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by

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ABSTRACT

The ability of a metal to cause toxicity in aquatic environments is highly dependant upon the water chemistry, which can influence metal bioavailability. Metal bioavailability is affected by the concentration of the metal, complexation of the metal by organic and inorganic ligands, and by the speciation of the metal. Predictive models of metal toxicity, such as the biotic ligand model (BLM) have mainly focused upon predicting the toxicity of individual metals rather than the toxicity of metals in mixtures, which are more commonly found in contaminated waters. Two metals that are commonly found together are Pb and Cd which can enter aquatic environments through anthropogenic sources such as mining and smelting operations, or naturally through the weathering of rock. The purpose of this research was to determine how low levels of Pb and Cd (25-2400 $nmolL^{-1}$ and 6-24 $nmolL^{-1}$ respectively) interacted with one another and the gills of rainbow trout (*Oncorhynchus mykiss*). Specific goals were to determine how acute (3-10 days) exposure influenced metal-gill binding and gill function, blood electrolytes (Na^+, Ca^{2+}, Cl^-), acid-base balance and survival in these fish. These interactions were studied using repetitive blood sampling, unidirectional Na^+ flux techniques, and gill Na^+/K^+-ATPase activity. Studies using a toxic unit approach (1TU of exposure = LC50) were also used to determine if it was possible to predict the toxicity of Pb plus Cd mixtures. The cannulation studies suggested that Pb plus Cd mixtures resulted in greater than additive reductions in plasma Ca^{2+} and Na^+ concentrations, but the decreases in plasma Na^+ were corrected within 5 d. Measurements of unidirectional Na^+ fluxes using the radiotracer $^{24}Na^+$ revealed that the decreases in Na^+ uptake caused by Pb or Cd alone at low levels were temporary and not observed during exposure to Pb plus Cd

mixtures. This seemingly protective effect of Pb against Na^+ loss was explained by competition between Pb and Cd for binding sites at the gill and accompanying increases in Na^+/K^+-ATPase activity. To conclude, low levels of Pb plus Cd can cause greater than additive reductions to internal Ca^{2+} balance, but trout are able to acclimate to these low concentrations. At higher concentrations, Pb appears to be protective against Cd toxicity. These findings underscore the need to better understand the mechanisms of Pb and Cd uptake by the gill alone and in mixtures, and when establishing water quality guidelines for these metals and for site specific risk assessments.

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Chapter 1

Background on the Toxicity of Pb and Cd and the Development of the BLM

Background

Due to the contamination of aquatic ecosystems by metals, considerable effort has been invested in the development of water quality criteria and guidelines over the last 30 years (reviewed in Paquin *et al.*, 2002). Furthermore, the evolution of the science behind creating ambient water quality criteria (AWQC) and guidelines has become more precise and considerate of site specific characteristics. Historically, AWQC were developed solely from total metal concentrations in aquatic ecosystems, but this approach failed to address the importance of other water chemistry parameters (Niyogi and Wood, 2004). However, many advances have been made about the critical importance that water chemistry parameters such as hardness, pH and dissolved organic carbon (DOC) play in determining the bioavailability of the free (charged) metal ion, which is considered to be the most toxic form in aquatic systems. Moreover, the development of models such as the Biotic Ligand model (BLM) consider the mechanism of toxicity for specific metals based on the specific affinity and interactions at the site of toxic action (i.e. the biotic ligand) (Playle, 1998; Di Toro *et al.* 2001). Currently, BLMs developed for individual metals such as copper and cadmium are being considered in the development of AWQC. However, there is no such developed BLM that accounts for metal mixtures such as Pb and Cd, which are more likely to be co-released into contaminated waters.

Both lead (Pb) and cadmium (Cd) are non-essential metals that are introduced into aquatic ecosystems through both natural and anthropogenic sources. The majority of anthropogenic inputs originate from activities that include the combustion of coal, mining and smelting operations, and through the disposal of electronic equipment and accessories (e.g. batteries, circuit boards) (Sorensen 1991; Zabel; 1993). Both Pb and Cd

are released into waters naturally as a result of volcanic activity (World Health Organization, 1995; Zabel, 1993), but Cd is also released through the natural weathering of rock (World Health Organization, 1992; Klassen *et al.* 2002). The levels of Cd and Pb in the Great Lakes remain relatively low ranging from 3.2-11 ngL^{-1} (0.01 – 0.05 $nmolL^{-1}$) and 2.8 – 4.5 ngL^{-1} (0.025 – 0.04 $nmolL^{-1}$; Nriagu *et al.* 1996), respectively. However, many contaminated lakes in North America and parts of Scandinavia have Pb and Cd concentrations that are substantially higher (0-250$nmolL^{-1}$ Pb and 10-50$nmolL^{-1}$ Cd) (Campbell *et al.* 1985; Reader *et al.* 1989; USEPA, 1980; 2001). The ecotoxicological effects that such metal contamination has on aquatic organisms have been elucidated in fish exposed to both Pb and Cd individually (e.g. Hodson *et al.* 1982; Rogers *et al.* 2003, 2004; Chowdhury *et al.* 2004). However, fish and other aquatic organisms are more likely to be exposed to mixtures of metals, rather individual metals, in metal contaminated waters (Grippo and Dunson, 1996; Norwood *et al.* 2003).

Effects of Water Chemistry on Metal Bioavailability

The ability of a metal to cause toxicity in aquatic environments is highly dependent on the water chemistry, which can influence metal bioavailability. Metal bioavailability is affected by the concentration of the metal, complexation of the metal by organic and inorganic ligands, and by the speciation of the metal (Playle 1998; Niyogi and Wood 2004). Factors such as water hardness, ionic strength, pH, temperature and dissolved organic carbon (DOC) all influence metal bioavailability and toxicity. High concentrations of ions such as Ca^{2+}, Na^{+} and Mg^{2+} can decrease the toxicity of certain metals due to competition for binding sites on the gill (Figure 1.1). By influencing metal solubility and speciation, changes in water pH and temperature may increase or decrease

metal bioavailability and toxicity (Grosell *et al* 2006). By complexing metals, both inorganic matter and DOC can bind metals in the water column, making them less available for uptake by aquatic organisms (Figure 1.1; Paquin *et al.* 2000; Playle 1998).

The effects that water chemistry has on metal speciation and bioavailability are considered by the Free Ion Activity Model (FIAM), Gill Surface Interaction Model and most recently the Biotic Ligand Model (BLM; Pagenkopf, 1983; Paquin *et al.* 2003). Along with previously established metal bioavailability models, the BLM is a tool used in ecological risk assessment (ERA) and in the development of water quality criteria for metals in some jurisdictions. Presently, Canadian ambient water quality guidelines for metals are based on water-hardness calculations that fail to consider how metal mixtures or different sources of natural organic matter (NOM) affect aquatic biota (Norwood *et al.* 2003; Niyogi and Wood, 2004; Playle, 2004). The BLM uses metal-gill stability constants to predict how water metal concentrations, complexation with organic and inorganic substances, and competition with similarly charged ions, affects metal-gill binding to biological membranes such as the epithelium or gills (the biotic ligands) of aquatic animals (Playle, 1993; Paquin *et al.* 2003). The biotic ligand is a site(s) within an organism that readily binds metals, and toxicity is considered to be directly proportional to the amount of metal-gill binding (Playle, 2004). In BLM models it is assumed that the amount of metal bound to a biologically sensitive membrane (fish gills) can accurately determine the metal's acute toxicity (Playle, 2004).

Concentrations of different metals within the water column do not necessarily have to be equal to induce similar toxicological effects on fish. This is because each metal has a specific conditional binding co-efficient to the gill, a constant which

measures the strength (affinity) to which different metals binds to a particular ligand (i.e. Ca^{2+} channels on the gill). The binding co-efficient for the metal may reflect a metal's relative toxicity compared to other metals (Playle, 2004). For instance, the greater binding co-efficient of Cd ($LogK_{Cd\text{-}gill} = 7.3$; Hollis *et al.* 2000) compared to Pb ($LogK_{Pb\text{-}gill} = 7.05$; Birceanu *et al.* 2008) reflects the greater toxicity of Cd (Playle, 2004). The BLM therefore considers how the affinity of metals for specific ligands ultimately influences toxicity. The BLM is based on the assumption that the free metal ion (charged state) is the metal species that causes toxicity to fish, by binding to their gills. However, the bioavailability (the ability of metals to cause toxicity) of the free metal ion is affected by a variety of water chemistry parameters. Metals in their ionic state can form complexes with organic and inorganic substances, rendering them less available to bind to the gill therefore decreasing toxicity. Parameters such as pH and alkalinity can alter the speciation of metals within the water, therefore also affecting their bioavailability. In addition, metals compete for specific binding sites necessary for ionoregulation (i.e. specific Ca^{2+}, Na^{+} and Mg^{2+} channels) therefore higher concentrations of these ions within the water column can subsequently decrease the toxicity of metals (Niyogi and Wood, 2004).

Water hardness is particularly important in predicting metal toxicity in the soft, low pH (5.5-6.5) waters that are found in the Canadian Shield. These very soft waters have low conductivity (< 50 μS), Ca^{2+} and Mg^{2+} (< 1mmolL^{-1}), alkalinity (<50 μeqL^{-1}) and DOC (<3 mg C L^{-1}) (Neary *et al.* 1990; Spry and Weiner, 1991; Flik and Verbost, 1993). Soft, acidic waters are thought to be more prone to metal contamination because for a given concentration of total metal (dissolved plus undissolved), bioavailability is

greater compared to harder, circumneutral pH waters (Campbell and Stokes, 1985; as reviewed in Spry and Weiner, 1991). Metal toxicity in fishes is also thought to be higher in soft water due to greater electrochemical gradients across the gills which favour Ca^{2+} loss in water with low environmental calcium. The diffusive loss divalent ions (e.g. Ca^{2+}) is thought to be inversely proportional to external Ca^{2+} concentration (Rodgers and Beamish, 1983), necessitating greater uptake of calcium to maintain ionic homeostasis (Perry and Wood, 1985; as reviewed in Evans *et al.* 2005), however it also increases the amount of metal that crosses into the gill (Rodgers and Beamish, 1983; Spry and Weiner, 1991).

Predicting the Toxicity of Metal Mixtures

The BLM and other mathematical-based speciation programs (i.e. WHAM, MINTEQA2) have been developed to predict and model the toxicity of metals to aquatic organisms. However, the development of different BLMs and the majority of metals research have been conducted on individual metals and not metal mixtures. It is therefore imperative to better understand the complex interactions between different metals in aquatic ecosystems, as well as their impacts on the health of fish (Playle, 1998, 2004; Borgmann *et al.* 2008).

The combined effects of toxicants can generally be predicted using the addition effects model (Norwood *et al.* 2003; Playle 2004). Using this model, the toxicity of each individual component of the mixture is expressed as a toxic unit (TU). Any standard end-point (e.g. 96-h LC50, gill metal binding) can be used, but the end-point needs to be identical for all components of the mixture, and each component must have the same mechanism of action (Sprague, 1970; Norwood *et al.* 2003; Playle, 2004). Toxicity can

then be predicted by determining the total TUs to which an organism is exposed to in mixtures using the following equation:

$$\text{Total Toxic Response } (\sum TU) = TU_1 + TU_2 + TU_3 + ...TU_n \quad (1)$$

Where $\sum TU$ is the predicted total toxic response and TU_n represent the toxicity of each component of the mixture expressed in toxic units. The interactions can be either strictly additive, in which $\sum TU$ equals the sum of the toxic responses for all the components in the mixture; less than additive (antagonistic), where the $\sum TU$ is less than that of sum of the effects of all components, or greater than additive (synergistic) where the $\sum TU$ is greater than the sum of all the individual components added together (Playle, 2004; Newman and Unger, 2002).

Playle (2004) modeled the combined effects of Pb plus Cd at the gill, and predicted that the two would interact in a less than additive manner due to competition for the same binding sites (Ca^{2+} channels) on the gills. However, Birceanu *et al* (2008) measured actual Pb- and Cd-gill binding during 3 h exposures to Pb (0-125 $nmolL^{-1}$) plus Cd (0-50$nmolL^{-1}$) mixtures , and observed that while metal-gill binding was less than additive, the effects on gill mediated Na^+ and Ca^{2+} uptake were greater than additive. It is not known, however, if such ionic disturbances persist during longer-term (several days) Pb plus Cd exposure and whether or not actual toxicity is exacerbated. These latter findings suggested that toxicity of Pb plus Cd could also be greater than additive. Winter (2008) examined metal-gill binding and toxicity in trout exposed to Pb plus Cd mixtures in the presence or absence of DOC, and also found that the interactions with the gills

were complex, making it difficult to predict toxicity. There is therefore a need to determine how combinations of Pb plus Cd affect the physiology and survival of fish when they are exposed to Pb plus Cd mixtures for longer periods. One goal of my thesis was to expose to trout to environmentally relevant concentrations of Pb plus Cd and monitor blood and whole body ion status, and rates of ion exchange across the gills in trout exposed to Pb plus Cd mixtures over several days.

The Gills: The Biotic Ligand in Fish

The gills of freshwater fish are a multi-functional organ that mediates not only gas exchange, but also nitrogenous waste excretion, acid-base balance and ionoregulation (Wilson and Laurent, 2002; Evans *et al.* 2005). Due to their dilute surroundings, freshwater fish continuously lose ions (e.g. Na^+, Cl^-, Ca^{2+}) across the gills down large blood-water electrochemical gradients, as well as via the urine. These ion losses are countered by gill-mediated ion uptake, which is carried out by specialized cells known as mitochondria rich cells (MRC) or chloride cells. The MRCs have extensive tubular networks and apical microvilli to increase the surface area for ion uptake, along with high densities of mitochondria to produce the ATP needed to fuel active transport of ions by the gill (Wilson and Laurent, 2002).

The MRCs are thought to mediate Na^+, Ca^{2+} and Cl^- uptake by primary and/or secondary active transport. The uptake of Na^+ via apical channels is coupled to H^+ extrusion via nearby proton pumps (H^+-ATPases), which along with basolateral Na^+/K^+ ATPase, generates the electrochemical gradients down which Na^+ moves (Figure 1.2). Similarly, Ca^{2+} uptake is by secondary active transport via apical Ca^{2+}channels (eCac - Shahsavarani *et al.* 2006) down an inward gradient that is generated by basolateral Ca^{2+}-

ATPases (Figure 1.2). Chloride-uptake is facilitated by apical Cl^-/HCO_3^- exchange (Figure 1.2); Wilson and Laurent, 2002). Because the apical membrane of the MRC is continually exposed to the ventilatory flow of water crossing the gills, it is also the target for many toxicants, including metals, which are found in contaminated aquatic systems.

Due to the similar valences and ionic radii of many metals to Na^+ and Ca^{2+}, the branchial uptake sites for these metals are particularly vulnerable. Both Pb^{2+} and Cd^{2+}, and other divalent metals (e.g. Zn^{2+}) are thought to interfere with Ca^{2+} uptake due to their +2 oxidation state (valence). Interference with Ca^{2+} uptake can be detrimental due to the important role that Ca^{2+} plays as a constituent of scales and bone, the requirement for Ca^{2+} to facilitate muscle twitches (contractions), and Ca^{2+}'s role as an important extracellular and intracellular chemical messenger for numerous processes critical to vertebrate life (e.g. neurotransmitter release). Sodium is a critical ion needed to maintain osmotic balance in the extracellular fluid, but it is also needed to drive the depolarization of nerve cells undergoing action potentials in the body. Metals such as Cu^+, Ag^+, interfere with Na^+ uptake by entering the gill via Na^+-specific channels and interacting with the basolateral Na^+/K^+-ATPase (Pelgrom *et al.* 1995; Richards *et al.* 1999; Schjolden *et al.* 2007). Recent research by Rogers *et al* (2004; 2005) suggests that Pb may also cause interference with Na^+ and Cl^- balance, despite its divalent charge.

Although the toxicity of metals was once thought to be solely due to respiratory impairment, recent research has proven that the competition between metals and ions inhibits gill-mediated ion transport mechanisms (reviewed in Paquin *et al.* 2002). The physiological and toxic effects of individual metals have also been well documented, but the mechanisms of how different metal mixture combinations exert their toxic effects

have not been studied in detail. Two metals that are commonly found together in contaminated waters are Pb and Cd. Because each metal has a similar hydrated radius to Ca^{2+}, they are thought to compete with Ca^{2+} for apical binding sites on the gills. Cadmium has been demonstrated to interfere with Ca^{2+} uptake via the branchial Ca^{2+}-channels found on the gill MRCs (Verbost *et al.* 1987). Cadmium also inhibits the movement of calcium through direct inhibition of basolateral Ca^{2+}-ATPases, which also reduces the electrochemical gradient across the apical membrane of the MRCs (Verbost *et al.* 1989).

Lead is also thought to compete with Ca^{2+} ions at calcium channels on the apical membrane of the fish gill (Rogers and Wood 2004). However, Pb^{2+} is also believed to affect Na^+ and Cl^- homeostasis due to the disruption of Na^+- K^+-ATPase activity (Figure 1.2; Rogers *et al.* 2003; 2005). Although Pb significantly reduces the influx of Na^+ across the gill, Na^+ channel blockers (Bafilomycin) caused no decline in Pb binding to the gill (Rogers *et al.* 2005). However, Pb inhibits cytosolic carbonic anhydrase, which provides H^+ and HCO_3^- from the hydration of CO_2 for the Na^+-H^+ transport system and Cl^-/HCO_3^- exchange at the apical membrane of the gill. As a result, Pb causes reductions in both Cl^- and Na^+ influx (Figure 1.2; Morgan *et al.* 2004, Rogers *et al.* 2005, Wilson *et al.* 2000).

Although detailed research has been conducted on the effects of Pb and Cd on fish exposed to these metals individually (Rogers *et al.* 2003; 2005; Grosell *et al.* 2006; Verbost, 1987; 1989; Niyogi *et al.* 2004; Niyogi and Wood, 2004; Playle, 1993), metal mixtures have not been as thoroughly studied (Winter, 2009; Birceanu *et al.* 2008). In addition, many earlier studies conducted used high metal concentrations that are less likely to occur in natural environments (e.g. Rogers *et al*, 2004, Chowdhury *et al*, 2004).

Here, rainbow trout were exposed to mixtures of Pb and Cd, at more environmentally relevant concentrations (Pb <500nmol L^{-1} and Cd <100nmol L^{-1}) in soft water, to determine how these metal mixtures influence gill-metal accumulation, ionoregulation and toxicity in these fish.

Objectives - Effects of Pb plus Cd mixtures on Fish

The objectives of my thesis were to test the following hypotheses that mixtures of Pb plus Cd would cause:

1. Greater than additive decreases in plasma and whole body ions (i.e. Ca^{2+} and Na^{+}) due to impaired ionoregulation at the gill (Chapter 2).
2. Persistent decreases of Na^{+} uptake at the gill, that explain Pb and Cd induced ionic disturbances during longer term metal exposure (Chapter 3).
3. Substantial metal-gill accumulation after 3h and that this accumulation would be predictive of longer term toxicity (Chapter 4).

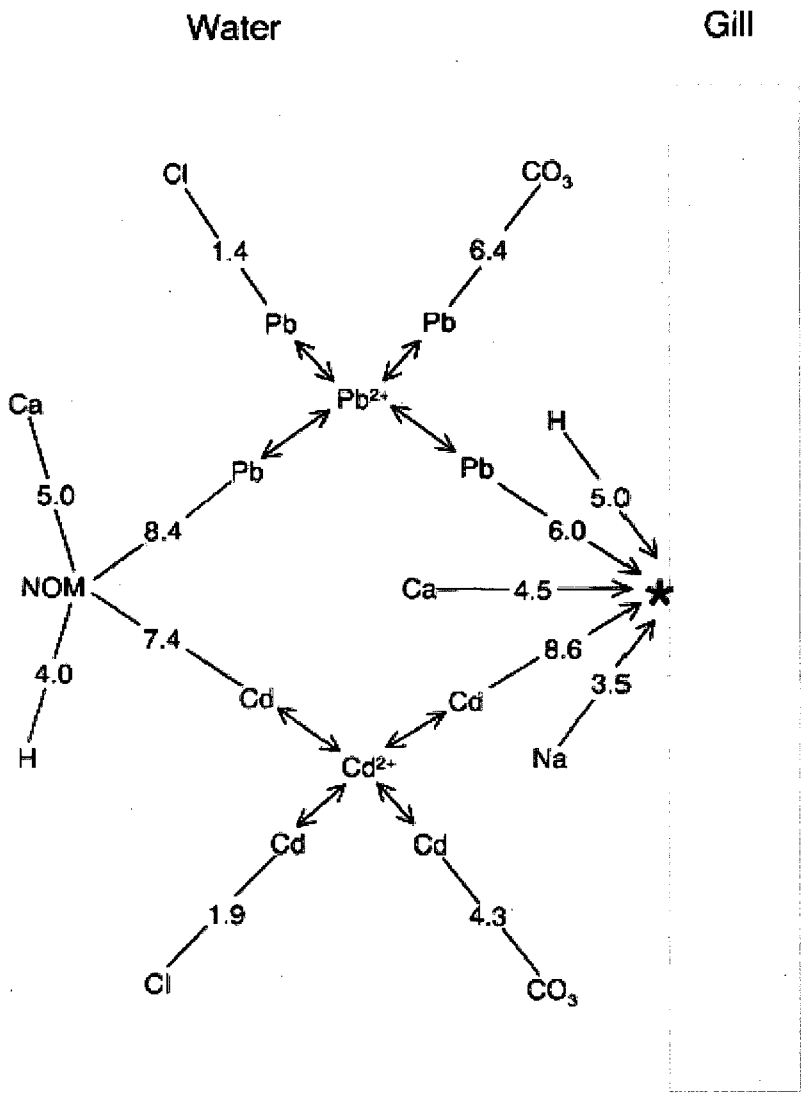
Short-term (3 h) exposure of trout to Pb and Cd mixtures inhibits Na^{+} and Ca^{2+} influx in a greater than additive manner (Birceanu *et al.* 2008). Because it is not clear if such disturbances persist during longer-term Pb plus Cd exposure, it was predicted that the total body ion pool would be decreased in trout exposed to these metals. To test this hypothesis, rainbow trout were exposed to nominal concentrations of 250nmol L^{-1} and 125nmol L^{-1} Pb in combination with 10nmolL^{-1} and 50nmolL^{-1} Cd (along with Pb only, Cd only and control fish) (Objective 1). Fish were then sampled at regular intervals to measure the concentrations of total body ions after various exposure times (1, 3 and 5 days). Whole body digests were then completed and the ions (Ca^{2+}, Na^{+}, Cl^{-} and K^{+})

quantified. Fish were also fitted with surgically implanted dorsal aorta cannulas to permit for repetitive measurement of plasma ion concentrations and osmolality as an index of metal-induced ionic or osmotic disturbances. Haemoglobin and haematocrit was determined to further define any ionic or osmotic disturbances that resulted from Pb plus Cd exposure. Measurements of acid-base regulation such as the arterial partial pressure of O_2 (Pa_{O2}), arterial pH (pHa) and blood lactate were also made. Although Pb and Cd are not known to impair gas exchange, these measurements were necessary to determine if gas exchange or acid-base disturbances also contributed to toxicity during longer-term Pb plus Cd exposure. Many toxicants including metals (i.e. Pb), are known to stimulate increased mucous production as a protective measure, which could subsequently compromise gas exchange and therefore acid-base balance by impairing CO_2 excretion or O_2 uptake (Sorensen, 1991).

To test the hypothesis that Pb plus Cd exposure would result in greater than additive disturbances to gill mediated Na^+ uptake and to determine if this disturbance can be corrected (Objective 2), the radiotracer $^{24}Na^+$ was used to track the unidirectional (influx, efflux, net flux) movements of Na^+ movements across the gills of trout exposed to metal mixtures for 72 h. Gill tissue was also sampled to relate gill metal binding directly to changes in Na^+ movements, and branchial Na^+/K^+-ATPase activity was measured to determine if this basolateral transporter was also a target of Pb and/or Cd at the low concentrations of metals that were used.

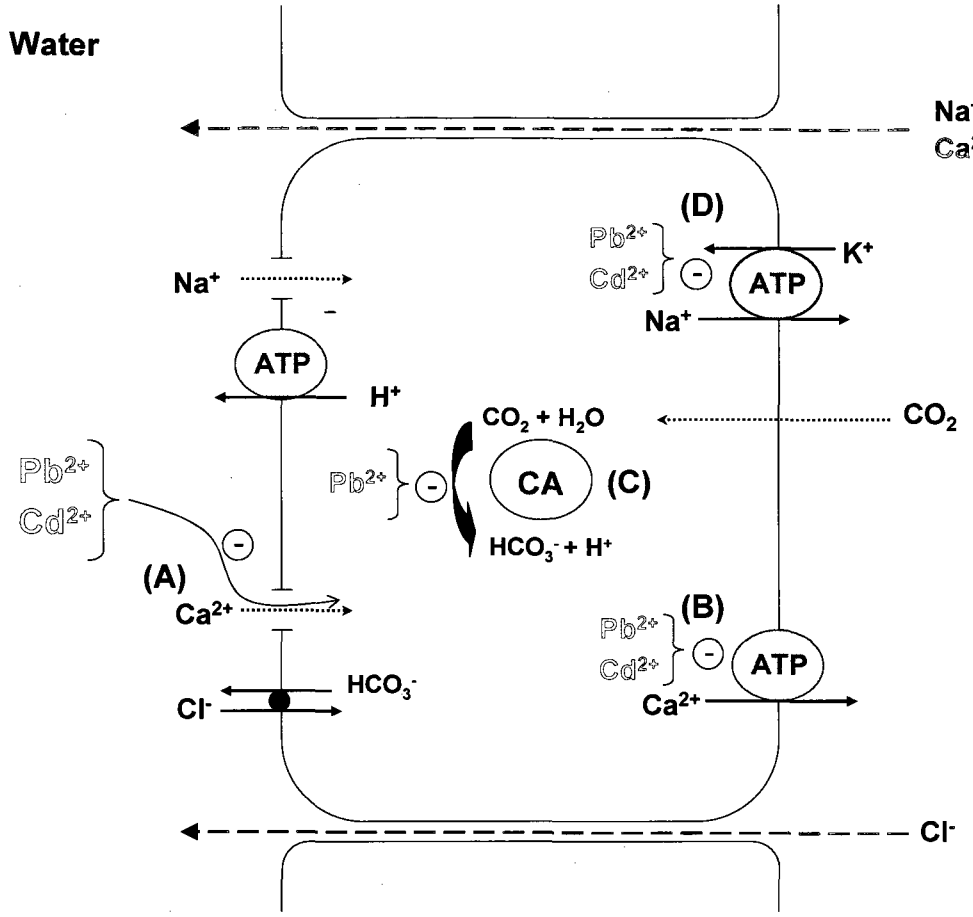
Finally, to determine if gill-metal accumulation was predictive of toxicity, interactions of Pb and Cd at the gills were modeled using methods previously developed to predict and describe the toxicity of metals in mixtures as it relates to metal-gill

accumulation (Objective 3) (Playle, 2004; Norwood *et al* 2004; Borgmann *et al* 2008). Assuming concentration addition, a toxic unit approach was used to complete 3 h gill accumulation exposures and subsequent 10 d LT50 exposures. This was completed to relate 3 h gill accumulation during Pb plus Cd exposures to the 10 d LT50.



(Adapted from Playle 2004)

Figure 1.1 The Pb and Cd gill-binding model depicting the competition for the same binding site (i.e. apical Ca^{2+} channels) on the biotic ligand (i.e. fish gill) and the respective affinities (binding strength; LogK) of each metal for the ligand.



