to 2.0 μg Cd/L ML fraction Cd concentrations fluctuated and were higher than control values on days 7 and 30 (Fig. 3.7).

3.4.2.2 Metal-sensitive vs. metabolically detoxified pools

Figure 3.8 displays the overall change in proportion of Cd (%) partitioned into metal-sensitive pool (MSP) which includes mitochondria and HSP; and metabolically detoxified pool (MDP) which is comprised of MTLP, ML and MRG, in each tissue throughout 30-day exposure to waterborne Cd. Fish gills exposed to both sublethal Cd concentrations attained comparable amounts of Cd bound to MSP and MDP, and partitioning into both pools varied with time and exposure (Fig. 3.8). In comparison to control values (MSP: 37-42%; MDP: 39-43%), Cd bound to MSP in fish exposed to 2.0 μg Cd/L was similar (33-43%), however partitioning of Cd into MDP was lower (29-45%) on days 1 and 7. In the livers of fish exposed to low and high Cd concentrations, it was observed that the proportion of Cd bound to MSP

ranged from 21-52% and 37-61% respectively, while the proportion of Cd bound to MDP ranged from 44-62% and 32-49% respectively (Fig. 3.8). Fish exposed to 0.75 μg Cd/L appeared to partition Cd into MDP, whereas fish exposed to 2.0 appeared to partition Cd into MSP, particularly during the first week of exposure. The proportion of Cd bound to MSP and MDP in kidneys of fish exposed to low and high Cd concentrations ranged from 26-48% and 12-26% (MSP), 44-56% and 29-69% (MDP) respectively (Fig. 3.8). In comparison to control values (MSP: 29-31%), fish exposed to 2.0 μg Cd/L partitioned significantly less Cd into MSP during each sampling day, whereas fish exposed to 0.75 μg Cd/L partitioned comparable amounts of Cd. Although partitioning of Cd into MSP was low in fish exposed to high Cd, there was no shift in partitioning into MDP during the first week of exposure. However, after one week, more Cd was bound to MDP in fish exposed to 2.0 μg Cd/L.

3.4.3 Metallothionein concentrations

Metallothionein concentrations in all tissues increased during 30 day sublethal Cd exposure (Fig. 3.9). Absolute metallothionein concentrations were greatest in livers followed by kidneys then gills. However relative increases were greatest in kidney, with levels increasing 10 times from control values (6.10 ± 0.77 (30) μM GSH/g), followed by levels increasing 3 times from gill control values (4.96 ± 0.48 (30) μM GSH/g), then 2 times liver control values (6.71 ± 0.84 (30) μM GSH/g) after 30 days of exposure to 2.0 μg Cd/L (Fig. 3.9). Metallothionien concentrations did not differ in kidneys of fish exposed to 0.75 μg Cd/L although concentrations were significantly higher than control values on day 7 only, whereas concentrations in kidneys of fish exposed to 2.0 μg Cd/L increased significantly with time, higher than control levels after one week of exposure. Liver metallothionien concentrations of Cd exposed fish were comparable to control values however fish exposed to 2.0 μg Cd/L Cd gradually synthesized metallothionein and levels were higher than control values at day 30. Metallothionein concentrations in gills of trout exposed to 0.75 μg Cd/L peaked higher then control values on day 4, then remained unchanged, whereas concentrations in trout exposed to 2.0 μg Cd/L significantly increased between days 4 and 7 and remained higher than control levels after one week.

3.4.4 Correlation between fraction Cd and whole tissue concentrations

In order to indentify which subcellular fractions responded to the increases in Cd accumulation, concentrations in each fraction (μg/g; y-axis) were regressed against total tissue burdens (μg/g; x-axis) and Cd concentrations in MTLP fractions were regressed against tissue metallothionein concentrations (μg/g; x-axis). Tables 3.2 – 3.4 show the coefficients of determination for the relationships between Cd concentrations in cell fractions and the respective whole liver, kidney and gill concentrations. Correlations were determined throughout the 30-day exposure, during the first week (days 1, 4 and 7) and after one week (days 7, 17 and 29) of exposure to observe potential differences in fractions that become particularly enriched with Cd early in the exposure and vice versa. In the liver, only the MRG fraction Cd concentrations were not correlated with hepatic Cd, which was also true for the gill HSP fraction throughout 30-day exposure to sublethal Cd. However, coefficients of determination (r^2) changed between the first week of exposure and after one week of exposure within each subcellular fraction from all tissues. Correlations between each fraction and the total hepatic Cd concentration demonstrated a stronger relationship within all fractions except HSP after one week of exposure; in contrast the gills displayed stronger correlations among all subcellular fractions during the first week of exposure. Gill r^2 values were less than 0.33 for all fractions compared to total gill Cd burdens. Kidney fractions that became particularly enriched with Cd during the first week of exposure include ML, MTLP and mitochondria, whereas nuclei-debris and MRG displayed greater enrichment after one week of exposure. The relationship between MTLP fraction Cd concentration and measured metallothionein concentrations were not significant during the first week of exposure; however the relationship was strongest in the kidney, then liver throughout the 30-day exposure and after one week of exposure. Gill MTLP fraction Cd concentrations were not related to metallothionein concentrations.

3.5 Discussion

3.5.1 Overview

The purpose of the current study was to observe tissue and subcellular Cd accumulation, and physiological impacts of chronic Cd exposure in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed

to sublethal Cd concentrations in moderately soft water. Particular attention was focused on partitioning of Cd into metal-sensitive and detoxified compartments, in comparison to the previous moderately hard water study. In moderately soft water (present study) Cd partitioning into MSP (mitochondria, HSP) was detected more frequently in all tissues than the study performed in moderately hard water. The sublethal concentrations (0.75 and 2.0 μg Cd/L) to which the fish were chronically exposed in both moderately hard and moderately soft water studies were high enough to result in significant amounts of Cd accumulation in all tissues, particularly in fish exposed to 2.0 μg Cd/L. In the present moderately soft water study, gill Cd burdens were the highest amongst tissues and displayed a biphasic pattern of accumulation (rapid initial increase followed by saturation and/or decline), whereas in the previous study, kidney Cd burdens were higher than gills after one month of exposure and both tissues accumulated Cd with time.

3.5.2 Effects of Cd exposure

Fish exposed to Cd experienced the characteristic damage-repair phases in response to metal exposure (McDonald and Wood, 1993), with acute symptoms such as Ca^{2+} ion loss (Fig. 3.2), tissue Cd accumulation and lethality, followed by a recovery of the ionoregulatory system and/or physiological changes (i.e. metallothionein induction) that result in increased tolerance to Cd accumulation. Plasma Na concentrations also displayed a similar trend of ion loss-recovery although it was not significant (Fig. 3.3). Similar results of reduced plasma Ca levels in rainbow trout (*Oncorhynchus mykiss*) after 24 hr of Cd exposure (10 μg/L) in moderately hard water were obtained by Chowdhury et al. (2004). However, a study done by Hollis et al. (2000b) reported no significant changes in plasma Ca concentrations to rainbow trout (*Oncorhynchus mykiss*) exposed chronically to 2.0 μg Cd/L in four different Ca concentrations (260 – 1200 μM Ca) in synthetic soft water. Reduced plasma Ca concentrations are most likely due to competition with Cd^{2+} for apical Ca^{2+} channels, and non-competitive inhibition of the basolateral Ca^{2+}-ATPase exchanger in gill mitochondrial-rich cells (Verbost et al., 1987; 1988; 1989).

A successive distribution of Cd from the gills to the liver and kidney was observed after one week (Fig. 3.4), when Cd concentrations in the gill became saturated and/or declined in fish exposed to both Cd concentrations, which has been well documented in other chronic studies (Zia and McDonald, 1994; Hollis et al., 2001; Szebedinszky et al., 2001; McGeer et al., 2007). Gills accumulated the most Cd throughout the exposure, followed by kidney, then liver and at day 30 gills contained 5-2x and 7-5x and more Cd than kidney and liver, for fish exposed to 0.75 and 2.0 μg Cd/L respectively.

In the previous chapter, acute toxic effects (i.e. mortality and ion loss) appeared to be more severe, as serum Ca concentrations did not recover in moderately hard water fish exposed to 2.0 μg Cd/L (Fig. 2.5) and experienced 9% and 20 % mortality (Fig 2.4) during the first 4 days of exposure (0.75 and 2.0 μg Cd/L) in comparison to the present study which resulted in 3% and 15% mortality (Fig. 3.1). However, these effects may be due to differences in temperature between moderately hard (11.0 °C) and moderately soft water (9.3 °C) Cd exposures. Also, fish acclimated to increased ambient Ca in moderately hard water may not have been taking up as much Ca into the body. This is most likely due to decreased unidirectional influx of Ca, and a decrease in affinity and binding sites at the gills for Ca, and indirectly Cd, which has been demonstrated in the presence of Ca ions (medium and high concentrations) by the work of Hollis et al. (2000b). Rainbow trout (*Oncorhynchus mykiss*) were exposed to background (257-270 μM), low (257-270 μM), medium (600-938 μM) and high (1218-1235 μM) Ca concentrations, with (2.0 μg Cd/L) or without Cd, in synthetic soft water. However, Hollis et al. (2000b) did not observe a decrease in whole-body or plasma Ca concentrations. In comparison to total Cd burdens in both moderately hard (Fig. 2.7) and moderately soft water (Fig. 3.4), the protective effect of Ca against Cd uptake into the organs was strongest for the gills, followed by the kidney and then liver. With regard to sublethal chronic Cd exposure in moderately soft water study, gill Cd burdens were the highest amongst tissue burdens and displayed a biphasic pattern, and accumulated 2-4x more Cd in comparison to gills from fish exposed to 2.0 μg Cd/L in moderately hard water, after one week of exposure.

Increased Cd uptake in gills of moderately soft water acclimated fish is most likely attributed to changes that occur at the gill during soft water acclimation in order to compensate for the low availability of ambient Ca. These changes include; proliferation of mitochondrial-rich cells and increase in gill surface Ca binding (both affinity and capacity; Reid et al., 1989), increased branchial Ca^{2+} -ATPase activity (Filk and Perry, 1989), increased calmodulin concentrations in mucus (Filk et al., 1984), and an increase in the affinity and capacity of the Ca transport mechanisms (Perry and Wood, 1985; Zia and McDonald, 1994). For example, the work of Verbost et al., (1988; 1989) demonstrated that the affinity of Ca^{2+} -ATPase in rainbow trout (*Oncorhynchus mykiss*) is at least 100 times higher for Cd^{2+} than for Ca which help maintain Ca^{2+} homeostasis and limit branchial Ca^{2+} uptake in fish. Verbost et al., (1988; 1989) also demonstrated that Ca^{2+} -ATPase activity is inhibited during Cd exposure. Consequently, this could enhance the role of the gill to act as a barrier by limiting internalization of Cd into the bloodstream and subsequent distribution to internal organs. This is particularly evident in the kidney; fish exposed to 2.0 μg Cd/L in soft water did not attain higher concentrations relative to controls until day 30, whereas moderately hard water fish attained higher amounts of Cd after one week of exposure. These results were consistent with another hard water and soft water comparison study done by Hollis et al., (1999; 2000) in juvenile rainbow trout (*Oncorhynchus mykiss*), however sublethal concentrations of Cd were much lower during the soft water exposure.

3.5.3 Subcellular Cd partitioning

Increased partitioning of Cd into metal-sensitive pool (MSP; includes mitochondria and HSP); was observed during the first week of exposure, particularly at the higher exposure concentration (Fig. 3.8). Shifts in Cd partitioning into MSP did not necessarily coincide with significant partitioning of Cd into metabolically detoxified pool in comparison to control values, although Cd concentrations in fish exposed to both sublethal concentrations in MDP significantly increased (MDP; includes MRG, ML and MTLP). This is most like due to substantial amounts of Cd bound to cellular debris in all tissues (Fig. 3.5-3.7), which was not accounted for when grouping fractions into generalized pools. Fish exposed to both Cd exposures displayed comparable concentrations of Cd bound to MSP in gills compared to control values,

however Cd partitioning into MDP was low, particularly in fish exposed to 2.0 μg Cd/L. In contrast, Cd bound to MSP in kidneys of 2.0 μg Cd/L fish was lower than control values, and more Cd was bound to MDP after one week of exposure. The liver displayed an intermediate response and partitioning of Cd into MSP and MDP varied with time and exposure.

In comparison to the previous chapter, similar patterns of Cd partitioning were observed within each tissue except dampened (Fig 2.11). Less Cd partitioning into MSP was observed, particularly within the liver and kidney. Elevated binding of Cd to intracellular metal-sensitive compartments is most likely related to changes in Cd uptake at the gills mentioned previously in fish acclimated to soft water which delineate that Cd uptake in fish involves entry through apical Ca channels, binding to intracellular proteins, and then diffusing through the basolateral membrane. Additionally, mitochondrial-rich cells are densely packed with mitochondria and rich in tubular endoplasmic reticulum (Laurent, 1984), and increased levels of intracellular Ca^{2+} binding proteins (i.e. calmodulin) may result in inevitable uptake of Cd into sensitive fractions throughout the duration of the exposure. Evidently, accumulation in metal-sensitive compartments may not be associated with adverse effects and tissues may be employing other defense mechanisms that impart tolerance (discussed in detoxification section below). Alternatively, threshold concentrations associated with adverse effects within gill MSP may not have been exceeded. It is likely that Cd is absorbed into internal tissues from plasma via any of the same transporters/mechanisms that are involved in branchial Cd uptake (McGeer at al., 2010). Indeed, stronger relationships between fraction enrichment and total tissue Cd concentrations were apparent in the gills and liver of fish exposed in moderately soft water (Tables 2.2-2.4 and Tables 3.2-3.4), which could reflect increased available binding sites for Cd.

With regard to known metal-sensitive cell targets, mitochondria are considered toxicologically sensitive subcellular targets for Cd toxicity (Belyaeva et al., 2001; Sokolova, 2004) and both studies indicated that this fraction has the potential to become enriched with Cd in liver, kidney and gills during chronic exposure (Tables 2.2-2.4 and Tables 3.2-3.4). According to the literature, Cd can inhibit the

multi-enzyme mitochondrial electron transport chain (ETC), resulting in lower ATP production and excess ROS production (Belyaeva et al., 2001; Sokolova, 2004). Additionally, both studies have demonstrated a strong relationship between Cd bound to HSP and organ-level Cd accumulation (Tables 2.2-2.4 and Tables 3.2-3.4), particularly in the liver. HSP can be enzymatic or serve other cell functions (e.g. structure), and are also considered sensitive to Cd. Potentially, Cd may compete with essential metals for binding sites on HSP and induce cell disruption by damaging the integrity of the protein (Pruell and Engelnardt, 1980).

The consequence, if any, of Cd accumulation in ML fraction is poorly understood. This is due to the presence of two organelles with different roles. Some studies regard Cd accumulation in ML as sensitive because microsomes contain fragmented endoplasmic reticulum which is responsible for protein synthesis and transport, and glycogen storage (Fowler et al., 1989), while others claim sequestration of metal in lysosomes is a primary detoxification mechanism and the ML fraction generally represents lysosomes (Viarengo et al, 1985b; Barka et al., 2000; Nassiri et al., 2000). Indeed, fraction enrichment with lysosomes was observed in the previous study (Fig. 2.1-2.3) in all tissues. Likewise, the accumulation of Cd in cellular debris fraction is difficult to interpret since the debris pellet contains a heterogeneous mixture of tissue fragments, cell membranes and un-broken cells (Wallace et al., 2003) However, both chronic studies confirmed that these fractions have the potential to become enriched with Cd, particularly in the liver and kidney (Tables 2-2-2.4 and Tables 3.2-3.4).

