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Cd-Induced Responses in Plasma Ionic Regulation and Oxidative Stress in Rainbow Trout (*O. mykiss*) or Lake Whitefish (*C. clupeaformis*) During Chronic Waterborne Exposure

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by

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Abstract

During chronic exposure to Cd or other metals, freshwater fish generally undergo physiological changes that results in acclimation, following a phase of damage and subsequent repair. The mechanisms associated with deleterious cellular effects induced by Cd throughout long-term exposures are less understood than those of acute toxicity, and may relate to an over-production of reactive oxygen species. The objectives of the present study were to examine the changes in, and relationship between, tissue-specific oxidative damage and antioxidant response, as well as plasma ionic regulation and tissue accumulation, in two freshwater salmonids throughout chronic Cd exposure. Rainbow trout (*O. mykiss*) or lake whitefish (*C. clupeaformis*) were exposed to either 0 (control), 0.8 or 2.0 μg Cd/L in moderately hard water (140 mg CaCO$_3$/L) for one month, and gills, liver, kidney and plasma were sampled throughout exposure. Measured responses included plasma Ca^{2+} and Na$^+$, as well as total tissue Cd burden and indicators of oxidative stress (lipid peroxidation, protein damage and enzymatic antioxidant defense (as catalase activity)) in tissues. Fish of either species experienced initial disruptions in plasma ion levels and/or mortality, which were associated with elevated Cd burdens in the gills. Exposure to sublethal Cd was sufficient to cause early oxidative lipid and/or protein damage in the liver and kidney, and subsequent recovery may relate to the elevated and sustained CAT response in these tissues, in addition to other cellular defenses. Long-term lipid peroxidation was found only in the gills of exposed rainbow trout, suggesting that the antioxidant capacities and/or detoxification mechanisms of the gills may be different than those in other tissues or of lake whitefish. Overall, this study demonstrates that the degree and pattern of oxidative damage and enzymatic antioxidant defense induced by Cd during chronic waterborne exposure varies amongst tissues and species.

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Chapter 1: Introduction

1.1 Cadmium in the Environment

Metals such as Cd are ubiquitous in aquatic environments due to natural and anthropogenic sources and are usually present in trace amounts. Cd is naturally found on the earth's crust, occurring in ore deposits with other elements such as Zn and Cu (Dobson, 1992; Canadian Council of Ministers of the Environment (or CCME), 1999). River run-off, erosion and abrasion of rocks and soils can contribute to Cd leaching into ground and surface waters (GESAMP, 1984; Dias and Edwards, 2003). Other natural sources include atmospheric deposition from forest fires and volcanic eruptions, as well as diatom deposition into marine sediment. Major anthropogenic releases of Cd occur from the mining, smelting and refining of Zn, Pb and Cu (Wilson, 1988; CCME, 1999). Cd is primarily used for electroplating other metals and in nickel-Cd batteries because of its relative resistance to corrosion and high electrical and thermal conductivity. Other industrial purposes include stabilizers for polyvinyl chloride, pigments in plastics or coatings and components in alloys (Wilson, 1988; CCME, 1999). Small amounts of Cd are also found in television picture tubes, telephone and trolley wires, automobile radiators and curing agents for tires. These inputs may result in above-background Cd levels of in aquatic ecosystems, which can be potentially toxic to organisms such as fish.

In aquatic environments, the potential of Cd to exert toxic effects on fish is dependent on its bioavailability or speciation (review by Niyogi and Wood, 2004). Whether in solution or bound to suspended particulates or sediment, Cd may be present in different chemical forms or species, such as $CdCl_2$, $CdCO_3$ or $CdSO_4$ (Calamari and Marchetti, 1978 in Wright and Welbourn, 1994). However, it is the free ionic form, Cd^{2+}, which is considered the most toxic to fish because of its potential for uptake across the biotic ligand (i.e. gill) (DiToro et al., 2001).

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1.2 Mechanism of uptake during waterborne exposure

Cd-gill binding in freshwater fish occurs via high-affinity, low-capacity sites (Playle et al., 1993), as well as low-affinity, high-capacity sites (Reid and McDonald, 1991). The high-affinity, low-capacity sites most likely represent the "toxic sites" that play an important role in maintaining Ca^{2+} homeostasis, as these sites were found to exhibit saturated binding during short-term (3h) assays (Playle et al, 1993). At the gill surface of fish, Cd^{2+} competes with Ca^{2+} for high-affinity Ca^{2+}-binding sites, such as the apical Ca^{2+} channels of chloride cells (Playle et al, 1993; Playle, 1998). In addition to disruption of apical Ca^{2+} uptake by direct competition for the Ca^{2+} channel, Cd further blocks Ca^{2+} uptake processes once in the ionocyte by inhibiting basolateral Ca^{2+} ATPases (Verbost et al, 1987, 1989). The result of these processes can lead to hypocalcemia in the fish (Wong and Wong, 2000) and potentially lethal consequences. Acute Cd uptake and toxicity are influenced by several water quality parameters, such as pH, hardness and alkalinity. Among these, water hardness appears to be the major factor affecting acute Cd toxicity, with Ca^{2+} having a greater protective effect than Mg^{2+} (review by Niyogi and Wood, 2003). At high water hardness, elevated concentrations of Ca^{2+} compete with Cd for binding sites on the gill (Playle et al., 1993), resulting in reduced accumulation and uptake in the gills (Hollis et al., 2000), thereby reducing Cd toxicity.

1.3 Cd distribution and accumulation in tissues

As with acute waterborne exposures, the main pathway/mechanism for Cd uptake into chronically-exposed fish is the gills. Once taken up branchially, Cd is transported to internal

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organs via specific proteins in blood plasma (Scott and Bradwell, 1984; Golaz et al., 1993). Because it is not required by the fish for metabolic needs, Cd is distributed to tissues for possible excretion and storage. Initial build-up occurs at the gills, however during extended exposures, Cd accumulates primarily in the kidney over time as well as in the liver, but not significantly in the brain or muscle (Norey et al., 1990; Harrison and Klaverkamp, 1989). In fish chronically exposed to Cd, the kidney appears to be the main storage organ, as Cd accumulates to high levels over time and is cleared relatively slower upon depuration (Hogstrand and Haux, 1991; McGeer et al., 2000b). The distribution to, and accumulation patterns within, the tissues is most likely related to specific roles associated with protective mechanisms. Some of these mechanisms involve Cd binding to cysteine-rich proteins such as metallothionein that aid sequestering non-essential metals (Kagi and Nordberg, 1979), thereby reducing potential toxicity at a cellular level (Roesijadi, 1992). Therefore, the progressive Cd burden within the liver and kidney may be linked to the induction/mobilization of metal-binding proteins, which is suggestive of roles in detoxification and long-term storage in these tissues. Strategies such as these are known to contribute to the fish's ability to handle (or tolerate) metal burden during chronic Cd exposures (Fu et al., 1990; De Smet et al., 2001; Hollis et al., 2001).

To better understand the mechanisms of chronic toxicity at a cellular level, studies have focused on relating overt toxic effects to the internal compartmentalization (or subcellular distribution) of metals within tissues, rather than the relation to total tissue burdens. Accumulated metal can be considered as existing in two pools—the biologically- or metabolically-active pool (referred to as biologically active metal (BAM)) and the biologically inactive pool (BIM). The BAM is available to participate or interact with cellular machinery, whereas the BIM is considered detoxified and therefore not able to interrupt normal metabolic

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function (Steen Redeker and Blust, 2004; Vijver et al., 2004). Because it is not required for metabolic needs, Cd is considered to be in a biologically- or metabolically-available form upon uptake (Rainbow, 2002). Thus, accumulated Cd within the tissues of fish must be made unavailable (or detoxified) in order to avoid possible physiological disruption and deleterious effects. In fish, one of the major mechanisms of detoxification involves the binding/ sequestering of Cd to soluble metal-binding proteins in the cytosol, such as metallothionein (Kagi and Schaffer, 1988; Roesijadi, 1992). Metallothionein is a cysteine-rich, low-molecular weight protein with a strong affinity for Cd. It has been shown to contribute to increased Cd tolerance in fathead minnows (Benson and Birge, 1985) and rainbow trout (Fu et al., 1990; Hollis et al., 2001; Chowdhury et al., 2005) chronically exposed to Cd. However, if Cd burden exceeds the detoxification capacities of the fish, for instance, metallothionein is insufficient for continued metal binding, Cd can remain biologically reactive. Therefore, if the BAM concentration is not maintained below a certain threshold level, this can, in theory, lead to disturbances and other toxic effects in sensitive tissues.

1.4 Acclimation

The pattern of acclimation to metals during chronic exposure is considered to occur in three phases, following a model of damage-repair (McDonald and Wood, 1993). The initial shock phase involves physical damage and accumulation at the primary uptake site (the gills) and is characterized by disturbances in ionoregulatory homeostasis (Wicklund Glynn et al., 1991; McDonald and Wood, 1993). This is a result of reduced influx of essential elements, such as Ca^{2+} and Na^+, and subsequent blood ion loss develops (Verbost et al., 1987, 1989; McGeer et al.,

2000a). The subsequent recovery phase involves enhanced biosynthetic pathways to help correct

the damage and disturbances (McDonald and Wood, 1993). This typically results in a re-

establishment of ionoregulation and changes in physiological status, as new steady states are

achieved and the fish develop an increased tolerance (or resistance) to further acute Cd

challenges (McDonald and Wood, 1993; Stubblefield et al., 1999; Hollis et al., 1999; McGeer et

al., 2000a).

1.5 ROS generation and oxidative stress

Reactive oxygen species (ROS) are produced by the incomplete (or partial) reduction of

molecular oxygen, O_2, and include radicals and non-radicals (review by Livingstone, 2001).

Examples of radicals include superoxide anion (O_2^-), hydroxyl ($OH^.$), hydroperoxyl ($HO_2^.$),

peroxyl ($RO_2^.$) and alkoxyl ($RO^.$). Non-radical species include hydrogen peroxide (H_2O_2),

hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). Some species, such as $OH^.$, are highly

reactive in aqueous solutions in comparison to others (Halliwell and Gutteridge, 1984;

Livingstone, 2001). This highly-reactive and non-discriminate nature allows them to interact

with macromolecules and potentially interfere with normal cellular function. Partially toxic ROS

are continuously produced in animals, mostly as unwanted by-products from various endogenous

processes (including enzymes and heme proteins) along the electron transport chain of

membranes, particularly the inner mitochondria (Raha and Robinson, 2000 in Cannino et al.,

2009). Under normal conditions, ROS are monitored and converted into less reactive species by

the fish's antioxidant defense system (Fig 2.1). Major non-enzymatic defenses include reduced

glutathione (GSH) and vitamin E, whereas enzymatic defenses include catalase (CAT),

superoxide dismutase (SOD), and glutathione peroxidase (GP_x) (Mather-Mihaich and DiGiulio, 1986 in Rand, 2003). Catalase, for example, is primarily associated with peroxisomes and responsible for the cytosolic degradation of H_2O_2 (into H_2O and O_2), which is formed as a by-product of fatty acid oxidation (Fahim and Sies, 1987 in Rand, 2003). Exposure to Cd or other toxicants may induce an increased rate or amount of ROS production, which can exceed the capacities of antioxidant defense. The resulting redox imbalance (i.e. oxidative stress) can lead to lipid, protein and/or DNA damage (Halliwell and Gutteridge, 1984; Livingstone, 2001).

Lipid peroxidation is the result of ROS-targeted oxidative degradation of polyunsaturated fatty acids (Rand, 2003). These fatty acids are susceptible to lipid peroxidation breakdown due to their high quantity of methylene-interrupted double bonds (Heam et al., 1987 in Palace et al, 1993). Radical-induced damage is propagated in a chain-reaction fashion across membranes, resulting in the formation of toxic by-products, such as malondialdehyde (or MDA). The measurement of the MDA content within the tissues of metal-exposed fish and other aquatic organisms has been commonly used as an indicator of oxidative stress (Viarengo et al., 1989; Rand, 2003). Another consequence of oxidative stress can be the carbonylation of proteins, which is induced by a metal-catalyzed oxidation of side-chain amino acids (Stadtman and Levine, 2000). Protein carbonyls are normally marked for proteolysis by proteasomes, however, they may escape degradation and form large, toxic aggregates within cells. Studies have demonstrated that carbonyl build-up increases with cellular age or with oxidative stress induced by exposure to toxicants, particularly redox-cycling metals, such as Cu^{2+} or Fe^{3+} (Stadtman 1992 and Levine, 2002 in Nystrom, 2005).

In freshwater fish, exposure to waterborne Cd has been linked to increased oxidative damage and/or changes in antioxidant response measured in the blood or tissues. The molecular

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mechanisms accounting for the Cd-induced ROS-related effects in fish is not yet clear, however recent research (Sokolova et al., 2005) found that Cd impaired mitochondrial ATP production in eastern oysters (*C. virginica*) during sublethal exposure. This suggests that Cd-induced ROS production and resulting oxidative stress may be a mechanism of chronic toxicity in fish. The majority of studies demonstrating ROS-related effects, such as significant lipid peroxidation, in Cd-exposed freshwater fish (Palace et al., 1993; Bashi and Rani, 2003; Pandey et al., 2008; Almeida et al., 2009) focused on acute to sub-chronic (up to 15 days) exposure to Cd, via waterborne or injectory routes. Limited research has been conducted on investigating the effects of solely sublethal waterborne Cd on oxidative damage and defense responses in salmonids during a chronic exposure period.

1.6 Objectives and Hypotheses

The objectives of the present study were to:

(1) Characterize the oxidative damage and response in the gill, liver and kidney of two freshwater salmonids, rainbow trout (*Oncorhynchus mykiss*) or lake whitefish (*Coregonus clupeaformis*) during a 30-day sublethal waterborne Cd exposure

(2) Investigate potential relationships between these ROS-related responses, accumulation and plasma ionic regulation in the context of the damage-repair-acclimation pattern Measured responses during exposure will include plasma Ca^{2+} and Na^{+}, as well as total tissue Cd burden and indicators of oxidative stress (lipid peroxidation, protein damage and enzymatic antioxidant defense (as catalase activity)) in the gill, liver and kidney

(3) Evaluate the tissue-specific oxidative damage and defense responses in the context of potential differences in their respective detoxification abilities

(4) Investigate whether variations in the ROS-related response amongst different species of fish can help explain differences in their relative sensitivity to Cd

It was hypothesized that any mortality or ionoregulatory disturbances experienced during the damage phase would be accompanied by elevated ROS generation, as indicated by increased malondialdehyde and/or carbonyl levels. Recovery and resulting acclimation would be characterized by a re-establishment of plasma levels, as well as an increased activity of the fish's antioxidant defense (as catalase) to help in repairing any cellular damage. Furthermore, when comparing patterns within the tissues, it was predicted that the most prominent (and initial) oxidative damage would occur in the gills corresponding to high accumulation in the damage phase, whereas the liver (typically associated with a role in detoxification) would be responsible for the majority of defense (or repair) response. In addition, the tissues of rainbow trout will experience a more significant elevation in lipid peroxidation initially in comparison to lake whitefish, the less sensitive of the two species to acute waterborne Cd.
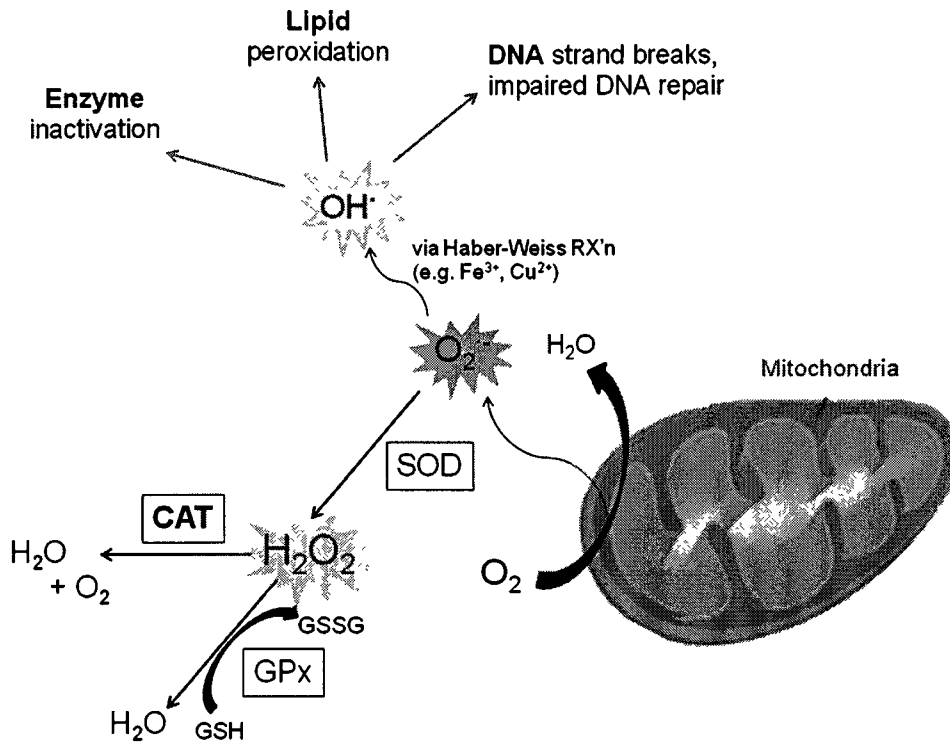
Fig 1.1 Schematic diagram depicting various examples of reactive oxygen species (ROS) and antioxidant defenses, as well as potential cellular oxidative damage that can arise. A high quantity of ROS generation is from reactions along the electron transport chain of the inner mitochondrial membrane. Figure adapted from Rand (2003).

Chapter 2: Cd-induced responses in plasma ionic regulation and oxidative stress during chronic waterborne exposure in rainbow trout (*Oncorhynchus mykiss*)

2.1 Abstract

Freshwater fish exposed to waterborne sublethal Cd can experience initial physiological disruption, such as Ca imbalance, during a phase of damage-repair prior to acclimation. The mechanisms associated with chronic toxicity in fish are not well understood, however, exposure to Cd has been linked to increased oxidative stress. The purpose of this study was to characterize the oxidative damage and antioxidant response in the tissues of juvenile rainbow trout (and in relation to accumulation and plasma ion regulation) during chronic Cd exposure. Trout were exposed to a sublethal concentration of either 0 (control), 0.75 or 2.0 μg Cd/L in moderately hard water (hardness of 140 mg/L $CaCO_3$) for 29 days. Gills, liver, kidney and plasma were sampled throughout exposure. Tissue malondialdehyde (MDA) and protein carbonyl levels, as well as catalase activity, were measured as indicators of oxidative stress. In both treatment groups, there was an initial reduction in plasma Ca by day 4, with levels re-establishing only in low-dose fish. Kidney MDA content was significantly elevated above controls in low-dose fish by day 4 after which levels declined. This 3-fold rise in above-background MDA (1.67 ± 0.18 nmol/mg protein) was the highest overall increase in all observed tissues. In the gill, MDA significantly increased above control levels at the end of exposure (day 29) in fish exposed to 2.0 μg Cd/L. The gills of unexposed fish had higher background MDA levels than the liver (~4-times) and kidney (~7-times) throughout the exposure. Liver catalase activity in high-dose fish significantly increased above controls (5-fold) on the first day of exposure and remained elevated up to day 17. Catalase activity in the gill was either significantly inhibited (in low-dose fish) compared to control basal levels or remained unaffected (in high-dose fish) by Cd exposure throughout the 29 days. This study demonstrates that the degree and pattern of oxidative damage and enzymatic defense induced by Cd during chronic waterborne exposure varies amongst tissues.

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2.2 Introduction

Cadmium is a metal with no known biological function in aquatic animals. When it is present in surface waters at elevated levels, it can exert toxic effects in fish (Sorenson, 1991; Vallee and Falchuk, 1993; Wood, 2001). In freshwater fish, waterborne Cd^{2+} competes with Ca^{2+} at the gill surface for binding and subsequent uptake into chloride cells, via apical ion channels (Playle et al., 1993; Playle, 1998). This initial competition and the resulting inhibition of basolateral Ca^{2+} ATPases within the cell can lead to hypocalcemia, which is considered the primary mechanism of acute Cd toxicity in freshwater fish (Verbost et al., 1987, 1989; Wong and Wong, 2000). Similar to acute exposures, disruption of Ca balance occurs during chronic exposure to waterborne Cd and is linked with gill accumulation. However, the mechanisms associated with chronic effects are not understood.

Fish chronically exposed to sublethal Cd show a variety of physiological effects including: decreased whole-body or plasma ion (such as Ca^{2+} and Na^+) levels (Haux and Larsson, 1984; Pratap et al., 1989; McGeer et al., 2000a; Baldisserotto et al., 2004), increased cardiac and ventilation rates, impaired oxygen transfer across the gills (Majewski and Giles, 1981), erythrocyte destruction, decreased hematocrit and haemoglobin (Gill and Epple, 1993; Zikic et al., 2001) as well as impaired glucose and cortisol response (Fu et al., 1990; Pratap and Wendelaar Bonga, 1990; Brodeur et al, 1998; Lacroix and Hontela, 2004).

Many of the above-mentioned effects were found to be short-lived (or able to 'recover') during the course of extended exposure periods (Haux and Larsson, 1984; Giles, 1984; Fu et al., 1990; McGeer et al., 2000a), which suggests the ability of fish to acclimate to low-level waterborne Cd, following a pattern of damage-repair (McDonald and Wood, 1993). The initial

shock phase in this damage-repair model involves damage and accumulation at the primary uptake site (the gills) and is characterized by disturbances in ionoregulatory homeostasis (Wicklund Glynn, 1991; McDonald and Wood, 1993). This is a result of reduced influx of essential elements, such as Ca^{2+} and Na^+, and a subsequent blood ion loss develops as efflux continues without replacement (Verbost et al., 1987, 1989). This is typically followed by a re-establishment of plasma ion concentrations in the recovery phase and the development of a new steady state which includes an increased tolerance to acute Cd challenges in the acclimation phase (McDonald and Wood, 1993; Stubblefield et al., 1999; Hollis et al., 1999; McGeer et al., 2000a).

