Metal Uptake and Toxicity in Rainbow Trout (*Oncorhynchus mykiss*) When Exposed to Metal

Mixtures Plus Natural Organic Matter.

By

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any final required revisions, as accepted by my examiners.

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Abstract

Multiple metal-gill modelling based on the toxic unit concept suggests that metals with the same toxic actions (e.g. Pb and Cd interacting at Ca-gill channels) will exhibit strictly additive binding at the gills of fish if the concentrations of the two metals sum to one toxic unit. Due to the non-linear nature in which metals bind to fish gills, the metal mixture will be more than additive below one toxic unit and less than additive above one toxic unit. This research tested the models by exposing rainbow trout to mixtures of Cd and Pb to investigate the metal-gill binding of these two metals when present in mixtures with fish. The relationship between Cd and Pb in mixtures was also investigated when natural organic matter (NOM) was added to the treatment solutions.

Juvenile rainbow trout (5 g) were exposed to equal mixtures of Cd and Pb at 0.75, 1.5, 2.25 and 3.0 μM each. Metal accumulation by trout gills was non-linear as suggested by the model and approximated additivity at 1.5 μM Cd + 1.5 μM Pb. There was some evidence of competitive effects at the higher metal concentrations (3.0 μM Cd + 3.0 μM Pb) because gill Cd for trout exposed to mixture treatments were lower than that for trout exposed to Cd alone, while gill-Pb remained high. 96 h toxicity experiments revealed that Cd and Pb in mixtures were more toxic than either Cd or Pb alone. The high gill-Pb accumulation in relation to gill-Cd was unexpected and suggested an additional gill-binding site for Pb aside from the apical Ca channels on the gill.

The bioaccumulation and partitioning of Cd and Pb within the body of 50 g rainbow trout was investigated when these fish were exposed to mixtures of Cd and Pb. Trout were exposed to 3 μM Pb, 3 μM Cd and a mixture of 3 μM Pb and Cd for 1, 3 and 7 d. The accumulation of metals within the trout body did not change between the single metal and

mixture exposures. Trout accumulated the greatest amount of Pb on the gills with some

accumulation also in the stomach. The greatest Cd accumulation was in the liver and trunk

kidney. Accumulation of Pb was not limited to the gills of trout resulting in the rejection of the

hypothesis that the gills were sequestering Pb while Cd was being transported into the body of

the fish.

To investigate how Pb affects Cd binding to fish gills, and how these metals exert

toxicity when present in mixtures, trout (2 g) were exposed to a range of Cd concentrations

(0.75, 1.5, 2.25 and 3.0 µM) while maintaining constant Pb concentrations over the same

concentration range. At the lowest metal concentrations (0.75 µM Cd and Pb), gill-Pb and gill-

Cd were about equal, suggesting strict additivity. Also, gill-Cd exceeded that of gill-Pb when

0.75 µM Pb was added to higher concentrations of Cd. In the presence of 10 mg C/L natural

organic matter (NOM) collected from Luther Marsh, gill-Cd always exceeded gill-Pb,

regardless of the mixture concentrations used.

LT50 results for fish exposed to metal mixtures for 96 h revealed that without NOM,

the mixture toxicity was dependent on Pb concentration. As Pb concentration increased, the

LT50 for the mixtures decreased. With the addition of NOM, mixture toxicity decreased with

increasing Pb. Toxicity results did not always agree with metal-gill binding, especially in

experiments with added NOM.

It is concluded that metal mixtures of Cd and Pb interact in a far more complex manner

than that suggested by the multiple-metal model mentioned above. The addition of NOM

further complicates the interactions of Cd and Pb in mixtures with fish gills by possibly

increasing the bioavailability of Cd.

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Finally, I thank my family for their much-needed support during the completion of this degree, especially my husband Kevin and my son Corson.

Dedication

This research is dedicated to my late supervisor, Dr. Richard C. Playle, who unexpectedly passed away July 8, 2005. His guidance, support, encouragement and patience encouraged me to pursue this degree and have been greatly missed. He was an excellent mentor, teacher and friend.

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Chapter 1

General Introduction

Metal contamination in the environment is an ongoing problem, particularly in aquatic environments, and there has been extensive investigation of metal effects on aquatic organisms (Playle, 1998; Niyogi and Wood, 2004). The goal of much previous research was to develop a model predicting how a particular metal will interact with an organism in any given aquatic environment, taking into account the water chemistry and the organisms present in that system. The model that was developed is now called the Biotic Ligand Model (BLM).

The BLM recognizes that metal speciation is important in aquatic environments as it is directly related to the bioavailability and toxicity of a metal to aquatic organisms. Usually, the free metal ion is the primary bioreactive form. The BLM is primarily used to predict the amount of metal that will bind to the site of toxic action (also known as the biotic ligand), which in turn is related to a toxicological response in an organism. This model also recognizes that other ions and ligands in the water influence metal toxicity by reducing the free ion activity of the metal through complexation or competition at the site of toxic action. Therefore, the predictions that are generated through this model will vary depending on the water chemistry (Paquin *et al.*, 2002).

For fish, research has identified the gill as the primary site of acute toxic action for most metals. The gills are responsible for ion and acid-base regulation, gas exchange and nitrogen excretion. Ion and acid-base regulation occur through negatively charged proteins on the surface of the gill that can bind with positively charged metals. There are three main groups of metals that exert toxicity on aquatic organisms. Monovalent metals (i.e., Ag^+, Cu^+)

disrupt Na^+ transport in the gill. Divalet metals (i.e., Cd^{2+}, Zn^{2+}) disrupt Ca^{2+} transport and there are metals that will cross the gill and act centrally (i.e., Pb^{2+}, Hg^{2+}) (Paquin *et al.*, 2002).

The BLM is an equilibrium model that functions on the assumption that all metals, ions, and ligands in the water are at chemical equilibrium with each other (Playle, 1998; Paquin *et al.*, 2002). Predictions of toxicity and metal-gill binding are made possible through the use of binding affinities of each metal to the biotic ligand and other ions and ligands in the water. Metal binding affinities are calculated from results of metal experiments involving fish or invertebrates using modelling programs such as MINEQL+ (Environmental Research Software, 1998) or WHAM (Natural Environment Research Council, 2001) and are usually expressed as log values. From these calculations, a model can be developed that illustrates how a given metal will interact with the biotic ligand (Playle, 1998).

As mentioned before, metal toxicity is dependent on the water chemistry; in particular pH, and water ion concentrations (Ca and Na) will affect metal binding to the biotic ligand. The model therefore also incorporates how the metal will interact with major cations and other ligands in the water to investigate metal complexation or competition for binding sites at the biotic ligand. Negatively charged anions in the water can also complex metals, reducing the bioavailability of the metal for the biotic ligand (Playle, 1993b; Paquin *et al.*, 2002; Niyogi and Wood, 2004). Interactions of metals with H^+ are also included to explain how water pH can affect the speciation of the metal. In addition, H^+ can compete with metal ions for binding sites at the biotic ligand. Figure 1.1 illustrates a combined Cd and Pb biotic ligand model. This model shows the affinities (log K values) for which Cd and Pb will bind to the gill in relation to other ions in the water. The model also shows the affinities that Cd and Pb have for

Figure 1.1 A combined Pb and Cd gill binding model from Playle (2004) which shows Pb and Cd competing for a common binding site (the biotic ligand) on the gill (*). Cationic competition at the gill surface and complexation of Pb and Cd in the water column also occur. The numbers represent the log conditional stability constants for Pb and Cd binding to the gill and to natural organic matter. A higher number means a stronger binding affinity between the metals and the gill, natural organic matter or other ligands in the water.
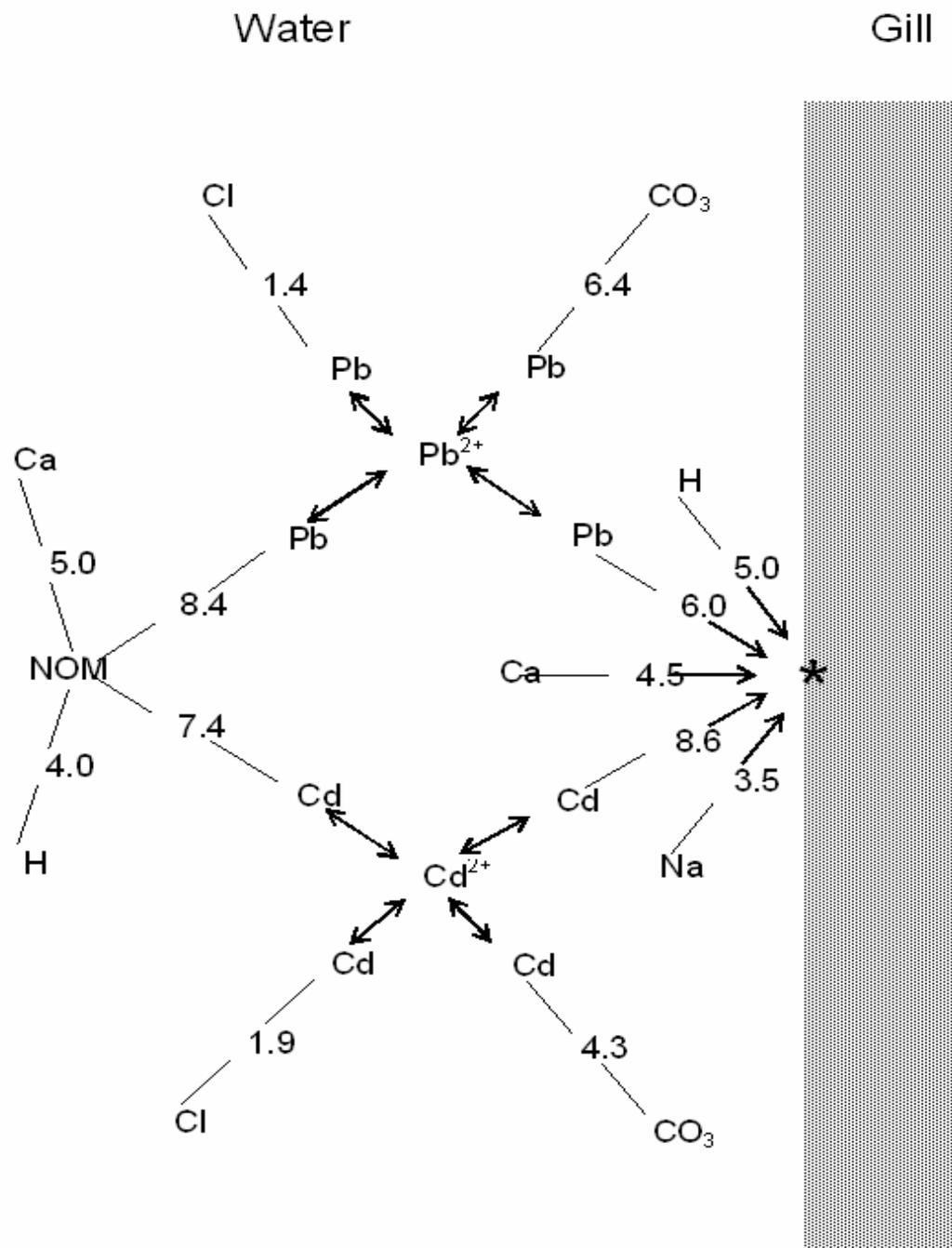
these other ions in the water and for natural organic matter, a competing ligand in the water (Playle, 2004).

Natural organic matter (NOM) is found in all natural water bodies and its chemical composition varies depending on its source. NOM is equivalent to dissolved organic carbon (DOC) or dissolved organic matter (DOM). In general, NOM is a mixture of organic compounds which includes humic and fulvic acids (Richards *et al*., 2001). Humic acids are the fraction of NOM that precipitate out of solution at low pH and have a high molecular weight. In contrast, fulvic acids are soluble under all pH conditions and lower in molecular weight (Schwartz *et al*., 2004). The chemical composition of NOM depends on its input source. NOM can be produced within the water column by phytoplankton and is termed autochthonous. NOM can also be washed into a catchment basin from terrestrial sources and is termed allochthonous. Autochthonous NOM is typically simple in chemical composition, being composed of mainly open chained carbon compounds such as carbohydrates. Allochthonous NOM is more complex, being enriched with humic and fulvic acids, which are closed chain carbon compounds. Allochthonous NOM is also typically dark in colour compared to autochthonous NOM (Richards *et al*., 2001). Many studies have shown that dark, allochthonous NOM is effective in binding metals in solution, reducing the metal bioavailability for the biotic ligand (Playle *et al*., 1993a; Hollis *et al*., 1997; Richards *et al*., 2001; Macdonald *et al*., 2002; Niyogi and Wood, 2004; Schwartz *et al*., 2004; Klinck *et al*., 2005; Winter *et al*., 2005).

The BLM faces some challenges with its use, in that it must be recognized that the biotic ligand itself is part of a living organism and will be under tight biological control. The biotic ligand exhibits changes to respond to environmental stressors, which can change the

dynamics of metal binding to the ligand. Another challenge the BLM faces is the prediction of metal binding when organisms are exposed to metal mixtures. Metal contamination is most often a mix of two or more metals and very rarely exists as a single metal. As a result, it is important to understand how aquatic organisms will respond to metals in mixtures, how those metals will interact with each other and the biotic ligand, and to ultimately generate a model that will be able to predict metal-gill binding when fish are exposed to metals in mixtures (Paquin *et al.*, 2002; Playle, 2004).

A theoretical multiple-metal model was postulated by Playle (2004) in an attempt to predict how metal mixtures would interact at the gills of fish. This model was constructed by combining existing BLMs for single metals in MINEQL+ to generate predictions on metal mixture toxicity. The multiple-metal model was based on the toxic unit concept. The toxic unit concept assumes concentration addition (Playle, 2004), which occurs when substances have the same sites of toxic action and similar modes of expressing toxicity. The alternate model is independent action in which there is a general lack of influence of one substance on the toxic effects of another. There is not always a clear difference in experimental results between concentration addition and independent action, especially when the substances being studied are at low concentrations (Dresher and Boedeker, 1995). Since the multiple-metal model is based on the toxic unit concept, it is assumed that the metals involved will interact in a purely competitive fashion. The main question the model was designed to answer was, using the toxic unit concept, will metals in mixtures exert strict additivity in their binding to the biotic ligand or will their combined actions be synergistic (more than additive) or antagonistic (less than additive) (Playle, 2004).

The multiple-metal model was based on metals that act as Ca analogues to fish. As a result, the metals in the model were assumed to be competing with one another for binding sites at the Ca channel on the fish gill. The model was based on a single 1 g fish in 1 L of water, which is equivalent to a gill site concentration of about 5 nM. For simplicity, according to this version of the model, when half of the gill sites of the fish were filled with metals, half of the organisms in the test were assumed to die. The concentration of metal at this point was considered the LC50 (lethal concentration required to produce 50% mortality) and equivalent to one toxic unit (Playle, 2004).

The multiple-metal model designed by Playle (2004) illustrated that the gill sites of fish fill with metals in a non-linear fashion (figure 1.2 A and 1.2B). As the gill sites filled with metals, the curve began to level off. When half of the gill sites were filled, the metal-binding curve crossed the line of strict additivity (straight diagonal line in figures 1.2 A-C). At this point, the concentration required to achieve metal binding to half the gill sites was the LC50 and equivalent to 1 toxic unit as explained above. When the Pb-gill and Cd-gill models were combined, their binding curves overlapped and both crossed the line of strict additivity at half a toxic unit each. This means that when a total of one toxic unit of metal was present, strict additivity would occur with respect to metal binding to the gills (figure 1.2 C). At lower metal concentrations, below a total of one toxic unit, the gill-binding curve rose above the line of strict additivity. Therefore, total metal bioaccumulation in the metal mixture was predicted to behave in a synergistic fashion (more than additive). At higher metal concentrations, the gill-binding curve fell below the line of strict additivity, resulting in a prediction that total metal bioaccumulation in the metal mixture would act antagonistically (less than additive). The same results were generated with three, four and five metals. As long as a total of one toxic unit of

Figure 1.2 From Playle (2004) (A) A calculated Pb-gill binding curve which illustrates Pb binding to gill sites (total gill sites = 5 nM). The gill was titrated with Pb from 0 to 12 µM. At 6 µM Pb, half (2.5 nM) the gill sites were filled with Pb, which was defined as 1 toxic unit. The straight diagonal line represents a linear binding curve of strict additivity. Below 6 µM Pb, gill sites filled in a synergistic manner (more than additive) and above 6 µM Pb, gill sites filled in an antagonistic manner (less than additive). (B) A calculated Cd-gill binding curve which illustrates Cd binding to gill sites as the gill is titrated with 0 to 28 nM Cd. At 14 nM, half the gill sites were filled with Cd and therefore, this was defined as 1 toxic unit. Similar to the Pb-gill binding curve, below 14 nM Cd, the gill sites filled in a synergistic manner and above 14 nM Cd, the gill sites filled in an antagonistic manner. (C) A combination of the Pb and Cd gill-binding curves. These curves illustrate Pb and Cd binding to the fish gill as the metal concentrations of both are increased from 0 to 1 toxic unit (6 µM Pb and 14 nM Cd). When half the gill sites were filled with Pb and Cd, strict additivity in metal binding was achieved and occurred at ½ toxic unit of each metal (i.e. 1 toxic unit total). Above a total of one toxic unit, the Pb and Cd mixture bound to the gill in a synergistic manner and below a total of one toxic unit, the Pb and Cd mixture bound to the gill in an antagonistic manner (Playle, 2004).
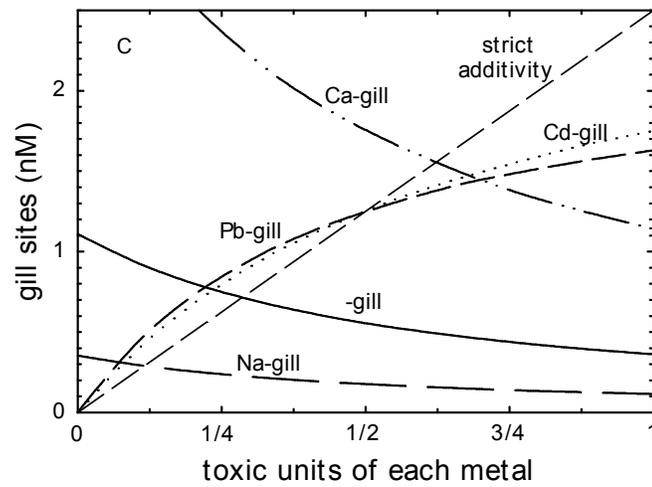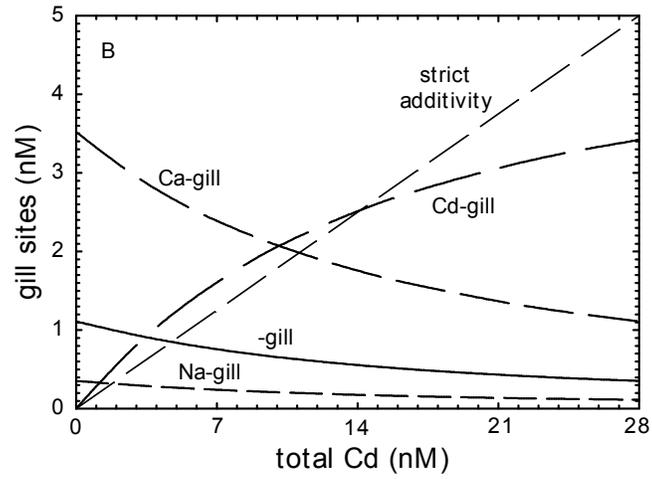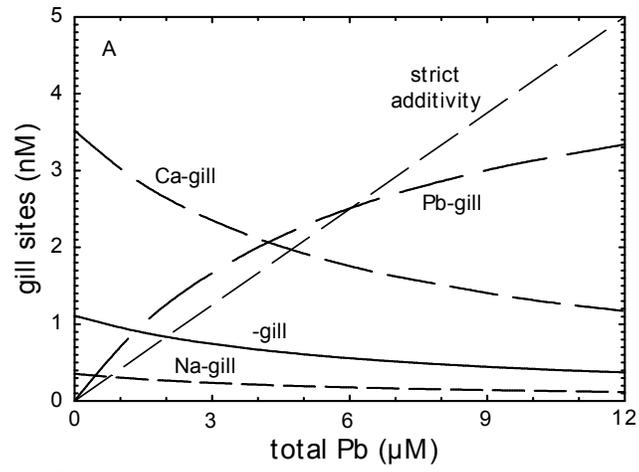
metal was present in the system, the total metal binding to the gill was predicted to be strictly additive (Playle, 2004).

The objectives for this thesis were to test the multiple metal model using Cd and Pb. Cd and Pb often appear in the environment together and are both known to affect Ca transport across the fish gill (Rogers and Wood, 2003; Rogers *et al*., 2004; Macdonald *et al*., 2002, Playle *et al*., 1993a, 1993b; Playle, 1998). These metals enter the environment though mining and smelting operations, as well as the production and disposal of batteries, automobiles, electronic hardware and paints (Fan, 1996; Newman, 1998; Carroll, 2008).