3.5.4 Metabolically detoxified Cd and metallothionein induction

Cd did not accumulate appreciably in liver MRG fractions, hence partitioning of Cd into MRG was not related to total liver Cd accumulation in both moderately hard and moderately soft water studies (Table 2.2-2.4 and Tables 3.2-3.4), which is consistent with other studies examining the fate of subcellular Cd in fish exposed to metal contamination gradients in the field (Campbell et al., 2005; Kraemer et al., 2005; Giguère et al., 2006). However relationships were observed in kidney and gill tissue. Although gill tissue may not be directly responsible for MRG formation, Cd exposure may induce granulocyte migration to gills, which are potentially capable of Cd sequestration. The presence of Cd accumulation in granulocytes

in the gills were observed by Zia and McDonald (1994) as indicated by the presence of dense labelling (Cd^{109}) on the eosinophilic granulocytes within gill tissue and inside the blood vessels of the secondary lamellae. Although the role of these white blood cells is poorly understood, the work of Powell et al., (1990) demonstrated that granulocytes migrate through blood capillaries within gill tissue of rainbow trout (*Oncorhynchus mykiss*) and suggest that this migration may aid in immunological or defense mechanisms.

Although previous studies have not examined subcellular distribution of Cd in kidneys, this study and the previous study (Chapter 2) demonstrated that fish kidneys may also employ granule sequestration to detoxify Cd, as indicated by increased Cd partitioning into MRG with time (Fig. 2.10 and Fig 3.7). Additionally, a study done by Oronsaye (1989) examined histological changes in kidneys of stickleback (*Gasterostreus aculeatus*) chronically exposed to dissolved Cd and found excessive formation of granular masses, particularly in the tubule lumina, suggesting that the kidneys were eliminating detoxified Cd.

The detoxified cytosolic fraction is comprised of MTLP and thiol-rich peptides (gluthathione: GSH). However, numerous studies examining detoxification mechanisms in liver revealed that metals bound within the detoxified cytosol are largely bound to MTLP (Kraemer et al., 2005; Giguère et al., 2006). Studies have confirmed that GSH levels decline in yellow perch (*Perca flavescens*) liver (Giguère et al., 2005) and oyster (*Crassostra virginica gmelin*) hepatopancreas (Ivanina et al., 2008) with increasing hepatic Cd and Zn concentrations along a metal contamination gradient. When fish are exposed to Cd, they synthesize MTLP in kidney and liver (Kagi and Schaffer, 1988) to detoxify Cd and protect against reactive oxygen species (Kagi and Schaffer, 1988; Norey et al., 1990; Bremner, 1991), thus metallothionein induction is a key step in the transition from the damage phase to repair and acclimation.

The present study showed that Cd accumulation in MTLP and induction of metallothionien and Cd accumulation in MTLP was greatest in the kidney, followed by liver then gills (Fig. 3.5-3.7; Fig. 3.9).

Although metallothionein concentrations were not measured in the previous study, concentrations of Cd partitioning into MTLP were similar in the gill and kidney, and slightly higher in the liver of fish exposed in moderately hard water, thus water hardness did not appear to influence metallothionein detoxification during chronic Cd exposure. Additionally, literature values of Cd partitioning into MTLP (Kraemer et al., 2005; Giguère et al., 2006; Kamunde 2009) and metallothionein induction (Hollis et al., 2001; Chowdhury et al., 2005; Wagsongsak et al., 2007) are consistent with the results obtained in the present studies.

It is believed that specific metal metabolisms exist within each tissue (Cinier et al., 1999). It is well known that the liver is the primary organ for detoxification; however the present study indicated that Cd accumulation was greatest in MTLP in the kidney during prolonged Cd exposure. A study done by Wagsongsak et al., (2007) with sivler barb (*Puntius gonionotus*) revealed that MTLP mRNA expression occurred faster in the liver than in the kidney at low Cd exposure concentration however MTLP expression in the kidney was higher than that of the liver at higher exposure concentrations, thus liver MTLP expression may be time-dependent whereas the kidney is likely to show dose-dependent expression. Indeed, these findings may attribute to observations in the present studies, partitioning of Cd into MTLP fractions in the liver and kidney gradually increased during both chronic sublethal exposures to 0.75 μg Cd/L. Additionally, in the present moderately soft water exposure, the kidney appeared to be more capable to protect accumulation in MSP and attained higher partitioning of Cd into MDP after one week in fish exposed to 2.0 μg Cd/L in comparison to low Cd exposed fish. These results confirm that each tissue will have different capacities to induce MTLP biosynthesis.

In comparison to the liver and kidney, gills have been shown to be the least capable of sequestering or detoxifying Cd in the present studies and other studies (Hollis et al., 2001; Chowdhury et al., 2005; Hansen et al., 2007; Wangsongak et al., 2007). However, is has been shown that fish are able to recover from gill Cd burdens by reducing apical uptake into the gill during chronic exposures which may have alleviated the strain on detoxification mechanisms in the present study (Hollis et al., 1999). The results obtained in the present study indicated that detoxification offered by metallothionein becomes

saturated after one week of exposure as metallothionein concentrations remained elevated but unchanged, similar to total gill Cd burden, however Cd partitioning into MTLP increased linearly. Potentially, these results suggest that MTLP in the gills initially bind to other molecules such as reactive oxygen species during acute stages of Cd exposure, and is gradually replaced by Cd. An alternative approach is that the stress protein response to Cd exposure in fish gill and liver is analogous to oysters (*Crassostra virginica gmelin*). For example, a study done by Ivanina et al., (2008) revealed that in hepatopancreas (analogous to fish liver) expression of MTLP mRNA increased 2-8 fold in response to Cd exposure whereas no such increases were found in Cd-exposed gills. In contrast, heat-shock protein levels increased in Cd-exposed gills but not in hepatopancreas. Furthermore, a study done by Hansen et al., (2007) examined the induction and activity of oxidative stress proteins in brown trout (*Salmo trutta*) transplanted from a river with low levels of metal to a high Cd/Zn contaminated river for 15 days and found that metalothionein levels decreased whereas superoxide dismutase and catalase increased in gills. Thus future research comparing the inducibility of different protective systems in different tissues is required in order fully characterize the cellular fate and consequence of accumulated metal.

This objective of this project was to characterize the bioaccumulation of Cd during sublethal (0.75 and 2.0 μg Cd/L) chronic waterborne exposure to juvenile rainbow trout (*Oncorhynchus mykiss*) in moderately soft water (50 mg/L as $CaCO_3$). Accumulation was studied at both tissue and subcellular levels of organization in order to establish linkages between exposure, uptake, detoxification and accumulation in toxicologically sensitive pools within the cell. This study will also examine metallothionein induction in relation to tissue specific accumulation and subcellular partitioning of Cd.

Examining patterns of tissue and subcellular distribution when rainbow trout (*Oncrohynchus mykiss*) are chronically exposed to sublethal concentrations of waterborne Cd revealed that the protection offered by metallothionein was greatest in kidney, followed by liver and gills during chronic sublethal Cd exposures to trout in moderately soft water. However, detoxification by metallothionein and other mechanisms (granule and lysosome sequestration) was imperfect, as Cd partitioned into metal-sensitive

fractions (mitochondria and HSP). In moderately soft water (present study) Cd partitioning into metal-sensitive fractions was detected more frequently in all tissues (particularly internal tissues) than the study performed in moderately hard water. This may be attributed to fish increasing available uptake sites for Ca, which indirectly increases uptake mechanisms of Cd, during soft water acclimation in fish. Both chronic Cd exposures in moderately hard and moderately soft water revealed that mitochondrial and MTLP fractions were enriched with Cd in all tissues and should continued to be studied when examining chronic toxicity. Also, Cd bound to MSP pools throughout the duration of the exposure, thus acute spill-over into fractions persists in most tissues (particularly the gills, then liver), however kidneys were able to dissipate Cd bound to MSP with time and appear to exhibit a dose dependent response. Lastly, the protective effects of Ca^{2+} were observed on both a tissue and subcellular levels, as lower amounts of Cd accumulated in tissues and sensitive fractions during chronic sublethal exposures in moderately hard water. This may be attributed to fish increasing available uptake sites for Ca, which indirectly increases uptake mechanisms of Cd, during soft water acclimation in fish. Future research should use radio-labelled Cd to monitor uptake and intracellular trafficking within MSP and MDP to gain a better understand of the dynamics of subcellular partitioning.

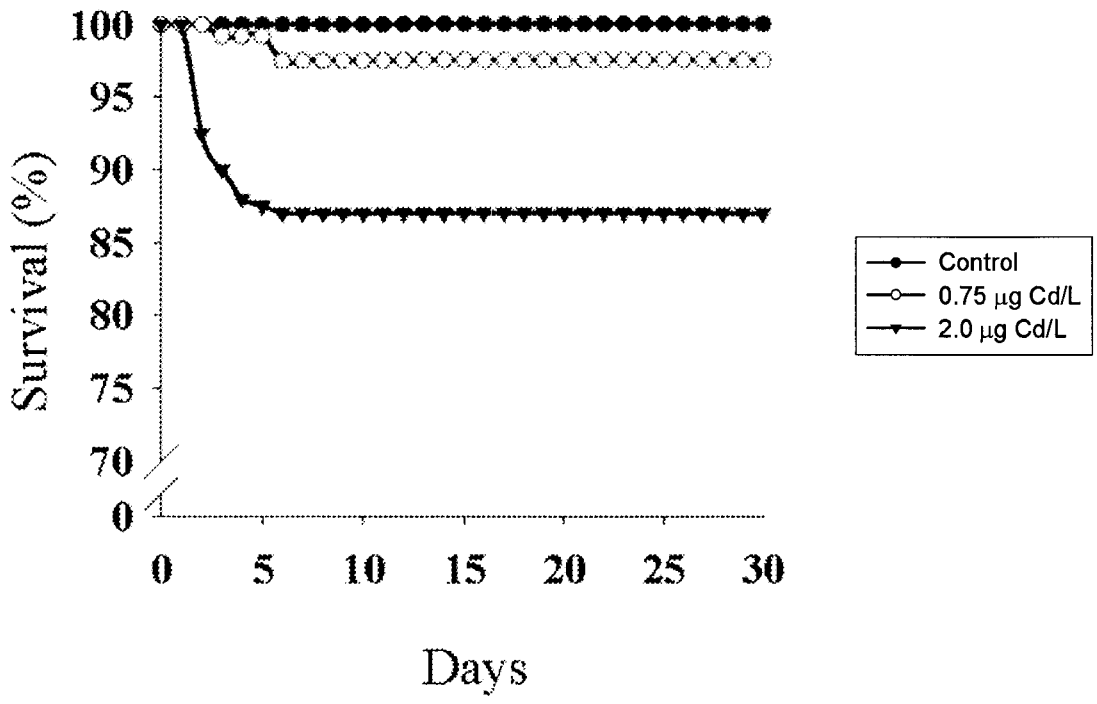

Fig. 3.1 The effect Cd exposure to rainbow trout (*Oncorhynchus mykiss*) (32.4 g ± 12.2 SD, n = 100) survival (%) throughout 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately soft water. Initially, n = 100 for control fish; n = 120 fish per treatment.

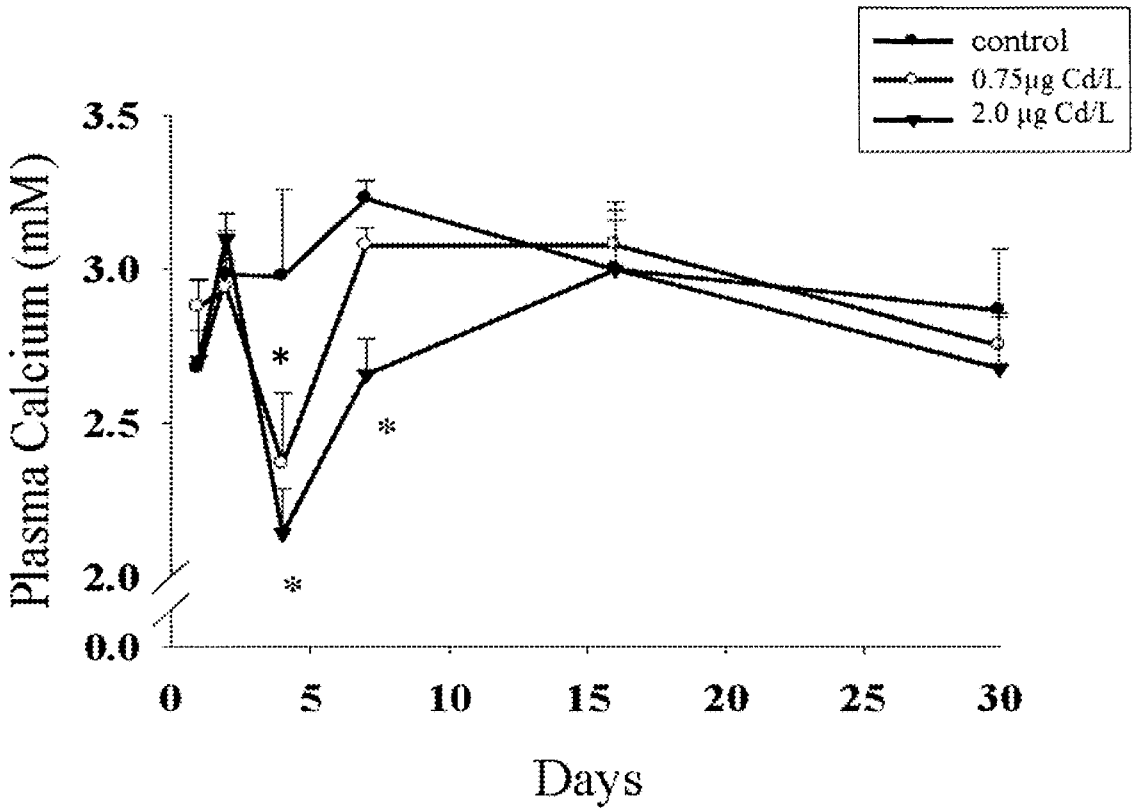

Fig. 3.2 The effect of waterborne Cd exposure to plasma Ca^{2+} concentrations in rainbow trout (*Oncorhynchus mykiss*) during 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L in moderately soft water . Values are means ± 1 SEM, n = 6. * indicates a significant difference compared to controls (ANOVA; Dunnet's test, $p < 0.05$)

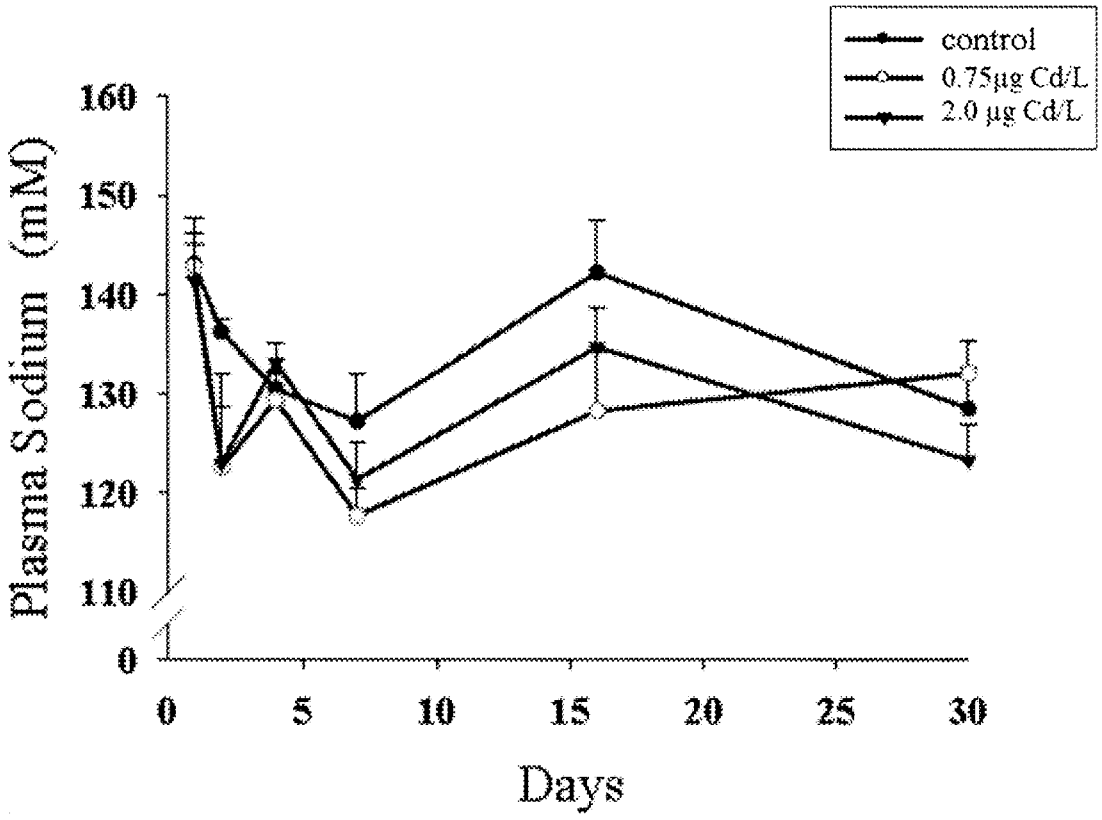

Fig. 3.3 The effect of waterborne Cd exposure to plasma Na^+ concentrations in rainbow trout (*Oncorhynchus mykiss*) during 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L in moderately soft water. Values are means ± 1 SEM, n = 6. Statistical comparisons made against control values at each sampling day were not significant (ANOVA; Dunnet's test, $p < 0.05$).