Adapted from Wood, (1992) and Marshall, (2002)

Figure 1.2 Mechanisms of toxic action for Pb and Cd. Dashed lines indicate passive mediated ion diffusion and solid lines indicate carrier mediated processes. A ⊖ indicates a possible target for Pb and/or Cd. (A) Pb and Cd are both believed to enter voltage-independant calcium channels on the apical membrane. (B) Pb and Cd both inhibit Ca^{2+} - ATPase on the basolateral membrane, thus reducing influx of Ca^{2+}. (C) Pb may also have inhibitory effects on carbonic anhydrase which hydrates CO_2 to H^+ and HCO_3^-. By decreasing H^+ and HCO_3^- generation via carbonic anhydrase (CA), Pb is thought to reduce Na^+ and Cl^- influx via the Na^+-H^+ transport system and Cl^-- HCO_3^- exchanger respectively. (D) Pb also disrupts Na^+/K^+-ATPase activity thus decreasing the Na^+ influx due to a change in the electrochemical gradient of Na^+. See text for further details.

Chapter 2

Greater than Additive Effects of Pb plus Cd Mixtures in Rainbow Trout: Effects on Blood & Tissue Ions, Osmotic and Acid-base Balance

ABSTRACT

The physiological and toxicological effects of lead and cadmium alone have been studied in depth in fishes, but at relatively high, non-environmentally relevant concentrations. Little is known about how mixtures of these metals in soft (< 100μmolL^{-1} Ca^{2+}) exert their toxic effects in fish living in slightly acidic (pH ~ 6) waters typical of lakes in the Canadian Shield. Pb and Cd individually, are thought to disrupt homeostasis by inhibiting ionic regulation leading to hypocalcaemia and eventual death. Birceanu *et al*, (2008) suggested that exposure to Pb and Cd mixtures (3 h) caused greater than additive reductions of internal Ca^{2+} and Na^{+} balance. To assess the physiological and toxicological effects of Pb and Cd mixtures over longer time periods (5 days) fish were exposed to Cd alone, Pb alone and a Cd plus Pb mixture. Accordingly, measurements of blood gases and acid-base regulation (PaO_2, pH_a), haematology (Ht, Hb, MCHC, and Protein) and ionic composition (whole body ions and plasma Ca^{2+}, Na^{+}, Cl^{-}, osmolality) were made in trout fitted with dorsal aortic catheters. There were no effects on whole body ions after 5 days of exposure, but there was a greater than additive reductions in plasma Ca^{2+}, Na^{+} and osmolality in the Cd plus Pb mixture compared to Cd or Pb alone. Osmolality and plasma Na^{+} appeared to recover by 120 h to control levels. Further investigation on the mechanisms of these effects is needed to fully understand the interactions between Pb and Cd at the gill.

INTRODUCTION

The effects of individual metals on fish physiology and toxicity have been thoroughly researched (Verbost *et al.* 1987; Rogers *et al.* 2003; 2004; MacDonald *et al.* 2002; Niyogi *et al.* 2008), but metal mixtures are usually more common in the natural environment and in contaminated waters (Nriagu and Pacyna, 1988; Sayer *et al.* 1991). Two metals that are co-released into the environment are Pb and Cd, which may be present in effluents arising from mining and smelting operations, coal combustion, and the disposal of electronic wastes (Nordic Council of Ministers, 2003; Nriagu and Pacyna, 1988). Both Pb and Cd are calcium analogs which compete with Ca^{2+} for binding sites on voltage independent calcium channels, which are found on the apical membranes of fish gill mitochondria rich cells and possibly pavement cells (Shahsavarani *et al.* 2006). This competitive binding is thought to cause ionoregulatory disturbances characterized by decreased rates of Ca^{2+} influx, leading to net Ca^{2+} losses that may eventually contribute to fish death (Verbost *et al.* 1989; Birceanu *et al.* 2008). Many metals such as Pb, Cd and Zn are more toxic in Ca^{2+}-poor soft water than in Ca^{2+}-rich hard waters due to decreased competition with Ca^{2+} ions for binding sites on the gill (Hollis *et al.* 2000; Niyogi *et al.* 2003; Birceanu *et al.* 2008). There remains a need, however, to learn more about the effects of metals and metal-mixtures on aquatic organisms that live in the slightly acidic, ion-poor waters that are found in the Canadian Shield (Campbell and Stokes, 1985). The goal of this study was to therefore learn how Pb and Cd mixtures interacted with the fish gill and caused toxicity in low pH (~6.0), soft (Ca^{2+} < 100μM) waters that have similar composition to those of vulnerable Canadian Shield waters.

The toxicity of metals can be predicted using models such as the Biotic Ligand Model (BLM), which determines how water chemistry influences the bioavailability of free metal ions (e.g. Cd^{2+}, Pb^{2+}). Differences in water chemistry can influence metal speciation, but also the availability of other cations (e.g. H^{+}, Ca^{2+}, Na^{+}) which can compete with metals for binding sites on the gill (e.g. the biotic ligand). The BLM also considers metal-complexation by inorganic and organic matter in the water column (see Playle 1998; Paquin *et al.* 2002; Niyogi and Wood 2004 for reviews). The BLM's utility in predicting the toxicity of single metals (e.g. Cu, Cd, Zn) to aquatic organisms is well established, but it is unclear if the BLM can be used to effectively predict the toxicity to metals in mixtures (Playle, 2004). Moreover, the BLM is based on the metal binding to the biotic ligand during short-term exposures, before any subsequent physiological changes or acclimation take place at the biotic ligand or in the blood, tissues or organs of target organisms (Niyogi and Wood, 2004).

By competing with cations for binding sites at the gill, the basis of the BLM is that metal binding at the gill will result in reduced rates of ion uptake (e.g. Ca^{2+}, Na^{+}), culminating in internal ionic and osmotic disturbances that contribute to toxicity (Playle 2004). Because Pb and Cd compete with each other for access to Ca^{2+}-binding sites on the gill, it was predicted that the combined effects on ion uptake by the gill would exacerbate internal ionic disturbances and toxicity in trout.

The interactions of multiple toxicants such as metals at the site of toxic action (i.e. biotic ligand) can be described as strictly additive, less than additive and greater than additive, relative to the effects observed during exposure to each constituent toxicant alone (Newman and Unger, 2000). These descriptions typically use the classical toxic

unit approach in modeling the toxic effects of each constituent in a mixture (Newman and Unger, 2000; Playle, 2004). Using the toxic unit approach for metals, it is assumed that the amount of each constituent metal bound to the biotic ligand determines acute toxicity (Playle, 2004). Toxic units (TU) can be classified as a value of toxicity (i.e. 96 h LC50) that is unique to a particular metal. When fish are exposed to 1 TU it is assumed that after 3 h that 50% of the gill sites for that metal will be filled thus killing 50% of the exposed group (Paquin *et al*, 2002). The concentration addition model uses the assumption that for metals in combination, any combination of concentrations (i.e. ½ TU + ½ TU) will cause the same toxicity. Moreover, the effects of metal mixtures can behave in a strictly additive fashion when the toxic effects of the metal mixture equal the sum of effects caused by the metals alone (see example above), less than additive when the sum of the effects seen in mixtures is less than the sum of the individuals; or greater than additive where the sum of the effects seen in mixtures are greater than the sum of the individual metal exposures. However, whether the assumptions of gill accumulation and toxicity of Pb and Cd can be predicted this way is uncertain. Moreover, it is not clear if the toxicity predicted by the concentration addition model can be predictive of internal (non-lethal) ionoregulatory disruption which is a more sensitive measure of toxicity especially in lower, more environmentally relevant concentrations found in natural aquatic ecosystems (Birceanu *et al.* 2008).

Birceanu *et al.* (2008) demonstrated that short-term (3 h) exposure of trout to Pb plus Cd metal mixtures resulted in greater than additive inhibition of Na^+ and Ca^{2+} uptake, but it was not clear if such disturbances lead to greater toxicity if the fish were exposed to Pb-Cd mixtures over longer periods (days). A key objective of this research

was to test the hypothesis that longer periods of Pb plus Cd mixture exposure resulted in greater disturbances to internal ionic and osmotic balance, leading to greater toxicity in fish. To test this hypothesis, rainbow trout were fitted with chronic, indwelling dorsal aortic catheters to allow for the collection of blood samples at regular intervals during longer-term (5 d) exposure to Pb plus Cd mixtures at concentrations closer to those that would be found in actual contaminated waters (0-250nmolL^{-1} Pb and 10-50nmolL^{-1} Cd; Beamish and Van Loon, 1977; Reader *et al.* 1989; Nriagu and Pacyna, 1988, USEPA, 2001). Whole body and plasma ions, hematological parameters (haematocrit and hemoglobin) and osmolality were measured to characterize any internal disturbances to internal electrolyte or osmotic balance. Arterial P_{O2} (Pa_{O2}), pH (pHa) and blood lactate were measured to determine if the combined effects of Pb plus Cd exposure interfered with gas exchange or metabolic pathways.

MATERIAL AND METHODS

Experimental Animals and Holding

Rainbow trout (*Oncorhynchus mykiss*) were purchased from Rainbow Springs Trout Farm, Thamesford, ON. Fingerling trout (3.2 ± 0.1g; n= 175) were used to quantify how whole body ion concentrations changed during Pb plus Cd exposure, and larger trout (236.9 ± 9 g; n = 85) were used for repetitive blood-sampling experiments. All fish were held in 180 L tanks supplied with well-aerated flowing water. Initially, the fish were held in 1:1 mixture of hard well water and soft water produced by reverse osmosis, resulting in a water composition of ~ 1.6mmolL^{-1} Ca^{2+}, ~500μmolL^{-1} Na^{+}, ~500 μmolL^{-1} Cl^{-}, conductivity of 400μS, at 9-11°C. The fish were gradually acclimated to softer water over one week by increasing the ratio of soft water to well water resulting in water with the following chemical composition: ~100-200 μmolL^{-1} Ca^{2+}, Na^{+} ~ 200 μmolL^{-1}, pH ~ 6 and 8-14°C. The fish were left in this water for a minimum of 2 weeks prior to experiments. Fish were fed commercial trout feed (Corey Mills Aquafeeds, Fredericton, New Brunswick) 3-times per week until satiation, but were fasted 72 h prior to experiments. All fish husbandry procedures and experiments were approved by the Wilfrid Laurier Animal Care Committee and followed the Canadian Council of Animal Care (CCAC) guidelines.

Experimental Protocols

Series I: Whole Body Ion Experiments

To test the hypothesis that exposure to Cd plus Pb mixtures caused greater than additive reductions to whole body ions, rainbow trout were exposed to a matrix of Cd

plus Pb concentrations (0-250 $nmolL^{-1}$ Pb and 0-50 $nmolL^{-1}$ Cd) and surviving fish were terminally sampled at 24, 72 and 120 h of exposure for later measurement of whole body ions. The fingerling trout were randomly distributed to experimental polyethylene buckets containing 10L of ion-poor, slightly acidic water (Ca^{2+} = 90.0 ± 0.3 $\mu molL^{-1}$, Na^{+} = 137.3 ± 7.2 $\mu molL^{-1}$, Cl^{-} = 61.4 ± 3.4 $\mu molL^{-1}$, pH = 6.0 ± 0.0, temperature =10-11 °C) and left to acclimate to these conditions for 12-24 h prior to the addition of metals. Separate groups of fish were subsequently exposed to Cd and/or Pb at nominal concentrations of 0, 10, 50 $nmolL^{-1}$ Cd and 0, 125, 250 $nmolL^{-1}$ Pb for 5 d (120 h) in a static system. Water pH was monitored every 3 h for the first 24 h, and every 24 h thereafter, to minimize increases in water pH that would result in the formation of $PbCO_3$ (cerrusite), and subsequent reductions in Pb^{2+} bioavailability. Surviving fish (target n = 8) were sampled at 24, 72, and 120 h, after being killed by a blow to the head, wrapped in aluminum foil, frozen in liquid nitrogen, and stored at -80 °C until processed for whole body ion analysis.

Water samples (7mL) were taken at the beginning of each experiment and at 24 h intervals thereafter. An additional water sample (7mL) was filtered by passing it through a 45μm Supor® membrane (Pall Life Sciences, Ann Arbor, MI) to obtain water samples for measurements of dissolved metal concentration. Filtered and unfiltered samples were acidified to 1% using 16N trace metal grade HNO_3 (Trace Metal Grade; EMD Chemicals Inc, Germany) and stored until metal content was determined. Additional unacidified, unfiltered samples (7mL) were used to measure water Cl^{-} and were stored in borosilicate scintillation vials with no head space for measurement of dissolved organic carbon (DOC).

Series II: Effects of Longer-Term Pb plus Cd Exposure on Blood Chemistry

Longer-term (5 d) exposures of larger trout to Pb plus Cd mixtures place in a re-circulating system comprised of an over-head tank (head tank) which drained via a flow splitter into individual fish-holding containers that were set-up within a large (32" x 57") PVC tray. Water leaving the containers drained into the tray and then into a lower reservoir from which the water was pumped via submersible pump back-up to the head tank. Water pH was maintained using a PHM82 pH meter (Radiometer-Copenhagen, Copenhagen, Denmark), connected to an autotitrator (TTT80; Radiometer-Copenhagen, Copenhagen) which was activated when pH exceeded a set-point of 6.3. Activation of the titrator led to the drop-wise addition of nitric acid (0.125 N HNO_3) via a solenoid valve (Cole Parmer Instrument Co., Quebec) into a well-aerated bucket contained within the head tank (e.g. Wilkie and Wood, 1991). Approximately 1/3 of the exposure water (~40 L) was replaced every 24 h to offset water loss due to evaporation, and to prevent nitrogenous wastes (ammonia) from accumulating. Water samples (7mL) were collected every 24 h for measurement of dissolved metals and ions throughout the exposure.

To determine if the 5 d exposure to Pb plus Cd mixtures disrupted plasma ion balance, hematology, blood oxygen or acid-base balance, rainbow trout (~200-500g) were fitted with surgically implanted dorsal aortic catheters. Prior to surgery, the fish were anaesthetized in buffered tricaine methane sulfonate (0.1 g L^{-1} buffered with 0.2 g L^{-1} $NaHCO_3$; Syndel Labs, Vancouver, BC), at which time the catheters (PE50 tubing) were implanted into the dorsal aorta of the fish according to Soivio and Nynolm (1975). The catheters were filled with heparanized Cortland's saline (Wolf, 1963) and the end of

the catheter was plugged with a needle. The fish were carefully placed into their individual PVC holding containers, and allowed to recover for 12-24 h, before initial control (pre-exposure) blood samples were collected.

Blood samples (500 μL) were collected under control conditions, and at regular intervals (pre-exposure; 8, 24, 48, 72, 96, 120 h) during exposure to control (metal-free) water, or water containing Cd alone (7.4 ± 0.3 $nmolL^{-1}$ dissolved), Pb alone (26.1 ± 1.6 $nmolL^{-1}$) or Cd plus Pb (6.9 ± 0.4 $nmolL^{-1}$ and 45.5 ± 1.9 $nmolL^{-1}$ respectively, dissolved) in the soft water ($[Ca^{2+}]$ ~ 100 $\mu molL^{-1}$) and maintained at pH 6 using the pH-stat set-up.

Blood Sampling

Blood samples (500μL) were collected from each fish before metal exposure (pre-exposure) or after 8, 24, 48, 72, 96, 120 h from the dorsal aorta catheter using a heparinized, ice cold, gas tight Hamilton syringe. Once the blood was sampled, arterial pH (pHa) and partial pressure of oxygen (Pa_{O2}) was measured using a Blood Gas Meter (Cameron Instrument Co. Texas, U.S.A). An aliquot of whole blood was also transferred into heparinized haematocrit tubes and centrifuged for five minutes at 10,000 x g for haematocrit determination. A drop of plasma from the haematocrit tubes was used to determine total plasma protein using a clinical refractometer (Sper Scientific). Whole blood was added to Drabkins reagent (20 μL in 5mL Drabkins; Sigma Aldrich) for later determination of hemoglobin concentration, and to 8% perchloric acid (25 μL in 50 μL acid) and stored at -80°C for later measurement of blood lactate. The remaining whole blood (~400μL) was centrifuged for 3 min at 10,000 x g, and the plasma frozen, and

saved at -80 °C for later determination of plasma Ca^{2+}, Na^{+}, and Cl^{-}. The remaining red blood cell pellet was then gently re-suspended (by flicking the bottom of the centrifuge tube lightly) in Cortland's saline (unheparinized) back-up to a volume of approximately 500μL and re-injected back into the fish via the dorsal aortic catheter to minimize reductions in plasma haematocrit and haemoglobin that could take place as a result of the repetitive blood sampling procedure. The cannula was then re-filled with heparinized saline.

At the end of the exposure (120 h) the surviving fish were killed by overdose with tricaine methane sulfonate ($0.5gL^{-1}$ buffered with $1gL^{-1}$ $NaHCO_3$) and the first gill arch on the right hand side of the fish was removed, rinsed for ten seconds in de-ionized water, snap-frozen in liquid N_2, and stored at -80 °C for later determination of gill-metal concentration.

Analytical Techniques

Tissue Processing

Whole fish from series I experiments were thawed, weighed and placed in 10 mL centrifuge tubes containing 1N HNO_3 at 5 times the fish's mass, and then digested at 60°C for 48-72 h. The resulting homogenate was then vortexed and divided into 1.5 mL aliquots that were centrifuged at 10,000 x g for 2 min. The supernatant was then diluted 600 times using E-Pure deionized water and then acidified to 1% HNO_3. The Na^{+} concentrations in the supernatants were subsequently determined by flame atomic absorption (FAA) spectrophotometry (Spectra 880 Atomic Absorption, Varian, Mississauga, ON.). The concentrations of Ca^{2+} and K^{+} were also determined by flame

atomic absorption spectrophotometry (AAS), after first diluting the samples with 1% $LaCl_3$ (Sigma-Aldrich) and 0.1% CsCl (Alfa Aesar, Ward Hill, MA) (Chowdhury, J. and Nudella, S., personal communication). Whole body chloride concentration was determined spectrophotometrically using the mercuric thiocyanate assay (Zall *et al.*, 1956).

Gills from series II experiments to be used for metal analysis were first thawed, weighed and digested in 5 times their weight in 1N Trace Metal Grade HNO_3, and then baked for 3 h at 80°C. The resulting digests were vortexed and centrifuged for 2 min at 12 000 rpm, and the supernatant collected and diluted ~10 times (as appropriate) using 1% HNO_3 (Trace Metal Grade; EMD Chemicals Inc, Germany) for subsequent Cd and Pb analysis.

Metal and Ion Analysis

Water and gill Cd and Pb were quantified using a graphite furnace (GTA100 atomizer, SpectrAA 880, N_2 gas; Varian, Mississauga, ON), and concentrations were validated using precision standards (TM28.3; Environment Canada). Total Ca^{2+} and Na^+ concentrations in the water were measured by flame AAS (see above), and water Cl^- concentration determined spectrophotometrically using the mercuric thiocyanate assay (Zall *et al.* 1956). Dissolved organic carbon was determined using a Shimadzu TOC 5050A Analyzer (Shimadzu Corporation, Kyoto, Japan).

Blood hemoglobin concentration was determined using the cyanomethaemoglobin method and sample absorbances were read on a plate spectrophotometer (SpectraMax 190, Molecular Devices, CA) at a wavelength of 540 nm. Whole blood lactate concentrations were measured enzymatically [lactate dehydrogenase (Sigma-Aldrich)] on

the supernatant of the PCA treated samples (Bergmeyer, 1983) using a plate spectrophotometer set to a wavelength of 340 nm. Plasma Na^+, Ca^{2+} were measured by FAA (see above), Cl^- was measured spectrophotometrically (see above) and plasma osmolality was measured using a Vapor Pressure Osmometer (Vapro®, Wescor Inc, Utah, U.S.A.).

Calculations and Statistical Analysis

All data is presented as the mean ± one Standard Error of the Mean (SEM) (N). Comparisons between control and experimental fish in Series I, experiments were analyzed using one-tailed t-test comparing each group with their respective control. In Series II experiments, in which the blood chemistry of the trout was followed over a 5 day exposure to Pb plus Cd mixtures, data where analyzed using one-way repeated-measures ANOVA. In instances where significant variation was observed, the ANOVA was followed by a Dunnett's post-test to determine if there were significant differences between the pre-exposure and Pb plus Cd mixture treatment. Unpaired *t*-tests were used to compare treatment means at $t = 0$ to the simultaneous control (non-exposed fish) means at $t = 0$. All significant differences were determined at the $P < 0.05$ level.

RESULTS

Series I: Effects of Cd plus Cd Mixtures on Whole Body Ion Balance

To determine if mixtures of Pb and Cd have an impact on whole body ions, rainbow trout were exposed to nominal Pb and Cd concentrations of 10 and 50 $nmolL^{-1}$ Cd, 125 and 250 $nmolL^{-1}$ Pb alone and in combination for 120 h. Although the measured Cd concentrations were comparable to nominal concentrations (Table 2.1), the measured Pb concentrations were 50-80 % lower than the nominal values, ranging from approximately 25-150 $nmolL^{-1}$ (Table 2.1). Regardless of the concentrations of Pb and/or Cd, however, there were few differences in whole body calcium, sodium, chloride and potassium measured after 24, 72 and 120 h of exposure compared to control fish held in metal-free water (Table 2.2). There was a significant decrease in Ca^{2+} after 24 h of exposure to10 $nmolL^{-1}$ of Cd only, but Ca^{2+} concentration rebounded and had returned to control levels by 5 d (Table 2.2). Whole body sodium increased after 24 h in most exposures (Table 2.2) but was not significantly different from controls at 120 h. Chloride decreased significantly after 72 h of exposure to125 $nmolL^{-1}$ Pb, but this trend was not seen in other treatments.

Despite the absence of major changes in the whole body ion concentrations, there were mortalities during exposure to Pb and Cd alone, and in mixtures. No mortality was observed in the control (non-exposed) fish, 67 % survival was observed in fish exposed to 50 $nmolL^{-1}$ Cd alone, and 96 % survival was observed in fish exposed to 10 $nmolL^{-1}$ Cd + 250 $nmolL^{-1}$ Pb. Survival was 41% in fish exposed to 50 $nmolL^{-1}$ Cd + 125 $nmolL^{-1}$ Pb, with slightly higher survival in the group of fish exposed to 50 $nmolL^{-1}$ Cd plus 250 $nmolL^{-1}$ Pb (66%) (data not shown).

Series II: Effect of Pb plus Cd Exposure upon Blood Ion, Osmotic, and Acid-base Balance

In these experiments, rainbow trout fitted with chronic, indwelling dorsal aortic catheters were exposed to nominal concentrations of 10 $nmolL^{-1}$ Cd alone, 50 $nmolL^{-1}$ Pb alone and a 10 $nmolL^{-1}$ Cd plus 50 $nmolL^{-1}$ Pb mixture. Measured Cd concentrations were more or less in agreement with the nominal values (Table 2.3), but the actual Pb concentrations were about 50 % lower in the fish exposed to a nominal concentration of 50$nmolL^{-1}$ (Table 2.3). However, in the fish exposed to mixtures of the metals, the concentrations of Cd and Pb were slightly less than the target values of 10 $nmolL^{-1}$ and 50 $nmolL^{-1}$, respectively (Table 2.3).

Metal Accumulation on the Gills

Metal-gill accumulation was measured from gills of fish that had survived to 120 h of each experiment (Figure 2.1). In fish not exposed to metals, background gill-Cd and gill-Pb remained low near 1.5 and 1.0 $nmolg^{-1}$ wet weight, respectively. There was substantial Pb-gill accumulation in fish exposed to Pb alone and Pb plus Cd mixture, which approached 25 nmol g^{-1} wet weight in both cases, but there was no difference in the gill-Pb accumulation between the two treatments (Figure 2.1). There was a significant increase in Cd-gill accumulation in the Cd only, and the Pb plus Cd exposed fish, but gill-Cd accumulation was lower (~40%) in the fish exposed to Pb plus Cd compared to Cd alone (Figure 2.1).

Mortality

Fish were considered to have died as a result of metal exposure provided there were no obvious mishaps with the cannula implanted in the dorsal aorta (i.e. pulled it out, major mishaps with blood re-injection etc.). Based on these criteria, control fish experienced 100% survival until 120 h, where one fish (out of twenty-four fish) died unexpectedly (Figure 2.2). However, only 48 % of the fish exposed to Cd alone survived for 120 h, compared to 85% that survived in the presence of Pb only. Survival was least, however, in the fish exposed to the Pb plus Cd mixture, in which only 16 % of the fish survived for the entire 120 h metal exposure period (Figure 2.2).

Gas Exchange and Acid-Base Balance

Exposure to Pb or Cd alone, or in combination, did not appear to cause any respiratory distress at the exposure concentrations used. Rainbow trout exposed to Cd alone, Pb alone, or the Cd plus Pb mixture had relatively stable Pa_{O2} values that fluctuated around 100 mm Hg (Figure 2.3), and pHa remained near pH 7.7 to 7.8 (Figure 2.4). Consistent with the lack of change in pH_a was whole blood lactate, which was unaffected by exposure to Pb only and Cd only (Figure 2.5A, B). There was a significant increase in lactate at 48 h in the fish exposed to Pb plus Cd mixtures (Figure 2.5C). However, the increase in lactate was slight, less than 1 $mmolL^{-1}$ compared to controls, further suggesting the Pb or Cd alone, or in mixtures, had minimal impact on the respiratory physiology of rainbow trout.

Ion Balance

Exposure to Cd alone, and Pb plus Cd mixtures resulted in marked disturbances to internal ion and osmotic balance. The fish exposed to Cd alone and to the Cd plus Pb mixtures experienced significant hypocalcaemia. In the fish exposed to Cd alone, plasma Ca^{2+} concentration began to decline immediately, and by 24 h it was significantly reduced by 22 %, and then stabilized through 72 h of exposure (Figure 2.6A). By 120 h, however, plasma Ca^{2+} concentration appeared to recover slightly in the fish surviving the Cd only exposure (Figure 2.4A). In contrast to Cd only, the Pb only exposure did not significantly affect plasma Ca^{2+} concentration (Figure 2.6B), but the presence of Pb exacerbated Cd-induced reductions in plasma Ca^{2+}; after 24 h, plasma Ca^{2+} concentrations were reduced by approximately 20 %, and declined further as exposure to the Pb plus Cd mixture continued (Figure 2.6C). By 120 h, plasma Ca^{2+} concentrations were 28 % lower compared to pre-exposure concentrations (Figure 2.6C).

Plasma Na^{+} was affected by Cd only exposures and the Cd plus Pb mixtures, but not the Pb only exposure (Figure 2.7). Exposure to Cd alone resulted in a non-significant 25% decrease in plasma Na^{+} after 72 h but for the most part was not different from the control levels of approximately 120mmolL^{-1}(Figures 2.7A, B). Exposure to Pb only resulted in no change in plasma Na^{+} concentration, which fluctuated around 120-130 mmolL^{-1} throughout the exposure period (Figure 2.7B). Plasma Na^{+} concentrations were significantly decreased by approximately 20 % after 24 h and 48 h of exposure to the Pb plus Cd mixture, but had returned to pre-exposure levels by 72 h (Figure 2.7C).

Exposure to Cd alone resulted in a gradual decline in plasma Cl^{-}, which was significantly reduced by 25 % by 72 h (Figure 2.8A), which coincided with a similar drop

in Na^+ at the same time (Compare Figure 2.7A to 2.8A). In both cases, however, Na^+ and Cl^- balance were restored by 120 h (Figure 2.7A, 2.8A). Exposure to Pb alone, however, had no effect on plasma Cl^- (Figure 2.8 B), nor did exposure to Pb plus Cd, despite a slight downward trend in plasma Cl^- over the course of the experiment (Figure 2.8C).

In addition to the transient ionoregulatory disruption observed, plasma osmolality was markedly reduced by 25% after 96 h of Cd only exposure (Figure 2.9A). Although Pb alone caused no change in plasma osmolality, the Pb plus Cd mixture caused a significant, persistent 10-15% decrease in plasma osmolality after 24 h of exposure (Figure 2.9).