To investigate the total metal bioaccumulation of Pb and Cd on fish gills when exposed to mixtures, rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne mixtures of Pb and Cd. It was predicted, due to the binding affinities of Pb and Cd for fish gills as discussed above, that when fish were exposed to mixtures of Cd and Pb in the water at similar concentrations, that Cd-gill accumulation would be greater than Pb-gill accumulation. Metal-gill binding of Cd and Pb was also investigated when natural organic matter (NOM) was present in the water. As mentioned above, dark allochthonous NOM is effective in reducing metal-gill binding for many metals. As a result, this type of NOM was used to investigate how metal-gill binding would be affected by the addition of NOM when fish were exposed to mixtures of Cd and Pb. Since Pb has a higher binding affinity for NOM than fish gills (figure 1.1) it was predicted that the addition of NOM would complex the Pb in solution, effectively reducing the bioavailability of the Pb to bind to the fish gills. The binding affinity of Cd for NOM is less than that for fish gills (figure 1.1) and therefore, when fish were exposed to mixtures of Cd and Pb with NOM, it was predicted that fish would accumulate Cd on their gills and very minimal Pb. Full experimental procedures are outlined in chapters two and three.

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Chapter Two

The investigation of metal-gill binding and body partitioning of Cd and Pb when rainbow trout (*Oncorhynchus mykiss*) are exposed to metal mixtures.

Abstract

Multiple metal-gill modeling using the toxic unit concept indicates that metals with the same toxic actions will bind to the gills in a strictly additive fashion if the concentrations of the two metals sum to one toxic unit. Due to the non-linear nature of the models, below one toxic unit, gill-metal binding will be more-than-additive and above one toxic unit gill-metal binding will be less-than-additive. This research tests the model by exposing juvenile rainbow trout to mixtures of Pb and Cd at 0.75, 1.5, 2.25 and 3.0 µM each. Accumulation by trout gills approximated additivity at lower metal concentrations (1.5 µM Pb + 1.5 µM Cd). There was some evidence of competitive effects at the higher metal concentrations (3.0 µM Pb + 3.0 µM Cd), because gill Cd for trout exposed to mixture treatments was lower than for trout exposed to Cd alone, while gill Pb remained high. 96 h experiments revealed a greater metal toxicity to trout when exposed to Pb and Cd in mixtures compared to fish exposed to Pb or Cd alone. The bioaccumulation and partitioning of Cd and Pb within the body of 50 g rainbow trout when exposed to mixtures of these two metals was also investigated. Trout were exposed to 3 µM Pb, 3 µM Cd and a mixture of 3 µM Pb and Cd for 1, 3 and 7 d. The accumulation of metals within the trout body did not change between the single metal and mixture exposures. Trout accumulated the greatest concentration of Pb in the gills with some accumulation also in the stomach. The greatest Cd accumulations were in the liver and trunk kidney.

2.1 Introduction

Extensive work has been done to investigate the toxic effects of single metals on fish (Playle *et al*., 1993a, 1993b; Janes and Playle, 1995; Richards and Playle, 1998; Macdonald *et al*., 2002; Paquin *et al*., 2002; Niyogi and Wood, 2004; Winter *et al.*, 2005). Metals however, rarely appear singly in the environment. It is important to understand how metals in mixtures will interact with each other and with aquatic organisms. A theoretical multiple-metal gill-binding model, developed by Playle (2004) investigated how metals in mixtures would interact with fish. The model was developed by combining existing biotic ligand models for single metals in MINEQL+ to predict how these metals would bind to fish gills when present in mixtures. This model was based on the toxic unit concept and follows the kinetics of a concentration-addition model (see introduction; Playle, 2004).

Biotic ligand models operate under the assumption that the amount of metal bound to the gills of fish (or other sensitive membranes in other organisms) is directly proportional to that metal's toxic action (Playle, 2004). Competition between metals and other ions or ligands in the water decrease metal binding to the gills and therefore decreases the metal toxicity. These models view biological membranes as ligands with particular binding strengths for metals and other ions, allowing for metal toxicity to be estimated from the surrounding water chemistry (Playle, 1998; Paquin *et al.*, 2002; Playle, 2004).

The multiple-metal model developed by Playle (2004) worked under the presumption that all the metals used in the model reacted in the same way with the gills of fish. In this case, the model treated all the metals involved as Ca analogues, which would therefore compete for binding sites on the fish gills at the Ca channels. For simplicity, the model was designed so that when half of the gill sites on the fish were filled with metal, half of the fish in the experiment would die. This was equivalent to the LA50 (lethal accumulation that produces

50% mortality) and the concentration at this point was the LC50 (lethal concentration that produces 50% mortality). The LC50 was therefore seen as being equivalent to one toxic unit (Playle, 2004). According to the multiple-metal model, when two metals with the same toxic action are mixed together so that the sum of their concentrations is equal to one toxic unit, these two metals will accumulate on the gills in a purely additive fashion. When the sum of their toxic units is greater than one, the gill accumulation will be greater than strictly additive (synergistic) and when the sum of their toxic units is less than one, the gill accumulation will be less than additive (antagonistic) (see introduction for more details; Playle 2004).

The individual biotic ligand models for Cd and Pb provide a relative binding strength for each metal to fish gills and other ligands and ions in the water. The relative strength with which Cd binds to fish gills ($logK_{Cd-gill}$=8.6) is about 400 times stronger than that with which Pb binds to fish gills ($logK_{Pb-gill}$=6.0; Playle *et al.*, 1993b; Macdonald *et al.*, 2002; Playle, 2004). Based on these numbers, when fish are exposed to mixtures of Cd and Pb at comparable concentrations, it can be predicted that the fish will accumulate more Cd on their gills than Pb since Cd binds more strongly to fish gills than Pb.

Cadmium and Pb enter aquatic environments through mining and smelting operations, as well as through the manufacture and disposal of electronics, batteries and automobiles (Sorensen, 1991; Newman, 1998). Lead has been found to disrupt Ca homeostasis in fish, especially in soft water, and to cause respiratory distress (Rogers and Wood, 2004). Cadmium has also been found to disrupt plasma Ca balance in fish and to result in a decrease in plasma Na, K and Cl in chronic exposures. It is also thought that Cd will target the muscular and nervous systems of fish resulting in the paralysis of the respiratory muscles (Sorensen, 1991).

The objectives of this study were to test the multiple-metal model by exposing rainbow trout to mixtures of Pb and Cd and then analyzing the amount of metal bound to the gills. The metal gill burden was also compared to the toxicity of the metal mixtures by investigating the LT50 (time to 50% mortality) results for fish exposed to Cd and Pb in mixtures for 96 h. The body burden and partitioning of Cd and Pb when trout were exposed to mixtures of these metals was also investigated to determine if the relatively large amount of Pb found on the gills of the trout could be compared to the amount of Cd found within the trout body.

2.2 Experimental Design

2.2.1 Fish Husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*; approximately 1 g and 45 g) were obtained from Rainbow Springs Trout Hatchery (Thamesford, ON). The 1 g trout were housed in 50:50 well water and soft water with well water cooling (pH 7.5, Ca 0.5 mM). The 45 g trout were housed in a living stream supplied with aerated well water (pH 7.8 – 8.0, Ca 1 mM). All fish husbandry was in compliance with the guidelines provided by the Canadian Council of Animal Care. Trout were fed three times a week to satiation with ground or whole three pt Corey Aquaculture Trout and Charr grower (Corey Feed Mills, Fredericton N.B.). At least one week before experimentation, trout were acclimated to ion-poor water supplied by a reverse osmosis system (Culligan, Series E Reverse Osmosis System) and supplemented with a Ca drip. The chemical composition of the water was approximately: 31 μM Ca, 623 μM Na, 313 μM Cl, pH 7.0, 13 – 15 °C. To avoid fouling of the experimental water, trout were not fed 24 h before each experiment.

2.2.2 Gill Accumulation and LT50 Test

Trout (3.8 ± 1.5 g) were exposed to 3.0 µM Cd, 3.0 µM Pb, and equal mixtures of these two metals in the following concentrations: 0 (control), 0.75, 1.5, 2.25 and 3.0 µM for 3 h and 96 h; impacts were assessed in terms of mortality and gill metal concentrations. Five 1 g trout were placed into each exposure container with 10 L of ion-poor water and 50 µM Ca (added as $CaSO_2 \bullet 2H_2O$, Sigma Aldrich, Oakville ON). A GK2401C combination pH electrode (Bach-Simpson Ltd, London ON) was used to measure water pH, which was adjusted to pH 6.0 by drop-wise addition of HNO_3 or KOH.   Metals were added as chloride salt solutions ($CdCl_2 \bullet 2.5\ H_2O$, Fisher Scientific, Fair Lawn NJ; $PbCl_2$, Mallinckrodt Specialty Chemicals Company, Paris Kentucky).

Sample Collection

Fish were sampled at 3 h, death or 96 h. Fish were killed by stunning and their gills were excised. Gills were rinsed in 100 mL of ultra-pure water for 10 s to remove any loosely bound metals and then placed into clean microcentrifuge tubes. Water samples were taken at 1 and 3 h for the gill binding experiment and daily for the 96 h acute toxicity experiment. Filtered and unfiltered samples were collected for metal and organic carbon analysis. Filtered samples were processed using a 0.45 µm Millex-HV syringe filter (Millipore, Bedford, Massachusetts) that had been rinsed with 20 mL of ultra-pure water and 20 mL of sample water. Samples for metal, Ca and Na analysis were collected into 7 mL plastic scintillation vials and acidified with one drop of 16 N HNO_3 (Fisher Scientific). Samples for organic carbon analysis were collected into 7 mL boroscilicate vials that had been previously heated to 450 °C for 2 h. Water samples for chloride analysis were collected into separate 7 mL plastic scintillation vials and were not acidified. Water samples were stored at 4 °C until analysis.

2.2.3 Body Partitioning Experiment

Trout (45 ± 12 g) were exposed to treatments of 3.0 µM Cd, 3.0 µM Pb, 3.0 µM Cd + 3.0 µM Pb and control (no metals) in 20 L of ion-poor water for 7 d. Each treatment was duplicated and contained nine trout. In this experiment, impacts were assessed after 1, 3, and 7 d in terms of tissue metal accumulation. Water pH was measured using a GK2401C combination electrode (Bach-Simpson Ltd, London ON) and adjusted to pH 6.0 with the drop-wise addition of HNO_3 or KOH, and each treatment was supplemented with 50 µM Ca (as $CaSO_4 \bullet 2H_2O$; Sigma Aldrich). Metals were added as chloride salt solutions ($CdCl_2 \bullet 2.5 \ H_2O$, Fisher Scientific, Fair Lawn NJ; $PbCl_2$, Mallinckrodt Specialty Chemicals Company, Paris Kentucky). A 50% water replacement was performed daily with water containing the appropriate metal concentrations and Ca, at a pH of 6.0. Water samples were taken daily as above and analyzed for metals, ions and organic carbon. Three fish from each treatment were sampled at 1, 3, and 7 d.

Sample Collection

At each sampling time, fish were anesthetized using MS-222 (1 g of 3-Aminobenzoic acid ethyl ester into 12 L of ion-poor water, Sigma Aldrich Canada, Oakville, ON, pH adjusted to 7 with KOH), and then weighed. A 1.0 mL Luerlock syringe was rinsed with heparinized saline (0.008 g heparine sodium salt, Sigma Aldrich, in 25 mL of Cortland Saline; Nichols, 2003) and used to collect blood by caudal puncture. After mixing, two heparinized hematocrit tubes were filled and centrifuged for three minutes (Readacrit centrifuge, Clay Adams, NJ) to determine blood hematocrit (pack cell length divided by total sample length). The plasma from those samples was measured for plasma proteins using a refractometer. The remaining blood was placed into a clean microcentrifuge tube and centrifuged for two minutes at 12 700 g

(IECMicro-MB Centrifuge, VWR) and the plasma was collected and stored at -40°C until analysis.

Immediately after blood collection, esophagus, stomach, liver, gall bladder, intestine, head kidney, trunk kidney and white muscle were removed from the fish, rinsed in 10 mL of ultra pure water, blotted dry and placed into clean, pre-weighed microcentrifuge tubes. Tissue samples were frozen at -40°C until analysis.

Sample Analysis

Plasma, tissue digests and water samples were measured for metals using a graphite furnace atomic absorption spectrophotometer (GFAAS, SpectrAA-880 with GTA-100 atomizer, Varian Canada Inc, Mississauga ON). For all metal analysis, all samples were measured against radiometer standards that were used to generate standard curves (Fisher Scientific). Operating conditions for Pb analysis as specified by Varian were: 5 s at 85 °C, 40 s at 95 °C, 10 s at 120 °C, 8 s at 400 °C and 5 s at 2100 °C. Operating conditions for Cd analysis as specified by Varian were: 5 s at 85 °C, 40 s at 95 °C, 10 s at 120 °C, 8 s at 250 °C, and 4.8 s at 1800 °C.

For metal analysis, 50 µL of plasma was mixed with 50 µL of 1 N trace metal grade HNO_3 (Fisher Scientific) and 400 µL of ultra pure water before analysis by GFAAS. Tissue samples were weighed and digested with 5 x their weight in 1 N trace metal grade HNO_3 at 80 °C for 3 h. Each sample digest was diluted 10 x and analyzed for Cd and Pb by GFAAS. Bile samples were diluted 100 x with ultra pure water and measured for metals by GFAAS. Water metals were measured by GFAAS, and water Na and Ca by flame AAS (Varian Canada Inc, Mississauga ON). Water Cl was measured colourimetrically using a Spectronic 301 spectrophotmeter (Milton Roy, Rochester NY). 1 mL of unacidified water sample was added

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to 1 mL of chloride reagent (Biopacific Diagnostics, North Vancouver, BC), and measured for absorbance at 460 nm. Water chloride was calculated from a standard curve, generated by measuring chloride standards (Fisher Scientific), prepared in the same way as the unknown water samples. Water organic carbon was measured using a Schimadzu 5050A Total Organic Carbon Analyzer (Mandel Scientific Co. Ltd.).

2.2.4 Statistical Analysis

All data were presented as the mean ± 1 S.D. (n = 5 for 3 and 96 h tests, n= 2 means of 3 fish each for body partitioning experiment). LT50's (h) from the 96 h experiment were determined graphically by plotting percent survival against time (h). One and two-way ANOVAs were calculated by hand on the tissue metal accumulation data from the body partitioning experiment to determine if there was any heterogeneity in the means. Where differences occurred, Tukey post-hoc test were applied (Zar, 1984).

2.3 Results

2.3.1 Acute Gill Accumulation and LT50 Test

When trout were exposed for 3 h to 3 µM Pb and 3 µM Cd alone, the gills accumulated approximately the same amount of metal, 20 nmol/g wet weight (figure 2.1). When exposed to a mixture of 3 µM Pb and Cd, the fish accumulated the same amount of Pb on their gills as the Pb alone treatment, but the gill-Cd was reduced to about half of the gill-Cd accumulation when trout were exposed to Cd alone. When trout were exposed to the other mixtures of Cd and Pb, gill Pb was higher than gill Cd in the 2.25 µM mixture, gill metals were the same for the 1.5 µM mixture and gill Cd exceeded that of gill Pb for the 0.75 µM mixture (figure 2.1).

When trout were exposed for 96 h to the same metal concentrations mentioned above, the LT50 values decreased when trout were exposed to mixtures of Pb and Cd at the higher

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Figure 2.1 The 3 h metal-gill binding of Pb and Cd after exposure of fish to mixtures of Pb

and Cd. Black bars represent gill Pb accumulation and grey bars represent gill Cd

accumulation. Data are presented as means ± 1 S.D. (n=5). Water treatment concentrations

are nominal. Actual measured water metal concentrations are illustrated in table 2.1. Numbers

in brackets are the LT50 results for fish exposed to the same treatment concentrations for 96 h.
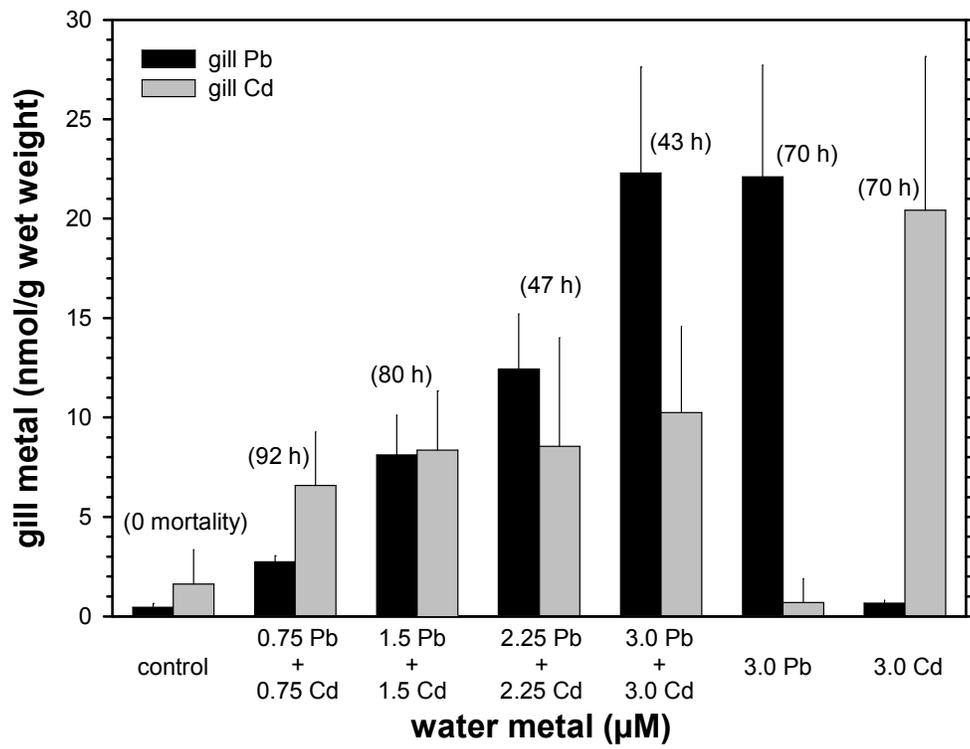
Table 2.1 – Water chemistry for the 3 h acute metal accumulation and 96 h toxicity test. All values are means ± 1 S.D. Water samples were taken at 1 h and 3 h for acute experiments and daily for 96 h experiments.

Treatment	experiment	Pb (µM)		Cd (µM)		Ca (µM)	Na (µM)	Cl (µM)	TOC (mg C/L)	DOC (mg C/L)
		total	dissolved	total	dissolved					
3 µM Pb	3 h	3.02 ± 0.00	2.94 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	44 ± 1	465 ± 1	58 ± 6	0.8 ± 0.1	0.8 ± 0.1
	96 h	3.13 ± 0.45	2.96 ± 0.32	0.01 ± 0.01	0.01 ± 0.01	48 ± 1	520 ± 7	66 ± 8	1.3 ± 0.4	1.2 ± 0.6
3 µM Cd	3 h	0.00 ± 0.01	0.01 ± 0.00	3.25 ± 0.08	3.28 ± 0.04	45 ± 3	473 ± 3	60 ± 11	1.7 ± 0.8	0.8 ± 0.2
	96 h	0.00 ± 0.00	0.00 ± 0.00	3.20 ± 0.10	3.11 ± 0.07	48 ± 1	512 ± 14	68 ± 13	1.3 ± 0.4	1.1 ± 0.4
3 µM Pb + 3 µM Cd	3 h	3.02 ± 0.06	2.97 ± 0.04	3.26 ± 0.01	3.30 ± 0.16	48 ± 2	474 ± 6	56 ± 5	0.7 ± 0.1	0.8 ± 0.1
	96 h	2.92 ± 0.23	2.79 ± 0.23	3.32 ± 0.10	3.27 ± 0.09	48 ± 1	538 ± 12	87 ± 19	1.4 ± 0.3	1.3 ± 0.3
2.25 µM Pb + 2.25 µM Cd	3 h	2.54 ± 0.01	2.40 ± 0.04	2.50 ± 0.06	2.45 ± 0.14	46 ± 1	470 ± 2	56 ± 3	0.7 ± 0.1	0.9 ± 0.1
	96 h	2.33 ± 0.31	2.13 ± 0.41	2.56 ± 0.05	2.54 ± 0.05	51 ± 1	566 ± 12	83 ± 23	1.2 ± 0.4	1.1 ± 0.4
1.5 µM Pb + 1.5 µM Cd	3 h	1.74 ± 0.05	1.54 ± 0.06	1.64 ± 0.01	1.70 ± 0.02	46 ± 1	474 ± 2	59 ± 11	0.6 ± 0.2	0.8 ± 0.0
	96 h	1.61 ± 0.08	1.38 ± 0.16	1.70 ± 0.04	1.70 ± 0.05	50 ± 1	551 ± 11	70 ± 14	1.3 ± 0.3	1.4 ± 0.6
0.75 µM Pb + 0.75 µM Cd	3 h	0.74 ± 0.02	0.50 ± 0.02	0.78 ± 0.02	0.76 ± 0.05	46 ± 2	468 ± 1	56 ± 2	1.0 ± 0.1	0.8 ± 0.1
	96 h	0.52 ± 0.12	0.27 ± 0.11	0.84 ± 0.03	0.80 ± 0.03	51 ± 1	549 ± 5	70 ± 13	1.7 ± 0.9	1.6 ± 0.6
control	3 h	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	44 ± 1	466 ± 4	55 ± 8	0.75 ± 0.1	0.9 ± 0.0
	96 h	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	49 ± 1	522 ± 9	58 ± 3	1.0 ± 0.2	1.0 ± 0.1

concentrations (figure 2.1). When exposed to either 3.0 μM Pb or Cd alone, the LT50 was 70

h. When trout were exposed to a mixture of 3.0 μM Pb and Cd, the LT50 decreased to 43 h.