This pattern of damage, repair and acclimation during chronic Cd exposure may be related to the internal compartmentalization strategies of fish. Accumulated metal can be considered as existing in two functionally distinct pools, that which is a metabolically active pool (referred to as biologically active metal (BAM)) and that which is not biologically active ((biologically inactive or inert pool (BIM)). The BAM is available to participate or interact with cellular processes, whereas the BIM is considered detoxified and therefore not able to interrupt normal metabolic function (Steen Redeker and Blust, 2004; Vijver et al., 2004). Cd is considered to be in the BAM form upon uptake (Rainbow, 2002) and therefore accumulated Cd must be made unavailable (or detoxified) in order to avoid possible physiological disruption and deleterious effects.

In fish, one of the major mechanisms of detoxification involves the binding/ sequestering of Cd to soluble metal-binding proteins in the cytosol, such as metallothionein (Kagi and Schaffer, 1988; Roesijadi, 1992). Metallothionein is a cysteine-rich, low-molecular weight protein with a strong affinity for Cd. It has been shown to contribute to increased Cd tolerance

in fathead minnows (Benson and Birge, 1985) and rainbow trout (Fu et al., 1990; Hollis et al., 2001; Chowdhury et al., 2005) chronically exposed to Cd. However, if Cd accumulation exceeds the detoxification capacities, Cd can remain biologically reactive, potentially leading to deleterious effects. The spill-over theory of chronic metal effects postulates that if the BAM concentration is not maintained below a certain threshold level, then metabolic disturbances and other toxic effects in will be observed in sensitive tissues.

Reactive oxygen species are formed by the partial reduction of molecular oxygen and are found in high quantities along the inner mitochondrial membrane (Raha and Robinson, 2000; Cannino et al., 2009). Their highly-reactive and non-discriminate nature allows them to interact with macromolecules and potentially interfere with normal cellular function. ROS are normally monitored and converted into less reactive species by the fish's antioxidant defense system (Halliwell and Gutteridge, 1984; Livingstone, 2001). Examples of ROS include superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). Catalase, for example, detoxifies hydrogen peroxide enzymatically into water. Exposure to Cd may induce an increased rate or amount of ROS production, which can exceed the capacities of antioxidant defense. The resulting redox imbalance (i.e. oxidative stress) can lead to lipid, protein and/or DNA damage (Halliwell and Gutteridge, 1984; Livingstone, 2001). Lipid peroxidation is the result of ROS-targeted oxidative fatty acid degradation that produces toxic by-products, such as malondialdehyde (Girotti, 1985). Another consequence of oxidative stress can be enzyme inactivation, induced by the carbonylation of side-chain amino acids (Stadtman and Levine, 2000).

In freshwater fish, exposure to waterborne Cd has been linked to increased oxidative damage and/or changes in antioxidant response measured in the blood or tissues. Recent

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research has provided a better understanding of the molecular mechanisms accounting for these Cd-induced ROS-related effects. *In vitro* studies have demonstrated that Cd induces necroptosis in gill and liver cell lines of rainbow trout during acute Cd exposure (Krumschnabel et al., 2010), as well as apoptosis and oxidative stress in hepatocytes via mitochondrial pathways (Risso-de Faverney et al., 2001, 2004). Even though there is limited research on the *in vivo* effect of Cd on mitochondrial function in fish, Sokolova et al. (2005) found that eastern oysters (*C. virginica*) exposed *in vivo* to sublethal Cd concentrations experienced an impaired capacity for mitochondrial ATP production despite a significant reduced proton leak rate. This suggests that Cd-induced ROS production and resulting oxidative stress may be a mechanism of chronic toxicity in fish.

ROS-related effects, such as significant lipid peroxidation and/or increased catalase activity, have been reported in the tissues of several freshwater fish species, such as tilapia (*O. niloticus*), cichlids (*C. punctata*) and yellow perch (*P. flavescens*) during exposure to waterborne Cd (Palace et al., 1993; Bashi and Rani, 2003; Pandey et al., 2008; Almeida et al., 2009). However, the majority of studies have focused on acute to sub-chronic (up to 15 days) exposure to Cd, via waterborne route or direct intraperitoneal administration. Limited research has been conducted on investigating the time course of effects of sublethal waterborne Cd on oxidative damage and defense responses in salmonids during a chronic exposure period.

The objectives of the present study therefore were to (1) characterize the oxidative damage and response in the gill, liver and kidney in rainbow trout during a 29-day sublethal waterborne Cd exposure and (2) evaluate relationships between these ROS-related responses, accumulation and plasma ionic regulation in the context of the damage-repair-acclimation pattern. Responses measured during exposure included plasma Ca^{2+}, Na^+ and glucose, total

tissue Cd burden (in gill, liver and kidney) and indicators of oxidative stress (lipid peroxidation, protein damage and enzymatic antioxidant defense (as catalase activity)). It was hypothesized that any mortality or ionoregulatory disturbances experienced during the damage phase would be accompanied by elevated ROS generation, as indicated by increased malondialdehyde and/or carbonyl levels. Recovery and resulting acclimation would be characterized to a re-establishment of plasma levels, as well as an increased activity of the fish's antioxidant defense (as catalase) to help in repairing any cellular damage. Furthermore, when comparing patterns within the tissues, it was predicted that the most prominent (and initial) oxidative damage would occur in the gills corresponding to high accumulation in the damage phase, whereas the liver (typically associated with a role in detoxification) would be responsible for the majority of defense (or repair) response.

2.3 Materials and Methods

2.3.1 Fish husbandry and acclimation

A group of 420 juvenile rainbow trout (*Oncorhynchus mykiss*, 30.4 ± 12.3g (mean ± 1 SD), n= 117) were purchased from a local supplier (Rainbow Springs Trout Hatchery, Thamesford, ON). Fish were non-selectively distributed across six 200-L tanks (70 fish per). Tanks were supplied with flowing water ($0.75 - 1$ L min^{-1}) from a central mixing tank where reverse osmosis deionized water and well water were mixed to achieve a hardness of 140 mg CaCO$_3$ L^{-1} (with 868 ± 34 Ca, 480 ± 16 Mg, 339 ± 16 Na (μM, means ± 1 SD, n=37), conductivity of 220 μS cm^{-1}, pH 7.1 and temperature 11.0°C). Water from the central header tank was directed to one of three 10L dosing head tanks before delivery to replicate exposure tanks. All tanks were

vigorously aerated and fish were fed a commercial feed (Bio Oregon Protein Inc, Warrenton, OR) at 2% of biomass daily except on the days prior to sampling.

2.3.2 Exposure to Cd

Fish were exposed to nominal concentrations of 0.75 or 2.0 µg Cd L^{-1} (measured concentrations of 0.71 and 1.85, see Table 2.1) in duplicate by delivering (QG6 pump, Fluid Metering Inc., Syosset, NY) appropriate volumes of Cd stock solution ($CdCl_2$ $2.5H_2O$, VWR International, Mississauga, ON) to dosing head tanks. The pair of fish tanks was fed from a third dosing head tank, but this one received no Cd and served as controls (0 added Cd). Therefore a total of 140 (n = 2 x 70) fish were exposed in each treatment. Exposure duration was 29 d.

2.3.3 Sampling

Water samples were collected from the mixing head tank for daily measurement of conductivity, temperature and pH. Water samples, both unfiltered and 0.45 µm filtered (Acrodisc HT Tuffryn, Pall, Ann Arbor, MI) were collected at least twice weekly from each exposure (10 mL) and acidified to 1% with HNO_3 (Trace metals grade, Fisher Scientific, Nepean, ON). Four fish were sampled from each tank (n=8 per exposure) on days 1, 4, 7, 17 and 29 of exposure and euthanized in $NaHCO_3$ buffered solution of 0.3 g L^{-1} tricaine methanesulfonate (Syndel Laboratories, Qualicum Beach, BC). Fish were blotted dry, weighed and a blood sample of approximately 0.2 to 0.4 mL collected from the caudal vasculature into vials containing 10 µL of lithium-heparinised cortland saline, centrifuged (Spectrafuge 16M, Mandel Scientific Inc, Guelph, ON) at 13,000 rpm for 3 minutes and plasma was isolated and then stored in -20^0C until

analysis for glucose, Na^+ and Ca^{2+} content. Gill arches were collected, rinsed for 10 s in Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), blotted dry, divided into two subsamples, placed into microtubes, frozen in liquid nitrogen and stored in -80^0C. Fish were dissected, liver and kidney collected and then similarly subdivided into two samples and stored with gill samples. One set of the tissue subsamples was used for total Cd concentration, the other for oxidative stress endpoints.

2.3.4 Analyses

Daily measurements for water conductivity and temperature were done using a portable meter (YSI 30, YSI Ltd., Yellow Springs, OH), as was pH (SG2-ELK-SevenGo, Mettler, Mississauga, ON). Acidified water samples were measured for Cd by graphite furnace atomic absorption spectroscopy (GFAAS, SpectraAA-880, Varian Inc., Mississauga, ON) with appropriate standards matched with certified reference standards (TMDA 28.3 and TM 26.3, National Water Research Institute, Burlington, ON).

2.3.4.1 Plasma measurements

Plasma glucose was determined using the glucose hexokinase assay, available as a commercial kit (GAHK-20, Sigma-Aldrich Canada, Oakville, ON). Additional glucose standard were developed by dilution of the kit standard, and absorbance of standards and samples was measured spectrophotometrically (SpectraMax M2, Molecular Devices, Downingtown, PA) at 340 nm in 96 well microplates. Results are expressed as mg dL^{-1} glucose. The remaining plasma was used for determining Ca^{2+} and Na^+ content. Samples were diluted (50-fold for Ca^{2+}

and 1000-fold for Na^+) and analyzed using flame atomic absorption spectroscopy (SpectraAA-880, Varian Inc., Mississauga, ON).

2.3.4.2 Tissue-specific total Cd burden

Gills, liver and kidney tissues were weighed, acidified with a 5- fold (wt : vol) volume of 1N HNO_3 (trace metal grade, Fisher Scientific, Nepean ON) and then digested for three h at 80^0C (Playle et al., 1993a). Following digestion, remixing and centrifuging, the supernatant was appropriately diluted and Cd was measured via GFAAS and results are expressed as µg Cd per g wet weight.

2.3.4.3 Biochemical assays

Lipid peroxidation in gill, liver and kidney was determined by measuring malondialdehyde (or MDA, also known as the TBARS) assay (Ohkawa et al., 1979), as outlined by a commercial kit (10009055, Cayman Chemical Company, Ann Arbor, Michigan) with modifications. Approximately 25 mg of each tissue was isolated and then homogenized in 1 mL of homogenization buffer solution (137 mM NaCl, 0.27 mM KCl, 10 mM Na_2HPO_4, 0.18 mM KH_2PO_4, 0.5 mM EDTA, pH 8.2) at 22,000 rpm (Omni THQ digital homogenizer, Omni International, Marietta, Georgia) and kept on ice at all times. Resulting tissue homogenates were then centrifuged at 10,000 x g for 5 minutes at 4^0C and 25 µL of supernatant mixed with 25 µL of sodium dodecyl sulphate and 1 mL of colour reagent. The reaction mixture was then placed in a boiling water bath and subsequently centrifuged at 10,000 x g for 5 minutes at 4^0C before absorbance was measured spectrophotometrically at 532 nm.

Remaining tissues were used for analysis of protein carbonyl content and catalase activity. Tissues were homogenized (1:15, wt : vol) in buffer solution (137 mM NaCl, 0.27 mM KCl, 10 mM Na_2HPO_4, 0.18 mM KH_2PO_4, 1 mM EDTA, pH 6.7) then centrifuged at 10,000 x g for 15 minutes at 4^0C. Supernatant was isolated and stored at -80^0C prior to analysis. Protein carbonyl content was quantified using the method of Levine et al. (1994), as outlined by a commercial kit (10005020, Cayman Chemical Company). Briefly, 2,4-dinitrohydrazine or hydrochloric acid (as a control) was added to each sample. Following dark incubation, 20% and 10% trichloroacetic acid were added prior to several pellet washes with ethanol/ethyl acetate. Samples were thoroughly remixed and centrifuged between the reagent additions. Absorbance was measured spectrophotometrically at 375 nm.

Catalase activity was quantified using the method described by Wheeler et al. (1990), as outlined in a commercial kit (707002, Cayman Chemical Company). Samples were first diluted if necessary (20- to 100-fold for the kidney and 200-fold for the liver) using buffer (25 mM potassium phosphate, 1 mM EDTA, pH 7.5) prior to being transferred to a 96-well plate and the addition of reagents. The spectrophotometer was used for the series of incubations and shaking of plates, and absorbance was measured at 540 nm.

For each sample, the final obtained values for all biochemical assays were normalized based on the total protein concentration. Results are expressed for gills, liver or kidney as: nmol MDA per mg protein for lipid peroxidation, nmol carbonyl per mg protein for protein carbonylation, and nmol of hydrogen peroxide consumed per min per mg protein for catalase activity. Protein content was determined by the Bradford method (Bradford, 1976), using protein reagent dye and bovine serum albumin (both from Sigma-Aldrich Canada) as protein standard.

2.3.5 Statistical Analysis

Data is expressed as mean ± 1 SEM. Statistical analysis was performed using SigmaPlot 11.0 computer software (Systat Software, Inc., San Jose, CA). All data was subjected to a one-way analysis of variance (ANOVA), in which normality and equal variance were initially tested. Statistical comparisons were made between treatment groups (control (0), 0.75 and 2.0 µg Cd L^{-1}) at each time interval (days 1, 4, 7, 17 and 29). When the ANOVA indicated significant difference ($P<0.05$) then the Tukey's HSD test was used to compare the means.

2.4 Results

2.4.1 Mortality and changes in plasma content

During the 29-day exposure, only acute mortality occurred, between days 1 and 4 of exposure for the 2.0 µg Cd/L group and days 1 and 5 for the 0.75 µg Cd/L group of exposed fish (Fig. 2.1). Total mortalities for fish exposed to 0.75 and 2.0 µg Cd/L were 9% and 20% respectively. There were no mortalities for unexposed controls and for each of the measured parameters, there were no significant differences among controls over time throughout the exposure. The mean of all control values was calculated and is included on figures as day 0.

There were no significant changes in plasma glucose over the 29 days in either exposed group compared to controls (Fig. 2.2). A disturbance of plasma Ca^{2+} was observed in both Cd-exposed groups, but the effect was initiated earlier and was persisted longer in fish exposed to 2.0 µg Cd/L (Fig. 2.3A). At the lower dose, plasma Ca^{2+} levels were significantly reduced from control levels only on day 4 and then returned to control levels until the end of the exposure (Fig.

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2.3A). In fish exposed to 2.0 μg Cd/L, the plasma Ca^{2+} levels remained significantly reduced for

the remainder of the exposure (Fig. 2.3A). Cd exposure had no effect on plasma Na^+ levels for

fish exposed to 2.0 μg Cd/L but at 0.75 μg Cd/L, there was a transient reduction of plasma Na^+

on day 4 only (Fig. 2.3B).

2.4.2 Tissue-specific total Cd burden

Cd accumulated in gills, liver and kidney of exposed trout in a dose- and time- dependent

manner (Fig. 2.4). Fish exposed to 2.0 μg Cd/L had significant increases in Cd concentrations

above control amounts by day 4 in the gill (Fig 2.4A) and kidney (Fig. 2.4C) and day 1 in the

liver (Fig. 2.4B). It took longer for significant increases in tissue Cd to develop in fish exposed

to 0.75 μg Cd/L and the degree of accumulation was less compared to fish at the higher exposure

level (Fig. 2.4). By day 29, Cd accumulated the most, on a tissue-weight basis, in the kidney of

the high-dose fish (3.2 ± 0.2 μg/g), followed closely by the gill (2.5 ± 0.1 μg/g), and to a lesser

degree the liver, which had approximately half the amount of Cd (1.3 ± 0.2 μg/g) by the end of

the exposure. In comparison to the unexposed fish, tissue Cd levels of fish exposed to 2.0 μg

Cd/L were increased above controls by 88-, 59- and 54-fold in the liver (Fig. 2.4B), gill (Fig.

2.4A) and kidney (Fig. 2.4C), respectively. By contrast, at the lower exposure, Cd

accumulations were increased above controls concentrations by 59-, 16- and 12-fold in the gill,

liver and kidney, respectively.

2.4.3 Effect of Cd on lipid peroxidation

MDA was significantly increased above control levels in the liver of fish exposed to 2.0 µg Cd/L on day 4 and day 7, then declined for the remainder of the exposure (Fig 2.5B). Gill MDA levels (Fig. 2.5A) in the fish in the high exposure were significantly elevated above controls (and above levels in fish in the lower exposure) only by day 29. In comparison to the other tissues, the gills of unexposed fish had the highest background MDA levels (12.0 ± 1.0 nmol/mg protein (mean ± SEM, n=30)) throughout the 29 days, approximately 4- and 7-fold higher than in liver and kidney, respectively. There were no significant changes in MDA levels in the gill (Fig. 2.5A) or liver (Fig. 2.5B) of fish exposed to 0.75 µg Cd/L compared to controls but in the kidney (Fig. 2.5C), there was a significant increase in MDA on day 4 of exposure. This 3-fold rise above controls (Fig. 2.5C; 1.67 ± 0.18 nmol/mg protein (n=30)) was the highest overall increase in MDA levels when comparing the three tissues.

2.4.4 Effect of Cd on protein carbonyl content

In the gills (Fig. 2.6A) of fish exposed to 0.75 µg Cd/L, protein carbonyl content was significantly elevated above control values on day 4, after which levels returned to controls for the remainder of the exposure. The average background protein carbonyl content of the gills was 15.14 ± 0.55 nmol/mg protein (n=30; Fig. 2.6A), which was approximately 8-fold higher than levels in the liver (Fig. 2.6B) of unexposed fish. There were no significant changes in protein carbonyl content compared to controls in the liver of either exposed groups of fish during the 29 days (Fig. 2.6B).

2.4.5 Effect of Cd on catalase activity

There was a significant decrease in gill catalase activity by day 4 in fish exposed to 0.75 μg Cd/L, and levels remained reduced from controls until day 17, after which activity recovered to basal levels (Fig. 2.7A). Catalase activity in the gill was unaffected by exposure to 2.0 μg Cd/L throughout the 29 days (Fig. 2.7A). In the liver (Fig. 2.7B) of fish exposed to 2.0 μg Cd/L, there was an initial significant 5-fold increase in catalase activity above levels in unexposed (control) fish after the first day of exposure. Liver catalase activity in fish in the higher exposure remained significantly elevated above control levels up to day 17 (Fig. 2.7B). By contrast, activity in the liver of fish exposed to 0.75 μg Cd/L followed a general increasing trend after one week, however, levels were significantly increased above controls by day 29. The average basal catalase activity in the liver (Fig. 2.7B) throughout exposure was 260.69 ± 13.19 nmol of H_2O_2 decomposed/min/mg protein (n=35), which was approximately 42- and 3-fold higher than in the gill (Fig. 2.7A) and kidney (Fig. 2.7C), respectively. In the kidney (Fig. 2.7C), there was a significant elevation in activity above control levels only in fish in the higher exposure and on day 7, after which levels had returned to controls by day 17 and remained so until the end of the exposure.

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2.5 Discussion

2.5.1 Effect of Cd on plasma ion regulation

There was a disruption in plasma Ca^{2+} (Fig. 2.3A) and Na^+ (Fig. 2.3B) levels in fish chronically exposed to waterborne Cd. In fish exposed to 0.75 µg Cd/L, plasma Ca^{2+} levels were significantly reduced from those of controls on day 4 only, after which levels recovered until the end of the exposure (Fig. 2.3A). Numerous studies have reported significant reductions in whole body or plasma Ca^{2+} regulation with subsequent recovery in rainbow trout during chronic exposure to sublethal Cd (Haux and Larsson, 1984; Giles, 1984; Fu et al., 1990; McGeer et al., 2000a). Waterborne Cd^{2+} induces acute toxicity in freshwater fish by competing with Ca^{2+} for uptake at the gill, via apical high-affinity Ca^{2+} channels (Playle et al., 1993; Playle, 1998) of chloride cells (Wong and Wong, 2000). In addition to the direct competition at the gill surface, Cd further blocks Ca^{2+} uptake processes by inhibiting basolateral Ca^{2+} ATPases (Verbost et al., 1987, 1989), which can lead to hypocalcemia (Wong and Wong, 2000). It has been suggested that the recovery of ionoregulatory capacity may be related to increased chloride cell density and apical surface area in the gills of fish (Wendelaar Bonga and Locke, 1992), which may facilitate in gas transfer and Ca^{2+} uptake (Wong and Wong, 2000).

For fish in the higher exposure, a re-establishment of plasma Ca^{2+} levels to those of controls was not observed, as levels remained significantly reduced throughout the 29 days (Fig. 2.3A). This finding, which has also been reported in gibel carp (*C. auratus gibelio*) collected from a Cd-contaminated river, was related to an incomplete detoxification of Cd, most likely within the gills because of their role in Ca^{2+} homeostasis (Van Campenhout et al., 2010). Kaley (2006) found that plasma Ca^{2+} levels in Nile tilapia (*O. niloticus*) exposed to waterborne Cd remained significantly below controls for 45 days but then recovered by day 60. The authors related the return to basal levels to the possible transition of Ca^{2+} from bone tissue to the circulatory system, to compensate for the lack of Ca^{2+} (Flik et al., 1986 in Kaley, 2006). Hollis et al. (2000) reported that Ca^{2+} influx, as well as the availability for Ca^{2+} binding, at the gills of rainbow trout had decreased with increasing waterborne Ca^{2+} concentrations. Hence, the observed lack of plasma Ca^{2+} re-establishment (Fig. 2.3A) may be related to changes in Ca^{2+} flux rates and/or affinity at the gills of fish exposed to 2.0 µg Cd/L.