Haematological Parameters

Although haematocrit and haemoglobin concentrations dropped over the course of exposure to Cd alone, Pb alone, and Cd plus Pb, haematocrit and haemoglobin declined in a similar manner in fish not exposed to metal but subjected to the same blood sampling regimen (Table 2.4). Mean cell haemoglobin was not affected by exposure to any of the metal treatments; however there was some variability in the control fish data (Table 2.4). Similarly, plasma protein was unchanged compared to control and t = 0 during control and all metal exposures, fluctuating around values of 2.0 to 2.5 g dL^{-1} in all treatments (Figure 2.10).

DISCUSSION

Metal-Gill Binding, Toxicity and Water Chemistry

The present study indicates that exposure of trout to Pb plus Cd mixtures exacerbates Cd-induced reductions to internal Ca^{2+} balance, causing hypocalcaemia that likely contributes to greater mortality compared to fish exposed to Cd or Pb alone. Disturbances to internal Na^{+} balance, however, do not appear to result from exposure to Pb alone. It should be noted, that there appears to be a transient reduction of plasma Na^{+} concentration during Pb plus Cd exposure. Because both Pb and Cd are known to be Ca^{2+} analogues (Verbost *et al.* 1989; Rogers *et al.* 2004), the presence of Pb was expected to exacerbate Cd-induced disturbances to internal Ca^{2+} balance.

Decreases in both Cd-gill and Pb-gill accumulation due to competition with calcium ions have been reported (Verbost *et al.* 1987; 1989; MacDonald, *et al.* 2002; Grosell *et al.* 2006; Niyogi *et al.* 2008). The decrease in Cd-gill accumulation observed in this study in the presence of Pb suggests there was a competitive interaction between Pb and Cd for a similar binding site on the gill (most probably the apical Ca^{2+} channels). However, the opposite was not true; the presence of Cd in the metal mixture did not lower Pb-gill binding as expected if the two metals were competing for identical binding sites. This finding was unexpected because Pb and Cd are both Ca^{2+} analogs and are thought to compete for the same binding site on the gill (Verbost *et al.* 1987; 1989; Rogers *et al.* 2004). Therefore, a decrease in the accumulation of both metals was expected (MacDonald *et al.* 2002; Rogers *et al.* 2003). The lack of impact on gill-Pb accumulation in the presence of Cd in this study may be explained by multiple binding

sites for Pb as described by Birceanu *et al* (2008). They identified a high affinity, low capacity population that was saturated at 18.2nmol g^{-1} (B_{max}). It could be possible that Pb has an alternate site to which Cd does not bind within the gill. In addition, the second high capacity, low affinity population of Pb binding sites could not be saturated by exposure water concentrations up to 4000nmolL^{-1} (Birceanu *et al.* 2008). It is, therefore, possible that if Pb is out competed by Cd for a common binding site (i.e. Ca^{2+} channels), it may bind to its alternate lower affinity, high capacity population of binding sites instead.

Despite the decrease in gill Cd accumulation during exposure to Pb plus Cd mixtures, mortality was always greater in exposures to mixtures compared to either individual metal exposure. This finding was expected, based on the idea that more metal should be more toxic or "the dose makes the poison". In other words, because the total metal (sum of Pb and Cd) was greater with metal mixture exposure, greater toxicity was expected. Surprisingly, the increase in mortality in the Pb plus Cd mixtures (Figure 2.2) was accompanied by no change in Pb-gill accumulation and a decrease in Cd-gill accumulation.

The combined effects of toxicants can be predicted using the concentration addition model which uses the Toxic Unit (TU) approach to predict toxicity, provided they have the same mechanism of action (Sprague 1970; Newman 1998; Norwood *et al.* 2003). For metals, 1 TU would be the metal concentration required to kill 50 % of the fish over a defined period. Thus, if fish are exposed to two or more metals in a mixture, toxicity could be quantitatively predicted by adding up the individual TUs for each metal. If the sum of the effects of two metals equals 1 TU, the interaction is considered to be

strictly additive, but if the total toxic effect is greater than 1 TU, toxicity is considered to be greater than additive ("synergistic"). Effects are considered less than additive, if the combined toxic effect is less than 1 TU ("antagonistic") (Norwood *et al.* 2003; Playle, 2004). These assumptions were tested by Playle (2004), who used a mathematical modeling program (MINEQL) to predict toxicity and metal accumulation of metals in mixtures. With these assumptions it is predicted, that both Pb and Cd compete for the same binding sites on the gill (i.e. Ca-channels), and that exposing fish to mixture concentrations above one toxic unit (LC50) results in metal-gill accumulation that is less than additive (Playle, 2004). Thus, toxicity should also be less than additive if there was competition for Ca^{2+} binding sites on the gill between Pb and Cd.

Birceanu *et al.* (2008) noted that short-term Pb plus Cd exposure (3 h) did result in Pb-gill and Cd gill-binding that was less than additive, but that disturbances to ion uptake were greater than additive. The present findings support those of Birceanu *et al.* (2008), because Cd-induced reductions in plasma Ca^{2+} observed in the present study were Ca^{2+} uptake greatly exacerbated in the presence of Pb, which is consistent with the exacerbation of Cd-induced reductions reported by Birceanu *et al* (2008). Similar to Birceanu *et al* (2008), in this study gill-Cd accumulation was less than additive when fish were exposed to Cd in the presence of Pb. However, in contrast, gill-Pb accumulation remained unchanged in the presence of Cd compared to the levels measured in fish exposed to Pb only. Despite the less than additive gill-metal accumulation in the presence of Cd and Pb in combination, greater than additive reductions were seen in the plasma Ca^{2+}, Na^{+} and osmolality of fish exposed to Pb and Cd mixtures compared to Pb or Cd alone.

Effects of Pb plus Cd Mixtures on Whole Body and Blood Ion, Osmotic and Acid-base Balance

For these experiments rainbow trout were exposed to either Cd alone, Pb alone, or Pb plus Cd mixtures, at environmentally relevant concentrations but within a range that elicited physiological disturbances in the fish. The metal concentrations and the water chemistry used in these experiments were similar to those reported by Birceanu *et al.* (2008), who reported that short-term (3 h) exposure to Pb plus Cd mixtures had greater than additive effects on Ca^{2+} and Na^{+} influx in trout. They reported that Ca^{2+} influx was inhibited by Cd, but not Pb, and that Pb, but not Cd, inhibited Na^{+} influx. However, the presence of Pb exacerbated the Cd-induced inhibition of Ca^{2+} influx in a greater than additive manner, and the presence of Cd exacerbated Pb-induced reductions in Na^{+} influx. These results demonstrate that greater than additive ionoregulatory effects of Pb plus Cd exposure observed during short-term metal exposure (3 h) persist during longer term metal exposure and result in pronounced disturbances to internal ionic and osmotic balance.

There were no definite trends of Pb plus Cd exposure on whole body ion concentrations. There was a slight decrease in whole body Ca^{2+} with subsequent recovery (significant) during 10 $nmolL^{-1}$ Cd exposure. Despite the lack of change in whole body ions, there was significant mortality throughout the experiments, especially in the higher concentrations of Pb and Cd mixtures. Cd and Pb have both been shown to be calcium antagonists at the site of uptake on the gill. However, it is likely that a decrease in calcium uptake and even plasma calcium may be too small to be reflected by changes in whole body ion stores (Stubblefield *et al.* 1995). Fleming (1974; as cited in Pelgrom *et al.*

1995) demonstrated that plasma calcium in teleosts comprises approximately 3-6% of the whole body calcium reserves. Thus, even a large change in plasma calcium may not be detectable in measurements of whole body calcium. Hollis *et al* (2000) did not see an effect on whole body calcium during chronic (30 day) exposure to Cd in both soft and hard waters. Plasma Na^+ was significantly higher after 24 h during most exposures. Grosell *et al.* (2006) saw a similar trend in fathead minnows during a 10 day exposure to 2-5$\mu molL^{-1}$ dissolved Pb. It was suggested that a recovery of plasma ions could be caused by a reduction in ion efflux due to a decrease in the gradient necessary for apical entry of Na^+ or a reduction in gill epithelium permeability (Laurent and McDonald, 1987). In addition, an up-regulation or increased transport capacity of active Na^+ or Ca^{2+} transport mechanisms could explain a subsequent recovery from metal exposure (Laurent and McDonald, 1987; 1987; Fu *et al.* 1990).

The observed constancy of PaO_2 and pH_a seen during these mixture experiments indicates that both Pb and Cd individually, and in combination do not cause toxicity via respiratory disruption in contrast to other metals such as zinc and aluminum in acidic water (Spry and Wood, 1983; Neville, 1985; Playle *et al.* 1989; Walker *et al.* 1991). Rogers and Wood (2003) also showed that respiratory distress is not present during Pb exposure in moderately hard water. However, in the past it has been thought that the production of excess mucus on the gill epithelium in the presence of low ambient water calcium and metals (including Pb and Cd) may cause asphyxiation in fish (Sorenson, 1991). However, more recent work (Rogers and Wood, 2003; Chowdhury *et al.* 2004) and the present study suggest that Pb, Cd or Pb plus Cd mixtures have minimal effects on respiration in trout.

While parameters of respiratory and acid-base regulation remained stable through out all metal exposures in this study, measurements of ionoregulatory disruption did not show this same trend. Plasma Cl^- remained at control levels; however plasma Ca^{2+} and Na^+ were differently impacted during the course of the individual exposures. Cd alone caused a significant decrease in plasma Ca^{2+} after 24 h of exposure but returned to control levels. The effect on plasma Ca^{2+} was expected, because Cd is a well known Ca^{2+}-channel antagonist (Niyogi and Wood, 2004; Rogers *et al.* 2003; Playle *et al.* 1993). Pb was also thought to be a Ca^{2+} antagonist (Playle 2004; Rogers and Wood, 2003; Grosell *et al.* 2006; Rogers and Wood, 2004), but no time dependent effects on Ca^{2+} balance with Pb only exposure were observed at the concentrations used. This can be explained by the low dissolved Pb concentration in the water (30-45 $nmolL^{-1}$), which was approximately 6 % of the observed LC50 of 482 $nmolL^{-1}$for rainbow trout in waters of similar chemistry (Birceanu *et al.* 2008). However, it should be noted that the Pb concentrations to which these fish were exposed are more environmentally realistic than those reported in many earlier studies (Nriagu *et al.* 1996).

The persistent and significant decrease (28 %) in plasma Ca^{2+}during Cd plus Pb exposure was greater than expected given the low concentration of Pb that was used. In other words, the presence of Pb exacerbated Cd-induced disturbances to plasma Ca^{2+} balance, suggesting that the hypocalcaemic effects of the two metals were greater than additive. The two metals also appeared to act in an additive fashion to lower plasma Na^+ concentration during the early (first 48 h) stages of metal mixture exposure, but due to the large reductions in plasma Na^+ that were observed in the presence of Cd alone, this effect cannot be considered greater than additive. Evidence of a greater than additive impact

(the final outcome is more than the two individuals added together) on both Na^+ and Ca^{2+} influx was reported by Birceanu *et al* (2008) during 3 h exposures to Pb plus Cd mixtures (10-50 $nmolL^{-1}$ Cd and 25-110 $nmolL^{-1}$ Pb). The present work suggests that is likely that the greater than additive effects on Ca^{2+} balance persists over a much longer time frame (5 d) and likely contributes to toxicity. The short-term disturbance to Na^+ uptake observed by Birceanu *et al* (2008) may be corrected over the longer time frame (5 d). It will be necessary, however, to confirm these interpretations by performing longer-term (5-10 d) measurements of Ca^{2+} and Na^+ uptake in fish exposed to Pb plus Cd mixtures.

Mechanism of Toxicity at the Gill

The results seen in this study can be explained by Pb and Cd causing hypocalcaemia via more than one mechanism. Toxicity caused by exposure to both Cd and Pb is believed to be mainly caused by competition between Pb, Cd and Ca^{2+} for entrance across the gill via apical calcium channels (Playle, 1998; 2004; Verbost *et al*, 1987, 1988). The mechanisms of toxic action for both Pb and Cd have been shown to be more complex than the direct competition that is assumed in modeling parameters. Research by Birceanu *et al* (2008) suggests that there are multiple binding sites for Cd on the fish gill. One population is believed to have a high affinity (high $logK_{me\text{-}gill}$) for each respective metal, but a low binding capacity (low B_{max}) as there are for Pb (see above). It is likely that these high affinity sites are saturated first during metal exposure, while the low affinity high capacity sites are only occupied if the high affinity sites are unavailable. Although these multiple binding sites have been identified for each metal individually, it

is not known whether they are similar for both metals. This further complicates matters in trying to relate physiological disruption to toxicity.

In addition to binding at apical channels on the gill, it has been shown that metals can have direct impacts on ATP dependent pumps on the basolateral membranes of the gill and on cytosolic enzymes responsible for ion homeostasis. For example, Lionetto *et al* (2000) showed an inhibition of the cytosolic enzyme carbonic anhydrase (CA) and basolateral Ca^{2+}-ATPase in eel gill homogenates after 1 h exposure to Cd. ATP dependent processes such as the Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase have been shown to be inhibited in the gill and intestines by exposure to metals (Pb, Cd, Cu, Zn and Cr) in both the mudskipper (Thaker *et al.* 1996) and freshwater Tilapia (Atli and Canli, 2007). Impacts on gill energy dependent transporters could reflect the greater than additive impacts on calcium ionoregulation as seen in this study.

The decrease in plasma Na^{+} during exposure to Pb plus Cd mixtures can be explained by both direct competition of Pb and possibly Cd for binding to apical Na^{+} channels on the gill and by indirect inhibition of internal Na^{+}/K^{+}-ATPase. The inhibition of Na^{+}/K^{+}-ATPase and the decrease of plasma Na^{+} have been demonstrated for Pb by Rogers *et al* (2003; 2005). In addition Rogers *et al.* (2005) demonstrated that Pb also inhibits CA. A decrease in CA efficiency would also cause a decrease in Na^{+} uptake due to a decrease in the production of H^{+}, an ion that aids in the creation of an electrochemical gradient (via H^{+}-ATPase) that helps facilitate Na^{+} uptake across the apical membrane. However, in this study a subsequent recovery of plasma Na^{+} back to control levels was observed. This recovery could be due to up-regulation of certain ionoregulatory processes (i.e. Na^{+}/K^{+}-ATPase) or a proliferation of chloride cells (Mallat,

1985; McDonald and Wood, 1993). Kundu *et al,* (1995) observed an initial stimulation of Ca^{2+} and Mg^{2+} –ATPases in addition to Na^{+}/K^{+}-ATPase found in brain and muscle tissue of the mudskipper (*Boleophthalmus dentatus*) during exposures (3 d) to Chromium. Further description of the possible mechanisms of acclimation is described in detail below.

Possible Acclimation to Metals

Although there was a significant and persistent decrease in plasma Ca^{2+} during the metal mixture exposure, the decrease seen in the Cd only exposure was only transient, and returned towards control levels in remaining fish by 120 h (Figure 2.6). The recovery of plasma Ca^{2+} levels seen in this study and "recovery" of plasma Na^{+} during Pb plus Cd mixture exposure may suggest a mechanism of acclimation to the metals or a stimulation of subsequent damage-repair mechanisms by surviving fish. Acclimation to metals can occur fairly quickly (as fast as 5-7 days). This has been seen in restored Na^{+} influx across the body during aluminum exposure in brook trout (*Salvelinus fontinalis*) (Mueller *et al.* 1991). One of the major causes of ionic loss due to metal exposure is physical damage to gill lamellar cells, but this damage is more commonly seen during exposure to higher concentrations of metals (as reviewed in Mallatt, 1985) and therefore may not play a major role in this study. This type of damage can be repaired by the thickening of lamellar and filament epithelia (McDonald and Wood, 1993). Alternate mechanisms of acclimation to lower levels of metals (as used in this study) have been demonstrated. The production of mucus on the gills of stressed or metal exposed fish has been observed in a variety of studies (Pärt and Lock, 1983; Handy *et al.* 1989). Mucus has been shown to

have a capacity to bind metals without reducing the uptake rates of essential ions (i.e. Na^+, Ca^{2+}, or Cl^-) into fish (Marshall, 1978; Part and Lock, 1983). This mechanism of acclimation has been shown in trout exposed to aluminum (Mueller *et al.* 1991; McDonald *et al.* 1991; Reid *et al* 1991). If the metal concentrations used in this experiment were sufficient to illicit mucus production, this mechanism could at least partially explain the recovery of plasma Ca^{2+} and Na^+ seen in this study. Further studies including measurements of plasma Cd (initial attempts did not show any detectable accumulation) or mucus production, or analysis of gill morphology are needed to substantiate these hypotheses. This recovery effect occurred for plasma Na^+ and not plasma Ca^{2+} in the Pb plus Cd exposure. Perhaps the exposure concentrations in the mixture exceeded a threshold necessary for response mechanisms for acclimation or elicited more extensive gill damage (Mallat, 1985). This possibility too, requires further investigation.

Another possible mechanism for the observed acclimation to metals seen here is a possible up-regulation of active transport mechanisms within the gill (i.e. Na^+/K^+-ATPase and Ca^{2+}-ATPase) or increases in the ion uptake capacity, through an increase in MRC density (McDonald and Wood, 1993). Fu *et al* (1990) demonstrated such a trend in the opercular epithelium of Cd exposed tilapia. Results indicated a two-fold increase in MRC density was observed after 4 d of exposure to $10 \mu gL^{-1}$ Cd (~89 $nmolL^{-1}$). The proliferation of MRCs during metal or exposure to low calcium levels is hypothesized to be responsible for increased active transport mechanisms in freshwater rainbow trout therefore suggesting a mechanism of acclimation to ionoregulatory stressors (Perry and Wood, 1985; Perry and Laurent, 1989). Based on this evidence it is possible that the

recovery of plasma calcium and sodium (in the Cd alone and Cd plus Pb exposures respectively) is initiated by an increase in MRC density and subsequent increases in active ion transport across the gill. Moreover it is possible for these physiological changes to occur in short periods of time <7 d, such as during the 5 d exposures examined in the present study. However, measurements of unidirectional ion movements and Na^+/K^+- ATPase activity are required to test this hypothesis (See Chapter 3).

Conclusions and Implications

The goal of this study was to determine how metals in combination act upon the rainbow trout physiology. Toxicity of metal mixtures is typically modeled using the predicted gill-metal accumulation in relation to exposure concentration. However, prediction models often do not consider the direct and subsequent physiological responses of aquatic organisms beyond competition at the site of action for individual metals (Paquin *et al.* 2002). In this study, there were no effects on acid-base homeostasis or haematological parameters caused by exposures to Pb or Cd individually or in mixtures. However, ionoregulatory disruption, as demonstrated by decreases of plasma Ca^{2+} of Cd only exposed fish, was likely one mechanism of toxicity, despite the recovery of calcium in surviving fish. The recovery from decreased plasma calcium could be a result of increased mucus production at the gill, ultimately decreasing metal accumulation while allowing the fish to maintain divalent cation uptake at the gill (Marshall, 1978). The persistent decrease in plasma Ca^{2+}, however, was not overcome by surviving fish (only 3) during exposures to Pb and Cd in combination indicating a possible threshold concentration for acclimation of these fish to metals. Similarly, there was a greater impact on plasma Na^{+} and osmolarity in fish exposed to the Pb and Cd mixture, which did not reflect any trend seen in the individual metal exposures. This study underlines the complexity of metals in mixtures on the physiology and toxicity of rainbow trout and the difficulties in predicting specific physiological responses in fish. Furthermore, this research may outline the necessity for more research on the physiological impacts of low level contaminants and in mixtures, which are not considered in toxicity prediction programs (BLM) (Paquin *et al.* 2002). For example, exposure of fish to Pb plus Cd

mixtures may have greater acute physiological impacts than can be predicted by gill metal accumulation alone. Thus, it may be necessary to use physiological endpoints, in addition to metal-gill binding assays, to predict toxicity in fish exposed to metal mixtures.

Table 2.1 Measured water chemistry throughout a 5 d (120 h) exposure of rainbow trout to nominal Cd concentrations of 10 and 50 $nmolL^{-1}$ and/or nominal Pb concentrations of 125 and 250$nmolL^{-1}$. All data is expressed as the mean ± 1 SEM.

Treatment (Nominal)	Dissolved Cd (nM)	Dissolved Pb (nM)	Ca^{2+} (µM)	Na^{+} (µM)	Cl^{-} (µM)
Control	0.1 ± 0.0	0.0 ± 0.0	88.7 ± 1.3	127.7 ± 1.5	47.9 ± 1.0
10 nM Cd	12.9 ± 1.3	0.0 ± 0.0	96.7 ± 1.5	92.3 ± 2.5	19.6 ± 2.1
50nM Cd	49.4 ± 1	0.0 ± 0.0	86.0 ± 0.4	168.1 ± 2.7	76.8 ± 10.3
125nM Pb	0.0 ± 0.0	25.1 ± 4.7	93.8 ± 2.3	88.7 ± 0.7	56.3 ± 11.2
250nM Pb	0.3 ± 0.1	170.6 ± 19	81.9 ± 1.6	169.9 ± 5.4	62.6 ± 0.6
10 nM Cd +125 nM Pb	10.7 ± 0.4	31.9 ± 6.7	97.8 ± 2.6	103.7 ± 10.7	21.7 ± 0.4
10 nM Cd + 250 nM Pb	5.3 ± 0.4	152.5 ± 9.6	85.1 ± 0.8	165.0 ± 2.3	60.4 ± 1.5
50 nM Cd + 125 nM Pb	49.2 ± 0.4	25.6 ± 2.4	96.5 ± 1.0	91.2 ± 0.6	28.7 ± 0.8
50 nM Cd + 250nM Pb	50.0 ± 0.1	154.0 ± 10.4	83.5 ± 0.8	197.0 ± 20.3	64.3 ± 0.3

Table 2.2 Whole body ion concentrations from control and fish exposed to Pb and Cd individually and in mixtures in soft water. Whole fish were sampled at 24, 72 and 120 h. All values are represented as mean ± 1 S.E.M (n). An Asterisk (*) indicates significant difference from respective controls at the same time interval.

Ion	Time	Control	10 nM Cd	50 nM Cd	125 nM Pb	250 nM Pb	10 nM Cd +125nM Pb	10 nM Cd + 250 nM Pb	50nM Cd + 125nM Pb	50nM Cd + 250nM Pb
	24	98.6 ± 1.7 (15)	90.8 ± 1.5 (8)*	98.4 ± 1.4 (7)	100.7±2.6(8)	101.9 ± 3.5(7)	91.0 ± 2.5 (8)*	101.2 ± 2.3 (7)	98.8 ± 5.6 (6)	99.7 ± 2.4 (6)
Calcium	**72**	96.2 ± 2.6 (13)	100.0 ± 1.3 (7)	-	96.1 ± 3 (8)	102.9 ± 3.2(7)	91.1 ± 3.2 (8)	107.4 ± 2.8 (7)*	93.7 ± 4.3(4)	100.6 ± 2.2 (6)*
	120	96.0 ± 3.4 (15)	105.2 ± 5.7 (8)	-	90.3 ± 1.9(8)	105.1 ± 5.1(7)	99.6 ± 1.7 (8)	103.0 ± 3.3 (7)	-	-
	24	47.0 ± 2.1 (15)	52.0 ± 1.5 (8)	40.7 ± 1(7)*	56.1 ± 1.5(8)*	41.7 ± 1.2 (7)	55.1 ± 2.8(8)*	38.1 ± 2 (7)*	49.6 ± 1.4(6)	40.8 ± 1.1 (6)*
Sodium	**72**	47.3 ± 2.5 (13)	57.9 ±1.3 (7)*	-	55.0 ± 1.8(8)*	41.6 ± 1.7 (7)	51.6 ± 3.5 (8)	42.6 ± 1.6(7)	45.9 ± 5.8 (4)	41.6 ± 2.2 (6)
	120	47.9 ± 1.8 (15)	47.3 ± 5.7 (8)	-	51.8 ± 1.5(8)	41.9 ± 4.5 (7)	77.3 ± 0.5(7)*	46.0 ± 1.8 (7)	-	-
	24	76.6 ± 1.6 (15)	77.2 ± 1.6 (8)	76.8 ± 1.5 (7)	76.9 ± 2.7(8)	77.3 ± 0.5(7)	76.2 ± 1.8 (8)	75.7 ± 1.8(7)	75.6 ± 3.7(6)	74.4 ± 1.3 (6)
Potassium	**72**	78.5 ± 2 (13)	81.4 ± 1.7 (7)	-	84.6 ± 2.2(8)*	72.6 ± 2.5 (7)*	78.8 ± 2.1 (8)	75.2 ± 0.5(7)	73.0 ± 2.1 (4)	71.6 ± 2.5 (6)
	120	76.0 ± 1.2 (15)	85.7 ± 7.9 (8)	-	97.0 ± 0.2(8)*	79.6 ± 6.4 (7)	81.5 ± 4.7 (8)*	69.6 ± 1.7(7)*	-	-
	24	36.0 ± 1.6 (15)	39.8 ± 4.7 (8)*	52.6 ± 10.5(7)	45.2 ± 2.9(8)*	37.1 ± 1.6 (7)	20.5 ± 5.1(8)*	32.5 ± 1.7 (7)	27.8 ± 3.8 (6)	34.4 ± 2.1(6)
Chloride	**72**	33.9 ± 3.2 (13)	52.5 ± 7.2 (7)*	-	47.2 ± 1.6(8)*	36.6 ± 2 (7)	54.5 ± 4.1(8)*	35.5 ± 5.7(7)	25.2 ± 8.1(4)	31.4 ± 2.7(6)
	120	40.1 ± 3.0 (15)	34.5 ± 11.7 (8)	-	49.1 ± 3.2(8)	30.8 ± 4.2(7)*	48.4 ± 6.8 (8)	30.5 ± 2.2(7)*	-	-

Table 2.3 Average water quality parameters from the re-circulating experimental set-up over the course of the 5 d metal and control exposures on cannulated rainbow trout. Control water Pb and Cd represent background levels from the system prior to the addition of metals.

Exposure	Cd (nM)	Pb (nM)	Ca^{2+} (μM)	Na^{+} (μM)	pH	Temp (°C)	DOC (mg C/L)
Control	1.4 ± 1	0.2 ± 0.7	108.7 ± 1.2	98.1 ± 7.4	6.1 ± 0.1	16.2 ±0.1	1.8 ± 0.1
10 nM Cd	7.4 ± 0.3	0.18 ± 0.9	84.3 ± 1.5	146.4 ± 4.8	6.2 ± 0.1	15.9 ± 0.1	1.3 ± 0.1
50 nM Pb	2.8 ± 2	26.1 ± 1.6	108.2 ± 1.4	216.8 ± 21.3	5.9 ± 0.1	14.4 ± 0.5	2.2 ± 0.2
10 nM Cd + 50 nM Pb	6.9 ± 0.4	45.5 ± 1.9	111.1 ± 2.2	179.9 ± 14.1	6.1 ± 0.0	15.8 ± 0.3	2.8 ± 0.3

Table 2.4 Haematological parameters (Ht, Hb and MCHC) measured from rainbow trout exposed to control, Cd only, Pb only, and Cd plus Pb mixtures for 120 h. Data is represented as the mean ± 1 S.E.M (N). An asterisk "*" indicates significance from the pre-exposure levels prior to exposure. A cross "+" indicates significant difference from control group at t = 0.