The LT50 for fish exposed to 2.25 μM Pb and Cd was 47 h, also more toxic than either Pb or

Cd alone. Fish exposed to mixtures of 1.5 μM and 0.75 μM Pb and Cd survived longer than

fish exposed to 3.0 μM Pb or Cd alone with LT50s of 80 h and 92 h respectively.

For clarity, the gill-binding and LT50 results are displayed using the nominal water

metal concentrations. The measured water chemistry for the 3 h gill accumulation and 96 h

acute toxicity tests is illustrated in table 2.1. Little variation in water metal concentration

occurred over the 3 h experiment with Cd or Pb. There was some variation in water Pb

concentrations over the 96 h experiment for exposures with higher Pb concentrations (2.25 and

3.0 μM; table 2.1). Organic carbon concentrations for the 96 h experiments were also slightly

higher than the 3 h experiments presumably due to mucus secretions over a longer exposure

time.

2.3.2 Body Partitioning Experiment

Metal analysis was completed for plasma, gills, esophagus, liver, stomach,

intestine, head kidney, trunk kidney, bile and white muscle to determine the body partitioning

of metals when fish were exposed to 3.0 μM Pb, 3.0 μM Cd and a mixture of 3.0 μM Pb and

Cd for 1, 3, and 7 d. The data displayed are the 1 d tissue metal concentrations. After 1 d, due

to unexpected mortality, it was not possible to collect relevant blood data from some fish. The

tissue metal accumulation for 3 d and 7 d followed the same trend as the 1 d results. The tissue

metal results illustrated here are gill, esophagus, liver, stomach, intestine, head kidney, and

trunk kidney (figure 2.2) and plasma (figure 2.3). There was no metal accumulation above

Figure 2.2 Tissue metal concentrations in the gills, esophagus, liver, stomach, intestine, head kidney, and trunk kidney of trout exposed to 3 µM Pb (A), 3 µM Cd (B), and a mixture of 3 µM Pb and Cd (C) for 1 d. Each bar represents the mean ± 1 S.D. of two replicates, in which there were three fish each. There was no metal accumulation above the controls for white muscle or bile so this data is not shown.
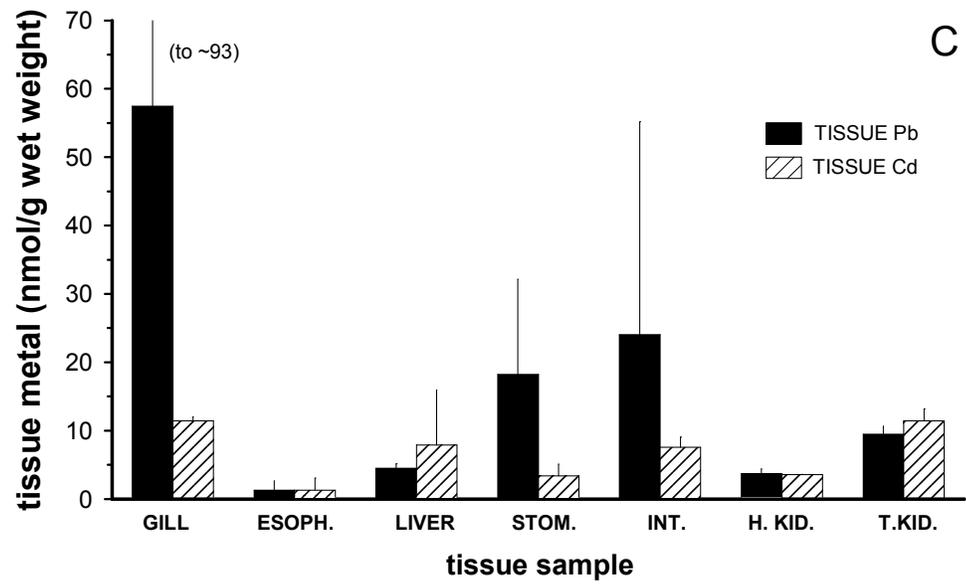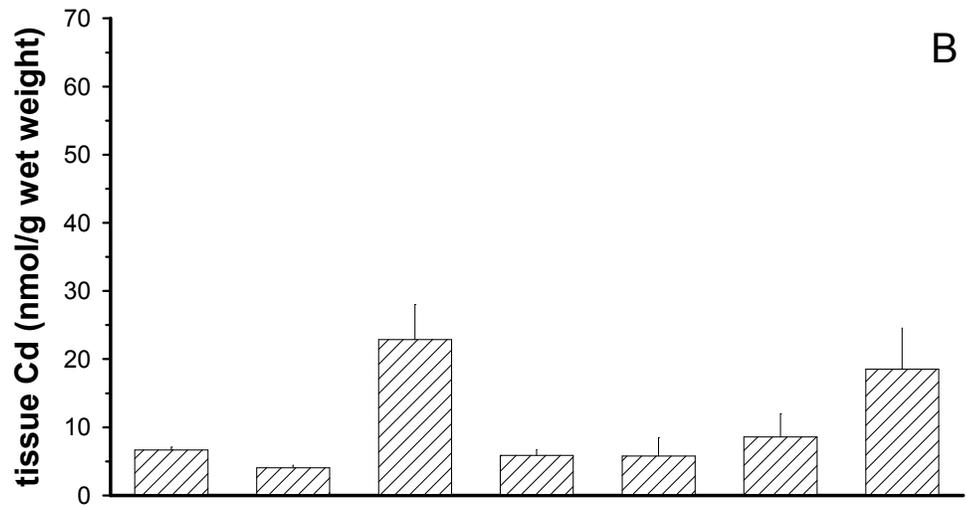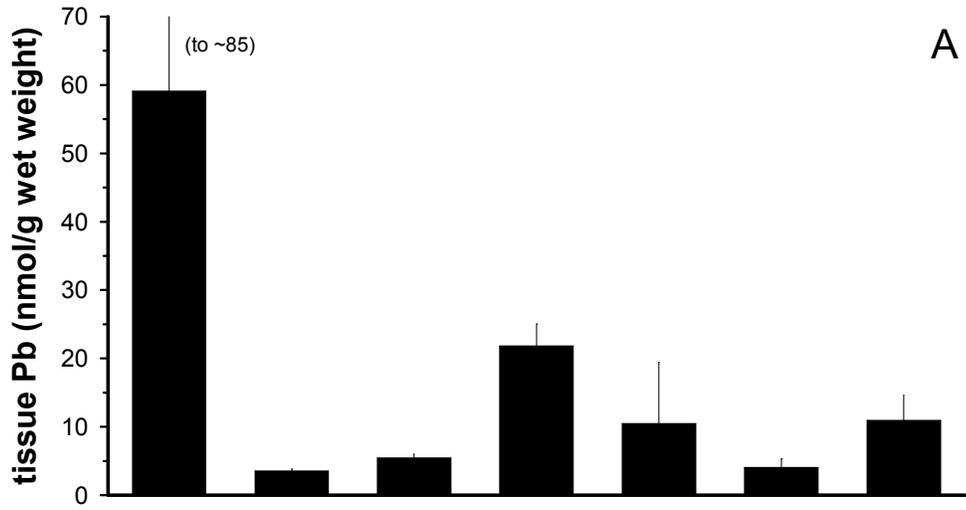
Figure 2.3 – Plasma metal concentrations for fish exposed to 3 μM Pb, 3 μM Cd and a mixture of these two metals for 1 d. Plasma Pb concentration is represented by black bars, and plasma Cd concentration is represented by hatched bars. Data are expressed as the mean ± 1 S.D. (n=2 replicates of 3 fish each).
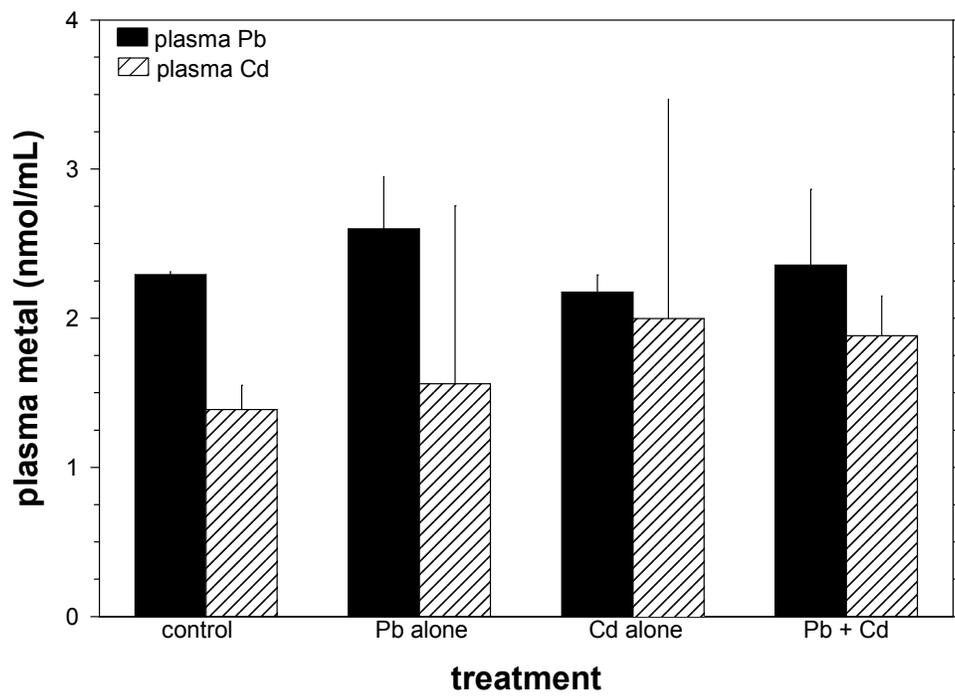
Table 2.2 – Mean water chemistry for the body partitioning experiment. Each treatment has two values to represent the replicate exposures. Water samples were taken daily over a 7 d period. Values are means ± 1 S.D. Values in brackets illustrate the range for each mean.

Treatment	Cd (μM)		Pb (μM)		Ca (μM)	Na (μM)	Cl (μM)	TOC (mg C/L)	DOC (mg C/L)
	total	dissolved	total	dissolved					
3.0 μM Pb	0.03 ± 0.09 (-0.09 - 0.17)	0.04 ± 0.11 (-0.13 - 0.12)	2.40 ± 0.27 (2.10 - 2.66)	0.48 ± 1.04 (-0.09 - 2.56)	57 ± 14 (43 - 80)	541 ± 51 (496 - 634)	247 ± 78 (163 - 385)	2.9 ± 0.8 (1.6 - 3.7)	2.8 ± 1.4 (1.2 - 4.8)
	0.06 ± 0.15 (-0.12 - 0.18)	0.08 ± 0.15 (-0.13 - 0.24)	2.36 ± 0.42) (1.56 - 2.60)	0.40 ± 1.00 (-0.10 - 2.43)	55 ± 12 (39 - 72)	534 ± 26 (506 - 574)	265 ± 54 (202 - 334)	3.6 ± 2.3 (1.5 - 8.0)	3.0 ± 1.3 (1.3 - 4.4)
3.0 μM Cd	2.93 ± 0.36 (2.55 - 3.33)	2.85 ± 0.37 (2.40 - 3.31)	-0.01 ± 0.01 (-0.01 - 0.00)	0.00 ± 0.01 (-0.01 - 0.00)	52 ± 15 (37 - 76)	532 ± 25 (500 - 558)	249 ± 33 (206 - 299)	2.6 ± 0.8 (1.2 - 3.2)	2.6 ± 1.1 (1.1 - 4.1)
	2.88 ± 0.33 (2.58 - 3.27)	2.73 ± 0.34 (2.35 - 3.10)	-0.01 ± 0.01 (-0.01 - 0.00)	0.00 ± 0.01 (-0.01 - 0.00)	53 ± 14 (41 - 76)	536 ± 37 (496 - 578)	260 ± 48 (186 - 303)	2.9 ± 1.1 (1.4 - 3.8)	2.8 ± 1.2 (1.4 - 4.2)
3.0 μM Pb + 3.0 μM Cd	2.86 ± 0.23 (2.68 - 3.32)	2.74 ± 0.31 (2.39 - 3.29)	2.30 ± 0.44 (1.47 - 2.68)	0.56 ± 0.97 (-0.04 - 2.50)	55 ± 14 (41 - 78)	556 ± 44 (516 - 610)	277 ± 60 (197 - 368)	2.4 ± 1.0 (1.4 - 2.6)	2.4 ± 1.1 (1.4 - 3.7)
	2.77 ± 0.21 (-0.11 - 0.25)	2.66 ± 0.23 (2.35 - 3.04)	2.21 ± 0.1 (1.72 - 2.64)	0.36 ± 0.88 (-0.12 - 2.14)	55 ± 12 (39 - 75)	551 ± 33 (505 - 604)	270 ± 45 (213 - 347)	3.0 ± 0.9 (1.5 - 3.9)	3.2 ± 1.0 (1.5 - 4.0)
control	0.11 ± 0.13 (-0.11 - 0.25)	0.07 ± 0.15 (-0.12 - 0.25)	-0.01 ± 0.02 (-0.06 - 0.01)	0.00 ± 0.01 (-0.01 - 0.00)	53 ± 11 (38 - 73)	486 ± 51 (417 - 551)	189 ± 78 (86 - 306)	2.5 ± 0.4 (1.7 - 3.0)	2.5 ± 0.8 (1.6 - 4.0)
	0.08 ± 0.16 (-0.11 - 0.24)	0.08 ± 0.16 (-0.13 - 0.26)	0.00 ± 0.01 (-0.01 - 0.00)	0.00 ± 0.01 (-0.01 - 0.01)	53 ± 9 (39 - 69)	503 ± 42 (433 - 543)	193 ± 85 (94 - 317)	2.7 ± 0.9 (1.7 - 3.6)	2.9 ± 1.6 (1.5 - 5.3)

the controls for white muscle and bile, and no change in blood hematocrit or proteins over the course of the experiment so these values are not reported.

When trout were exposed to Pb and Cd individually for 1 d, Pb showed a significantly greater concentration on the gills when compared with the other tissues (figure 2.2; $p<0.025$). Stomach Pb levels appeared to be higher than other tissues, although this result was not significant. Cd accumulation was significantly greater in the liver and trunk kidney when compared with the other tissues ($p<0.05$). Interestingly, the gills did not accumulate a significantly greater concentration of Cd when compared with the other tissues. There was no significant accumulation of Pb or Cd in the plasma above the controls for fish exposed to Pb or Cd individually (figure 2.3). Mean Pb tissue concentrations for control fish were as follows: gill: below detection, esophagus: 0.334 ± 0.091, liver: 2.430 ± 0.307, stomach: 7.668 ± 0.367, intestine: 2.043 ± 0.080, head kidney: 1.712 ± 0.045 and trunk kidney: 1.989 ± 0.066. Mean Cd tissue concentrations for control fish were as follows: gill: 2.456 ± 0.715, esophagus: 0.540 ± 1.354, liver: 4.849 ± 1.812, stomach: 1.249 ± 0.004, intestine: 3.164 ± 0.812, head kidney: 2.397 ± 0.226 and trunk kidney: 2.759 ± 1.886.

When fish were exposed to a mixture of Pb and Cd, most of the metal accumulation results were similar to those obtained in the single metal treatments. When the metal accumulation within a single tissue was compared, the gills and stomach accumulated significantly greater concentrations of Pb than Cd ($p<0.05$). The liver and trunk kidney accumulated significantly greater concentrations of Cd than Pb ($p<0.05$). The plasma did not accumulate significant concentrations of Pb or Cd when fish were exposed to mixtures of these two metals (figure 2.3).

For clarity, figures 2.2 and 2.3 were constructed using the nominal water metal concentrations. Measured water chemistry is illustrated in table 2.2 and is reported as the mean ± 1 S.D for water samples taken daily over 7 d. Due to the length of this experiment, there was some variation in measured water Cd and Pb. Water pH was monitored often, however it varied by one pH unit between adjustments due to bucket aeration and fish metabolic activities. This pH variation was enough to allow some Pb and Cd to fall out of solution and to greatly reduce the concentration of dissolved Pb in the water when compared to total Pb concentrations (table 2.2)

A two-way ANOVA was performed to determine if there were any significant decreases in Pb or Cd accumulation from the single metal treatments, for any given tissue exposed to the metal mixture treatment. The liver and trunk kidney Cd levels appeared to be reduced in the mixture treatment compared with the Cd alone treatment, however, there were no significant differences in metal accumulation between the treatments.

2.4 Discussion

2.4.1 Acute Gill Binding and LT50 Experiments

When juvenile rainbow trout were exposed to mixtures of Pb and Cd, gill-Pb exceeded that of gill-Cd for mixtures of 2.25 and 3.0 µM Pb and Cd (figure 2.1). When trout were exposed to a mixture of 3.0 µM Pb and Cd, gill-Cd accumulation was half of the gill-Pb accumulation, and half of the gill-Cd accumulation for fish exposed to 3.0 µM Cd alone. This result is different from the predicted results for fish exposed to mixtures of Cd and Pb: based on the multiple metal model of Playle (2004), Pb and Cd should interact at the gills of fish in a purely competitive fashion. Both metals are known to be Ca analogues and presumably, both will interact at the gills of fish only at the Ca channels (Verbost *et al.*, 1989; Macdonald *et al.*,

2002; Playle, 2004). When the previously determined binding affinities for both metals were taken into account (log $K_{Cd-gill}$ = 8.6, log $K_{Pb-gill}$=6.0), it was presumed that the metal gill binding results would yield a greater amount of Cd on the gills than Pb due to the higher binding affinity of Cd for fish gills than Pb (Playle, 2004). The metal gill binding results for fish exposed to 1.5 µM Cd and Pb revealed equal amounts of gill-Cd and gill-Pb. This result suggested that Pb and Cd were interacting in a strictly additive fashion, with equal attraction to the gill. Trout exposed to 0.75 µM Cd and Pb displayed a greater amount of gill-Cd than gill-Pb. This result was expected due to the relative binding affinities of each metal for fish gills, as mentioned above.

Based on these results, it would appear that at low concentrations, Pb and Cd compete for binding sites on the gill and that since Cd has a higher binding affinity for gills than Pb, more Cd accumulates on the fish gills. At higher metal concentrations, it appears than Pb is out-competing Cd for gill binding sites. This result was unexpected and suggests that multiple metal models cannot rely solely on the pre-determined relative binding affinities for metals to gills calculated from single metal exposures.

The results from the LT50 experiment revealed that Pb and Cd are more toxic in mixtures than either metal alone at higher concentrations (figure 2.1). The LT50 for both 3.0 µM Cd and 3.0 µM Pb alone was 70 h. When 3 µM Pb and Cd were mixed together, the LT50 was 43 h which is very close to half of the LT50 for 3 µM Pb or Cd alone (i.e. 35 h), suggesting that strict additivity with regards to toxicity was occurring, as predicted by the multiple-metal model (Playle, 2004). The 1.5 µM mixture treatment produced an LT50 of 80 h. Again, this result was close to the LT50 for both the 3.0 µM Cd or Pb alone treatments (LT50=70 h), suggesting strict additivity with regards to toxicity. The LT50 for the 0.75 µM

mixture treatment was greater (92 h) than that achieved for 3 µM Cd or Pb alone, however, this result was expected since the total metal concentration in the 0.75 µM mixture treatment is less than 3.0 µM.

When metal-gill binding was compared with the LT50 results for mixtures of Pb and Cd, the treatment exposures that resulted in the greatest toxicity also resulted in the greatest concentration of metal on the gills (figure 2.1). The metal-gill binding results for fish exposed to 1.5 µM Pb and Cd suggested strict additivity, similar to the LT50 data. The toxicity of Cd and Pb in the 2.25 and 3.0 µM mixtures was greater than that of 3.0 µM Cd or Pb alone, and the gill binding experiment resulted in greater metal-gill binding for fish exposed to these mixtures when compared to 3.0 µM Pb or Cd alone. The gill binding data for these experiments is perplexing however, since the gill-metal results are different from what was expected and do not clearly illustrate pure competition between the metals for binding sites, as predicted by the multiple-metal model (Playle, 2004).