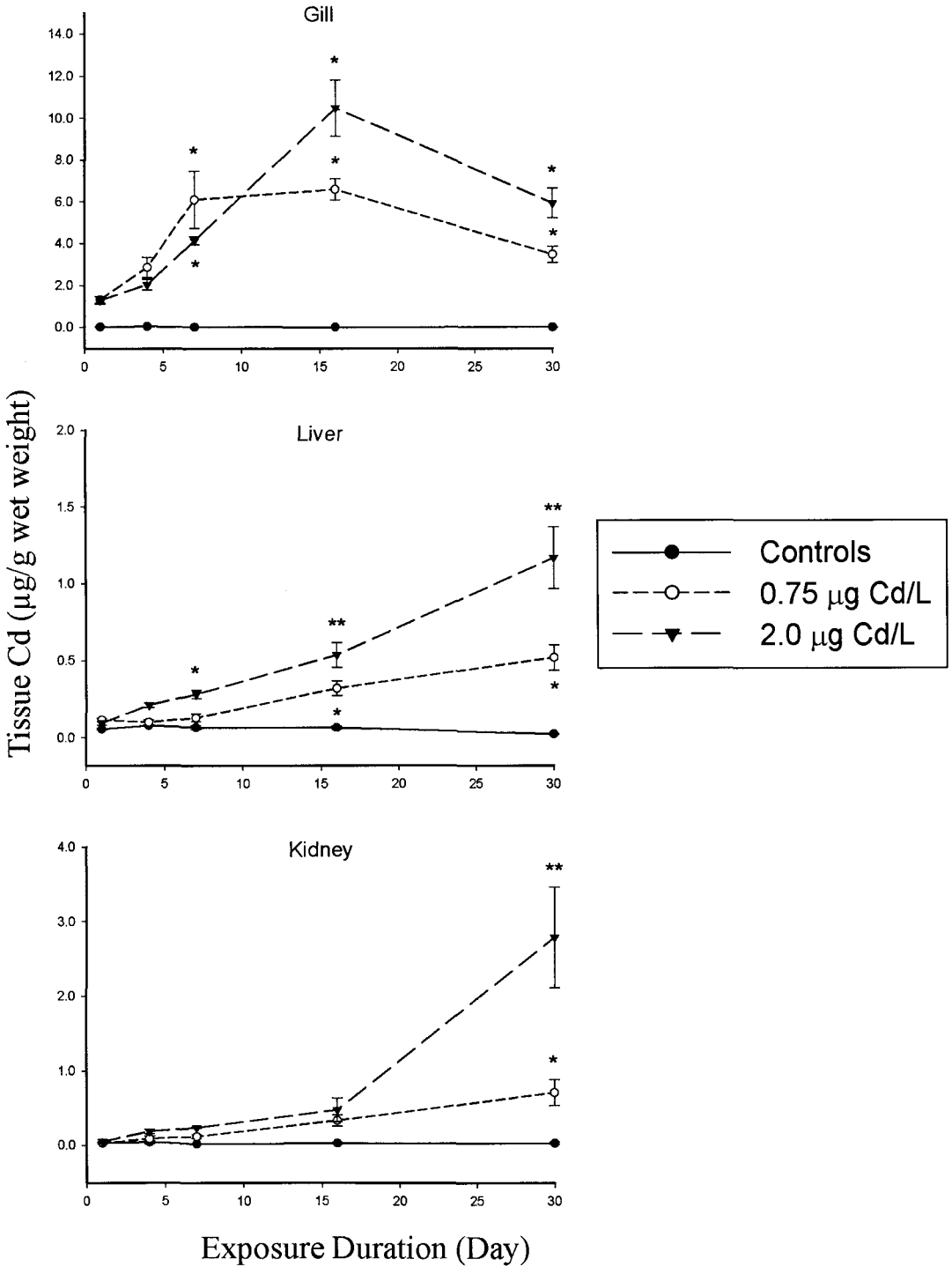

Fig. 3.4 Dose- and time-course of Cd accumulation in rainbow trout (*Oncorhynchus mykiss*) gill, liver and kidney during 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately soft water. Values are means ± 1 SEM, n = 6 fish. * indicate significance against control values whereas ** indicate significance from both control values and between treatment groups at each time point (day) (Anova; Tukey's HSD, $p < 0.05$)

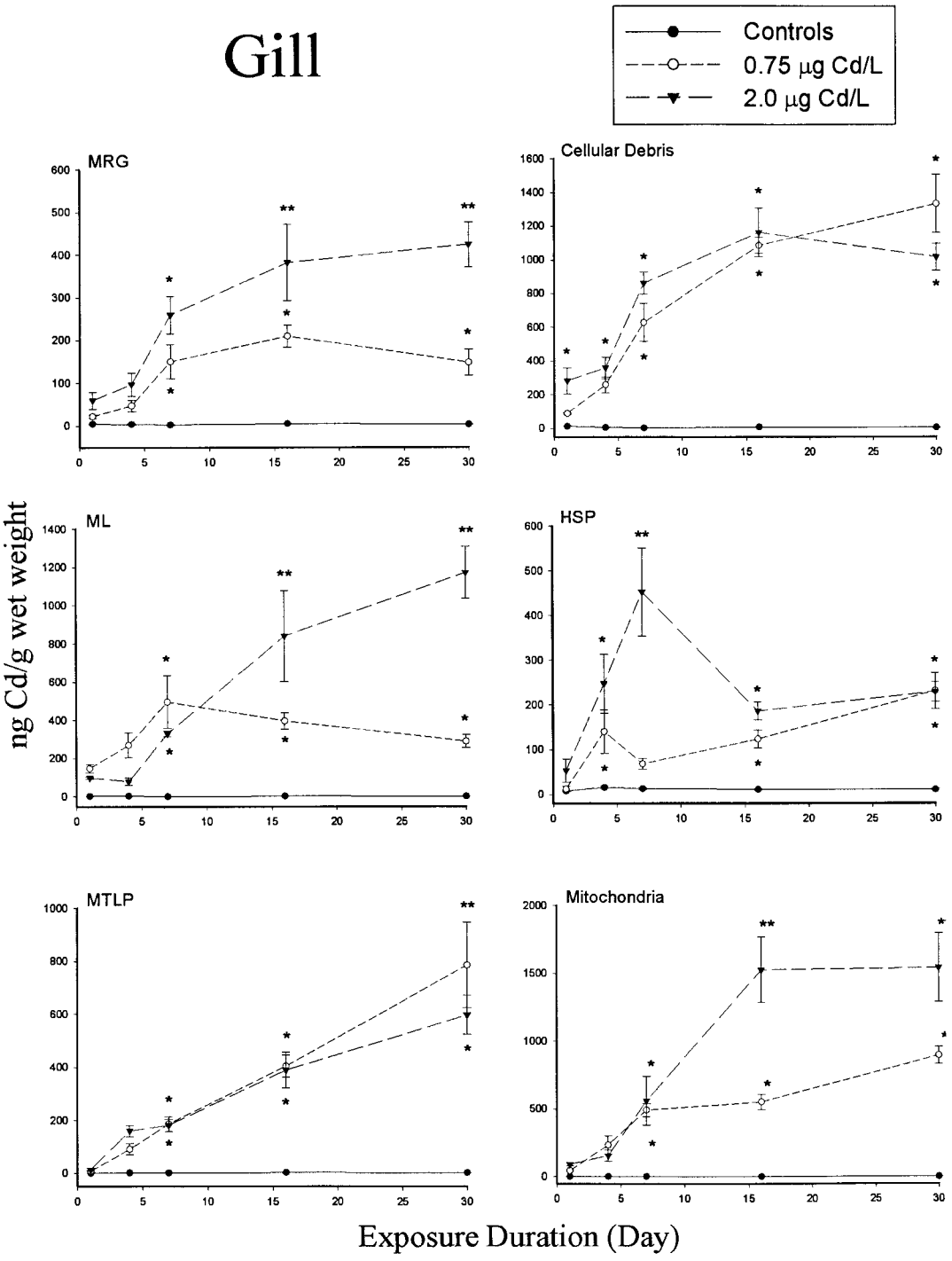

Fig. 3.5 Dose- and time-course of Cd accumulation in gill subcellular fractions of rainbow trout (*Oncorhynchus mykiss*) during 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately soft water. Values are means ± 1SEM, n = 6 fish. * indicate significance against control values whereas ** indicate significance from both control values and between treatment groups at each time point (day) (Anova; Tukey's HSD, $p < 0.05$)

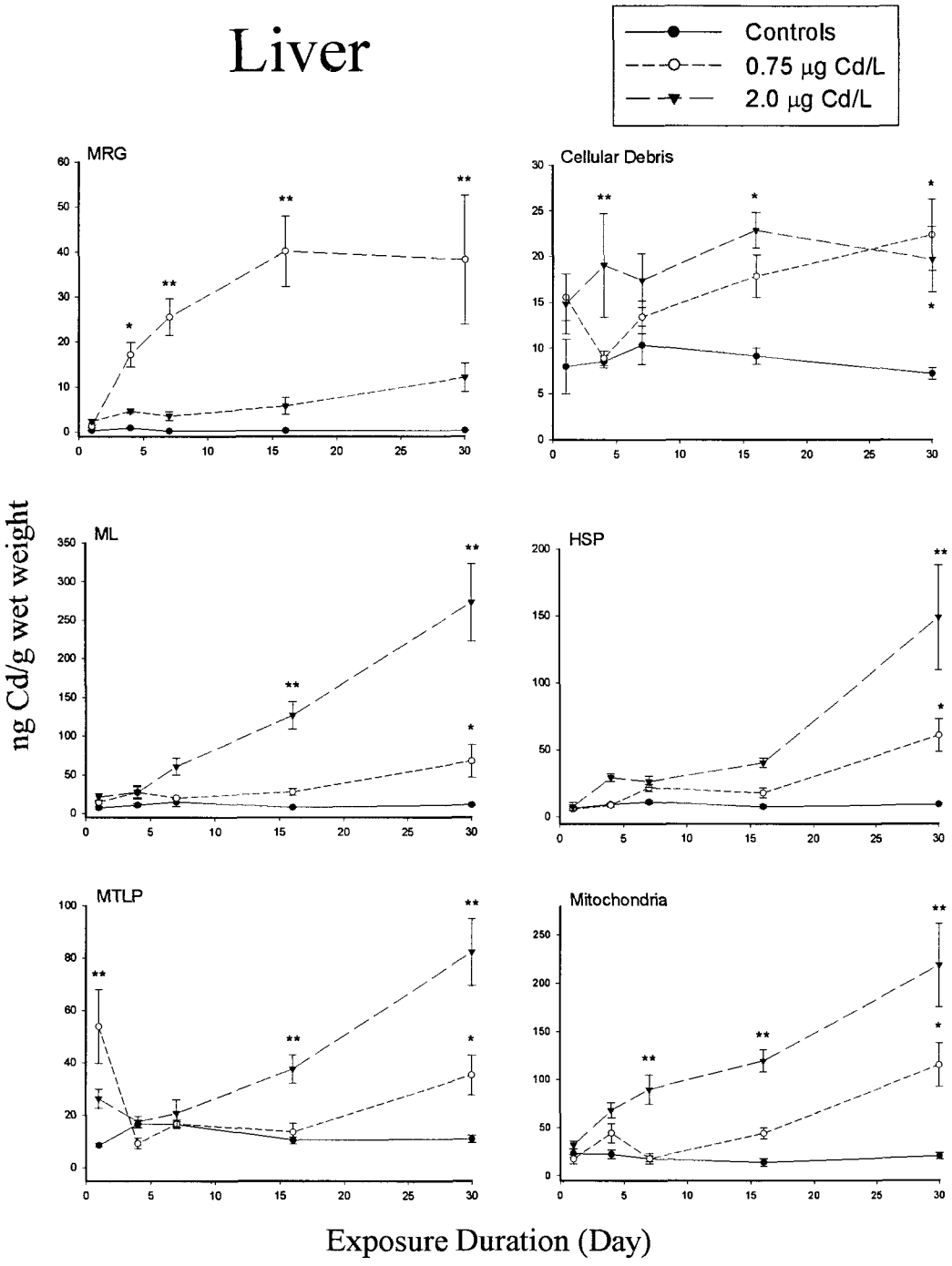

Fig. 3.6 Dose- and time-course of Cd accumulation in liver subcellular fractions of rainbow trout (*Oncorhynchus mykiss*) during 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately soft water. Values are means ± 1SEM, n = 6 fish. * indicate significance against control values whereas ** indicate significance from both control values and between treatment groups at each time point (day) (Anova; Tukey's HSD, $p < 0.05$)