	Ht (% RBC)				Hb (g dL^{-1})				MCHC (g Hb mL^{-1})			
Time (h)	**Control**	**Cd**	**Pb**	**Mix**	**Control**	**Cd**	**Pb**	**Mix**	**Control**	**Cd**	**Pb**	**Mix**
0	20.2 ± 1.4 (15)	24.0 ± 1.3 (22)	22.7 ± 1.2 (14)	21.3 ± 0.8 (14)	10.7 ± 0.7 (15)	9.0 ± 0.5 (22)+	7.6 ± 0.6 (14)+	6.7 ± 0.3 (14)	0.55 ± 0.04 (15)	0.27 ± 0.02 (22)+	0.34 ± 0.02 (14)+	0.32 ± 0.01 (14)+
8	18.8 ± 1.1 (15)	21.7 ± 0.8 (19)	20.0 ± 1.5 (13)	19.3 ± 1.0 (13)	9.1 ± 0.6 (15)	7.6 ± 0.3 (19)	7.1 ± 0.6 (13)	6.1 ± 0.2 (13)	0.49 ± 0.02 (15)	0.23 ± 0.01 (19)	0.35 ± 0.02 (13)	0.32 ± 0.02 (13)
24	14.7 ± 1.1 (12)*	18.9 ± 1.1 (17)	18.9 ± 1.4 (15)	17.8 ± 1.2 (12)	6.3 ± 0.6 (12)	6.9 ± 0.3 (17)	6.3 ± 0.4 (15)	6.1 ± 0.4 (12)	0.43 ± 0.01 (12)*	0.22 ± 0.02 (17)	0.34 ± 0.02 (15)	0.34 ± 0.01 (12)
48	16.2 ± 1.7 (8)	20.7 ± 1.6 (13)	16.1 ± 1.4 (15)*	17.2 ± 2.8 (7)	7.6 ± 0.7 (8)	6.7 ± 0.4 (13)*	5.6 ± 0.4 (15)	4.9 ± 0.5 (7)*	0.48 ± 0.03 (8)	0.22 ± 0.02 (13)	0.37 ± 0.02 (15)	0.31 ± 0.02 (7)
72	12.9 ± 1.4 (8)*	20.47 ± 2.6 (11)	13.8 ± 1.5 (14)*	15.8 ± 2.2 (7)*	5.4 ± 0.7 (8)	6.9 ± 0.7 (11)	4.9 ± 0.5 (14)*	5.3 ± 0.4 (7)	0.42 ± 0.02 (8)*	0.22 ± 0.03 (11)	0.39 ± 0.04 (14)	0.30 ± 0.02 (7)
96	10.8 ± 1.3 (7)*	16.6 ± 3.3 (7)*	13.3 ± 1.3 (12)*	12.4 ± 2.5 (5)*	5.5 ± 0.8 (7)	5.8 ± 0.8 (7)*	4.5 ± 0.5 (12)*	4.9 ± 0.7 (5)	0.45 ± 0.06 (7)	0.20 ± 0.05 (7)	0.34 ± 0.02 (12)	0.37 ± 0.02 (5)
120	8.9 ± 1.4 (3)*	19.6 ± 3.4 (7)	13.3 ± 1.5 (13)*	10.7 ± 0.5 (3)*	5.6 ± 1.4 (3)	5.7 ± 1.0 (7)*	4.7 ± 0.5 (13)*	3.5 ± 0.2 (3)*	0.39 ± 0.07 (3)*	0.19 ± 0.03 (7)	0.37 ± 0.02 (13)	0.32 ± 0.04 (3)

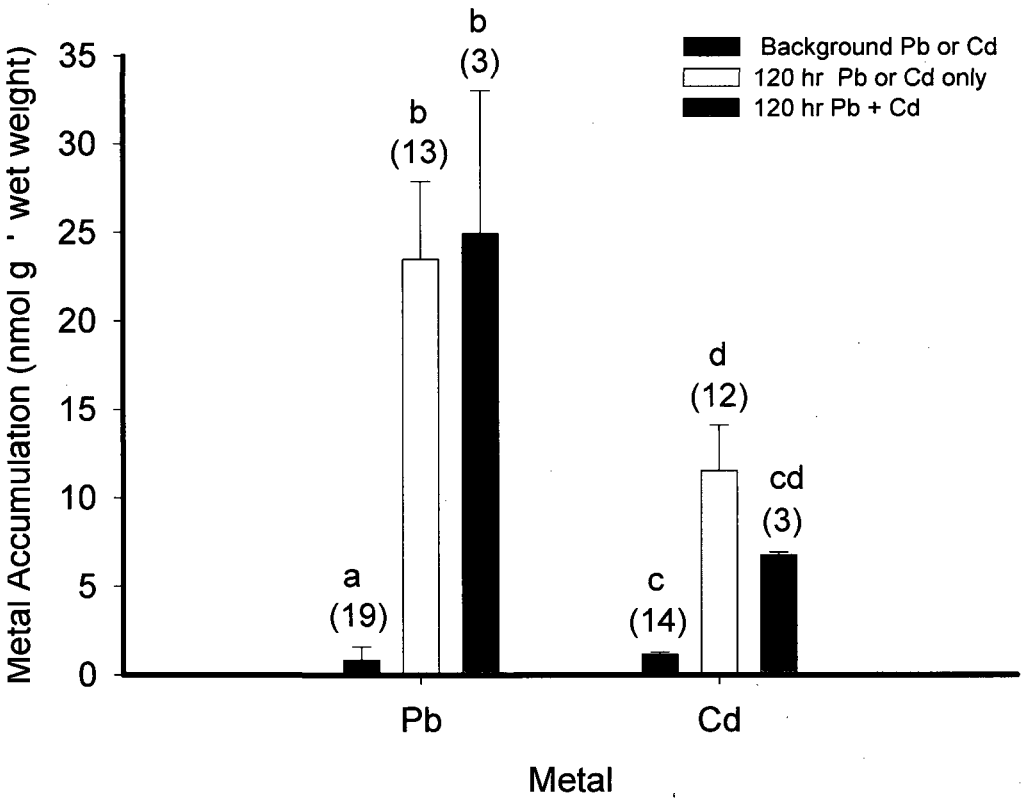


Figure 2.1 Gill-metal accumulation of rainbow trout after 120 h of exposure to control, Cd only (10nmolL^{-1}), Pb only (50nmolL^{-1}), or a Cd plus Pb mixture (10nmolL^{-1} Cd plus 50nmolL^{-1} Pb). Background accumulation was measured in experiments with no exposure to that particular metal. Data represented as mean ± 1 S.E.M (N). Bars sharing the same letter are not significantly different from each other (within measurements of each metal).

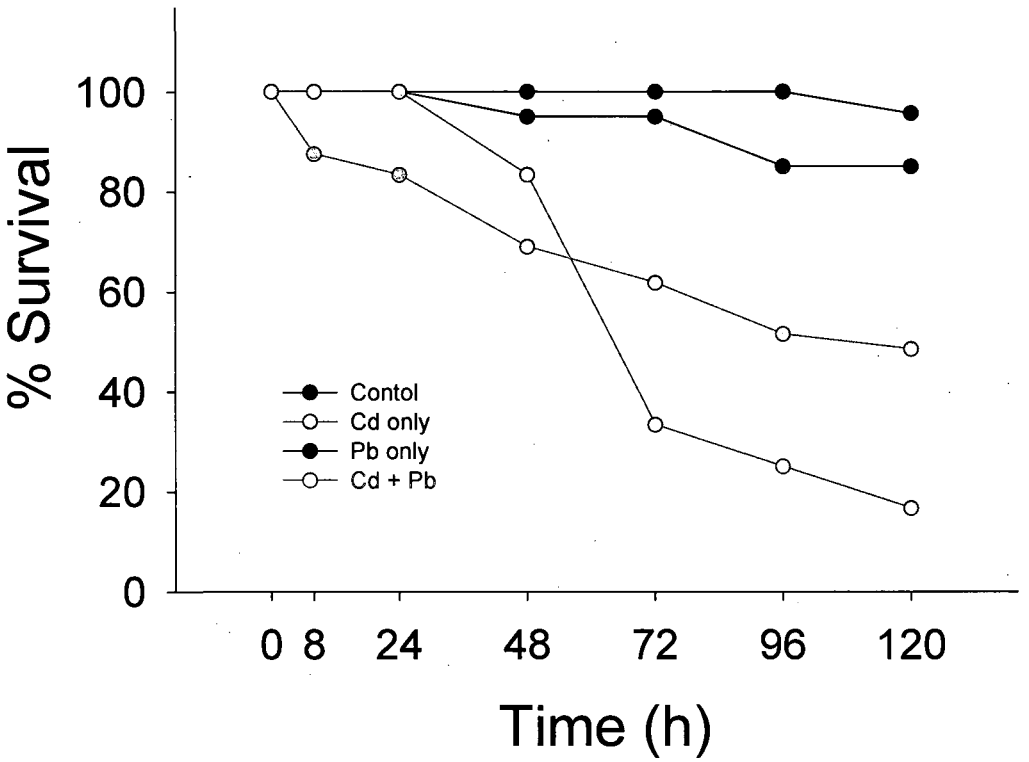


Figure 2.2 Percent survival of rainbow trout exposed to control and **(a)** 7.4 ± 0.3 $nmolL^{-1}$ Cd alone **(b)** 26.1 ± 1.6 $nmolL^{-1}$ Pb alone or **(c)** 6.9 ± 0.4 $nmolL^{-1}$ Cd plus 45.4 ±1.9 $nmolL^{-1}$ Pb. Only mortality due to metal exposure was considered.

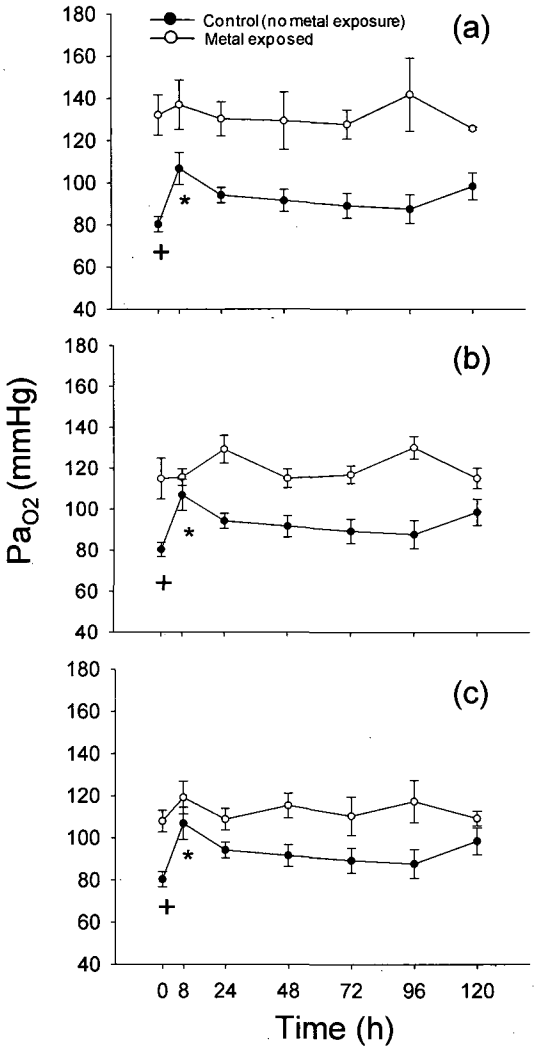


Figure 2.3 Arterial Pa_{O2} from rainbow trout exposed to **(a)** 7.4 ± 0.3 $nmolL^{-1}$ Cd alone **(b)** 26.1 ± 1.6 $nmolL^{-1}$ Pb alone or **(c)** 6.9 ± 0.4 $nmolL^{-1}$ Cd plus 45.4 ±1.9 $nmolL^{-1}$ Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. An asterisk (*) indicates significant differences from the pre-exposure condition for that treatment ($P < 0.05$; Dunnett's one way ANOVA). A cross (+) indicates significant differences from controls ($P < 0.05$; unpaired t-test). N = 7-14 under pre-exposure conditions; N = 7-13 at 8 h; 6-14 at 24-72 h, and 3-5 at 96 and 120 h.

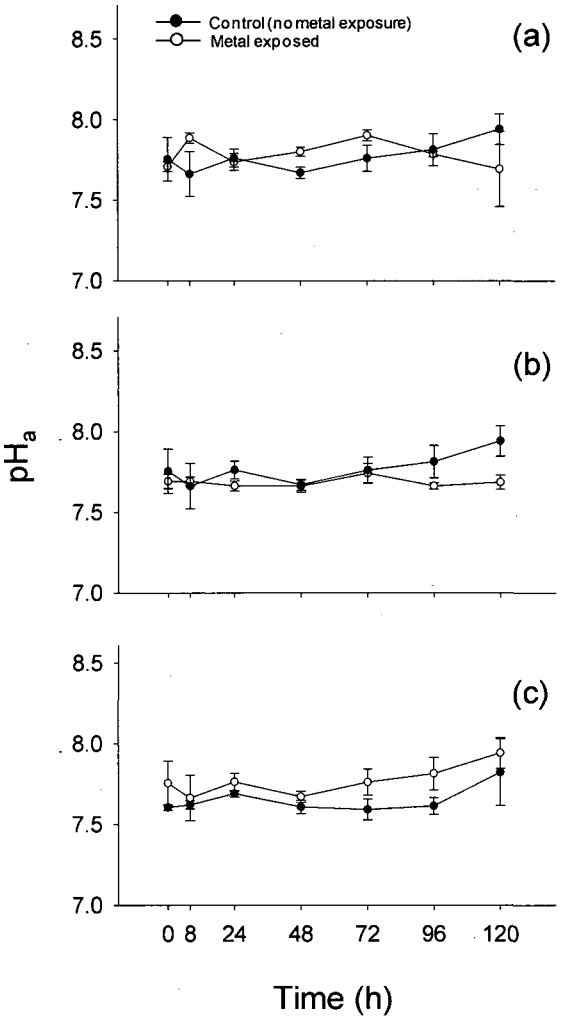


Figure 2.4 Arterial pH (pHa) of rainbow trout exposed to control conditions or metals exposure. **(a)** 7.4 ± 0.3 nmolL^{-1} Cd alone **(b)** 26.1 ± 1.6 nmolL^{-1} Pb alone or **(c)** 6.9 ± 0.4 nmolL^{-1} Cd plus 45.4 ±1.9 nmolL^{-1} Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. There were no significant differences ($P < 0.05$). N = 7-14 under pre-exposure conditions; N = 7-13 at 8 h; 6-14 at 24-72 h, and 3-5 at 96 and 120 h.

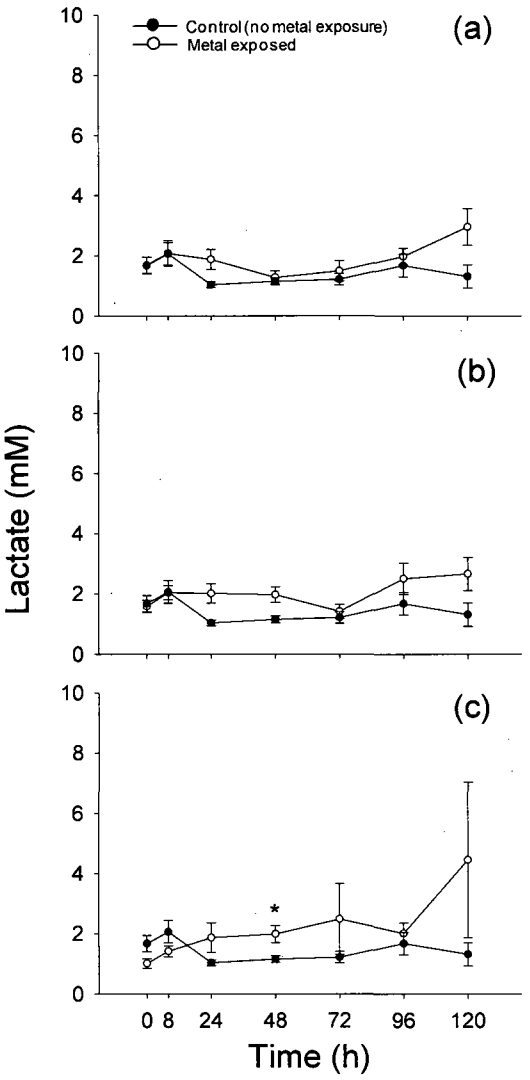


Figure 2.5 Lactate from rainbow trout exposed to control and **(a)** 7.4 ± 0.3 nmolL^{-1} Cd alone **(b)** 26.1 ± 1.6 nmolL^{-I} Pb alone or **(c)** 6.9 ± 0.4 nmolL^{-1} Cd plus 45.4 ±1.9 nmolL^{-1} Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. An asterisk (*) indicates significant differences from the pre-exposure condition for that treatment ($P < 0.05$; Dunnett's one way ANOVA). A cross (+) indicates significant differences from control group at $t = 0$ ($P < 0.05$; unpaired t-test). N = 14-22 for pre-exposure conditions; N = 13-19 at 8 h, 12-17 at 24 h, 7-15 at 48 h, 7-14 at 72 h, 5-12 at 96 h and 3-13 at 120 h.

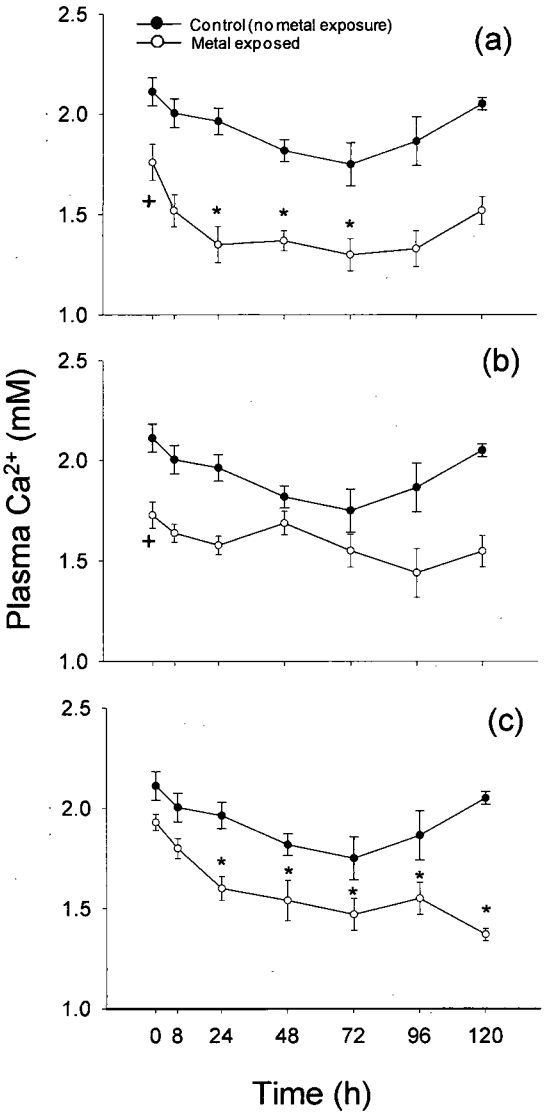


Figure 2.6 Plasma Ca^{2+} from rainbow trout exposed to **(a)** 7.4 ± 0.3 $nmolL^{-1}$ Cd alone **(b)** 26.1 ± 1.6 $nmolL^{-1}$ Pb alone or **(c)** 6.9 ± 0.4 $nmolL^{-1}$ Cd plus 45.4 ±1.9 $nmolL^{-1}$ Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. An asterisk (*) indicates significant differences from the pre-exposure condition for that treatment ($P < 0.05$; Dunnett's one way ANOVA). A cross (+) indicates a significant difference from control group at $t = 0$ ($P < 0.05$; unpaired t-test). N = 14-22 for pre-exposure conditions; N = 13-19 at 8 h, 12-17 at 24 h, 7-15 at 48 h, 7-14 at 72 h, 5-12 at 96 h and 3-13 at 120 h.

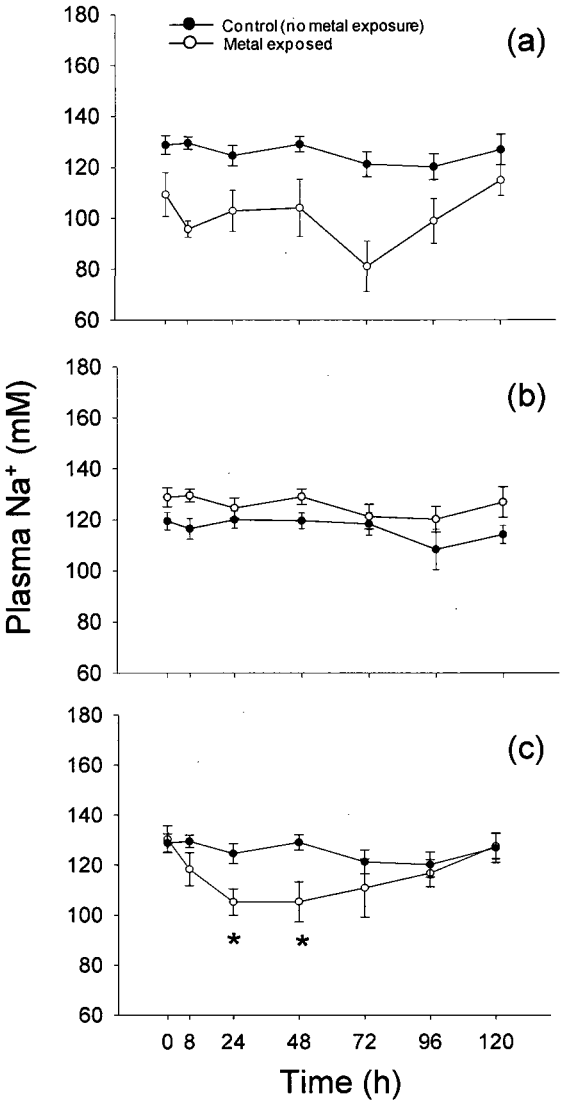


Figure 2.7 Plasma Na^+ from rainbow trout exposed to control and **(a)** 7.4 ± 0.3 $nmolL^{-1}$ Cd alone **(b)** 26.1 ± 1.6 $nmolL^{-1}$ Pb alone or **(c)** 6.9 ± 0.4 $nmolL^{-1}$ Cd plus 45.4 ±1.9 $nmolL^{-1}$ Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. An asterisk (*) indicates significant differences from the pre-exposure condition for that treatment ($P < 0.05$; Dunnett's one way ANOVA). A cross (+) indicates significant differences from control group at $t = 0$ ($P < 0.05$; unpaired t-test). N = 14-22 for pre-exposure conditions; N = 13-19 at 8 h, 12-17 at 24 h, 7-15 at 48 h, 7-14 at 72 h, 5-12 at 96 h and 3-13 at 120 h.

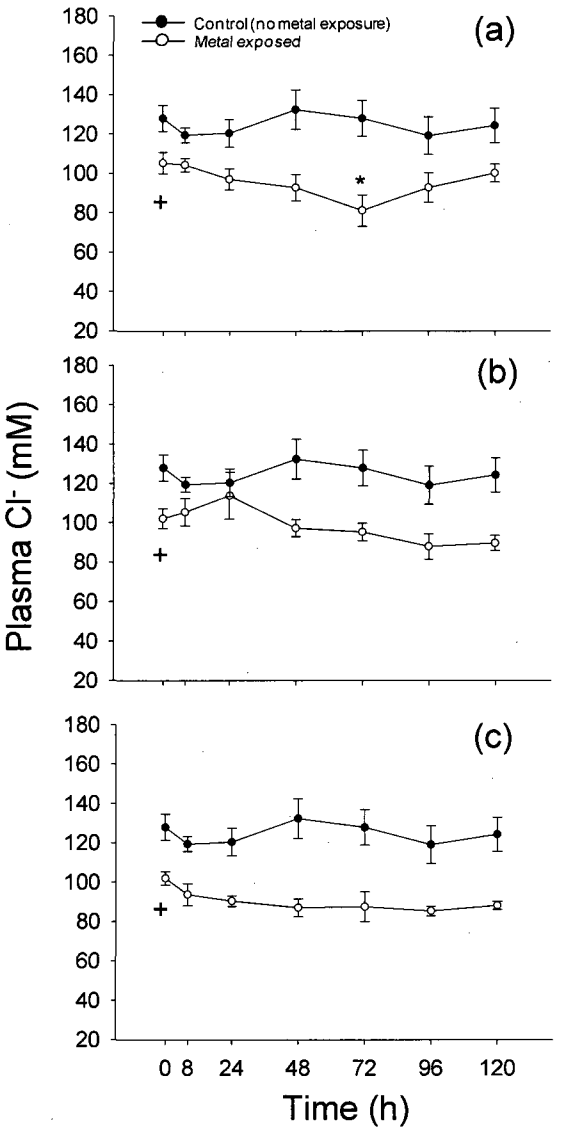


Figure 2.8 Plasma Cl^- from rainbow trout exposed to control and **(a)** 7.4 ± 0.3 $nmolL^{-1}$ Cd alone **(b)** 26.1 ± 1.6 $nmolL^{-1}$ Pb alone or **(c)** 6.9 ± 0.4 $nmolL^{-1}$ Cd plus 45.4 ±1.9 $nmolL^{-1}$ Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. An asterisk (*) indicates significant differences from pre-exposure condition for that treatment ($P < 0.05$; Dunnett's one way ANOVA). A cross (+) indicates a significant difference from the control group at $t = 0$ ($P < 0.05$; unpaired t-test). N = 14-22 for pre-exposure conditions; N = 13-19 at 8 h, 12-17 at 24 h, 7-15 at 48 h, 7-14 at 72 h, 5-12 at 96 h and 3-13 at 120 h.

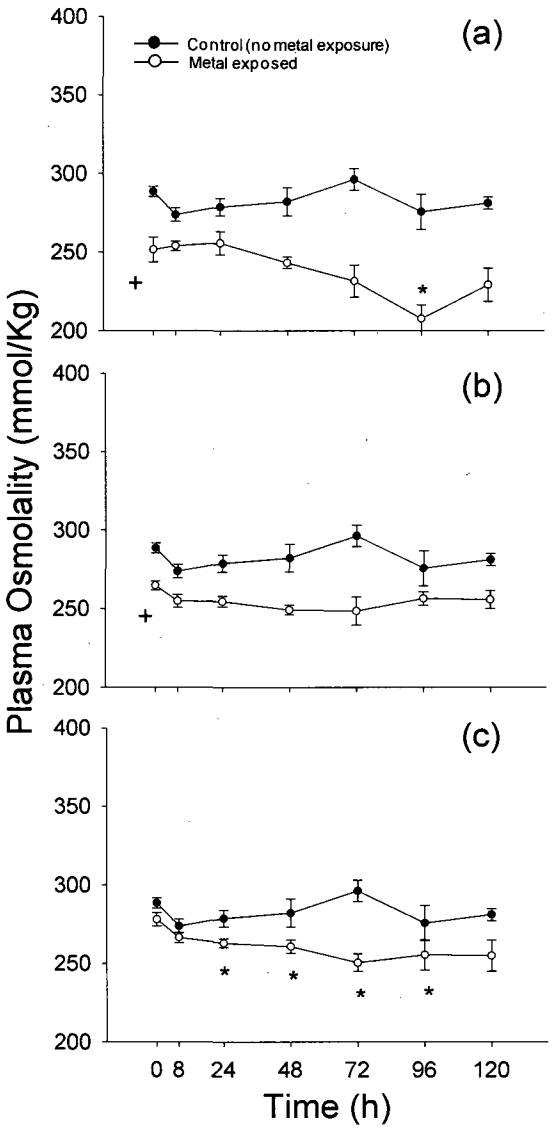


Figure 2.9 Plasma osmolality from rainbow trout exposed to control and **(a)** 7.4 ± 0.3 $nmolL^{-1}$ Cd alone **(b)** 26.1 ± 1.6 $nmolL^{-1}$ Pb alone or **(c)** 6.9 ± 0.4 $nmolL^{-1}$ Cd plus 45.4 ±1.9 $nmolL^{-1}$ Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. An asterisk (*) indicates significant difference from pre-exposure condition for that treatment ($P < 0.05$; Dunnett's one way ANOVA). A cross (+) indicates significant differences from control group at $t = 0$ ($P < 0.05$; unpaired t-test). N = 7-14 for pre-exposure conditions; N = 7-13 at 8 h, 6-12 at 24 h, 6-15 at 48 h, 6-14 at 72 h, 3-12 at 96 h, and 2-13 at 120 h.