The binding affinities for Cd and Pb for fish gills were calculated from experiments that used lower metal concentrations than those used here. The $logK_{Cd-gill}$ was calculated to be 8.6 by Playle *et al* (1993b), using 50 nM Cd and fathead minnows. The $logK_{Pb-gill}$ was calculated to be 6.0 by Macdonald *et al,* (2002) using 0.6 to 1.0 µM Pb and rainbow trout. In contrast, the experiments conducted here exposed trout to Cd and Pb concentrations of 0.75 to 3.0 µM. With regards to the higher gill-Pb binding compared to gill-Cd binding seen here, it is possible that at higher metal concentrations there are actually multiple sites for metal binding to the gills. Evidence of multiple binding curves has been found by Rogers *et al.* (2005) and Birceanu *et al.* (submitted) when fish are exposed to Pb at higher concentrations. It is thought that there are high-affinity, low-capacity sites that fill up first, and low-affinity, high-capacity

sites that fill with Pb once the other sites are saturated (Rogers *et al.*, 2005; Birceanu *et al.*, submitted). If this is indeed occurring when fish are exposed to higher metal concentrations, this may explain the presence of more Pb on the gills than Cd when fish are exposed to mixtures of these two metals. If there are multiple binding sites on the gills for Pb to bind to, then Cd and Pb are not interacting in a purely competitive fashion when present in mixtures. The multiple-metal model, using the toxic unit concept, was based on concentration-addition, which is a purely competitive system with regards to metal interactions with the biotic ligand (Playle, 2004). Since it appears that Pb and Cd are not solely interacting in a competitive fashion, this theory cannot be used when investigating multiple metal interactions of Cd and Pb with regards to gill binding.

2.4.2 Body Partitioning Experiment

The body partitioning experiment was performed to investigate the differences in Cd and Pb accumulation within the trout body. The previous experiments resulted in higher gill-Pb than gill-Cd when trout were exposed to metal mixtures. To reconcile this result, it was hypothesized that Cd was being transported into the body of the trout while Pb was binding to the gill only.

When trout were exposed to 3.0 µM Pb, the gills of the fish accumulated the highest concentration of Pb. There was also some accumulation in the stomach, intestine and trunk kidney. The concentration of Pb found in the gills, however, was significantly greater than that found in the other tissues (figure 2.2). The accumulation of Pb in the stomach, intestine and trunk kidney of the trout was surprising since there was no Pb accumulation in the plasma of the fish (figure 2.3). These fish were not fed during the experiment and it is unlikely that these fish were drinking the water as salt-water fish do (Evans, 1993). According to Sorensen

(1991), Pb is absorbed into the fish body through the gills and intestinal epithelium and the tissues that accumulate the most Pb during either water or dietary exposure are the kidney, gill and liver. It was hypothesized that Pb must be binding to the erythrocytes in the blood and being transported through the body that way. This actually has been found to be true in other studies (Sorensen, 1991). It was also hypothesized, that as Pb was binding to the gills of the fish, the fish were able to clear their gills of mucus and thus, clear away any Pb that was bound by the gills. In order to clear mucus from the gills, fish reverse the flow of water over their gills (Moyle and Cech, 2000). This flow reversal could have resulted in the cleared mucus entering the mouth of the fish. If this mucus was swallowed, any Pb that was bound to that mucus would have ended up in the stomach of the fish, possibly explaining the Pb accumulation found in the stomach tissue (Borgmann and Dixon, personal communication).

When exposed to 3.0 µM Cd, trout accumulated Cd in the liver and trunk kidney. There was no significant Cd accumulation in any of the other tissues, including the gills (figure 2.2). Similar to the Pb accumulation, there was also no Cd accumulation in the plasma of the fish (figure 2.3). It has been found that Cd is transported through the body by metallothionein and other Cd binding proteins (Sorensen 1991). Cadmium has been shown to have a high affinity for sulfhydryl groups which increases its lipid solubility. It has also been found that Cd will displace Zn in large proteins and it is thought that Cd will move across plasma membranes using the same carrier proteins as Zn (Sorensen, 1991). This suggests that Cd, like Pb may have an alternate route of entry into the gills of fish, other than the apical Ca channel, allowing Cd to enter the body of the fish both through the apical and basolateral membranes of the gills.

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The low Cd accumulation on the gills of trout when exposed to Cd alone was unexpected since the gills are known to be one of the main pathways for Cd to enter into the body (Sorensen, 1991). However, it has been found that exposure times greater than 3 h results in a build-up and sloughing of surface mucus which aids in Cd secretion and increases survival among fish (Sorensen 1991). The 24 h exposure period of this experiment may have been long enough to remove most of the Cd bound to the gill, resulting in low gill Cd accumulation. Similarly, this 24 h exposure period could also have been long enough to transport much of the Cd bound on or within the gill to other parts of the fish body.

It was hypothesized that the difference in Pb and Cd accumulation on the gills of rainbow trout when trout were exposed to mixtures of Pb and Cd was a result of Pb remaining on the gills of the fish and Cd being transported into the fish body. If this were the case, it would have been expected that Pb would have only been found on the gills and not within the body of the trout. However, Pb accumulated in the stomach, intestine and trunk kidney as well as the gills. Therefore, Pb is not being sequestered only by the gills but is also being transported into the body. The hypothesis that Pb was remaining on the gills while Cd was being transported into the body of the trout was therefore not supported by these results.

The results of this experiment illustrate that when fish are exposed to mixtures of Cd and Pb at higher concentrations, these two metals are not interacting in a purely competitive fashion. If these metals were competing for binding sites, it would be expected that more Cd would bind to the gill and within the body than Pb based on their relative binding affinities for fish gills as stated above. It should be noted that Pb does interact at the gills of fish in other ways than just through the apical Ca channels. Studies done by Rogers *et al.* (2003) and Rogers *et al.* (2005) have shown that Pb will disrupt the Na, K, and Cl balance in the fish

suggesting alternative modes of toxic action that just the Ca channels. Also, as mentioned above, Pb has been shown to produce multiple binding curves when fish are exposed to this metal in higher concentrations, suggesting multiple binding sites on the gill (Rogers *et al.*, 2005, Birceanu *et al.*, submitted). It is also possible that Cd can interact at the gill at more than one site if it can displace Zn in major proteins, allowing it to cross plasma membranes, as mentioned above (Sorensen, 1991). It is clear that the toxic interactions of Pb and Cd in mixtures, with respect to fish are not as simple as initially thought. These two metals seem to interact with each other to some degree but not in a purely competitive fashion. It is necessary to investigate how these two metals interact with each other and how each metal affects the gill binding of the other.

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Chapter 3

The influence of Pb on Cd-gill binding and toxicity when rainbow trout (*Oncorhynchus mykiss*) are exposed to metal mixtures with and without organic matter.

Abstract

Multiple metal-gill modelling based on the toxic unit concept suggests that metals with the same toxic actions (e.g. Pb and Cd interacting at Ca-gill channels) will exhibit strictly additive bioaccumulation if the concentrations of the two metals sum to one toxic unit. Due to the non-linear nature of the models, the metal mixture will be more than strictly additive below one toxic unit and less than strictly additive above one toxic unit. This research tests this model against reality by exposing juvenile rainbow trout (~1 g) to mixtures of Cd and Pb. Previous research revealed that mixtures of Pb and Cd often result in higher gill-Pb than gill-Cd even though Cd has a stronger binding affinity to trout gills that Pb. To investigate how Pb affects Cd binding to fish gills, trout were exposed to a range of Cd concentrations (0.75, 1.5, 2.25 and 3.0 µM) while maintaining constant Pb concentrations. At the lowest metal concentrations (0.75 µM Cd and Pb), gill-Pb and gill-Cd were about equal suggesting strict additivity. Gill-Cd exceeded gill-Pb however, when 0.75 µM Pb was added to higher concentrations of Cd (1.5-3.0 µM). In the presence of 10 mg C/L of natural organic matter (NOM) collected from Luther Marsh, gill-Cd always exceeded gill-Pb, regardless of the mixture concentration used. Acute 96 h toxicity tests were also conducted to compare metal-gill binding with toxicity. Toxicity results did not always agree with metal-gill binding, especially in experiments with added NOM. It is concluded that metal mixtures of Cd and Pb interact in a far more complex manner than that suggested by the multiple metal model mentioned above. The addition of NOM further complicates the interactions of Cd and Pb in mixtures with fish gills by possibly increasing the bioavailability of Cd.

3.1 Introduction

Extensive work has been done on the effects of individual metals on aquatic organisms and many biotic ligand models have been developed as a result (see Playle, 1998; Paquin *et al.*, 2002; Niyogi and Wood, 2004 for reviews). While these models are thorough and useful in predicting individual metal-gill binding, they fall short of being able to predict the effects of metal mixtures on aquatic organisms. Playle (2004) developed a theoretical multiple metal model by combining existing biotic ligand models for individual metals into MINEQL+ to predict how metals in mixtures would interact with each other and with fish. The multiple metal model was based on the toxic unit concept and is therefore a purely competitive model. Consequently, the metals included in the model are assumed to all have the same sites and modes of toxic action and to compete for binding sites on the fish gill.

The research presented here has focused on two of the metals included in the model, Cd and Pb. As mentioned above, it was assumed in the model that both of these metals would bind to, and exert toxicity at the same site on the gill, in this case, the Ca channels (Playle, 2004). Based on the previously determined log binding affinities for Cd and Pb to the gill, ($logK_{Cd-gill}$=8.6, $logK_{Pb-gill}$=6.0; see chapter 1 for further information) it was expected that when fish were exposed to mixtures of Cd and Pb in comparable concentrations, there would be a greater amount of Cd binding to the gills than Pb. Previous experiments have found that when juvenile rainbow trout were exposed to equal concentrations of Cd and Pb in mixtures (0.75, 1.5, 2.25 and 3.0 μM), the concentration of Pb bound to the gills was greater than that of Cd (see chapter 2 for full details).

Since Pb was binding to the gills of 1 g trout in much greater amounts than Cd when trout were exposed to these two metals in mixtures, a body partitioning experiment was conducted to investigate how these metals were distributed throughout the body when 50 g

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trout were exposed to a mixture of 3.0 µM Cd and Pb. The results again displayed a very high concentration of Pb on the gills when compared with Cd, and a higher concentration of Pb bound on and within the trout body compared to Cd (see chapter 2 for full details).

Due to the unexpected high concentrations of Pb binding to the gills of trout when compared to Cd in the previous experiments as mentioned above, a matrix of experiments was run to investigate how Pb affected Cd binding to the gills. Fish were exposed to a range of Cd concentrations with constant amounts of Pb, repeated over the same concentration range. These experiments were conducted as 3 h acute binding tests and LT50 tests with a duration of 96 h. The entire experimental protocol was repeated with the addition of natural organic matter (NOM) to investigate how NOM affected metal binding to the gills when fish were exposed to Cd and Pb in mixtures.

According to the multiple metal model developed by Playle (2004), the addition of NOM was expected to completely change the relationship between the metals in the mixture and the organisms involved. This is because each metal has it's own relative binding affinity to NOM as well as the biotic ligand. Some metals bind to NOM stronger than to the biotic ligand. Other metals have a stronger binding affinity for the biotic ligand than for NOM. As a result, NOM would provide protection against some metals while not protecting against others. With respect to Cd and Pb, Cd has a stronger binding affinity to the biotic ligand than to NOM. Pb however, has a stronger binding affinity to NOM than the biotic ligand. Therefore, when trout were exposed to Cd and Pb in mixtures with NOM, it was expected that the gills would accumulate Cd while the Pb would be bound by the NOM, making it unavailable to the fish (Macdonald *et al.*, 2002; Playle, 2004; Schwartz *et al.*, 2004).

The objectives of this study were to investigate the effects that Pb had on Cd binding to the gills of juvenile rainbow trout (1 g) and to evaluate how the addition of NOM affected this relationship. The results of this study were then modelled to compare these results with those of the multiple metal model with respect to Cd and Pb. In particular, it was necessary to determine if Cd and Pb were interacting in competitive or non-competitive ways. The basic formula for a metal and competing ion bound competitively to a ligand in the BLM is:

$$ML= \frac{Lt*Km*M}{1+Km*M+Kx*X} \qquad \text{(equation 1)}$$

where M is the concentration of the first metal, X is a competing metal, Km and Kx are the binding constants for each metal to the biotic ligand and Lt is the total concentration of the ligand. When the binding curves for the metal to the ligand do not level off much, Lt is very large (infinite in some situations) and Km is very small (0 in some situations). Therefore, the competitive model can be written as:

$$ML= \frac{LtKm*M}{1+Km*M+Kx*X} \qquad \text{(equation 2)}$$

where LtKm=Lt*Km from equation 1 and is equivalent to the slope of the line at low M. LtKm can often be estimated with much greater precision than either Lt or Km individually.

When the two metals under investigation are not competing, the formula is as follows:

$$ML= \frac{LtKm*M}{(1+Km*M)*(1+Kx*X)} \qquad \text{(equation 3)}$$

To determine whether Cd and Pb were interacting in competitive or non-competitive ways, both equations 2 and 3 were used when modelling results (Borgmann *et al.*, 2004; Borgmann *et al.*, 2008 in press).

3.2 Experimental Design

3.2.1 Fish Husbandry

Juvenile rainbow trout (1.90 ± 1.67 g) were obtained from Rainbow Springs trout hatchery in Thamesford, Ontario. Trout were housed in a 50:50 mix of well water and soft (ion-poor) water and fed ground 3pt trout char (Corey Feed Mills, Fredericton, NB) three times a week. Trout were acclimated to ion-poor water at least one week before experimentation, supplied by a reverse osmosis unit (Series E Reverse Osmosis System, Culligan of Canada, Mississauga, Ontario) with an added Ca drip to maintain fish ion homeostasis. Water chemistry of the acclimation water was approximately: Ca = 53 μM, Na = 400 μM, Cl = 196 μM, pH = 7.0, 13 − 17 °C. Trout were starved for 24 h before any experiments to avoid fouling of the experimental water.

3.2.2 3 h Matrix Experiments

Fish were exposed for 3 h to a range of Cd concentrations (nominal 0.75, 1.5, 2.25 and 3.0 μM) with constant Pb concentrations over the same concentration range. Metals were added as chloride salt solutions to 10 L of ion-poor water with 50 μM Ca (added as $CaSO_4$ $2H_2O$, Sigma-Aldrich, St Louis, MO). Water pH was measured using a Sper Scientific Advanced pH meter (model 840035, Scottsdale, AZ) and adjusted to pH 6.0 with HNO_3 or KOH to keep the Pb in solution. Exposure containers were randomized to prevent position effects and 5 fish were added randomly to each container. Water temperature was 12 °C. Filtered and unfiltered water samples were taken at 1 h and 3h and analyzed for total and dissolved Cd and Pb, total Ca^{2+}, Na^+, and Cl^-, and organic carbon. Water samples were filtered using 0.45 μM Millex-HV syringe filters (Millipore, Bedford, Massachusetts).

All treatments mentioned above were repeated with the addition of 10 mg C/L natural organic matter (NOM). NOM was concentrated in the field from Luther Marsh, near Guelph

Ontario in June of 2005. Source water was filtered through 144 mm diameter glass fibre filters (pore size 1μm, Geotech Environmental Equipment, Denver CO) and pumped into a stainless steel portable reverse osmosis unit (Limnological Research Corporation, Kelowna B.C.). Water was concentrated about 40 fold (200 L to 5 L) to produce the NOM concentrate. The NOM concentrate was treated in the laboratory with a H$^+$ cation exchange resin to pH 2.0 (USF C-211 H cation resin, U.S. Filter Corporation, Rockford IL). See Schwartz *et al.* (2004) for full details.

3.2.3 96 h Matrix Experiments

To compare the toxicity of Pb and Cd in mixtures with and without NOM, the above experiments were repeated as 96 h toxicity experiments. Water metal, Ca and pH conditions were kept the same as the 3 h experiments. Fish gills were sampled at fish death or 96 h. Filtered and unfiltered water samples were taken daily.

3.2.4 Analytical Methods

After 3 h of metal exposure, fish were individually removed from the exposure containers and killed by stunning. Fish gills were removed and rinsed in 100 mL of ultra-pure water for 10 s to remove any loosely bound metals and placed into individual, clean microcentrifuge tubes. Each gill was weighed and mixed with 5 times its weight in 1 N trace-metal grade HNO$_3$. Gills were digested by heating them for 3 h at 80 °C. Digested gills were agitated, vortexed and diluted with ultra-pure water for metal analysis. Gills for Pb analysis were diluted 10 times and gills for Cd analysis were diluted 20 times. Gill and water metal concentrations were measured using a graphite furnace atomic absorption spectrophotometer (Varian SpetrAA-880 with GTA 100 atomizer; Varian Canada Inc., Mississauga ON.). Operating conditions for Pb analysis as specified by Varian were: 5 s at 85 °C, 40 s at 95 °C,

10 s at 120 °C, 8 s at 400 °C and 5 s at 2100 °C. Operating conditions for Cd analysis as specified by Varian were: 5 s at 85 °C, 40 s at 95 °C, 10 s at 120 °C, 8 s at 250 °C, and 4.8 s at 1800 °C. Metal standards were prepared from Fisher certified reference solutions (Fisher Scientific, Nepean ON).

Water Ca and Na were measured from acidified water samples by flame AAS (SpectrAA 880, Varian Canada Inc., Mississauga ON) against standards prepared from Fisher certified reference solutions (Fisher Scientific, Nepean ON). Water chloride was measured by adding 1 mL of chloride reagent (Biopacific Diagnostics, North Vancouver, B.C.) to 1 mL of unacidifed water sample. Prepared samples were measured for absorbance at 460 nm on a Spectronic 301 spectrophotometer (Milton Roy, Rochester NY).   Chloride standards were made using an analytical grade chloride standard (Fisher Scientific, Nepean ON). Total and dissolved organic carbon was measured using a Schimadzu 5050A Total Organic Carbon Analyzer (Mandel Scientific). Each water sample was sparged with ultra pure compressed N_2 to remove inorganic carbon.

3.2.5 Modelling
Modelling of experimental results was performed using Systat to determine if Pb and Cd were behaving in a competitive or non-competitive manner. Measured water metal concentrations were converted to free ion activity in WHAM (Windermere Humic Aqueous Model) using the other measured water parameters mentioned above, and setting the system open to the atmosphere. Metal-gill results from the 3 h matrix experiments were plotted against the metal free ion activity. Both the competitive and non-competitive models were used (as mentioned above in introduction) to calculate the K values for each metal to

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investigate how Cd and Pb were interacting in mixtures with rainbow trout (see appendix A for full modelling methods).

3.3 Results

3.3.1 3 h Matrix Experiments

When trout were exposed to Cd in the presence of Pb, Cd binding to the gills was reduced when compared with fish exposed to Cd alone (figure 3.1). Fish exposed to the lowest amount of Pb (0.75 μM) with Cd experienced the smallest decrease in Cd binding. Fish exposed to higher amounts of Pb (1.5 to 3.0 μM) showed a much greater decrease in gill-Cd binding.

When the same data was viewed as gill-Pb binding in the presence of Cd, the amount of Pb accumulated on the gill was not affected by Cd in the water (figure 3.2). In fact, the presence of Cd in the water with Pb seems to have increased gill-Pb binding in the 1.5 and 3.0 μM Pb treatments. The gill binding figures have been constructed using the nominal water chemistry for clarity. Tables 3.1 to 3.6 illustrate measured water metals and ions. Dissolved metal concentrations were very close to the total metal concentrations for all treatments.