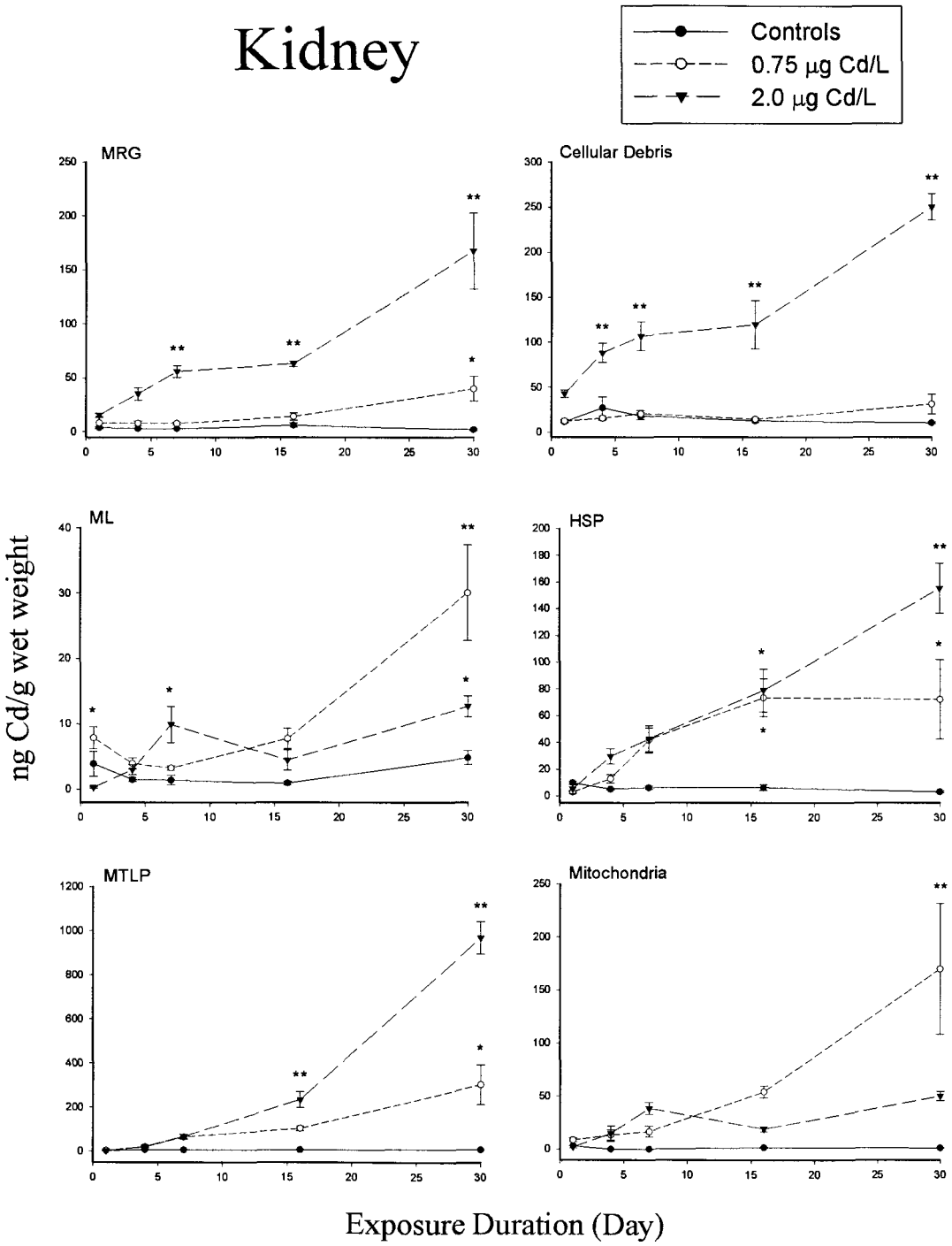

Fig. 3.7 Dose- and time-course of Cd accumulation in kidney subcellular fractions of rainbow trout (*Oncorhynchus mykiss*) during 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately soft water. Values are means ± 1SEM, n = 6 fish. * indicate significance against control values whereas ** indicate significance from both control and between treatment groups at each time point (day) (Anova; Tukey's HSD, $p < 0.05$)

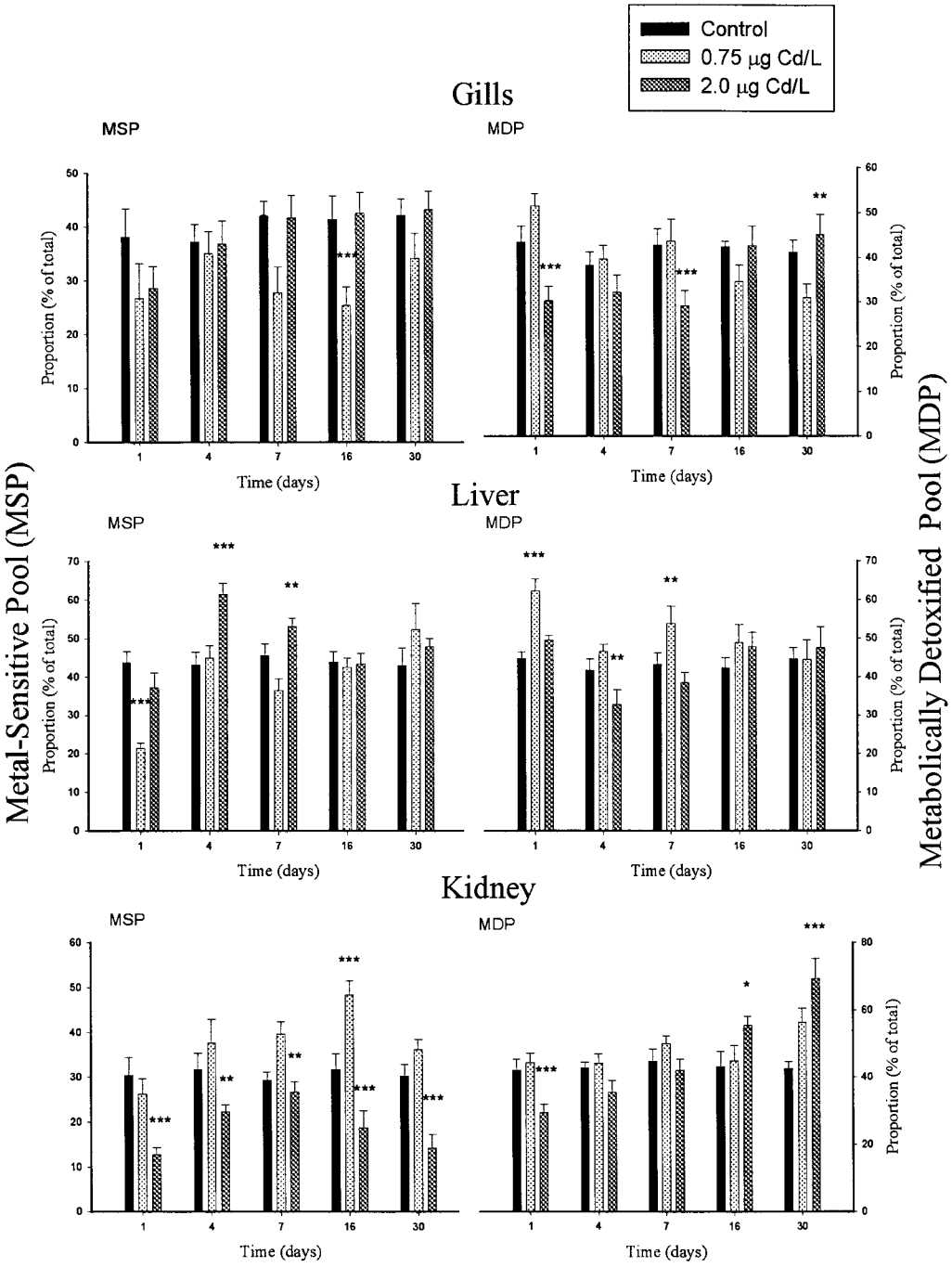

Fig. 3.8 Relative proportions (%) of Cd distributed between metal-sensitive and detoxified pools (MSP and MDP) in rainbow trout (*Oncorhynchus mykiss*) during 30-day exposure to control (nominal 0), 0.75 and 2.0 µg/L of waterborne Cd in moderately soft water. Values are means ± 1SEM, n = 6 fish. * indicate significance against control values whereas ** indicate significance between treatments and *** indicate significance from both control values and between treatment groups at each time point (day) (Anova; Tukey's HSD, $p < 0.05$)

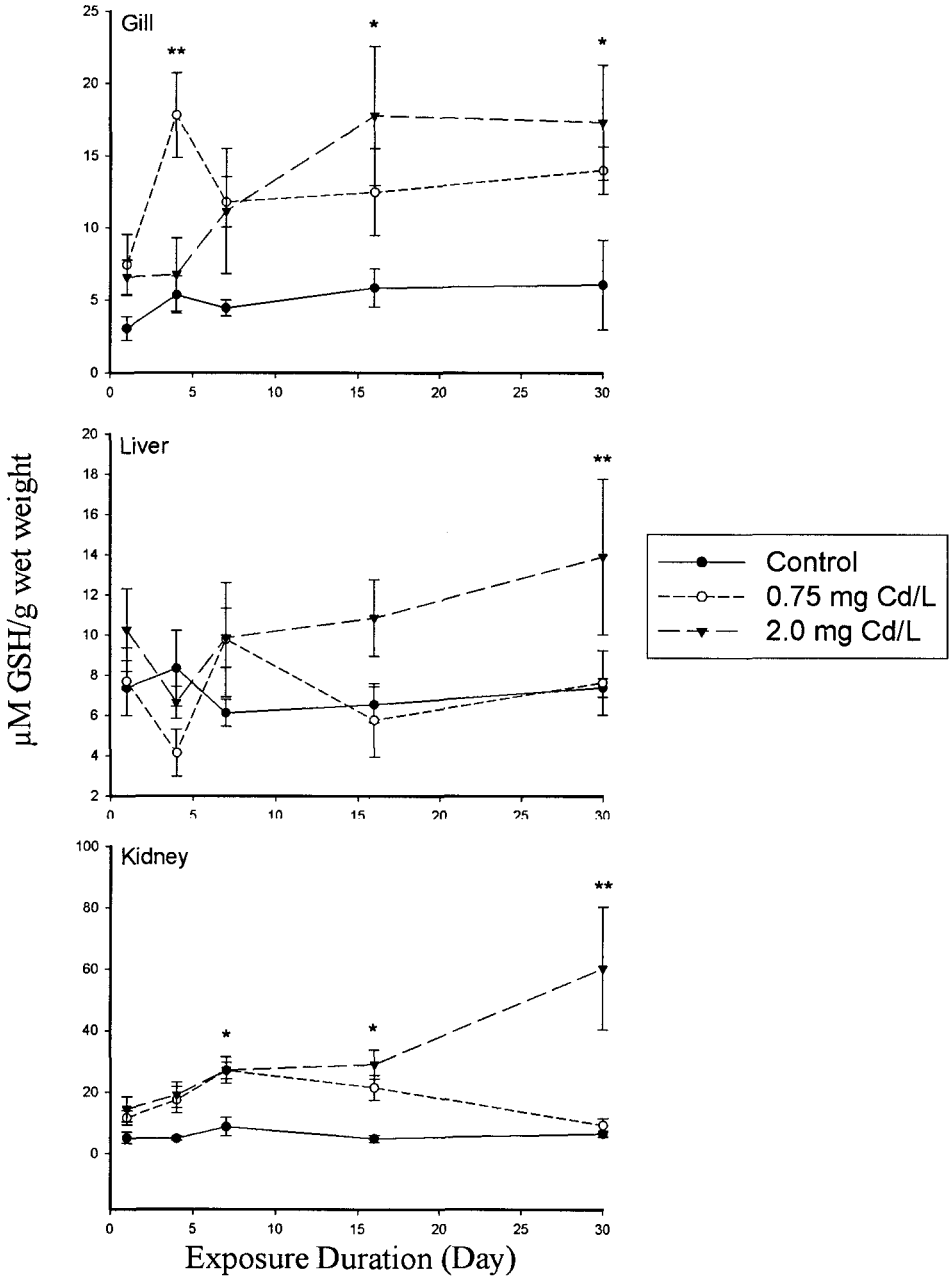

Fig. 3.9 Concentrations of metallothionein-like proteins (μM GSH/g wet weight) in gill, liver and kidney of rainbow trout (*Oncorhynchus mykiss*) during 30-day exposure to control (nominal 0), 0.75 and 2.0 μg/L of waterborne Cd in moderately soft water. Values are means ± 1SEM, n = 6 fish. * indicate significance against control values whereas ** indicate significance from both control values and between treatment groups at each time point (day) (Anova; Tukey's HSD, $p < 0.05$)

Table 3.1 Measured exposure concentrations of Cd from head tanks and fish tanks taken during the 30-day exposure to juvenile rainbow trout (*Oncorhynchus mykiss*). Temperature of exposure water ranged from 9.9 – 11.6 °C. Values are expressed as means ± 1SEM (n); HT-F: filtered water samples from head tanks; HT-UF: un-filtered water samples from head tanks; FT-F: filtered water samples from fish tanks; FT-UF: un-filtered water samples from fish tanks.

Exposure	HT-F	HT-UF	FT-F	FT-UF
Control (0)	0.01 ± 0.003 (5)	0.04 ± 0.031 (5)	0.01 ± 0.033 (4)	0.05 ± 0.006 (6)
0.75 µg Cd/L	0.77 ± 0.211 (5)	0.87 ± 0.093 (7)	0.73 ± 0.063 (6)	0.81 ± 0.190 (6)
2.0 µg Cd/L	2.09 ± 0.163 (5)	2.25 ± 0.191 (7)	1.82 ± 0.212 (6)	2.19 ± 0.226 (6)

Table 3.2 Linear regression analyses of Cd concentrations in rainbow trout (*Oncorhynchus mykiss*) gill subcellular fractions against the total gill Cd concentrations throughout all sampling days (1-30), within the first week (1-7) and after one week (7-30) of Cd exposure (0.75 and 2.0 μg Cd/L) in moderately soft water. The Cd concentrations in the MTLP fractions and total liver Cd also were regressed against the respective MT concentrations. Values are coefficients of determination (r^2) and asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS: not significant; MT: metallothionein; M + L: microsomes + lysosomes; MTLP: metallothionein-like proteins; Mito: mitochondria; MRG: metal-rich granules.

Subcellular Fraction	Days 1-29	Days 1-7	Days 7-29
M + L	0.189***	0.286***	NS
MTLP	0.0733*	0.305***	NS
Mito	0.149**	0.192**	NS
Enzymes	NS	NS	NS
Nuclei-debris	0.211***	0.327***	NS
MRG	0.222***	0.309***	NS
MTLP vs. MT	NS	NS	NS

Table 3.3 Linear regression analyses of Cd concentrations in rainbow trout (*Oncorhynchus mykiss*) liver subcellular fractions against the total liver Cd concentrations throughout all sampling days (1-30), within the first week (1-7) and after one week (7-30) of Cd exposure (0.75 and 2.0 μg Cd/L) in moderately soft water. The Cd concentrations in the MTLP fractions and total kidney Cd also were regressed against the respective MT concentrations. Values are coefficients of determination (r^2) and asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS: not significant; MT: metallothionein; M + L: microsomes + lysosomes; MTLP: metallothionein-like proteins; Mito: mitochondria; MRG: metal-rich granules.

Subcellular Fraction	Days 1-29	Days 1-7	Days 7-29
M + L	0.787*	0.531***	0.754***
MTLP	0.486***	NS	0.807***
Mito	0.877***	0.406***	0.810***
Enzymes	0.744***	0.520***	0.703***
Nuclei-debris	0.152**	NS	0.168*
MRG	NS	NS	NS
MTLP vs. MT	0.0858*	NS	0.115*

Table 3.4 Linear regression analyses of Cd concentrations in rainbow trout (*Oncorhynchus mykiss*) kidney subcellular fractions against the total kidney Cd concentrations throughout all sampling days (1-30), within the first week (1-7) and after one week (7-30) of Cd exposure (0.75 and 2.0 μg Cd/L) in moderately soft water. The Cd concentrations in the MTLP fractions and total gill Cd also were regressed against the respective MT concentrations. Values are coefficients of determination (r^2) and asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS: not significant; MT: metallothionein; M + L: microsomes + lysosomes; MTLP: metallothionein-like proteins; Mito: mitochondria; MRG: metal-rich granules.