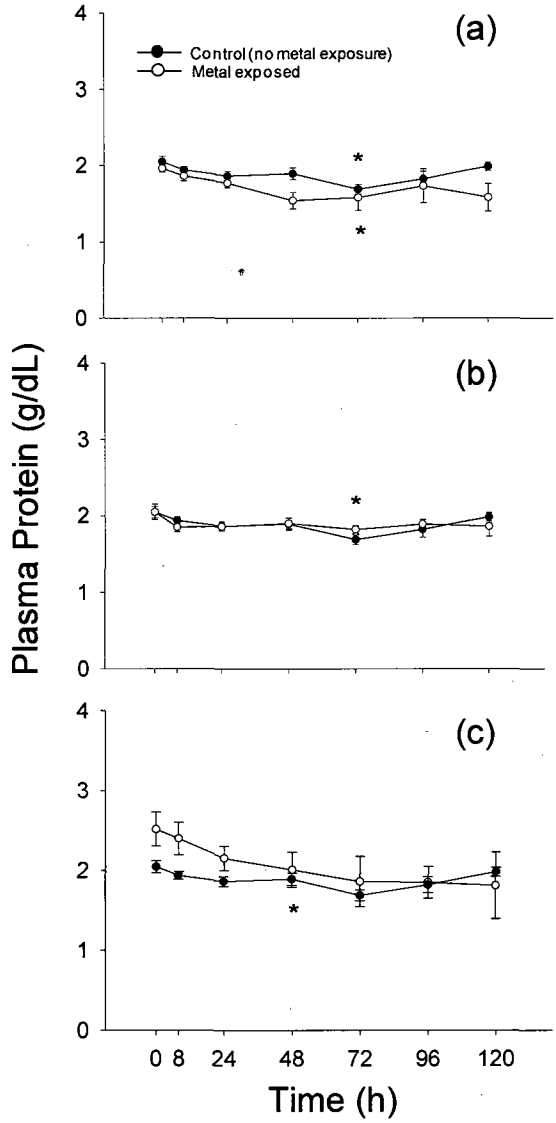


Figure 2.10 Plasma protein from rainbow trout exposed to control and **(a)** 7.4 ± 0.3 $nmolL^{-1}$ Cd alone **(b)** 26.1 ± 1.6 $nmolL^{-1}$ Pb alone or **(c)** 6.9 ± 0.4 $nmolL^{-1}$ Cd plus 45.4 ±1.9 $nmolL^{-1}$ Pb. Shaded circles represent control fish not exposed to metals while open circles represent fish exposed to metals. An asterisk (*) indicates significant difference from a pre-exposure condition for that treatment ($P < 0.05$; Dunnett's one way ANOVA). A cross (+) indicates significant differences from control group at $t = 0$ ($P < 0.05$; unpaired t-test). N = 14-22 for pre-exposure conditions; N = 13-19 at 8 h, 12-17 at 24 h, 7-15 at 48 h, 7-14 at 72 h, 5-12 at 96 h and 3-13 at 120 h.

Chapter 3

Changes in Ion Uptake, Outflux and Net flux in Rainbow Trout Exposed to Pb plus Cd Mixtures

ABSTRACT

Exposure of fish to Pb and Cd individually is known to disrupt Ca^{2+} homeostasis due to competition with Ca^{2+} for binding sites on the apical membrane of fish gills and the subsequent inhibition of active Ca^{2+} uptake mechanisms at the gill. Pb however is also known to inhibit Na^+ uptake mechanisms. Little is known about combined Pb and Cd interactions at the level of the gill. Indeed, fish are more likely to be exposed to metal mixtures in contaminated aquatic systems. The purpose of this study was to monitor Na^+ influx, net flux and outflux at the gills of rainbow trout in soft acidic water using the radiotracer $^{24}Na^+$ to describe how Pb plus Cd mixtures interfered with gill-mediated ion uptake and loss. Exposure to either Pb alone ($40nmolL^{-1}$) and Cd alone ($6nmolL^{-1}$) caused an immediate significant decrease (50% and 33% respectively) to Na^+ influx. However this effect was not observed at higher Cd concentrations, or during Pb plus Cd exposures. Branchial Na^+/K^+-ATPase activity was not inhibited by Pb, Cd, or Pb plus Cd mixtures. Nevertheless, it remains likely that the inhibition of Na^+ influx by Cd was due to inhibition of this protein (Na^+/K^+-ATPase) *in vivo,* while Pb-induced inhibition of intracellular carbonic anhydrase inhibited Na^+ uptake. Decreased binding of Pb to high affinity, low capacity binding sites due to competition with Cd may explain the lack of impact on Na^+ uptake observed during Pb plus Cd exposures. Moreover, the results provided here suggest a protective effect of Pb and Cd in combination caused by the possible differences in the affinity of physiological active Pb-gill and Cd-gill binding sites within the gill and give further insights to the complex gill interactions of Pb and Cd.

INTRODUCTION

Lead and cadmium are two metals that are commonly co-released into aquatic ecosystems. The majority of input is from anthropogenic sources such as mining and smelting operations, and due the improper disposal of batteries and transistor boards (Sorensen 1991). The weathering of rock and volcanic activity are natural sources of these metals (World Health Organization, 1995; Zabel, 1993). The toxic mechanisms of action of Pb and Cd have been studied thoroughly (Pb: MacDonald *et al.* 2002; Rogers *et al.* 2003, 2004; Cd: Verbost 1987; Hollis *et al.* 1997; Chowdhury *et al.* 2004), with less attention paid to understanding how these metals act in combination (c.f. Reader *et al.* 1989; Birceanu *et al.* 2008; Komjarova and Blust, 2009). In most metal contaminated waters, aquatic organisms are more likely to be exposed to a mixture of metals, rather than a single metal (see Norwood *et al.* 2003 for review).

Both Pb and Cd are thought to act as Ca^{2+} antagonists which compete with Ca^{2+} for apical calcium channel sites on the gill (Verbost *et al.* 1989; Pratap and Wendelaar-Bonga, 1993; McDonald *et al.* 2002; Playle *et al.* 1993; Niyogi and Wood, 2004; Niyogi *et al.* 2008; Rogers *et al.* 2004). In rainbow trout (*Oncorhynchus mykiss*), Ca^{2+} uptake takes place via epithelial Ca^{2+} channels (eCaC) that may be found on both mitochondria rich cells (MRCs) and possibly pavement cells in the gills (Shahsavarani *et al.* 2006). Thus, both Pb and Cd would be expected to compete with Ca^{2+} and inhibit active Ca^{2+} uptake to the blood causing hypocalcaemia and eventual death. This also involves the impairment of Ca^{2+}-ATPases (Verbost *et al.* 1988; Rogers *et al.* 2004).

In addition to the disruption to calcium homeostasis, Pb is known to interfere with Na^+ uptake via inhibition of the basolateral Na^+/K^+-ATPase (Rogers *et al.* 2003). This

effect is compounded by reductions to both Na^+ and Cl^- uptake via Pb-induced inhibition of the cytosolic carbonic anhydrase (Rogers *et al.* 2003, 2005), which decreases the rate of intracellular CO_2 hydration and the subsequent H^+ and HCO_3^- generation needed to sustain apical Na^+ uptake via the Na^+ channel-H^+-ATPase system and Cl^- uptake via Cl^- /HCO_3^-exchange (see Marshall 2002, Evans *et al.* 2005 for reviews of branchial ion uptake mechanisms).

Birceanu *et al.* (2008) reported that rainbow trout exposed to Pb alone (approximately ~ 125nmolL^{-1}) experienced a significant decrease in Na^+ uptake, while exposure to Cd alone (~ 50nmolL^{-1}) caused a significant decrease in Ca^{2+} influx. Furthermore, when the fish were exposed to Pb and Cd in combination, the respective Pb and Cd induced effects on Na^+ and Ca^{2+} uptake were greater than when exposed to each metal individually. The purpose of this study was to determine if these greater than additive effects on Na^+ influx seen during Pb and Cd exposure persist over longer periods of time (~5 days) and to establish if the effects seen can be explained by gill-metal accumulation. It was hypothesized that over longer periods of time that there would be a persistent decrease of Ca^{2+} and Na^+ influx in the presence of Pb and Cd mixtures, and that a decrease in Na influx would be reflective of greater Pb and Cd gill binding.

To determine how longer-term Pb plus Cd exposure affected ionoregulation in fish, trout were exposed to Pb plus Cd mixtures and the rates of Na^+ influx, outflux (efflux), and net flux was measured using the radio-tracer $^{24}Na^+$. This unidirectional ion flux technique is a useful tool for quantifying the inward, outward and net movements of ions across the gill. To mimic the waters found in the Canadian Shield, all experiments were done in soft, slightly acidic (pH ~ 6.2) water.

MATERIAL AND METHODS

Fish Husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*) (7.9 ± 0.4g) purchased from Rainbow Springs Trout Hatchery, Thamesford, ON, were initially held in a 180 L flow through aerated tank supplied with well water (400-500mL min^{-1}) in which the measured conductivity was ~1000μS. Over the course of 1 week the fish were acclimated to soft water by increasing the ratio of reverse osmosis (RO) generated soft water to well water. At the end of one week, the trout were ultimately held in a combination of 400-500 $mLmin^{-1}$ flowing RO water and 13-14 $mLmin^{-1}$ flowing well water to obtain holding water with a conductivity of approximately 50μS. The final holding conditions were approximately ~ 35-50μS (~100μmolL^{-1} Ca^{2+}), Na^{+} ~ 200 μmolL^{-1}, pH ~ 6-6.5, 8-14 °C. The fish were allowed to acclimate under these conditions for at least 2 weeks prior to experiments. Fish were fed to satiation three times per week with commercial trout feed (Corey Mills Aquafeeds, Fredericton, New Brunswick) but were fasted 72 h prior to experiments. All fish husbandry procedures and experiments were approved by the Wilfrid Laurier Animal Care Committee, and followed Canadian Council of Animal Care (CCAC) guidelines.

Unidirectional Fluxes

The unidirectional movements (influx, outflux, net flux) of Na^{+} across the gills of the trout during Pb, Cd, or Cd plus Pb mixture exposure were measured using the radio-tracer $^{24}Na^{+}$. The radio-tracer $^{45}Ca^{2+}$ (2.5 μCi) was also added to each container because an original objective of these experiments was to simultaneously measure Ca^{2+}

unidirectional flux movements across the gills. Unfortunately, due to unforeseen technical problems, this was not possible. Accordingly, only unidirectional Na^+ movements are reported in this thesis.

Approximately 12-24 h prior to an experiment, 8 fish were transferred into individual, aerated darkened plastic containers (approximate volume 450 mL) receiving control water (62.5 ± 2.0 $\mu molL^{-1}$ Ca^{2+}, 185.3 ± 1.7$\mu molL^{-1}$ Na^+, pH 6.2 ± 0.01, conductivity 47.7 ± 0.7 μS and 13.7 ± 0.2 °C). The fish holding boxes were held within a 100 L recirculation system maintained at pH 6-6.2 using the same pH titration set-up described in Chapter 2.

Rates of Na^+ influx, outflux and net flux were measured under control (nominally metal-free) conditions and at regular intervals (0-3 h, 6-9 h, 22-25 h, 46-49 h, 70-73 h) during Pb, Cd, or Pb plus Cd exposure. At the beginning of each 3 h flux period water flow to the boxes (flux chamber) was stopped and the water volume adjusted to 250-300 mL in each flux chamber. This was followed by the addition of 2.5 μCi of ^{45}Ca and 2.5 μCi of ^{24}Na to each chamber. After a 10 minute mixing period, 15 mL water samples were taken hourly using a 20 mL plastic disposable syringe during 3 h flux measurement periods. At the end of each 3 h flux an additional 5 mL water sample was taken for determination of dissolved metal concentrations, and water flow was re-established to each container. At the end of the control flux, however, the boxes were not flushed, but water volume re-adjusted to 250 mL, and extra isotope added to the container to compensate for $^{24}Na^+$ loss due to radioactive decay (the half-life of $^{24}Na^+$ is 15 h) and sampling. The boxes were then spiked with the appropriate metals (final measured concentrations of 6.3 ± 0.6$nmolL^{-1}$ and 14.1 ± 0.8$nmolL^{-1}$ Cd alone, 40.2 ± 2$nmolL^{-1}$ Pb

alone, 6.9 ± 0.9nmolL^{-1} Cd plus 57.3 ± 8 nmolL^{-1} Pb and 17.6 ± 0.7nmolL^{-1} Cd plus 45.6 ± 4 nmolL^{-1} Pb) followed by the addition of the appropriate metals to the remainder of the system which was still in recirculation mode.

Water samples were then processed for determination of total cpm (counts per minute) of both ^{45}Ca and ^{24}Na by adding 4 mL of aqueous counting scintillant (Amersham Biosciences, England) to 2 mL water samples. Total Beta cpms were counted in triplicate using a Beckman-Coulter Multi Purpose Scintillation counter (Model LS6500, USA). Due to the fact that ^{45}Ca and ^{24}Na produce radiation spectra within a similar range, total counts were first measured immediately after the 3 hour flux period and then the samples were left to allow the ^{24}Na to decay (8 days) before counts were determined again for ^{45}Ca. Accordingly, ^{24}Na radioactivity was determined by subtracting the cpm due to ^{45}Ca from the total cpm measured immediately following the flux period. To ensure that chemilumenescence was not affecting the measured cpm, trial samples that were radioactive (n = 3) were counted immediately after sampling (within 30 min) and then measured again 24 h later in a preliminary experiment to ensure that the cpm did not change. The remaining water sample was saved for quantification of non-radioactive ("cold") Na^+, Ca^{2+}, Pb and Cd.

At the end of the 72 h metal exposure period, surviving fish were euthanized by a blow to the head and the whole gill basket was removed, rinsed in deionized water for 10 seconds and cut in half. Each half was placed in separately labeled micro centrifuge tubes, frozen in liquid N_2 and stored at -80°C for later determination of Na^+/K^+-ATPase activity and metal-gill accumulation.

Analytical Techniques

Gill Na^+/K^+-ATPase Activity Determination

To determine the effects of Cd and/or Pb on branchial Na^+/K^+-ATPase activity, gills were processed following the methods described by McCormick *et al.* (1993) in which Na^+/K^+-ATPase activity was calculated based on the difference between total branchial ATPase activity and ouabain inhibited branchial Na^+/K^+-ATPase activity. The assay solutions consisted of 2.8 $mmolL^{-1}$ phosphoenolpyruvate (PEP), 3.5 $mmolL^{-1}$ ATP, 0.495 $mmolL^{-1}$ NADH, LDH (4 U mL^{-1}) pyruvate kinase (PK; 5 U mL^{-1}) in imidazole buffer (50$mmolL^{-1}$) at pH 7.5. A salt solution containing 189 $mmolL^{-1}$ NaCl, 10.5 $mmolL^{-1}$ MgCl ·6H2O, 42$mmolL^{-1}$ KCl prepared in de-ionized water was added to the assay solution (containing enzymes) in a 3:1 ratio (with and without the addition of 0.5$mmolL^{-1}$ouabain the day of the experiment). The Na^+/K^+-ATPase activity was then determined on gill homogenates.

The gills were first weighed (approximately 30mg of tissue) and homogenized in a 4:1 (400µL:100µL) ratio of SEI buffer (150$mmolL^{-1}$ sucrose, 10$mmolL^{-1}$ EDTA and 50$mmolL^{-1}$ imidazole buffer) to SEID buffer (SEI with the addition of 0.1g sodium deoxycholate), and homogenized on ice using a hand-held motorized pestle. Total activity was then measured by monitoring the decrease in absorbance (at 340 nm) over 10 min in the presence or absence of ouabain (0.5 $mmolL^{-1}$) when 10µL of sample was added to micro-well plates with the addition of 200µL of salt solution. The protein concentration in the homogenates was determined by the Bradford assay (Bradford, 1976).

Water and Gill Analysis

Gills used for metal analysis were first thawed, weighed and digested in 5 times their weight in 1N Trace Metal Grade HNO_3, and then baked for 3 h at 80°C. The resulting digests were vortexed and centrifuged for 2 minutes at 12 000 rpm, and the supernatant collected and diluted ~10-100 times (as appropriate) using 1% HNO_3 (Trace Metal Grade; EMD Chemicals Inc, Germany) for subsequent Cd and Pb analysis. Total Ca^{2+} and Na^+ in the water was determined by flame atomic absorption (FAA) spectrophotometry (Spectra 880 Atomic Absorption, Varian, Mississauga, ON.). Water and gill Cd and Pb were quantified using graphite furnace (GTA100 atomizer, SpectrAA 880, N_2 gas; Varian, Mississauga, ON), and concentrations validated using precision standards (Environment Canada).

Calculations and Statistical Analysis

To differentiate between the individual isotopes within the dual labeled water samples, the total counts (cpm due to $^{24}Na^+$ plus cpm due to $^{45}Ca^{2+}$) were measured immediately after sampling. Because ^{24}Na has such a short half life (~15 h) the samples were counted again after the ^{24}Na cpms were fully exhausted (~14 d), which yielded the radioactivity (cpm) due to ^{45}Ca. The ^{45}Ca counts were then subtracted from the total counts to get the ^{24}Na cpm.

To account for decay of $^{24}Na^+$ (half-life = 15 h) between sampling and the time of measurement, each sample was half-life corrected back to the time that each 3 h flux period began (t = 0) using the following calculation:

$$A = A_o \times e^{-0.693(t/t1/2)} \quad (1)$$

Where A, is the activity (cpm/mL) at time t, A_o is the activity at t = 0 h, $t_{1/2}$ is the half-life of $^{24}Na^+$ in h, and t is the elapsed time in h.

The Na^+ influx rates were calculated using the following formula:

$$Na^+ \text{ influx rate} = \frac{(\text{initial CPM} - \text{final CPM})*V}{MSA * M * t} \quad (2)$$

Where CPM is counts per minute per mL of sample, V is the volume of water in each flux chamber, M is the mass of the fish in kg, t is the time elapsed during the flux period (h), MSA is the mean specific activity in which:

$$MSA = ((cpm)/[Na^+]) \quad (3)$$

where MSA is the respective cpm due to $^{24}Na^+$ per μmol of "cold" Na^+.

The net flux rates of Na^+ were determined using the following formula:

$$\text{Net Flux} = \frac{[([Na^+]_i - [Na^+]_f) \times V]}{M \times t} \quad (4)$$

Where $[Na^+]_i$ and $[Na^+]_f$ refer to the initial and final concentrations of $^{24}Na^+$ in the water, V, t, and M are as previously described (Wood 1992; Matsuo *et al.* 2004).

The outflux rates of Na^+ were calculated as the difference between net flux and influx rates respectively using the formula:

$$\text{Outflux} = (\text{Net flux} - \text{Influx}) \quad (5)$$

Where the net flux and influx rates were calculated using formulas 2 and 4 above.

All data is presented as the mean ± one S.E.M (N). Comparisons between control and experimental fish were made using one-way ANOVA. Where significant variability was observed, significant differences between the means were determined using a Tukey-Kramer post-test for gill accumulation and Na^+/K^+-ATPase activity, and Dunnett's post-test for flux measurements at the $p<0.05$ level. Where necessary, data was log transformed prior to statistical analysis.

RESULTS

Gill Cd and Pb Accumulation and Mortality

Control measurements of gill-metal accumulation were made on nine fish that were held under the same low pH, soft water conditions as metal exposed fish. Background gill-Pb and gill-Cd concentrations in control fish were near 1.8 $nmolg^{-1}$ wet weight and 5 $nmolg^{-1}$ wet weight, respectively (Figure 3.1). There was substantial Pb-gill accumulation in fish exposed to Pb alone, and the two Pb plus Cd mixtures, in which Pb-gill concentrations approached 26 $nmolg^{-1}$ wet weight, but there were no significant differences in Pb-gill binding between the two treatments (Figure 3.1A). Exposure to Cd resulted in significantly greater Cd-gill accumulation in the low Cd exposure group (nominal Cd = 6 $nmolL^{-1}$), with slightly greater accumulation seen (~7 nmol g^{-1} wet weight) in fish exposed to low Cd plus Pb mixture (Figure 3.1B). Only one fish died during exposure to high Cd (~ 17 $nmolL^{-1}$) plus Pb (~ 45 $nmolL^{-1}$) treatment after 48 h of exposure. There were no other mortalities throughout the experiments.

Na^+ Influx, Net flux and Outflux

Measurements of Na^+ influx, net flux and outflux were measured in rainbow trout exposed to Cd alone (6.3 and 15 $nmolL^{-1}$), Pb alone (40 $nmol^{-1}$), and Cd plus Pb mixtures. Low levels of Cd alone (6.3 $nmolL^{-1}$) significantly reduced Na^+ influx by 33% after 6-9 h, and this inhibition worsened through to 70-73 h where Na^+ influx was completely blocked. Despite the inhibited Na^+ influx, no significant net losses of Na^+ were observed due to accompanying decreases in Na^+ outflux (Figure 3.2).

Exposure to Pb alone (40nmolL^{-1}) significantly decreased Na^+ influx by 50% after 0-3 h of exposure leading to an increase in net Na^+ loss at 46-49 h (Figure 3.3). However, this net loss was corrected by 70-73 h due to a corresponding reduction in Na^+ outflux at this time (not significant) (Figure 3.3)

Exposure to a mixture of low Cd plus Pb (6 and 57 nmolL^{-1}, respectively) had no significant effect on Na^+ influx, but there was a slight change in net Na^+ flux from negative to positive (not significant) after 70-73 h of exposure (Figure 3.4). The higher concentration of Cd alone (14 nmolL^{-1}) also had no effect on Na^+ influx or net Na^+ flux, and Na^+ outflux was significantly lower at 6-9 h compared to controls (Figure 3.5). This was followed by a recovery of Na^+ influx to control levels by 22-25 h. The highest mixture combination of 17nmolL^{-1} Cd plus 40nmolL^{-1} Pb exposure also had no effect on Na^+ influx, outflux or net flux (Figure 3.6).

Impact on Active Transport

Na^+/K^+-ATPase activity was measured in fish exposed to Cd and Pb individually and in combination for 72 h. Total activity increased in all exposure groups compared to controls after 72 h. Notably, the mean Na^+/K^+-ATPase activity was significantly (65%) greater in the high Cd plus Pb mixture treatment compared to controls (Figure 3.7).

DISCUSSION

The present study demonstrates that exposure to Cd and Pb individually can significantly inhibit gill-mediated Na^+-uptake by fishes, but that the disturbances are less pronounced when the fish are exposed to mixtures of these metals. These findings do not support the hypothesis that mixtures of Cd plus Pb would have a greater than additive impact on Na^+ and Ca^{2+} influx in trout, or that this response would be concentration dependent (i.e. the greater the total metal, the greater the effect). Thus, the greater than additive decreases in Na^+ uptake compared to Pb alone observed by Birceanu *et al.* (2008) do not appear to persist beyond the first few hours of metal mixture exposure, despite substantial Cd- and Pb-gill accumulation during the exposure period.

Gill-metal Accumulation

Decreases in both Cd-gill and Pb-gill accumulation due to competition with calcium ions have been reported (Verbost *et al.* 1987; Verbost *et al.* 1989; MacDonald, *et al.* 2002; Grosell *et al.* 2006; Niyogi *et al.* 2008). The gill-Cd accumulation seen after 72 h of exposure to low (6.3 $nmolL^{-1}$) and high (15 $nmolL^{-1}$) Cd concentrations in this study would suggest the saturation of only the high-affinity low-capacity binding population (Bmax~1.73 $nmolg^{-1}$; Birceanu *et al.* 2008). When "new" gill-Cd is calculated by subtracting background metal from exposure metal, the highest Cd concentration causes only ~ 4 nmol g^{-1} wet weight of Cd-binding. The B_{max} calculated by Birceanu *et al* (2008) for the second low-affinity high capacity population of binding sites is ~ 13.7 nmol g^{-1}, a level that is not reached in this study. Furthermore, Pb accumulation also reaches the respective calculated B_{max} for the high affinity low capacity binding sites

(18.2 nmol g^{-1}) (Birceanu *et al.* 2008). It is these low capacity high affinity binding sites that are considered to represent the physiologically active sites within the gill such as apical Ca^{2+} channels and basolateral Ca^{2+}-ATPases (Niyogi and Wood. 2004a,b). The second low affinity population of Pb and Cd binding sites is suggested to bind metals less specifically and therefore accumulate metals slowly over longer exposure periods (Niyogi and Wood, 2004; Niyogi *et al.* 2008). These high affinity sites are assumed to accumulate metals quickly, and only after they are saturated do metals bind to the low affinity, high capacity sites, which are considered to be less biologically active and contribute to the gradual increase of metal burden over longer periods of exposure (Niyogi *et al.* 2008).

Using the assumption that both Pb and Cd compete for similar binding sites on the gill, the amount of Cd and Pb accumulation seen in this study (greater than the B_{max} for Cd and the B_{max} for Pb) should have been enough to elicit a physiological effect, but this was not necessarily the case with Pb plus Cd mixtures.

Effects of Pb-gill and Cd-gill Binding on Unidirectional Movements of Na^+ Across the Gill

Unidirectional Na^+ fluxes were measured on rainbow trout exposed to low (~ 6 $nmolL^{-1}$) and high (~15 $nmolL^{-1}$) concentrations of Cd alone, and in combination with Pb (~ 40$nmolL^{-1}$) to study the interactions of more environmentally relevant metal concentrations at the gill. Exposure to low Cd caused a significant decrease in Na^+ uptake (33%) after 8 h of exposure (Figure 3.2). The effect was persistent and led to a complete inhibition of Na^+ influx after 72 h of exposure. This result contradicts some studies that have found Cd to have no effect on internal Na^+ balance or Na^+ uptake (Reid and

McDonald, 1988; Verbost, 1989; Chowdhury *et al.* 2004). The lack of evidence suggesting that Cd disturbs Na^+ homeostasis could be because Cd is normally thought of as a Ca^{2+} antagonist, and therefore less attention has been given to its possible effects on Na^+ uptake in aquatic organisms (Komjarova and Blust, 2009). Moreover, the majority of studies have focused on high concentrations of Cd in fish acclimated to harder waters (high Ca^{2+}) that may not be representative of the situation in soft (low Ca^{2+}) more acidic waters (e.g. pH 6-6.5).

It has been suggested that Na^+ uptake occurs via differing mechanisms in soft compared to hard water situations (Boisen *et al.* 2003). Boisen *et al* (2003) observed that the proton-ATPase inhibitor bafilomycin and Na^+-channel blocker amiloride had no effect on Na^+ influx in soft water acclimated zebrafish (*Danio rerio*) , but inhibited Na^+ uptake in hard water acclimated zebrafish. This finding therefore raises the possibility that the mechanism(s) of toxicity for metals, such as Cd or Pb, could differ in soft versus hard water. Indeed the possibility that lower concentrations of metals in soft water may have differing mechanisms of toxicity has been raised previously (Wood, 1992). Further evidence is provided by Komjarova and Blust (2009) who demonstrated a 50 % decrease in Cd uptake rates with increasing external Na^+ levels in trout exposed to low Cd concentration ($12 nmol L^{-1}$) in soft water. These Cd/Na interactions at low Cd levels outlined by Komjarova and Blust (2009), and the absence of Na^+ uptake inhibition at higher levels seen in this study may suggest that low levels of Cd can cause unexpected effects on Na^+ uptake compared to higher Cd concentrations.

Cd is also known to affect intracellular enzymes and energy dependent ion channels (Verbost *et al.* 1989; Atli and Canli, 2007, Lionetto *et al.* 2000). A decrease in

Na^+/K^+-ATPase activity caused by Cd has been demonstrated in different fish species over a range of concentrations ($0.1nmolL^{-1}$-$5\mu molL^{-1}$) (Atli and Canli, 2007; Pratap and Wendelaar Bonga, 1993). Moreover, Atli and Canli (2007) observed a greater decrease in gill Na^+/K^+-ATPase activity in tilapia (*Oreochromis niloticus*) exposed to lower ($5\mu molL^{-1}$) Cd concentrations compared to higher concentrations (up to 20 $\mu molL^{-1}$) in freshwater, but no explanation was provided for this observation. Lionetto *et al* (2000) also observed a decrease in CA activity in eel (*Anguilla anguilla*) gill homogenates during exposure to Cd with a calculated IC50 (~$9.97nmolL^{-1}$) for branchial CA in hard water. Cd-induced inhibition of intracellular CA in trout may therefore be an alternate or additional mechanism to explain reduced Na^+ influx caused by low Cd in trout because CA provides the H^+ needed to drive Na^+ uptake at the gill via the hydration of CO_2 into HCO_3^- and H^+ (see Perry, 1997 for a review). Such intracellular mechanisms may explain why a divalent metal such as Cd^{2+} can inhibit the movements of a univalent ion such as Na^+.

