BIOAVAILABILITY AND INTERACTION OF METALS VIA THE GASTROINTESTINAL TRACT OF THE RAINBOW TROUT (*Oncorhynchus mykiss*)

BIOAVAILABILITY AND INTERACTIONS OF METALS VIA THE GASTROINTESTINAL TRACT OF RANBOW TROUT (*Oncorhynchus mykiss*)

By

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TITLE: Bioavailability and Interaction of Metals via the Gastrointestinal Tract of the Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

Knowledge into uptake rate and interactions of the metals via the gastrointestinal tract of freshwater fish is vital, in order to provide tools to protect and to sustain aquatic biota. An *in vitro* stomach and gut sac technique was utilized to investigate uptake rates of essential metals (copper, zinc and nickel) and non-essential metals (cadmium, lead and nickel) at luminal concentrations of 50μM via the gastrointestinal tract. Metals had no effect on the fluid transport rates via the gastrointestinal tract except for copper at the stomach. The stomach emerged as small but important site for metal absorption and interaction. Essential metals were absorbed at approximately the same rate as non-essential metals via the gastrointestinal tract. Copper, zinc, nickel, silver, and lead showed statistical correlation between rate of absorption and mucus binding via gastrointestinal tract, an important first finding for the development of a Biotic Ligand Model (BLM) for the gastrointestinal tract of trout.

There was an antagonistic effect of calcium on cadmium uptake at the stomach but not at the intestine. Zinc and calcium exhibited synergistic interaction at the stomach but no interaction at the intestine. These results showed the possibility of the transporters DMT1 to mediate copper and cadmium uptake via the intestine; hZip 2 to mediate copper and zinc uptake at the stomach; and Mzip 4 or ZTL1 to mediate zinc uptake via the gastrointestinal tract of trout. These results can be used to develop a BLM for the gastrointestinal tract of fish.

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Thesis Organisation and Format

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

Chapter 1: **Introduction and Focus of the Projects**

Chapter 2: **Bioavailability of Metals via the Gastrointestinal tract of Rainbow Trout (*Oncorhynchus mykiss*)**

Authors: Adeola. A. Ojo and Chris M. Wood

Comments: This study was conducted by AAO under the supervision of CMW. This paper will be submitted to the *Journal Comparative Biochemistry and Physiology*

Chapter 3: **Interactions of Metals via the Gastrointestinal Tract of Rainbow Trout (*Oncorhynchus mykiss*)**

Authors: Adeola. A. Ojo and Chris M. Wood

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

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

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Chapter 1

General Introduction

Metals in the environment

Copper, zinc, cadmium, silver, lead and nickel are important metals that need to be regulated for environmental protection. Copper, zinc, and nickel are nutritionally essential metals, while cadmium, silver and lead are non-essential metals. These metals can originate from domestic, agricultural/aquacultural, mining, industrial sources, leaching and geological weathering.

Mechanism of metals toxicity: Indication for BLM at the gill.

Many authors, including Meyer et al. (1999), Di Toro et al. (2001), and Niyogi and Wood (2003) have helped to develop the Biotic Ligand Model (BLM) at the branchial epithelium for water quality criteria (WQC) derivation and ecological risk assessment (ERA) of metals. This model assumes all toxicity results from metal damage to the gills. Playle (2004) has recently developed multiple-metal modeling at the gill, using the classic toxic unit concept for interpretation of multiple metal toxicity results. The potential mechanisms of toxicity for copper (Lauren and Mcdonald, 1986) and silver (Morgan et al., 1997) are Na^+ and Cl^- antagonism by inhibition of Na^+ uptake, for zinc, (Spry and Wood, 1985), cadmium (Verbost et al., 1989) and lead (Mac Donald et al.,

2002, Rogers et al., 2003) are inhibition of Ca^{2+} uptake, and for nickel (Pane et al., 2003), a respiratory mechanism at the gill. But to date, there is no comparable BLM at the gastrointestinal tract of fish.

Branchial epithelium

Gills of fish serve as a multifunctional organ, in ionoregulation, respiration, acid-base regulation, and nitrogenous waste excretion.

The branchial epithelium consists of four or five cell types (Laurent and Dunel, 1980). Pavement cells appear to function as active acid-base and ion transporters, specifically in H^+ excretion and coupled Na^+ uptake (Goss et al., 1992), while chloride cells serve as sites for active uptake of Cl^-, Ca^{2+} and probably other divalent metals in freshwater fish (Wood, 2001). Accessory cells are considered immature chloride or a modified type of chloride cell, and generally occur only in seawater fish. Mucous cells secret mucus which functions to increase Na^+, K^+, Cl^- and Ca^{2+} concentrations close to the gill surface, to complex metals, to slow diffusion rate of metals, and to provide the respiratory enzyme carbonic anhydrase (Handy et al., 1989). Neuroepithelial cells act as putative chemoreceptors, important in providing afferent information to the central nervous system for the control of ventilation (Burleson and Milsom, 1995).

Gastrointestinal tract

The gastrointestinal tract of fish can function in digestion and nutrient absorption. This can result into breaking down of foodstuffs, transport of the by-products, and excretion of the unused waste products.

In some fishes, the digestive canal constitutes a straight tube from the mouth to the anus. More often, however, the canal makes loops and is structurally divided into functionally different parts. Thus, one can usually distinguish oesophagus, stomach and intestine and often subdivisions of these. Valves or sphincters often separate different parts of the digestive canal.

Gut is an anatomical term that specifically refers to the developing stomach and intestine in the embryo, but which is also commonly used to refer to the entire gastrointestinal tract. The principal layers of the gut wall are the mucosa (inner epithelium and adjacent tissues), submucosa, muscularis (usually double layered), and serosa. Associated with the canal are two glands, the liver and the pancreas, which deliver their secretions into the intestinal lumen through special ducts.

Structure of the gastrointestinal tract

Oesophagus: It lies next to the buccal cavity and then leads to the stomach. It is muscular and contains mucus secreting cells and a few taste buds and is lined with stratified epithelium (Kapoor et al., 1975).

Stomach: The typical pH of the stomach in many fish is about 4 (Bergman et al., 2003). The principal layers of the stomach are the mucosa, which consists of columnar epithelial cells and mucus cells (Kapoor et al., 1975), submucosa, stratum compactum and muscularis (which consists of circular and longitudinal smooth muscles).

Intestine: The following description is based partially on material summarized by Horn (1998) in a recent review. The intestine is the tube lying between the stomach and rectum. It functions mainly in the digestion of food and in the absorption of by-products

from food and water. The first segment of intestine next to pyloric stomach is the anterior intestine and it contains finger-like projections called pyloric ceca, which can also serve as an additional site for absorption and digestion of food and water (Dimes et al., 1994). The length of the intestine in fishes varies according to type of fish (i.e. whether carnivore, omnivore or herbivore) (Gerking, 1994). This is related specifically to the type of food that each fish eats. For example, the longer intestine in herbivores and detritivorous fishes functions to increase the absorptive area for the digestion and absorption of food (Buddington et al., 1987). The intestine may be topographically laid out more or less in a straight line, as exemplified by the rainbow trout, arranged in a convoluted fashion filling all the available space, as with the goldfish, or ordered in tightly opposing circular arrays, as with the winter flounder (Ferraris and Ahearn, 1983).

Rectum: The primary function of rectum is for elimination of waste products of food through the anus. It is the terminal part of the intestine and can be demarcated from the intestine by an ilio-rectal valve formed by smooth muscles (Mohsin, 1962).

Ion and water transport via the gastrointestinal tract

The intestine has been shown to function importantly in water absorption (House and Green, 1965; Buddington et al., 1997). The anterior intestine was the highest absorptive region for water absorption compared to other parts of the intestine (Bergman et al., 2003; Nadella, unpublished results). Studies have related the transport of electrolytes (such as Na^+, Cl^-) and base (HCO_3^-) to water absorption via the intestine of fish, suggesting that water absorption could be driven by these ionic fluxes (Loretz, 1995; Grosell et al., 2001; Bergman et al., 2003; Grosell et al., 2005). The mechanism of water

transport via the intestine can be through active trans-epithelial transport of Na^+ and Cl^- (Grosell et al. 2001). Recent work from Grosell et al. (2005) also showed that Cl^- absorption can occur via an anion exchange (Cl^-/HCO_3^-) system that can partly drive fluid absorption across the intestine. Previous and recent studies have also considered that Na^+ and Cl^- transport across the intestine produce an osmotic driving force for water movement in fish (Curran and Solomon, 1957; Curran, 1960; Bucking et al. unpublished). Similarly in mammals, studies have related the absorption of water to the transport of electrolytes in the small intestine in their both starved and fed states (Cizek, 1954; Gotch et al., 1957). In humans, water has been shown to be co-transported through the K^+/Cl^- co-transporter, the lactate or monocarboxylate transporter, and the Na^+/glucose transporter (Zeuthen, 1994; Wright, 1993).

Copper

Copper (Cu) is an essential micronutrient and potent toxicant. Copper is used as a prophylactic agent; it is released into the environment from industrial and domestic sources. Copper has high solubility at low pH (Wood, 2001).

Soloman and Lowery (1993) reported that the redox nature of copper is utilized in a large number of enzymatic processes, like that catalyzed by mitochondrial cytochrome c oxidase, which makes copper an essential element for all aerobic organisms. The different inorganic copper species are $CuCO_3$, $Cu(CO_3)_2$, $CuOH^+$, $Cu(OH)_2$, Cu^{2+} and $CuHCO_3$ (De –Schamphelaere and Janssen, 2002). The free Cu^{2+} ions and the $CuOH^+$ species appear to be the most bioavailable and toxic forms of copper (Allen and Hansen, 1996). Leland and Kuwabara (1985) reported that concentrations of copper in freshwater

environments range from < 1 to 9000 μg Cu/L depending on the level of natural and anthropogenic inputs. Typical levels would be < 30 μg/L.

Copper homeostasis entails regulated uptake, distribution and excretion, and occurs by coordinated interactions of several organ systems. Mechanisms of copper homeostasis in mammals have received considerable attention, particularly because of two genetically linked fatal disorders in copper metabolism, Menke's disease and Wilson's disease (Camakaris et al., 1999). Grosell et al. (1998a,b, 2001b) and Kamunde et al. (2001) reported that, as in mammals, the liver is the major homeostatic organ for copper in fish and biliary copper excretion is elevated in situations of elevated copper uptake.