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There was a transient reduction of plasma Na^+ levels in fish exposed 0.75 µg Cd/L and on day 4 only (Fig. 2.3B). The effects of waterborne Cd on plasma Na^+ levels in exposed fish are variable among studies, however, minor and/or less pronounced effects in relation to Ca^{2+} have been reported. Previous studies have similarly reported significant reductions in plasma Na^+ levels (Reid and McDonald, 1988; Fu et al., 1989; Pelgrom et al., 1995) and subsequent recovery to control levels (Giles, 1984; Fu et al., 1990; McGeer et al., 2000a) in fish during sub-acute to chronic Cd exposure. Cd has been shown to inhibit gill Na^+/K^+ ATPase activity in exposed tilapia (*O. mossambicus, O.niloticus*; Pratap and Wendelaar Bonga, 1993; Atli and Canli, 2007) and European eel (*A. angilla*; Lionetto et al., 2000). Lionetto et al. (2000) also reported an inhibition of carbonic anhydrase in the epithelium of the gills and intestine in Cd-exposed eel. Carbonic anhydrase is highly-active in the gills of fish and indirectly linked to apical Na^+ and Cl^- uptake and HCO_3^- exchanger sites. It is possible that Cd uses mechanisms similar to those of Pb (Rogers et al., 2003, 2005) or other metals (e.g. Cu; Morgan et al., 1997; Li et al.; 1998) that may directly or indirectly decrease carbonic anhydrase activity in the gills. Therefore, although the mechanisms remain unclear, the transient reduction of plasma Na^+ (Fig. 2.3B) may be related to the inhibition of multiple aspects of ionoregulation at the gill.

2.5.2 Tissue-specific total Cd burden

The accumulation and distribution of Cd within the tissues of exposed fish was dose- and time-dependent throughout the 29-d period (Fig. 2.4A-C). During the first week of exposure, Cd burden was higher (~2-3-fold) in the gills of exposed fish than in the liver and kidney (Fig. 2.4A-C). This may be related to the fact that the gills are highly branched, are in direct exchange with the external environment and are the primary site of waterborne metal uptake in freshwater fish (Mallett et al., 1985; Evans et al., 1987). An initial build-up of Cd in the gills, and delay of burden in other organs, has similarly been reported in rainbow trout (McGeer et al., 2000b; Hollis et al., 2001; Szebedinsky et al., 2001; Chowdhury et al., 2005) and catfish (*C. gariepinus*; Asagba et al., 2008) during chronic exposure to waterborne Cd. It has been suggested that the retention of Cd in the gills at the onset of exposure functions as a barrier to limit the internal Cd load (Hollis et al., 1999).

For the latter phase of the exposure period, there was a stabilization of Cd burden in the gills of fish exposed to 2.0 μg Cd/L, whereas a linear increase in Cd concentration was observed in the kidneys (Fig. 2.4A, C). Hence, it appears there was a shift in the distribution of Cd from the gills to the liver and kidney following day 7. This may indicate an increased emphasis on the detoxification and/or storage of Cd within internal tissues, which could relate to the fish's tolerance to Cd during extended exposure. Studies have similarly concluded that while the gills are the primary site for immediate damage, the induction/mobilization of metal-binding proteins (such as MT) in other tissues of exposed fish may contribute to its ability to acclimate (Fu et al., 1990; Hollis et al., 2001; Chowdhury et al., 2005). The reallocation of Cd among the tissues of exposed fish observed in the present study (Fig 2.4) may also be a result of reduced Cd uptake at the gill surface (Hollis et al., 1999).

By the end of the 29-day period, the kidney of fish in the higher exposure had accumulated the highest concentration of Cd (Fig. 2.4C), followed more closely by the gills (Fig. 2.4A) than the liver (Fig. 2.4B). This finding, which is in agreement with previous studies on rainbow trout in similar exposure conditions (McGeer et al., 2000b; Hollis et al., 2001; Chowdhury et al., 2005), suggests that the kidneys are a target organ for Cd accumulation during chronic waterborne exposure. Kidney Cd levels in exposed fish have been found to remain elevated after depuration (Kumada et al., 1980; Harrison and Klaverkamp, 1989; Asagba et al., 2008), which further emphasizes the possible role of this tissue in the long-term storage of Cd. Additionally, in mammals, the kidneys are regarded as the final destination of Cd from various tissues and therefore are associated with chronic Cd toxicity (Wito, 1992). Cd-MT complexes, in mammals, are released from the liver, delivered via blood to the kidneys (Nordberg and Nordberg, 1987) filtered through the glomerulus and ultimately reabsorbed in the proximal tubules and retained (Timbrell, 1991). This may be related to the elevated Cd levels found in the kidney of fish (Fig. 2.4C) after exposure to 2.0 μg Cd/L for 29 days.

2.5.3 Effect of Cd on lipid peroxidation

In the liver of exposed fish, MDA levels were unaffected by exposure to 0.75 μg Cd/L, whereas fish in the higher exposure had significantly elevated liver MDA on days 4 and 7 (Fig. 2.5B).

The increase in MDA above control levels suggests that exposure to 2.0 μg Cd/L was sufficient to cause significant lipid peroxidation (LPO) in the liver during the first week of the 29-d period. Previous studies have reported increases in LPO in the liver of Nile tilapia (Almeida et al., 2009), Atlantic croaker and striped mullet (*M. undulatus* and *M. cephalus*, respectively; Wofford and Thomas, 1988) during sub-chronic or chronic exposure to waterborne Cd. Pretto et al. (2011) found that MDA levels in the liver of silver catfish (*R. quelen*) were significantly elevated after a 7- and 14-d exposure to waterborne Cd and concluded that oxidative stress was induced due to the observed sustained LPO.

Redox-inactive metals, such as Cd, Hg and Pb, most likely induce oxidative stress by indirect mechanisms which are not clearly understood (Halliwell and Gutteridge, 1984). It is suspected that Cd depletes the cell's major sulfhydryl reserves (for example, via binding to reduced glutathione), thereby reducing its ability to combat unstable free radicals and other ROS (Rand, 2003). An increased production of ROS could lead to cellular damage to lipids and/or proteins (Halliwell and Gutteridge, 1989; Livingstone, 2001). The significant elevation in liver LPO observed on days 4 and 7 may be a result of an overproduction of ROS induced indirectly by exposure to 2.0 μg Cd/L. This may relate to the increased liver Cd accumulation that occurs as the retention capacity of the gill for Cd is overwhelmed and distribution and uptake to internal tissues develops during the first week of exposure (Fig 2.4A-B).

The significant LPO in the liver on days 4 and 7 (Fig. 2.5B) may be a result of an overwhelming of the liver's antioxidant defense capabilities. Fish possess antioxidant defenses, enzymatic and non-enzymatic, capable of maintaining background ROS levels under normal conditions. The findings suggest that exposure to 2.0 μg Cd/L was sufficient to induce higher than background ROS that could not be maintained by the defense system. However, it appears as though the oxidative stress in the liver was temporary, as MDA levels recovered to those in unexposed fish by day 17 (Fig. 2.5B). This suggests that despite an initial stress, the antioxidant defenses were able to effectively maintain ROS, thereby preventing further lipid damage, during chronic exposure to Cd. Asagba et al. (2008) similarly demonstrated that MDA levels in the liver of catfish (*C. gariepinus*) had significantly decreased by the end of a 21-d exposure to waterborne Cd. This was related to a corresponding elevated activation of superoxide dismutase (SOD), a key component of the enzymatic CAT-SOD antioxidant system.

In the kidney (Fig. 2.5C) of fish exposed to 0.75 µg Cd/L, MDA levels were significantly elevated on day 4. Interestingly, this demonstrates that there was LPO in the kidney during the initial days of the chronic exposure, despite no significantly elevated Cd burden (Fig. 2.4C). Significant LPO has similarly been reported in the kidney of catfish after sub-chronic to chronic exposure to waterborne Cd (Asagba et al., 2008; Pretto et al., 2011). It was concluded that this may have related to the observed decrease in antioxidant enzymatic defense (Asagba et al., 2008). In the present study, kidney MDA levels in fish in the lower exposure recovered to those of controls by day 7 (Fig. 2.5C). This suggests there may have been a lag in the response of the antioxidant defense system that would allow for excess ROS and subsequent lipid damage in the kidney during the first 4 days of exposure. Asagba et al. (2008) concluded that no significant LPO in the kidney of catfish after 7 days of Cd exposure may have corresponded to the observed increase in SOD activity. This suggests that in the present study, antioxidant enzymatic defenses may be responsible for the subsequent recovery in kidney MDA to basal levels by day 7. In addition, the fact that initial LPO was found in the kidney of fish in the lower exposure only (as compared to in the liver) could indicate that the defense mechanisms associated with the kidney are different, and perhaps weaker, than those of the liver of rainbow trout.

In the gills (Fig. 2.5A), MDA levels were significantly elevated above controls in fish in the higher exposure and on day 29. This suggests that lipid peroxidation in the gill is induced only after chronic exposure to 2.0 µg Cd/L. A similar ~2-fold elevation in MDA levels was found in the gills of *C. punctata* fish after 30-d exposure to waterborne Cd, Cu, Fe and Ni (Pandey et al., 2008). The authors suggested that this demonstrated the persistent nature of metal-induced oxidative damage in the gill. Significantly elevated gill MDA has also been reported in silver catfish (*R. quelen*) exposed to waterborne Cd for 14 days, and levels remained elevated following recovery in Cd-free water (Pretto et al., 2011). It was concluded that the sustained LPO in the gill (in comparison to an observed recovery in the kidney) indicated tissue-specific oxidative stress caused by Cd.

In the present study, the lack of initial lipid peroxidation (Fig. 2.5A), despite high Cd burden (Fig. 2.4A), in the gills of exposed fish may be the result of an antioxidant defense response. The significantly increased MDA levels on day 29 could relate to an eventual overwhelming of the defense mechanisms in the gills of rainbow trout, resulting in oxidative

stress. The gills may also be more susceptible to oxidative attack than other tissues due to their direct external contact with waterborne metals (Laurent et al., 1984; Wong and Wong, 2000), large surface area and high density of polyunsaturated fatty acids (Mallett et al., 1985; Evans et al., 1987). These characteristics could also relate to the finding that the gills of unexposed fish had higher basal MDA levels than the liver (~4-fold) and kidney (~7-fold), which is consistent with other studies (Pretto et al., 2011).

2.5.4 Effect of Cd on protein carbonyl content

Another consequence of ROS-related damage induced by metals is protein carbonylation. The use of protein carbonyls as markers of oxidative stress has more recently (in relation to lipid peroxidation, for example) been applied to fish species (Almroth et al., 2005; Ferreira et al., 2005; 2007). In the present study, exposure to Cd did not cause any significant changes in hepatic protein carbonyl content (Fig. 2.6B) throughout the 29 days. This may relate to the efficiency of the liver's antioxidant defense system in detoxifying ROS and thereby preventing significant damage to proteins. Previous studies have reported similar findings in the hepatopancreas of estuarine crab (*C. granulata*; Sabatini et al., 2009) and brain of fish (*C. bratachus*; Maiti et al., 2010) after chronic exposure to Cu or Pb, respectively. Although there was no observed protein carbonylation in the liver (Fig. 2.6B), other studies have reported significant increases in protein carbonyl content in the plasma, liver and gills of fish after exposure to Cu or Cd (Craig et al., 2007; Almroth et al., 2008; Pretto et al., 2011).

The gills of fish (Fig. 2.6A) exposed to 0.75 µg Cd/L had significantly elevated protein carbonyl content on day 4, after which levels recovered for the remainder of the exposure. Almroth et al. (2005) concluded that an observed significant increase and subsequent decrease in protein carbonyl content indicates the complicated relationship between pro-oxidant exposure and accumulation of carbonyls. In addition, it has been shown that the proteolytic degradation of damaged proteins increases with exposure to moderate oxidants (Grune, 2003). This suggests that in the present study, exposure to 0.75 µg Cd/L was sufficient to cause relatively mild protein damage initially, which may have stimulated a cellular degradation response by day 7. Interestingly, Pretto et al. (2011) reported that protein carbonyl content in the liver of silver

catfish was significantly elevated only at day 7 (not after 14 days) and that levels returned to those of unexposed fish following depuration. These findings, and the results demonstrated in the present study, suggest that protein carbonyl content is not a reliable indicator of Cd toxicity.

2.5.5 Effect of Cd on catalase (CAT) activity

The activity of antioxidant enzymes, such as CAT, has been shown to be induced or inhibited by exposure to metals (Pruell and Engelhardt, 1980; Shukla et al., 1987), and response is dependent on dose, route and duration of exposure and species sensitivity (Van der Oost et al., 2003; Sanchez et al., 2005; Gravato et al., 2006). These free radical scavengers constitute a major component of the fish's defense system and protect cells against potential ROS-related damage (Halliwell and Gutteridge, 1989; Livingstone, 2001). In the liver (Fig. 2.7B) of fish exposed to 2.0 μg Cd/L, there was a significant (5-fold) increase in CAT activity by day 1, and levels remained elevated up to day 17. Liver CAT activity was also significantly elevated in fish in the lower exposure (Fig. 2.7B) by day 29, following a continual increasing trend. The changes in CAT activity in exposed fish suggest that exposure to waterborne Cd induces a response in antioxidant defenses, most likely to counteract an increased ROS production. Significant elevations in CAT activity have been reported in the liver of tilapia (*O. mossambicus*; Basha and Rani, 2003; *O. niloticus*; Atli and Canli, 2006; Almeida et al., 2009), silver catfish (Pretto et al., 2011) and marine *S. basilisca* (Messaoudi et al., 2009) during acute to chronic Cd. Atli and Canli (2010) suggested that increased CAT activity may indicate an effective activation of the antioxidant defense system against metal-induced oxidative stress and/or to compensate for a decrease in other enzymes (e.g. SOD or glutathione peroxidase (GPx)).

The CAT-SOD system is considered the first line of defense against oxyradical formation (Winston and Guilio, 1991; Sies, 1993). The liver of unexposed and exposed groups of fish (Fig. 2.7B) had the highest basal and stimulated, respectively, CAT activity compared to the kidney and gill (Fig. 2.7A, C). The finding that the liver had the strongest CAT activation in response to metal exposure is in agreement with numerous studies (Hidalgo et al., 2002; Gul et al., 2004; Avci et al., 2005; Atli and Canli, 2006). The liver is known to have a significant role in the detoxification of metals and other toxicants (Goering et al., 1995). It is the site of multiple

oxidative reactions and the organ associated with maximal free radical generation (Winston and Guilio, 1991). The sustained significant increase in liver CAT activity in fish exposed to 2.0 μg Cd/L may be why there was an observed recovery of MDA to basal levels (Fig. 2.4B). In addition, the lack of significant protein carbonylation in the liver (Fig. 2.6B) may also relate to the antioxidant defense capabilities of this organ. Ferreira et al. (2008) correlated high ambient tissue Cu, Cd, Pb and Ag residues in the liver of wild white seabream (*D. sargus*) with elevated antioxidant enzymatic activity and reduced lipid peroxidation. The authors also reported a higher correlation between CAT activity and Cd (and Cu) residues in the liver, compared to the other metals.

Additional protection against ROS-related damage may be provided by non-enzymatic defenses, such as intracellular sulfhydryl-rich proteins. Metallothionein (MT) and reduced glutathione (GSH), for instance, are synthesized in the liver (Wito 1992; Boelsterli, 2009) and known to act as metal chelators (Rabestein et al., 1985; Kagi and Schaffer, 1988) as well as free radical scavengers (Stegeman et al., 1989; Sato and Bremner, 1993). Previous studies have reported increased levels of Cd associated with the MT fraction in the liver of tilapia (Ueng et al., 1996; Wu et al., 2002; Atli and Canli, 2003) or with elevated MT synthesis in the liver of rainbow trout (Hollis et al., 2001; Chowdhury et al., 2005) during chronic exposure to waterborne Cd. MT has also been shown to contribute to increased Cd tolerance in fathead minnows (Benson and Birge, 1985) chronically exposed to Cd.

Glutathione is most abundant in its reduced form (GSH) and can become oxidized (GSSG) via the conversion of free radicals (Boelsterli, 2009). Levels of GSH, and of its ratio to its oxidized form (i.e. GSSG/GSH) have been found to be significantly altered in the liver of numerous fish species after acute (Atli and Canli, 2006) or chronic (Lima et al., 2006; Zirong and Shijun, 2007; Atli and Canli, 2008) Cd exposure. Zirong and Shijun (2007) concluded that GSH appears to make a rapid protection against Cd-induced oxidative stress in the liver of Nile tilapia. It has also been suggested that changes in GSH redox status in the liver of fish may be an antioxidant adaptation to chronic Cd exposure (Lima et al., 2006). Hence, the lack of sustained damage to lipids (Fig. 2.5B) and/or proteins (Fig. 2.6B) in the liver of exposed fish after the 29-d period may relate to the presence of MT and/or GSH. Overall, the results suggest that the antioxidant CAT response in the liver (Fig. 2.7B) may have attributed to the lack of long-term

oxidative stress in this tissue. Additionally, the significant and maintained increase in CAT activity emphasizes the role of the liver as the main detoxification organ in rainbow trout and its enhanced ability to combat potential Cd-induced ROS generation.

In the kidney (Fig. 2.7C), there was a significant increase in CAT activity on day 7 in fish exposed to 2.0 µg Cd/L, after which levels recovered for the remainder of the exposure. The elevation in kidney CAT activity by the end of the first week may be why there was no significant LPO (Fig. 2.5C) throughout the 29 days. Considering the eventual high Cd burden in the kidney of these fish (Fig. 2.4C), the observed initial CAT response, in addition to other defenses, may have prevented long-term oxidative damage. Basha and Rani (2003) reported significant (and sustained) increases in CAT, SOD and glutathione peroxidase (GPx) activity by day 7 onwards in the kidney of tilapia during a 30-d exposure. GPx catalyzes both the reduction of H_2O_2 and lipid peroxides, thus providing additional cellular defense against oxidative lipid damage (Winstin and DiGiulio, 1991). The authors also found that in comparison to the liver, the kidney had relatively lower activities of all antioxidant enzymes except GPx, suggesting that the kidney is the main site for GPx activation. Previous studies have demonstrated a simultaneous induction of enzymes (such as CAT and GPx) in the kidney of tilapia in the latter phase of the exposure to Cd, indicating a potential shift towards a detoxification mechanism during chronic periods (Basha and Rani, 2003; Atli and Canli, 2010).

In the kidney of fish exposed to 0.75 µg Cd/L, there were no significant changes in CAT activity throughout exposure (Fig. 2.7C). A lack of CAT response has similarly been reported in the kidney of tilapia and catfish after acute (Atli and Canli, 2006), sub-chronic (Pretto et al., 2011) or chronic (Asagba et al., 2008) exposure to waterborne Cd. Atli and Canli (2006) suggested that this finding may relate to elevated levels of MT synthesis, and/or retention of Cd-MT, in the kidney of exposed fish during extended periods. Hansen et al. (2007) found significantly increased levels of MT and MT transcription in the kidney (and liver) of wild brown trout (*S. trutta*) after transfer and exposure to a lake with elevated waterborne Cd and Zn levels. The authors concluded that this is most likely related to the redistribution of Cd from the gill to these tissues. These findings suggest that the observed lack of long-term lipid peroxidation in the kidney (Fig. 2.5C) of exposed fish may be related to the activation of additional antioxidant enzymatic (e.g. GPx) and/or non-enzymatic defenses, such as MT. The

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short-term LPO (on day 4 only) in the kidney of fish in the lower exposure may be due to an initial lag in defense response.

In the gills (Fig. 2.7A) of fish exposed to 0.75 µg Cd/L, there was a significant decrease in CAT activity up to day 17, after which levels recovered to those in controls. A reduction in CAT activity has similarly been reported in the gills of *C. punctata* fish during chronic exposure to Cd, Cu, Fe and Ni (Pandey et al., 2008). The authors found that CAT activity was significantly decreased throughout the 30 days, despite a gradual increasing trend after day 7. Pretto et al. (2011) also reported a significant inhibition in gill CAT in silver catfish after a 7- and 14-d Cd exposure. It has been suggested that an observed inhibition of CAT may be due to the direct binding of Cd^{2+} to sulfhydryl groups on the enzyme molecule (Pruell and Engelhardt, 1980; Reddy et al., 1981). Cd is also known to alter the mitochondrial production of O_2^- (Wang et al., 2004), which has been shown to inhibit CAT activity (Kono and Fridovich, 1982). The findings of the present study suggest that exposure to 0.75 µg Cd/L may have been sufficient to cause an initial inhibition of CAT (via direct or indirect mechanisms) in the gill.

Despite the CAT inhibition in the gills of fish in the lower exposure, no lipid peroxidation (as well as transient protein damage only) was observed throughout the 29 days (Fig. 2.5A; 2.6A). Interestingly, exposure to 2.0 µg Cd/L caused no significant changes in gill CAT activity (Fig. 2.7A) and LPO was observed after 29 days (Fig. 2.5A). The lack of initial LPO in exposed fish may be related to other defense mechanisms in the gill, such as the activation of additional antioxidant enzymes and/or a non-enzymatic response (e.g. MT and/or GSH). Hansen et al. (2007) related an initial (non-significant) decrease in gill CAT activity in exposed brown trout to Cd-mediated oxidative stress. The authors also noted that the initial reduction in CAT activity was found with a simultaneous increase in SOD activity during sub-chronic exposure to Cd and Zn. It has been shown that the activity of multiple antioxidant enzymes can work in a synergistic fashion to combat oxidative stress (Michaels et al., 1994; Bagnyukova et al., 2006) Bagnyukova et al. (2005) demonstrated that CAT inhibition in goldfish (*C. auratus*) during oxidative stress resulted in compensatory changes in glutathione-dependent enzyme activity. These findings suggest that in the present study, a response in other antioxidant defenses, may have compensated for that of CAT. In addition, the eventual LPO observed in the gills of fish in the higher exposure may be related to an overwhelming of the defense capabilities, such as the

depletion of GSH. It has also been suggested that a lack of initial LPO during chronic Cd exposure may be the result of a higher renovation of gill epithelium (Hansen et al., 2007). The findings of the present study suggest that the relationship between the oxidative cellular damage (to lipids and proteins) and antioxidant enzymatic defense (as CAT activity) appears to be the most complex in the gills of exposed rainbow trout than in the liver or kidney.