Subcellular Fraction	Days 1-29	Days 1-7	Days 7-29
M + L	0.0897**	0.412***	0.0672*
MTLP	0.701***	0.769***	0.688***
Mito	0.0852**	0.578***	0.0663*
Enzymes	0.470***	0.331***	0.453***
Nuclei-debris	0.537***	NS	0.574***
MRG	0.473***	0.0935*	0.466***
MTLP vs. MT	0.236***	NS	0.241***

Chapter 4

General Discussion

The objective of this project was to characterize the pattern of accumulation and subcellular distribution within tissues (gill, liver and kidney) of rainbow trout (*Oncorhynchus mykiss*) over the course of exposure to understand the relationships to damage-repair-acclimation events in the organism. Both chronic sublethal Cd exposures in moderately hard and moderately soft water resulted in increases accumulation of Cd in tissues with time and exposure. Examination of patterns of tissue and subcellular distribution in rainbow trout (*Oncorhynchus mykiss*) chronically exposed to sublethal concentrations of waterborne Cd revealed that protection from Cd accumulation in metal-sensitive fractions was greatest in kidneys, followed by liver and gill. This was attributed to high amounts of Cd bound to MTLP and metallothionein induction in kidneys, compared to liver and gills after one week of exposure. Both Cd exposures in moderately hard and moderately soft water revealed that mitochondrial and MTLP fractions become enriched during chronic Cd exposure and are important fractions that could help link consequences, if any, of Cd accumulation in tissues during prolonged exposure. Furthermore, both studies revealed that both low and high Cd exposed fish accumulated excessive amounts of Cd in MSP on day 1, which could be interpreted as acute spill-over in the kidneys, however Cd accumulation in MSP in liver and gills throughout the duration of the exposure . Lastly, the protective effects of Ca^{2+} were observed on both a tissue and subcellular level, as lower amounts of Cd accumulated in gills and metal-sensitive fractions during chronic sublethal exposures in moderately hard water (see Table. 4.1 for comparison of observed effects of Cd exposure).

The exposure concentrations of Cd (0.75 and 2.0 μg Cd/L) used in both chronic Cd exposures were within an environmentally realistic range. Currently, freshwater-quality criteria for aquatic life in water hardnesses ranging from 20 – 120 mg/L as $CaCO_3$, recommends an acute limit of 0.6 – 4.8 μg Cd/L for acute exposures and a chronic limit of 0.3 – 1.3 μg Cd/L for chronic exposures, according to the U.S. Environmental Protection Agency (Thornton, U.S. EPA-822-R-01-001, 2001). Furthermore, concentrations up to 5 μg Cd/L exist in North American surface waters (Spry and Wiener, 1991). Fish exposed to the same sublethal Cd concentrations in both moderately soft (50 mg/L as $CaCO_3$; 360 ± 22

μM Ca) and moderately hard water (140 mg/L as $CaCO_3$; 868 ± 28 μM Ca) displayed physiological impacts associated with chronic Cd exposure. These include disruptions of ionoregulation (particularly plasma Ca), increased accumulation of Cd in tissues and binding to metal-sensitive pools (particularly during first week of exposure).

Mortality and plasma ion loss occurred early during both sublethal chronic Cd exposures in moderately hard and moderately soft water. Plasma ion concentrations of fish exposed to both water hardnesses alone were comparable, thus additional ions present in the exposure medium did not have an impact on internal ionoregulation in fish. However, trout exposed to 2.0 μg Cd/L in moderately hard water had significantly reduced plasma Na levels on day 4 (similar reduction was observed but not significant in moderately soft water exposure), whereas fish exposed to both low and high Cd exposures in moderately hard and moderately soft water displayed lower plasma Ca concentrations during the first week of exposure. Interestingly, plasma Ca^{2+} concentrations did not recover back to control values in fish exposed to Cd in moderately hard water which contains more Ca ions that are available to compete with Cd uptake at the gills. Reduced plasma Ca concentrations are most likely due to competition with Cd^{2+} for apical Ca^{2+} channels, and non-competitive inhibition of the basolateral Ca^{2+}-ATPase exchanger in gill mitochondrial-rich cells (Verbost et al., 1987; 1988; 1989). Also, decreased unidirectional influx of Ca, and a decrease in affinity and binding sites at the gills for Ca, and indirectly Cd, which has been demonstrated in the presence of Ca ions (medium and high concentrations) by the work of Hollis et al. (2000b) may contribute to reductions in plasma Ca. Rainbow trout (*Oncorhynchus mykiss*) were exposed to background (257-270 μM), low (257-270 μM), medium (600-938 μM) and high (1218-1235 μM) Ca concentrations, with (2.0 μg Cd/L) or without Cd, in synthetic soft water. However, Hollis et al. (2000b) did not observe a decrease in whole-body or plasma Ca concentrations. Presumably, plasma Na^+ influx and binding at the gill may also be lowered in response to increases in ambient Na however studies have yet to report that Na^+ concentrations can influence Cd binding to fish gills (Niyogi et al. 2008).

The sublethal concentrations (0.75 and 2.0 μg Cd/L) to which the fish were chronically exposed in both moderately hard and moderately soft water studies were high enough to result in significant amounts of Cd accumulation in all tissues, particularly in fish exposed to 2.0 μg Cd/L. The protective effect of Ca against Cd uptake into the organs was strongest for the gills, followed by the kidney and then liver. With regard to sublethal chronic Cd exposure in moderately soft water study, gill Cd burdens were the highest amongst tissue burdens and displayed a biphasic pattern (rapid initial increase followed by saturation and/or decline), and accumulated 2-4x more Cd in comparison to gills from fish exposed to 2.0 μg Cd/L in moderately hard water, after one week of exposure. This is most likely due to changes in gill binding characteristics that occur when rainbow trout (*Oncorhynchus mykiss*) acclimate to low ambient Ca^{2+}, such as a significant increase in binding sites (Hollis et al., 2000a, b; Niyogi et al., 2004). Increased binding sites on the gill are believed to help compensate for ion loss of Ca from the fish to ion-poor water. Furthermore, studies with chronically exposed trout that have been acclimated to Cd have also displayed an increase in binding sites available at the gill, which is believed to reflect increased detoxification or storage of Cd (McGeer et al., 2010)

In comparison, all tissues accumulated Cd with time; however kidney concentrations in exposed fish were the greatest after 30-days of sublethal Cd exposure in moderately hard water. Fish exposed to 2.0 μg Cd/L accumulated 2-6x and 2-3x less Cd in kidney and liver respectively, in comparsion to fish exposed to 2.0 μg Cd/L in moderately soft water during the first week of exposure. These results are consistent with previous studies (Wicklund and Runn, 1988; Hollis et al., 2000b) that demonstrated similar protective effects of Ca against Cd uptake with slower uptake of Cd into the gills with increasing water Ca levels. This is likely due to competition between Cd and Ca for apical Ca^{2+} channels/exchangers and non-competitive inhibition of Ca^{2+}-ATPase activity at the basolateral membrane of the gill (Verbost et al., 1987; 1988; 1989).

All tissues initially accumulated Cd in MSP (mitochondria and HSP) during chronic sublethal Cd exposures in both moderately hard and moderately soft water. In moderately soft water, Cd spill-over into

metal-sensitive fractions was detected more frequently in all tissues (particularly internal tissues) in comparison to chronic sublethal Cd exposure in moderately hard water. This may be attributed to increased uptake mechanisms of Cd, which share common pathways with Ca that are enhanced to compensate for Ca ion loss during soft water acclimation in fish (e.g. increased binding sites on the gill and/or increased intracellular calmodulin synthesis).

Both chronic studies revealed that the gills of rainbow trout (*Oncorhynchus mykiss*) are sensitive to chronic sublethal waterborne Cd and displayed the lowest amounts of Cd partitioning into detoxified fractions during the first week of exposure, whereas partitioning into metal-sensitive compartments varied and may link to acute toxic effects (mortality and ion loss). However, this study only evaluated the protective mechanisms offered by metallothionein, and it is highly likely that gills employ other protective mechanisms that are involved with repairing any damage induced by Cd accumulation in sensitive fractions. In contrast, the present chronic studies revealed that the kidney was most efficient at detoxification via sequestration by MTLP (and to a lesser extent ML and MRG), and contained relatively low amounts of Cd bound to mitochondria and HSP.

With regard to the spill-over hypothesis, the results indicated that acute spill-over occurs particularly within the kidney, however accumulation in MSP was observed throughout the duration of both sublethal Cd exposures in moderately hard and moderately soft water. Similar to previous studies (Kraemer et al., 2005; Giguère et al., 2006; Campbell et al., 2005, 2008), the spill-over hypothesis may be too simplistic to describe chronic accumulation or may only be applied to acute metal exposures. An alternate spill-over hypothesis that can be applied to chronic metal exposure is depicted in Fig. 4.2. The adjusted spill-over hypothesis predicts that metal-binding capacity is initially overwhelmed and is unable to match metal uptake into the cell, thus excessive accumulation results in MSP. Future studies examining the relationship between subcellular Cd partitioning and detoxification mechanisms should use a suite of biomarkers, and also usedradio-labelled Cd^{109} in order to obtain more accurate measurements if the

dynamics of Cd partitioning into and/or between subcellular fractions and consequences of accumulated metal.

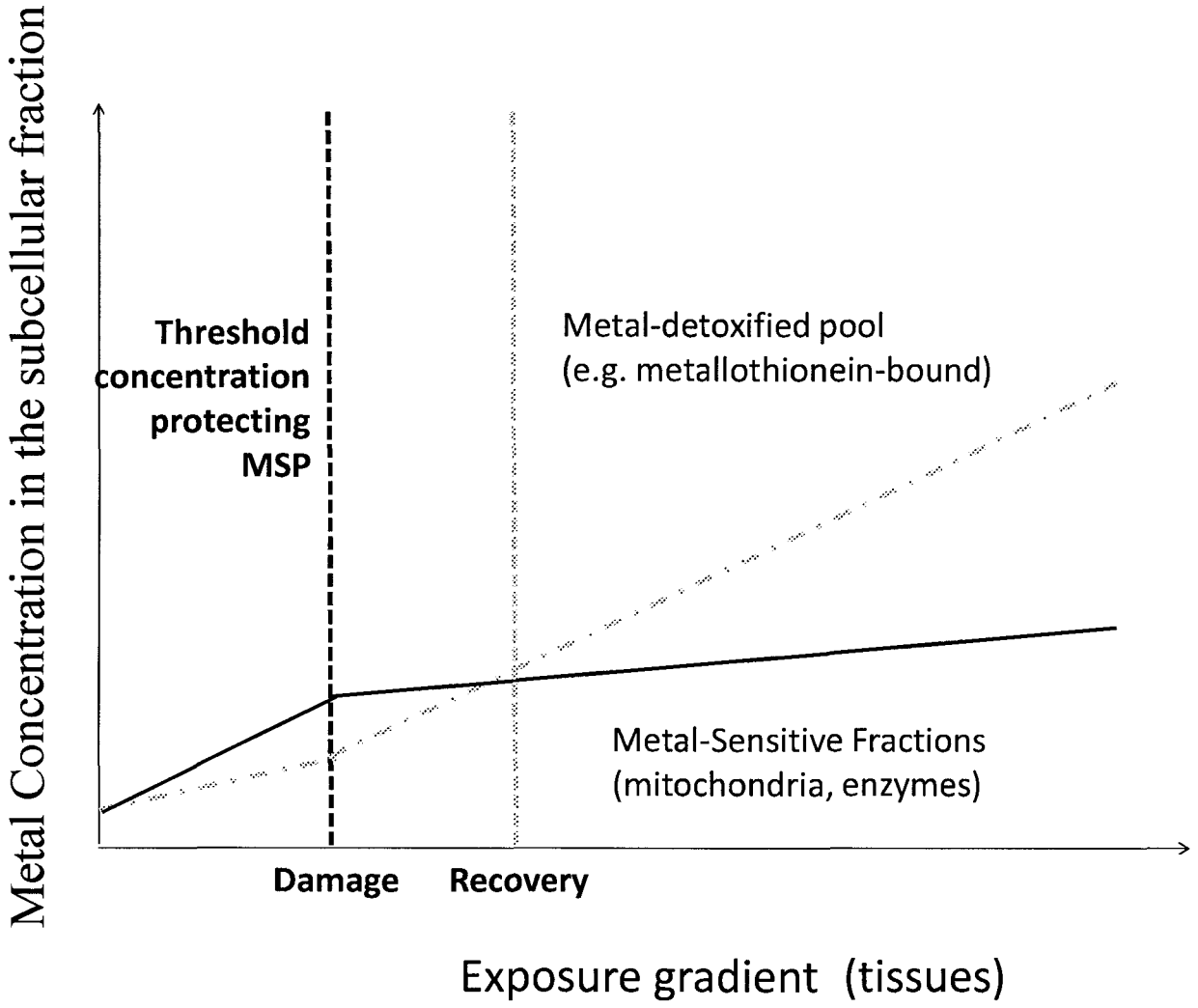

Fig. 4.1 Adapted from Kraemer, 2005. Graphical representation of the adjusted spill-over hypothesis for prolonged metal exposure that predicts metal detoxification mechanisms (e.g. metallothionein-binding; dashed gray line) are unable to match initial uptake of Cd into the cell, thus metal accumulates in MSP and exceeds the threshold concentration (vertical dashed line) of metal accumulation which could be linked to acute toxic effects. However, detoxification capacity increases with time and exposure; recovery from initial damage occurs when Cd accumulation in detoxified pool exceeds uptake into the cell and metal-sensitive fractions (solid black line).

Table 4.1 Comparison of observed effects of Cd exposure in moderately hard water versus moderately soft water exposures for juvenile rainbow trout (*Oncorhynchus mykiss*) exposed for one month

Measured Parameter	Moderately hard water exposure to Cd (0.75 or 2.0 μg/L	Moderately soft water exposure to Cd (0.75 or 2.0 μg/L
Mortality	9% and 20% at low and high Cd exposures	3% and 15% at low and high Cd exposures
Plasma ions	Acute Ca and Na ion loss reductions in plasma Ca in fish exposed to 2.0 μg Cd/L	Acute Ca ion loss only in fish exposed to both low and high Cd exposures
Tissue Cd burdens	Greatest in kidney > gill > liver at day 30	Greatest in gill > kidney > liver at day 30
Gill subcellular partitioning	Trend of lower partitioning into MDP	Trend of lower partitioning into MDP
Liver subcellular partitioning	Less Cd partitioning into MSP	More Cd partitioning into MSP
Kidney subcellular partitioning	Trend of decreased partitioning into MSP with time and dose	Trend of decreased partitioning into MSP with dose

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Appendix A: Subcellular Distribuiton Protocol

Adapted from Kamunde and MacPhail (2008; 2009)

Note: Keep everything frozen until ready. Use 1% HNO_3 to sterilize tools. When sampling store tissues in liquid nitrogen. Weigh all tubes that will contain pellets prior to beginning analyses. If the tissue weighs less than 200 mg then add buffer 3-fold tissue wet weight. If tissue weighs more than 200 mg add buffer 4-fold tissue wet weight. Keep tissues on ice before first homogenization step and during subsequent steps prior to heat denaturation.

1) Homogenize liver in 0.9% NaCl OR PBS solution using Omni THQ set at ~ 20 RPM until uniform. Use the same settings to each tissue to maintain consistency.
2) Subject tissue to 800 x g for 15 mins at 4 °C

Isolation of MRG

Notes: Decant supernatant (S1) into weighed bullet tube, store S1 in freezer (-40 or -80). After adding ultra pure water vortex each tube before heating to make sure pellet isn't at the bottom. After you obtain a pellet, rinse 1-2 times (in buffer) to increase purity of pellet.