Branchial copper uptake

Uptake of copper from water has been extensively studied, particularly as it relates to toxicology (McDonald and Wood, 1993; Wood, 2001). Lauren and McDonald (1987a) and Grosell et al. (1997) reported that copper readily enters across the gills and builds up in the internal organs, but the entry mechanism was unknown at that time. The log K_D value for Cu- gill binding is about 7.4 (Playle et al., 1993a,b). The log K_D value means the affinity or binding strength at the gill sites.

Several factors such as water chemistry and body size influence the rate of copper uptake in freshwater fish. Water hardness and alkalinity are strongly protective against copper toxicity, whereas the reported influence of pH is variable, ranging from antagonism to synergism at very low pH (< 5.0) and from no effect to protection by pH elevations in the circumneutral and alkaline pH range (Chakoumakos et al., 1979; Miller

and Mackay, 1980). Recently, Taylor et al. (2002) showed that uptake of copper into the gill also declines exponentially with increasing body mass.

Lauren and McDonald (1987b) reported two mechanisms of action by which copper induces ionoregulatory dysfunction. One is a mixed competitive (i.e. increased K_m) and non-competitive (reduced J_{max}) inhibition of Na^+ and Cl^- influx, which occurs at a much lower threshold than the other which is a stimulation of passive effluxes of these ions. K_m means inverse of affinity while J_{max} means maximum transport rate. Recently, Grosell and Wood (2002) reported that a portion of branchial copper uptake occurs via an apical Na^+ channel, based on competitive interactions between sodium and copper uptake, inhibition of copper uptake by a proton pump inhibitor (bafilomycinA1, 2 μmol^{-1}) and a Na^+ channel blocker (phenamil, 100 μmol^{-1}). This sodium pathway is via an apical, H^+-ATPase-coupled, Na^+ channel (Fenwick et al., 1999) and a basolateral Na^+/K^+-ATPase extruding sodium from the gill epithelial cells to the blood plasma (Wood, 2001) (Fig. 1). In rainbow trout acclimated to ion poor soft water, the sodium–sensitive copper uptake demonstrates saturation kinetics, with a K_m of 7.1 $nmolL^{-1}$ and a J_{max} of 21.2 pmol g^{-1} h^{-1}, and is characterized by an IC50 of 104 $\mu molL^{-1}$ sodium. However there also appeared to be a second, sodium-insensitive pathway of copper uptake with similar kinetic characteristics (K_m = 9.6 $nmolL^{-1}$, J_{max} = 3.5 pmol g^{-1} h^{-1}). The nature of this second pathway is at present unknown (Grosell and Wood, 2002).

Gastrointestinal uptake of copper

In humans, dietary sources of copper include: liver, shellfish, oysters, nuts, cocoa, eggs, beans, fresh - fruit, and cheese (Pennington, 1973). Normal dietary sources of

copper for fish would be copper -rich invertebrates such as molluscs and crustaceans. The bioavailability and assimilation efficiency of dietary copper are affected by several factors including diet quantity, quality and the concentration of copper in diet. The threshold for diet-borne copper toxicity to rainbow trout is approximately 664-730mg Cu kg^{-1} dry diet or at doses of ~ 44mg Cu kg^{-1} body weight d^{-1} (Julshamn et al., 1988; Knox et al., 1984). The assimilation/retention efficiency of copper decreases with increasing dietary concentration or dose (Handy, 1996; Clearwater et al., 2000, 2002; Kamunde et al., 2001, 2002b; Kamunde and Wood, 2003) indicating that the uptake mechanism saturates at higher levels of copper, and that a copper transport mechanism exists in the gut. Handy et al. (2000) using gut sacs, described concentration-dependent changes in basolateral copper absorption across the catfish gut and postulated the presence of a Cu-ATPase and a Cu/anion symport. Clearwater et al. (2000) using cannulation to deliver a bolus of $CuSO_4$ into the stomach of intact trout suggested that intestinal apical entry was by diffusion and the basolateral exit was transport- mediated based on Q_{10} analysis. In other words, these studies suggested that gastrointestinal apical entry is passive while the basolateral export is the rate–limiting step in intestinal uptake of copper. Recently, Clearwater et al. (2002), in a review of literature, concluded that most copper is probably absorbed in the pyloric ceca and intestine in a manner homologous to mammalian copper absorption, and copper uptake across the basolateral membrane of the intestine is probably the rate-limiting step. Recent research from Handy (2002) suggested that apparent sodium- dependent copper uptake across epithelial tissue such as frog skin, fish gills and vertebrate intestine, is possible when external Na^+ is only a few millimoles (fish gills, frogs in freshwater). This might involve a Cu^{2+} leak through epithelial Na^+

channels. Conversely, Cu ions inhibit basolateral Na^+ K^+-ATPase and may increase $[Na^+]_i$ (Fig. 2). However, a copper-specific transporter such as CTR-1 that has been found in yeast and humans (Lee et al., 2002) is a likely route of Cu^{2+} entry when external Na^+ is higher (e.g. intestinal epithelia).

Recent research in our lab using the isolated gut sac technique on trout intestine has shown that copper uptake was stimulated with increasing sodium concentration. Even after replacement of chloride with sulphate salts in the saline, there was an identical stimulation by increasing sodium levels, indicating that copper uptake was insensitive to chloride. In the presence of phenamil (sodium-channel blocker), copper uptake was inhibited by 40% in the mid and posterior intestine, providing evidence for a link between sodium and copper transport at the gut (Nadella et al., unpublished).

Competitive studies with 500$\mu molL^{-1}$ of $ZnSO_4$ and Fe $(NO_3)_2$, a ten-fold excess in relation to copper, showed inhibition of copper uptake by 60-80% in the presence of these cations. This indicated that part of copper-uptake could potentially occur through the zinc or iron transport pathway, possibly implicating the DMT1 mediated mechanism originally described by Gunshin et al. (1997).

Interaction between branchial and gastrointestinal uptake of copper

Branchial copper uptake plays an important role in the normal metabolism of copper in fish. Recently Bury et al. (2003) highlighted the importance of branchial uptake in the metabolism of essential metals. Normally, the major portion of copper uptake comes from the diet, rather than from the water. Kamunde et al. (2001, 2002) investigated interactions between branchial and gastrointestinal uptake of copper, and demonstrated

that under normal levels of copper in the water and food, rainbow trout derive over 80% of their copper requirement from food. Also, the rate of internalization of dietary copper is more than 10 times greater than that of waterborne copper. Kamunde et al. (2002 a, b) reported that whole body copper status and previous exposure to copper through the diet influenced branchial copper uptake, while gastrointestinal copper uptake from food was not influenced by previous exposure to waterborne copper. McGeer et al. (2002) reported that, during long-term dietary exposures, whole body and internal copper burdens continue to increase with time and do not appear to saturate, while during long-term waterborne copper exposures, whole body and tissue copper burdens saturate. These have led to the proposition that dietary uptake serves for large-scale acquisition of copper, while waterborne uptake is focused on fine adjustment of copper homeostasis based on requirements. Pyle et al. (2003) and Kamunde et al. (2003) reported that elevated dietary Na^+ effectively reduces waterborne copper uptake in rainbow trout, during short-term copper exposure and under copper chronic exposure conditions, presumably because the Na^+-dependent pathway at the gills is down - regulated.

Zinc

Zinc (Zn) is both a micronutrient and toxicant that is released diffusely from industrial and domestic sources (e.g. galvanized metals). The concentrations of total zinc in water have been reported to be $1 \mu g L^{-1}$ or less in unpolluted areas (Spry et al., 1981, Hogstrand et al., 1991), though it can be as high as 30 μg/L in local waters in southern Ontario. Keilin and Mann (1940) were the first to recognize the involvement of zinc in enzymes, such as carbonic anhydrase. Zinc is not reduced or oxidized under

physiological conditions. Zinc is taken up, probably in the form of Zn^{2+}, by an active saturable transport system, which displays standard Michaelis-Menten kinetics in the gills (Spry and Wood, 1989). A few proteins use zinc to stabilize or modulate the structure of the protein (Williams, 1984), like the trans-acting factors responsible for metallothionen (MT) induction (Imbert et al., 1989). The erythrocyte zinc concentration is similar in rainbow trout to that in mammals, and the whole body content of zinc does not differ between fish and man (Bettger et al., 1987). The processes of zinc uptake, distribution, intracellular metabolism and excretion are so well regulated that zinc deficiency is next to unknown in fish and zinc toxicity is uncommon. Uptake of zinc from the environment can occur both through the gills and through the gastrointestinal system (Renfro et al., 1975; Spry et al., 1988).

Branchial uptake of zinc

Zinc uptake at the gill appears to be a normal branchial function, occurring via the chloride cells. The log K_D value for Zn-gill binding is about 5.0 (Galvez et al., 1998). The uptake of waterborne zinc by the gills is dependent on the chemical composition of the water. Increased hardness and decreased pH are both protective against zinc uptake, whereas carbonate alkalinity has only a very small ameliorative influence (Bradley and Sprague, 1985 a, b). A high calcium concentration of the water greatly reduces the rate of zinc uptake and the accumulation of zinc by gill tissue. The effects are likely due to H^+ and Ca^{2+} competition for Zn^{2+} binding sites on the gill. Bradley and Sprague (1985a) and Bentley (1992) provided evidence that reduced water pH decreases the accumulation rate of zinc in gills, while the influx of zinc to the whole animal may actually be increased.

Decreasing the pH of the pond water from 7.3 to 5.0 resulted in a 1.7 fold increase in zinc influx in channel catfish (*Ictalurus puntatus*) (Bentley, 1992).

The hypocalcemic effect of zinc suggest that Ca^{2+} and Zn^{2+} share, at least partially, a common uptake pathway via the chloride cells, such that zinc is a potent inhibitor of calcium uptake and vice-versa (Spry and Wood, 1989a; Bentley, 1992). The interactions are largely competitive with large effects on respective K_m values and small effects on J_{max} values. Hogstrand et al. (1996) demonstrated that Zn^{2+} could enter the apical gill epithelium through the same channels as Ca^{2+}. The route for zinc transfer across the basolateral membrane is unknown; one of the calcium transporters could be used, or a separate transport mechanism for zinc may occur (Hogstrand and Wood, 1996).

At very high zinc concentrations (e.g.1250-40 000 $\mu g\ l^{-1}$), rapidly developing hypoxemia occurs, due to gross morphologic damage of the gills as the mechanism of lethality (Skidmore, 1970; Skidmore and Tovell, 1972), but this is not an environmentally realistic situation.