2.5.5 Relation to Acclimation & Concluding Remarks

During chronic exposure to Cd or other metals, freshwater fish generally undergo physiological changes, following an initial damage phase and a subsequent recovery (McDonald and Wood, 1993) that results in an increased tolerance to acute challenges (Stubblefield et al., 1999; Hollis et al., 1999; McGeer et al., 2000a). During the first week of exposure to 0.75 or 2.0 µg Cd/L, the gills (Fig. 2.5A) of exposed fish had significantly higher Cd burdens than controls, associated with the disruption of plasma Ca^{2+} and, to a lesser degree, Na^+ levels (Fig. 2.3A-B). Previous studies have similarly linked immediate physiological responses, such as transient ion losses, with elevated metal loads in the gills of fish during chronic exposure to waterborne metals (McGeer et al., 2000a; Monteiro et al., 2005).

A significant increase in protein carbonyl content (Fig. 2.6A) was found in the gills of fish in the lower exposure on day 4, which may be related to changes induced in the epithelial tissue during Cd^{2+} uptake. The gills of freshwater fish are considered the primary site of acute toxic impact (i.e. initial damage) by metals during waterborne exposure (Evans, 1987). Therefore, it may be concluded that the significant Cd accumulation in the gills (Fig. 2.4A) of exposed fish during the initial days of exposure is most likely related to the transient reduction in plasma Ca^{2+} and Na^+ levels (Fig. 2.3A-B) and perhaps to the oxidative protein damage observed on day 4 (Fig. 2.6A). Therefore, in exposed fish, the responses that characterize the 'damage phase' of the 29-d period included plasma Ca^{2+} and Na^+ disruption and elevated protein carbonylation in the gills, most likely related to the high Cd burdens within this tissue. In addition, the mortality (Fig. 2.1) of exposed fish up to day 5 was most likely a result of the experienced hypocalcemia (Fig. 2.3A), induced by the competitive uptake of Cd^{2+} (Playle et al., 1993; Playle, 1998) and subsequent enzyme inhibition (Verbost et al., 1987, 1989). Other

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deleterious effects observed during the onset of the exposure was the significant ROS-related lipid damage in the liver and kidney (Fig. 2.5B-C) of fish exposed to 0.75 or 2.0 µg Cd/L, respectively.

The oxidative damage to lipids in the liver and kidney, as well as to proteins in the gills, however was temporary, as respective MDA and protein carbonyl content had returned to basal levels by day 17. Hence, the transient ROS-related cellular damage was related to an initial overwhelming or lag of the fish's antioxidant defense response. The liver of exposed fish had the highest, and most immediate, elevation in CAT activity (Fig. 2.7B), compared to the response in other tissues. The subsequent recovery of liver MDA levels (Fig. 2.5B) in fish exposed to 2.0 µg Cd/L was most likely related to the sustained CAT activation (Fig. 2.7B) in this tissue. The findings of the present study emphasize the liver's known role in the detoxification of excessive amounts of ROS (Winston and DiGuilio, 1991). In addition to CAT, the elevated activity of other cellular defenses, such as SOD and/or MT, may have attributed to the lack of long-term lipid or protein damage in the liver of these fish.

There appeared to be a relatively weaker (i.e. short-lived) CAT response in the kidneys (Fig. 2.7C) of exposed fish (compared to that in the liver), however, no long-lasting lipid damage was found given the eventual high Cd burden (Fig. 2.4C) in this tissue. The redistribution of Cd from other tissues to the kidney (Fig. 2.4A-C) during the latter phase of exposure suggests that the kidney acts as the major storage organ of Cd in rainbow trout. Hence, the lack of significant ROS-related damage and the continual retention of Cd in the kidneys of fish exposed to 2.0 µg Cd/L over the course of 29 days may be related to the presence of Cd-MT complexes. Despite increasing its biological half-life within the fish (Norey et al., 1990), Cd bound to MT and subsequently retained in the kidneys in this form renders it unavailable (i.e. biologically-inactive (BIM)) to induce potential cellular damage.

In fish in the lower exposure, a subsequent recovery of plasma Ca^{2+} and Na^+ levels (Fig. 2.3A-B) to those of controls by the end of the first week was also observed. However, fish exposed to 2.0 µg Cd/L had significantly reduced plasma Ca^{2+} levels throughout the 29-d exposure (Fig. 2.3A), despite an eventual stabilization of accumulated Cd in the gills (Fig. 2.4A). It has been suggested that the re-establishment of ion balance may relate to the stabilization of tissue Cd burdens (McGeer et al., 2000a, b). Therefore, this suggests that the prolonged Ca^{2+}

disruption in the plasma of fish in the higher exposure may be linked to the elevated levels of accumulated Cd in the kidney particularly (Fig. 2.4C). Although there was a recovery of MDA to basal levels in the liver and kidney of exposed fish, there was significant lipid peroxidation in the gills at the end of the exposure period (day 29) only (Fig. 2.5A-C). The observed lack of oxidative lipid damage in the gills during the initial days of exposure is surprising, given the relatively higher Cd burden (and lack or inhibition of CAT response) in this tissue. The lag of lipid peroxidation in the gills of fish exposed to 2.0 µg Cd/L may relate to an eventual overwhelming of other antioxidant defenses, such as the depletion of GSH, and would require further investigation.

In conclusion, this study suggests that:

1) The relationship between tissue-specific total Cd burden, oxidative damage (to lipids and/or proteins) and antioxidant enzymatic defense (as CAT activity) in rainbow trout chronically exposed to waterborne Cd can be complicated, particularly in the gills.

2) Examining the ROS-related effects, accumulation of Cd and plasma ionic regulation throughout a chronic exposure period allows for the assessment of changes in the responses, as the fish undergoes the process of acclimating to Cd.

3) Regarding the damage-repair-acclimation pattern:

   a) During the damage phase, exposed fish experienced mortality, plasma Ca^{2+} and Na^{+} disruption and protein carbonylation in the gills (most likely be a result of the high Cd burden) as well as lipid peroxidation in the liver and kidney.

   b) Subsequent recovery of responses to levels found in unexposed fish was associated with sustained or elevated CAT activity in the liver and kidney.

   c) The resulting acclimation was characterized by a re-establishment and/or stabilization of plasma ion levels and CAT activity, respectively, and a lack of sustained oxidative damage to proteins and lipids (in the liver and kidney only).

4) Future investigations of additional responses and/or mechanisms induced during chronic exposure to waterborne Cd in freshwater is needed, and additional measurements could include: GSH metabolism (and/or GPx activity), transcriptional rates of antioxidant

enzymatic (e.g. CAT) or non-enzymatic (e.g. MT) defenses, and indicators of health, such as swimming performance or cortisol.

Table 2.1 Measured exposure concentrations of total dissolved Cd from head tanks and fish tanks taken during the 29-day exposure to rainbow trout. Temperature of exposure water ranged from 9.9 – 11.6 °C. Values are expressed as means ± 1 SD (n); HT-uf: unfiltered water samples from head tanks; HT-f: filtered water samples from head tanks; FT-uf: unfiltered water samples from fish tanks; FT-f: filtered water samples from fish tanks.

Exposure	HT-uf	HT-f	FT-uf	FT-f
Control (0)	0.06 ± 0.019 (6)	0.02 ± 0.003 (7)	0.08 ± 0.026 (6)	0.03 ± 0.002 (6)
0.75 µg Cd/L	0.81 ± 0.198 (6)	0.66 ± 0.135 (7)	0.78 ± 0.069 (6)	0.71 ± 0.101 (6)
2.0 µg Cd/L	2.15 ± 0.310 (6)	1.87 ± 0.214 (7)	2.02 ± 0.106 (6)	1.85 ± 0.119 (6)
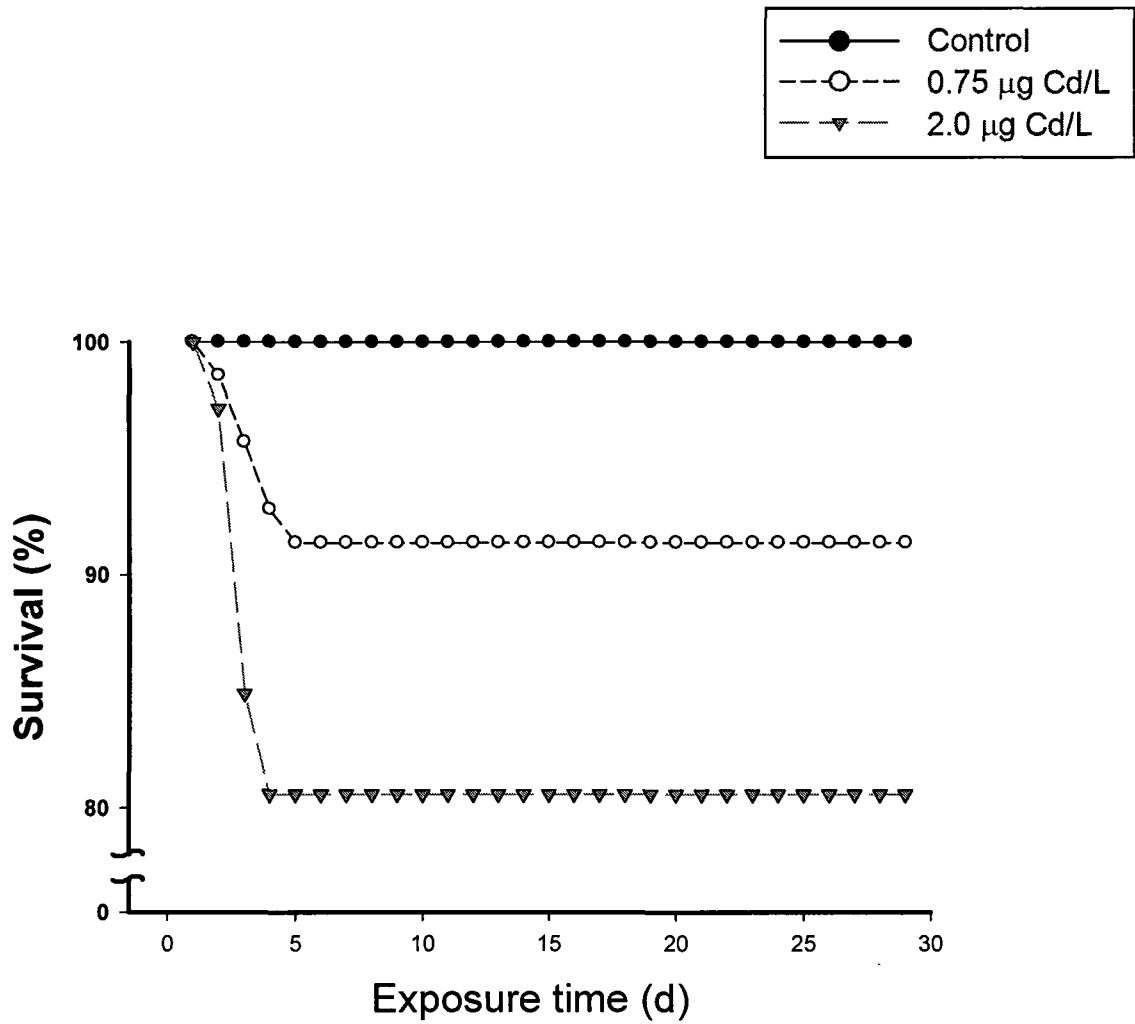
Fig. 2.1 The effect of waterborne Cd exposure on the survival of rainbow trout exposed to 0.75 or 2.0 µg Cd/L over 29 days. A group of unexposed fish (controls) is also included.
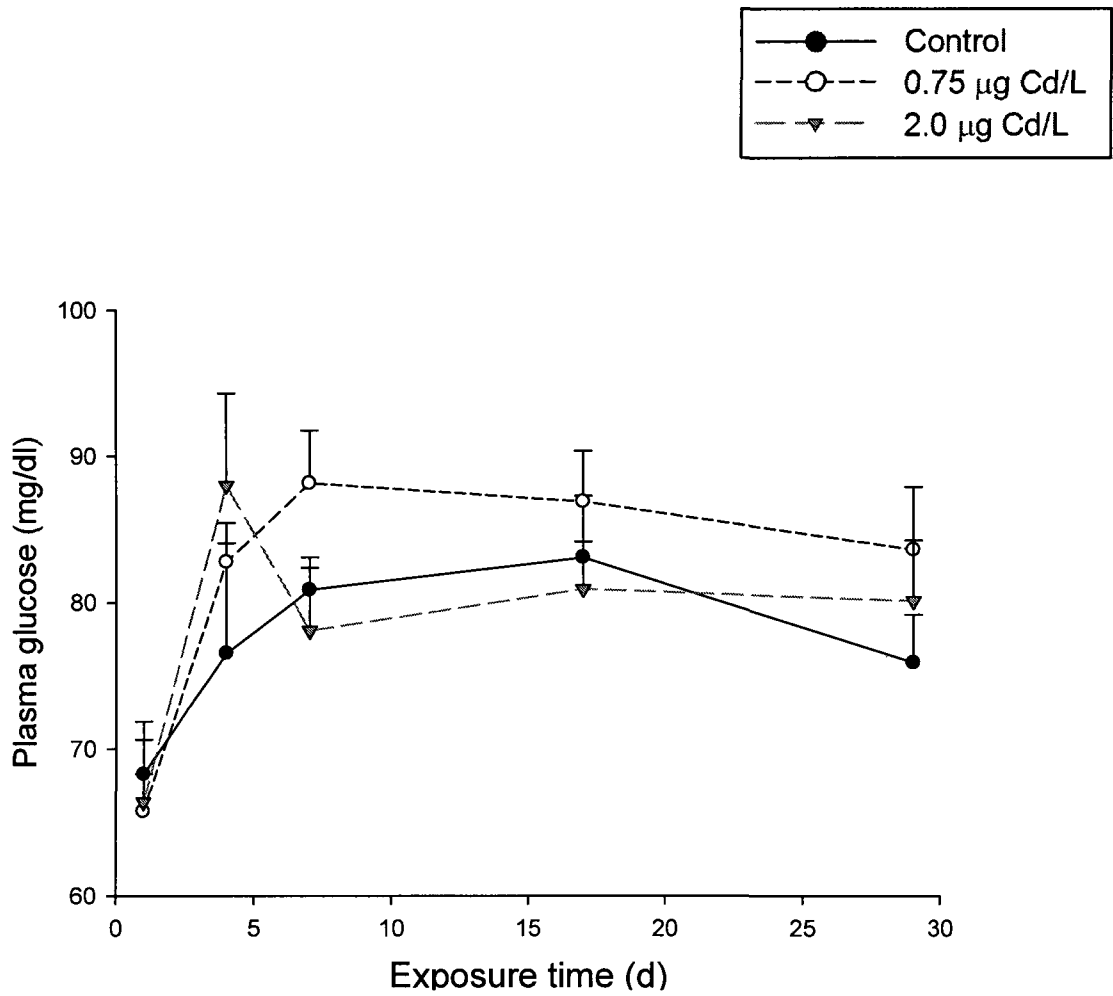
Fig. 2.2 The effect of waterborne Cd exposure on plasma glucose in rainbow trout exposed to 0.75 or 2.0 µg Cd/L over 29 days. Means ± 1 SEM (n=6) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.
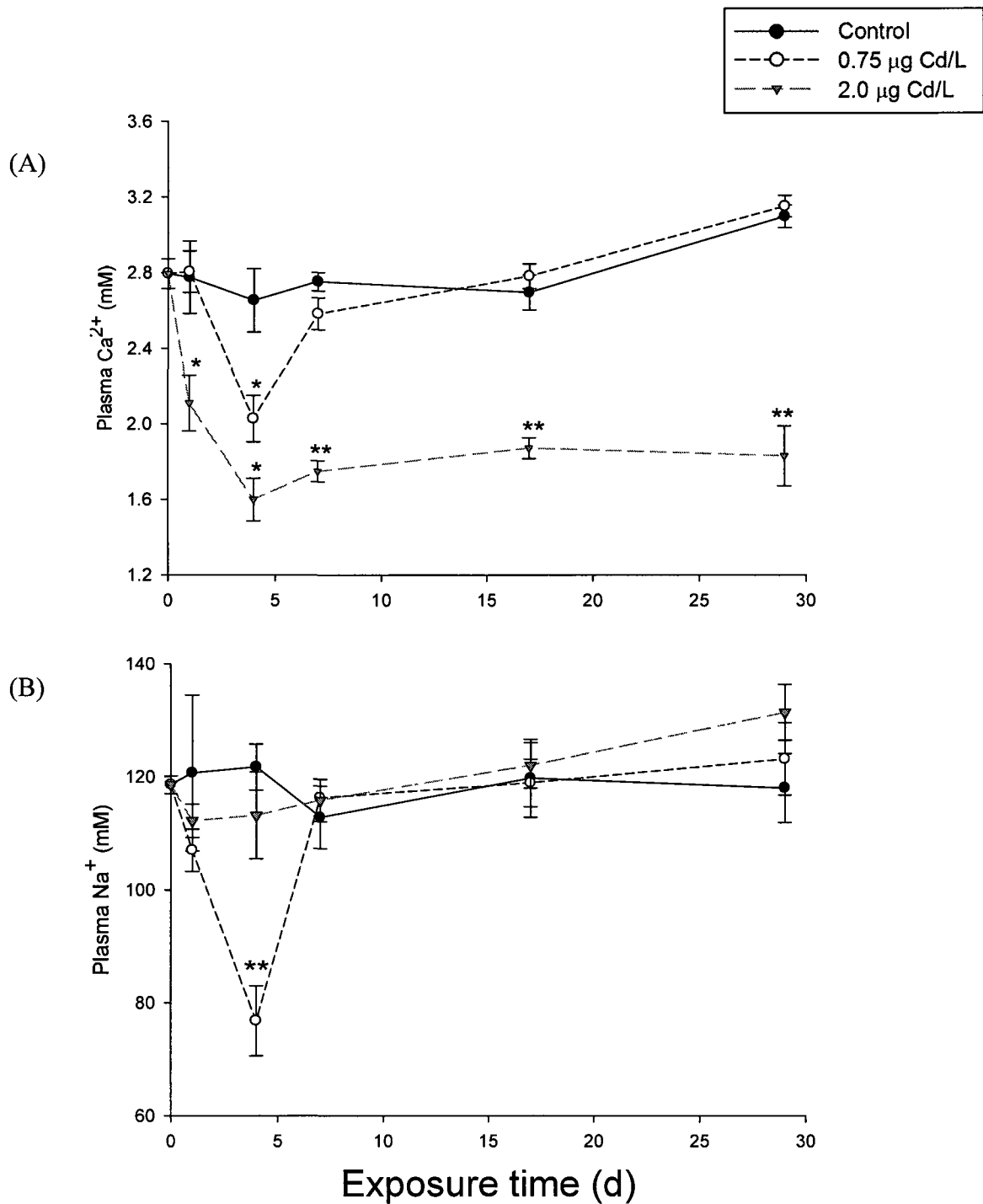
Fig. 2.3 The effect of waterborne Cd exposure on plasma Ca (A) and Na (B) in rainbow trout exposed to 0.75 or 2.0 μg Cd/L over 29 days. Means ± 1 SEM (n=6) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.
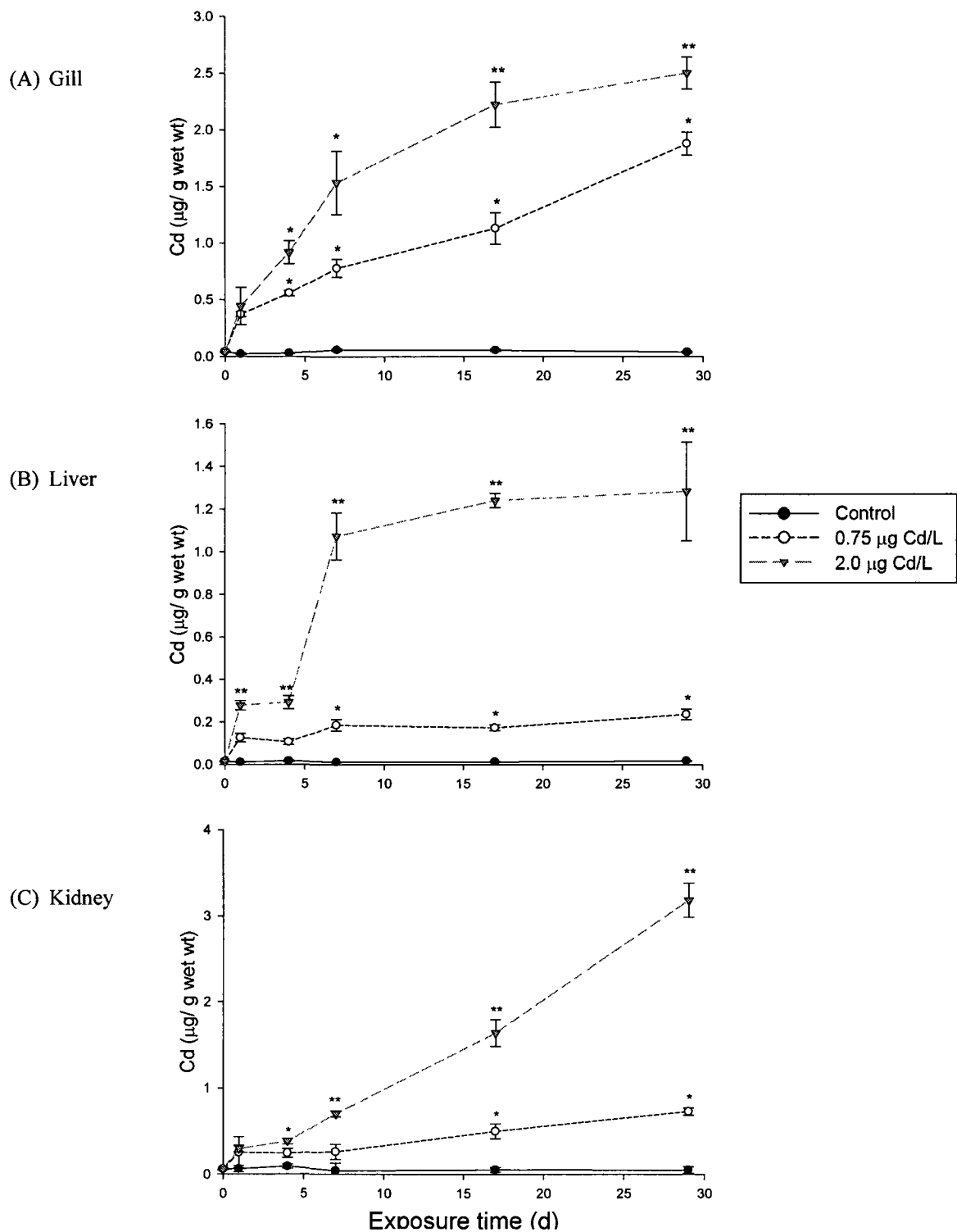
Fig. 2.4 Dose- and time-course of total Cd burden in the gill (A), liver (B) and kidney (C) of rainbow trout exposed to 0.75 or 2.0 μg Cd/L over 29 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.
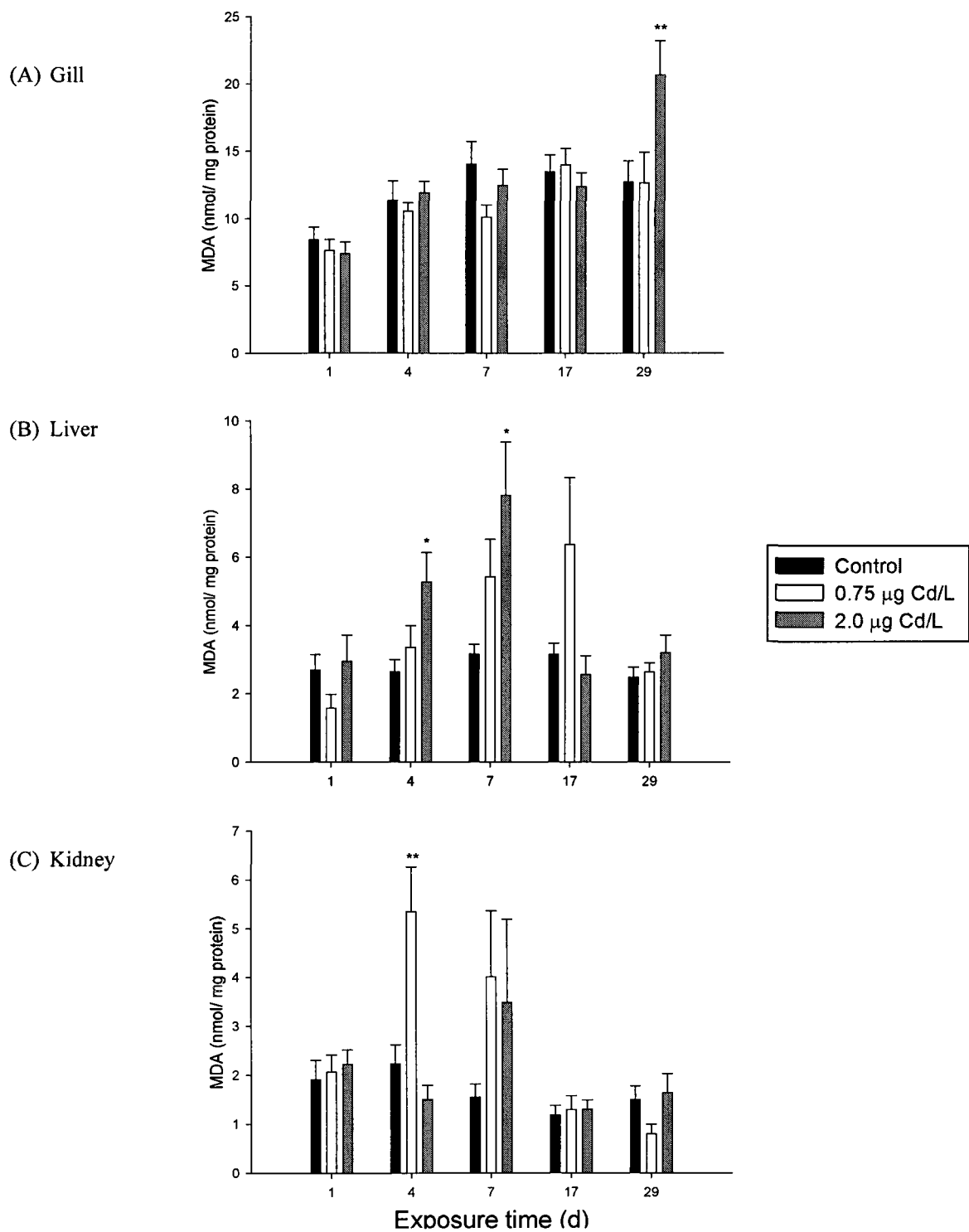
Fig. 2.5 The effect of waterborne Cd exposure on lipid peroxidation in the gill (A), liver (B) and kidney (C) of rainbow trout exposed to 0.75 or 2.0 μg Cd/L over 29 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.
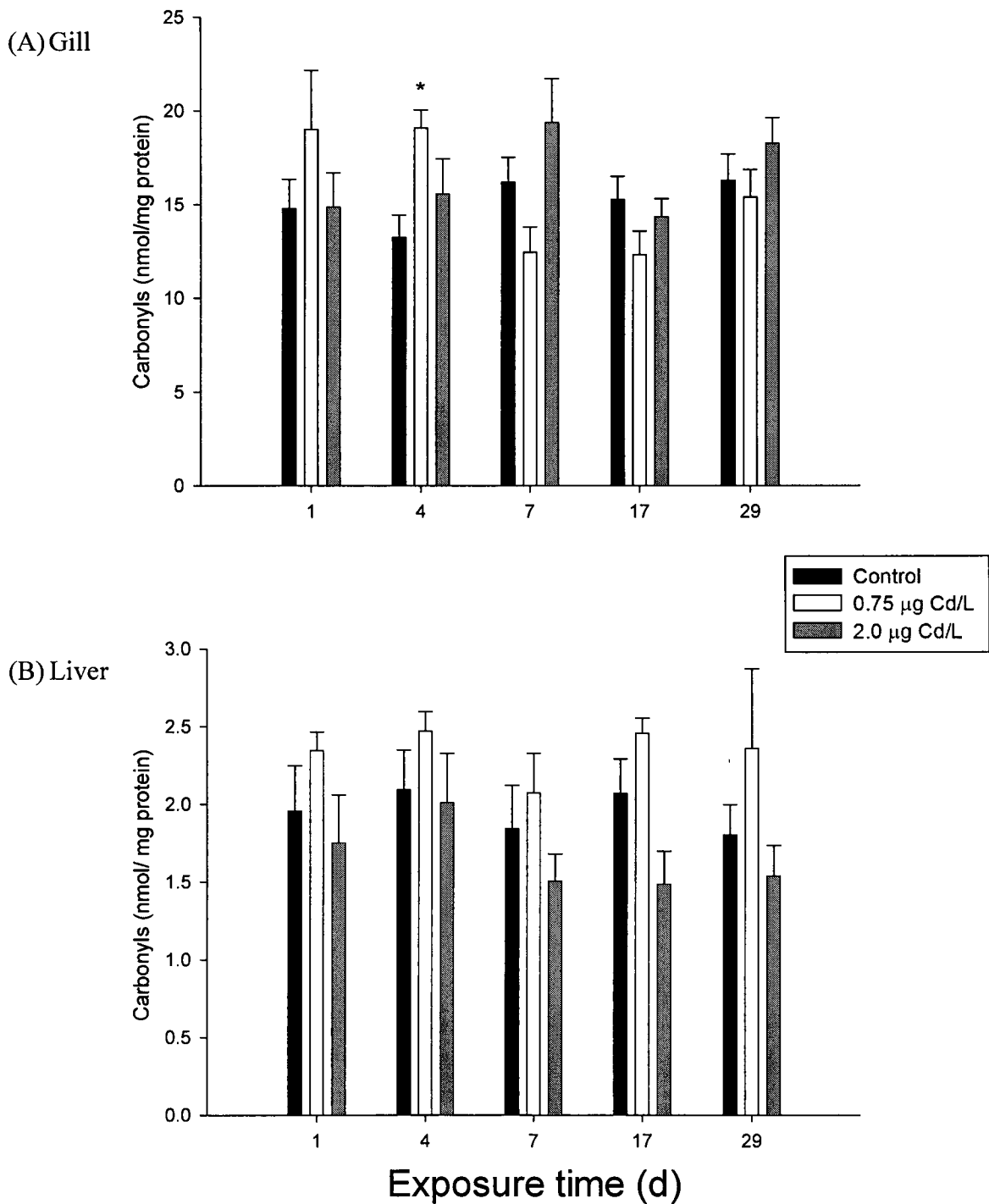
Fig. 2.6 The effect of waterborne Cd exposure on protein carbonyl content in the gill (A) and liver (B) of rainbow trout exposed to 0.75 or 2.0 μg Cd/L over 29 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.
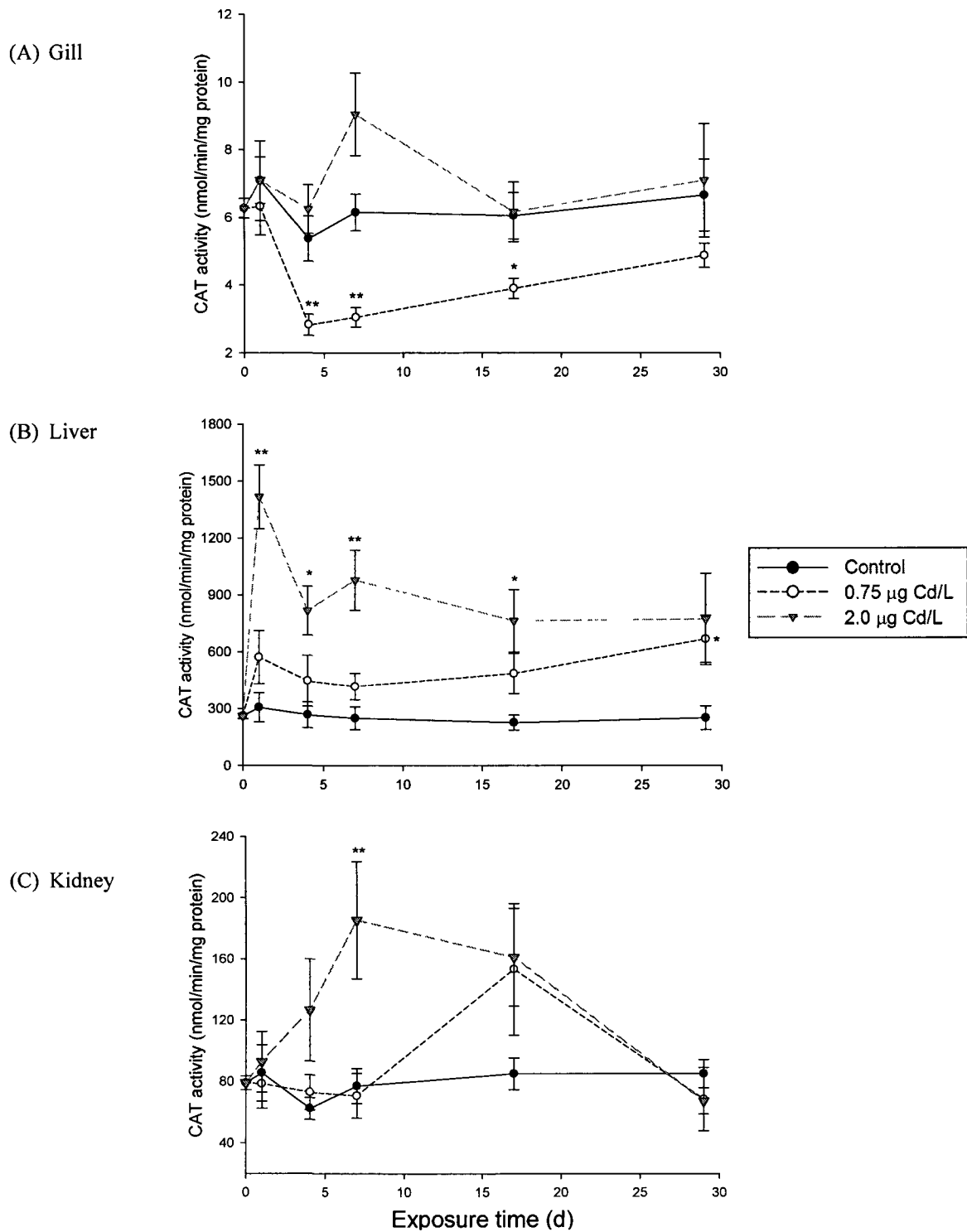
Fig. 2.7 The effect of waterborne Cd exposure on catalase activity in the gill (A), liver (B) and kidney (C) of rainbow trout exposed to 0.75 or 2.0 µg Cd/L over 29 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.