A variety of metals such as Cu^{2+} (Reid and McDonald, 1988; Schjolden *et al.* 2007) and Hg^+ (Klinck *et al.* 2005) interfere with Na^+ regulation through competitive binding at the site of Na^+ uptake into the gill (i.e. Na^+-channels). Most metals however, do not have specific transporters associated with metal uptake such as the ones identified for copper (Grosell and Wood, 2002). Like Cd^{2+}, Pb^{2+} is also one of the metals that is known to have an impact on Na^+/Cl^- regulation caused by its effects on Na^+/K^+-ATPase and cytosolic CA (Rogers *et al.* 2004). Because of the additional impacts of Pb seen on Ca^{2+} homeostasis are due to interactions with apical Ca^{2+}-channels and basolateral Ca^{2+}-

ATPase, Pb is considered to have characteristics half-way between the Ca^{2+} and Na^{+} inhibiting metals (Rogers *et al.* 2003; 2004; 2005; MacDonald *et al.* 2002).

The immediate decrease in Na^{+} influx at the gill (after 3 h) in trout exposed to low levels of Pb ($40nmolL^{-1}$) agrees with studies that have shown Pb to interfere with Na^{+} and Cl^{-} homeostasis. For example, Rogers *et al* (2003, 2005) observed a decrease in Na^{+} influx accompanied by an increase in outflux in fish exposed to Pb ($\sim 0.5\mu molL^{-1}$). This was likely to be caused by interactions with cytosolic CA, which subsequently caused a decrease in Na^{+} uptake. In addition these metals have been shown to have effects on the active uptake sites on the basolateral membrane (i.e. Na^{+}/K^{+}-ATPase) (Laurent and McDonald, 1987; Atli and Canli, 2007).

Although the movements of Na^{+} across the gill are clearly disturbed by both low Pb and low Cd alone, similar disturbances were not observed when trout were exposed to mixtures of these metals (Figures 3.4-3.6) despite significant Cd- and Pb-gill accumulation. This result does not support the hypothesis that Cd and Pb in combination would have greater than additive effects on Na^{+} uptake at the gills as reported by Birceanu *et al* (2008) during short term (3 h) Pb plus Cd exposures. However, Birceanu *et al* (2008) only observed a significant decrease in Na^{+} uptake (3 h) at higher Pb and Cd concentrations ($50nmolL^{-1}$ Cd and $30nmolL^{-1}$ Pb) and not the lower ones used in this study. This difference may not, however, explain the reduction in toxicity seen during Pb plus Cd exposures (addressed in Chapter 4).

Entrance of both Cd and Pb into the gill occurs via apical Ca^{2+}-channels (Rogers *et al.* 2005; Verbost *et al.* 1987; 1988; 1989). Therefore, competition in the presence of both metals at this site of entry is expected to cause a decrease in gill accumulation

during acute exposures (Playle, 2004). However, greater accumulation of Cd in the presence of Pb, and no change in Pb accumulation with the addition of Cd suggests that instead of binding to the same site on the gill (i.e. high affinity, low capacity binding sites) the metals also bind to different sites (i.e. higher capacity, lower affinity sites) (Birceanu *et al.* 2008; Niyogi *et al.* 2008). The competition of Pb and Cd may explain the lack of disruption to Na^+ uptake in this study. Because of the competition, Pb and Cd cannot both enter the gill at the same rate, therefore delaying the effects caused by the individual metals. Perhaps Pb is out competed by Cd due to the higher affinity of Cd for binding sites on the gill (LogK Cd ~ 7.33; Pb ~7.05) (Birceanu *et al.* 2008). If Cd is not as toxic internally (shown by the more gradual time to decrease Na^+ uptake compared to Pb; compare Figures 3.2-3.3) than perhaps this can also explain the lesser effects on Na^+ uptake in the fish exposed to Pb plus Cd compared to Pb only.

As described above, both Pb and Cd inhibit CA activity thereby causing a decrease in the electrochemical gradient necessary for Na^+ uptake (Rogers *et al.* 2005; Lionetto *et al.* 2000). Normally, the Na^+ electrochemical gradient is generated by the hydration of CO_2, which produces H^+ that is ultimately pumped out of the gill via H^+-ATPases (see above). Although both Pb and Cd disrupt Na^+/K^+-ATPases (Rogers *et al.*2004; Pratap and Wendelaar Bonga. 1993), there was a tendency to increase rates of Na^+/K^+-ATPase activity in fish exposed to Pb plus Cd for 72 h (Figure 3.7). Observations of increased Na^+/K^+-ATPase activity during Pb exposure is in accordance with studies that have demonstrated an increase in Na^+/K^+-ATPase activity in the presence of external or diet borne Pb (Atli and Canli, 2007; Alves and Wood, 2006). Atli and Canli (2007) demonstrated increased branchial Na^+/K^+-ATPase activity in Tilapia exposed to

$20\mu molL^{-1}$ Pb in hard water. In addition, Alves and Wood, (2006) observed an increase in Na^+/K^+-ATPase activity in the anterior intestine of trout exposed to a diet high in Pb. A Pb-induced increase of Na^+/K^+-ATPase activity during Pb plus Cd exposure may have maintained the electrochemical gradient needed to drive Na^+ uptake by the gill, and therefore overcome the decreased CA activity caused by Pb and possibly Cd. This mechanism of acclimation to Pb and Cd however may only be a temporary fix, because CA is also necessary for acid-base regulation within the gill (Wood, 1992; Perry, 1997).

In both the individual exposures to low levels of Cd or Pb, there was a decrease in outflux of Na^+ across the gill over time. This reduction may be explained by a decrease in permeability of paracellular junctions (regulated by hormones such as prolactin and cortisol), to regulate diffusive losses of ions, a process similarly seen in fish adapting to low pH environments (McDonald *et al.* 1991; Perry and Laurent, 1989). Ca^{2+} is thought to play an important role in stabilizing these tight junctions, thus reducing permeability (McDonald *et al.* 1991). At higher concentrations (threshold), diffusive outflux can increase via displacement of Ca^{2+} ions within tight junctions, however, an increase in diffusive Na^+ loss over time was not evident in this study (Wood, 1992).

Conclusions and Implications

The Pb-induced disruption of Na^+ uptake seen in this study is in accordance with studies that have identified Pb as having characteristics in between those metals which interfere with Na^+ or Ca^{2+} homeostasis. The Cd-induced disruption of calcium homeostasis has been well documented, but the impacts of Cd on Na^+ regulation at the gill have been for the most part neglected. This study has outlined how possible Cd^{2+}/Na^+ interactions may be more important in soft acidic waters. One explanation is that is the

mechanisms of Na^+ uptake could be different in soft vs. hard waters (Boisen *et al.* 2003) but further investigation is needed. The lesser inhibition of Na^+ uptake by Cd in the presence of Pb might be explained by competition between Pb and Cd for gill binding sites. Further investigations using radiotracers of Cd and Pb and subsequent gill morphology studies may help reconcile the exact location of metal accumulation within the gill. In addition, the use of Ca^{2+} and Na^+ channel blockers such as lanthanum and amiloride, and determining how the activity or expression of other active uptake mechanisms (i.e. Ca^{2+}-ATPase and CA) are affected by Pb and Cd, alone and in combination, could also make possible differences in uptake mechanisms clearer. Overall, the mechanisms of Pb and Cd uptake and toxicity examined here are very complex.

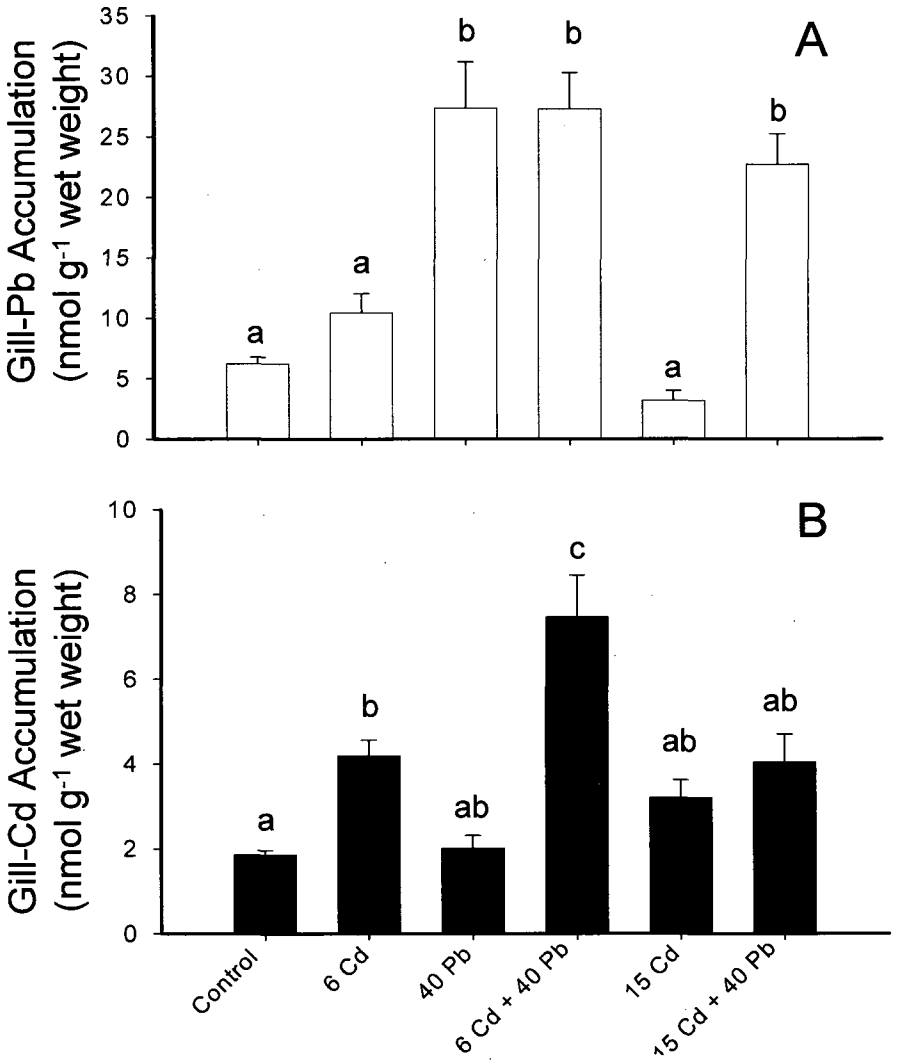


Figure 3.1 Pb-gill accumulation (A; grey bars) and Cd-gill accumulation (B; black bars) in rainbow trout exposed to Cd alone, Pb alone, and Cd plus Pb mixtures ($nmolL^{-1}$). All data is represented as the mean ± 1 S.E.M (n = 6-9). Lower case letters that are shared are not significantly different from one another within the same panel ($P < 0.05$).

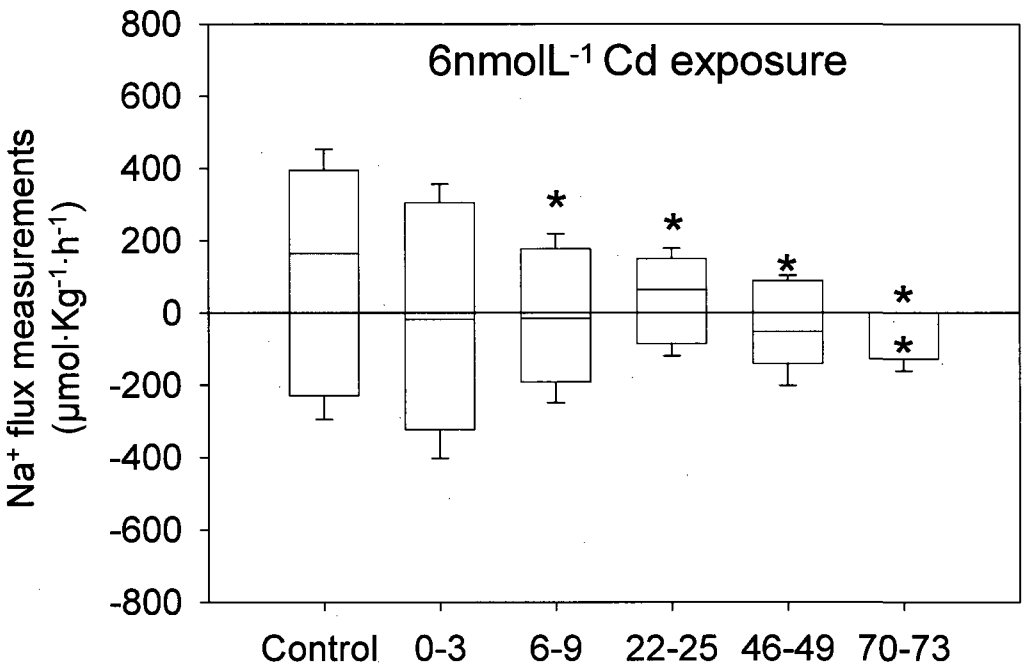


Figure 3.2 Unidirectional flux measurements of Na^+ in rainbow trout exposed to 6 $nmolL^{-1}$ Cd (low Cd) for 72 h. Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Error bars are not shown for net flux data. All other data presented as the mean ± 1 S.E.M (n = 4-8). An asterisk "*" indicates a significant difference from controls ($P < 0.05$).

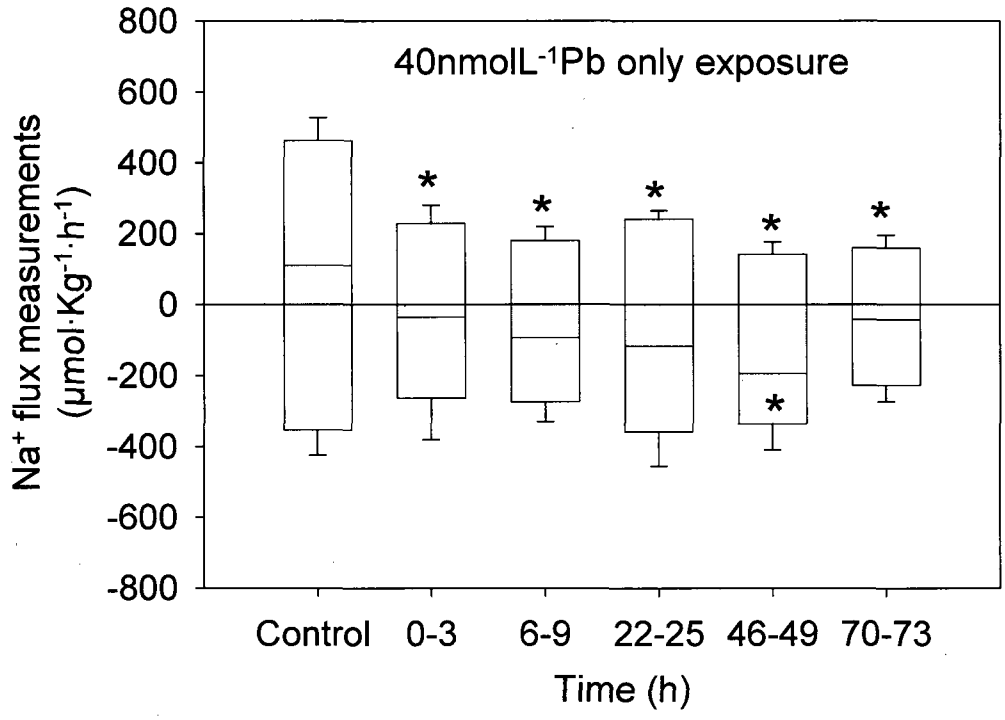


Figure 3.3 Unidirectional Na^+ flux of rainbow trout exposed to 40nmolL^{-1} Pb alone for 72 h. Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Error bars are not shown for net flux data. All other data presented as the mean ± 1 S.E.M (n = 6-8). An asterisk "*" indicates a significant difference from controls ($P < 0.05$).

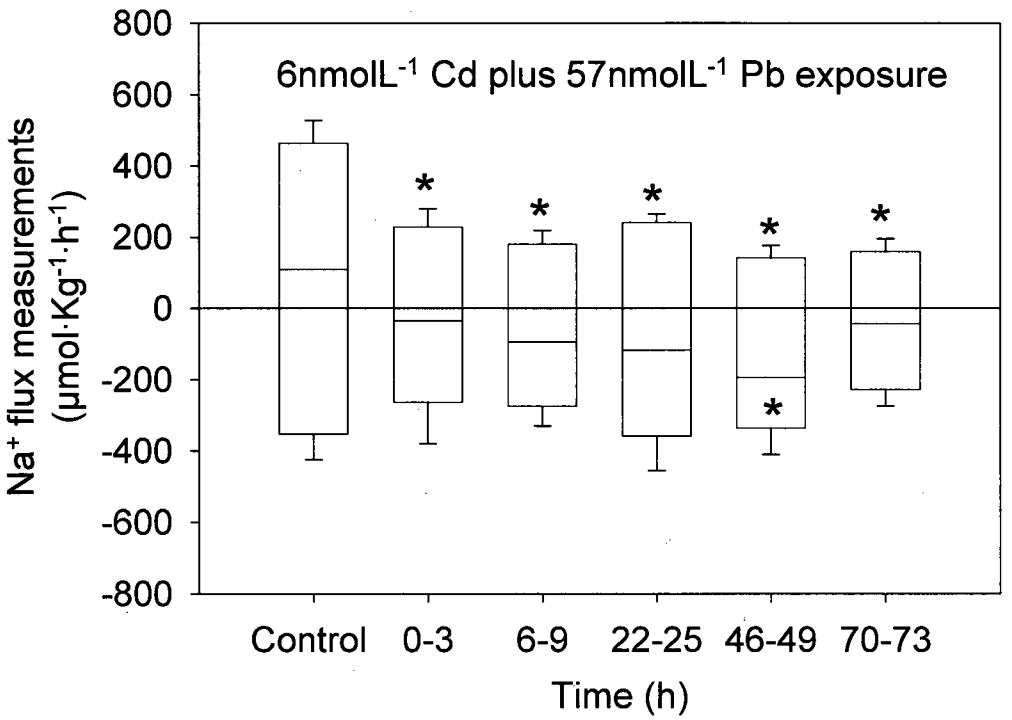


Figure 3.4 Unidirectional Na^+ flux of rainbow trout exposed to 6nmolL^{-1} Cd plus 57nmolL^{-1} Pb (low Cd plus Pb) for 72 h. Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Error bars are not shown for net flux data. All other data presented as the mean ± 1 S.E.M (n = 8). An asterisk "*" indicates a significant difference from controls ($P < 0.05$).

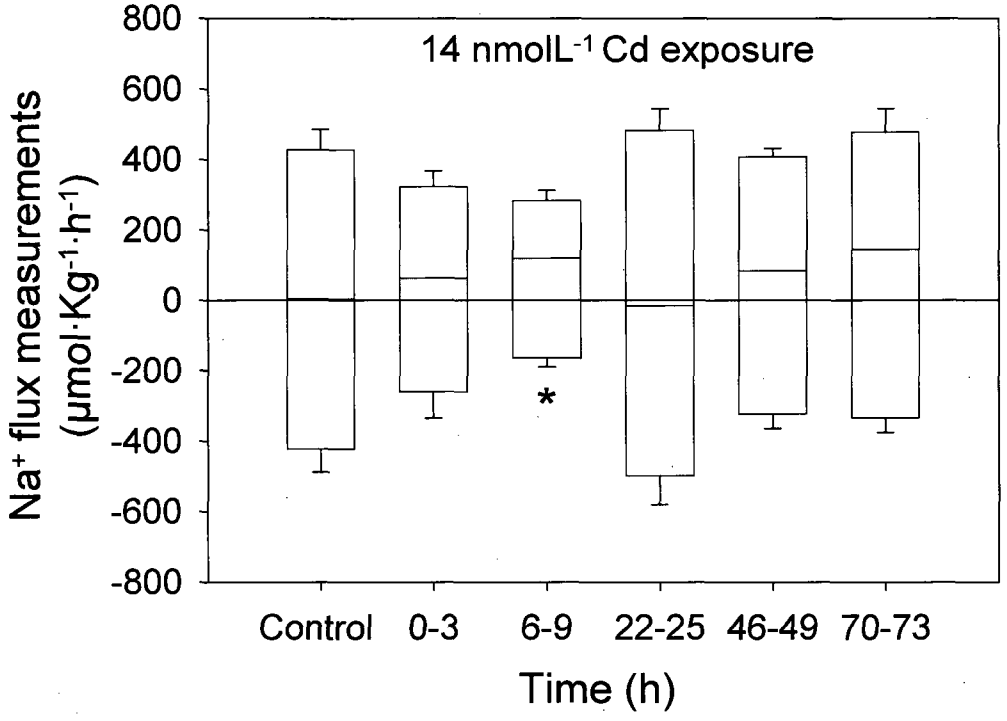


Figure 3.5 Unidirectional Na^+ flux of rainbow trout exposed to 14nmolL^{-1} Cd (high Cd) for 72 h. Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Error bars are not shown for net flux data. All other data presented as the mean ± 1 S.E.M (n = 7-8). An asterisk "*" indicates a significant difference from controls ($P < 0.05$).

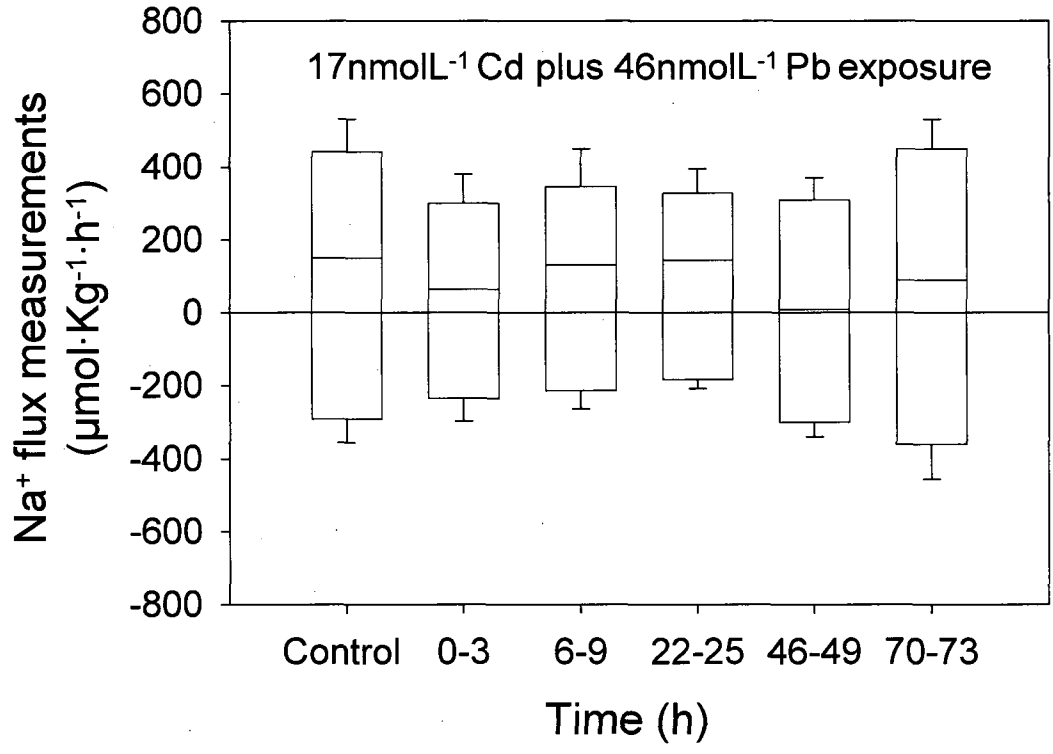


Figure 3.6 Unidirectional Na^+ flux of rainbow trout exposed to 17nmolL^{-1} Cd plus 45nmolL^{-1} Pb (high Cd plus Pb) for 72 h. Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Error bars are not shown for net flux data. All other data presented as the mean ± 1 S.E.M (n = 7-8). An asterisk "*" indicates a significant difference from controls ($P < 0.05$).

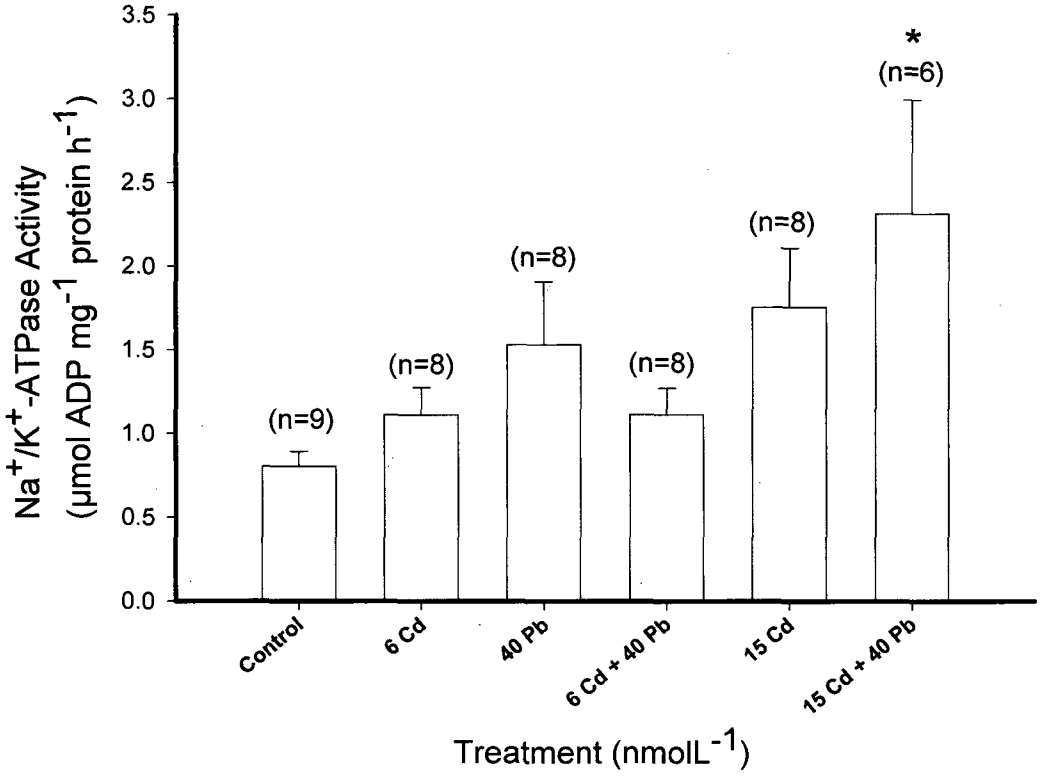


Figure 3.7 Na^+/K^+-ATPase activity measured in homogenates of rainbow trout gill following 72 h exposure to low (6 $nmolL^{-1}$) and high (15 $nmolL^{-1}$) Cd concentrations alone, Pb alone (40$nmolL^{-1}$), and in Pb (40$nmolL^{-1}$) plus Cd (15 $nmolL^{-1}$) mixtures. All data is represented as the mean ± 1 S.E.M (N). An asterisk "*" indicates a significant difference from controls ($P<0.05$).