Gastrointestinal uptake of zinc

Zinc is absorbed primarily in the upper gastrointestinal tract in mammals. In humans, dietary sources of zinc include sea food, meat, and various oils (Sandstead, 1973). For fish, molluscs and crustaceans are rich sources of dietary zinc (Severy, 1923). The zinc concentration for rainbow trout in commercial diets is 15-30 mg Zn kg^{-1} dry diets. Water quality affects diet-borne zinc requirement (Wekell et al., 1983). Diet- borne zinc uptake is similar in some respects to diet- borne copper uptake in rainbow trout (Clearwater et al., 2000).

Intestinal zinc uptake (like copper uptake) is a two-step process in the rainbow trout, with rapid zinc uptake into the gut tissue and then slower zinc distribution to the blood and internal organs. Recent studies from Glover and Hogstrand (2002b) described saturable zinc uptake by the cannulated intestine of rainbow trout perfused with ^{65}Zn in a saline solution .The addition of N-ethylmaleimide (NEM) significantly decreased zinc uptake into the blood and body, suggesting that the transfer of zinc to the post –intestinal compartments involves reactive sulfhydryl groups and/or that the process is energy – dependent, similar to copper uptake in rainbow trout (Clearwater et al., 2000). Glover and Hogstrand (2002b) suggested that mucus also promotes zinc uptake at low concentrations by trapping zinc in higher concentrations next to the mucosa. However, addition of zinc to the perfusate, resulted in increased mucus secretion, thereby protecting the gut tissue from zinc exposure (Glover and Hogstrand, 2002b) as observed in copper exposure of African walking catfish gut tissue (Handy et al., 2000). Glover et al. (2004) have shown that interaction between calcium and zinc uptake were not exerted at the level of a calcium channel at the intestine using an intestinal perfusion technique.

Ogino and Yang (1978) and Maage and Julshamn (1993) reported that significant amounts of diet-borne zinc accumulate in the intestinal tissues during both short term (days) and a long –term (weeks) exposure. Hardy et al. (1987) showed that the lower intestinal tract (pyloric caecae and intestine) accumulated the most ^{65}Zn and slightly less was accumulated in the stomach in trout. Overall, the digestive tract accumulated the most diet- borne zinc whether from short-term or long-term exposure, while the gills, kidney, skeletal tissues, liver and spleen were next in importance for accumulating diet –

borne zinc. Unlike copper, diet-borne zinc did not appear to accumulate preferentially in the liver and gall bladder (Knox et al., 1984; Wekell et al., 1986).

In mammals, transporters such as DMT-1 and ZIP-2 have been described to facilitate apical zinc uptake (Gunshin et al., 1997; Gaither and Eide, 2000). Possible basolateral zinc export mechanism in fish can be through the transporter ZnT-1 (Cousins and McMahon, 2000).

Interaction between branchial and gastrointestinal uptake of zinc

Spry et al. (1988b) showed that the relative importance of each exposure route for zinc uptake by rainbow trout depended mostly on the zinc concentration in each medium. Clearwater et al. (2002) similarly concluded that the relative importance of diet-borne and waterborne uptake is determined by the relative exposure from the different media.

Within certain concentrations of waterborne and diet-borne zinc, waterborne uptake appeared to be independent of diet-borne zinc content, even when diet -borne zinc concentration was increased. Thus, waterborne uptake did not appear to be well regulated. In contrast, uptake of diet-borne zinc is probably tightly regulated because the percentage retention of zinc tended to decrease as diet-borne concentrations increased (Spry et al., 1988b). Pentreath (1973, a, b,) and Renfro et al. (1975) reported that, at low waterborne zinc concentration, zinc is taken up mostly from diet, while when waterborne zinc concentration is high, zinc is taken up mostly from the water (Kock and Bucher, 1997).

Grosell et al (unpublished manuscript) reported that dietary exposure clearly influenced subsequent branchial zinc uptake kinetics by increasing transport capacity

(J_{max}), similar to the work of Szebedinszky et al. (2001) on dietary cadmium. He also reported that when there is increased branchial zinc uptake capacity caused by dietary and waterborne zinc exposure, there is also reduction in sensitivity to acute waterborne challenge tests. Niyogi (unpublished manuscript) reported that dietary calcium inhibits waterborne zinc uptake in target tissues (gill and gut).

Cadmium

Cadmium (Cd) is a toxic metal, which can be found in elevated levels in the water, sediment and benthos as a result of mining, industrial processes, forestry practices, waste disposal and fuel combustion; it has no essential function in physiological processes (Pratap et al., 1989; Farag et al., 1994). Cadmium can have severe toxic effects on aquatic organisms when present in excessive amounts (Alabaster and Lloyd, 1982; Sorensen, 1991). The acute toxic mechanism of cadmium to fish appears more similar to zinc than to copper (Sprague, 1987; Lauren, 1991).

Branchial uptake of cadmium

The gills rather than the diet appear to be usually the major route of cadmium uptake (Williams and Giesy, 1978). Cadmium has greater toxicity than zinc because the affinity of the gills for cadmium is relatively high (Log K_d = 8.6) and there is a strong relationship between gill cadmium burden and toxicity during acute exposures (Playle et al., 1993b). Factors affecting the acute toxicity of cadmium include temperature, dissolved oxygen, pH, salinity, and dissolved organic matter (Alabaster and Lloyd, 1982; Sprague, 1987). Carol et al. (1979) demonstrated that calcium, and not magnesium or

carbonate alkalinity, was the dominant protective factor against cadmium toxicity to brook trout.

Low concentrations of cadmium effectively block calcium uptake (Verbost et al., 1987, 1989) and higher concentrations of calcium effectively block cadmium uptake (Part et al., 1985). Plasma and /or whole body hypocalcemia are the classic symptoms of cadmium exposure in freshwater fish and likely the direct cause of death (Roch and Maly, 1979).

Verbost et al. (1987, 1988, 1989) described the details of cadmium versus calcium interaction at the levels of the chloride cell. The cadmium in the form of Cd^{2+} enters through the Ca^{2+}-selective channels in the apical membrane without appreciably blocking simultaneous Ca^{2+} influx. Calmodulin and other intracellular binding proteins initially sequester both ions. As intracellular free Cd^{2+}gradually builds up, it potently and competitively inhibits the basolateral Ca^{2+} pump, the high- affinity Ca^{2+}-ATPase. The resulting blockade of Ca^{2+} export causes a rise in intracellular free Ca^{2+} which acts as a signal to close the apical Ca^{2+}-selective channels, thereby greatly decreasing the entry of both ions (Wicklund Glynn, 1996). Cd^{2+} is not pumped by the high–affinity Ca^{2+}- ATP ase, but instead inhibits the enzyme, and the blockade appears to be more or less irreversible (Reid and McDonald, 1988).

Cadmium appears to be particularly effective in binding to pre- existing proteins (Olsson and Hogstrand, 1987, Wicklund-Glynn and Olsson, 1991) as well as in inducing the synthesis of new metal-binding protein in the gills. These proteins include both metallothionein (Benson and Birge, 1985) and non-metallothionein low molecular weight (<3000Da) polypeptides (Thomas et al., 1983, 1985). Giles (1988) and Farag et al. (1994)

reported that during chronic sub lethal exposures, the cadmium- binding characteristics of the gills appear to change.

Recent research from Wicklund-Glynn (2001) reported that waterborne zinc also competitively inhibits apical cadmium uptake in zebra fish (*Danio rerio*) and suggested that zinc and cadmium influx occur through a common pathway. Bentley (1992) reported that when calcium concentration was increased from 0.1mM to 3mM, the influx of zinc (from 10^{-6} M) was decreased by 85%. The presence of cadmium (10^{-5} M) similarly decreased zinc uptake.

Gastrointestinal uptake of cadmium

Uptake of cadmium via the alimentary canal occurs with an initial transfer from food to the gut tissue followed by movement into the blood and subsequent internal distribution via the circulation (Thomann et al., 1997). The uptake of cadmium across the gut of rainbow trout during dietary exposures resulted in substantial accumulation of cadmium in internal organs, particularly the gastrointestinal tract, liver, and especially kidney (Szebedinszky et al., 2001).

Cadmium is often described as a nephrotoxicant due to its selective accumulation in the kidney tissue. Kumada et al. (1980) suggested that the kidney has a central role in the elimination of cadmium during chronic sublethal exposure. The highest tissue cadmium accumulations for dietary-exposed fish were at the site of uptake, the posterior and anterior intestine (Handy, 1992). Accumulation of cadmium in internal organs was directly related to dietary-exposure concentration; it was not related to the total accumulation of cadmium in intestinal tissue (Szebedinszky et al., 2001).

The mechanism of cadmium uptake from diet is not understood, but Handy (1996) suggests that cadmium binds to the luminal surface of the mucosal cells by electrostatic attraction and, subsequently is taken up into the blood. It is possible that Cd^{2+} is taken up from the gut via Ca^{2+} or Mg^{2+} uptake and transport mechanisms, because Pratap et al. (1989) reported that dietary cadmium caused hypermagnesaemia and hypocalcaemia in tilapia adapted to water with low Ca^{2+} levels. Schoenmakers et al. (1992) suggested that dietary cadmium not only accumulates in the tissues but likely is transported into the blood via a Na^{+}/Ca^{+} exchanger in the basolateral membrane of intestinal cells. Franklin et al. (in press) have shown that dietary calcium inhibited dietary cadmium accumulation in stomach but not in the intestinal tissue. However, in mammals there is a possibility for cadmium uptake to occur through CTR1 or DMT-1 (Lee et al., 2002; Elisma and Jumarie, 2001) and basolateral export can be through ZnT-1 (Palmiter and Findley, 1995).

Shears and Fletcher (1983) have provided evidence for competitive interaction between zinc and cadmium uptake through the intestinal tract of the marine winter flounder (*Pseudoplueronectes americanus*). These authors hypothesized that the interaction occurred at the level of the mucosal epithelium rather than at the level of a cellular (cytosolic) protein such as MT. Also competitive interaction between cadmium and zinc has been shown in mammalian intestine (Foulkes, 1985; Tacnet et al., 1991).

Interaction between waterborne and dietary cadmium exposure

In rainbow trout, during waterborne exposure, the gills and kidney were the organs with the highest cadmium accumulation (Hollis et al., 1999; McGeer et al., 2000) while

food-borne cadmium accumulated mostly in the gastrointestinal tract and kidney (Kumada et al., 1980; Harrison and Klaverkamp, 1989; Szebedinszky et al., 2001). The latter authors reported that chronic sublethal cadmium exposure via the diet can alter the uptake of cadmium from water into the gill and chronic exposure to dietary or waterborne cadmium results in different tissue–specific accumulations.