Chapter 3: Cd-induced responses in plasma ionic regulation and oxidative stress in lake whitefish (*Coregonus clupeaformis*) during chronic waterborne exposure

3.1 Abstract

The objectives of the present study were to evaluate the oxidative damage and antioxidant response in the tissues of lake whitefish (*C. clupeaformis*), and in relation to accumulation and plasma ionic regulation, during chronic Cd exposure. Fish were exposed to either 0 (control), 0.85 or 2.0 µg Cd/L in moderately hard water (hardness of 140 mg CaCO$_3$/L) for 32 days, and gills, liver, kidney and plasma were sampled throughout exposure. Tissue malondialdehyde (MDA) and protein carbonyl levels, as well as catalase (CAT) activity, were measured as indicators of oxidative stress. Throughout the initial 16 days, exposed fish experienced a reduction in plasma Ca^{2+} levels, in addition to significant elevations in MDA levels in the gills, liver and kidney. Increased protein carbonyl content was also observed in the liver of higher dose fish on day 7. Basal levels of hepatic CAT activity were higher than those of the gill (~13-fold) and kidney (~7-fold). CAT activity in the liver and kidney of fish exposed to 2.0 µg Cd/L was significantly elevated and maintained throughout exposure. Cd accumulation (on a tissue-wt basis) was the highest in the gill and kidney, then liver, in fish of either exposure. In exposed fish, there was a recovery of MDA and protein carbonyl levels in all tissues, as well as a re-establishment of plasma Ca^{2+}, by day 32. These findings suggest that exposure to sublethal levels of waterborne Cd was sufficient to induce initial oxidative stress in the tissues of lake whitefish, and that the lack of long-term lipid or protein damage may be related to an antioxidant enzymatic response.

3.2 Introduction

Cd has no known biological function in fish and can exert toxic effects if present in the environment at concentrations above natural background levels (Sorenson, 1991; Vallee and Falchuk, 1993; Wood, 2001). Within aquatic ecosystems, Cd concentrations result from natural sources as well as to anthropogenic releases, such as industrial effluents associated with metal extraction and refining (Wilson, 1988; CCME, 1999). For freshwater fish, waterborne Cd^{2+} competes with Ca^{2+} for uptake sites at the gill surface (Playle et al., 1993; Playle, 1998). This initial competition and the resulting inhibition of basolateral Ca^{2+} ATPase within the cell can lead to hypocalcemia, which is considered the primary mechanism of acute Cd toxicity in freshwater fish (Verbost et al., 1987, 1989; Wong and Wong, 2000).

Although the disruption of Ca^{2+} balance resulting from accumulation of Cd in the gill also occurs during chronic exposure to sublethal Cd, the mechanisms associated with these chronic effects are not understood. Other physiological effects in fish exposed chronically to Cd include decreased hematocrit and haemoglobin (Gill and Epple, 1993; Zikic et al., 2001) as well as impaired glucose and cortisol response (Fu et al., 1990; Brodeur et al, 1998; Lacroix and Hontela, 2004). During extended exposure to sublethal concentrations, the transient nature of some of these effects (particularly disruption of Ca^{2+} homeostasis) suggests the ability of fish to acclimate to waterborne Cd. The process of acclimation follows the damage-repair model characterized by McDonald and Wood (1993). Initial physiological disruption, particularly ion regulation, and accumulation associated with the gill are followed by a restoration phase during prolonged exposure periods. During the resulting acclimation, fish undergo changes to achieve a new steady state and can develop an increased tolerance to acute Cd challenges (McDonald and Wood, 1993; Stubblefield et al., 1999; Hollis et al., 1999; McGeer et al., 2000a).

The internal mobilization of metal-binding proteins, such as metallothionein, in the tissues of fish (Bradley et al., 1985; Hogstrand and Wood, 1996), may be an important feature of the recovery phase associated with extended exposures. Upon uptake into fish, Cd is considered to be in a biologically-active (or BA) form (Rainbow, 2002), thus accumulated Cd must be made unavailable (or detoxified) in order to avoid possible deleterious cellular effects (Steen Redeker and Blust, 2004; Vijver et al., 2004). In fish, a major mechanism in the detoxification of Cd involves its sequestration by metallothionein, cysteine-rich protein (Hogstrand and Haux, 1991; Roesijadi and Robinson 1994; Wu and Hwang 2003). This has been shown to contribute to increased Cd tolerance in fathead minnows (Benson and Birge, 1985) and rainbow trout (Fu et al., 1990; Hollis et al., 2001; Chowdhury et al., 2005) exposed chronically to Cd. However, if the Cd detoxification capacities of fish are overwhelmed by Cd burden, this may lead to toxic effects in sensitive tissues. These effects may be related to adverse cellular interactions involving the increased production of reactive oxygen species.

Reactive oxygen species (or ROS) are derived from the incomplete reduction of molecular oxygen and include highly-reactive radicals and non-radicals (Halliwell and Gutteridge, 1984; Livingstone, 2001). Despite their potentially damaging nature, ROS are continually produced under normal conditions, with quantities being monitored by the fish's antioxidant defense systems. Catalase, for example, is an enzymatic defense responsible for the degradation of H_2O_2, a non-radical species (Fahim and Sies, 1987; Rand, 2003). Exposure to Cd, however, may induce increased ROS production in fish, which can potentially exceed the capacities of cellular antioxidant defences. The resulting oxidative stress can lead to damage to lipids, proteins and/or DNA (Halliwell and Gutteridge, 1984; Livingstone, 2001).

Malondialdehyde (or MDA), for example, is a toxic by-product arising from ROS-directed fatty acid breakdown, and it is used as an indicator of oxidative lipid damage (Girotti, 1985; Christie and Costa, 1987). With regards to proteins, ROS-related interactions with side-chain amino acids can result in their transformation to carbonyls, leading to toxic aggregate build-up and/or enzyme inactivation (Stadtman and Levine, 2000). In freshwater fish, cellular ROS-related effects, such as increased oxidative damage and/or changes in antioxidant response have been linked to waterborne Cd exposure. Although the mechanisms accounting for these effects in fish are not yet known, eastern oysters (*C. virginica*) exposed *in vivo* to sublethal Cd had an impaired capacity for mitochondrial ATP production (Sokolova et al., 2005). This suggests that Cd-induced ROS production and resulting oxidative stress may be a mechanism of chronic toxicity in fish.

ROS-related effects, such as significant lipid peroxidation and/or increased catalase activity, have been reported in the tissues of several freshwater fish species, such as tilapia (*O. niloticus*), cichlids (*C. punctata*) and yellow perch (*P. flavescens*) during exposure to waterborne Cd (Palace et al., 1993; Bashi and Rani, 2003; Pandey et al., 2008; Almeida et al., 2009). However, the majority of studies have focused on acute to sub-chronic (up to 15 days) exposure to Cd, via waterborne route or direct intraperitoneal administration. Limited research has been conducted on investigating the time course of effects of sublethal waterborne Cd on oxidative damage and defense responses in salmonids during a chronic exposure period.

The objectives of the present study therefore were to (1) characterize the oxidative damage and response in the gill, liver and kidney in lake whitefish during a 32-day sublethal waterborne Cd exposure and (2) evaluate relationships between these ROS-related responses, accumulation and plasma ionic regulation in the context of the damage-repair-acclimation

pattern. Responses measured during exposure included plasma Ca^{2+} and Na^+, total tissue Cd

burden (in gill, liver and kidney) and indicators of oxidative stress (lipid peroxidation, protein

damage and enzymatic antioxidant defense (as catalase activity)).

3.3 Materials and Methods

3.3.1 Fish husbandry and acclimation

A group of 300 juvenile lake whitefish (*Coregonus clupeaformis*, 44.15 ± 14.4g (mean $\pm$ 1 SD),

n= 179) were provided by the Ontario Ministry of Natural Resources (White Lake Hatchery,

ON). Fish were non-selectively distributed across six 200-L tanks (50 fish per). Tanks were

supplied with flowing water ($0.75 - 1$ L min^{-1}) from a central mixing tank where reverse osmosis

deionized water and well water were mixed to achieve a hardness of 140 mg $CaCO_3$ L^{-1} (with

800 ± 21 Ca, 453 ± 20 Mg, 369 ± 30 Na (μM, means $\pm$ 1 SD, n=16), conductivity of 220 μS

cm^{-1}, pH 7.3 and temperature 13.0°C). Water from the central header tank was directed to one of

three 10L dosing head tanks before delivery to replicate exposure tanks. All tanks were

vigorously aerated and fish were fed a commercial feed (Bio Oregon Protein Inc, Warrenton,

OR) at 2% of biomass daily except on the days prior to sampling.

3.3.2 Exposure to Cd

Fish were exposed to nominal concentrations of 0.85 or 2.0 μg L^{-1} Cd (measured concentrations

of 0.82 and 2.2, see Table 3.1) in duplicate by delivering (QG6 pump, Fluid Metering Inc.,

Syosset, NY) appropriate volumes of Cd stock solution ($CdCl_2$ $2.5H_2O$, VWR International,

Mississauga, ON) to dosing head tanks. A pair of fish tanks was fed from a third dosing head tank but this one received no Cd and served as controls (0 added Cd). Therefore a total of 100 (n = 2 x 50) fish were exposed in each treatment. Exposure duration was 32 d.