3) Re-suspend P1 in 0.5 ml ultra pure water and heat at 100 °C for 2 mins
4) Add 0.5 ml of 1N NaOH and heat to 60 -70 °C for 1 hr *Vortex before heating
5) Centrifuge at 10,000 g for 10 min at 20 °C
6) Decant S2 (cellular debris) into new tube. Re-suspend pellet (P2) in 500-1000 µL buffer and rinse for 10 mins at 10 000 g at 20 °C *Weigh Pellet

Isolation of MTLP and HSP

Unthaw (on ice or in waterbath) supernatant (S1) and subject it to 3000 g for 15 mins at 4 °C to isolate light mitochondria (P3). Decant supernatant (S3) into a new tube and spin for 10,000 g for 30 mins at 4°C to isolate heavy mitochondria (P10). Rinse pellets twice with 500-1000 µl of buffer at 10,000 g for 10 min at 4 °C. Vortex each time after buffer is added. Decant and store pellet in fridge or freezer. * Weigh pellet

7) Decant supernatant S10 into 3 mL Beckman centrifuge tubes and spin at 100,000 g (43,000 RRM) for 60 min at 4 °C to obtain P4 (microsomes + lysosomes).
8) Decant supernatant (S4) back into bullet tube and denature it with heat at 80 °C for 10 mins. Place on ice for 1 hr. Poor supernatant back into 3 ml tube and centrifuge at 50,000 g (30,000 RPM) for 10 min at 4 °C to obtain P5 (HSP). Decant S5 (MTLP) into bullet tube and store in freezer.
9) For P4 and P5, add 1 ml of buffer to 3 ml tube after decanting supernatant and break up the pellet via flicking or vortexing quickly before transferring back to 1.5 ml bullet tubes. Rinse pellets with 500-1000 µl ml buffer at 10,000 g for 10 mins at 4°C before storing.

Cd Analyses

Pellet:

Unthaw pellet

Digest with 1:5 1N HNO_3 and vortex

Place in oven at 80 °C for 3 hrs

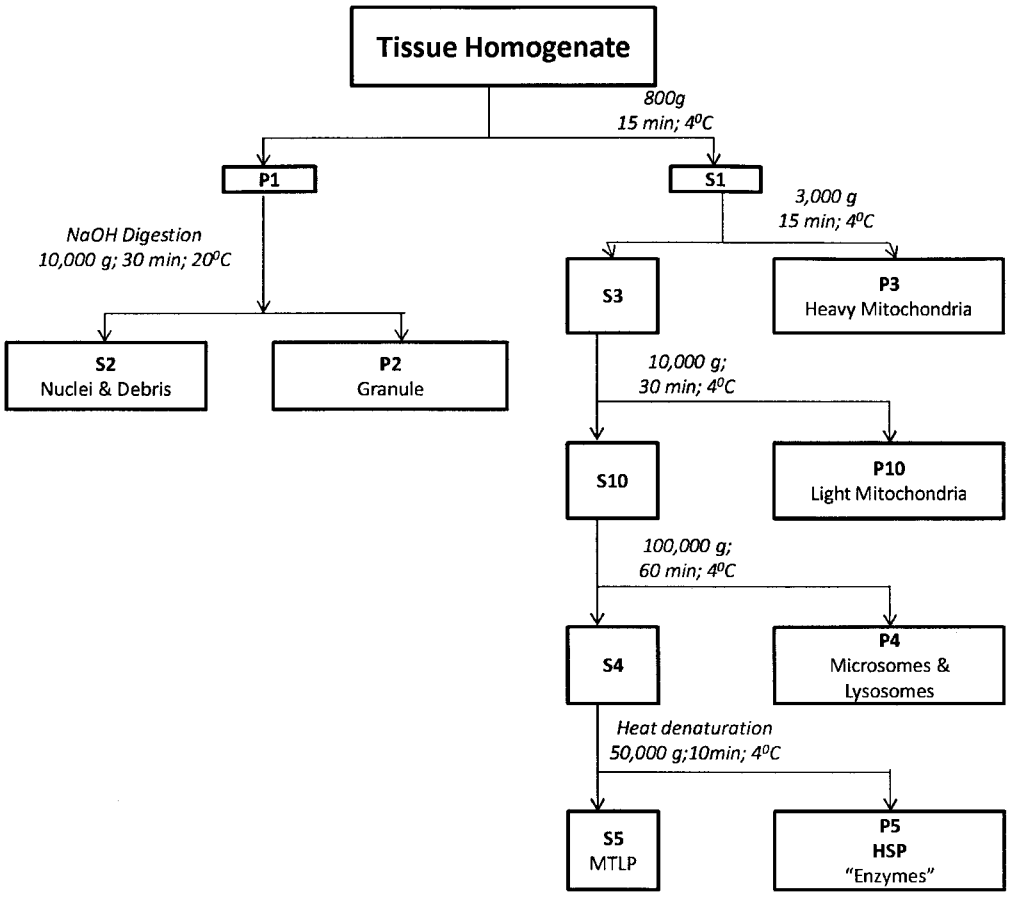
Spin at 10,000 g for 10 min at 20 °C

Supernatant:

Equate µL with mg and perform a 1:2 or 1:3 acid digest. Usually take 200 to 500 µL of sample.

Note: To convert measured fraction absorbance into µg Cd/g wet tissue weight:

1) Convert absorbance to concentration using equation of the standard curve
2) Multiply concentrations by dilution factor
3) Add fraction weight with amount of acid added to obtain the total volume in the tube
4) Multiply the total volume by concentration to obtain total amount of Cd in tube, divide by 1000 to obtain µg
5) Divide amount of Cd in tube by the tissue weight to obtain µg Cd/g wet tissue weight (multiply by 1000 to convert to ng Cd/g wet tissue weight


Tissue Homogenate
800g
15 min; 4°C
P1
S1
NaOH Digestion
10,000 g; 30 min; 20°C
3,000 g
15 min; 4°C
S3
P3
Heavy Mitochondria
S2
Nuclei & Debris
P2
Granule
10,000 g;
30 min; 4°C
S10
P10
Light Mitochondria
100,000 g;
60 min; 4°C
S4
P4
Microsomes &
Lysosomes
Heat denaturation
50,000 g;10min; 4°C
S5
MTLP
P5
HSP
"Enzymes"

Appendix B: Quality Control Assays

Acid Phosphatase (Lysosomes)

Adapted from Bergman, (1969), Dr. Tania Ng and Sigma acid phosphatase protocol (CS0740)

Solutions:

- Citrate Buffer - 0.1 M, pH 4.8, 4 °C (sodium citrate: 0.1M 36.3 ml + citric acid: 0.1 M 13.7 ml, diluted to 100 ml and adjusted with 1N HCl)
- Substrate solution – 0.042 g 4-nitrophenyl phosphate salt D(Tris) salt (-20 °C)into 6 ml buffer (15.2 mM) (enough for 12 std + 60 samples)

Standards:

- Original stock (200 µl; 180 mU/ µl): take 28 µl of acid phosphatase enzyme (1285.7 mU/ µl) into 172 µl buffer
- Diluted substock (200 µl; 4.5 mU/ µl): take 5 µl from original stock into 195 µl buffer

From diluted substock: Std 5 (0.49 mU/ µl): 33 µl diluted substock into 270 µl buffer

Std 4 (0.26 mU/ µl): 17 µl substock into 280 µl buffer

Std 3 (0.1225 mU/ µl): 75 µl std 5 into 225 µl buffer

Std 2 (0.645 mU/ µl): 75 µl std 4 into 225 µl buffer

Std 1 (0.0306 mU/ µl): 75 µl std 3 into 225 µl buffer

Blank: 300 µl buffer

Protocol:

1) Pre-set the waterbath temperature to 37 °C and microplate reader to 37 °C, end point absorbance at 405 nm
2) Put 0.5 N NaOH solution in water bath
3) Dilute samples by adding 10 µl sample into 240 µl buffer (25x)
4) Note: For pellets, resuspend in buffer via sonication (low). Sonicate supernatants
5) Transfer 50 µl standard or sample into each well, 2 replicates each
6) Prepare fresh substrate solution and transfer 50 µl into each well by repeator pipet
7) Shake for 10 sec, then incubate at 37 °C for 30 mins
8) Pipet 200 µl NaOH to each well (yellow colour formed) to stop reactions
9) Shake for 10 sec and read at 405 nm

Calculations:

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

DF = dilution factor of original sample

Time = time of incubation at 37 °C in mins

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

0.05 concentration (µmole/ml) of 4-nitrophenol in the std. solution

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

Unit definition: one unit of acid phosphatase will hydrolyze 1 µmole of 4-nitrophenol phosphate per minute at pH 4.8, 37 °C

Citrate Synthase (mitochondria)

Adapted from Srere (1969), Dr. Tania Ng and Sigma citrate synthase protocol (CS0720)

Solutions:

- Tris-HCl buffer (125 mM, pH 8)
- Oxaloacetic acid (10 mM in buffer/1.32 mg/ml, 1 week use only, -20 °C, usually prepare 10 x 200 µl
- Acetyl Co-A trilithium salt (30 mM in ulra-pure water, 40 µl each centrifuge tube, -20 °C) -5, 5 dithiobis-2-nitrobenzoic acid (DTNB, 10 mM in 100 % ethanol, 40 µl each centrifuge tube, -20 °C)
- Citrate synthase substock (8.8 mU/µl in buffer, 50 µl each bullet tube, -20 °C)

Protocol (followed by Sigma Citrate Assay kit):

1) Set microplate reader to 25 °C, kinetic reading at 10 sec interval for 90 sec, 412 nm
2) Set waterbath to 25 °C and defrost chemicals in waterbath
3) Dilute samples by adding 10 µl sample into 490 µl buffer (50x)
4) Note: For pellets, re-suspend in buffer and sonicate, supernatants are sonicated as well
5) Preparde standard concentrations:

Std 5 (2.75 mU/µl): 50 µl standard substock into 110 µl buffer

Std 4 (1.375 mU/µl): 80 µl std 5 into 80 µl buffer

Std 3 (0.567 mU/µl): 13 µl std 5 into 50 µl buffer

Std 2 (0.284 mU/µl): 13 µl std 4 into 50 µl buffer

Std 1 (0.153 mU/µl): 10 µl std 4 into 80 µl buffer

Blank: 50 µl buffer

6) Pipet 10 µl standard or sample into each 96-well plate cell (3 replicates)
7) Mix 40:40 3520 µl (18 reactions) acetyl co-A, DTNB and buffer as the reaction mixture solution (each well: 2 µl acetyl co-A, 2 µl DTNB, 176 µl buffer)
8) Pipet 180 µl reaction solution into each well using repeator pipet (200 µl for 18 reactions)
9) Shake 10 sec in microplate reader
10) Read absorbance at 10 sec for 90 sec at 412 nm (total activy: change of abs/sec)
11) Calculate the net activity (total activity – endogenous activity)

Calculations: units (µmole/ml/min) = $\frac{(\Delta A_{412})/\text{min} \times V(\text{ml}) \times DF}{\varepsilon^{mM} \times L(\text{cm}) \times \text{Venz (ml)}}$

DF: dilution factor of the original sample

V (ml): the reaction volume (0.2 ml for 96-well plate)

Venz(ml): volume of the enzyme sample in ml

ε^{mM} (mM^{-1} cm^{-1}): the extinction coefficient of TNB at 412 nm is 13.6

L (cm): pathlength for absorbance measurement (for 96 well-plates, pathlength = 0.552 cm)

Appendix C: Metallothionein Protocol

Adapted from Viarengo (1997) and Dr. Tania Ng

Solutions to prepare or acquire prior to performing the assay:

Homogenization Buffer, pH 8.6 – Total volume: 100 ml or 0.**100L** milliQ

Note: Final volume is 101 ml. PMFS is photosensitive and must be stored in the dark at -4 °C. Ethanol must be cold (store at -40 °C). β-mercaptoethanol must be used in the fumehood.

Solution	**Final Concentration**	**Calculations**
Sucrose	500 mM MW: 342.31	Mass (g) = 342.31 g * 0.**100 L** * 0.5 M Mass (g) = 17.1155 g
Tris HCl	20 mM MW: 157.2	Mass (g) = 157,6 g * 0.**100 L** * 0.02 M Mass (g) = 0.3152 g
PMFS *Ethanol as solvent	100 mM MW: 174.2	Mass (g) = 174.2 g * 0.001 L * 0.1 M Mass (g) = 0.01722 g
β-mercaptoethanol	% MW 78.13	From 98% pure solution (14.3 M) 9.13 µL into 100 ml solution: 1094.8 g/L = 109.48 g/ 100 ml 109.48 g/ 100 ml = 0.01 g/X X = .009134 ml/**100 ml**

NaCl

NaCl	250 mM MW: 58.44	Mass (g) = 58.44 g * 0.050 L * 0.250 M Mass (g) = 0.7305 g
NaCl	2.0 M MW: 58.44	Mass (g) = 58.44 g * 0.200 L * 2 M Mass (g) = 23.376 g
NaCl	125 mM MW: 58.44	Mass (g) = 58.44 g * 0.200 L * 0.125 M Mass (g) = 1.461 g

Tris-HCl

Tris-HCl	20mM MW: 157.6	Mass (g) = 157.6 * 0.100 L * 0.02 M Mass (g) = 0.3152 g

Ethanol - (100%) at -20 °C

Chloroform – (100%) at -20 °C

HCl - (37 %)

Ethanol – Chloroformium and Tris-HCl (20 mM) – pH 8.6

- Combine 3, 4, and 5 to make a solution containing:
  a) 12 % Tris-HCl
  b) 87 % Ethanol
  c) 1 % Chloroform

Solutions to prepare the day of analyses:

EDTA Solution (50 ml)

EDTA	4 mM PM 292.2	Mass (g) = 292.2 g * 0.50 L Mass (g) = 0.05844
HCl	1N (37%) MW 36.46	4.94 ml into 45.06 ml milliQ H_2O

DTNB

DTNB *Methanol as solvent	20 mM MW: 396. 3	Mass (g) = 396.3 g * 0.050 L * 0.02 M Mass (g) = 0.3963 g

Working Buffer – pH 8.0

Phosphate Buffer Na2HPO4.7H2O	200 mM MW: 268.07	Mass (g) = 268.07 * 0.200 L * .2M Mass (g) = 10.7228 g

Note: Must be made at room temperature. Combine with NaCl (b) for a total volume of 200 mL MilliQ H_2O

Standard Preparation:

EDTA solution (200ml) 100 ml – blank 100 ml – GSH	EDTA 2mM MW: 292.2	Mass (g) = 292.2 * 0.200 L * 0.002 M Mass (g) = .11688 g
	HCl 0.5 N= 0.5M 37% MW: 36.46	9.87 ml into 190.13 ml MillQ H_2O
	NaCl (c)	
GSH solution	500 µM MW: 307.3	Mass (g) = 307.3 * 0.100 L (EDTA sol'n) * 0.0005M Mass (g) = 0.015365 g

Dilutions: Total volume = 10 ml

Concentration (µM)	EDTA solution (ml)	GSH solution (ml)
15	9.7	0.3
30	9.4	0.6
50	9	1
90	8.2	1.8
200	6	4
300	4	6
400	2	8

Protocol

1) Homogenize (minimum of 0.075 g) tissue: 5 ml of homogenization buffer/ g wet tissue
2) Centrifuge at 30,000 g for 45 mins at 4 °C
3) Take 250 µl of supernatant and add 265 µl of cold ethanol (100%) and 20 µ of cold chloroform (100%). *Vortex
4) Centrifuge at 6000 g for 10 mins at 4 °C
5) Take 300 µl of supernatant and add 20 µl of HCl (37%) plus 900 µl cold ethanol (100%), vortex, and incubate at -20 °C for 1 hour

Note: During one hour break, add 21.5 µl of DNTB solution every 1 ml of working buffer required (usually 10 ml will suffice)

6) Centrifuge at 6000 g for 10 mins at 4 °C and keep the pellet
7) Add to the pellet 200 µl of ethanol-chloroform (and Tris-HCl) solution, homogenize, then add 800 µl of the same solution and vortex
8) Centrifuge at 6000 g for 10 mins at 4 °C and keep the pellet
9) Add to the pellet 150 µl of (a) NaCl 250 mM solution and 150 µl of EDTA 4 mM + HCl 1n solution, homogenize

10) Add 100 µl of each sample to bullet tubes filled with 1.2 ml of working buffer + DNTB solution and vortex
11) Add 100 µl of each standard (GSH) to 1.2 ml of working buffer + DNTB solution and vortex
12) Add 350 µl of each sample and standard mixed with the working buffer + DNTB to 96-well plates (3 replicates each)
13) Read at 412 nm (stable for several hrs)