Dietary calcium protected against cadmium accumulation in gill, liver, and kidney but did not protect against the inhibition of calcium uptake into the gill or plasma hypocalcemia (Zohouri et al., 2001). Dietary–exposed trout exhibited carcass accumulations of cadmium that were approximately 5-10 fold greater than the level found as a result of the waterborne cadmium exposure (Szebedinszky et al., 2001). Baldisserotto et al. (2004) reported that dietary Ca^{2+} reduced waterborne cadmium uptake and internalization.

Silver

Most silver (Ag) in surface waters originates from natural leaching. Elevated concentrations are usually associated with anthropogenic activities such as mining and photographic processing. Silver complexes found in freshwater include silver chloride, silver sulfide, silver DOM (dissolved organic matter), and silver thiosulfate (Hogstrand and Wood, 1998; Purcell and Peters, 1998; Wood et al., 1999).

Silver is considered to be relatively non-toxic to humans and other mammals (Hollinger, 1996), but in the aquatic environment, the silver ion Ag^{+} can be extremely toxic, and indeed in this form it appears to be the most toxic of the metals, with LC_{50} values in the range of a few $\mu g\ L^{-1}$. In rainbow trout, the toxicity is associated only with

the fraction which is present as the free silver ion (Ag^+) and not with silver bound to chloride, dissolved organic matter, or other naturally occurring anions (Hogstrand et al., 1996; Galvez and Wood, 1997; McGeer and Wood, 1998; Bury et al., 1999a, b).

Branchial uptake of silver

Gills serve as the primary site for acute silver toxicity (Wood et al., 1999). The log K_D value for Ag-gill binding is very high, about 10.0 (Janes and Playle, 1995). Hardness exerts a weak beneficial influence against silver toxicity, whereas pH and alkalinity are of negligible influence. Natural anionic ligands with a strong affinity for Ag^+, such as Cl^-, dissolved organic carbon, sulfide and thiosulfate provide almost complete protection when present in excess.

Wood et al. (1996) reported that, in fresh water, silver is a highly specific ionoregulatory toxicant to the gill with resulting internal effects in the fish reminiscent of those of low pH exposure, i.e. progressive decline in blood Na^+ and Cl^- levels, fluid volume disturbance, and circulatory failure. The mechanism of toxic action involves an extremely potent, non –competitive inhibition of the active uptake of both Na^+ and Cl^-, which is explained by a potent inhibition of Na^+, K^+ ATPase activity in the ion transporting cells (Morgan et al., 1997). Recently, Morgan et al. (2004a) reported that the initial, rapid phase of inhibition of whole body Na^+ uptake observed during silver exposure occurs by inhibition of carbonic anhydrase activity, blockage of the apical Na^+ channel by Ag^+ or competition between Na^+ and Ag^+ for uptake at the apical Na^+ channel, rather than by Na^+ K^+ ATPase inhibition. Morgan et al. (2004b) subsequently concluded that carbonic anhydrase inhibition could explain the early decline in Na^+ and

Cl^- uptake, while the later decline is probably related to Na^+ K^+- ATPase blockade, and that Ag^+ did not inhibit its own apical uptake in the short term. Bury and Wood (1999), showed that silver, probably in the ionic form Ag^+, can enter the gills of freshwater rainbow trout via a Na^+ channel situated on the branchial apical membrane, and the entry step was powered by a H^+ ATPase because the entry was blocked by phenamil and by bafilomycin A1. The silver uptake rate across the gills and into the body exhibited Michaelis-Menten kinetics (which gives a measure of K_m and J_{max}), suggesting that part of the uptake process is carrier-mediated. Bury et al. (1999) reported the presence of a P-type ATPase at the basolateral membrane of the gills of rainbow trout that can actively transport silver.

Gastrointestinal uptake of silver

Assimilation efficiency of silver associated with various ingested particles (e.g. algae, natural suspended particles, oxic and anoxic sediments) determined for several organisms such as zooplankon, polychaetes and bivalves, clearly demonstrated that uptake of silver can occur through the diet (Luoma and Fisher, 1997; Wang and Fisher, 1999).

Bioavailability of silver from ingested food is strongly influenced by the nature of silver- food association and differs considerably among animal species. During digestion, silver bound to organic molecules could be released or leached from food particles and the silver could then react with amino acids or proteins. Silver bound to these organic molecules could be transported into individual cells via specific transporters for macromolecules in the gut.

Galvez and Wood (1999) reported that juvenile rainbow trout fed a diet containing Ag_2S (3000 mg Ag kg^{-1} or 28 mmol kg^{-1} diet) accumulated silver in liver 4-fold higher than control trout after 56 days feeding. However, juvenile rainbow trout provided a diet containing a much lower concentration of biologically incorporated silver (approximately 3 mg Ag kg^{-1} or 0.03 mmol kg^{-1} diet) displayed liver concentrations 12-fold higher than those of controls after three months (Galvez et al., 1996), suggesting that biologically incorporated silver is much more bioavailable to trout than Ag_2S mixed with food. Hepatic metallothionein concentrations remained unchanged, but silver concentrations were significantly elevated in the kidneys, gills and plasma of fish fed the diet with biologically incorporated silver. Nothing is known about silver transport across the intestine but Clearwater et al. (2005) suggested that it might be through an osmotic gradient, i.e. via a solvent drag phenomenon.

Lead

Lead (Pb) is an ubiquitous constituent of the environment. Elevated concentrations of lead now occur in the food, water and air for most of the world's population. Lead is highly useful in many industrial processes. Organic lead compounds such as tetraethyl or tetra methyl lead are highly toxic compared to inorganic lead compounds.

Hodson and Blunt (1975) indicated that ALA-D (∂ -amino levulinic acid dehydratase) inhibition in fish was specific for lead, since copper, zinc, cadmium or mercury exposures did not inhibit the enzyme. Hodson (1976) reported that ALA-D inhibition was associated with a reduction in hemoglobin synthesis and red blood cell density, which is an indication of a harmful lead exposure in fish in the same way as it is in mammals.

Under normal conditions, waterborne lead falls within the range of 0.6-120 :g. L^{-1} (Demayo et al., 1982) though the Research Triangle Park (1999) reported concentrations as high as 890 :g l^{-1} in contaminated freshwater.

Lead precipitates from water as insoluble carbonate, phosphate or hydroxide salts, so high concentrations in static solutions are not constant and estimates of toxicity may be imprecise. Hodson et al. (1978) reported that lead species such as Pb^{2+} and Pb $(OH)^{+}$ occur commonly in soft, low pH waters and are more available and toxic. However in hard water, lead readily complexes to form Pb (CO_3); such complexes are less available for uptake, therefore less toxic to fish (Davies et al., 1976), and indeed may precipitate out of solution. Lead could also have direct or indirect influence on the central nervous system of the fish.

Branchial uptake of lead

The log K_D value for lead is 6.0 (MacDonald et al., 2002). Calcium, a prominent component of hard water, competes with lead for uptake contributing to protective effects of water hardness. As hardness of water increases, more lead precipitates due to the greater availability of anions such as carbonate and hydroxide. Recent research (MacDonald et al., 2002; Rogers and Wood, 2004) indicates that lead disrupts Ca^{2+} homeostasis by competitive inhibition at apical Ca^{2+} channels in the fish gill, therefore entering the fish by the same mechanism as Ca^{2+}.

Branchial uptake of Ca^{2+} is thought to be primarily by passive movement through apical voltage–insensitive channels in the chloride cells of the fish gill (Flik et al., 1993). Once entering the chloride cell, Ca^{2+} is transported via Ca^{2+} - binding proteins to the

basolateral membrane where it is actively extruded into the circulation by way of a high – affinity Ca^{2+}-ATPase enzyme (Flik et al., 1985a, Verbost et al., 1994, Marshall, 2002) and /or a Na^{+} /Ca^{2+} exchange mechanism (Flik et al., 1994, Flik et al., 1997). Lead could potentially have an impact at any one of these steps of calcium entry.

Recent research by Rogers et al. (2003) provides evidence that the acute toxic mechanism for waterborne lead in rainbow trout (*Oncorhynchus mykiss*) is ionoregulatory disruption, with observed effects on Ca^{2+} homeostasis and Na^{+} and Cl^{-} balance. Rogers and Wood (2004) reported that the uptake of waterborne lead by the fish gill is by the same mechanism as Ca^{2+}, which involves competitive inhibition of apical entry at lanthanum-sensitive Ca^{2+} channels and interference with the function of the ATP-driven basolateral Ca^{2+} pump, similar to cadmium (Verbost et al., 1989) and zinc (Spry and Wood, 1985).

Gastrointestinal uptake of lead

Gastrointestinal absorption of lead by adult subjects is only about 5-10%, so fecal excretion provides a rough estimate of intake. Kolbye et al. (1974) indicated that lead content of the diet of young adults generally averages 150-250 μg/day. Tepper (1971) reported estimates of total dietary intake of lead to be 137 μg/day in humans. In mammals there is possibility that lead transport to be through DMT1 (Bressler et al., 2004).

Hodson et al. (1978) exposed rainbow trout to dietary lead concentrations as high as 61 mg/kg dw for 32 weeks, but dietary lead was not assimilated, with >90% being excreted in fecal matter and no significant lead accumulation was observed in fish tissue.

Mount et al. (1994) reported that dietary lead concentrations as high as 170mg/kg dw showed no effects on survival or growth of rainbow trout fed for two 60-day studies. However, Draves and Fox (1998) related the substantial lead concentration in juvenile yellow perch (*Perca flavescens*) to high levels of lead in the invertebrate prey (from a contaminated region in the Montreal river) in which yellow perch were fed, and suggested that dietary uptake is the main route for lead accumulation in yellow perch. In addition, Farag et al. (2000) related the concentration of lead in a contaminated natural diet from Coeur d' Alene river, with the distribution of lead in the particulate fraction of the intestinal contents and protein fraction of the intestinal fluid in cutthroat trout.

Nickel

Nickel (Ni) is ubiquitous in the biosphere. Nickel and its compounds are regulated by USEPA for many industrial point sources, including the processing of iron, steel, non-ferrous metals, and batteries.

Nickel is a transition metal that is generally considered to be an essential micronutrient (Nielsen, 1971), because it is consistently found in animal tissues at low levels that appear to be well regulated. Nickel is essential for the normal growth of many species of microorganisms and plants and numerous species of vertebrates (NAS, 1975; USEPA, 1980; WHO, 1991).