3.3.3 Sampling

Water in the mixing head tanks was measured daily for conductivity, temperature and pH. Water samples(10 mL), both unfiltered and 0.45 μm filtered (Acrodisc HT Tuffryn, Pall, Ann Arbor, MI) were collected at least twice weekly from each exposure and acidified to 1% with HNO_3 (Trace metals grade, Fisher Scientific, Nepean, ON) for subsequent Cd measurement. On days 3, 7, 16 and 32 of exposure, four fish were sampled from each tank (n=8 per exposure) and euthanized in a $NaHCO_3$ buffered solution of 0.3 g L^{-1} tricaine methanesulfonate (Syndel Laboratories, Qualicum Beach, BC). Fish were blotted dry, weighed and a blood sample of approximately 0.2 to 0.4 mL collected from the caudal vasculature into vials containing 10 μL of lithium-heparinised cortland saline, centrifuged (Spectrafuge 16M, Mandel Scientific Inc, Guelph, ON) at 13,000 rpm for 3 minutes and plasma was isolated and then stored in -20^0C (or -80^0C) until analysis for Na and Ca content. Gill arches were collected, rinsed for 10 s in Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), blotted dry, divided into two subsamples, placed into microtubes, frozen in liquid nitrogen and stored in -80^0C. Fish were dissected, liver and kidney collected and then similarly subdivided into two samples and stored with gill samples. One set of the tissue subsamples was used for total Cd concentration, the other for oxidative stress endpoints.

3.3.4 Analyses

Daily measurements for water conductivity and temperature were done using a portable meter (YSI 30, YSI Ltd., Yellow Springs, OH), as was pH (SG2-ELK-SevenGo, Mettler, Mississauga, ON). Acidified water samples were measured for Cd by graphite furnace atomic absorption spectroscopy (GFAAS, SpectraAA-880, Varian Inc., Mississauga, ON) with appropriate standards matched with certified reference standards (TMDA 28.3 and TM 26.3, National Water Research Institute, Burlington, ON).

Plasma samples were analyzed using flame atomic absorption spectroscopy (SpectraAA-880, Varian Inc., Mississauga, ON). Gills, liver and kidney tissues were weighed, acidified with a 5- fold (wt: vol) volume of 1N HNO_3 (trace metal grade, Fisher Scientific, Nepean ON) and then digested for three h at 80^0C (Playle et al., 1993a). Following digestion, remixing and centrifuging, the supernatant was appropriately diluted and Cd was measured via GFAAS. Cd tissue burdens are expressed as μg Cd per g wet weight.

Biochemical assays

The gill, liver or kidney were homogenized (1:10, wt: vol) in ice-cold potassium phosphate buffer (50 mM KH_2PO_4, 1 mM EDTA, pH 7.2) at 22,500 rpm (Omni THQ digital tissue homogenizer, Omni International, Marietta, Georgia). Homogenates were centrifuged at 1600 x g for 10 minutes at 4^0C and supernatant was isolated and used for analysis of lipid peroxidation. Samples were then resuspended, centrifuged at 10,000 x g for 15 minutes at 4^0C and this supernatant was used for characterization of protein carbonyl content and catalase activity. All supernatant was stored in -80^0C prior to analysis.

Lipid peroxidation in tissues was determined by measuring malondialdehyde (or MDA, also known as the TBARS) assay (Ohkawa et al., 1979), as outlined by a commercial kit (10009055, Cayman Chemical Company, Ann Arbor, Michigan) with modifications. The modifications were as follows: 25 μL of supernatant was mixed with 25 μL of sodium dodecyl sulphate and 1 mL of colour reagent before being placed in a boiling water bath. Absorbance was measured spectrophotometrically (SpectraMax M2, Molecular Devices, Downingtown, PA) at 532 nm.

Protein carbonyl content was quantified using the method of Levine et al. (1994), as outlined by a commercial kit (10005020, Cayman Chemical Company). Briefly, 2,4-dinitrohydrazine or hydrochloric acid (as a control) was added to each sample, prior to a series of reagent additions, centrifugations and pellet washes. Samples were thoroughly resuspended by vortexing and/or manually between the reagent additions. Absorbance was measured spectrophotometrically at 375 nm.

Catalase activity was quantified using the method described by Wheeler et al. (1990), as outlined in a commercial kit (707002, Cayman Chemical Company). Samples were first diluted (10-, 20-, and 200-500-fold for the gill, kidney and liver, respectively) using buffer (25 mM potassium phosphate, 1 mM EDTA, pH 7.5) prior to being transferred to a 96-well plate and the addition of reagents. The spectrophotometer was used for the series of incubations and shaking of plates, and absorbance was measured at 540 nm.

For each sample, the final obtained values for all biochemical assays were normalized based on the total protein concentration. Protein content was determined by the Bradford method (Bradford, 1976), using protein reagent dye and bovine serum albumin (both from

Sigma-Aldrich Canada) as protein standard. Results for MDA, protein carbonyl content and catalase activity are expressed for gills, liver or kidney as nmol MDA per mg protein, nmol carbonyl per mg protein and nmol of hydrogen peroxide consumed per min per mg protein, respectively.

3.3.5 Statistical Analysis

Data is expressed as mean ± 1 SEM. Statistical analysis was performed using SigmaPlot 11.0 computer software (Systat Software, Inc., San Jose, CA). All data was subjected to a one-way analysis of variance (ANOVA), in which normality and equal variance were initially tested. Statistical comparisons were made between treatment groups (control (0), 0.85 and 2.0 µg/L) at the time intervals on days 3, 7, 16 and 32). When the ANOVA indicated significant difference (P<0.05) then the Tukey's HSD test was used to compare the means.

3.4 Results

3.4.1 Mortality and changes in plasma content

There were no mortalities for Cd-exposed groups throughout the exposure nor for unexposed (controls). For each of the measured parameters, there were no significant differences over time in controls. Plasma Ca^{2+} levels in fish exposed to 2.0 μg Cd/L followed an initial decreasing trend, with levels significantly reduced from controls by day 16, prior to recovery and on day 32, there was no significant difference compared to control levels (Fig. 3.1A). Cd exposure had no effect on plasma Na^+ levels compared to controls in either of the exposed groups (Fig. 3.1B), although there was a trend toward Na^+ disruption in Cd-exposed whitefish.

3.4.2 Tissue-specific total Cd burden

Cd accumulated in gills, liver and kidney of exposed lake whitefish in a dose- and time-dependent manner (Fig. 3.2). In the kidney (Fig. 3.2C), Cd concentrations appeared to increase in a linear-fashion after an initial delay, in contrast to the gill (Fig. 3.2A), where saturation appeared to occur in both exposed groups by day 16. In fish exposed to 2.0 μg Cd/L, Cd accumulated the most (on a tissue-wt basis) in the gill (3.9 ± 0.32 μg/g), which had a 2- and 8-fold higher burden than the kidney and liver, respectively. In comparison to the levels in unexposed fish, tissue Cd amounts in the fish exposed to the higher exposure were increased above controls by 36-, 9- and 3-fold in the gill (Fig. 3.2A), kidney (Fig. 3.2C) and liver (Fig. 3.2B), respectively. Cd concentrations increased above control values in a similar pattern in the tissues of fish in the lower exposure, with a 16-, 6- and 2-fold increase in the gill, kidney and liver, respectively.

3.4.3 Effect of Cd on lipid peroxidation

In the gills of fish exposed to 0.85 μg Cd/L, MDA was significantly increased above control levels by day 3 and remained elevated up to day 16 (Fig. 3.3A). In unexposed fish, average background MDA levels in the gill (Fig 3.3A; 27.72 ± 0.37 nmol/mg protein (mean ± 1 SEM, n=40)) were comparable to those in the kidney (Fig. 3.3C; 24.57 ± 0.82 nmol/mg protein (n=40)) and both were approximately 15-fold higher than levels in the liver (Fig. 3.3B). In the liver of fish exposed to 2.0 μg Cd/L (Fig. 3.3B), MDA was significantly elevated from control levels by day 3 and remained so until day 16, after which levels declined. This 4.5-fold rise above controls on day 3 (Fig. 3.3B) was the highest overall increase in MDA levels when comparing the three tissues. Liver MDA levels in fish in the lower exposure were significantly elevated above controls on day 16 only. In the kidney (Fig. 3.3C), exposure to 2.0 μg Cd/L caused a significant increase in MDA above control levels during the first week of exposure.

3.4.4 Effect of Cd on protein carbonyl content

In the gills, exposure to 2.0 μg Cd/L did not cause any significant changes in protein carbonyl content compared to controls during the first week of exposure or by day 32 (Fig. 3.4A). In unexposed fish, average background protein carbonyl levels in the gill were 13.68 ± 0.55 nmol/mg protein (n=24; Fig. 3.4A), which was comparable to those in the kidney (Fig. 3.4B; 7.76 ± 0.17 nmol/mg protein (n=24)). In the kidney of fish in the higher exposure (Fig. 3.4B), protein carbonyl levels were significantly elevated (2.5-fold) above controls on day 7, however, levels had returned to controls by the end of the exposure.

3.4.5 Effect of Cd on catalase activity

There were no significant changes in gill catalase activity (compared to control levels) for fish in either exposure throughout the 32 days, despite a decreasing trend over time in activity in the fish exposed to 2.0 μg Cd/L (Fig. 3.5A). In the liver (Fig. 3.5B) of fish exposed to 0.85 μg Cd/L, there was an initial significant 3-fold increase in catalase activity above levels in unexposed fish on day 3 and activity levels remained significantly elevated above controls for the remainder of the exposure (Fig. 3.5B). Liver catalase activity in fish in the higher exposure (Fig. 3.5B) was significantly elevated above control levels on day 3 and remained so throughout the 32 days. This average 3.5-fold rise above controls throughout exposure was the highest overall increase in catalase activity levels when comparing the three tissues. The average basal catalase activity in the liver (Fig. 3.5B) throughout exposure was 269.88 ± 5.63 nmol of H_2O_2 decomposed/min/mg protein (n=40), which was approximately 13- and 7-fold higher than in the gill (Fig. 3.5A) and kidney (Fig. 3.5C), respectively. In the kidney (Fig. 3.5C) of fish exposed to 2.0 μg Cd/L, there was a significant elevation in activity above control levels throughout the exposure.

3.5 Discussion

3.5.1 Effect of Cd on plasma ion regulation

There was an initial disruption in plasma Ca^{2+} levels in fish exposed to 2.0 µg Cd/L (up to day 16), with levels re-establishing to those in unexposed fish by the end of the exposure (Fig. 3.1A). Numerous studies have reported significant reductions in whole body or plasma Ca^{2+} levels with subsequent recovery in rainbow trout during chronic exposure to sublethal Cd (Haux and Larsson, 1984; Giles, 1984; Fu et al., 1990; McGeer et al., 2000a). Exposure to waterborne Cd^{2+} induces hypocalcemia in freshwater fish via the competitive uptake with Ca^{2+} at the gill surface (Playle et al., 1993; Playle, 1998) and subsequent inhibition of basolateral Ca^{2+} ATPases (Verbost et al., 1987, 1989). Additionally, the successive recovery of plasma Ca^{2+} ions to basal levels in Cd-exposed fish has previously been the result of an increased number of chloride cells (Wendelaar Bonga and Locke, 1992). This finding suggests that the ability of fish to acclimate to Cd during extended exposure may be linked to changes in the structure of, and/or uptake kinetics in, gill epithelium.

Exposure to Cd did not cause any significant changes in plasma Na^+ levels, despite an initial decreasing trend (Fig. 3.1B). The effects of waterborne Cd on plasma Na^+ balance in exposed fish are variable among studies. Undisturbed plasma or whole-body Na^+ levels have similarly been reported in rainbow trout (*O. mykiss*; Hollis et al., 1999; Baldisserrotto et al., 2004; Chowdhury et al., 2004) and tilapia (*O. niloticus*, *O. mossambicus*; Pratap et al., 1989; Garcia-Santos et al., 2005) during acute to chronic Cd exposure. The disruption of plasma Na^+ regulation in Cd-exposed fish has, in some studies, been linked to an inhibition of gill Na^+/K^+ ATPase activity (Pratap and Wendelaar Bonga, 1993; Lionetto et al., 2000; Atli and Canli, 2007), wheres others found no change in activity despite a transient reduction in whole-body Na^+ (McGeer et al., 2000a). Cd has also been shown to inhibit another enzyme related to ionic regulation, carbonic anhydrase, in the gills of European eel (*A. angilla*; Lionetto et al., 2000) during waterborne exposure. Hence, the mechanisms underlying the observed trends in plasma Ca^{2+} (Fig. 3.1A) and Na^+ (Fig. 3.1B) regulation are unclear and would require further investigation, for example, by evaluating ion flux rates and/or ionoregulatory enzyme activity within the gills.

3.5.2 Tissue-specific total Cd burden

Throughout the 32 days, Cd accumulated in the gills, liver and kidney of exposed fish in a dose- and time-dependent manner (Fig. 3.2A-C). During the initial week of exposure, the gills of fish in both exposed groups (Fig. 3.2A) had accumulated significant amounts of Cd, and concentrations were ~3-fold higher than in the liver and kidney (Fig. 3.2B, C). This finding, which has also been reported in rainbow trout (McGeer et al., 2000b; Hollis et al., 2001; Szebedinsky et al., 2001; Chowdhury et al., 2005), catfish (*C. gariepinus;* Asagba et al., 2008), and olive flounder (*P. olivaceus*; Kim et al., 2004) during chronic Cd exposure, suggests that the retention of Cd in the gills may act as a barrier to reduce internalization into other tissues (Hollis et al., 1999). Following day 16, it appears that Cd concentrations in the gills of exposed fish (Fig. 3.2A) had stabilized for the remainder of the exposure period, in contrast to levels in the kidney (Fig. 3.2C), which increased in a linear-fashion. This suggests that during the latter phase of exposure, Cd is distributed from the gills to internal tissues, indicating an increased emphasis on Cd detoxification and/or storage (Harrison and Klaverkamp, 1989).

While the gills of exposed freshwater fish are considered the site of immediate damage, Cd sequestration in the liver and kidney via metallothionein has been shown to contribute to the fish's ability to acclimate to Cd during extended exposure (Fu et al., 1990; Hollis et al., 2001; Chowdhury et al., 2005). The continual increase in kidney Cd levels throughout the latter phase of the exposure (Fig. 3.2C) suggests that this tissue may have a significant role in the long-term storage of Cd in lake whitefish. Previous studies have similarly concluded that the kidneys are a target organ for Cd accumulation in freshwater fish during chronic waterborne exposure (McGeer et al., 2000b; Hollis et al., 2001; Chowdhury et al., 2005). Cd levels have also been shown to remain elevated in the kidneys of exposed fish even after depuration (Kumada et al., 1980; Harrison and Klaverkamp, 1989; Asagba et al., 2008). This may be related to the accumulation/ retention of Cd-MT complexes within this tissue. In mammals, for instance, Cd-MT complexes are released from the liver and delivered to the kidneys (Nordberg and Nordberg, 1987), where they are filtered through the glomerulus, reabsorbed through the proximal tubules and retained (Timbrell, 1991).

By the end of the 32-d period, the gills of fish exposed to 2.0 µg Cd/L had accumulated higher levels of Cd (~8- and 4-fold) than the liver and kidney, respectively (Fig. 3.2A-C). This pattern of accumulation has similarly been reported in the tissues of rainbow trout after chronic exposure to similar concentrations of waterborne Cd (Szebedinsky et al., 2001; Franklin et al., 2005). Absolute Cd concentrations were highest in the gills of these fish at day 32 (Fig. 3.2A), which is in agreement with previous studies on lake whitefish (Harrison and Klaverkamp, 1989) and rainbow trout (Harrison and Klaverkamp, 1989; Szebedinsky et al., 2001; Franklin et al., 2005). The main location of Cd accumulation in tissues can vary strongly with fish species (Kraal et al., 1995), as well as with other factors, such as water chemistry, and fish age and metabolic activity (Hawkins et al., 1980; Pagenkopf, 1983 in Kim et al., 2004). The observed elevated Cd concentrations in the gills of lake whitefish may also relate to the amount of mucus on the gill surface (Reid and McDonald, 1991), which has previously been shown to increase in fish during exposure to waterborne Cd (Wu et al., 2007; Pandey et al., 2008).

3.5.3 Effect of Cd on tissue-specific oxidative damage and antioxidant enzymatic defense

Gill

MDA levels were significantly elevated in the gills of fish in the lower exposure up to day 16 of the chronic period (Fig. 3.3A). Significant elevations in gill MDA has similarly been reported in the gills of marine mussels (*B. azoricus*; Company et al., 2004) and catfish (Farombi et al., 2007) after acute or chronic exposure to Cd and/or other metals. The increase in MDA above control levels (Fig. 3.3A) suggests that exposure to 0.85 µg Cd/L was sufficient to cause significant oxidative damage to lipids (i.e. lipid peroxidation (LPO)) in the gills during the initial weeks of the 32-d period. Oxidative damage to lipids, as well as proteins, is the result of cellular attack by above-background levels of reactive oxygen species (ROS). It is suspected that Cd induces oxidative stress by indirect mechanisms, such as by depleting the cell's major sulfhydryl reserves (e.g. via binding to reduced glutathione), which would reduce the cell's ability to combat ROS and lead to potential damage (Rand, 2003). Therefore, the significant elevation in gill LPO observed for 16 days (Fig. 3.3A) may be a result of an overproduction of ROS, induced indirectly by exposure to 0.85 µg Cd/L. Basal levels of ROS are normally maintained by the

fish's antioxidant defense system, which include enzymatic and non-enzymatic sources. Hence, the resulting LPO in the gills of these fish (Fig. 3.3A) may be related to an overwhelming or inhibition of antioxidant cellular defenses. The observed oxidative stress in the gills was most likely associated with the significant Cd burden in this tissue (Fig. 3.2A), particularly as concentrations of Cd continued to increase throughout the initial 16 days of exposure.

Gill LPO was not sustained, however, as MDA levels had recovered to those of unexposed fish by the end of day 32 (Fig. 3.3A). This suggests that despite an initial stress, the antioxidant defenses of lake whitefish were able to manage, over time, ROS production in the gills of fish exposed to sublethal Cd. Previous research has reported LPO in the gills of silver catfish (*R. quelen;* Pretto et al., 2011) and rainbow trout (see Chapter 2) during sub- to chronic exposure to waterborne Cd. Pretto et al. (2011) also found that gill MDA levels in silver catfish remained significantly elevated even after depuration in Cd-free water. Hence, these findings suggest that the defenses in the gills of lake whitefish may be able to counteract Cd-induced oxidative stress more effectively, and/or within a shorter time span, than those of other freshwater fish.

Throughout the 32 days, there were no significant changes in gill MDA levels in fish exposed to 2.0 µg Cd/L (Fig. 3.3A), which has similarly been reported in catfish during a 21-d exposure to waterborne Cd (Asagba et al., 2008). The authors suggested that the lack of LPO in the gills, despite an observed inhibition of antioxidant enzymatic defense (as superoxide dismutase (SOD)) may be due to the action of metallothionein (MT). MT has been shown to contribute to increased Cd tolerance in fathead minnows (Benson and Birge, 1985) and rainbow trout (Fu et al., 1990; Hollis et al., 2001; Chowdhury et al., 2005) chronically exposed to Cd. In addition to the observed lack of LPO (Fig. 3.3A), exposure to 2.0 µg Cd/L did not cause any significant changes in protein carbonyl content in the gills of these fish (Fig. 3.4A) during the first week of exposure or by day 32. The carbonylation of side-chain amino acids is another consequence of ROS-related cellular damage that can potentially be induced in fish during metal exposure (Stadtman and Levine, 2000). A lack of significant changes in protein carbonyl levels has also been reported in the hepatopancreas of estuarine crab (*C. granulata;* Sabatini et al., 2009) and brain of fish (*C. bratachus;* Maiti et al., 2010) after chronic exposure to Cu or Pb, respectively. Increases in gill protein carbonyls have been found in zebrafish (*D. rerio*) exposed

to sublethal Cu, however, at the higher dose and after acute (48-h) exposure only (Craig et al., 2007). These findings further emphasize the possibility that the capabilities of antioxidant defenses in the gills of whitefish are effective in detoxifying ROS and/or sequestering Cd, thereby preventing long-term, if any, significant oxidative lipids and/or protein damage in this tissue.

Examples of major enzymatic defenses in the tissues of fish include catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Mather-Mihaich and DiGiulio, 1986 in Rand, 2003). The activity of antioxidant enzymes, such as CAT, has been shown to be induced or inhibited by exposure to metals (Pruell and Engelhardt, 1980; Shukla et al., 1987), and response is dependent on dose, route and duration of exposure and species sensitivity (Van der Oost et al., 2003; Sanchez et al., 2005; Gravato et al., 2006). In the gills of fish in either exposure, there were no significant changes in catalase activity (Fig. 3.5A) throughout the 32 days, despite a decreasing trend over time in activity in the fish exposed to 2.0 μg Cd/L. A lack of significant CAT response has similarly been reported in the gills of tilapia (Atli and Canli, 2006) and catfish (Asagba et al., 2008) after acute or chronic exposure to waterborne Cd. In addition, no significant changes in gill CAT transcriptional levels were found in wild brown trout (*S. trutta*) after transfer and 15-d exposure to a lake with elevated waterborne Cd and Zn levels (Hansen et al., 2007).

Interestingly, Hansen et al. (2007) also noted a non-significant decreasing trend in CAT activity levels in the gills of exposed brown trout, which was similarly observed in whitefish exposed to 2.0 μg Cd/L (Fig. 3.5A). Exposure to waterborne Cd has been shown to inhibit gill CAT activity in *C. punctata* fish (Pandey et al., 2008) and silver catfish (*R. quelen*; Pretto et al., 2011) during sub- to chronic periods. Although the mechanisms of Cd-induced CAT inhibition are not yet clear, it has been suggested that Cd^{2+} directly binds to sulfhydryl groups on the enzyme molecule (Pruell and Engelhardt, 1980; Reddy et al., 1981). Cd is also known to alter the mitochondrial production of O_2^- (Wang et al., 2004), which has been shown to inhibit CAT activity (Kono and Fridovich, 1982).