Calculations: 0.12 * [GSH]/1000) * (1/0.05) * (535/300) * (300/100) = µM GSH/g wet tissue

Appendix D: ANOVA Tables

1) Sublethal chronic Cd exposure in moderately hard water

Table 1.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for total Cd in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	S	S		
17	S	S	NS	
29	S	S	NS	NS

Table 1.0.1 Results from two-way ANOVA for total Cd in the liver. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	11.301	5.651	127.649	<0.001
Day	4	2.392	0.598	13.509	<0.001
Exposure x Day	8	3.762	0.470	10.622	<0.001
Residual	75	3.320	0.0443		
Total	89	20.775	0.233		

Table 1.1-1.6 Results from ANOVA post-hoc tests (Tukey's HSD) comparing individual subcellular fraction Cd concentrations in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

Metal-rich granules (MRG)

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	S	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

Table 1.1.1 Results from two-way ANOVA for total Cd in the liver MRG fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.00241	0.00120	9.650	<0.001
Day	4	0.000594	0.000149	1.191	0.322
Exposure x Day	8	0.00101	0.000126	1.009	0.437
Residual	75	0.00935	0.000125		
Total	89	0.0134	0.000150		

Microsomes + Lysosomes (ML)

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	S	S		
17	S	S	NS	
29	S	S	S	S

Table 1.2.1 Results from two-way ANOVA for total Cd in the liver ML fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0867	0.0434	40.472	<0.001
Day	4	0.0221	0.00552	5.155	0.001
Exposure x Day	8	0.0565	0.00706	6.587	<0.001
Residual	75	0.0804	0.00107		
Total	89	0.246	0.00276		

Metallothionein-like proteins (MTLP)

	control					0.75 µg Cd /L					2.0 µg/L				
Days	1	4	7	17		Days	1	4	7	17		Days	1	4	7	17
4	NS					4	NS					4	NS			
7	NS	NS				7	NS	NS				7	S	S		
17	NS	NS	NS			17	NS	NS	NS			17	S	S	S	
29	NS	NS	NS	NS		29	NS	NS	NS	NS		29	S	S	S	NS

Table 1.3.1 Results from two-way ANOVA for total Cd in the liver MTLP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.530	0.265	203.906	<0.001
Day	4	0.0934	0.0233	17.950	<0.001
Exposure x Day	8	0.234	0.0292	22.462	<0.001
Residual	75	0.0975	0.00130		
Total	89	0.955	0.0107		

Cellular-debris

	control					0.75 µg Cd /L					2.0 µg/L				
Days	1	4	7	17		Days	1	4	7	17		Days	1	4	7	17
4	NS					4	NS					4	S			
7	NS	NS				7	NS	NS				7	NS	NS		
17	NS	NS	NS			17	NS	NS	NS			17	S	NS	NS	
29	NS	NS	NS	NS		29	NS	NS	NS	NS		29	S	NS	NS	NS

Table 1.4.1 Results from two-way ANOVA for total Cd in the liver cellular-debris fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0991	0.0495	85.743	<0.001
Day	4	0.00285	0.000713	1.234	0.304
Exposure x Day	8	0.00508	0.000635	1.098	0.374
Residual	75	0.0433	0.000578		
Total	89	0.150	0.00169		

Mitochondria

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	S	S		
17	S	S	NS	
29	S	S	S	NS

Table 1.5.1 Results from two-way ANOVA for total Cd in the liver mitochondria fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.372	0.186	95.794	<0.001
Day	4	0.144	0.0359	18.501	<0.001
Exposure x Day	8	0.164	0.0205	10.530	<0.001
Residual	75	0.146	0.00194		
Total	89	0.825	0.00927		

Heat-sensitive proteins (HSP)

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	S	S		
17	NS	NS	NS	
29	S	S	S	NS

Table 1.6.1 Results from two-way ANOVA for total Cd in the liver HSP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.275	0.138	123.821	<0.001
Day	4	0.0333	0.00833	7.498	<0.001
Exposure x Day	8	0.0555	0.00693	6.235	<0.001
Residual	75	0.0834	0.00111		
Total	89	0.447	0.00503		

Table 2.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for total Cd in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	S	S	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	S	NS		
17	S	S	S	
29	S	S	S	S

Table 2.0.1 Results from two-way ANOVA for total Cd in the kidney. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	24.296	12.148	175.857	<0.001
Day	4	16.212	4.053	58.672	<0.001
Exposure x Day	8	20.622	2.578	37.316	<0.001
Residual	75	5.181	0.0691		
Total	89	66.312	0.745		

Table 2.1-2.6 Results from ANOVA post-hoc tests (Tukey's HSD) comparing individual subcellular fraction Cd concentrations in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

Metal-rich granules (MRG)

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	S	S		
17	S	S	NS	
29	S	S	S	S

Table 2.1.1 Results from two-way ANOVA for total Cd in the kidney MRG fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.379	0.190	84.113	<0.001
Day	4	0.116	0.0290	12.855	<0.001
Exposure x Day	8	0.159	0.0198	8.800	<0.001
Residual	75	0.169	0.00225		
Total	89	0.823	0.00925		

Microsomes + Lysosomes (ML)

A) control

Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

B) 0.75 µg Cd /L

Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	S	NS	NS

C) 2.0 µg/L

Days	1	4	7	17
4	NS			
7	S	NS		
17	S	S	S	
29	S	S	S	NS

Table 2.2.1 Results from two-way ANOVA for total Cd in the kidney ML fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.243	0.121	77.127	<0.001
Day	4	0.191	0.0477	30.274	<0.001
Exposure x Day	8	0.227	0.0284	18.043	<0.001
Residual	75	0.118	0.00157		
Total	89	0.779	0.00875		

Metallothionein like-protein fraction (MTLP)

	control					0.75 µg Cd /L					2.0 µg/L				
Days	1	4	7	17		Days	1	4	7	17	Days	1	4	7	17
4	NS					4	NS				4	NS			
7	NS	NS				7	NS	NS			7	NS	NS		
17	NS	NS	NS			17	NS	NS	NS		17	S	S	S	
29	NS	NS	NS	NS		29	S	S	S	S	29	S	S	S	S

Table 2.3.1 Results from two-way ANOVA for total Cd in the kidney MTLP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	1.589	0.795	56.356	<0.001
Day	4	2.422	0.606	42.950	<0.001
Exposure x Day	8	1.759	0.220	15.599	<0.001
Residual	75	1.057	0.0141		
Total	89	6.828	0.0767		

Cellular-debris

	control					0.75 µg Cd /L					2.0 µg/L				
Days	1	4	7	17		Days	1	4	7	17	Days	1	4	7	17
4	NS					4	NS				4	NS			
7	NS	NS				7	NS	NS			7	NS	NS		
17	NS	NS	NS			17	S	S	S		17	S	S	NS	
29	NS	NS	NS	NS		29	S	S	NS	NS	29	S	S	S	S

Table 2.4.1 Results from two-way ANOVA for total Cd in the kidney cellular-debris fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	1.960	0.980	65.616	<0.001
Day	4	1.188	0.297	19.895	<0.001
Exposure x Day	8	1.058	0.132	8.860	<0.001
Residual	75	1.120	0.0149		
Total	89	5.327	0.0598		

Mitochondria

control				
Days	1	4	7	17
4	S			
7	NS	S		
17	NS	NS	S	
29	S	NS	S	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	S	S	S	
29	S	S	S	NS

Table 2.5.1 Results from two-way ANOVA for total Cd in the kidney mitochondria fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0120	0.00598	22.190	<0.001
Day	4	0.00584	0.00146	5.420	<0.001
Exposure x Day	8	0.0292	0.00365	13.550	<0.001
Residual	75	0.0202	0.000269		
Total	89	0.0672	0.000755		

Heat-sensitive proteins (HSP)

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	17
4	S			
7	S	NS		
17	S	NS	NS	
29	S	NS	NS	NS

Table 2.6.1 Results from two-way ANOVA for total Cd in the kidney HSP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0836	0.0418	25.388	<0.001
Day	4	0.0584	0.0146	8.865	<0.001
Exposure x Day	8	0.0499	0.00623	3.787	<0.001
Residual	75	0.123	0.00165		
Total	89	0.315	0.00354		

3.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for total Cd in the gills within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	S	NS	
29	S	S	S	S

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	S	S	S	
29	S	S	S	NS

Table 3.0.1 Results from two-way ANOVA for total Cd in the gills. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.737	0.369	65.348	<0.001
Day	4	0.388	0.0969	17.189	<0.001
Exposure x Day	8	0.253	0.0317	5.614	<0.001
Residual 75	0.423	0.00564			
Total 89	1.801	0.0202			

Table 3.1-3.6 Results from ANOVA post-hoc tests (Tukey's HSD) comparing individual subcellular fraction Cd concentrations in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

3.1 Metal-rich granules (MRG)

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	17
4	S			
7	NS	S		
17	NS	NS	NS	
29	NS	S	NS	NS

Table 3.1.1 Results from two-way ANOVA for total Cd in the gill MRG fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0393	0.0196	57.308	<0.001
Day	4	0.00239	0.000597	1.741	0.150
Exposure x Day	8	0.00738	0.000923	2.692	0.012
Residual	75	0.0257	0.000343		
Total	89	0.0748	0.000840		

3.2 Microsomes + Lysosomes (ML)

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	S	NS	NS	
29	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	S	S		
17	S	S	S	
29	S	S	S	NS

Table 3.2.1 Results from two-way ANOVA for total Cd in the gill ML fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.307	0.154	43.123	<0.001
Day	4	0.213	0.0532	14.919	<0.001
Exposure x Day	8	0.258	0.0323	9.064	<0.001
Residual	75	0.267	0.00356		
Total	89	1.046	0.0118		

3.3 Metallothionein-like proteins (MTLP)

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	S	S	S	S

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	S	S	S	S

Table 3.3.1 Results from two-way ANOVA for total Cd in the gill MTLP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.926	0.463	15.266	<0.001
Day	4	3.419	0.855	28.167	<0.001
Exposure x Day	8	1.824	0.228	7.513	<0.001
Residual	75	2.276	0.0303		
Total	89	8.444	0.0949		

3.4 Cellular-debris

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

Table 3.4.1 Results from two-way ANOVA for total Cd in the gill cellular-debris fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	1.895	0.947	89.630	<0.001
Day	4	0.0497	0.0124	1.174	0.329
Exposure x Day	8	0.0581	0.00726	0.687	0.701
Residual	75	0.793	0.0106		
Total	89	2.796	0.0314		

3.5 Mitochondria

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	S	S	S	S

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	S	NS		
17	S	S	NS	
29	S	S	S	NS

Table 3.5.1 Results from two-way ANOVA for total Cd in the gill mitochondria fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	2.952	1.476	42.236	<0.001
Day	4	2.534	0.633	18.124	<0.001
Exposure x Day	8	1.521	0.190	5.439	<0.001
Residual	75	2.621	0.0349		
Total	89	9.627	0.108		

3.6 Heat-sensitive proteins (HSP)

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	S	S		
17	S	S	S	
29	S	S	S	NS

Table 3.6.1 Results from two-way ANOVA for total Cd in the gill mitochondria fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	1.759	0.880	78.537	<0.001
Day	4	0.495	0.124	11.045	<0.001
Exposure x Day	8	1.178	0.147	13.143	<0.001
Residual	75	0.840	0.0112		
Total	89	4.272	0.0480		

2) Sublethal chronic Cd exposure in moderately soft water

Table 4.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for total Cd in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	S	
30	S	S	S	S

Table 4.0.1 Results from two-way ANOVA for total Cd in the liver. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	2.416	1.208	51.529	<0.001
Day	4	2.785	0.696	29.699	<0.001
Exposure x Day	8	2.430	0.304	12.959	<0.001
Residual	75	1.758	0.0234		
Total	89	9.389	0.105		

Table 4.1-4.6 Results from ANOVA post-hoc tests (Tukey's HSD) comparing individual subcellular fraction Cd concentrations in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

4.1 Metal-rich granules (MRG)

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 µg/L			
	1	4	7	16
4	NS			
7	S	NS		
16	S	S	NS	
30	S	S	NS	NS

Table 4.1.1 Results from two-way ANOVA for total Cd in the liver MRG fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.00954	0.00477	38.605	<0.001
Day	4	0.00269	0.000672	5.445	<0.001
Exposure x Day	8	0.00380	0.000475	3.843	<0.001
Residual	75	0.00926	0.000123		
Total	89	0.0253	0.000284		

4.2 Microsomes + Lysosomes (ML)

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	S	
30	S	S	S	S

Table 4.2.1 Results from two-way ANOVA for total Cd in the liver ML fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.140	0.0700	48.656	<0.001
Day	4	0.124	0.0309	21.498	<0.001
Exposure x Day	8	0.149	0.0186	12.903	<0.001
Residual	75	0.108	0.00144		
Total	89	0.520	0.00585		

4.3 Metallothionein-like proteins (MTLP)

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	S			
7	S	NS		
16	S	NS	NS	
30	NS	S	NS	NS

	2.0 µg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

Table 4.3.1 Results from two-way ANOVA for total Cd in the liver MTLP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.00886	0.00443	21.256	<0.001
Day	4	0.00939	0.00235	11.263	<0.001
Exposure x Day	8	0.0161	0.00202	9.684	<0.001
Residual 75	0.0156	0.000208			
Total 89	0.0500	0.000562			

4.4 Cellular-debris

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	S	NS	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

Table 4.4.1 Results from two-way ANOVA for total Cd in the liver cellular-debris fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.00162	0.000809	17.712	<0.001
Day	4	0.000299	0.0000748	1.638	0.174
Exposure x Day	8	0.000523	0.0000654	1.431	0.198
Residual	75	0.00343	0.0000457		
Total	89	0.00587	0.0000659		

4.5 Mitochondria

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	S	NS		
16	S	NS	NS	
30	S	S	S	S

Table 4.5.1 Results from two-way ANOVA for total Cd in the liver mitochondria fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.116	0.0579	47.339	<0.001
Day	4	0.0939	0.0235	19.204	<0.001
Exposure x Day	8	0.0658	0.00822	6.730	<0.001
Residual	75	0.0917	0.00122		
Total	89	0.367	0.00412		

4.6 Heat-sensitive proteins (HSP)

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	NS	S

	2.0 µg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

Table 4.6.1 Results from two-way ANOVA for total Cd in the liver HSP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0270	0.0135	19.181	<0.001
Day	4	0.0493	0.0123	17.517	<0.001
Exposure x Day	8	0.0386	0.00482	6.855	<0.001
Residual	75	0.0527	0.000703		
Total	89	0.168	0.00188		

Table 4.7 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for metallothionein concentrations in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	S	NS	NS

Table 4.7.1 Results from two-way ANOVA for liver metallothionein concentrations. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	489.492	244.746	14.337	<0.001
Day	4	101.777	25.444	1.490	0.214
Exposure x Day	8	347.936	43.492	2.548	0.016
Residual	75	1280.347	17.071		
Total	89	2219.551	24.939		

Table 5.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for total Cd in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

Table 5.0.1 Results from two-way ANOVA for total Cd in the kidney. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	7.312	3.656	17.389	<0.001
Day	4	16.681	4.170	19.834	<0.001
Exposure x Day	8	18.181	2.273	10.809	<0.001
Residual	75	15.769	0.210		
Total	89	57.943	0.651		

Table 5.1-5.6 Results from ANOVA post-hoc tests (Tukey's HSD) comparing individual subcellular fraction Cd concentrations in the kidney within each treatment (control, 0.75 and 2.0 μg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

5.1 Metal-rich granules (MRG)

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 μg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