The addition of 10 mg C/L NOM to the matrix experiments changed the dynamics of metal gill binding in relation to those experiments with no added NOM. The addition of NOM decreased gill-Cd binding (figure 3.3) when compared with fish exposed to Cd without NOM (figure 3.1). Gill-Cd accumulation was not decreased by the presence of Pb in the water when NOM was present (figure 3.3). When 0.75 and 1.5 μM Pb was added to the treatments, gill Cd was actually enhanced when compared to fish exposed to Cd alone with NOM. When 2.25 and 3.0 μM Pb was present, the gill-Cd binding was not different from fish exposed to Cd alone with NOM

Figure 3.1 – Gill-Cd concentrations of trout exposed to Cd alone (0.75 to 3.0 µM) and Cd with

constant Pb over the same concentration range for 3 h. Each data point represents the mean of

5 fish ± S.E. Open circles represent fish exposed to Cd only. Trout exposed to Cd with 0.75,

1.5, 2.25 and 3.0 µM Pb are represented by solid squares, triangles, diamonds and hexagons

respectively.

gill Cd, 3 h, soft water, pH 6

Cd only

0.75 µM Pb

1.5 µM Pb

3.0 µM Pb

2.25 µM Pb

gill Cd (nmol/g wet weight)

water Cd (µM)
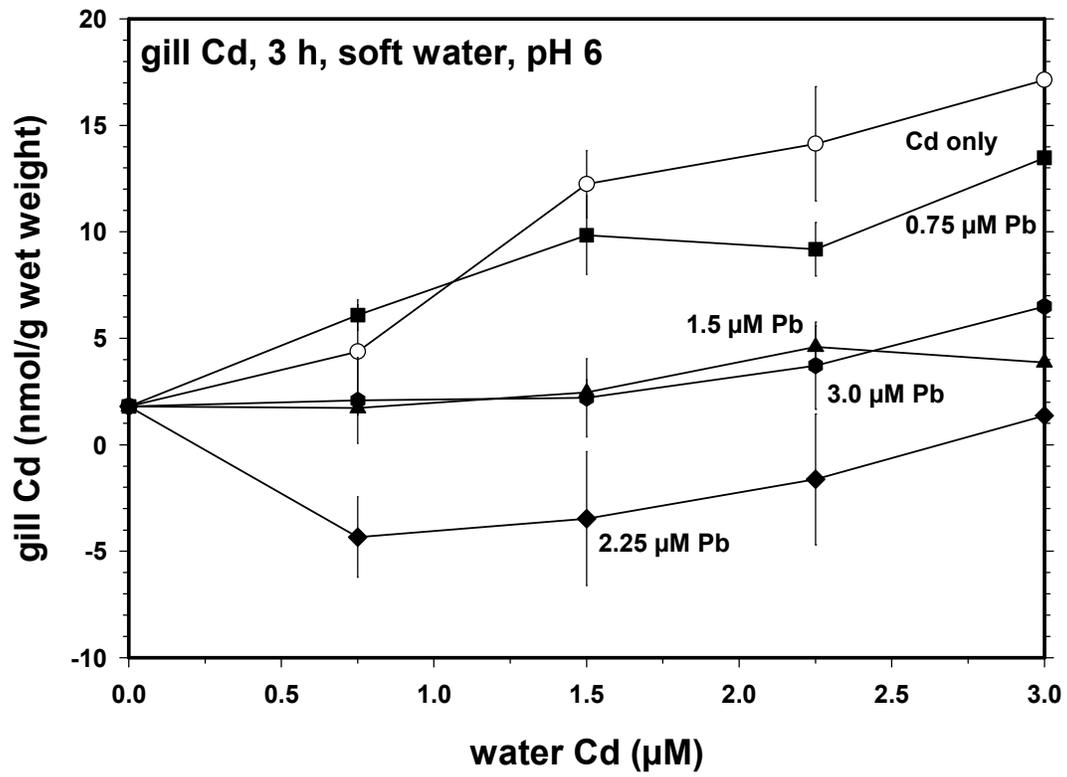
Figure 3.2 – Gill-Pb concentrations of trout exposed to Pb alone (0.75 to 3.0 µM) and Pb with

constant Cd over the same concentration range for 3 h. Each data point represents the mean of

5 fish ± S.E. Open circles represent fish exposed to Pb only. Trout exposed to Pb with 0.75,

1.5, 2.25 and 3.0 µM Cd are represented by solid squares, triangles, diamonds and hexagons

respectively.

gill Pb, 3h, soft water, pH 6

1.5 µM Cd

0.75 µM Cd

2.25 µM Cd

3.0 µM Cd

Pb only

gill Pb (nmol/g wet weight)

water Pb (µM)
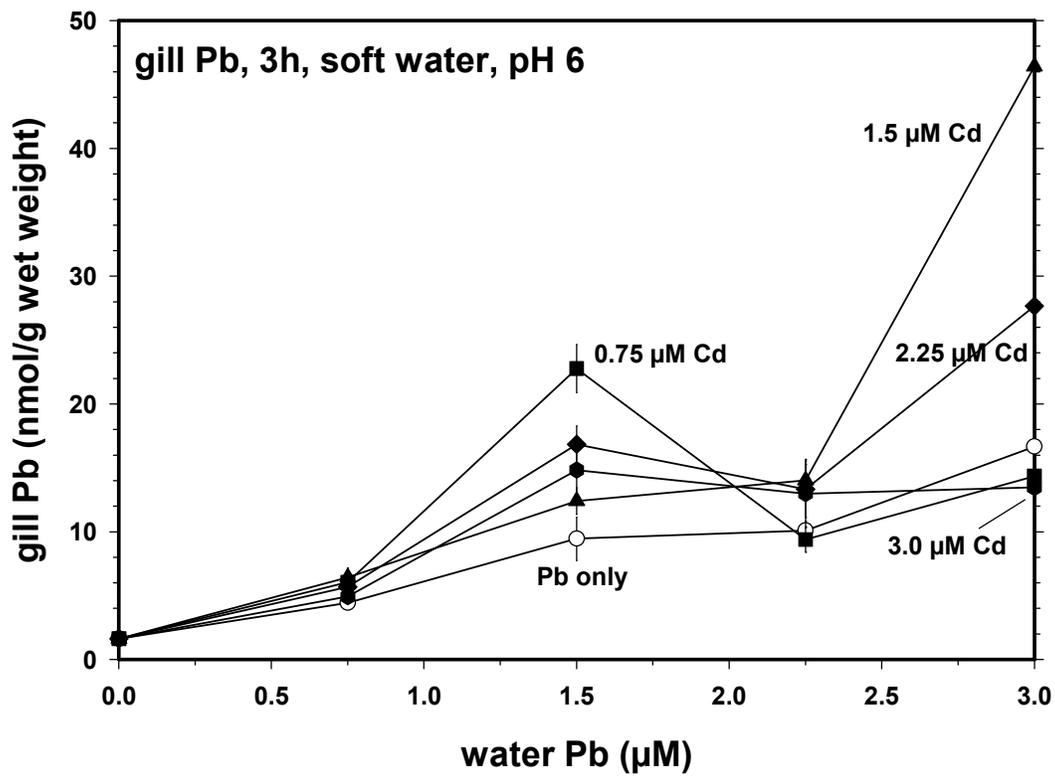
The presence of NOM had a profound effect on the gill-Pb binding results for fish exposed to Cd and Pb with NOM. For fish exposed to Pb alone with NOM (figure 3.4), gill Pb was reduced when compared to fish exposed to Pb only (figure 3.2). When Cd was present in the water with Pb and NOM, gill-Pb was greatly reduced when compared to fish exposed to Pb alone with NOM (see figure 3.4). As mentioned above, the gill binding figures were constructed using nominal water chemistry. Tables 3.1 to 3.6 illustrate the measured water metals, ions and organic carbon. The addition of NOM to the treatment water aided in keeping Pb in solution, to a greater degree than experiments with no NOM added.

3.3.2 Modelling

The metal gill binding modelling results for the matrix experiments failed to provide any clear indication about whether or not Cd and Pb are competing for the same binding sites on the fish gills at these experimental metal concentrations. With respect to gill-Cd binding, Pb does inhibit Cd uptake at the gill. When the gill-Cd results were modelled with water Pb, both the competitive (equation 2) and non-competitive (equation 3) models worked equally well, producing very similar $\log K_{Cd}$, $\log K_{Pb}$, and R^2 values. All modelling calculations were performed using μM water concentrations. Therefore, the K values generated, of a molar basis are all x 10^6 (Appendix A). For the competitive model, $\log K_{Cd}$ was calculated to be 5.4 and $\log K_{Pb}$ was calculated to be 6.5 with an R^2 of 0.514. For the non-competitive model, $\log K_{Cd}$ was calculated to be 5.3 and $\log K_{Pb}$ was calculated to be 6.3 with an R^2 of 0.512.

With respect to gill-Pb binding, Cd did not inhibit Pb uptake at the fish gills. Therefore, for both the competitive and non-competitive models, K_{Cd} was 0 and as a result, $\log K_{Cd}$ was insignificant. For both models, the $\log K_{Pb}$ was calculated to be 5.6 with an R^2 of 0.800. To model Pb binding to the gills, the gill-Pb values had to be log transformed due to a

Figure 3.3 – Gill-Cd concentrations of trout exposed to Cd alone (0.75 to 3.0 µM) and Cd with

constant Pb over the same concentration range with the addition of 10 mg C/L NOM for 3 h.

Each data point represents the mean of 5 fish ± S.E. Open circles represent fish exposed to Cd

only + NOM. Trout exposed to Cd with 0.75, 1.5, 2.25 and 3.0 µM Pb + NOM are represented

by solid squares, triangles, diamonds and hexagons respectively.

gill Cd, 3 h, soft water, pH 6, 10 mg C/L

0.75 µM Pb

1.5 µM Pb

Cd only

3.0 µM Pb

2.25 µM Pb

gill Cd (nmol/g wet weight)

water Cd (µM)
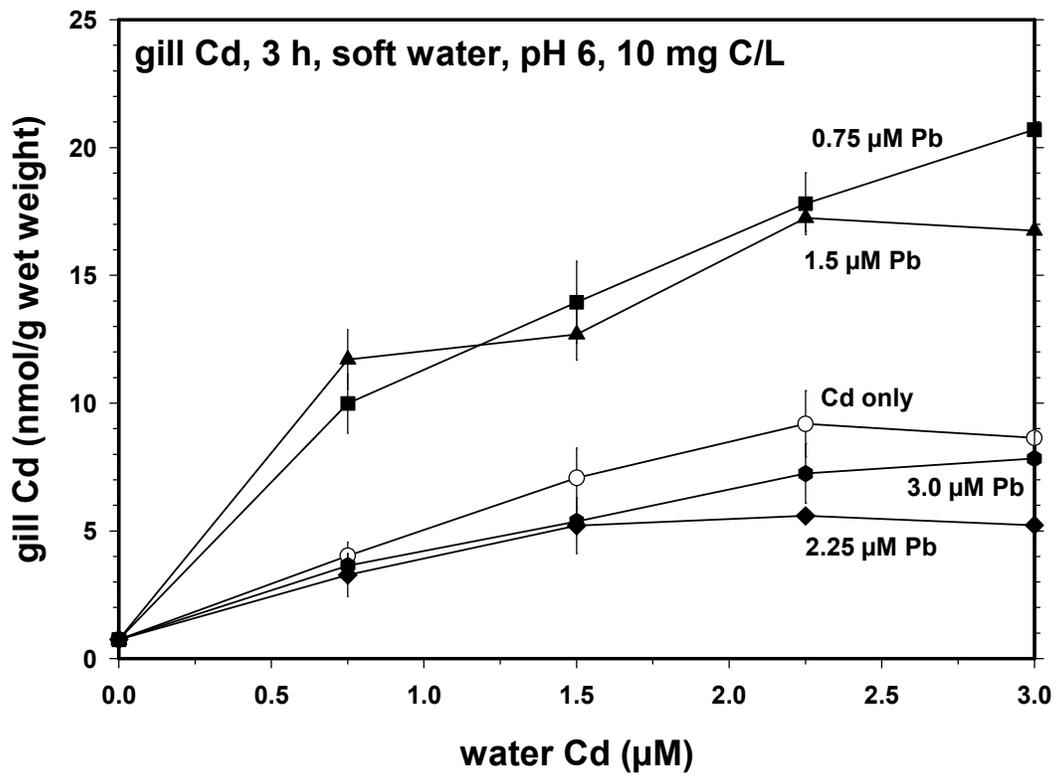
Figure 3.4 – Gill-Pb concentrations of trout exposed to Pb alone (0.75 to 3.0 µM) and Pb with

constant Cd over the same concentration range with the addition of 10 mg C/L NOM for 3 h.

Each data point represents the mean of 5 fish ± S.E. Open circles represent fish exposed to Pb

only + NOM. Trout exposed to Pb with 0.75, 1.5, 2.25 and 3.0 µM Cd + NOM are represented

by solid squares, triangles, diamonds and hexagons respectively.

gill Pb, 3h, soft water, pH 6, 10 mg C/L

Pb only

1.5 µM Cd

2.25 µM Cd

0.75 µM Cd

3.0 µM Cd

gill Pb (nmol/g wet weight)

water Pb (µM)
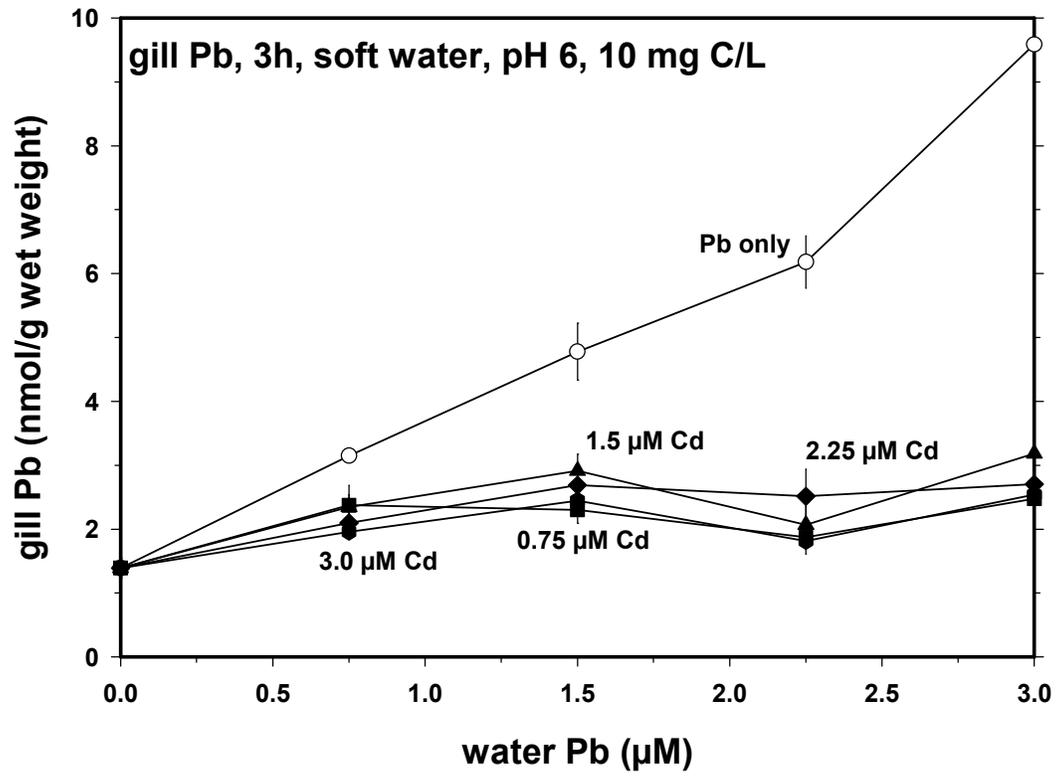
Table 3.1 – Water chemistry for the 3 h acute metal accumulation and 96 h toxicity test for fish exposed to Cd only. Water samples were taken at 1 h and 3 h for acute experiments and daily for 96 h experiments. All values are means ± 1 S.D. Treatments with (*) are with added NOM.

Treatment	test	Cd (µM)		Ca (µM)	Na (µM)	Cl (µM)	TOC mg C/L	DOC mg C/L
		total	dissolved					
0.75 µM Cd	3 h	0.65 ± 0.03	0.62 ± 0.03	48 ± 1	498 ± 8	57 ± 21	1.1 ± 0.2	1.1 ± 0.0
	96 h	0.70 ± 0.05	0.67 ± 0.04	44 ± 4	204 ± 10	67 ± 14	0.7 ± 0.2	0.8 ± 0.3
1.5 µM Cd	3 h	1.38 ± 0.01	1.36 ± 0.11	46 ± 1	528 ± 11	76 ± 9	0.9 ± 0.9	1.6 ± 0.2
	96 h	1.34 ± 0.02	1.33 ± 0.06	48 ± 6	203 ± 12	69 ± 13	0.7 ± 0.3	0.8 ± 0.3
2.25 µM Cd	3 h	1.66 ± 0.65	1.78 ± 0.52	48 ± 2	498 ± 8	52 ± 1	1.4 ± 0.3	1.3 ± 0.1
	96 h	1.98 ± 0.05	2.03 ± 0.07	47 ± 3	177 ± 33	64 ± 8	0.6 ± 0.3	0.7 ± 0.3
3.0 µM Cd	3 h	2.70 ± 0.21	2.51 ± 0.18	46 ± 2	502 ± 1	54 ± 6	1.0 ± 0.2	1.3 ± 0.1
	96 h	2.68 ± 0.07	2.66 ± 0.07	48 ± 4	214 ± 14	72 ± 16	0.7 ± 0.3	0.7 ± 0.1
control	3 h	0.00 ± 0.00	0.00 ± 0.00	48 ± 1	508 ± 2	56 ± 5	0.9 ± 0.3	0.4 ± 0.1
	96 h	0.12 ± 0.06	0.11 ± 0.07	44 ± 7	200 ± 14	60 ± 10	0.4 ± 0.2	0.6 ± 0.2
0.75 µM Cd *	3 h	1.00 ± 0.13	0.97 ± 0.09	56 ± 3	412 ± 8	195 ± 0	8.3 ± 0.0	8.5 ± 0.0
	96 h	0.54 ± 0.18	0.66 ± 0.06	34 ± 13	65 ± 18	49 ± 2	11.0 ± 1.6	15.0 ± 5.3
1.5 µM Cd *	3 h	2.00 ± 0.45	2.02 ± 0.47	60 ± 7	428 ± 8	212 ± 4	8.9 ± 0.1	9.1 ± 0.0
	96 h	1.40 ± 0.07	1.39 ± 0.06	40 ± 4	80 ± 6	48 ± 4	14.3 ± 2.6	13.8 ± 2.8
2.25 µM Cd *	3 h	2.75 ± 0.74	2.74 ± 0.71	54 ± 4	416 ± 12	200 ± 1	8.5 ± 0.1	8.4 ± 0.1
	96 h	2.22 ± 0.10	2.15 ± 0.14	48 ± 3	82 ± 5	53 ± 5	14.0 ± 3.4	13.1 ± 1.8
3.0 µM Cd *	3 h	3.72 ± 1.46	3.73 ± 1.44	60 ± 1	422 ± 25	221 ± 8	7.8 ± 0.1	8.4 ± 0.1
	96 h	3.04 ± 0.06	3.13 ± 0.25	44 ± 7	78 ± 7	49 ± 3	13.6 ± 2.1	13.0 ± 2.0
control *	3 h	-0.02 ± 0.04	-0.02 ± 0.03	52 ± 8	426 ± 3	248 ± 79	8.3 ± 0.1	8.4 ± 0.1
	96 h	0.02 ± 0.03	0.01 ± 0.00	73 ± 60	75 ± 17	49 ± 7	13.0 ± 2.0	13.0 ± 1.4

Table 3.2 – Water chemistry for the 3 h acute metal accumulation and 96 h toxicity test for fish exposed to Pb only. Water samples were taken at 1 h and 3 h for acute experiments and daily for 96 h experiments. All values are means ± 1 S.D. Treatments with (*) are with added NOM.

Treatment	test	Pb (µM)		Ca (µM)	Na (µM)	Cl (µM)	TOC (mg C/L)	DOC (mg C/L)
		total	dissolved					
0.75 µM Pb	3 h	0.74 ± 0.04	0.49 ± 0.01	46 ± 1	578 ± 3	150 ± 6	3.1 ± 0.3	3.1 ± 0.1
	96 h	0.34 ± 0.11	0.34 ± 0.15	46 ± 5	211 ± 15	63 ± 13	0.6 ± 0.2	0.8 ± 1.1
1.5 µM Pb	3 h	1.41 ± 0.08	1.20 ± 0.06	49 ± 0	564 ± 7	144 ± 8	1.9 ± 0.1	3.0 ± 0.0
	96 h	0.99 ± 0.16	0.84 ± 0.36	45 ± 3	132 ± 12	43 ± 8	0.6 ± 0.3	0.5 ± 0.2
2.25 µM Pb	3 h	2.30 ± 0.02	1.85 ± 0.00	46 ± 0	564 ± 6	154 ± 8	3.5 ± 2.6	2.8 ± 0.6
	96 h	1.43 ± 0.58	1.21 ± 0.56	47 ± 4	209 ± 17	72 ± 15	0.7 ± 0.3	0.7 ± 0.3
3.0 µM Pb	3 h	2.83 ± 0.03	2.72 ± 0.08	46 ± 2	576 ± 6	149 ± 1	2.0 ± 1.1	2.6 ± 0.1
	96 h	1.75 ± 0.83	1.54 ± 1.26	44 ± 9	207 ± 23	79 ± 18	0.7 ± 0.2	0.8 ± 0.4
control	3 h	0.03 ± 0.00	0.02 ± 0.01	48 ± 1	548 ± 0	138 ± 11	2.0 ± 0.1	1.6 ± 0.9
	96 h	0.00 ± 0.01	0.00 ± 0.01	44 ± 7	209 ± 14	60 ± 10	0.4 ± 0.2	0.6 ± 0.2
0.75 µM Pb *	3 h	1.02 ± 0.01	1.00 ± 0.04	59 ± 1	392 ± 73	198 ± 9	8.4 ± 0.2	8.6 ± 0.1
	96 h	0.61 ± 0.05	0.57 ± 0.10	41 ± 2	78 ± 3	44 ± 2	12.2 ± 1.8	13.5 ± 2.0
1.5 µM Pb *	3 h	2.15 ± 0.04	2.04 ± 0.21	54 ± 2	446 ± 3	214 ± 2	8.7 ± 0.3	8.7 ± 0.6
	96 h	1.23 ± 0.12	1.22 ± 0.08	44 ± 4	71 ± 7	48 ± 3	13.9 ± 2.7	13.5 ± 2.2
2.25 µM Pb *	3 h	2.98 ± 0.06	3.01 ± 0.04	56 ± 3	340 ± 120	206 ± 6	8.3 ± 0.0	8.4 ± 0.6
	96 h	2.46 ± 0.28	2.39 ± 0.33	46 ± 2	80 ± 7	55 ± 7	13.7 ± 2.2	12.7 ± 1.0
3.0 µM Pb *	3 h	2.00 ± 0.05	2.00 ± 0.01	58 ± 1	426 ± 9	207 ± 0	8.6 ± 0.1	8.8 ± 0.1
	96 h	2.85 ± 0.44	2.82 ± 0.44	46 ± 1	82 ± 6	58 ± 8	13.1 ± 2.9	13.6 ± 2.2
control *	3 h	0.01 ± 0.00	0.01 ± 0.00	52 ± 8	426 ± 3	248 ± 79	8.4 ± 0.0	8.4 ± 0.1
	96 h	0.01 ± 0.01	0.01 ± 0.00	73 ± 60	75 ± 17	49 ± 7	13.0 ± 2.0	13.0 ± 1.4

Table 3.3 – Water chemistry for the 3 h acute metal accumulation and 96 h toxicity test for fish exposed to Cd + 0.75 μM Pb. Water samples were taken at 1 h and 3 h for acute experiments and daily for 96 h experiments. All values are means ± 1 S.D. Treatments with (*) are with added NOM.