The observed lack of, or slight reduction in, CAT response in the gills of exposed fish (Fig. 3.5A) may be related to the significant increase in MDA levels in fish in the lower exposure (Fig. 3.3A). The lack of long-term LPO (Fig. 3.3A) or protein damage (Fig. 3.4A), in exposed

fish suggests that additional defenses in the gills, such as other antioxidant enzymes (e.g. SOD) and/or non-enzymatic sources (e.g. MT and/or reduced glutathione (GSH)) may mediate ROS production during extended Cd exposure. Hansen et al. (2007) noted that the non-significant reduction in CAT activity was found with a simultaneous increase in SOD activity during sub-chronic exposure. It has been shown that the activity of multiple antioxidant enzymes can work in a synergistic fashion to combat oxidative stress (Michaels et al., 1994; Bagnyukova et al., 2006). Bagnyukova et al. (2005) demonstrated that CAT inhibition in goldfish (*C. auratus*) during oxidative stress resulted in compensatory changes in glutathione-dependent enzyme activity. These findings suggest that in the present study, a response in other antioxidant defenses in the gills of lake whitefish may have compensated for that of CAT and contribute to the lack of long-term ROS-related cellular damage induced by sublethal levels of Cd.

Liver

Significant elevations in MDA levels were found in the liver of fish exposed to 2.0 μg Cd/L throughout the first week of exposure, as well as on day 16 only in fish in the lower exposure (Fig. 3.3B). This suggests that exposure to sublethal levels of Cd was sufficient to cause LPO in the liver of lake whitefish during the initial weeks of the 32-d period. Previous studies have similarly reported increases in LPO in the liver of tilapia (Almeida et al., 2009), silver catfish (Pretto et al., 2011), Atlantic croaker and striped mullet (*M. undulatus* and *M. cephalus*, respectively; Wofford and Thomas, 1988) during sub- or chronic exposure to waterborne Cd. The significant hepatic lipid damage in exposed whitefish (Fig. 3.3B) may relate to the increased liver Cd accumulation that occurs as the retention capacity of the gill for Cd is overwhelmed and distribution and uptake to internal tissues develops during the first week of exposure (Fig 3.2A-B). The recovery of liver MDA to basal levels in fish of either exposure by day 32 (Fig. 3.3B) indicates that LPO was not sustained. This may suggest that despite an initial stress, the antioxidant defenses in the liver were able to eventually maintain ROS, thereby preventing further lipid damage, during chronic exposure to Cd. Asagba et al. (2008) similarly demonstrated that MDA levels in the liver of catfish had significantly decreased by the end of a

21-d exposure to waterborne Cd, and the authors related this to a corresponding elevated activation of SOD.

The CAT-SOD system is considered the first line of defense against oxyradical formation (Winston and Guilio, 1991; Sies, 1993). In the present study, CAT activity was significantly elevated in the liver of fish exposed to 0.75 or 2.0 µg Cd/L (Fig. 3.5B), and levels remained so throughout the 32-d period. Significant increases in CAT activity has also been reported in the liver of tilapia (Basha and Rani, 2003; Atli and Canli, 2006; Almeida et al., 2009), catfish (Pretto et al., 2011) and marine *S. basilisca* (Messaoudi et al., 2009) during acute to chronic Cd exposure, and has been attributed to an effective activation of the antioxidant defense system (Atli and Canli, 2010). The observed elevation in liver CAT activity in exposed whitefish (Fig. 3.5B) suggests that exposure to waterborne Cd induced an antioxidant defense response in this tissue to counteract oxidative stress. The liver is known for its significant role in metal detoxification (Goering et al., 1995), and is considered the site of maximal pro-oxidant activity and antioxidant (such as MT and GSH) synthesis (Winston and Guilio, 1991). The liver of exposed fish had the highest activation of CAT compared to other tissues (Fig. 3.5A, C), which is in agreement with previous research on rainbow trout (see Chapter 2) and numerous studies on metal-exposed fish (Hidalgo et al., 2002; Gul et al., 2004; Avci et al., 2005; Atli and Canli, 2006).

The observed sustained CAT response in the liver of exposed fish (Fig. 3.5B) throughout the 32 days may be why there was a lack of long-term LPO in this tissue (Fig. 3.3B). Elevated antioxidant enzymatic activity and reduced lipid peroxidation have been correlated with high ambient tissue Cu, Cd, Pb and Ag residues in the liver of wild white seabream (*D. sargus*; Ferreira et al., 2008). The recovery of MDA levels (Fig. 3.3B) may also relate to additional defense mechanisms in the liver, such as metal sequestration and/or free radical scavenging by MT and/or GSH. The tolerance of fish to waterborne Cd during extended exposure has previously been linked to elevated hepatic MT synthesis in rainbow trout (Hollis et al., 2001; Chowdhury et al., 2005), as well as to increased levels of Cd associated with the MT fraction in the liver of tilapia (Ueng et al., 1996; Wu et al., 2002; Atli and Canli, 2003).

It has also been suggested that changes in the redox status of GSH (via its conversion of free radicals) in the liver of fish may be an antioxidant adaptation to chronic Cd exposure

(Zirong and Shijun, 2007; Boelsterli, 2009). Previous studies have reported significantly altered levels of hepatic GSH, and of its ratio to its oxidized form (i.e. GSSG/GSH), in numerous fish species after acute (Atli and Canli, 2006) or chronic (Lima et al., 2006; Zirong and Shijun, 2007; Atli and Canli, 2008) Cd exposure. These findings and those of the present study, suggest that that the antioxidant defense capabilities of the liver, such as the activation of CAT (Fig. 3.5B) and other possible responses in MT and/or GSH, may have contributed to the lack of long-term oxidative stress in this tissue. Additionally, the significant and sustained hepatic CAT response throughout exposure emphasizes the significant role in detoxification of this organ in lake whitefish and its ability to combat potential Cd-induced ROS generation.

Kidney

MDA levels were significantly elevated in the kidney of fish exposed to 2.0 μg Cd/L during the first week of exposure only (Fig. 3.3C), a trend that was similarly observed in the liver of these fish (Fig. 3.3B). Previous studies have concluded that significant LPO in the kidney of catfish after sub- to chronic exposure to waterborne Cd may be related to an observed decrease in antioxidant enzymatic defense (Asagba et al., 2008; Pretto et al., 2011). The lack of sustained kidney LPO in fish in the higher exposure (Fig. 3.3C) suggests that there may have been a lag in the response of the antioxidant defense system that would allow for excess ROS and subsequent lipid damage. Asagba et al. (2008) suggested that an observed elevation in renal SOD activity may be why there was no significant LPO in catfish after 7 days of Cd exposure. This suggests that an enzymatic response, for example, of the CAT-SOD system, may be responsible for the recovery of MDA to basal levels (Fig. 3.3C) in the kidney of whitefish.

A significant increase in protein carbonyl content by the end of the first week (Fig. 3.4B) was also found in the kidney of fish in the higher exposure, however, levels had returned to controls by day 32. This suggests that sublethal levels of waterborne Cd may have been sufficient to cause short-term oxidative damage to renal proteins in whitefish. Significant increases in protein carbonyl content has similarly been reported in the liver, gill or plasma of silver catfish (Pretto et al., 2011), zebrafish (*D. rerio*; Craig et al., 2007) and marine corkwing wrasse (*S. melops*; Almroth et al., 2008), respectively, after acute to chronic exposure to Cd or

Cu. Interestingly, Pretto et al. (2011) reported that protein carbonyl content in the liver of catfish was significantly elevated only at day 7 (not after 14 days) and that levels returned to those of unexposed fish following depuration. The authors reported that these findings demonstrated that protein carbonyl content is not a reliable indicator of Cd toxicity. It has been also been concluded (Almroth et al., 2005) that a significant increase and subsequent decrease in protein carbonyl content indicates the complicated relationship between pro-oxidant exposure and accumulation of carbonyls. In addition, it has been shown that the proteolytic degradation of damaged proteins increases with exposure to moderate oxidants (Grune, 2003). This suggests that in the present study, exposure to 2.0 µg Cd/L was sufficient to cause relatively mild protein damage initially, which may have stimulated a cellular degradation response following day 7. The observed recovery in protein carbonyl levels (Fig. 3.4B) may also be related to an antioxidant defense response in the kidney of whitefish in the higher exposure.

In fish exposed to 2.0 µg Cd/L, kidney CAT activity (Fig. 3.5C) was significantly elevated by day 3 and levels were maintained throughout exposure. Hence, the lack of long-term oxidative damage to lipids (Fig. 3.3C) and/or proteins (Fig. 3.4B) in fish exposed to 2.0 µg Cd/L may relate to the sustained CAT response, in addition to other antioxidant defenses. Significant increases in kidney CAT, SOD and GPx activity have similarly been reported in tilapia during chronic Cd exposure (Basha and Rani, 2003; Atli and Canli, 2010). The authors also found that activity of CAT and GPx was elevated in the latter phase of exposure, and that this tissue had relatively higher GPx activity levels than those of the liver (Bashi and Rani, 2003). It was concluded that this may indicate a potential shift towards a detoxification mechanism in the kidney of tilapia during extended Cd exposure, and that this tissue is the main site for GPx activation. These findings suggest that the observed lack of long-term LPO in the kidney of exposed whitefish (Fig. 3.3C) may be related to the activation of additional antioxidant enzymatic defenses, such as GPx. Additionally, the transient oxidative renal damage to lipids and/or proteins (Fig. 3.3C, 3.4B) in fish in the higher exposure may be due to an initial lag (or latter activation) in the defense response.

3.5.4 Relation to Acclimation & Concluding Remarks

Freshwater fish are known to undergo changes in physiological status during extended exposure to sublethal levels of Cd and other metals. Following an initial phase of damage (primarily associated with the gills), there is a recovery (or 'repair;' McDonald and Wood, 1993) that results in an increased tolerance to acute challenges (Stubblefield et al., 1999; Hollis et al., 1999; McGeer et al., 2000a). Throughout the first two weeks of exposure to 0.85 or 2.0 μg Cd/L, the gills of lake whitefish accumulated significantly higher amounts of Cd than unexposed fish (Fig. 3.2A), which was associated with decreases in plasma Ca^{2+} levels (Fig. 3.1A). Waterborne Cd^{2+} induces hypocalcemia in freshwater fish via competitive uptake with Ca^{2+} at the gill surface (Playle et al., 1993; Playle, 1998) and subsequent enzyme inhibition (Verbost et al., 1987, 1989). Significant oxidative lipid damage was found in the gills (Fig. 3.3A) of fish in the lower exposure up to day 16, which may also be related to the elevated Cd burden. The gills of freshwater fish are considered the primary site of acute toxic impact (i.e. initial damage) by metals during waterborne exposure (Evans, 1987). Their direct external contact with waterborne metals (Laurent et al., 1984; Wong and Wong, 2000), large surface area and high density of polyunsaturated fatty acids (Mallett et al., 1985; Evans et al., 1987) most likely make them susceptible to oxidative attack. Therefore, in exposed fish, the responses that characterize the 'damage phase' of the 32-d period included plasma Ca^{2+} disruption and elevated LPO in the gills, most likely related to the significant Cd accumulation within this tissue.

During the initial week of exposure, LPO was similarly found in the liver and kidney (Fig. 3.3B, C) of fish exposed to 0.85 and/or 2.0 μg Cd/L, respectively, in addition to the observed increase in renal protein carbonyl content (Fig. 3.4B). The ROS-related damage to lipids and/or proteins in the gills, liver and kidney, however, was short-lived, and this was related to an initial overwhelming or lag of the fish's antioxidant defense responses. In the gills of fish in either exposure, there were no significant changes in the CAT activity (Fig. 3.5A) throughout the 32 days. This suggests that the recovery in gill MDA levels, perhaps the observed lack of long-term cellular damage (Fig. 3.3A, 3.4A), in this tissue of exposed fish may be related to the activity of additional defenses, such as SOD and/or GSH.

The liver and kidney of exposed fish had significant (and maintained) elevations in CAT activity (Fig. 3.5B, C) throughout the 32 days. The sustained CAT response may be why there is no long-term oxidative damage in these tissues. The findings of this study emphasize the liver's known role in the detoxification of excessive amounts of ROS (Winston and DiGuilio, 1991). Hence, the lack of long-term LPO in the liver (Fig. 3.3A) of exposed fish may be a result of the elevated CAT response (Fig. 3.5B), in addition to the activity of other cellular defenses, such as SOD and/or MT. In the kidneys of fish exposed to 2.0 µg Cd/L, there was a recovery of MDA and protein carbonyl levels (Fig. 3.3C, Fig. 3.4B) to those of controls by day 32, despite the eventual high Cd burden (Fig. 3.2C) in this tissue. The redistribution of Cd from other tissues to the kidney (Fig. 3.2A-C) during the latter phase of exposure suggests that the kidney acts as the major storage organ of Cd in lake whitefish. Hence, the observed continual retention of Cd in the kidney of exposed fish and lack of sustained ROS-related damage may be related to the maintained CAT response (Fig. 3.5C), and possibly to an accumulation of Cd-MT complexes. The retention of Cd in the kidneys via its binding to MT renders it unavailable (i.e. biologically-inactive (BIM)) to induce potential cellular damage, despite increasing its biological half-life within the fish (Norey et al., 1990).

To conclude, the damage phase in exposed fish was associated with plasma Ca^{2+} disruption and LPO in the gills (most likely a result of the high Cd burden), as well as protein carbonylation and/or LPO in the kidney and liver, respectively. Subsequent recovery of responses to levels found in unexposed fish was associated with elevated and sustained CAT activity in the liver and kidney. The resulting acclimation was characterized by a re-establishment of plasma Ca^{2+} levels and a lack of sustained oxidative damage to lipids and proteins in the gills, liver and kidney. Therefore, this study demonstrates that the degree and pattern of oxidative damage and antioxidant enzymatic defense in lake whitefish induced by sublethal levels of waterborne Cd varies with dose, tissue and time. In addition, the lack of sustained oxidative stress in the gills, liver and kidney suggests that the defense mechanisms of whitefish are capable of effectively mediating ROS, thus preventing long-term damage, during extended Cd exposure. Future investigations of additional responses and/or mechanisms in lake whitefish could include measuring GSH metabolism or GPx activity (particularly in the gills), transcriptional rates of non-enzymatic defenses (e.g. MT), and indicators of health, such as swimming performance or cortisol.

Table 3.1 Measured exposure concentrations of total dissolved Cd from head tanks and fish tanks taken during the 32-day exposure to lake whitefish. Temperature of exposure water ranged from 12.6 – 14.4 °C. Values are expressed as means ± 1 SD (n); HT-uf: unfiltered water samples from head tanks; FT-uf: unfiltered water samples from fish tanks; FT-f: filtered water samples from fish tanks.

Exposure	HT-uf	FT-uf	FT-f
Control (0)	0.02 ± 0.004 (7)	0.04 ± 0.006 (6)	0.03 ± 0.002 (6)
0.85 μg Cd/L	1.02 ± 0.070 (7)	0.86 ± 0.088 (6)	0.82 ± 0.063 (6)
2.0 μg Cd/L	2.49 ± 0.064 (7)	2.22 ± 0.295 (6)	2.20 ± 0.267 (6)
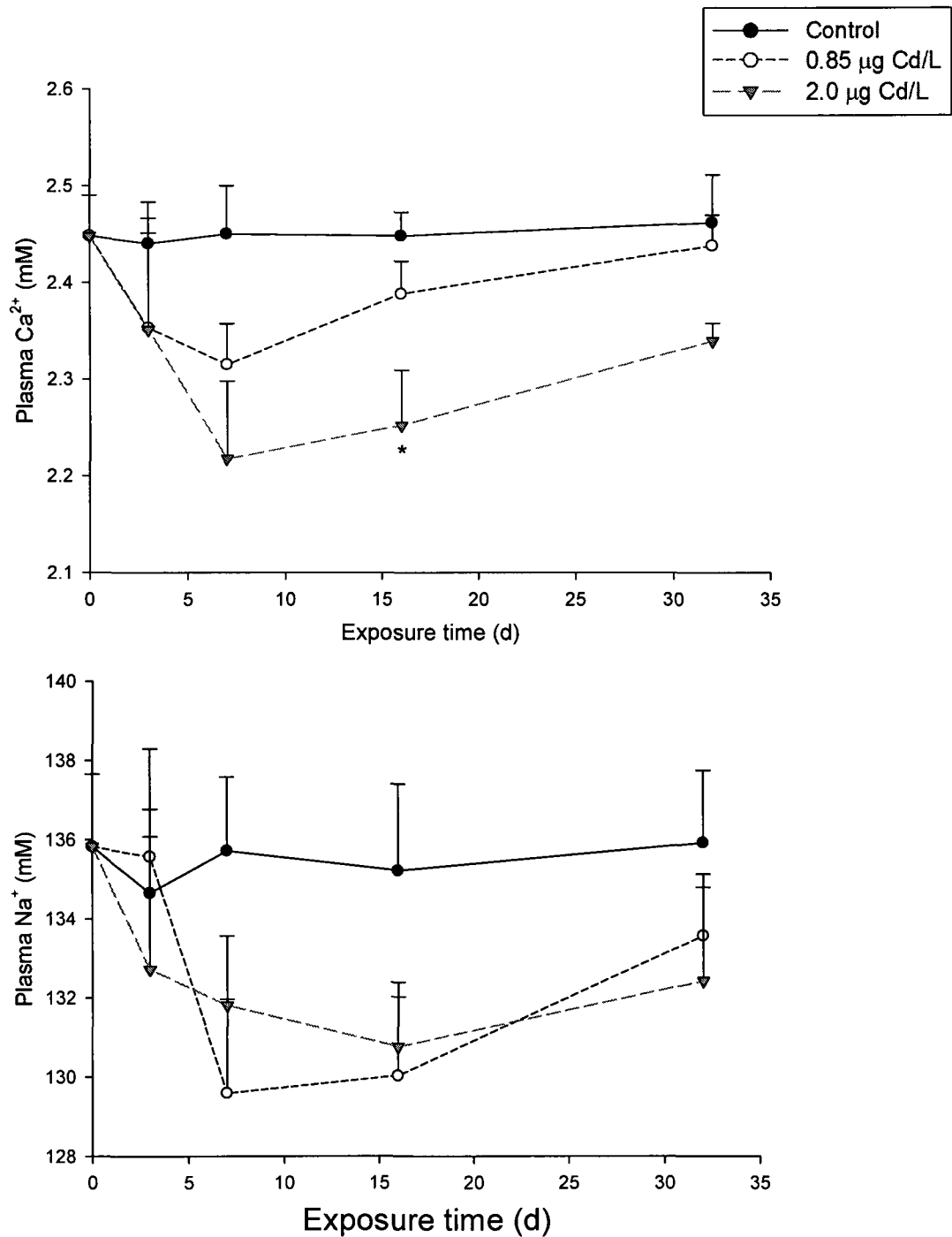
Fig. 3.1 The effect of waterborne Cd exposure on plasma Ca (A) and Na (B) in lake whitefish exposed to 0.85 or 2.0 μg Cd/L over 32 days. Means ± 1 SEM (n=6) are shown and * indicates significance from control values at that time (P<0.05). A group of unexposed fish (controls) is also included.
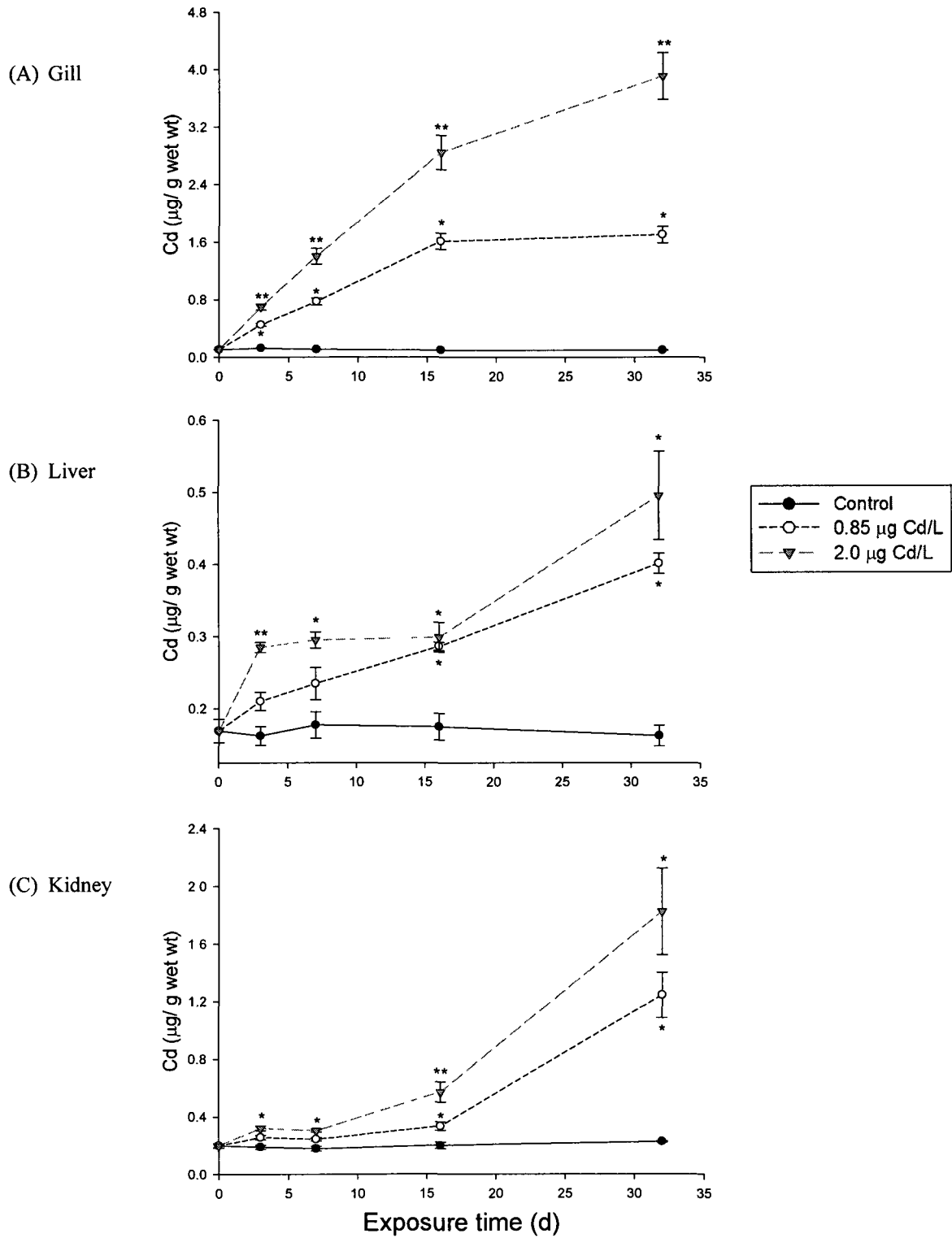
Fig. 3.2 Dose- and time-course of total Cd burden in the gill (A), liver (B) and kidney (C) of lake whitefish exposed to 0.85 or 2.0 µg Cd/L over 32 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.
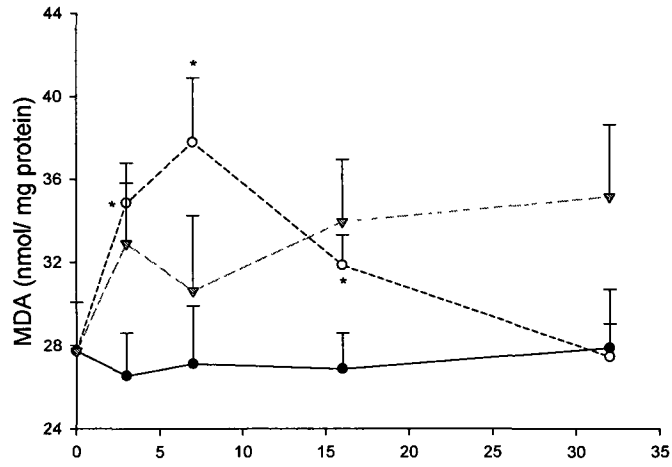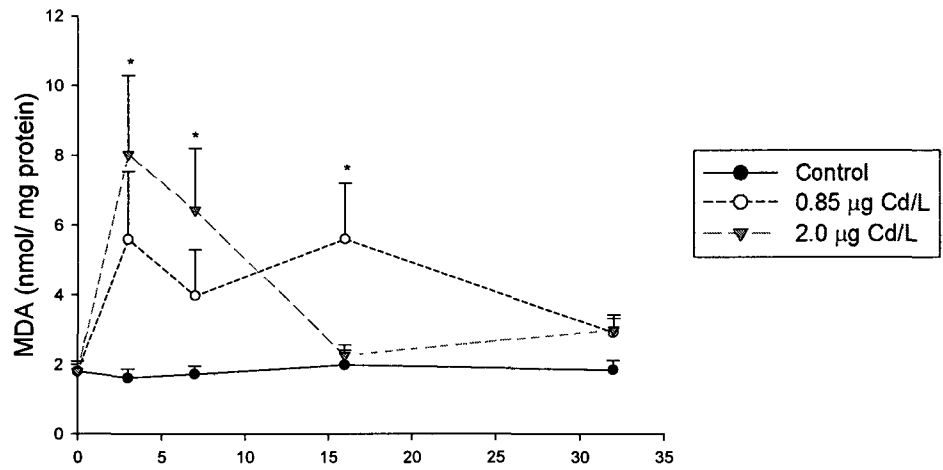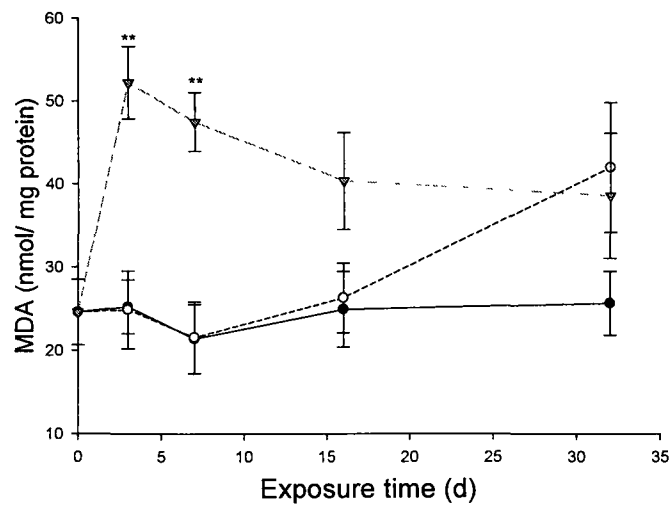
Fig. 3.3 The effect of waterborne Cd exposure on lipid peroxidation in the gill (A), liver (B) and kidney (C) of lake whitefish exposed to 0.85 or 2.0 µg Cd/L over 32 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.