2.0 μg/L				
Days	1	4	7	16
4	NS			
7	S	NS		
16	S	NS	NS	
30	S	S	S	S

Table 5.1.1 Results from two-way ANOVA for total Cd in the kidney MRG fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0694	0.0347	57.263	<0.001
Day	4	0.0412	0.0103	16.989	<0.001
Exposure x Day	8	0.0467	0.00584	9.641	<0.001
Residual	75	0.0454	0.000606		
Total	89	0.203	0.00228		

5.2 Microsomes + Lysosomes (ML)

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 μg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

2.0 μg/L				
Days	1	4	7	16
4	NS			
7	S	NS		
16	NS	NS	NS	
30	S	S	NS	NS

Table 5.2.1 Results from two-way ANOVA for total Cd in the kidney ML fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.000983	0.000491	15.553	<0.001
Day	4	0.00207	0.000517	16.353	<0.001
Exposure x Day	8	0.00159	0.000199	6.294	<0.001
Residual	75	0.00237	0.0000316		
Total	89	0.00701	0.0000788		

5.3 Metallothionein-like proteins (MTLP)

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

	2.0 µg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	S	
30	S	S	S	S

Table 5.3.1 Results from two-way ANOVA for total Cd in the kidney MTLP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.978	0.489	81.049	<0.001
Day	4	2.238	0.560	92.781	<0.001
Exposure x Day	8	2.100	0.262	43.526	<0.001
Residual	75	0.452	0.00603		
Total	89	5.768	0.0648		

5.4 Cellular-debris

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	16
4	S			
7	S	NS		
16	S	NS	NS	
30	S	S	S	S

Table 5.4.1 Results from two-way ANOVA for total Cd in the kidney cellular-debris fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.216	0.108	167.814	<0.001
Day	4	0.0545	0.0136	21.220	<0.001
Exposure x Day	8	0.0923	0.0115	17.960	<0.001
Residual	75	0.0482	0.000642		
Total	89	0.411	0.00461		

5.5 Mitochondria

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

Table 5.5.1 Results from two-way ANOVA for total Cd in the kidney mitochondria fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0395	0.0197	12.383	<0.001
Day	4	0.0551	0.0138	8.651	<0.001
Exposure x Day	8	0.0648	0.00810	5.083	<0.001
Residual	75	0.120	0.00159		
Total	89	0.279	0.00313		

5.6 Heat-sensitive proteins (HSP)

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	NS	
30	S	S	NS	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	NS	
30	S	S	S	S

Table 5.6.1 Results from two-way ANOVA for total Cd in the kidney HSP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.0482	0.0241	31.332	<0.001
Day	4	0.0595	0.0149	19.359	<0.001
Exposure x Day	8	0.0485	0.00606	7.889	<0.001
Residual	75	0.0576	0.000769		
Total	89	0.214	0.00240		

Table 5.7 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for metallothionein concentrations in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	S	S	S

Table 5.7.1 Results from two-way ANOVA for kidney metallothionein concentrations. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	3383.486	1691.743	6.360	0.003
Day	4	3370.993	842.748	3.168	0.018
Exposure x Day	8	7898.647	987.331	3.712	0.001
Residual	75	19950.980		266.013	
Total	89	34604.107		388.810	

Table 6.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for total Cd in the gill within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	S	NS		
16	S	S	S	
30	NS	NS	NS	S

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	S	
30	S	NS	NS	S

Table 6.0.1 Results from two-way ANOVA for total Cd concentrations in the gills. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	483.310	241.655	39.639	<0.001
Day	4	455.351	113.838	18.673	<0.001
Exposure x Day	8	263.722	32.965	5.407	<0.001
Residual	75	457.231	6.096		
Total	89	1659.614	18.647		

Table 6.1-6.6 Results from ANOVA post-hoc tests (Tukey's HSD) comparing individual subcellular fraction Cd concentrations in the gills within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

6.1 Metal-rich granules (MRG)

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	NS	
30	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	S	S		
16	S	S	NS	
30	S	S	S	NS

Table 6.1.1 Results from two-way ANOVA for total Cd concentrations in the gill MRG fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.871	0.435	63.557	<0.001
Day	4	0.449	0.112	16.375	<0.001
Exposure x Day	8	0.341	0.0426	6.220	<0.001
Residual	75	0.514	0.00685		
Total	89	2.174	0.0244		

6.2 Microsomes + Lysosomes (ML)

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	S	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	S	
30	S	S	S	S

Table 6.2.1 Results from two-way ANOVA for total Cd concentrations in the gill ML fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	3.863	1.931	47.141	<0.001
Day	4	2.264	0.566	13.818	<0.001
Exposure x Day	8	3.758	0.470	11.464	<0.001
Residual	75	3.073	0.0410		
Total	89	12.958	0.146		

6.3 Metallothionein-like proteins (MTLP)

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	S	
30	S	S	S	S

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	S	
30	S	S	S	S

Table 6.3.1 Results from two-way ANOVA for total Cd concentrations in the gill MTLP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	1.500	0.750	47.221	<0.001
Day	4	2.431	0.608	38.260	<0.001
Exposure x Day	8	1.285	0.161	10.113	<0.001
Residual	75	1.192	0.0159		
Total	89	6.409	0.0720		

6.4 Cellular-debris

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	S	S		
16	S	S	S	
30	S	S	S	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	S	S		
16	S	S	NS	
30	S	S	NS	NS

Table 6.4.1 Results from two-way ANOVA for total Cd concentrations in the gill cellular-debris fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	9.893	4.947	139.498	<0.001
Day	4	6.554	1.639	46.207	<0.001
Exposure x Day	8	3.898	0.487	13.739	<0.001
Residual	75	2.660	0.0355		
Total	89	23.004	0.258		

6.5 Mitochondria

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	S	NS		
16	S	NS	NS	
30	S	S	NS	NS

2.0 µg/L				
Days	1	4	7	16
4	NS			
7	S	NS		
16	S	S	S	
30	S	S	S	NS

Table 6.5.1 Results from two-way ANOVA for total Cd concentrations in the gill mitochondrial fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	9.028	4.514	66.946	<0.001
Day	4	8.210	2.052	30.440	<0.001
Exposure x Day	8	6.606	0.826	12.248	<0.001
Residual	75	5.057	0.0674		
Total	89	28.900	0.325		

6.6 Heat-sensitive proteins (HSP)

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	NS	S	NS

2.0 µg/L				
Days	1	4	7	16
4	S			
7	S	S		
16	NS	NS	S	
30	S	NS	S	NS

Table 6.6.1 Results from two-way ANOVA for total Cd concentrations in the gill HSP fraction. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	0.745	0.372	45.961	<0.001
Day	4	0.256	0.0640	7.898	<0.001
Exposure x Day	8	0.403	0.0504	6.223	<0.001
Residual	75	0.608	0.00810		
Total	89	2.011	0.0226		

Table 6.7 Results from ANOVA post-hoc tests (Tukey's HSD) comparing means for metallothionein concentrations in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	S			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 µg/L			
	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	NS	
30	S	S	NS	NS

Table 6.7.1 Results from two-way ANOVA for gill metallothionein concentrations. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
Exposure	2	631.740	315.870	7.388	0.001
Day	4	647.246	161.811	3.785	0.007
Exposure x Day	8	557.736	69.717	1.631	0.130
Residual	75	3206.617	42.755		
Total	89	5043.339	56.667		

3) Subcellular partitioning of Cd into MSP and MDP

Table 7.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MSP in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately hard water. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	S	S	S	NS

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

Table 7.0.1 Results from two-way ANOVA for Cd bound to MSP in the liver of fish exposed to sublethal concentrations of Cd in moderately hard water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.166	0.0414	2.309	0.066
exposure	2	0.176	0.0878	4.899	0.010
day x exposure	8	0.291	0.0364	2.028	0.054
Residual	75	1.345	0.0179		
Total	89	1.977	0.0222		

Table 7.1 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MDP in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately hard water. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	S	NS

2.0 µg/L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

Table 7.1.1 Results from two-way ANOVA for Cd bound to MDP in the liver of fish exposed to sublethal concentrations of Cd in moderately hard water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.120	0.0299	1.127	0.350
exposure	2	0.268	0.134	5.048	0.009
day x exposure	8	0.274	0.0342	1.288	0.263
Residual	75	1.991	0.0266		
Total	89	2.653	0.0298		

Table 7.2 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MSP in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately hard water. S indicates significant difference between means (P <0.05) and NS that they are not

	control					0.75 µg Cd /L					2.0 µg/L				
Days	1	4	7	17		Days	1	4	7	17		Days	1	4	7	17	
4	NS					4	S					4	NS				
7	NS	NS				7	S	NS				7	S	NS			
17	NS	NS	NS			17	S	S	NS			17	S	S	NS		
29	NS	NS	NS	NS		29	S	S	S	NS		29	S	S	NS	NS	

Table 7.2.1 Results from two-way ANOVA for Cd bound to MSP in the kidney of fish exposed to sublethal concentrations of Cd in moderately hard water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.463	0.116	17.254	<0.001
exposure	2	0.224	0.112	16.722	<0.001
day x exposure	8	0.310	0.0387	5.778	<0.001
Residual	75	0.503	0.00670		
Total	89	1.499	0.0168		

Table 7.3 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MDP in the kidney within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately hard water. S indicates significant difference between means (P <0.05) and NS that they are not

	control					0.75 µg Cd /L					2.0 µg/L				
Days	1	4	7	17		Days	1	4	7	17		Days	1	4	7	17	
4	NS					4	NS					4	NS				
7	NS	NS				7	NS	NS				7	NS	NS			
17	NS	NS	NS			17	NS	NS	NS			17	NS	NS	NS		
29	NS	NS	NS	NS		29	S	S	NS	NS		29	NS	NS	NS	NS	

Table 7.3.1 Results from two-way ANOVA for Cd bound to MDP in the kidney of fish exposed to sublethal concentrations of Cd in moderately hard water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.261	0.0653	3.624	0.009
exposure	2	0.0292	0.0146	0.810	0.449
day x exposure	8	0.237	0.0296	1.643	0.127
Residual	75	1.352	0.0180		
Total	89	1.879	0.0211		

Table 7.4 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MSP in the gill within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately hard water. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	S	S	NS

2.0 µg/L				
Days	1	4	7	17
4	S			
7	S	NS		
17	S	NS	NS	
29	S	NS	NS	NS

Table 7.4.1 Results from two-way ANOVA for Cd bound to MSP in the gill of fish exposed to sublethal concentrations of Cd in moderately hard water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.209	0.0523	4.790	0.002
exposure	2	0.0597	0.0299	2.737	0.071
day x exposure	8	0.558	0.0698	6.395	<0.001
Residual	75	0.818	0.0109		
Total	89	1.645	0.0185		

Table 7.5 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MDP in the gill within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately hard water. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	17
4	NS			
7	NS	NS		
17	NS	NS	NS	
29	S	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	17
4	NS			
7	NS	S		
17	NS	NS	NS	
29	NS	NS	NS	NS

Table 7.5.1 Results from two-way ANOVA for Cd bound to MDP in the gill of fish exposed to sublethal concentrations of Cd in moderately hard water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.217	0.0543	3.778	0.007
exposure	2	0.120	0.0601	4.177	0.019
day x exposure	8	0.330	0.0413	2.868	0.008
Residual	75	1.079	0.0144		
Total	89	1.747	0.0196		

Table 8.0 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MSP in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately soft water. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	S			
7	S	NS		
16	S	NS	NS	
30	S	NS	S	NS

	2.0 µg/L			
Days	1	4	7	16
4	S			
7	S	NS		
16	NS	S	NS	
30	S	S	NS	NS

Table 8.0.1 Results from two-way ANOVA for Cd bound to MSP in the liver of fish exposed to sublethal concentrations of Cd in moderately soft water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.265	0.0662	9.681	<0.001
exposure	2	0.123	0.0613	8.963	<0.001
day x exposure	8	0.266	0.0333	4.865	<0.001
Residual	75	0.513	0.00684		
Total	89	1.167	0.0131		

Table 8.1 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MDP in the liver within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately soft water. S indicates significant difference between means (P <0.05) and NS that they are not

control				
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

0.75 µg Cd /L				
Days	1	4	7	16
4	S			
7	NS	NS		
16	NS	NS	NS	
30	S	NS	NS	NS

2.0 µg/L				
Days	1	4	7	16
4	S			
7	NS	NS		
16	NS	S	NS	
30	NS	S	NS	NS

Table 8.1.1 Results from two-way ANOVA for Cd bound to MDP in the liver of fish exposed to sublethal concentrations of Cd in moderately soft water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.128	0.0321	4.492	0.003
exposure	2	0.126	0.0630	8.824	<0.001
day x exposure	8	0.124	0.0154	2.163	0.040
Residual	75	0.536	0.00714		
Total	89	0.914	0.0103		

Table 8.2 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MSP in the kidney within each treatment (control, 0.75 and 2.0 μg Cd/L) by day of exposure for fish exposed in moderately soft water. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 μg Cd /L			
Days	1	4	7	16
4	NS			
7	S	NS		
16	S	NS	NS	
30	NS	NS	NS	NS

	2.0 μg/L			
Days	1	4	7	16
4	NS			
7	S	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

Table 8.2.1 Results from two-way ANOVA for Cd bound to MSP in the kidney of fish exposed to sublethal concentrations of Cd in moderately soft water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.116	0.0290	4.880	0.001
exposure	2	0.533	0.266	44.827	<0.001
day x exposure	8	0.115	0.0144	2.418	0.022
Residual	75	0.446	0.00594		
Total	89	1.210	0.0136		

Table 8.3 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MDP in the kidney within each treatment (control, 0.75 and 2.0 μg Cd/L) by day of exposure for fish exposed in moderately soft water. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 μg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 μg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	S	NS	
30	S	S	S	S

Table 8.3.1 Results from two-way ANOVA for Cd bound to MDP in the kidney of fish exposed to sublethal concentrations of Cd in moderately soft water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.335	0.0838	11.191	<0.001
exposure	2	0.0376	0.0188	2.511	0.088
day x exposure	8	0.353	0.0441	5.889	<0.001
Residual	75	0.562	0.00749		
Total	89	1.287	0.0145		

Table 8.4 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MSP in the gill within each treatment (control, 0.75 and 2.0 µg Cd/L) by day of exposure for fish exposed in moderately soft water. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 µg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	2.0 µg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

Table 8.4.1 Results from two-way ANOVA for Cd bound to MSP in the gill of fish exposed to sublethal concentrations of Cd in moderately soft water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.0704	0.0176	1.640	0.173
exposure	2	0.187	0.0934	8.706	<0.001
day x exposure	8	0.0792	0.00990	0.922	0.503
Residual	75	0.805	0.0107		
Total	89	1.141	0.0128		

Table 8.5 Results from ANOVA post-hoc tests (Tukey's HSD) comparing partitioning of Cd into MDP in the gill within each treatment (control, 0.75 and 2.0 μg Cd/L) by day of exposure for fish exposed in moderately soft water. S indicates significant difference between means (P <0.05) and NS that they are not

	control			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	NS	NS	NS	NS

	0.75 μg Cd /L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	S	NS	NS	
30	S	NS	NS	NS

	2.0 μg/L			
Days	1	4	7	16
4	NS			
7	NS	NS		
16	NS	NS	NS	
30	S	NS	S	NS

Table 8.5.1 Results from two-way ANOVA for Cd bound to MDP in the gill of fish exposed to sublethal concentrations of Cd in moderately soft water. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, P = P value

Source of Variation	DF	SS	MS	F	P
day	4	0.0256	0.00639	0.870	0.486
exposure	2	0.0517	0.0259	3.521	0.035
day x exposure	8	0.271	0.0339	4.609	<0.001
Residual	75	0.551	0.00735		
Total	89	0.899	0.0101		