Treatment	test	Cd (μM)		Pb (μM)		Ca (μM)	Na (μM)	Cl (μM)	TOC (mg C/L)	DOC (mg C/L)
		total	dissolved	total	dissolved					
0.75 μM Cd+	3 h	0.69 ± 0.08	0.66 ± 0.06	0.68 ± 0.09	0.29 ± 0.06	45 ± 1	388 ± 3	48 ± 4	4.1 ± 1.8	2.4 ± 0.6
0.75 μM Pb	96 h	0.62 ± 0.02	0.63 ± 0.01	0.48 ± 0.13	0.31 ± 0.08	28 ± 5	236 ± 20	63 ± 9	0.6 ± 0.3	0.7 ± 0.1
1.5 μM Cd +	3 h	1.46 ± 0.06	1.36 ± 0.01	0.72 ± 0.01	0.28 ± 0.01	50 ± 1	398 ± 2	52 ± 4	2.7 ± 0.8	2.1 ± 0.6
0.75 μM Pb	96 h	1.37 ± 0.06	1.34 ± 0.03	0.41 ± 0.21	0.32 ± 0.18	34 ± 10	238 ± 17	62 ± 9	0.6 ± 0.2	0.7 ± 0.1
2.25 μM Cd +	3 h	2.17 ± 0.01	2.11 ± 0.06	0.70 ± 0.04	0.55 ± 0.00	47 ± 0	388 ± 1	48 ± 1	3.0 ± 0.4	2.3 ± 0.7
0.75 μM Pb	96 h	1.94 ± 0.10	1.96 ± 0.05	0.40 ± 0.12	0.26 ± 0.10	28 ± 4	245 ± 22	68 ± 12	0.5 ± 0.2	0.7 ± 0.1
3.0 μM Cd +	3 h	2.76 ± 0.1	2.69 ± 0.04	0.66 ± 0.04	0.32 ± 0.04	50 ± 0	384 ± 2	49 ± 4	2.6 ± 0.2	2.6 ± 0.6
0.75 μM Pb	96 h	2.50 ± 0.08	2.47 ± 0.03	0.35 ± 0.23	0.22 ± 0.16	29 ± 10	245 ± 28	75 ± 18	0.6 ± 0.2	0.7 ± 0.2
control	3 h	-0.06 ± 0.04	-0.07 ± 0.03	0.01 ± 0.01	0.01 ± 0.01	48 ± 1	384 ± 2	47 ± 1	3.4 ± 0.0	2.5 ± 1.3
	96 h	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	29 ± 6	229 ± 23	59 ± 9	0.7 ± 0.3	0.8 ± 0.2
0.75 μM Cd+	3 h	0.87 ± 0.00	0.86 ± 0.04	1.20 ± 0.02	1.22 ± 0.04	56 ± 4	172 ± 7	96 ± 8	8.2 ± 0.3	8.4 ± 0.4
0.75 μM Pb *	96 h	0.68 ± 0.07	0.71 ± 0.03	0.64 ± 0.08	0.65 ± 0.06	40 ± 5	77 ± 7	53 ± 4	13.1 ± 1.8	13.0 ± 2.2
1.5 μM Cd +	3 h	1.57 ± 0.04	1.54 ± 0.04	1.18 ± 0.01	1.16 ± 0.04	53 ± 0	172 ± 1	94 ± 1	9.8 ± 3.2	8.3 ± 0.3
0.75 μM Pb *	96 h	1.45 ± 0.15	1.37 ± 0.22	0.61 ± 0.04	0.61 ± 0.09	45 ± 5	75 ± 11	50 ± 3	11.4 ± 2.6	12.7 ± 2.3
2.25 μM Cd +	3 h	2.16 ± 0.18	2.17 ± 0.08	1.19 ± 0.01	1.18 ± 0.04	53 ± 1	320 ± 17	173 ± 0	8.0 ± 0.0	8.0 ± 0.1
0.75 μM Pb *	96 h	2.21 ± 0.15	1.98 ± 0.28	0.65 ± 0.02)	0.62 ± 0.13	46 ± 4	79 ± 9	54 ± 4	13.2 ± 1.5	13.1 ± 1.7
3.0 μM Cd +	3 h	2.78 ± 0.04	2.70 ± 0.19	1.13 ± 0.00	1.12 ± 0.04	50 ± 1	309 ± 4	176 ± 8	7.8 ± 0.1	7.7 ± 0.1
0.75 μM Pb *	96 h	2.84 ± 0.11	2.87 ± 0.05	0.63 - 0.09	0.63 ± 0.10	42 ± 2	78 ± 3	55 ± 3	13.0 ± 2.1	13.0 ± 1.5
control *	3 h	0.00 ± 0.05	0.01 ± 0.04	-0.02 ± 0.00	-0.02 ± 0.01	56 ± 1	170 ± 6	88 ± 2	8.2 ± 0.1	8.1 ± 0.0
	96 h	0.02 ± 0.03	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	73 ± 60	75 ± 17	49 ± 7	13.0 ± 2.0	13.0 ± 1.4

Table 3.4 – Water chemistry for the 3 h acute metal accumulation and 96 h toxicity test for fish exposed to Cd + 1.5 µM Pb. Water samples were taken at 1 h and 3 h for acute experiments and daily for 96 h experiments. All values are means ± 1 S.D. Treatments with (*) are with added NOM.

Treatment	test	Cd (µM)		Pb (µM)		Ca (µM)	Na (µM)	Cl (µM)	TOC (mg C/L)	DOC (mg C/L)
		total	dissolved	total	dissolved					
0.75 µM Cd +	3 h	0.74 ± 0.02	0.72 ± 0.04	1.60 ± 0.04	0.98 ± 0.09	44 ± 1	410 ± 2	62 ± 5	3.1 ± 0.1	3.8 ± 1.4
1.5 µM Pb	96 h	0.72 ± 0.03	0.72 ± 0.01	1.27 ± 0.20	0.86 ± 0.05	47 ± 2	314 ± 26	66 ± 13	3.6 ± 1.7	2.2 ± 1.1
1.5 µM Cd +	3 h	1.53 ± 0.04	1.46 ± 0.06	1.62 ± 0.06	1.28 ± 0.13	47 ± 0	417 ± 0	62 ± 1	3.4 ± 0.9	2.4 ± 0.8
1.5 µM Pb	96 h	1.39 ± 0.02	1.40 ± 0.03	1.41 ± 0.13	1.13 ± 0.10	44 ± 1	301 ± 16	60 ± 12	1.9 ± 1.0	2.9 ± 0.2
2.25 µM Cd +	3 h	2.19 ± 0.08	2.18 ± 0.13	1.52 ± 0.16	1.17 ± 0.01	46 ± 1	416 ± 1	60 ± 1	3.2 ± 0.8	3.0 ± 1.5
1.5 µM Pb	96 h	2.06 ± 0.03	2.04 ± 0.03	1.37 ± 0.15	0.93 ± 0.08	46 ± 1	309 ± 22	63 ± 7	3.1 ± 0.4	2.4 ± 1.7
3.0 µM Cd +	3 h	2.96 ± 0.08	2.92 ± 0.06	1.70 ± 0.08	1.12 ± 0.04	46 ± 1	405 ± 3	63 ± 0	3.2 ± 0.6	3.1 ± 0.3
1.5 µM Pb	96 h	2.70 ± 0.05	2.71 ± 0.04	1.41 ± 0.12	1.03 ± 0.13	45 ± 1	352 ± 78	66 ± 9	2.1 ± 1.4	3.0 ± 0.4
control	3 h	0.04 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	46 ± 1	408 ± 6	60 ± 7	4.2 ± 0.6	4.0 ± 0.6
	96 h	0.06 ± 0.02	0.06 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	46 ± 2	304 ± 16	56 ± 3	5.1 ± 3.6	2.8 ± 2.5
0.75 µM Cd +	3 h	0.79 ± 0.00	0.72 ± 0.06	1.92 ± 0.04	1.90 ± 0.06	51 ± 1	177 ± 6	91 ± 0	8.0 ± 0.1	8.0 ± 0.1
1.5 µM Pb *	96 h	0.87 ± 0.14	0.78 ± 0.10	1.53 ± 0.17	1.43 ± 0.24	35 ± 3	92 ± 18	32 ± 8	11.8 ± 0.7	11.3 ± 0.8
1.5 µM Cd +	3 h	1.38 ± 0.14	1.46 ± 0.11	1.90 ± 0.02	1.93 ± 0.01	52 ± 1	168 ± 5	92 ± 3	7.7 ± 0.1	7.9 ± 0.0
1.5 µM Pb *	96 h	1.69 ± 0.28	1.63 ± 0.26	1.44 ± 0.18	1.39 ± 0.17	34 ± 2	92 ± 22	39 ± 11	12.0 ± 1.0	11.9 ± 1.1
2.25 µM Cd +	3 h	2.16 ± 0.06	2.12 ± 0.04	1.97 ± 0.01	1.94 ± 0.01	54 ± 1	320 ± 5	180 ± 9	7.9 ± 0.4	7.6 ± 0.1
1.5 µM Pb *	96 h	2.47 ± 0.34	2.42 ± 0.34	1.56 ± 0.25	1.51 ± 0.21	35 ± 2	89 ± 20	31 ± 5	11.1 ± 0.6	11.6 ± 1.5
3.0 µM Cd +	3 h	2.84 ± 0.04	2.79 ± 0.08	1.94 ± 0.03	1.97 ± 0.03	56 ± 1	310 ± 19	176 ± 5	7.6 ± 0.1	7.8 ± 0.1
1.5 µM Pb *	96 h	2.24 ± 0.54	2.44 ± 0.49	1.47 ± 0.22	1.37 ± 0.30	34 ± 2	94 ± 25	36 ± 5	11.3 ± 0.5	11.0 ± 0.6
control *	3 h	0.00 ± 0.05	0.01 ± 0.04	-0.02 ± 0.00	-0.02 ± 0.01	56 ± 1	170 ± 6	88 ± 2	8.2 ± 0.1	8.1 ± 0.0
	96 h	0.10 ± 0.08	0.10 ± 0.10	0.05 ± 0.06	0.05 ± 0.06	36 ± 3	91 ± 20	28 ± 9	11.8 ± 0.6	11.7 ± 0.7

Table 3.5 – Water chemistry for the 3 h acute metal accumulation and 96 h toxicity test for fish exposed to Cd + 2.25 µM Pb. Water samples were taken at 1 h and 3 h for acute experiments and daily for 96 h experiments. All values are means ± 1 S.D. Treatments with (*) are with added NOM.

Treatment	test	Cd (µM)		Pb (µM)		Ca (µM)	Na (µM)	Cl (µM)	TOC (mg C/L)	DOC (mg C/L)
		total	dissolved	total	dissolved					
0.75 µM Cd +	3 h	0.74 ± 0.03	0.73 ± 0.03	2.28 ± 0.08	2.03 ± 0.13	48 ± 1	567 ± 1	139 ± 8	1.7 ± 0.2	2.1 ± 0.5
2.25 µM Pb	96 h	0.69 ± 0.06	0.72 ± 0.08	1.88 ± 0.34	1.62 ± 0.44	47 ± 2	653 ± 10	183 ± 40	7.6 ± 3.5	7.0 ± 4.3
1.5 µM Cd +	3 h	1.52 ± 0.04	1.44 ± 0.08	2.32 ± 0.13	1.85 ± 0.10	46 ± 0	571 ± 12	148 ± 10	2.0 ± 0.7	1.5 ± 0.4
2.25 µM Pb	96 h	1.25 ± 0.06	1.29 ± 0.08	1.90 ± 0.20	1.94 - 0.57	50 ± 2	556 ± 27	124 ± 16	7.1 ± 2.4	6.7 ± 2.0
2.25 µM Cd +	3 h	2.22 ± 0.11	2.15 ± 0.01	2.26 ± 0.06	1.87 ± 0.11	45 ± 0	573 ± 11	157 ± 6	2.6 ± 1.0	2.4 ± 0.9
2.25 µM Pb	96 h	2.02 ± 0.16	1.99 ± 0.10	1.92 ± 0.56	1.83 ± 0.54	50 ± 1	606 ± 25	142 ± 22	5.9 ± 2.7	5.5 ± 2.6
3.0 µM Cd +	3 h	2.85 ± 0.08	2.84 ± 0.07	2.30 ± 0.04	1.90 ± 0.08	47 ± 1	555 ± 10	170 ± 5	3.0 ± 0.2	3.1 ± 0.0
2.25 µM Pb	96 h	2.61 ± 0.10	2.53 ± 0.12	2.03 ± 0.29	1.28 ± 0.23	47 ± 3	587 ± 49	170 ± 71	5.1 ± 2.1	4.7 ± 1.2
control	3 h	0.04 ± 0.04	0.04 ± 0.04	0.03 ± 0.00	0.02 ± 0.01	48 ± 1	548 ± 0	138 ± 11	2.1 ± 0.2	1.4 ± 0.6
	96 h	0.05 ± 0.01	0.06 ± 0.02	0.18 ± 0.34	0.01 ± 0.00	46 ± 1	574 ± 8	110 ± 6	5.9 ± 2.7	2.7 ± 2.5
0.75 µM Cd +	3 h	0.71 ± 0.06	0.74 ± 0.03	3.37 ± 0.02	2.99 ± 0.44	43 ± 4	192 ± 5	70 ± 3	8.1 ± 0.0	8.2 ± 0.2
2.25 µM Pb *	96 h	0.84 ± 0.15	0.82 ± 0.13	2.3 ± 0.52	2.24 ± 0.48	36 ± 4	103 ± 29	39 ± 7	12.0 ± 0.6	11.3 ± 0.6
1.5 µM Cd +	3 h	1.39 ± 0.05	1.35 ± 0.17	3.37 ± 0.15	3.34 ± 0.16	43 ± 4	194 ± 11	74 ± 4	8.2 ± 0.1	8.2 ± 0.1
2.25 µM Pb *	96 h	1.70 ± 0.29	1.60 ± 0.28	2.29 ± 0.48	2.23 ± 0.50	34 ± 1	96 ± 25	33 ± 7	11.4 ± 0.7	11.7 ± 1.5
2.25 µM Cd +	3 h	2.14 ± 0.03	2.23 ± 0.05	3.19 ± 0.10	3.08 ± 0.41	40 ± 8	176 ± 23	80 ± 4	8.0 ± 0.1	8.1 ± 0.1
2.25 µM Pb *	96 h	2.35 ± 0.40	1.95 ± 0.70	2.21 ± 0.47	1.96 ± 0.72	32 ± 2	87 ± 19	30 ± 9	10.8 ± 0.9	10.6 ± 1.1
3.0 µM Cd +	3 h	2.42 ± 0.02	2.32 ± 0.14	3.24 ± 0.01	2.98 ± 0.04	44 ± 5	194 ± 6	74 ± 8	7.0 ± 0.2	7.6 ± 0.3
2.25 µM Pb *	96 h	2.29 ± 0.62	2.15 ± 0.69	2.10 ± 0.45	1.85 ± 0.52	32 ± 3	80 ± 25	31 ± 4	10.9 ± 0.5	10.7 ± 0.5
control *	3 h	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.01	0.04 ± 0.02	43 ± 4	182 ± 16	64 ± 4	8.0 ± 0.1	8.2 ± 0.2
	96 h	0.10 ± 0.08	0.10 ± 0.10	0.05 ± 0.06	0.05 ± 0.06	36 ± 3	91 ± 20	28 ± 9	11.8 ± 0.6	11.7 ± 0.7

Table 3.6 – Water chemistry for the 3 h acute metal accumulation and 96 h toxicity test for fish exposed to Cd + 3.0 µM Pb. Water samples were taken at 1 h and 3 h for acute experiments and daily for 96 h experiments. All values are means ± 1 S.D. Treatments with (*) are with added NOM.

Treatment	test	Cd (µM)		Pb (µM)		Ca (µM)	Na (µM)	Cl (µM)	TOC	DOC
		total	dissolved	total	dissolved				mg C/L	mg C/L
0.75 µM Cd +	3 h	0.67 ± 0.03	0.69 ± 0.01	2.84 ± 0.35	2.67 ± 0.13	48 ± 2	508 ± 6	77 ± 4	1.1 ± 0.5	1.7 ± 0.2
3.0 µM Pb	96 h	0.71 ± 0.02	0.71 ± 0.02	2.06 ± 0.95	2.39 ± 0.06	46 ± 1	528 ± 12	117 ± 28	5.0 ± 1.7	6.1 ± 3.6
1.5 µM Cd +	3 h	1.40 ± 0.01	1.46 ± 0.11	3.42 ± 0.50	2.94 ± 0.01	49 ± 0	496 ± 2	66 ± 4	0.9 ± 0.2	1.0 ± 0.2
3.0 µM Pb	96 h	1.41 ± 0.07	1.39 ± 0.02	2.06 ± 0.91	2.55 ± 0.01	45 ± 1	581 ± 21	137 ± 21	3.9 ± 1.6	7.8 ± 3.4
2.25 µM Cd +	3 h	2.20 ± 0.14	2.16 ± 0.07	3.41 ± 0.40	2.68 ± 0.20	47 ± 1	512 ± 6	85 ± 11	1.3 ± 0.5	1.3 ± 0.1
3.0 µM Pb	96 h	2.03 ± 0.08	2.04 ± 0.11	2.22 ± 0.73	2.49 ± 0.47	46 ± 2	617 ± 20	143 ± 26	4.9 ± 3.0	5.0 ± 2.0
3.0 µM Cd +	3 h	2.88 ± 0.21	2.81 ± 0.16	3.92 ± 0.19	2.88 ± 0.59	48 ± 1	508 ± 11	54 ± 13	1.4 ± 0.5	0.9 ± 0.2
3.0 µM Pb	96 h	2.78 ± 0.11	2.74 ± 0.07	2.30 ± 0.71	2.62 ± 0.26	49 ± 2	570 ± 22	143 ± 18	7.3 ± 2.5	7.6 ± 2.8
control	3 h	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.01	48 ± 1	508 ± 2	56 ± 5	0.9 ± 0.3	0.4 0.1
	96 h	0.05 ± 0.01	0.06 ± 0.02	0.18 ± 0.34	0.01 ± 0.00	46 ± 1	574 ± 8	110 ± 6	5.9 ± 2.7	2.7 ± 2.5
0.75 µM Cd +	3 h	0.63 ± 0.07	0.62 ± 0.10	2.42 ± 0.64	3.50 ± 0.69	40 ± 4	192 ± 3	67 ± 6	7.6 ± 0.2	7.6 ± 0.1
3.0 µM Pb *	96 h	0.89 ± 0.10	0.88 ± 0.07	2.49 ± 0.76	2.94 ± 0.78	38 ± 2	93 ± 21	41 ± 11	12.2 ± 0.1	11.9 ± 0.2
1.5 µM Cd +	3 h	1.27 ± 0.13	1.26 ± 0.12	3.68 ± 0.21	3.35 ± 0.10	37 ± 7	178 ± 15	76 ± 4	7.7 ± 0.1	7.8 ± 0.1
3.0 µM Pb *	96 h	1.65 ± 0.23	1.55 ± 0.19	2.84 ± 0.76	2.77 ± 0.76	33 ± 4	85 ± 17	29 ± 8	10.7 ± 0.5	10.9 ± 1.1
2.25 µM Cd +	3 h	2.29 ± 0.04	2.17 ± 0.21	3.78 ± 0.79	3.45 ± 0.14	45 ± 3	192 ± 4	78 ± 11	8.2 ± 0.1	8.4 ± 0.1
3.0 µM Pb *	96 h	2.44 ± 0.42	2.30 ± 0.28	2.98 ± 0.79	2.87 ± 0.79	34 ± 4	98 ± 26	37 ± 5	11.2 ± 0.7	11.4 ± 0.5
3.0 µM Cd +	3 h	2.77 ± 0.04	2.65 ± 0.18	4.11 ± 0.44	3.89 ± 0.52	42 ± 6	194 ± 15	84 ± 8	8.4 ± 0.1	8.5 ± 0.0
3.0 µM Pb *	96 h	2.31 ± 0.56	2.32 ± 0.60	2.78 ± 0.75	2.70 ± 0.74	33 ± 4	90 ± 21	33 ± 8	11.5 ± 0.7	11.3 ± 0.6
control *	3 h	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.01	0.04 ± 0.02	43 ± 4	182 ± 16	64 ± 4	8.0 ± 0.1	8.2 ± 0.2
	96 h	0.10 ± 0.08	0.10 ± 0.10	0.05 ± 0.06	0.05 ± 0.06	36 ± 3	91 ± 20	28 ± 9	11.8 ± 0.6	11.7 ± 0.7

spread in the data that increased with water metal concentration. Also, there was a positive Pb background of 1.501 nmol/g wet weight that also had to be considered when modelling.