Chapter 4

Using the Toxic Unit Approach to Predict Gill Accumulation and Toxicity of Pb and Cd mixtures

ABSTRACT

Biotic ligand models (BLM) in general are designed to predict acute toxicity in aquatic organisms exposed to metals in the environment. The BLMs use site specific water chemistry to predict the bioavailability and toxicity of metals to aquatic biota. To date however, such models do not account for metals in mixtures, a situation most commonly found in contaminated ecosystems. Predicting the toxicity of metals in mixtures is difficult, but there have been attempts to predict patterns of metal-mixture toxicity using the concentration addition model and the effects addition model. The concentration addition model uses the toxic unit approach, where one toxic unit (TU) represents the toxicity of each component in a mixture of toxicants (e.g. 96-h LC50). The total number of toxic units exposed to the organism is used to predict the toxicity (measured by gill accumulation) that the mixture will cause. The mixtures are assumed to behave in either a strictly additive manner if the effects (i.e. gill accumulation) of individual toxicants can be added together to produce the actual observed effect; less than additive manner, where the effect of toxicants in combination does not equal the sum of the effects caused by individual metals or greater than additive (synergistic), where the effects observed in exposure to mixtures is greater than the sum of the effects caused by toxicants. This research used the TU approach to describe the interactions between Pb and Cd, which are commonly released together in contaminated waters, and are thought to have a common site of toxic action on the gill (apical Ca^{2+} channels; eCac). It was hypothesized that exposure to a matrix of Pb plus Cd mixtures would result in less than additive Cd-gill metal accumulation due to competition between Pb and Cd for binding sites on the gills, and that toxicity would increase in a step-wise manner in accordance

with the total TU to which the fish were exposed. Accordingly, gill accumulation (3 h) and the 10 d LT50 were determined in rainbow trout (*Oncorhynchus mykiss*; 1-3g) exposed to a matrix of Pb and Cd concentrations (~0-24 $nmolL^{-1}$ Cd and ~0-2400$nmolL^{-1}$ Pb) in soft acidic water. Metal accumulation was not less than additive, however. Rather, Pb accumulation was augmented by Cd at during exposure to the two highest concentrations of Pb plus Cd while Cd accumulation was unchanged with the addition of Pb. Moreover, determination of the LT50s for the different metal mixture combinations indicated that Pb protected fish from Cd-induced toxicity. It is concluded that the interactions of Pb and Cd are more complex than expected and cannot be predicted by a simple concentration addition model. In addition, our results suggest some evidence for other types of interactions between Pb and Cd such as non- or anti-competitive inhibition. The presence of additional metal-binding sites, in addition to Ca^{2+} channels, on the gill for both Pb and Cd, may explain discrepancies in the ability to reliably relate the toxicity of Pb plus Cd mixtures to metal-gill binding.

INTRODUCTION

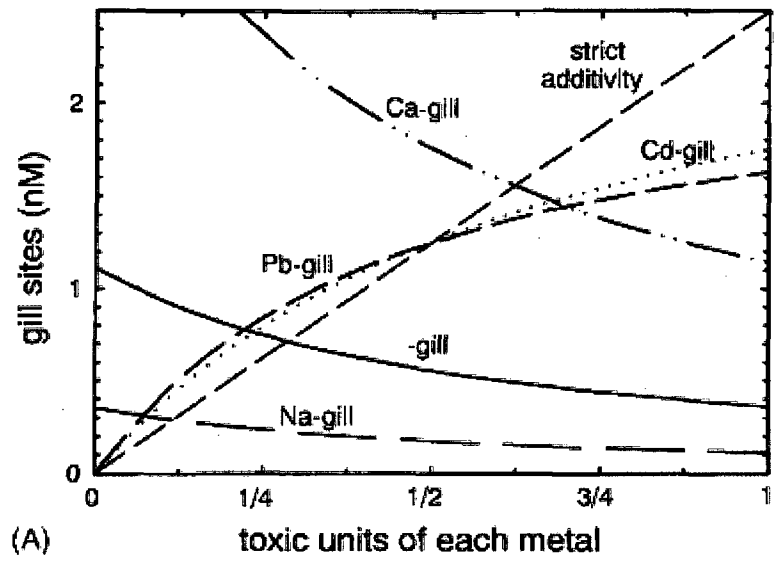
Metal contamination is an ongoing problem in aquatic environments, resulting in extensive research on the impacts of metals on aquatic organisms (e.g. Spry, 1991; Playle, 1998; Niyogi and Wood, 2004; Burger, 2008). The continuing goal of the BLM is to predict metals toxicity based on site specific criteria such as water chemistry, the affected aquatic biota at a specific site, and the bioavailable metal concentration (Paquin *et al.* 2000). The BLM integrates water chemistry parameters such as temperature, pH and hardness to determine metal bioavailability and therefore the potential toxicity of metals in a given aquatic environment. Toxicity predicted by the BLM is assumed to be directly related to the amount of metal bound to the biotic ligand (i.e. the gills in fish) and has been incorporated into the development of water quality guidelines in some jurisdictions (Paquin, 2002; Niyogi and Wood, 2004; Borgmann *et al.* 2008). Despite the usefulness of this detailed framework, the BLM has been developed only for individual metals, and it is not yet clear if it could be used to accurately predict the interactions and effects of metals in mixtures, which are more likely in contaminated waters (Playle, 2004). Moreover, the majority of metals research has focused on quantifying the responses of aquatic organisms to high concentrations of metals, which result in measurable physiological and toxicological effects but may not be representative of the lower metal concentrations that are typically found in metal contaminated aquatic ecosystems (e.g. Paquin *et al.* 2000).

Over many decades, different methodologies have been used to predict the toxicity of metals in mixtures (Sprague, 1969; Playle, 2004; Norwood *et al.* 2003; Borgmann, 2008). The most common method is the concentration addition model, which

is typically applied using the toxic unit approach (Newman and Unger, 1991). The Concentration Addition model suggests that there are three modes of interaction between multiple toxicants in an aquatic environment and these assumptions can be applied to multiple metals: 1) strict additivity, where the effects (i.e. gill accumulation) of individual toxicants can be added together to produce the actual observed effect. 2) Less than additive, where the effect of toxicants in combination does not equal the sum of the effects caused by individual metals or 3) Greater than additive, where the effects observed in exposure to mixtures is greater than the sum of the effects caused by the toxicants individually (Sprague, 1970).

Toxic units are frequently expressed as units of lethality (i.e. the 96-h LC50) (Newman and Unger, 1991). Using the TU approach, Playle (2004) attempted to model multiple metal interactions (including Pb and Cd) with the gills of fish. His simulations were based on the assumptions: 1) that both Pb and Cd compete for the same binding sites on the ligand and therefore have the same mode of toxicity (decrease Ca^{2+} uptake leading to hypocalcaemia); 2) acute toxicity is directly proportional to the amount of metal bound to the gill; and 3) when half of the gill binding sites are filled, half of the fish will die (Playle, 2004). Furthermore, each combination of metals added up to equal one toxic unit. His simulations revealed that metal-gill accumulation would be greater than additive when fish were exposed to metal mixture concentrations that were less than 1 TU, but that there would be less than additive gill binding when fish were exposed to metal concentrations greater than 1 TU due to the non-linear nature of the model (Figure 4.1; Playle, 2004).

To validate the predictions of Playle (2004) using the TU approach, rainbow trout (*Oncorhynchus mykiss*) were exposed to metal mixtures of Pb plus Cd comprising 1, 2 and 3 toxic units of metal (1 TU ~ 600 $nmolL^{-1}$ and 6 $nmolL^{-1}$ respectively) and metal-gill binding quantified. To test whether metal-gill accumulation was predictive of longer-term toxicity, metal-gill accumulation after 3 h of Pb plus Cd mixture exposure was compared to the LT50 for each metal combination over a 10 day time frame. Based on the concentration addition model, it was predicted that gill metal accumulation and toxicity would be less than additive due to the competition between Pb and Cd for binding sites on the gill.



(Playle, 2004)

Figure 4.1 Model of concentration additivity for Pb and Cd. Figures represent the amount of Pb and Cd predicted to accumulate on the gill based on external concentration. At lower concentrations, metals bind in a greater than additive manner, whereas at higher concentrations they are predicted to bind in a less than additive manner. These can be explained by the non-linear nature of the model as well as competition between metals for binding sites on the gill.

MATERIAL AND METHODS

Fish Husbandry

Rainbow trout (1-3g) were purchased from Rainbow Springs Trout Hatchery, Thamesford Ont., and held in a 100L aerated tanks in a 1:1 mixture of well water and reverse osmosis water ~ 1.5mmolL^{-1} Ca^{2+}, 500μmolL^{-1} Na^{+}, 500μmolL Cl^{-}, pH ~8, ~12°C as per Birceanu *et al* (2008). After 2 days, they were transferred to reverse osmosis only water supplied with a calcium ($CaCl_2$) drip to produce soft water (~ 100μM Ca^{2+}). The fish were fed ground commercial trout pellets (Corey Feed Mills Ltd., Fredericton, New Brunswick) 3 times per week and food was withheld for 72 h prior to experiments. Fish were allowed to acclimate to the soft water for at least 2 weeks before experiments. All fish husbandry procedures and experiments were approved by the Wilfrid Laurier Animal Care Committee, and followed the Canadian Council of Animal Care (CCAC) guidelines.

Experimental Protocol: Relating Gill Accumulation to the LT50 of Pb plus Cd mixtures

Rainbow trout (1-3g) were randomly distributed in groups of 16 to experimental buckets containing 10L of ion-poor, slightly acidic ([Ca^{2+}] ~103.7± 0.1μmolL^{-1}, pH ~ 6.18 ± 0.001) experimental water containing Cd and Pb. The fish were exposed for 240 h (10 days) to a matrix of different nominal Cd and Pb concentrations (6, 12 and 24nmolL^{-1}

Cd and 600, 1200 and 2400 nmolL^{-1} Pb for individual metal exposures as well as all combinations; Table 4.1). Fish mortality was then monitored for up to 10 days to determine the LT50 (time to reach 50% mortality) for each metal exposure combination. To relate 3 h metal-gill binding to metal toxicity after 3 h of exposure to the matrix of Cd and Pb concentrations, gills were collected from subsamples of fish (n = 6) for metal-gill analyses. The remaining 10 fish were left in the exposure buckets for the remainder of the 240 h toxicity experiment. Water pH and mortality were monitored during the experiment (every 3 h for the first 2 days and every 4-8 h for the remainder of the experiment) and pH was adjusted accordingly using 8N HNO_3 and KOH to maintain the desired pH of 6. Water samples (10mL) were sampled at the beginning of the experiment, and at 24 h intervals throughout. Filtered (45μm Supor® membrane, Pall Life Sciences, Ann Arbor, MI) and unfiltered water samples were acidified to 1% using 16N trace metal grade HNO_3 and saved for later measurement of water metals and ions (Ca^{2+} and Na^{+}). Additional un-acidified water samples were collected and saved for measurement of Cl^{-} or stored in borosilicate scintillation vials with no head space for measurement of dissolved organic carbon (DOC).

Sample Collection and Processing

Fish sampled at 3 h were killed by a blow to the head, and the entire gill basket was excised, rinsed in 100 mL of deionized water for 10 s to wash away unbound metals or mucous, and then stored in 1.5 mL micro-centrifuge tubes at -20°C until further digestion. The gills were digested in 5 times their mass in 1N Trace Metal Grade HNO_3

and baked for 3 h at 80°C. The digests were then vortexed and centrifuged for 2 min at 12 000 rpm. The supernatant was then collected, and diluted 10 times using 1% HNO_3 (Trace Metal Grade) for Cd and Pb analyses.

Metal and Ion Analysis

Water and gill Cd and Pb were quantified using graphite furnace atomic absorption (FAA) spectrophotometry (GTA100 atomizer, SpectrAA 880, N_2 gas; Varian, Mississauga, ON), and concentrations were validated using precision standards (Environment Canada). Total Ca^{2+} and Na^{+} concentrations in the water were measured by FAA spectrophotometry (see above), and water Cl^{-} concentration determined spectrophotometrically using the mercuric thiocyanate assay (Zall *et al.,* 1956). Dissolved organic carbon was determined with a Shimadzu TOC 5050A Analyzer (Shimadzu Corporation, Kyoto, Japan).

Statistical Analysis

All data is expressed as the mean ± one standard error of the mean (SEM; N). Significance between the metal-gill accumulation of different treatments was determined using a one-way ANOVA. In instances where significant variation was observed, the ANOVA was followed by a Tukey post-test to determine if there were significant differences between control and experimental groups. The LT50 for all exposures was determined by plotting the log % survival over log time as described by Litchfield and Wilcoxon (1948).

RESULTS

Toxicity of Metal Mixtures

To determine the toxicity of Pb and Cd mixtures compared to Pb or Cd alone, the LT50 (time to 50% mortality) was determined over a range of Pb and/or Cd concentrations (nominal concentrations: 0-24 $nmolL^{-1}$ Cd, 0-2400 $nmolL^{-1}$ Pb). All control fish survived the entire 10 d (240 h) experiment. Exposure to lower nominal Cd concentrations (6 and 12 $nmolL^{-1}$) yielded an LT50 of 168 h, compared to 24 $nmolL^{-1}$ Cd), which was more toxic with an LT50 of 96.7 h (Figure 4.2, column 1; A-D). In the presence of low Pb concentration (600 $nmolL^{-1}$) plus Cd (6-24 $nmolL^{-1}$), toxicity was greater compared to the individual Cd exposures, as indicated by a decrease in the LT50 to 60, 91, 76 and 80 h for 0, 6, 12 and 24 $nmolL^{-1}$ Cd, respectively (Figure 4.2, column 2). However, higher Pb concentrations (1200 and 2400 $nmolL^{-1}$) in the presence of Cd, decreased toxicity compared to the low Pb plus Cd combination. This trend was seen predominantly in the 6 and 12 $nmolL^{-1}$ Cd plus 1200 and 2400 $nmolL^{-1}$ Pb exposures (Figure 4.2B and C).

Exposure to Pb alone caused toxicity, but the LT50 increased (toxicity decreased) with increasing concentrations of Pb. The LT50s for Pb alone were 60, 100 and 75 h in fish expose to 600 1200 and 2400 $nmolL^{-1}$ Pb respectively, where the Pb toxicity was greatest at the lowest concentration of 600 $nmolL^{-1}$ (Figure 4.2A).

Metal-gill Accumulation

Background Pb on the gill was low at a concentration of 2.20 nmol g^{-1} $wetweight^{-1}$ (Figure 4.3). All Pb exposures caused significant step-wise increases in Pb-gill

accumulation compared to controls, increasing from 7.4 ± 0.8, 26.3 ± 6.5 and 51.1 ± 11.9 $nmolg^{-1}$ wet weight as the nominal Pb concentration increased from 600 to 2400 $nmolL^{-1}$ (Figure 4.3). Simultaneous exposure to nominal Cd concentrations of 6 and 10 $nmolL^{-1}$ had no significant effect on Pb accumulation. Only at the highest Cd plus Pb concentrations (24$nmolL^{-1}$ and 2400 $nmolL^{-1}$) was there significantly greater gill-Pb accumulation, approximately 125nmol g^{-1} wet $weight^{-1}$, compared to the lower Cd exposure concentrations (Figure 4.3).

Background gill Cd was around 0.5-0.7 nmol g^{-1} wet weight. There were no significant differences in gill-Cd accumulation of fish exposed to Cd (6, 12, 24 $nmolL^{-1}$) compared to controls (Figure 4.4). Gill-Cd accumulation for control, 6, 12 and 24$nmolL^{-1}$ exposures was 0.7 ± 0.1, 0.6 ± 0.1, 0.6 ± 0.2 and 0.8 ± 0.1 nmol $^{-1}$g wet weight respectively. Moreover, Gill-Cd accumulation was not affected by the addition of Pb (nominal 600, 1200 and 2400 $nmolL^{-1}$Pb) (Figure 4.4).

DISCUSSION

Toxicity of Metal Mixtures

Over the 10 d period, the toxicity of both Pb and Cd individually and in mixtures were observed through the measurement of respective LT50s (lethal time for 50% mortality). The Pb alone exposures were more toxic than control exposures. However, there seemed to be little differences between the LT50s derived for low (600 $nmolL^{-1}$) or the high (2400 $nmolL^{-1}$) Pb exposures (Figure 4.2). The Cd alone exposures showed a similar trend, where the two median concentrations had similar LT50s while the highest was most toxic (Figure 4.2). Similar observations were made by Winter (2008) for rainbow trout exposed to equal micromolar (0-3$\mu molL^{-1}$) concentrations of Pb and Cd. In this case the lower Cd and Pb concentrations seemed to be more toxic than the higher ones and the addition of NOM decreased overall toxicity of the metals to the fish.

The addition of Pb to the lower Cd exposures suggests that Pb protected fish from Cd toxicity, resulting in higher LT50s. Based on these results it would appear that the protective effects of Pb are concentration dependent because the amount of protection increases with the addition of increasing water Pb concentration. The approach that was taken in this experiment was to expose the fish to 1, 2 and 3 TU of each metal individually and in combination, with the TU being equal to the respective LC50 for each metal (~6.8 $nmolL^{-1}$ Cd and 492 $nmolL^{-1}$ Pb; Birceanu *et al.* 2008). The protective effects of Pb seen in these experiments would suggest less than additive toxicity. If the concentration addition model (i.e. predicting toxicity based on the concentrations in the water) is used to analyze this data, then toxicity is less than additive (Sorensen, 1991;

Norwood *et al.* 2003). However, this could be reflective of the decrease in Pb concentration over the 10 d period in which Pb concentrations fell by 20-40% between 3 h and 10 d (Table 4.1). Water was not replaced throughout the experiment therefore the metal became less bioavailable due to binding to experimental buckets and/or mucous within the test water. According to speciation calculations using Visual MINTEQ using water chemistry parameters and measured dissolved metals, more than 95% of both metals were calculated to be in the ionic form (Table 4.2).

Another method of determining the interactions of metal mixtures is the response addition model, where if the toxic effects are strictly additive, then the control corrected survival in the mixture can be predicted using the following equation:

$$S''_{mix} = S''_1 \times S''_2 \times S''_{n...} \quad (1)$$

S''_{mix} is the product of the survival rate observed when fish were exposed to each metal alone (S''_n) (Norwood *et al.* 2003). The response effect was calculated for the mixtures in our experiment (Table 4.3), but, control correction was not necessary because none was observed in the study. In most cases the predicted % survival was lower than the actual % survival observed over 10 days. This type of response caused by mixtures is also described as less than additive (Hewlett and Plackett, 1979 as referenced in Norwood *et al.* 2003). Like the concentration addition model, the response effects model does not appear to be an effective means to predict metal mixtures toxicity based on the results seen using Pb and Cd concentration (Table 4.3).

Gill-metal Burden

Gill-Pb accumulation during 3 h of exposure increased with increasing Pb concentration (Figure 4.2). At the lowest concentration of Pb (actual ~539nmolL^{-1}; similar to the respective LC50 = 1TU) gill-Pb accumulation was approximately 10nmol g^{-1} (wet weight). This amount of Pb on the gill corresponds to the LA50 for Pb as calculated by Birceanu *et al* (2008). Similarly, when the fish were exposed to 2 and 3 TU of Pb (actual ~15067 and ~3149.5nmolL^{-1}) the corresponding gill accumulation was 2 and 3 times greater than LA50 for Pb, which would be expected. Gill accumulation for Pb was directly proportional to the metal concentration. This proportional increase suggests the potential to predict Pb-gill accumulation during Pb only exposures by models such as the BLM (Paquin *et al.* 2002; Playle. 2004). Gill accumulation, however, typically does not occur in a linear fashion due to gill-binding kinetics so this linear trend seen in our experiments may only be observed within the concentration ranges tested here (Playle, 2004; Niyogi *et al.* 2008). This Pb-gill trend was not, however, evident in the presence of all Cd concentrations. Furthermore, Pb-gill accumulation was significantly greater at the highest Pb and Cd concentrations in combination. Augmentation of Pb accumulation was not expected due to the anticipated competitive nature of Pb and Cd for similar binding sites on the gill (i.e. apical Ca^{2+} channels and Ca^{2+}-ATPase) (Verbost *et al.* 1988; Grosell *et al.* 2006; Niyogi *et al.* 2003; 2008). Competitive inhibition between two metals can only be demonstrated if the two metals compete for the same binding site on the ligand, but this may not be true for most metals in combination (Borgmann *et al.* 2008). Recent studies, however, suggest multiple binding sites for both Pb and Cd which

may account for the discrepancy from the predicted strict additivity for Pb and Cd (Birceanu *et al.* 2008; Niyogi *et al.* 2008).

The possibility of two binding sites provides evidence for other types of inhibition: anti-competitive binding (where there could be enhancement of metal accumulation caused by another metal) and non-competitive binding (where there is no competition between metals at all sites) (Borgmann *et al.* 2008). The enhancement of Pb-gill accumulation in the presence of Cd indicates the possibility of these two inhibition types and although more research and modeling is needed to support one or the other (and could possibly be concentration-dependent) (Borgmann *et al.* 2008). Despite the slight impacts that Cd had on Pb-gill accumulation, Pb did not affect Cd-gill accumulation.

Unexpectedly, no significant Cd accumulation was observed on the gill after 3 h exposure to any of the chosen concentrations (Actual ~6, 13, 23.4nmolL^{-1}). At these concentrations, Cd typically binds to the gill at very low levels (0.1-0.5 nmol g^{-1} wet weight) compared to Pb despite its high affinity for the gill (~ $K_{Cd\text{-}gill}$ = 7.33 – 8.6; Birceanu *et al* 2008; Playle 1993). This discrepancy may be because the background gill-Cd was high (~ 0.3-0.8 nmolg^{-1}), making it difficult to determine any substantial differences between exposure groups. Further studies using the radiotracer ^{109}Cd may help reconcile small differences in Cd uptake during exposure to low concentrations of this metal (Hollis *et al.* 2000).

The BLM assumes that the amount of metal bound to the gill is directly related to the toxicity of each metal to the fish. This study, however, suggests that this may not be the case for Pb and Cd in combination. For higher metal exposures in combination,

greater toxicity and less than additive gill accumulation was expected. This was hypothesized due to the non-linear nature of metal binding to the biological ligand (Playle, 2004), and the assumed competition between Pb and Cd for the same binding sites on the gill (i.e. Ca^{2+} channels). The weight of current evidence now suggests, however, that there is more than one binding site for both Pb and Cd on the gill, which decreases the probability of strictly competitive interactions between Pb and Cd. Moreover, Pb and Cd are known to have slightly differing modes of toxicity.

Rogers *et al.* (2005) found that in addition to competition for Ca^{2+} channels on the gill, Pb causes a decrease in activity of both the basolateral Na^+/K^+-ATPase and cytosolic carbonic anhydrase, which inhibits Na^+ and Cl^- uptake by the gills. Whereas, Cd binds to Ca^{2+} apical channels as well as interfering with Ca^{2+}-ATPase within the gill membranes (Verbost *et al.* 1987; 1988). The present study suggests that the premise that gill accumulation is predictive of toxicity may not apply to metal mixtures, unlike the case for individual metals. Here, the most toxic concentrations (i.e. lowest LT50) were not necessarily the exposure in which metal-gill accumulation was highest (Figure 4.2). This discrepancy can be explained by the length of our study (10 d) compared to the typical time (96 h) used to relate gill accumulation to mortality.

Acclimation through damage repair mechanisms to metals such as the production of metallothionein-like proteins that sequester and bind metals as well as MRC proliferation can aid in the process required to maintain homeostasis within contaminated waters (Chowdhury *et al.* 2005; Silvestre *et al.* 2005; McGeer *et al.* 2007). Moreover, acclimation processes can begin to occur within days (<4 d) of initial exposure (Lauren and McDonald, 1987). With this in mind, there are many more variables to consider

when relating 3 h gill accumulation with 10 day toxicity. It should be noted that continued research is being conducted on the development of BLMs able to account for physiological responses of fish during chronic exposures (e.g. Hollis *et al.* 2000).

It is clear that Pb plus Cd mixture interactions are very complex. This complexity likely arises from the differences in the toxic mechanism(s) of action of these two metals. The enhancement of Pb accumulation in the presence of Cd is concentration-dependent and is evidence to support anti-competitive interactions between the two metals (Bormann *et al.* 2008). Moreover, the response effect calculations conducted support interactions different from strict competition. In addition to the prediction of toxicity based on % survival, the 3 h gill accumulation did not accurately reflect toxicity after 10 d of exposure. In recognition of the results observed in this study, the interactions of Pb and Cd at the gill and their ultimate impact on toxicity seem to be less predictable than once believed. Simple models of additivity including the effects addition model do not accurately predict the toxicity of Pb and Cd mixtures, perhaps due to the complexity of mechanistic action at the gill. Multiple binding sites for both Pb and Cd may explain the unpredictable toxicity and possibly the greater than additive gill accumulation. In conclusion, the interactions of Pb and Cd cannot be explained by the simple additivity model; however this evidence may suggest a more complex interaction such as non-competitive or anti competitive inhibition. The toxicity of Pb and Cd, as seen in this study is not predictable based on the acute (3 h) gill accumulation as described in the concentration addition model and this finding is important to note when using predictive models for use in the development of ambient water quality criteria.

Table 4.1 Water chemistry for the 3 h gill accumulation and 10 day LT50 experiments. Data represented as mean ± 1 S.E.M.

Treatment	Time	Cd ($nmolL^{-1}$) (dissolved)	Pb ($nmolL^{-1}$) (dissolved)	Ca^{2+}($\mu molL^{-1}$) (dissolved)	Na^{+} ($\mu molL^{-1}$) (dissolved)	Cl^{-}($\mu molL^{-1}$) (total)	pH
Control	3 h	0.0 ± 0.0	40.3 ± 6.6	106.1 ± 0.9	230.9 ± 1.2	44.8 ± 3.1	7.1 ± 0.7
	10 d	0.3 ± 0.4	0.0 ± 0.0	86.5 ± 4	281.8 ± 20.1	50.1 ± 13.5	6.6 ± 0.2
0 nM Cd + 600 nM Pb	3 h	0.0 ± 0.1	539.8 ± 119.2	99.3 ± 2.9	224.9 ± 1.54	34.1 ± 9.4	6.2 ± 0.1
	10 d	1.3 ± 0.8	441.2 ± 233	73.8 ± 2.2	254.2 ± 17.8	40.4 ± 14.0	6.2 ± 0.1
0 nM Cd + 1200 nM Pb	3 h	0.0 ± 0.0	15067 ± 81.2	101.1 ± 1.3	228.5 ± 1.2	44.6 ± 6.1	6.4 ± 0.1
	10 d	0.3 ± 0.4	839 ± 270	85.4 ± 2	283.2 ± 24	59.8 ± 13.0	6.2 ± 0.2
0 nM Cd + 2400 nM Pb	3 h	0.0 ± 0.1	3149.5 ± 383.8	105.1 ± 1.3	231.4 ± 1.1	39.6 ± 6.5	6.3 ± 0.1
	10 d	1.2 ± 0.7	1127.2 ± 450	85.0 ± 2.5	206.7 ± 52.4	57.1 ± 13.0	6.1 ± 0.1
6 nM Cd + 0 nM Pb	3 h	6.6 ± 01	40.4 ± 6.7	107.8 ± 2.7	224.4 ± 0.6	38.6 ± 4.9	6.3 ± 0.1
	10 d	3.3 ± 0.3	0.02 ± 0.0	89.0 ± 3.7	261.6 ± 28.5	47.7 ± 5.6	6.2 ± 0.0
6 nM Cd + 600nM Pb	3 h	6.0 ± 0.0	691.3 ± 60.7	105.9 ± 1.6	229.6 ± 1.4	48.5 ± 5.0	6.3 ± 0.1
	10 d	3.1 ± 0.5	512.6 ± 144	88.7 ± 2	288.1 ± 29.5	63.5 ± 8.1	6.1 ± 0.1
6 nM Cd + 1200 nM Pb	3 h	7.2 ± 0.3	1482.9 ± 5.6	104 ± 1	233.2 ± 1.1	32.8 ± 15.1	6.9 ± 0.1
	10 d	5.6 ± 2.2	729 ± 290	90.9 ± 2.8	287.9 ± 21.0	55.1 ± 14.8	6.2 ± 0.1
6 nM Cd + 2400 nM Pb	3 h	6.4 ± 0.2	2520.8 ± 277.1	101.8 ± 1.4	228.9 ± 1.5	47.2 ± 6.3	6.7 ± 0.1
	10 d	4.8 ± 1.9	970.3 ± 370	86.4 ± 1.3	284.0 ± 25.3	68.9 ± 7.9	6.2 ± 0.0
12 nM Cd + 0 nM Pb	3 h	13.1 ± 0.0	39.8 ± 6.3	109.4 ± 0.2	225.2 ± 2.5	37.8 ± 4.8	6.8 ± 0.1
	10 d	10.0 ± 2.4	0.02 ± 0.0	88.5 ± 1.2	247.4 ± 13.3	45.4 ± 12.4	6.2 ± 0.1
12 nM Cd + 600 nM Pb	3 h	11.9 ± 0.1	664.2 ± 8.5	101.0 ± 1.2	230.0 ± 0.9	35.6 ± 6.2	6.8 ± 0.1
	10 d	10.8 ± 3.9	433.9 ± 168	85.5 ± 1.4	248.3 ± 17.6	30.3 ± 18.2	6.2 ± 0.1
12 nM Cd + 1200 nM Pb	3 h	13.5 ± 0.1	1454.3 ± 127.5	101.7 ± 0.3	219.8 ± 2.2	41.4 ± 3.8	6.4 ± 0.1
	10 d	8.8 ± 0.8	845.4 ± 259	85.4 ± 0.9	290.2 ± 25.6	62.9 ± 9.6	6.1 ± 0.1
12 nM Cd + 2400 nM Pb	3 h	12.6 ± 0.3	3210.7 ± 318.5	101.1 ± 0.5	226.9 ± 3.7	43.1 ± 3.9	6.8 ± 0.1
	10 d	8.0 ± 1.0	1111.7 ± 346	90 ± 2.7	297.2 ± 31.7	60.4 ± 12.6	6.1 ± 0.1
24 nM Cd + 0 nM Pb	3 h	23.4 ± 0.2	39.8 ± 6.2	105.8 ± 0.9	227.4 ± 1.1	35.2 ± 9.2	6.8 ± 0.1
	10 d	23.8 ± 4.0	0.02 ± 0.0	86.7 ± 1.5	269.4 ± 20.9	63.8 ± 19.6	6.2 ± 0.1
24 nM Cd + 600 nM Pb	3 h	24.3 ± 0.7	647.9 ± 6.3	106.1 ± 1.0	219.6 ± 0.7	40.2 ± 4.1	6.8 ± 0.0
	10 d	21.4 ± 0.7	453.7 ± 140	88.5 ± 3	262.4 ± 17.6	56.3 ±7.5	6.1 ± 0.0
24 nM Cd +1200 nM Pb	3 h	22.9 ± 0.2	1586.4 ± 122.8	100.4 ± 0.2	220.5 ± 3.0	46.1 ± 1.8	6.8 ± 0.1
	10 d	19.7 ± 0.9	864.2 ± 260	85.2 ± 1	275.5 ± 28.0	63.5 ± 14.0	6.2 ± 0.0
24 nM Cd + 2400 nM Pb	3 h	23.0 ± 0.3	2730.8 ± 99.7	103.2 ± 2.5	230.3 ± 1.6	40.9 ± 11.6	6.8 ± 0.0
	10 d	23.0 ± 3.7	1032.9 ± 453	84.7 ± 1.2	279.8 ± 22.2	56.7 ± 18.2	6.1 ± 0.1

Table 4.2 Predicted Cd and Pb speciation (% of dissolved metal concentration) in exposure water after 10 d. Nominal metal concentrations are displayed here for clarity in $nmolL^{-1}$. Parameters used to calculate Cd and Pb speciation using Visual MINTEQ (Version 2.52) are found in Table 4.1 except for – Water: $PCO_2 = 0.00038atm$.