In mammalian blood, absorbed nickel is present as free hydrated Ni^{2+} ions, as small complexes, as protein complexes and as nickel bound to blood cells. The chemical and physical forms of nickel and its salts strongly influence bioavailability and toxicity (WHO, 1991). The observed redox properties of the nickel- histidine complex are crucial

for maximizing the toxicity and carcinogenicity of nickel (Datta et al., 1992, 1994). Nickel normally occurs in the 0 and +2 oxidation states, although other oxidation states are reported (NAS, 1975; Nriagu, 1980b, Higgins, 1995). In natural waters, Ni^{2+} is the dominant chemical species in the form of Ni $(H_2O)_6)^{2+}$ (WHO, 1991; Chau and Kulikovsky-Corderio, 1995). Pyle et al. (2000) reported that increasing water pH, hardness and total suspended solids all reduce nickel toxicity to larval fathead minnows. Increasing pH reduces toxicity by progressive formation of less bioavailable nickel species, such as nickel-carbonate. Water hardness (Ca^{2+}, Mg^{2+}) reduces toxicity by out-competing nickel for gill- surface binding sites. Total suspended solids remove free nickel from the water column by adsorption, thereby reducing nickel bioavailability to fish.

Branchial uptake of nickel

Recent research from Pane et al. (2003) reported that nickel is primarily a respiratory, rather than an ionoregulatory toxicant at exposure levels close to the 96-h LC_{50}. Pane et al. (2003) further showed that nickel is an acute respiratory toxicant, causing a decrease in mean arterial oxygen tension, increase in mean arterial carbon dioxide tension, increase in hematocrit (Ht), and plasma lactate, and a significant decrease in spleen hemoglobin (Hb), as for aluminium (Playle et al., 1989). The log K_D of nickel binding to the gill is 4.0 (Meyer et al., 1999), indicating that it binds less strongly than most other metals.

Nickel accumulates in fish tissues and causes alterations in gill structure, including hypertrophy of respiratory and mucus cells, separation of the epithelial layer from the

pillar cell system, cauterization and sloughing, and necrosis of the epithelium (Nath and Kumar, 1989). There is a possibility for nickel to diffuse in across the gills via an unknown mechanism (Pane et al., 2004).

Gastrointestinal uptake of nickel

In humans, average dietary intakes of nickel range from 300 to 500 μg daily, with very low absorption (1-10%). Similarly, in dogs and cats given nickel, nickel sulfate hexahydrate, or nickel chloride I, by diet or by gavage, all absorbed only 1-10% from the gastrointestinal tract (USEPA, 1980, 1986; Sigel and Siegl, 1988; USPHS, 1993). In humans, nearly 40 times more nickel was absorbed from the gastrointestinal tract when nickel sulfate was given in the drinking water with absorption efficiency of 27% than when it was given in the diet (0.7%) (WHO, 1991; USPHS, 1993), perhaps because nickel chloride hexahydrate and nickel sulfate hexahydrate are extremely soluble in water at 2,400-2,500 g/l.

Ptashynski et al. (2001) investigated the bioavailability and toxicity of dietary nickel in fish. They reported that histopathological alterations were observed in kidneys of adult lake whitefish (*Coregonus clupeaformis*) fed low and high Ni diets (1000 and 10 000μg Ni/g). Livers of white fish fed high dose diets (10 000 μg Ni/g) and intestine of lake white fish fed high dose diets (10 000 μg Ni/g) and lake trout (*Salvelinus namaycush*) fed low and high diets (1000 and 10 000 μg Ni/g) also showed the same effects as at the kidneys.

Conclusions about current knowledge, leading to hypotheses tested in this thesis.

Based on the information contained in this literature review, it appears that mechanisms of branchial uptake of copper, zinc, cadmium, lead and nickel are at least partially understood in fish. However, it appears that very little is known about the mechanisms of gastrointestinal uptake of copper, zinc and cadmium and that virtually nothing is known about the mechanisms of gastrointestinal uptake of silver, lead, and nickel in fish. More importantly, there is virtually nothing known about metal uptake at the stomach of fish. Furthermore, there is little or no knowledge about the possible toxic effects of metals on ion transport processes in the gastrointestinal tract, in contrast to the fairly detailed knowledge available about their effects on comparable processes at the gills, which have formed the basis of the gill BLM.

As a result of all these, the first hypothesis was, to test whether the presence of non-essential metals (such as cadmium, silver and lead) will be more toxic (i.e. whether they might affect ion transport) than essential metals (such as copper, zinc and nickel) on the fluid transport rates via the gastrointestinal tract. Since fluid transport is driven by ion transport (Bergman et al., 2003; Grosell et al., 2005), it might provide a broad index of the integrity of ion transport processes. A second hypothesis evaluated whether there were differences in regional locations of uptake and/or rates of uptake between essential metals and non-essential metals via the gastrointestinal tract. This hypothesis was based on fact that essential metals are required for normal growth of fish while non-essential metals have no physiological function in fish. Also from this report, it appears that there is little information on interaction between branchial and gastrointestinal uptake of copper, zinc and cadmium (Kamunde et al., 2001, 2002; Grosell et al., unpublished;

Szebedinszky et al., 2001) and that there is none for possible interactions involving silver, lead and nickel. The third hypothesis was made on whether there will be differences between metal uptake rates at the gut as compared to the gill. Note that the concentrations of each metal tested at the gut were several orders of magnitude higher than those usually tested in water for the gill, but this difference reflects the fact that typical metal levels in natural foods are this much higher than metal levels in natural freshwaters.

Furthermore, this literature review has shown that calcium, cadmium and zinc uptake interact at the gill (Verbost et al., 1987, 1988, 1989; Hogstrand et al., 1996b), but there appears to be little known about the effect of calcium on cadmium and zinc uptake via gastrointestinal tract (Franklin et al., In press; Glover et al., 2004). Based on this, I tested the fourth hypothesis, on whether there will be competitive interaction between calcium, cadmium and zinc uptake via the gastrointestinal tract as compared to the gill.

This literature review also bring into our attention that interactions between metals in fish could be comparable to those in mammals. For example, the documented interaction between zinc and cadmium at the intestine of fish using an *in situ* technique (Shears and Fletcher, 1983) is apparently similar to that seen in mammals (Foulkes, 1985; Tacnet et al., 1991). As a result of this, the fifth hypothesis was tested, whether there will be antagonistic interaction between zinc and cadmium via the gastrointestinal tract. Interaction between copper and zinc at the fish intestine was also included in this literature review (Nadella et al., unpublished). But, there is nothing known about the interaction between copper and zinc at the stomach. Based on this the sixth hypothesis was tested, whether there will be different interactions between zinc and copper in

different parts of the gut of fish as compared to other previous studies in fish and in mammals (Cousins, 1985).

Focus of the project

The main goal of this thesis was to both physiologically and toxicologically investigate metal bioavailability and interaction via the gastrointestinal tract of fish. In view of this, the following goals were set forth in two experimental chapters:

1. The goal of the experiments in Chapter 2 was to gain insight into the bioavailability of six metals via the gastrointestinal tract of the freshwater rainbow trout using a common and environmentally realistic concentration that would help show what was happening in a natural environment. An *in vitro* gut sac technique was followed, similar to that recently developed and validated by Nadella et al., (unpublished). This technique has been shown to yield results comparable with previous *in vivo* work in fish. It can also help to gain knowledge into mechanisms by which these metals are transported at higher concentration via the gut, as well as reveal whether uptake rates of these metals at the gut (at higher concentration) differ from uptake rates at the gill (at lower concentration). The preparation provides a sensitive measure of fluid transport rate, so measuring the fluid transport rate in the presence of each of the metals, should be indicative of potential toxicity to ion transport process, because fluid transport is driven by ion transport. This can also give results that can be of environmental importance.

The following topics were investigated in Chapter 2 (i) Effects of both essential (copper, zinc and nickel) and non-essential metals (cadmium, silver and lead) on the fluid transport rate via the gastrointestinal tract. (ii) Uptake rates of these metals at three different compartments (i.e. serosal fluid + muscle, rinse and mucosal epithelium) via the gastrointestinal tract. (iii) Partitioning among the three compartments for each of the metal at each segment and lastly, (iv) The possible correlation for each metal between the rate of absorption and mucus-binding, and between the rate of absorption and the rate of mucosal epithelium accumulation via the gastrointestinal tract of fish.

2. The experiments of Chapter 3 studied potential interaction between metals via the gastrointestinal tract of fish using the same techniques as used in Chapter 2. This approach can help to gain knowledge into the actual mechanisms by which copper, zinc and cadmium are transported via the gastrointestinal tract of fish as compared to mammalian work, as well as into mechanisms of homeostatic regulation of these metals in fish in comparison to mammals. These results can also help to show whether the mechanism by which zinc and cadmium are transported via the gill differ from that via gastrointestinal tract. The following topics were investigated in Chapter 3. (i) Effects of calcium on cadmium and zinc uptake via the gastrointestinal tract (ii) Interactions between an essential (zinc) and non-essential metal (cadmium) via the gastrointestinal tract and lastly (iii) Interactions between two essential metals (i.e. zinc and copper) via the gastro-

intestinal tract of fish. Potentially, these results could all be used to develop an intestinal BLM via the gastro-intestinal tract of fish.

Figures

Fig. 1-1. Diagrammatic model of the mechanisms of ionic (such as Na^+, Ca^{2+}, H^+, K^{2+}, Cl^-), acid-base (NH_4^+, NH_3, HCO_3^-, OH^-) and CO_2 transport, and their functional interrelationships at the gills freshwater teleost fish (Wood, 2001).