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Fig. 3.4 The effect of waterborne Cd exposure on protein carbonyl content in the gill (A) and kidney (B) of lake whitefish exposed to 0.85 or 2.0 µg Cd/L over 32 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.

Fig. 3.5 The effect of waterborne Cd exposure on catalase activity in the gill (A), liver (B) and kidney (C) of lake whitefish exposed to 0.85 or 2.0 μg Cd/L over 32 days. Means ± 1 SEM (n=8) are shown and * indicates significance from control values at that time while ** indicates significance from all other means at that time (P<0.05). A group of unexposed fish (controls) is also included.

Chapter 4: General Discussion
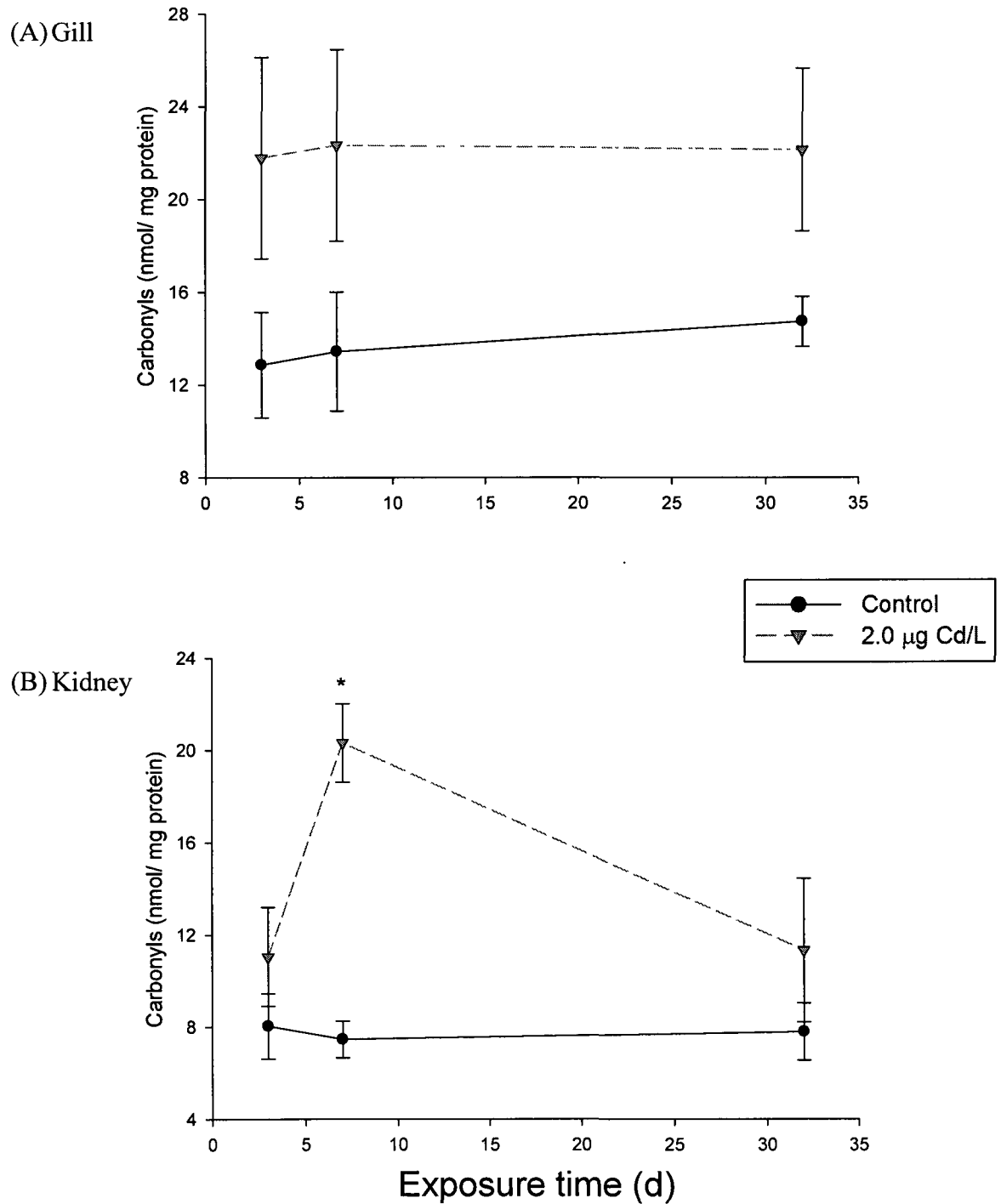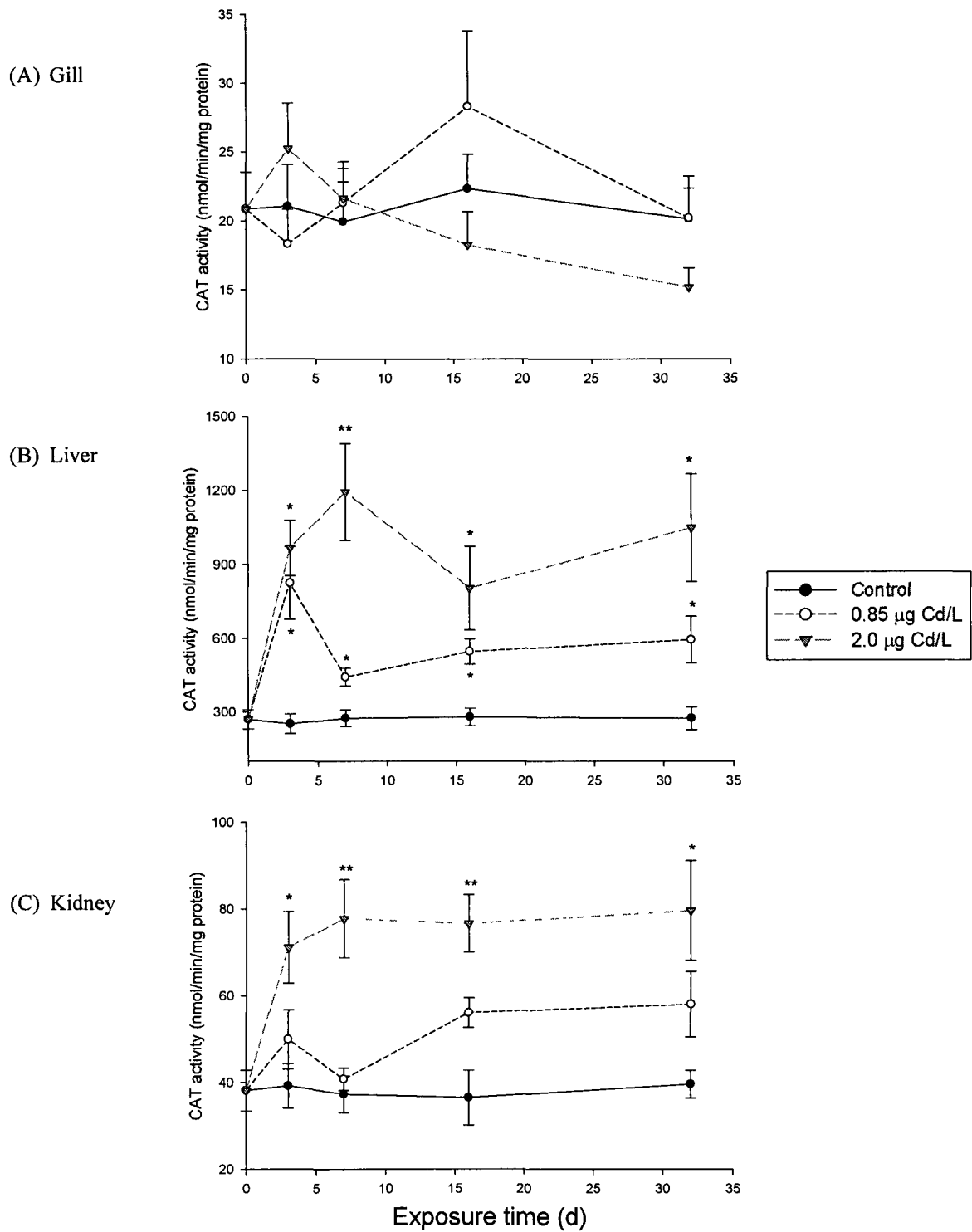
4.1 Species comparison of rainbow trout and lake whitefish & Conclusions

The studies outlined in Chapters 2 and 3 examined the relationship between the oxidative responses, the pattern of tissue Cd burden and plasma ion levels in exposed rainbow trout or lake whitefish (both salmonids) during acclimation to waterborne Cd. Preliminary research demonstrated that lake whitefish was more tolerant to Cd compared to rainbow trout during acute (96 h LC50s) waterborne exposure. The relative sensitivity of these two species to Cd may be related to differences in ROS-related responses induced during exposure.

Differences in detoxification processes, such as metallothionein synthesis, in the tissues of freshwater fish have been reported in studies investigating sensitivity amongst species or populations (Campbell et al., 2005; Kraemer et al, 2006; Wu et al., 2006). However, the majority of studies have focused on comparing indigenous fish (e.g. yellow perch, *P. flavescens*) with benthic bivalves (e.g. floater mollusc, *P. grandis*), or have used physiological stress responses, such as cortisol secretion, to assess differences in species sensitivity to Cd (Lacroix and Hontela, 2004; Campbell et al., 2005; Annabi et al., 2009). Limited research has been conducted on comparing ROS-related damage and defense responses amongst salmonid species during chronic exposure to sublethal waterborne Cd.

Additional objectives of this research therefore were to (1) compare the oxidative damage and defense responses in the gill, liver and kidney in rainbow trout to those in lake whitefish during respective month-long waterborne exposures and (2) investigate whether variations in the ROS-related response (and in the plasma ionic regulation and tissue accumulation) amongst the two species can help explain differences in their relative sensitivity to Cd.

The most pronounced differences in the Cd-induced effects during sublethal exposure among species (see Table 4.1) appear to be those that characterize the respective damage phases,

particularly related to the gills. Throughout the initial week of the month-long exposure, rainbow trout in either exposed group experienced mortality (Fig. 2.1) and significant hypocalcemia (Fig. 2.3A), which was associated with the high gill Cd burden (Fig. 2.4A). Changes in gill epithelium during Cd^{2+} uptake, and resulting hypocalcemia, may have attributed to the significant increase in protein carbonyl levels also observed in the gills (Fig. 2.6A) of rainbow trout at the onset of exposure. Additionally, plasma Ca^{2+} levels in trout (Fig. 2.3A) exposed to 2.0 μg Cd/L remained significantly reduced from controls throughout exposure. By comparison, exposed lake whitefish did not experience mortality, oxidative protein damage in the gills or sustained hypocalcemia (Fig. 3.1A, 3.4A) throughout the 30 days. Previous studies have concluded that relative sensitivity to Cd (*in vitro* or *vivo*) among exposed freshwater fish may be related to the differences in the competitive interactions between Ca and Cd for uptake pathways (LaCroix and Hontela, 2004; Raynal et al., 2005; Wu et al., 2006). Hence, the observed finding that extended exposure to waterborne Cd had a relatively more pronounced effect on plasma Ca^{2+} regulation in rainbow trout (Fig. 2.3A) may relate to this species being more sensitive during acute (96-h LC50s) exposure.

Short-term lipid peroxidation was observed in the gills of whitefish (Fig. 3.3A) in the lower exposure, with MDA levels recovered to those in unexposed fish by the end of the month. In exposed rainbow trout, gill MDA levels (Fig. 2.5A) were significantly elevated at the end of the exposure only, which was associated with a possible overwhelming of the antioxidant defense capabilities, such as the depletion of GSH and/or MT. In the gills of trout or whitefish, the observed lack of, or recovery in, respectively, gill LPO in the initial phase of exposure most likely related to the activity of other antioxidant defenses, to compensate for the lack of CAT response (Fig. 2.7A, 3.5A). Changes in erythrocyte antioxidant enzymatic activity have been

shown to vary among three cichlid species living in a metal-contaminated lake (Raus et al., 2008). Additionally, Olsson and Kille (1997) reported that MT gene induction could explain some of the observed differences in Cd sensitivity between rainbow trout and pike (*E. lucius*). MT synthesis in gills has also been shown to respond in a species-specific manner in three freshwater fish with varied tolerance during waterborne Cu exposure (De Boeck et al., 2003). Hence, in the present study, the species-specific pattern of oxidative lipid damage in the gills of exposed fish may relate to differences in defense mechanisms.

It has also been previously suggested (Norey et al., 1990; Lacroix and Hontela, 2004) that differences in sensitivity to Cd between fish species is relative to different rates of accumulation, in addition to various fates and patterns of distribution within tissues. During the first week of exposure to 2.0 µg Cd/L, Cd accumulation in the gills of rainbow trout (Fig. 2.4A) increased above controls by 23-fold, whereas there was a 10-fold increase in gill Cd levels in exposed whitefish (Fig. 3.2A). Despite the higher rate of accumulation in the gills of exposed trout, there was no initial gill LPO in this species (Fig. 2.5A), compared to the significant increase of gill MDA in whitefish. This may suggest that the defense response in the gills of rainbow trout is different, and perhaps more effective, in mediating ROS and thus preventing oxidative damage at the onset of exposure to waterborne Cd. By the last day of the month-long exposure, the relative increase in gill Cd levels above controls was higher in rainbow trout compared to the whitefish by ~2-fold. Interestingly, long-term LPO was observed in the gills of trout only (Fig. 2.5A). This suggests that during the latter phase of extended exposure to Cd, the antioxidant defense response in the gills of whitefish may be stronger, or perhaps experiences a later activation, than that in the trout.

In summary, during the initial phase of chronic exposure to Cd, rainbow trout (which had a lower 96-hr LC50), experienced mortality, sustained hypocalcemia, and transient oxidative damage to proteins in the gills, and these responses were not observed in whitefish. LPO was significant in the gills of trout at the end of exposure only. This suggests that establishing correlations between relative species sensitivity and the ROS-related damage induced in the tissues of rainbow trout and whitefish during chronic Cd exposure can be difficult. Therefore, it may be concluded that the interspecies difference in sensitivity to waterborne Cd may relate to some observed effects, particularly those relating to physiology, but not others, for example, specific ROS-related responses. In addition, the exposure concentrations (0.8 or 2.0 µg Cd/L) used were both within an environmentally realistic range , and current freshwater quality criteria for aquatic life (U.S. EPA, 2001) sets a range limit of 0.25—1.3 µg Cd/L for chronic Cd exposure in water of similar hardness (140 mg $CaCO_3$/L) to that used in this study. Hence, the results of the present study demonstrate that evaluating a multitude of parameters associated with oxidative stress in the tissues of fish may be useful in predicting and/or identifying the early effects of Cd exposure in environmental risk assessments.

With regards to other aspects of the integrative nature, this study was part of numerous collaborations within and between laboratories and fields of research. Throughout both of the respective month-long Cd exposures, fish of either species were also sampled by colleagues from the McGeer labratory, as well as from members of the Vijayian lab at the University of Waterloo. For instance, rainbow trout were used for research regarding the subcellular distribution of Cd within specific tissues, in addition to the effect of *in vivo* Cd exposure on the mechanisms/ pathways involving cortisol impairment. Lake whitefish were also part of a co-exposure with a labmate investigating the effect of waterborne Cd on overall swim performance.

Hence, this study, as well as the research conducted by collaborators, involves the assessment of numerous levels of biological organization, by assessing effects on the cellular-, tissue- and whole organism-level. In summary, the integrative evaluation of the numerous parameters measured throughout each exposure allows for a clearer (and more complete) understanding of the mechanisms underlying the acclimatization of fish chronically exposed to metals.

In conclusion, when addressing the hypotheses of the present study:

(1) Any mortality or ionoregulatory disturbances experienced during the damage phase would be accompanied by elevated ROS generation, as indicated by increased malondialdehyde and/or carbonyl levels.

During the damage phase, a reduction in Ca^{2+} levels was accompanied with significant LPO (as increased MDA levels) in the liver and kidney of both species, and in the gills of whitefish only. Initial elevations in protein carbonyl levels were observed in the kidney of whitefish, and, to a lesser degree, in the gills of rainbow trout.

(2) Recovery and resulting acclimation would be characterized by a re-establishment of plasma levels, as well as an increased activity of the fish's antioxidant defense (as catalase) to help in repairing any cellular damage.

This was observed in both species, as characterized by a re-establishment of plasma ions to basal levels and increased hepatic and renal CAT activity.

(3) The most prominent (and initial) oxidative damage would occur in the gills corresponding to high accumulation in the damage phase, whereas the liver (typically associated with a role in detoxification) would be responsible for the majority of defense (or repair) response.

This was observed in both species.

(4) The tissues of rainbow trout will experience a more significant elevation in lipid peroxidation initially in comparison to lake whitefish, the less sensitive of the two species to acute waterborne Cd.

This was not observed. The most significant difference in LPO was in the gills, with elevations in gill MDA found initially in the lake whitefish, and on the last day of exposure for the rainbow trout.

Table 4.1 Differences in the effects of waterborne Cd exposure in rainbow trout and lake whitefish exposed to 0.8 or 2.0 μg Cd/L over one month

Measured Parameter		Rainbow trout (*O. mykiss*)	Lake Whitefish (*C. clupeaformis*)
Mortality		9% and 20% in low and high exposures	0% in either exposure
Plasma ionic levels		$\downarrow Ca^{2+}$ at either dose, Sustained at 2.0 μg Cd/L Transient $\downarrow Na^{+}$	Transient $\downarrow Ca^{2+}$ at 2.0 μg Cd/L
Tissue Cd burden at day 30		K > G > L	G > K > L
Gill	Lipid peroxidation	Long-term	Transient
	Protein carbonyl levels	Transient $\downarrow$ at low dose	None
	CAT activity	No change at high dose, Inhibition at low dose	No change
Kidney	CAT activity	Transient ↑	Sustained ↑

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