Speciation	**Cd**	**6**	**12**	**24**	**-**	**-**	**-**	**6**	**6**	**6**	**12**	**12**	**12**	**24**	**24**	**24**
	Pb	**-**	**-**	**-**	**600**	**1200**	**2400**	**600**	**1200**	**2400**	**600**	**1200**	**2400**	**600**	**1200**	**2400**
Cd																
Cd^{2+}		99.53	99.64	99.40	-	-	-	99.42	99.49	99.39	99.69	99.42	99.44	99.48	99.41	99.42
$CdOH^{+}$		0.012	0.012	0.012	-	-	-	-	-	-	0.012	-	-	-	0.012	-
$CdCl^{+}$		0.417	0.397	0.556	-	-	-	0.553	0.553	0.599	0.266	0.548	0.525	0.491	0.553	0.553
$CdHCO_3$		0.027	0.027	0.027	-	-	-	0.021	0.021	0.021	0.027	0.021	0.021	0.021	0.027	0.021
Pb																
Pb^{2+}		-	-	-	95.41	95.37	96.28	96.28	96.30	96.26	95.45	96.28	96.29	96.29	95.36	96.27
$PbOH^{+}$		-	-	-	3.603	3.585	2.886	2.872	2.873	2.782	3.599	2.874	2.871	2.877	3.585	2.875
$PbCl^{+}$		-	-	-	0.129	0.19	0.184	0.203	0.177	0.221	0.097	0.202	0.193	0.181	0.202	0.204
$PbCO_3(aq)$		-	-	-	0.202	0.201	0.129	0.128	0.128	0.128	0.202	0.128	0.128	0.128	0.201	0.128
$PbHCO_3$		-	-	-	0.652	0.649	0.522	0.52	0.52	0.52	0.652	0.52	0.52	0.521	0.649	0.521

Table 4.3 Response addition approach to assess Pb and Cd toxicity over time. S''_{mix} (Predicted survival during exposure to metal mixtures) is the product of the actual survivals of fish (S''_i) during exposure to respective metal treatments (individual metals). The actual survival is the proportion of individuals that survived the exposure to each metal treatment.

Response Addition (effects addition)

	6 nM Cd + 600 nM Pb		6 nM Cd + 1200 nM Pb		6 nM Cd + 2400 nM Pb		12 nM Cd + 600 nM Pb		12 nM Cd + 1200 nM Pb		12nM Cd + 2400 nM Pb		24 nM Cd + 600 nM Pb		24nM Cd + 1200 nM Pb		24 nM Cd + 2400 nM Pb	
Time (h)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)	S"mix (Predicted survival)	Actual (Survival)
0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.9
48	1	0.9	1	0.9	0.7	1	1	1	1	0.56	0.8	0.56	1	0.56	0.56	1	0.56	0.8
72	0.3	0.8	0.9	0.8	0.6	0.9	0.3	0.9	0.9	0.36	0.6	0.36	0.7	0.36	0.36	0.7	0.36	0.4
96	0.09	0.4	0.36	0.5	0.27	0.9	0.1	0.4	0.7	0.18	0.6	0.18	0.7	0.18	0.18	0.7	0.18	0.3
120	0	0.4	0.27	0.5	0.27	0.8	0	0.27	0.6	0.12	0.2	0.12	0.5	0.12	0.12	0.5	0.12	0.3
144	0	0.3	0.16	0.5	0.24	0.6	0	0.18	0.4	0.12	0.2	0.12	0.5	0.12	0.12	0.5	0.12	0.2
168	0	0.2	0.16	0.3	0.24	0.5	0	0.18	0.4	0.09	0.2	0.09	0.5	0.09	0.09	0.5	0.09	0.2
192	0	0.2	0.16	0.3	0.16	0.4	0	0.18	0.3	0.06	0.1	0.06	0.4	0.06	0.06	0.4	0.06	0.2
216	0	0.2	0.16	0.3	0.16	0.2	0	0.18	0.3	0.04	0.1	0.04	0.4	0.04	0.04	0.4	0.04	0.2
240	0	0.2	0.16	0.3	0.16	0.2	0	0.18	0.1	0.02	0.1	0.02	0.4	0.02	0.02	0.4	0.02	0.2

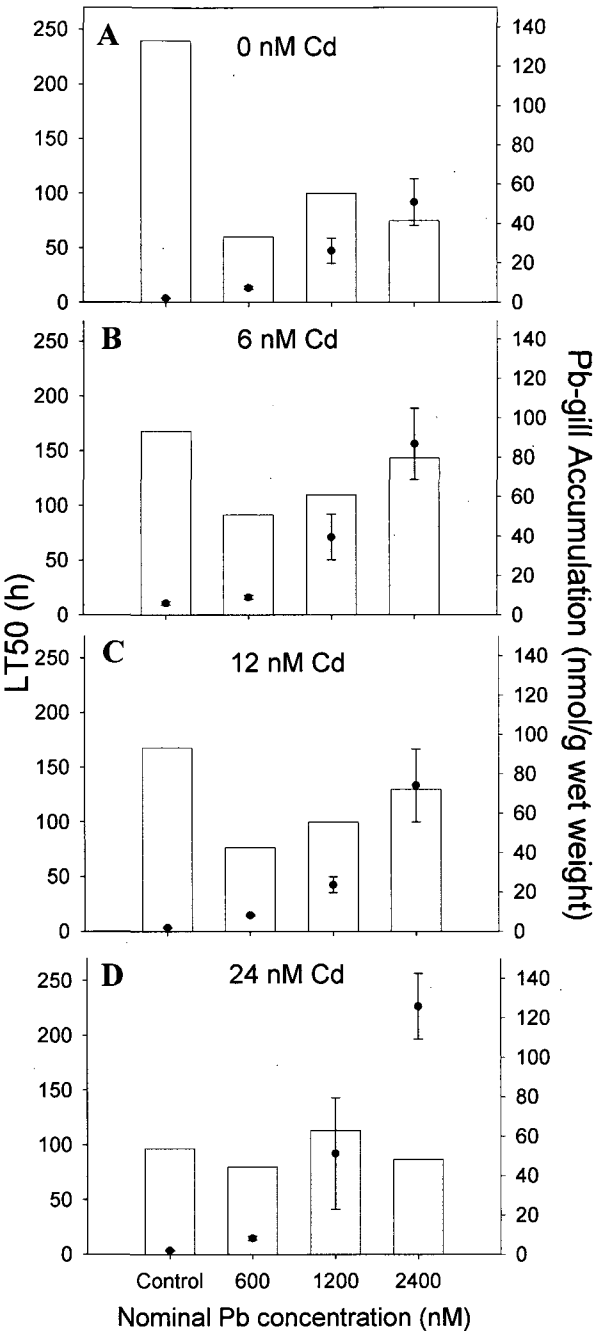


Figure 4.2 Toxicity (10 day = 240 h LT50 values) and metal-gill accumulation (3 h) in rainbow trout exposed to A) Pb alone, or to Pb plus nominal Cd concentrations of B) 6, C) 12 and D) 24 $nmolL^{-1}$. Data are presented as the mean ± 1 S.E.M. (n = 6 for all gill accumulation data).

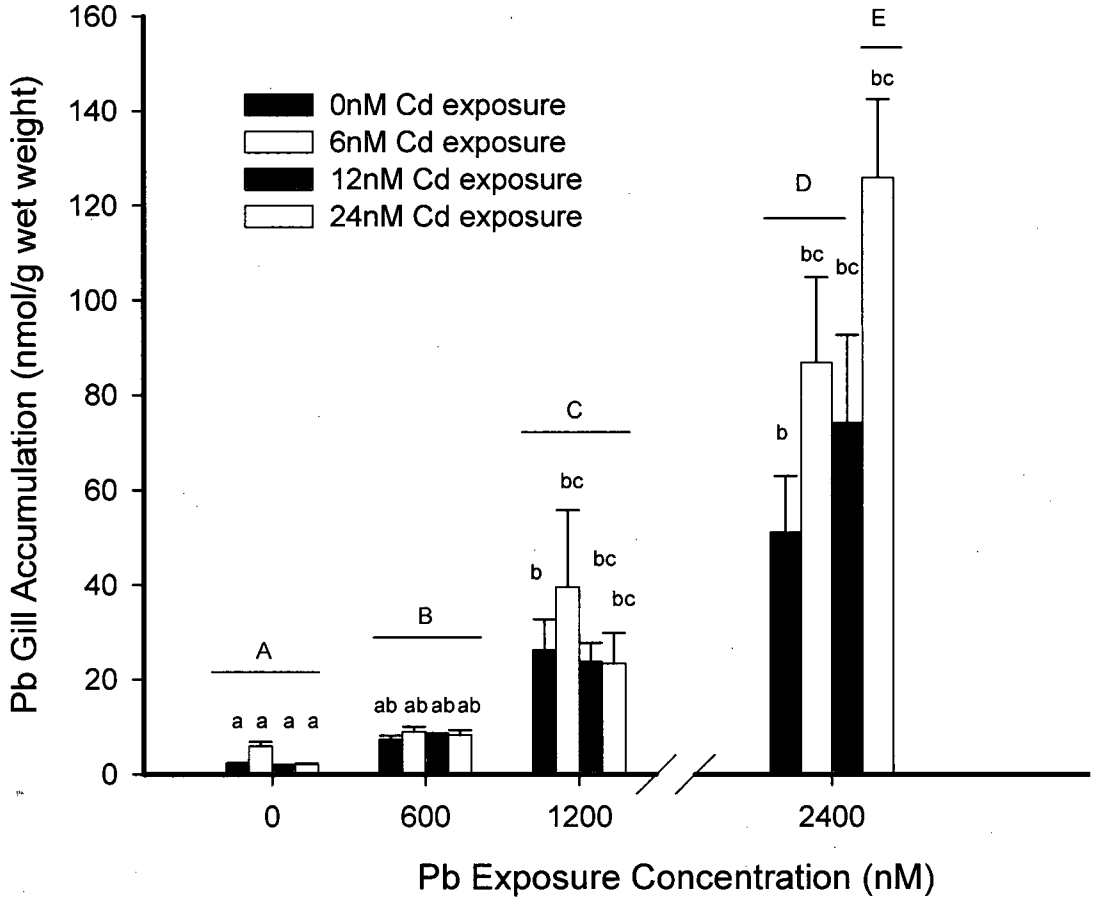


Figure 4.3 Pb-gill accumulation in rainbow trout exposed for 3 h to Cd concentrations of 6, 12 and 24 nmolL^{-1}Cd and Pb concentrations of 600, 1200 and 2400 nmolL^{-1}. Data is presented as the mean ± 1 S.E.M (n = 6 for each treatment). Capital letters over a line indicate significant differences between metal-gill accumulation for different Pb exposure concentrations, while lower case letters indicate significant differences between Cd exposures (same coloured bars). Bars sharing the same letter are not significantly different.

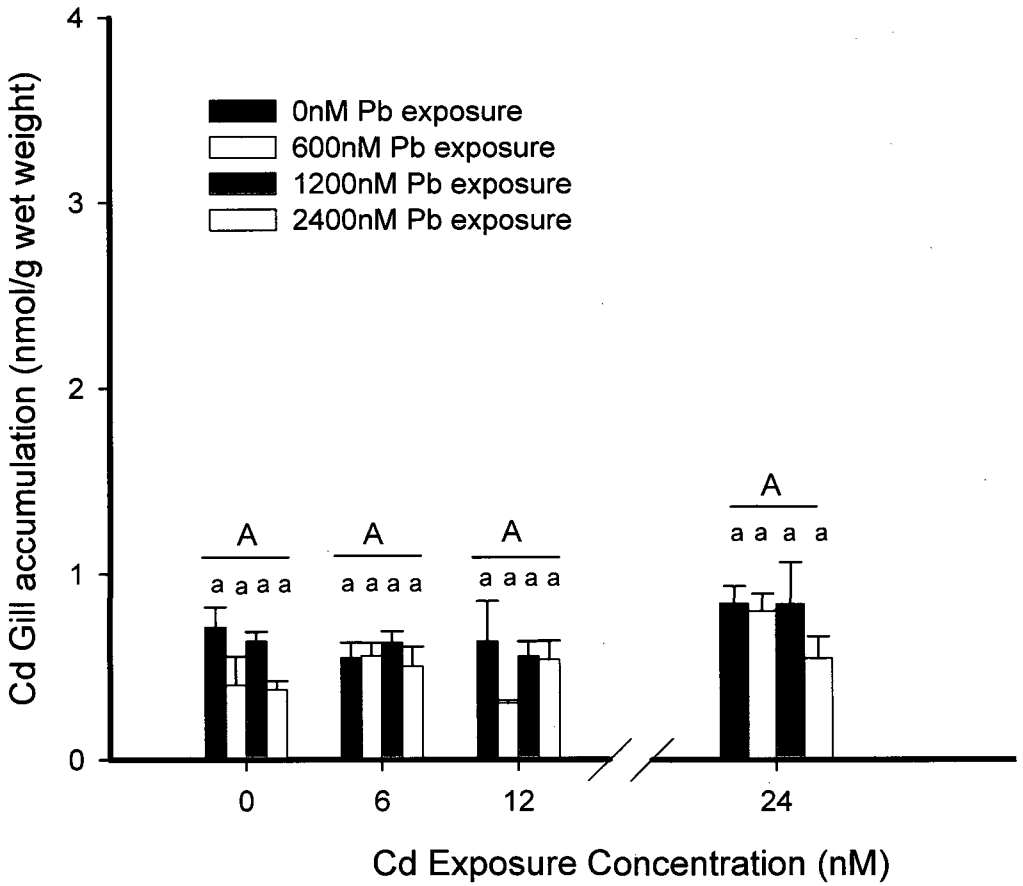


Figure 4.4 3 h gill-Cd accumulation of rainbow trout in the presence of nominal Cd concentrations of 6, 12 and 24 $nmolL^{-1}$ and nominal Pb concentrations of 600, 1200 and 2400 $nmolL^{-1}$. Data is represented as the mean ± 1 S.E.M (n = 6). Capital letters over a line indicate significant differences between metal-gill accumulation for different Cd exposure concentrations, while small letters indicate significant differences between Pb exposures (same coloured bars). Bars sharing the same letter are not significantly different.

Chapter 5

Mechanism(s) of Pb and Cd Toxicity on Rainbow Trout in Soft Acidic Water: A Synopsis

An Integrative Approach to Understanding Pb and Cd Toxicity

The inputs of both Pb and Cd into aquatic systems are derived from both natural and anthropogenic sources (Zabel, 1993; Chapter 1). Once these metals enter aquatic systems, their bioavailability, or their potential to cause toxicity to aquatic organisms, is affected by water chemistry parameters including hardness, pH, and complexing cations such as natural organic matter that may decrease this potential. The most sensitive of aquatic systems are considered to be the soft, slightly acidic waters found in the Canadian Shield and Scandinavia (Spry and Weiner. 1991). Due to their low hardness and slightly acidic nature, these waters are more sensitive to metal inputs. The majority of metals research in such waters had been conducted on individual metals at high concentrations (μmol – mgL^{-1}),however, which are not reflective of actual concentrations found in contaminated sites. In consideration of genuine environmental scenarios, the major focus of my thesis was to determine how low, environmentally realistic concentrations (3-50 $nmolL^{-1}$ Pb and 0.4-8 $nmolL^{-1}$ Cd; Reader *et al*, 1989) affect metal-gill binding, physiology and toxicity of rainbow trout in soft acidic water ($[Ca^{2+}] \sim 100$ $\mu molL^{-1}$, slightly acidic water (pH 6.0-6.2).

To better understand the integrative mechanisms of Pb plus Cd mixture toxicity in rainbow trout, three approaches were used: 1) Measurement of the physiological disruption caused by Pb plus Cd mixtures within the blood of trout where acid-base regulation, haematology, and ionoregulatory parameters were monitored by repetitive blood sampling using cannulas implanted in the dorsal aorta; 2) Monitoring of the uptake, net flux and outflux of Na^+ across the gill using the radiotracers ^{24}Na to determine how the presence of Pb and/or Cd caused internal ionic disturbances; and 3) Using the

concentration addition model to determine if Pb- plus Cd-gill accumulation was predictive of longer term toxicity (10 days) in the presence of metal mixtures.

Physiological Disruption Caused by Pb and Cd Mixtures

Plasma Ca^{2+} and Na^{+} concentration, and plasma osmolality decreased in a greater than additive manner when rainbow trout were exposed to Pb plus Cd mixtures. These findings were in accordance with those reported in Birceanu *et al.* (2008) who saw a greater than additive decrease in Ca^{2+} and Na^{+} influx in fish exposed to Pb plus Cd mixtures. Surprisingly, however, the greater than additive effects were only persistent for plasma calcium measurements, which may have contributed to the greater toxicity observed (Chapter 2). Plasma Na^{+} and osmolality, although significantly reduced within 48 h of exposure to Pb plus Cd mixtures, had been corrected by 120 h in the surviving trout, however. This recovery indicated that some of the fish were able to acclimate to the low metal concentrations used in these studies. The restoration of the plasma Na^{+} and osmolality may be related to an ability of the fish to restrict Na^{+} losses across gills as demonstrated by reductions in Na^{+} outflux, and by elevating branchial Na^{+}/K^{+}-ATPase activity. There were no disturbances to acid-base balance or haematology measurements (haematocrit, haemoglobin concentration), which is consistent with recent studies on Pb and Cd (Rogers *et al.* 2003; Chowdhury *et al.* 2004).

Unexpectedly, exposure to low Cd concentrations decreased Na^{+} uptake completely after 72 h. The exact mechanism by which Cd exerts its impacts on Na^{+} homeostasis cannot be determined by these studies, however studies by Atli and Canli (2007) and Kinne-Saffran *et al.* (1993) observed a decrease in Na^{+}/K^{+}-ATPase activity in the presence of Cd in Tilapia gills and Dogfish rectal glands, respectively. Moreover

Lionetto *et al.* (2000) reported a decrease in carbonic anhydrase activity in eel gill homogenates exposed to Cd. Komjarova and Blust (2009) described a decrease in Cd accumulation in the presence of high external Na, also suggesting that there were Na^+/Cd interactions. There was no significant decrease in Na^+/K^+-ATPase measured in fish exposed to any of the Pb and/or Cd treatments suggesting that the mechanism of Na^+ disruption is caused by inhibition of the cytosolic enzyme carbonic anhydrase in addition to an *in vivo* disruption to Na^+/K^+-ATPase efficiency.

In contrast unidirectional Na^+ flux measurements in the presence of Pb plus Cd did not have any observable effects on Na^+ uptake over 72 h. This finding was unexpected and contradicted the hypothesis that longer Pb plus Cd exposure would cause a greater than additive effect on Na^+ influx (Birceanu *et al.* 2008). The competition of Pb and Cd for binding sites (i.e. apical Ca^{2+}-channels) may have delayed the decreases on Na^+ influx, ultimately buying more time for the fish to acclimate to the metals. This acclimation was demonstrated by the up-regulation of basolateral Na^+/K^+-ATPase activity in the gills of trout exposed to Pb and Cd mixtures. Moreover, acclimation could possibly have been initiated by the decrease in gill Na^+ permeability that was observed (see above).

Predicting Toxicity of Pb and Cd Mixtures from Gill-metal Accumulation

Overall, these studies support the conclusion that gill-Cd or gill-Pb accumulation is not necessarily predictive of toxicity. This was seen in all three of the studies reported here. This discrepancy between the traditional views that gill metal accumulation is directly proportional to toxicity may be explained by the presence of multiple binding sites for both Pb and Cd on the trout gill. The identified high affinity, low capacity sites

are typically filled first, and are believed to be the physiological active sites (i.e. apical Ca^{2+}-channels and Ca^{2+}-ATPase; Niyogi *et al.* 2004), whereas the second, low affinity, high capacity population of binding sites are less understood, and likely of less physiological relevance (Niyogi and Wood. 2004; Niyogi *et al.* 2004). Metal-gill binding to this second population of binding sites may explain why it was difficult to establish clear relationships between metal-gill accumulation, physiological disturbances or toxicity.

Initial attempts at modeling the interactions of Pb and Cd toxicity (Playle, 2004) were based on the assumption that Pb and Cd compete for the same binding sites on the gill and that when 50% of the gill sites were filled, 50% of the population would die. Results presented in this study, however, do not support such a result. Measured gill-Pb and gill-Cd accumulation in all three of the reported studies here were at or near the calculated B_{max} for both Pb and Cd (e.g. metal-gill binding was greater than 50 % of B_{max}) for rainbow trout in similar water chemistry, but greater than 50% mortality was not observed in many of the exposures to low concentrations of Pb and Cd (Chapter 2, 3). During higher exposure concentrations (chapter 4) greater gill accumulation was not necessarily reflective of greater toxicity. Interpretation was likely further complicated by the presence of multiple binding sites for both Pb and Cd on the gill (see above; Birceanu *et al.* 2008; Niyogi *et al.* 2008), making it more difficult to simply model Pb plus Cd interactions at the gill. To assess this, it will be important to understand the mechanisms behind the interactions of the metals at the gill causing ionoregulatory disruption. A clear understanding of metal-gill binding and toxicity is therefore necessary to provide further insight into the development of predictive models for metal mixture toxicity to fish.

Implications of Understanding Pb and Cd Mechanisms of Toxicity in Soft Water

Water quality criteria and guidelines are intended to be based on scientific evidence to support the protection of aquatic life (Niyogi and Wood. 2004). The toxicity of metals is based on the idea that metals bind to physiological active ligands and this binding is dependent on the bioavailability, and the ability of a metal to bind to such a ligand. The formation of metal complexes with organic and inorganic ions cause a decrease in metal bioavailability, thereby decreasing their potential toxicity and competition with hardness divalent cations and univalent protons for binding sites on the biotic ligand (i.e. the fish gill for purposes demonstrated here). Moreover, the pH of aquatic systems can affect the bioavailability of metals by increasing the free metal concentration (low pH) or by increasing complexation of the metal with inorganic compounds (i.e. CO_3^-). These factors can make even relatively low concentrations of metals toxic under certain environmental conditions. The development of the BLM has focused on using site specific water chemistry to predict the toxicity of metals to aquatic organisms. The BLM presently predicts the amount of metal required to fill 50% of gill binding sites (i.e. physiologically active sites for Ca^{2+} uptake at the gill) after 3 h of exposure (LA50) which is assumed to be predictive of subsequent longer term toxicity (96 h LC50; Niyogi *et al.* 2004; Playle 2004). Although there have been great advances in the predictive power of the BLM for individual metals such as copper and Cd, the BLM is not used to predict the toxicity of metal mixtures, a situation more commonly found in contaminated waters. Moreover, the consideration of subtle, long-term internal effects caused by metal toxicity are not considered in the BLM, but likely play an important role in longer-term metal toxicity, and may potentially be a better indicator of

metals toxicity compared to the classical endpoint of death. Thus, physiological end-points rather than death might be a more appropriate means for predicting longer-term toxicity to Pb, Cd, or Pb plus Cd mixtures (Grossell *et al.* 2006).

Conclusions

This thesis used an integrative approach to study the interactions of Pb plus Cd mixtures at the gill, and to explain how the combined effects of these metals influence toxicity. Toxicity tests revealed that exposure to Pb and Cd in combination for longer periods of time causes greater than additive decreases in plasma ions Ca^{2+} and Na^{+}. The decrease in plasma Na^{+}, however, was not sustained over 3-5 d exposures suggesting that hypocalcaemia is the likely cause of death during metal exposure, but it is also likely that other toxic mechanisms such as neurological damage is caused by these metals (Weber *et al.* 1997). Despite the greater than additive effects on the regulation of internal calcium, recovery of plasma Na^{+} and studies of unidirectional Na^{+} fluxes in addition to a maintenance of Na^{+}/K^{+}-ATPase activity suggests a possible acclimation to such a disturbance during exposure to Pb plus Cd.

Results reported in this study have shown that, despite previously used assumptions, gill-metal accumulation is not entirely predictive of longer term Pb plus Cd toxicity and that internal physiological endpoints may be necessary to consider when predicting toxicity to mixtures. In addition, internal disruption caused by Pb and Cd mixtures may be overcome through acclimation processes. The results of this thesis will aid in the understanding of the complex interactions of Pb and Cd on gill mediated ionoregulation. This thesis further outlines the importance of considering mechanisms of action and differences in gill accumulation in understanding of metals toxicity. Finally, it also suggests that the mechanisms of Pb and Cd toxicity in soft water may be different to those in hard water due to possible differences in the ionoregulatory strategies used by fishes in these two media. Thus it will be necessary to better understand the

ionoregulatory mechanisms used by fish in soft acidic waters which are characteristic of those found in the Canadian Shield. Further knowledge of these strategies will allow for better understanding of the toxicity of metals in similar environments.

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