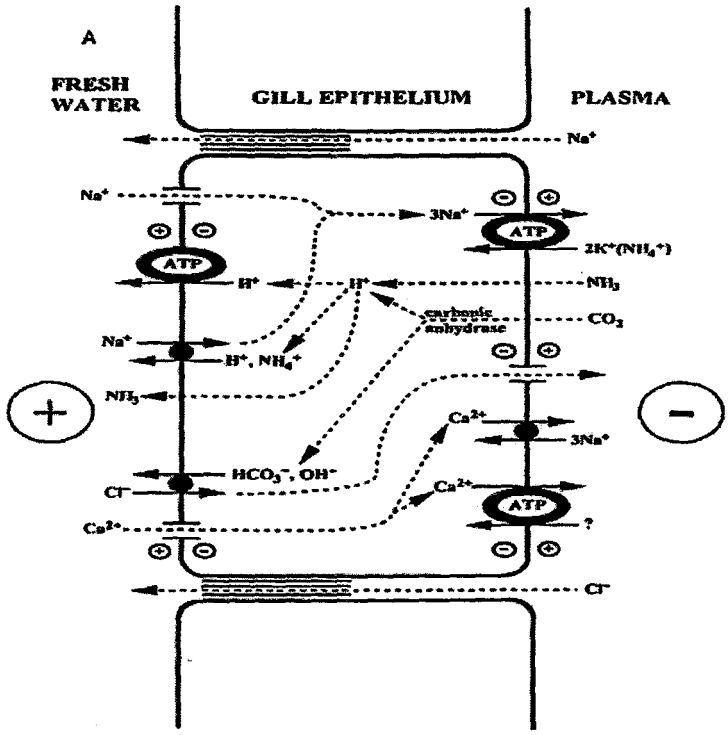

A
FRESH WATER
GILL EPITHELIUM
PLASMA
Na+
3Na+
ATP
2K+(NH4+)
H+
NH3
CO2
carbonic anhydrase
Na+
H+, NH4+
NH3
Ca2+
3Na+
HCO3-, OH-
Cl-
Ca2+
ATP
?
Ca2+
Cl-
+
-

Fig. 1-1

Fig. 1-2. Transcellular uptake pathways for sodium (a) and copper (b) in an idealized intestinal epithelial cell (Handy, 2002). Copper can enter the epithelial cell through sodium channel. Intracellular sodium can bind to the nucleus and mitochondria. Basolateral copper export can be through Na^+/K^+ ATPase (Fig. 1-2A). Copper can also enter the epithelial cell through Ctr1. Copper in the intracellular spaces can bind to Golgi apparatus, nucleus and mitochondria. Basolateral copper extrusion out of the cell can be through Cu/Cl^- co-transporter (Fig. 1-2B).

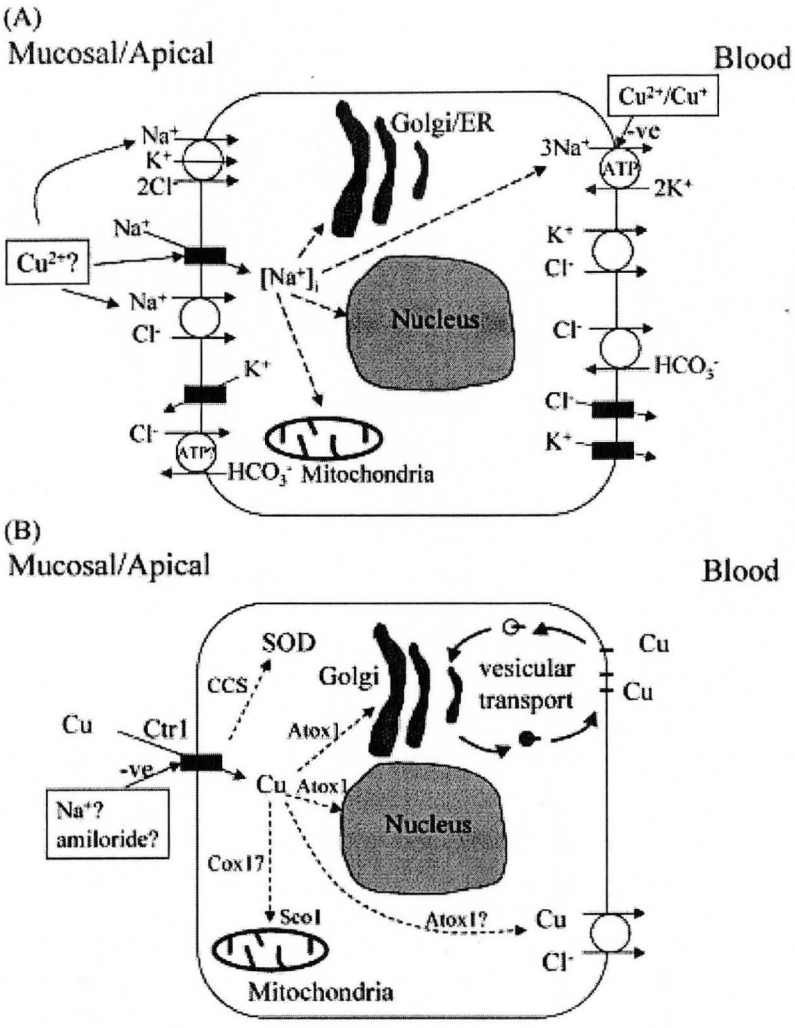

(A)
Mucosal/Apical
Blood
Golgi/ER
Na+
K+
2Cl-
Na+
Cu2+?
Na+
Cl-
K+
Cl-
ATP
HCO3-
Mitochondria
[Na+]i
Nucleus
Cu2+/Cu+
-ve
3Na+
ATP
2K+
K+
Cl-
Cl-
HCO3-
Cl-
K+
(B)
Mucosal/Apical
Blood
SOD
CCS
Golgi
vesicular
transport
Cu
Cu
Cu
Ctr1
Atox1
-ve
Na+?
amiloride?
Cu
Atox1
Nucleus
Cox17
Sco1
Atox1?
Cu
Cl-
Mitochondria

Fig.1-2

Chapter 2: Study 1

Bioavailability of Metals via the Gastrointestinal Tract of Rainbow trout (*Oncorhynchus mykiss*)

Abstract

Only a few studies have focused on the bioavailability of copper, zinc and cadmium via gut of fish, and almost nothing is known about the bioavailability of silver, lead and nickel via the gastrointestinal tract of fish. Uptake rates of essential metals (copper, zinc and nickel) and non-essential metals (cadmium, lead and silver) at 50μM each in the luminal saline were investigated using an *in vitro* stomach and gut sac technique. The fluid transport rate was highest at the anterior intestine compared to mid and posterior intestine. The stomach emerged as an important site for metal absorption. Rates of absorption for copper, silver, zinc and cadmium were highest at the anterior intestine compared to stomach, mid and posterior intestine, suggesting a similar spatial distribution of transport mechanisms. Lead absorption was highest at the mid-intestine, while nickel absorption was highest at the mid and posterior intestine, suggesting different spatial distributions for lead and nickel transport sites. Essential metals were taken up at almost the same rates as non-essential metals. Copper, zinc, nickel, silver and lead showed statistical significant correlation between rate of absorption and mucus binding indicating that they are important for BLM binding site via the gastrointestinal tract. Nickel, silver and lead showed statistical correlation between rate of absorption and rate of accumulation in the mucosal epithelium.

1. Introduction

Nutritionally essential metals are copper, zinc and nickel, while silver, cadmium and lead are non-essential metals. Essential metals are required for the normal growth of the fish, and the diet can be their main source (Bury et al., 2003). Non-essential metals have no physiological function in fish. Both types of metals can originate from domestic and agricultural/aquacultural sources for copper and zinc; industrial processes for copper, zinc, nickel, cadmium and lead; mining, forestry, waste disposal for cadmium (Pratap et al., 1989; Farag et al., 1994); natural leaching, photographic processing for silver (Purcell and Peters, 1998) and geological weathering, smelting, coal burning, batteries and paint for lead (World Health Organization, 1995).

The entire gastrointestinal tract of fish can be divided into oesophagus, stomach and intestine (anterior, mid and posterior). The principal layers of the gut wall consist of mucosa, submucosa, muscularis externa (circular and londitudinal muscle fibres) and serosa (Fig. 2-1; Clearwater et al., unpublished). The gut can function in the absorption of water in fish (Buddington et al., 1997; House and Green, 1965). The anterior intestine has been shown to absorb more water compared to other parts of the intestine in fish (Bergman et al., 2003; Nadella et al., unpublished). Solvent drag has been excluded from copper uptake through the perfused intestine of the African walking catfish (Handy et al., 2000), which indicates that, at least in this species, water uptake through the intestine does not have an effect on copper transport.

Metals can be absorbed from the diet through the gut of fish (Farag et al., 2000). Metal transport via the gut can involve three steps. The first step may be binding of metal to gut surface mucus, which may either facilitate or retard uptake (Part and Lock, 1983;

Whitehead et al., 1996). The second step involves transferring of metals from the gut lumen into the mucosal epithelium (apical entry). The third step involves movement of metals out of the epithelial cells through the basolateral membrane, then through the interstitial space into the blood side.

Knowledge of the regional uptake of metals might help gain insight into transport mechanisms for these metals via the gastrointestinal tract. Studies using both *in vivo* and *in vitro* approaches have shown the anterior intestine to be the highest absorptive region for copper, zinc and cadmium uptake compared to other parts of the intestine in fish, while the stomach in particular has been considered to have low copper, zinc and cadmium absorption (Hardy et al. 1987; Pentreath, 1976; Shears and Fletcher, 1983; Clearwater et al., 2002; Chowdhury et al., 2004; Nadella et al., unpublished). Similarly in mammals, zinc absorption appears to be most important at the upper intestine (Kowarski et al., 1974; Lee et al., 1989). However, Hodson et al. (1978) reported that there was no dietary lead assimilation, with no appreciable amount of lead accumulation in tissues of rainbow trout fed dietary lead for 32 weeks.

Mucus is a gelatinous layer that is both a defense barrier and potential transport medium at the mucosal surface. The ability of metal ions to penetrate this layer and reach the mucosal epithelium will depend on their mobility through mucus (Part and Lock, 1983; Whitehead et al., 1996). Morphological changes such as increased mucous cell activity, disruption of the intestinal brush border and an increased renewal rate of absorptive cells at the mid and posterior intestine have been reported in trout exposed to dietary cadmium and lead for 15 or 30-days (Crespo et al., 1986). Cadmium has also been shown to have a high affinity for epithelial mucus (Part and Lock, 1983) and to increase

mucus cell secretion and activity (Gardener and Yevich, 1970; Glover and Hogstrand, 2003). Glover and Hogstrand (2002b) have also shown that metals can accumulate on/in the epithelial cells of the gut mucosa, and cadmium, which is often considered to be a calcium analogue, has been shown to inhibit basolateral Ca-ATPase at the intestine of fish (Schoenmakers et al., 1992). This suggests that cadmium can accumulate in the mucosal epithelium because it blocks the basolateral exit mechanism.

In light of the above, only a little is known about the bioavailability of copper, zinc, and cadmium via the gastrointestinal tract of fish, and virtually nothing about silver, lead and nickel. Therefore, the main objectives of this study are to use an *in vitro* stomach and gut-sac technique from adult rainbow trout to:

(i) Investigate the effects of the six metals on the fluid transport rates.

(ii) Investigate regional uptake rates of these six metals in the stomach, anterior, mid and the posterior intestine.

(iii) Compare overall metal transport rates in all four segments of the gastrointestinal tract.

(iv) Compare metal surface binding to mucus in all four segments of the gastrointestinal tract.

(v) Compare apical entry (accumulation in the mucosal epithelium) of the six metals in all four segments of the gastrointestinal tract.

Based on these, I tested the hypotheses that:

(i) There may be regional differences between essential and non-essential metals.