To model gill-Cd binding in the presence of Pb and NOM, the calculated K_{Cd}, and K_{Pb} values from the competitive and non-competitive models without NOM were used. The best fit occurred when the data was modeled with competitive Pb and non-competitive NOM, with an R^2 of 0.694 and is described as follows:

Cdgill = 10.797 * Cddissp / [(1+0.241*Cddisp+2.932*Pbdissfree) * (1+0.166*DOC1free)

where LtKCd=10.797, $K_{Cd-gill}$=0.241, $K_{Pb-gill}$=2.932 and 0.166 is the binding constant for Cd to DOM. Cddissp is the predicted dissolved water Cd, and Pbdissfree is the predicted dissolved and free metal ion for water Pb. (see appendix A for full details).

With respect to gill-Pb binding in the presence of Cd and NOM, the same method was used as for Cd and NOM, in that the previously calculated K values from the gill-Pb binding experiments without NOM were used. Pb binding at the gills was inhibited by the presence of NOM. Cd also inhibited Pb binding at the gills but only if NOM was also present. The model with the best fit was a non-competitive inhibition as a result of a Cd-NOM complex (R^2=0.591) and was described as follows:

Pbgill = 12.190*Pbdissp / [(1+0.378*Pbdissp+0.619*DOC)*(1+0.388*Cddissp*DOC)+1.501]

Where LtPb=12.190, $K_{Pb-gill}$=0.378, 0.619 is the binding constant for Pb to DOM and 0.388 is the binding constant for the Cd-DOM complex to the ligand, with a background Pb correction of 1.501 nmol/g wet weight (see appendix A for full details).

3.3.3 96 h Matrix Experiments

The 96 h matrix experiments for trout exposed to Pb and Cd mixtures without NOM illustrated that metal mixtures of Pb and Cd are more toxic than either Pb or Cd alone. In

general, as the concentration of Pb in the mixtures increased, the LT50 decreased (table 3.7). Interestingly, at higher Pb concentrations, the mixtures with the highest toxicity contained lower Cd concentrations, i.e., mixtures with 2.25 µM Pb + 0.75 µM Cd and 3.0 µM Pb with 0.75 and 1.5 µM Cd were the most toxic with LT50's of 19 h, 18.5 h and 19 h respectively. The mixture treatment of Cd + 1.5 µM Pb showed moderate toxicity and the mixtures with the lowest toxicity were those with 0.75 µM Pb (table 3.7).

When trout were exposed to Pb and Cd in mixtures with NOM, the 96 h LT50 results were quite different than for fish exposed to metals without NOM. Trout exposed to Pb only or to the Cd range with 1.5, 2.25 and 3.0 µM Pb showed very little toxicity with most LT50 results greater than 96 h (table 3.8). Fish exposed to Cd only and Cd + 0.75 µM Pb displayed the greatest amount of toxicity with the most toxic treatments being 1.5 µM Cd, 1.5 µM Cd + 0.75 µM Pb and 2.25 µM Cd + 0.75 µM Pb with LT50's of 45 h, 43 h, and 45 h respectively (table 3.8).

Tables 3.1 to 3.6 illustrate the measured water metals, ions and organic carbon concentrations for these experiments. Water Cd showed very little variation over the 96 h experiments while water Pb did show some variability. The constant pH adjustments during these experiments would have affected the water Pb concentrations, allowing some Pb to fall out of solution between pH measurements. Water pH was set at pH 6.0 for each experiment however, through treatment aeration and fish metabolic processes, water pH often rose to pH 6.3-6.5 in between pH adjustments. TOC concentrations were higher for the 96 h experiments with NOM than the 3 h tests with NOM. The 96 h experiments often resulted in an increase in TOC over time, presumably due to fish mucus secretions.

Table 3.7: LT50 results (h) for fish exposed to Cd and Pb in mixtures for 96 h

		water Pb (μM)				
		0.00	**0.75**	**1.50**	**2.25**	**3.00**
water Cd (μM)	**0.00**	>96	>96	>96	94	90
	0.75	>96	>96	46	19	18
	1.50	>96	>96	46	42	20
	2.25	>96	74	22	26	22
	3.00	90	47	44	26	26

Table 3.8: LT50 results (h) for fish exposed to Cd and Pb in mixtures with NOM for 96 h

		water Pb (μM)				
		0.00	**0.75**	**1.50**	**2.25**	**3.00**
water Cd (μM)	**0.00**	>96	>96	>96	>96	>96
	0.75	91	50	>96	95	93
	1.50	45	43	91	73	>96
	2.25	69	45	>96	>96	>96
	3.00	68	69	>96	>96	93

3.4 Discussion

Similar to previous experiments (see chapter 2), when juvenile rainbow trout were exposed to Cd and Pb in mixtures, gill-Pb accumulation exceeded that of gill-Cd accumulation in all treatments except those with 0.75 μM Pb added to the Cd range. In addition, the presence of Pb in the water decreased gill-Cd binding in all treatments without the presence of added NOM (figure 3.1). When investigating the gill-Pb binding, the presence of Cd in the water did not affect the concentration of Pb binding to the gills. The metal-gill modelling results support the experimental results where Pb was found to decrease gill-Cd binding but Cd was found to have no effect on gill-Pb binding (see above). In the case of gill-Cd binding, the modelling was unable to determine if Cd and Pb are behaving in a competitive or non-competitive fashion as both methods of calculating the model worked equally well.

When taking into account the previously calculated individual binding affinities for Pb and Cd for fish gills (figure 1.1), it is surprising that the binding affinities calculated here for these metals in mixtures produced a binding affinity for Pb to the gills that is about 12 times greater than that for Cd, based on Cd uptake. The calculated binding affinity for Pb based on Pb uptake is eight fold lower than the binding affinity for Pb based on Cd uptake, however, both of these values are greater than the calculated binding affinity for Cd to the gills. It was expected that there would be more Cd on the gills than Pb when fish were exposed to Pb and Cd in mixtures, and that Cd would have a higher binding affinity for the gills than Pb. Work done by Birceanu *et al.* (submitted) found that there may be two different populations of sites of binding on the gills for Pb. When fish were exposed to 0.25 to 5.0 μM Pb, they found two different Pb-gill binding curves. The second binding curve, produced with higher concentrations of Pb could not be saturated at the Pb concentrations used. It has been

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suggested therefore that there are high affinity, low capacity sites and low affinity, high capacity sites for Pb to bind to (Birceanu *et al.*, submitted). Rogers *et al.* (2005) found similar results when they exposed fish to Pb (0.48 to 4.8 μM) in hard water. These researchers found a sudden increase in gill-Pb when fish were exposed to higher concentrations of Pb (Rogers *et al.*, 2005). With respect to the experimental results presented here, one explanation for the higher gill-Pb concentrations could be due to this second, low affinity, high capacity site being bound by Pb when fish were exposed to Pb and Cd in mixtures.

While a second binding site for Pb may explain excess Pb on the gills when compared to Cd, the question remains, how is Pb decreasing gill-Cd binding when fish are exposed to Cd and Pb in mixtures? When fish are exposed to Pb in water, the Pb may induce the production of excess mucus on the gills as a protective measure. In cases of short metal exposures, fish have been known to expel this mucus from their gills, thus also expelling any metal bound to the mucus (Sorensen 1991). It is possible that the mucus produced by Pb exposure is either binding Cd and then being expelled, or blocking Cd from binding to the gills of the fish. Fish mucus has been shown in this lab to bind metals in a similar fashion to NOM, reducing their bioavailability (unpublished data). The gill-Pb levels found on the fish may not be affected by the production of excess mucus as tissues with mucus membranes such as the gills have been shown to accumulate more Pb than other tissues (Sorensen, 1991).

Rogers and Wood (2004) found that Pb exposure decreased Ca^{2+} influx rates and that Pb binding to the gills could be decreased when fish were exposed to Lanthanum, a Ca^{2+}-channel blocker. They also found that when fish were exposed to Pb in hard water, the Pb reduced the affinity of the Ca channel for Ca (Rogers and Wood, 2004). The reason that Cd has such a high affinity for the Ca channels on the fish gill is that the ionic radius of Cd is so similar to

that of Ca (Radel and Navidi, 1994). Since Cd and Ca are so similar in the size of their ionic

radii, it may be possible that the Ca channels have a reduced affinity for Cd as well as Ca when

also exposed to Pb. Although in the same study, Rogers and Wood (2004) found that 1 µM Cd

was enough to decrease gill-Pb binding to fish exposed to 2.3 µM Pb, their experiments were

performed in hard water (1 mM Ca) at pH 8.0 which would have resulted in lower amount of

Pb^{2+} than the experiments presented here. This may explain why the experimental results

presented here are different from those produced by Rogers and Wood (2004).

The addition of NOM to the Cd and Pb mixtures used here dramatically changed the 3 h

gill binding results. The gill-Cd and gill-Pb accumulation results for fish exposed to Cd and Pb

alone with NOM were reduced when compared to fish exposed to Cd and Pb alone without

NOM (compare figures 3.1 and 3.2 to 3.3 and 3.4). Also, gill-Cd concentrations were higher

than those of gill-Pb when fish were exposed to Cd and Pb in mixtures with NOM, compared

to the higher gill-Pb concentrations found in fish exposed to these metals without NOM. These

results were expected due to the previously calculated individual binding affinities of Cd and

Pb for NOM (figure 1.1). When fish were exposed to mixtures of Cd and Pb with NOM, gill-

Cd was actually enhanced at lower Pb concentrations (0.75 and 1.5 µM, figure 3.3). It was

speculated that at lower Pb concentrations, the Pb bound to the NOM, thus displacing Cd and

increasing the bioavailability of Cd to interact with the fish gills. When fish were exposed to

Cd only with NOM or Cd + 2.25 or 3.0 µM Pb with NOM, there was still either some NOM or

Pb to interact with the Cd and compete for binding sites at the gills.

The NOM used for these experiments was collected from Luther Marsh and is very dark

and aromatic in nature. This NOM is allocthonous meaning that it has been degraded from

outside sources such as leaves, sticks etc and has a high lignin content. This NOM has a high

specific absorption coefficient (SAC; usually around 30 L/mg C) and has been previously

shown to greatly reduce the binding of Al, Cu, Ag, Hg and Pb to the gills of rainbow trout.

SAC in calculated by dividing the absorbance at 340 nm of a sample, by it's measured TOC

(Richards *et al.*, 2001; Schwartz *et al.*, 2004; Winter *et al.*, unpublished). These studies have

shown that NOM source has an affect on how protective the NOM is with regards to metal

binding to fish gills. In general, NOM that is optically dark, with a high SAC value has been

shown to be more protective against metal exposure than NOM that is optically light with a

low SAC value (Richards *et al.*, 2001; Schwartz *et al.*, 2004). With respect to Cd, it has been

shown that gill-Cd binding is not as affected by NOM source since Cd has a higher binding

affinity to fish gills than to NOM (chapter 1). When considering this information, it is not

surprising then that gill-Pb accumulation was affected much more by the addition of NOM

while gill-Cd accumulation was less affected when these metals were present in mixtures.

Interestingly, while gill-Pb accumulation was reduced in the presence of NOM, it was also

inhibited by the additional presence of Cd with the NOM (figure 3.4). This result was

unexpected but intriguing nonetheless. The modelling results indicated that Cd would only

inhibit gill-Pb accumulation in the presence of NOM. This interaction only worked well when

calculated as a non-competitive inhibition with a NOM-Cd complex (see above). It is unlikely

that a Cd-NOM complex was binding to the gills or that NOM itself was binding to the gills as

Pb would have also bound to the NOM at the gills and be measured as gill-Pb accumulation. It

appears therefore that in this capacity, the Cd was better suited to compete with Pb for gill

binding sites, resulting in greater gill-Cd accumulation and gill-Pb binding inhibition. This

ultimately was the expected result for fish exposed to mixtures of Cd and Pb without NOM. It

would appear that the presence of NOM is necessary for the multiple metal model developed

by Playle, (2004) to work with respect to Cd and Pb. This result is confounding and much

more work is required to understand how Cd and Pb are interacting with the gills of fish when

present in mixtures.

When trout were exposed to Cd and Pb in mixtures for 96 h, all the mixture treatments

were more toxic than either Cd or Pb alone. In general, in experiments without NOM, as the

concentration of Pb increased in the water containing the Cd range, the toxicity of the mixture

increased. Interestingly, the treatments that were the most toxic (0.75 µM Cd + 2.25 µM Pb,

0.75 µM Cd + 3.0 µM Pb and 1.5 µM Cd + 3.0 µM Pb) were not always the treatments that

resulted in the highest amount of gill-Pb accumulation (compare figure 3.2 with table 3.7).

Gill-Cd accumulation was the highest in trout exposed to Cd only and Cd + 0.75 µM Pb.

These two treatments also had very low toxicity results compared to the other treatments. The

biotic ligand model operates under the assumption that acute toxicity is directly proportional to

the amount of metal binding to the gills of fish (Paquin et $al.$, 2002; Playle, 2004). With

respect to the results presented here, it would appear that metal binding to the gills is not the

only source of toxicity, but rather some other physiological processes are also responsible for

fish death such as a loss in Ca^{2+} and Na^{+} homeostasis (Sorensen, 1991).

When trout were exposed to metal mixtures with NOM for 96 h, most of the treatments

had LT50's greater than 96 h. The two most toxic treatments were the Cd only and Cd + 0.75

µM Pb. When the LT50 results for mixture treatments with NOM are compared to the gill-Cd

binding results for the 3 h experiments with NOM, the Cd + 0.75 µM Pb treatment produced

the highest amount of gill-Cd binding (compare table 3.8 with figure 3.3). In contrast, the Cd

only treatments did not have higher gill binding than the other treatments. The Cd + 1.5 µM

Pb treatments also produced higher gill-Cd binding, however these treatments had a much

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lower toxicity than either the Cd alone or Cd + 0.75 μM Pb treatments. It appears that when trout are exposed to Cd and Pb with NOM, toxicity is dependent on the Cd concentration in the water as opposed to the water Pb concentration as seen in the 96 h tests without NOM. Also, as the Pb concentration in the water for experiments with NOM increased, toxicity decreased along with gill-Cd binding. Similar to above, these results suggest that metal binding to the gills of fish is not the only source of toxicity and must also be attributed to other physiological processes mentioned above.

When trout were exposed to Cd only and Cd + 0.75 μM Pb, the addition of NOM seemed to increase the toxicity of the Cd in the water, causing an increase in fish death over the 96 h. This result was also seen by Schwartz *et al.* (2004) when fish were exposed to a variety of organic matter sources and 1 μM Cd. While some forms of the organic matter that these researchers used did provide a protective effect to the fish, some sources seemed to increase Cd toxicity (Schwartz *et al.*, 2004). Although Cd has a stronger affinity for fish gills than for NOM, it was expected that the Luther Marsh NOM used in the experiments presented here would have provided more of a protective effect with regards to toxicity. Schwartz *et al.*, (2004) determined that NOM source does not have an effect on Cd toxicity, however, they did find that the darker, more aromatic NOM provided some sort of protective effect when fish were exposed to 1 μM Cd in soft water.

Studies performed on the uptake of metals in American oysters also revealed that organic matter increased Cd accumulation (Guo *et al.*, 2001). Guo *et al.* (2001) noticed not only an increase in Cd accumulation in the body of the oysters when organic matter was present in the water, but also an increase in the rate of uptake of Cd with increasing organic matter concentration. Since oysters are filter feeders, a high concentration of organic matter in the

water increased the bioavailability of the Cd (Guo *et al*., 2001). Cd bioavailability and toxicity has also been found to increase with the presence of organic matter in studies on nematodes (Hoss *et al*., 2001). These researchers found that the Cd in the water would bind to the organic matter, reducing the free ion activity of the metal, however, this metal-NOM binding seemed to facilitate Cd uptake by the nematodes (Hoss *et al*., 2001). It is possible therefore, that when trout were exposed to Cd only and Cd with low Pb, that the organic matter in the experiment facilitated in transporting the Cd in the water to the gills of the trout. The higher binding affinity for Cd to the gills in relation to the NOM would presumably have then resulted in the Cd transferring to the fish from the NOM.

Metal mixtures of Cd and Pb at the concentrations used here interacted with fish and each other in complex ways. The metal-gill binding of each metal to the fish gill proved to be dependent on the individual concentration of each metal and the presence or absence of organic matter. In particular, gill-Cd binding is inhibited by the presence of Pb when organic matter is not present in the water. In the presence of organic matter, Cd will inhibit gill-Pb binding. Metal-gill modelling conducted from the results of these experiments produced two different logK values for Pb, one for Pb inhibiting Cd and the other for Pb uptake and the gills. This suggests that there may be two different binding sites available for Pb on the fish gill. While this theory is exciting, these experimental and modelling results do not contain the data to validate this theory.

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Chapter 4

General discussion

Metal-gill modelling and the Biotic Ligand Model (BLM) have been developed to predict the effects of metal contamination on fish and other sensitive aquatic organisms (Playle, 1998; Di Toro *et al*., 2001; Paquin *et al*., 2002). It has been recognized, through extensive experimental study, that water chemistry is a very important factor in determining the degree of toxicity that any given metal has in an aquatic system (Di Toro *et al*., 2001). As a result, the BLM is not based on metal toxicity being equal to the aqueous metal concentration, but rather, to the degree of metal-biotic ligand complexation and the interactions between the metal and competing cations at the site of toxic action on the biotic ligand. As such, the BLM defines the bioavailability of metals by considering complexation with organic and inorganic ligands within the water column, which could potentially reduce metal bioavailability (Di Toro *et al*., 2001). The ultimate goal of the BLM is to be able to gain better predictions and understandings of metal-gill interactions by inserting biology into chemical equilibrium programs in order to produce better regulations for metals in aquatic environments (Playle, 1998).

Due to the nature of metal contamination, in that metals are quite often present in the environment in mixtures, it has become necessary to investigate how multiple metals will interact with each other and the biotic ligand. Multiple-metal modelling by Playle (2004) investigated the theoretical multiple metal interactions with fish by combining previously developed BLM's for individual metals. This model, similar to the BLM, was designed using the toxic unit concept and was based on concentration addition, that is, competition for the same site of toxic action on the gills of fish (Playle, 2004). The multiple-metal model focused

on metals that interact at the apical Ca channels on the fish gills such as Cd and Pb, which are often found in the environment together (Fan, 1996; Newman, 1998).

The objectives of the research presented here were to investigate the interactions of Cd and Pb with fish gills when fish were exposed to these metals in mixtures at comparable concentrations. The results of these experiments were then compared to the predictions made by the multiple-metal model, based on their individual BLM's. It was expected that, due to the stronger binding affinity that Cd has for fish gills (log $K_{Cd-gill}$=8.6; Playle et $al.$, 1993b) in relation to Pb (log $K_{Pb-gill}$=6.0; Macdonald et $al.$, 2002), that Cd would out compete Pb for binding sites on the gills, thereby resulting in a higher gill-Cd accumulation when fish were exposed to these metals in mixtures. This research also investigated how metal-gill interactions would change when fish were exposed to Cd and Pb in mixtures with the addition of natural organic matter (NOM). From the individual BLM's for Cd and Pb, Cd binds to NOM less well than Pb (log K_{Cd-NOM}=7.4, log K_{Pb-NOM}=8.4 respectively). As a result, it was expected that fish gills would accumulate much more Cd than Pb, as the Pb would bind to the NOM leaving the Cd free to bind to the gills of the fish.

Preliminary research exposed 1 g rainbow trout to equal amounts of Cd and Pb in mixtures (0.75, 1.5, 2.25 and 3.0 µM each) for 3 h and 96 h to investigate metal-gill binding and toxicity. It was found that trout exposed to 3.0 µM Cd and Pb in mixtures accumulated the same amount of Pb on their gills as 3.0 µM Pb alone, but that Cd accumulation on the gills was decreased by half when compared to fish exposed to 3.0 µM Cd alone (figure 2.1). Gill-Pb accumulation was also greater than gill-Cd accumulation when fish were exposed to 2.25 µM Cd and Pb in mixtures. Fish exposed to 1.5 µM Cd and Pb accumulated the same amount of metal, and fish exposed to 0.75 µM Cd and Pb accumulated more Cd on the gills than Pb.