(ii) Essential metals may be transported more rapidly than non-essential metals.

Knowledge about the rates and spatial distribution of transport for metals via the gut

might help to gain insight into mechanisms of absorption via the gut. It might also help to gain knowledge into what might be happening in the natural environment with respect to dietary metal loading. Finally, it might also help to give a general overview of what might be happening at the gut as compared to the gill, where metal binding and transport have been studied much more intensively in the last few years (e.g. Wood, 2001; McGeer et al., 2003; Playle, 2004).

2.0. **Materials and Methods.**

2.1. Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*; N = 101), 250g (~ 30cm) were obtained from Humber Springs Fish Hatchery (Orangeville, ON). Fish were maintained in 500L tanks with flowing aerated and dechlorinated Hamilton city tap water from Lake Ontario (approximate ionic composition in mM: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8-8.0, DOC ~ 3mg C L^{-1}, hardness ~ 140mgL^{-1} as $CaCO_3$). The fish were fed a maintenance ration of Martin's commercial dried trout pellet feed 5 times per week at 1% body weight per feeding (manufacturer's specifications: crude protein 41%; crude fat 11%; crude fibre 3.5%; calcium 1%; phosphorus 0.85%; sodium 0.45%; vitamin A 6,800 IU/kg; vitamin D 2,100 IU/kg; vitamin E 80 IU/kg; Martins Mills Inc Elmira, ON). Metals content of the food include 27 μg/g dry wt. for copper, 173 μg/g for zinc, 0.26 μg/g for cadmium, 10 μg/g for lead, 0.05 μg/g for silver and 3.86 μg/g for nickel. Water temperature was maintained between 11-13°C. Fish were starved for three days prior to the experiment.

2.2. Experimental Technique: *In vitro* Stomach and Gut Sac Technique

An *in vitro* gut sac technique similar to that used by Bury et al. (2001) and Grosell et al. (2001) was followed. This technique allows manipulation of both the apical (mucosal) and basolateral (serosal) media composition, thereby enabling the study of transport mechanisms and facilitating analysis of potential sites of regulation of copper, silver, zinc, cadmium, lead and nickel uptake via the gastrointestinal tract. This approach helps to identify the function of each segments of the gastrointestinal tract in metals absorption. It also allows for a quick analysis of transport mechanisms, and the effects of competitive ions, and provides relevant information on future directions for *in vivo* studies.

Rainbow trout were euthanised with an overdose of MS-222 (0.25 g L^{-1}). A ventral incision from the gills to the anus was made to remove the entire gastrointestinal tract. The tract was then placed immediately in a Petri dish for dissection on ice, and bathed with the modified Cortland saline solution (see below for composition). The liver and gall bladder were removed after tying off the bile duct that enters just posterior to the stomach. Gut contents were squeezed gently from the stomach and intestine. Visceral fats were removed from the entire gastrointestinal tract. The gastrointestinal tract was flushed with saline (see below for composition) to remove food and faeces. The cleaned gastrointestinal tract was sectioned into four segments, the stomach, anterior, mid and posterior intestine. The stomach gut sac was made by ligating with suture at the junction between the oesophagus and the stomach and between stomach and the anterior intestine at the pyloric aperture. For the other segments of the gastrointestinal tract, the regional division of the intestine was made along obvious morphological differences for the posterior section, while the remaining portion between the pyloric aperture and the

posterior region was split into anterior and mid intestinal sections based on the presence of cecae in the anterior section only. One end of each segment was sealed tightly with surgical silk and into the other end, a 5cm piece of PE-50 was inserted at the intestine (PE-160 at the stomach) and tied with surgical silk to allow for administration and sampling of luminal saline.

To these gut sacs, 1 ml of appropriate luminal saline containing 50 μmol L^{-1} of the metal of interest appropriately labelled with one of ^{64}Cu, ^{110m}Ag, ^{65}Zn, ^{109}Cd, or ^{63}Ni was injected (with a 3ml syringe and 18.5 - guage needle for the stomach gut sac and 3ml syringe and 21-guage needle for the three segments of the intestine) and a sub sample taken for initial analysis along with a sample of serosal saline (plasma saline). As there is no available radioisotope for lead, "cold" lead (50 μmol L^{-1}) was used. The PE tubing was sealed and the sacs were weighed (initial weight). The sacs were transferred into 40ml or 12ml Falcon tubes for incubation in serosal saline (40ml for stomach and anterior intestine, 12ml for mid and posterior intestine), which was bubbled constantly with 99.5% O_2, and 0.5% of CO_2 gas mixture because *in vivo* $\bar{P}co_2$ levels in the blood are approximately 3.75 torr. The temperature was maintained at 11-13°C.

After incubation for 4h for the stomach and 2h for the intestine, the gut sacs were re - weighed for final weight, the luminal saline was removed and taken for counting. A sub-sample of serosal saline was also analyzed for each of ^{64}Cu, ^{110m}Ag, ^{65}Zn, and ^{109}Cd activity by a Minaxi – γ Auto-gamma 5530 counter (Canberra Packard, Mississauga, Ontario, Canada); lead was measured by atomic absorption spectrophotometry (SpectrAA-220, Varian, Mississauga, Ont., Canada) with graphite furnace atomization; and ^{63}Ni activity was counted on a scintillation counter (LKB Wallac 1217 Rackbeta,

Pharmacia-LKB AB, Helsinki).

The gut sacs were cut open, washed in 5ml of modified Cortland saline and then with 1mM EDTA disodium salt saline, and then blotted dry with a small piece of paper towel. The washing solutions plus blotting paper were collected for analysis. The washing plus blotting procedure ensured removal of mucus and associated metal loosely bound to the surface of the gut lumen, so that the metal incorporated into the gut tissue could be considered to represent the actual copper, silver, zinc, cadmium, nickel or lead absorption. The mucosal epithelium was then scraped off gently with a glass slide and collected separately. This left behind the submucosa, muscle layers, and serosa, collectively referred to here as the "muscle layer''. The exposed surface area of each segment of the intestine was measured using graph paper; this was very similar to the method used by Grosell and Jensen (1999). For the anterior intestine, only the graph of the exposed luminal surface area could be measured; the surface area of the ceca could not be measured, so the surface area measurements for this segment are undoubtedly an underestimate. Then the wash solutions, blotting paper, epithelial scrapings and muscle layer were counted separately for radioactivity.

For lead, for which there is no readily available radioisotope, the blotting paper, and muscle layer were digested separately in five volumes of 1N HNO_3 (trace metal grade, Fisher Scientific) and then placed in an oven at 60°C for 48h in a sealed tube. The tube was then centrifuged and the supernatant was taken, along with serosal saline, luminal saline, saline rinse and EDTA rinse, and epithelial scrapings and assayed for lead on the graphite furnace (GFAAS).

For nickel, where the radioisotope is a beta-emitter rather than a gamma-emitter, 1ml

subsamples taken from serosal saline, luminal saline, saline rinse and EDTA rinse and epithelial tissue were acidified with 1N HNO_3 to solubilize all metals and vortexed. Five ml of the scintillant Ultima Gold (Perkin Elmer) was added and the samples were kept in the dark for 5h, to eliminate chemiluminescence before counting in the scintillation counter. The blotting paper and muscle layer were digested in five volumes of 1N HNO_3 and then placed in an oven at 60°C for 48h in a sealed tube. The tube was then centrifuged and 1ml of supernatant was added to five ml of Ultima Gold, which was again kept in the dark for 5h, before counting in the beta counter. After counting, I corrected for quenching by using a quench curve constructed for the tissues of interest based on the external standard ratio method. The dilution factor associated with digestion was taken into consideration in all cases.

2.3. Experimental Protocol

In vivo dietary studies have recently shown that the normal concentration of the essential metal copper in trout intestinal chyme is about 3 μg/ml = 50μM (Nadella *et al.* unpublished). Glover and Hogstrand (2003) had also studied intestinal zinc uptake at 50μM in trout using an *in vivo* intestinal perfusion technique, and this value appears to fall within the range found in the intestinal fluid of plaice (*Pleuronectes platessa*) from mildly contaminated estuarine water (Turner and Olsen, 2000) in a natural environment. This concentration (50μM) was now chosen for other metals (such as silver, cadmium, lead and nickel) to investigate their uptake rate via the gastrointestinal tract of fish.

Modified Cortland saline (sulphate-based saline) was used on both the luminal (mucosal) and serosal (plasma) surface because silver was found to visually precipitate

with chloride (cloudiness) in chloride-based saline, and there was a potential for other metals to form similar, perhaps undetectable precipitates. At least for copper, Nadella et al. (unpublished) have shown that this saline yields the same uptake rates in intestinal sac preparations as for traditional chloride-based saline.

Preparation of modified Cortland saline for the metals: (mmol.l^{-1}) Na_2SO_4, 66.5; K_2SO_4, 2.5; $CaSO_4$, 1; $MgSO_4.7H_2O$, 1.9; $NaHCO_3$, 1.9; $NaH_2PO_4.H_2O$, 2.9; Glucose, 5.5 pH 7.4.Osmolality was adjusted to 276 mOsm by adding mannitol.

(i) Preparation of luminal saline for copper: Dried Cu $(NO_3)_2$ (0.2mg) was irradiated (^{64}Cu, half life 12.9h) at McMaster Nuclear Reactor to achieve a radioactivity level of 21 MBq (600 μci). After irradiation, the Cu $(NO_3)_2$ was dissolved in 0.1 mol.l^{-1} HNO_3 (400μl) and 0.01mol.l^{-1} $NaHCO_3$ (400μl). 15μl of resuspended copper was then added to1 ml of modified Cortland saline to give a final concentration of 50μM. This gives about 3.0μg Cu ml^{-1} in 1 ml administered in each gut sac. The molecular weight of copper is 63.55.

(ii) Preparation of luminal saline for silver: 15μci of radioactive silver $^{110}AgNO_3$ (Risφ National Laboratory Radiation Research Department; Denmark) was added to a 50μM solution of $AgNO_3$ in modified Cortland saline with pH of 7.4. This gives about 5.4 μg Ag ml^{-1} in 1 ml administered in each gut sac. The molecular weight of silver is 107.87.

(iii) Preparation of luminal saline for zinc: 15μci of radioactive $^{65}ZnCl_2$ (Los Alamos National Laboratory, Los Alamos, NM; USA) was added to a 50μM solution of $ZnSO_4.7H_2O$ in modified Cortland saline; 1 drop of 1N NaOH was also added to raise the pH back to 7.4. After the addition of radioactive zinc to 50μM cold zinc solution, the solution was thoroughly stirred with a magnetic stirrer. This gives about 3.3 μg Zn ml^{-1} in

1 ml administered in each gut sac. The molecular weight of zinc is 65.38.