With regards to toxicity, metal mixtures of Cd and Pb at 2.25 and 3.0 µM were more toxic than either 3.0 µM Cd or Pb alone (figure 2.1). The metal mixture of 1.5 µM Cd and Pb produced about the same LT50 as Cd and Pb alone (80 h as compared to 70 h respectively) and the 0.75 µM mixture was less toxic than any other treatment.

The higher than expected Pb accumulation on the gills of trout lead to the hypothesis that when fish were exposed to Pb and Cd in mixtures, Pb was being sequestered by the gills and Cd was being transported into the body. To investigate this hypothesis, 50 g rainbow trout were exposed to Cd and Pb separately and in mixtures to observe the body partitioning of each metal. Similar to the above experiments, the greatest concentration of Pb was found on the gills of trout, followed by the stomach, intestine and trunk kidney. The greatest concentration of Cd was found in the liver and trunk kidney (figure 2.2). These tissue metal accumulations were the same for fish exposed to Pb and Cd alone and in mixtures. Since Pb was found not only on the gills but also within the trout body, the hypothesis that the gills were sequestering Pb while Cd was being transported within the trout body was not supported.

The results of the gill accumulation and body partitioning experiments presented above were unexpected in that the gills of trout accumulated a greater concentration of Pb than Cd when exposed to these metals in mixtures. It was expected that gill-Cd accumulation would be greater than that of Pb, based on the individual binding affinities for these metals and gills as mentioned above. The body partitioning of Cd and Pb did not change significantly between exposures of fish to these metals alone and in mixtures. These results suggest that Cd and Pb are not exhibiting strict competition for binding sites on the gills. It has been found that Pb will not only disrupt Ca homeostasis in trout, but also that of Na and Cl (Rogers *et al.*, 2005). This suggests that there may be other sites of toxic action at the gills of fish for Pb. This

theory is supported by the presence of multiple gill binding curves when fish were exposed to

higher concentrations of Pb, similar to those used for the experiments mentioned above.

Rogers *et al*., (2005) found that when rainbow trout were exposed to Pb (0.48 to 4.8 µM) in

hard water, there was a sudden increase in gill binding at the higher concentrations of Pb.

Birceanu *et al*. (submitted) also found two separate gill-binding curves when trout were

exposed to Pb (0.25 to 5.0 µM) in soft water. These researchers found that at the top of their

Pb concentration range, the gill could not be saturated with Pb. It was thought that the multiple

binding curves produced for Pb were due to two different populations of Pb binding sites:

those being high-affinity, low-capacity and low-affinity, high-capacity sites. The high-affinity

sites were thought to saturate with Pb at lower metal concentrations while the low-affinity sites

were thought to fill with Pb after the high-affinity sites have been filled (Rogers *et al*., 2005;

Birceanu *et al*., submitted). It was also thought, that since Pb and Cd do seem to compete at

lower metal concentrations, as found by the research presented here and by Birceanu *et al*.

(submitted), that Pb and Cd are actually competing for the high-affinity sites, most likely the

apical Ca channels. At higher metal concentrations however, since Pb will presumably also

bind to other low-affinity sites, the amount of Pb found on the gills exceeded that of Cd.

To investigate the influence that Pb seemed to have on Cd binding to the gills, a matrix

of experiments was performed in which 1 g rainbow trout were exposed to a range of Cd

concentrations (0.75, 1.5, 2.25 and 3.0 µM) while maintaining a constant Pb concentration,

over the same concentration range. These experiments were conducted as short, 3 h gill

binding experiments and as LT50 experiments with a duration of 96 h. The results of these

experiments revealed that in all mixture treatments except those with 0.75 µM Pb + Cd, gill Pb

accumulation was greater than gill-Cd accumulation. In addition, in all mixture treatments, the

presence of Pb decreased gill-Cd accumulation when compared with those fish exposed to Cd alone (figure 3.1). Alternatively, the presence of Cd in the water had no effect on gill-Pb binding (figure 3.2). Similar to the 96 h toxicity experiment presented in chapter 2, the 96 h matrix experiments were more toxic when Cd and Pb were present as mixtures when compared to Cd or Pb alone. Interestingly, the mixture toxicity seemed to be dependent on the water Pb concentration rather than Cd, as toxicity was greatest in mixtures with the highest water Pb concentrations and lower water Cd concentrations (table 3.7).

This set of matrix experiments was repeated with the addition of 10 mg C/L NOM collected from Luther Marsh. The metal-gill binding and toxicity of Pb and Cd both alone and as mixtures was investigated as NOM was added to the system. The addition of NOM resulted in greater gill-Cd accumulation than gill-Pb accumulation when trout were exposed to these metals in mixtures. Interestingly, when fish were exposed to Cd and Pb at lower concentrations (0.75 and 1.5 μM) with NOM, gill-Cd was increased when compared with fish exposed to Cd alone or with higher Pb concentrations (2.25 and 3.0 μM) and NOM (figure 3.3). Alternatively, gill-Pb binding was greatly reduced by the presence of Cd and NOM, when compared to trout exposed to Pb alone with NOM (figure 3.4).

The greater concentration of Pb binding to the gills of rainbow trout as opposed to Cd when exposed to mixtures of Cd and Pb without NOM were similar to the gill binding experiment presented in chapter 2. The presence of Pb in the water with Cd reduced gill-Cd accumulation, however, Cd had no effect on gill-Pb accumulation. Similar to the results reported for chapter 2, these results suggest that Cd and Pb are not just competing for the same binding sites on the gill, but rather, Pb at least seems to have another site for gill binding or route of entry. The modelling work done with these results revealed that both the competitive

and non-competitive models work equally well (equations 2 and 3 respectively, chapter 3) when investigating the interactions between Cd and Pb in mixtures with fish gills. As these experimental results indicated, the modelling also revealed that Pb inhibited Cd binding to the gills, while Cd had no effect on gill-Pb binding. The modelling also produced two different logK values for Pb, one for Pb competing with Cd and one for Pb binding to the fish gills ($logK_{Pb-gill}$=6.5 and $logK_{Pb-gill}$=5.6 respectively). These two different logK values may suggest two different binding sites, however, the experiments presented here do not have the data to support such a claim.

Lead has been found to disrupt Ca, Na and Cl homeostasis, and as such, it has been termed to have a toxic mechanism that is "midway" between other metals (Rogers $et\ al.$, 2005). While the only confirmed route for Pb to enter into the gill is through the apical Ca channels (Rogers and Wood, 2004), once inside the gill, Pb can interact with multiple enzymes involved with ionoregulation such as the basolateral Na^+/K^+ ATPase, Ca^{2+}-ATPase and carbonic anhydrase (Rogers $et\ al.$, 2005). The disruption of so many ionoregulatory processes within the fish gill cells may explain why Pb and Cd mixture toxicity was dependent on the water Pb concentration for the 96 h matrix experiments.

The internal pH of the gill cells of rainbow trout has been determined to be approximately 7.4 (Wood and LeMoigne, 1991). According to solubility curves for Pb generated in MINEQL+ using the experimental water parameters for the experiments presented in chapters 2 and 3 (data not shown), Pb precipitates out of solution between approximately pH 6.5 and 8.5. As a result, it is possible that as Pb entered the fish gill, the internal pH of the gill cells was high enough to promote Pb precipitation, which then became trapped within the gill. If this was the case, it would explain why the resulting gill-Pb accumulation for the gill

binding, body partitioning, and matrix experiments without NOM, was so much higher than that of Cd.

The addition of NOM to the matrix experiments not only resulted in an increase in gill-Cd accumulation at lower Pb concentrations (0.75 and 1.5 µM), there were also higher toxicity results for fish exposed to Cd alone and Cd + 0.75 µM Pb. The increase in gill-Cd binding for fish exposed to Cd at lower Pb concentrations was thought to be a result of the Pb in solution binding to the NOM, thus increasing the bioavailability of Cd in the water. At higher Pb concentrations, the gill-Cd accumulation was similar to Cd alone with NOM, suggesting that there was still some competition for gill binding either between Cd and Pb or Cd and NOM at these metal concentrations. The modelling results for these experiments, using the K values generated for Cd and Pb binding to the gills without NOM (chapter 3 and appendix A), revealed that NOM will inhibit Cd binding to the gills but that Pb at the right concentration will cancel out this inhibition. At higher Pb concentrations, the modelling showed that there is still some competition between Cd and either Pb or NOM, whichever is greater in concentration. The interactions of these metals with NOM was therefore best described using competitive binding for Pb and non-competitive binding for NOM (chapter 3 and appendix A). Gill-Pb accumulation when fish were exposed to Pb alone with NOM was reduced when compared to fish exposed to Pb without NOM. When Cd was present in the water with Pb and NOM, gill-Pb accumulation was inhibited. The model that gave the best fit, using the K values generated from the modelling with no NOM, was a non-competitive inhibition due to a Cd-NOM complex which inhibited Pb binding to the gills (chapter 3 and appendix A). The interactions of Cd and Pb with NOM were expected based on the individual binding affinities that each metal has for NOM as mentioned above. NOM has also been shown to provide

protective effects when present in solution with metals (Richards *et al*., 2001; Schwartz *et al*., 2004).

The LT50 results for fish exposed to Cd and Pb with NOM for 96 h seemed to be a function of the Cd concentration in the water as opposed to Pb, as found in the matrix experiments conducted without NOM. Also, the toxicity results did not completely agree with the gill-binding results.   The two most toxic exposures when NOM was added were Cd alone and Cd + 0.75 µM Pb (table 3.8). While there was an increase in Cd binding to the gills in the treatment with 0.75 µM Pb, the fish exposed to Cd alone did not accumulate more Cd on their gills than those in treatments with 2.25 and 3.0 µM Pb, most of which had LT50's greater than 96 h (table 3.7). It has been found by other researchers that the presence of NOM can actually increase the toxicity of a metal, in particular, Cd (Guo *et al*., 2001; Hoss *et al*., 2001; Schwartz *et al*., 2004). Since the treatments with the greatest toxicity did not necessarily have the highest gill-Cd accumulation, it would appear that Cd toxicity is not only a function of gill binding, but also some physiological effects as well.

The interactions of Cd and Pb in mixtures, at the experimental concentrations used here, were much more complex than those proposed by the multiple-metal model (Playle, 2004). The gill binding results from the preliminary 3 h gill binding and matrix experiments suggest that when fish are exposed to Cd and Pb in mixtures at comparable concentrations, Cd does not out-compete Pb for gill binding sites as would be expected based on the multiple metal model, constructed using the individual BLMs for these metals. The modelling results for the matrix experiments showed that the competitive binding affinity of Pb for fish gills when fish are exposed to Pb and Cd in mixtures ($logK_{Pb-gill}$=6.5) is about 12 fold greater than the competitive binding affinity for Cd ($logK_{Cd-gill}$=5.4). The difference in calculated binding

affinities for these metals in mixtures when compared to those calculated for these metals alone is intriguing.

The results of the body partitioning experiment suggest that Cd and Pb were interacting in non-competitive ways when trout were exposed to these metals in mixtures. A significant difference in tissue metal accumulation between fish exposed to Cd and Pb individually and those exposed to Cd and Pb in mixtures would have been expected if these two metals were solely competing for binding sites and entry at the gill.

It is proposed therefore, that since there is evidence of Pb inhibiting Cd binding at the gill and of non-competitive interactions, these two metals may be interacting in both competitive and non-competitive ways when present in mixtures. The suggestion that Pb may have multiple binding sites at the gill supports this theory. It is clear that these metals are not interacting in a purely competitive fashion at the concentrations used for the work presented here, and as such, the multiple-metal model, and the toxic unit concept cannot be used to predict their interactions or gill-binding when fish are exposed to them in mixtures. The modelling of the results presented here does not provide a clear indication of how exactly these metals are interacting with each other and fish. As a result, much more research is needed to understand the relationship between metals in mixtures and their potential for interactions at the biotic ligand.

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Appendix A

To determine if Cd and Pb were behaving in a competitive or non-competitive manner,
the gill binding results for the 3 h gill accumulation studies were modelled by using Systat.
The formula for the competitive model can be written as:

$$ML = \frac{LtKm*M}{1+Km*M+Kx*X}$$

were Km and Kx are binding constants, M is the metal, L is the ligand, X is the competing
metal and Lt is the total ligand available for binding. Alternatively, the non-competitive model
can be written as:

$$ML = \frac{LtKm*M}{(1+Km*M)*(1+Kx*X)}$$

These models are pH dependent and therefore the results generated are specific for the
experimental pH used, which in this case was pH 6.0.

The addition of NOM can inhibit metal uptake at the gills by binding to the metal in
solution. When there are more binding sites on the NOM than metal available in solution,

$$MDOC = a*Mfree*DOC$$

$$Mdiss = Mfree+MDOC = Mfree*(1+a*DOC)$$

$$Mfree = \frac{Mdiss}{(1+a*DOC)}$$

where a is the binding constant and Mdiss and Mfree are the dissolved and free metal ions that
are unbound. When the DOC factor was inserted into the competitive model as give above, the
model became:

$$ML = \frac{Lt*Km*M}{(1+Kx*X)*(1+a*DOC)+Km*M}$$

When metal "X" is not present, the equation appears to be competitive inhibition and it is not possible to distinguish between the NOM binding the metals in solution and the NOM binding to the gill.

To model these experimental results, the measured water chemistry was inserted into WHAM to obtain the predicted dissolved or free ion concentrations. These values were then transformed using polynomial equations to provide a more consistent estimate of the mean free ion concentrations as a function of the nominal water chemistry. All calculations were performed using the water chemistry values in μM. The generated K values, of a molar basis should therefore be multiplied by 10^6. For experiments without NOM, the predicted dissolved Pb was calculated as follows:

$$Pbdissp = 0.317617*Pbnom+0.355826*Pbnom^2-0.051295*Pbnom^3$$

where Pbdissp is the predicted dissolved Pb and Pbnom is the nominal Pb concentration. The predicted dissolved Cd was calculated as follows:

$$Cddissp = 0.923022*Cdnom$$

where Cddissp is the predicted dissolved Cd and Cdnom is the nominal Cd concentration. For experiments with NOM added, the predicted dissolved Pb was given by:

$$Pbdissp = 1.582564*Pbnom-0.12599*Pbnom^2.$$

The predicted dissolved Cd for experiments with added NOM was given by:

$$Cddissp = 1.111695*Cdnom-1.152731*Cdnom^2.$$

To calculate the predicted free metal ion as a function of nominal, for experiments without NOM, the predicted free Pb was calculated by:

$$Pbfreep = 1.852295*Pbdissp-0.688795*Pbdissp^2+0.146658*Pbdissp^3$$

where Pbfreep is the predicted Pb free ion concentration and Pbdissp is the predicted dissolved

Pb concentration as calculated above. The predicted free Cd was calculated by:

Cdfreep = 0.929122*Cddissp

where Cdfreep is the predicted free Cd ion concentration and Cddissp is the predicted

dissolved Cd concentration as calculated above. For experiments with NOM added, the

predicted free Pb ion was given by:

Pbfreep = -0.005198*Pbdissp+0.009812*Pbdissp^2.

The predicted free Cd ion was given by:

Cdfreep = 0.285877*Cddissp+0.157709*Cddissp^2-0.023158*Cddissp^3.

       The above equations were used to incorporate the measured water chemistry when

modelling gill-metal interactions for competitive and non-competitive inhibition. For

experiments without NOM, DOC was set at 0. Gill Cd had a uniform variance and was

therefore not log transformed. The variance in gill Pb increased with measured gill Pb and log

transformation of the data was required.

Cd uptake without NOM

       The presence of Pb inhibited Cd binding to the gills. Both the competitive and non-

competitive models worked equally well. For the competitive model, the equation used to

predict Cd binding to fish gills in the presence of Pb was given by:

Cdgill = LtKCd*Cddissp / (1+KCd*Cddissp+KPb*Pbdissp).

Using the transformed water chemistry as discussed above, LtKCd, KCd and KPb were

calculated to be 10.797, 0.241 and 2.932 respectively. The competitive model for Cd binding

to fish gills in the presence of Pb was therefore:

Cdgill = 10.797*Cddissp / (1+0.241*Cddissp+2.932*Pbdissp)

with an r^2 of 0.514.

$logK_{Cd-gill} = log(0.241x10^6) = 5.4$

$logK_{Pb-gill} = log(2.932x10^6) = 6.5$

The non-competitive model used to predict Cd binding to the gills in the presence of Pb was given by:

Cdgill = LtKCd*Cddissp / [(1+KCd*Cddissp)*(1+KPb*Pbdissp)].

The generated values for LtKCd, KCd and KPb were 9,925, 0.183 and 1.948 respectively. The non-competitive model to predict Cd binding to fish gills in the presence of Pb was therefore:

Cdgill = 9.925*Cddissp / [(1+0.183*Cddissp)*(1+1.948*Pbdissp)]

with an r^2 of 0.512.

$logK_{Cd-gill} = log(0.183x10^6) = 5.3$

$logK_{Pb-gill} = log(1.948x10^6) = 6.3$

Cd uptake with NOM

The LtKCd, KCd and KPb values generated from the competitive model given above were used to model Cd binding to the gills in the presence of NOM. The presence of NOM alone inhibited Cd binding to the gills. When Pb was present at lower concentrations (0.75 and 1.5 µM), this inhibition was cancelled. When 2.25 and 3.0 µM Pb was present with Cd and NOM, Cd binding was inhibited to the same degree as that of Cd alone with NOM. The binding between Pb and NOM were therefore thought to consume either all the NOM or Pb, whichever was less. The remaining NOM or Pb was then free to inhibit Cd binding.

To calculated gill-Cd binding with NOM, it was necessary to develop a DOC value, denoted as DOC1. This value was calculated as follows:

DOC1 = DOC*0.27

Where DOC is the measured DOC from the experiments and 0.27 is a constant that was varied manually, gave the best fit to the data, and the best r^2 value. The interactions between the Pb and NOM in the water were given by:

DOC1free = DOC1-Pbdissp

Pbdissfree = Pbdissp-DOC1

Where DOC1free and Pbdissfree were the unbound DOC and dissolved Pb. If either DOC1free or Pbdissfree were less than 0, they were set to 0. The best fit model for these experiments was a competitive model with respect to Cd binding inhibition due to Pb and a non-competitive inhibition model with respect to Cd and NOM, given by:

Cdgillp = LtKCd*Cddissp / [(1+KCd*Cddissp+KPb*Pbdissp)*(1+a*DOC1free)].

The binding constant for DOC1 (a) was calculated to be 0.1657. The model was therefore given as:

Cdgillp = 10.797*Cdidssp / [(1+0.241*Cddissp+2.932*Pbdissp)*(1+0.1657*DOC1free)]

with an r^2 of 0.694.

Pb uptake without NOM

Similar to the modelling of Cd uptake without NOM, both the competitive and non-competitive models worked equally well with regards to gill-Pb binding. There was no inhibition of Pb uptake by Cd and the KCd values were therefore calculated to be 0. This resulted in the same LtKPb, KPb and r^2 values for both models, which were 12.1899, 0.378 and 0.800 respectively. There was a positive Pb background of 1.501 that also had to be incorporated into the models. The competitive model for Pb binding to the gills in the presence of Cd was given by:

lPbgill = 110 (LtKPb*Pbdissp) / ((1+KPb*Pbdissp+ KCd*Cddissp)+bk).

The model had to be log transformed due to the transformation of the Pb-gill binding data as mentioned above. Using the generated LtKPb, KPb and bk values reported above, the competitive model was therefore:

lPbgill = l10 (12.1899*Pbdissp) / ((1+0.378*Pbdissp+0.0*Cddissp)+1.501).

The non-competitive model for Pb binding to the gills in the presence of Cd was given by:

lPbgill = l10 (LtkPbp*Pbdissp) / [(1+KPb*Pbdissp)*(1+KCd*Cddissp)]+ bk.

Again, using the values reported above, the non-competitive model was therefore:

lPbgill = l10 (12.1899*Pbdissp) / [(1+0.378*Pbdissp)*(1+0.0*Cddissp)]+1.501.

$\log K_{Pb\text{-}gill} = \log(0.378 \times 10^6) = 5.6$

Pb uptake with NOM

Gill-Pb binding was completely inhibited by the presence of Cd with the addition of NOM. As with Cd binding to the gills in the presence of NOM, gill-Pb binding was modelled using the fixed coefficients calculated above from gill-Pb binding without NOM. The best fit model was one of non-competitive inhibition due to a Cd-DOC complex with an r^2 of 0.591. The model was therefore given by:

Pbgill = LtKPb*Pbdissp / [(1+KPb*Pbdissp+a*DOC)*(1+KCdDOM*Cddissp*DOC)+bk].

The binding constant for DOC (a) was calculated to be 0.619, and for the Cd-DOM complex was 0.388. Using the coefficients calculated above, the model was given by:

Pbgill = 12.1899*Pbdissp /

[(1+0.378*Pbdissp+0.619*DOC)*(1+0.388*Cddissp*DOC)+1.501].

All modelling was performed by Dr. Uwe Borgmann at NWRI in Burlington.