(iv) Preparation of luminal saline for cadmium: 15μci of radioactive $^{109}CdCl_2$ (Perkin Elmer Life and Analytical Sciences, Boston, MA; USA) was added to a 50μM solution of $CdNO_3.H_2O$ in modified Cortland saline; 1 drop of 1N NaOH was added to raise the pH back to 7.4. After the addition of radioactive cadmium to 50μM cold cadmium solution, the solution was thoroughly stirred with a magnetic stirrer. This gives about 5.6 μg Cd ml^{-1} in 1 ml administered in each gut sac. The molecular weight of cadmium is 112.41.

(iii)**Preparation of luminal saline for lead:** 50mM of Pb $(NO_3)_2$ was dissolved in 1% HNO_3, to avoid the lead precipitating with Na_2SO_4 in the saline, after which 50 μl was taken and dissolved in 50ml of the modified Cortland saline to make 50μM of cold Pb with pH of 7.4. This gives about 10.4 μg Pb ml^{-1} in 1 ml administered in each gut sac. The molecular weight of lead is 207.2.

(vi) **Preparation of luminal saline for nickel:** 15μci of radioactive $^{63}NiCl_2$ (Perkin-Elmer Life Sciences, Boston, MA; U.S.A) was added to a 50μM solution of $NiCl_2.6H_2O$ in modified Cortland saline with pH of 7.4. This gives about 2.9 μg Ni ml^{-1} in 1 ml administered in each gut sac. The molecular weight of nickel is 58.69.

In each case, the exact final total concentration of the metal of interest in the luminal saline stock was measured directly by graphite furnace atomic absorption spectrophotometry. For copper it was 2.77 μg/ml, zinc = 3.75 μg/ml, silver = 5.26 μg/ml, cadmium = 5.26 μg/ml, lead = 7.82 μg/ml and nickel = 4.53 μg/ml. These values are all close to 50μM, and for the purpose of comparison, final rates were normalized to 50μM.

2.4. Sample Calculations:

The fluid transport rate (FTR) was calculated in a very similar manner to House and Green (1965) and Grosell and Jensen (1999) as:

$$FTR = (\text{Difference in weight of the gut sac} / ISA*T). \quad (1)$$

Difference in weight (in mg) of the gut sac = (initial weight of the sac (before incubation) – final weight of the sac (after incubation).

ISA is the intestinal surface area in square centimeters. T is time in hours. This produced the fluid transport rate expressed as $\mu l/cm^2/hr$.

For each preparation, three compartments of metal fate were measured. Firstly, the rinse (i.e. wash solutions and blotting paper) represents metals that were loosely bound to the mucus. Secondly, epithelial scrapings represent metals in surface mucosal epithelial cells. Thirdly, muscle and serosal fluid represent a conservative estimate of true metals absorption (i.e. metals that had been transported through the mucosal epithelial cells and into the extracellular fluid). Radioactivity in the wash solutions, epithelial scrapings, tissue and serosal fluid for the radiolabelled metal (copper, silver, cadmium, zinc and nickel) were calculated in a very similar manner to that used by Glover et al. (2003) for zinc uptake, as follows:

$$\text{Metal uptake (copper, silver, zinc, cadmium and nickel)} = ((\text{Tissue cpm}/SA*ISA*T)* 1000/M.W). \quad (2)$$

Tissue cpm represents the total ^{64}Cu, ^{110}Ag, ^{109}Cd, ^{65}Zn and ^{63}Ni activity of the compartment measured. Cpm for copper, zinc, cadmium and nickel were counted in the gamma or scintillation counter.

SA is the initial specific activity of the introduced mucosal fluid (cpm /μg) calculated as:

$$SA = Activity / [M] \quad (3)$$

Where activity is in cpm/ml as counted on the gamma or beta counter, [M] is the concentration of metals (copper, silver, zinc, cadmium and nickel) in μg/ml as measured on the graphite furnace. This gives specific activity (SA) in cpm/μg. ISA is the intestinal surface area in square centimeters, T is time in hours and M.W. is the molecular weight of each metal. This produced copper, silver, cadmium, zinc and nickel uptake rates expressed as nM cm^{-2} h^{-1} in equation (2).

Lead uptake rate, measured by GFAAs was, calculated as:

$$Pb\ uptake = ((tissue\ Pb/ISA*T)*1000/M.W. \quad (4)$$

$$Tissue\ Pb\ (\mu g\ /total\ ml) = (Pb\ (\mu g/l)*D.F*Dg.F)/1000 \quad (5)$$

where Pb (μg/l) is the lead concentration in the compartment measured, D.F is the dilution factor, and Dg. F is the digestion factor.

ISA is the intestinal surface area in square centimeters, T is the time in hours and M.W. is the molecular weight of lead. This produced lead uptake rate expressed as nM cm^{-2} h^{-1} in equation (4).

2.5. Graphite Furnace Atomic Absorption Spectrophotometry

The concentrations of each metal in luminal saline were measured by graphite furnace atomic absorption spectroscopy for copper, silver, cadmium, lead and nickel (GFAAS; Varian SpectrAA-220 with graphite tube atomizer [GTA-110], Mulgrave, Australia) or by flame atomic absorption spectroscopy for zinc (FAAS; Varian SpectrAA –220 FS, Mulgrave, Australia). National Research Council of Canada certified analytical standards run at the same time were within the specified range. Standards for zinc, copper, silver and nickel, were manufactured by Fisher Scientific, cadmium by Sigma Chemical Company, and lead by Aldrich Chemical Company.

2.6. Radioactivity Counting

Radioisotopes such as ^{64}Cu, ^{65}Zn, ^{109}Cd and ^{110m}Ag are gamma- emitters, while ^{63}Ni is a beta -emitter. The gamma radioactivities were counted using an energy window 433-2000KeV for ^{64}Cu, 15-2000KeV for ^{65}Zn and 15-150KeV for ^{109}Cd. For ^{110m}Ag the gamma radioactivity was counted in a specialized window 1050-2000KeV so as to prevent contamination of ^{110m}Ag counts with trace ^{109}Cd that is present in commercially prepared ^{110m}Ag (Hansen et al. 2002). The beta radioactivity for ^{63}Ni was counted in the 8-110KeV window on the scintillation counter and quench correction was taken into consideration using a quench curve. ^{64}Cu was corrected for decay to a common reference time, because it has a very short half-life (12.9h).

2.7. Statistical Analysis

Plotted values represent the means (± SEM) with N = 10 for copper and cadmium, N = 9 for lead, N = 8 for zinc, silver, nickel at the stomach and N = 8 for each treatment at the intestine. The significance of differences (at $P < 0.05$) for each metal among the four segments of the gastrointestinal tract was assessed using a one way analysis of variance (ANOVA), followed by a post-hoc least significant differences (LSD) test to identify individual differences. Similarly, the significance of differences (at $P < 0.05$) among the six metals within each segment of the gastrointestinal tract was assessed using the same approach. Analysis was performed using SPSS software. For correlation between the compartments (i.e. serosal fluid + muscle, mucus-binding and mucosal epithelium) linear regression relationship was assessed and the significance level was tested using Vassar stats.

3.0. Results

3.1. Fluid transport rates in the presence of all the metals in different segments of the gastrointestinal tract.

Fluid transport rates were higher in the anterior intestine than in the other three compartments (stomach, mid-intestine and posterior intestine) where they were generally similar (Fig. 2-2 A). The only significant difference in the latter occurred in the presence of copper and nickel, where the fluid transport rate was higher in the mid-intestine than in the stomach. Overall control mean values were 1.72 ± 0.18 $\mu l/cm^2/h$ at the stomach, 9.88 ± 0.68 $\mu l/cm^2/h$ at the anterior intestine, 2.95 ± 0.15 $\mu l/cm^2/h$ at the mid-intestine, and 2.07 ± 0.59 $\mu l/cm^2/h$ at the posterior intestine respectively. These values were compared

to the fluid transport rate in the presence of each metal (50μM) in each section. There were no significant effects associated with the presence of any of the metals in any segment with the single exception of copper in the stomach, which caused a mild inhibition (Fig. 2-2 B).

3.2. Basolateral metal net transport rates in different segment of gastrointestinal tract : serosal fluid + muscle

Transport of metal through the basolateral membrane of the enterocyte into the muscle tissue layer and serosal fluid was taken as a conservative measure of true absorption (i.e. all the way through the enterocytes, see Methods). For copper, zinc and silver there was a common pattern with highest rates in the anterior intestine, lower and approximately equal rates in the mid and posterior intestine, and lowest rates in the stomach though not all of the differences were significant (Fig. 2-3 A). For cadmium uptake, there was significant difference between stomach and anterior intestine, stomach and mid intestine, stomach and posterior intestine, anterior and mid intestine and between anterior and posterior intestine (Fig. 2-3 A).

For copper, zinc, silver and cadmium typical transport rates were about 0.20 ± 0.04 nmol/cm^2/h in the anterior intestine, 0.05 ± 0.01 nmol/cm^2/h in the mid-intestine, and 0.03 ± 0.01 nmol/cm^2/h in the posterior intestine. Typical transport rates for copper, zinc, silver and cadmium in the stomach were 0.012 ± 0.002 nmol/cm^2/h, 0.004 ± 0.001 nmol/cm^2/h, 0.008 ± 0.001 nmol/cm^2/h and 0.012 ± 0.003 nmol/cm^2/h respectively. At the stomach, zinc had lower transport rate than copper, cadmium and lead but transport rates among other metals (such as silver and nickel) were more or less the same (Fig. 2-3

B). At the intestine, there were no significant differences among these four metals (copper, zinc, silver and cadmium) (Fig. 2-3 B). Lead, on the other hand, exhibited the highest absorptive rate in the mid-intestine (about 0.35 ± 0.09 nmol/cm^2/h), 8-fold higher than in the anterior intestine (about 0.04 ± 0.01 nmol/cm^2/h), 2-fold higher than in posterior segments (about 0.15 ± 0.04 nmol/cm^2/h) and about 35-fold higher than in the stomach (about 0.01 ± 0.001 nmol/cm^2/h) (Fig. 2-3 A). Finally, for nickel net transport rate was highest in the posterior intestine (about 0.27 ± 0.02 nmol/cm^2/h), intermediate at the mid-intestine (about 0.22 ± 0.03 nmol/cm^2/h), lower at the anterior intestine (about 0.13 ± 0.04 nmol/cm^2/h) and lowest at the stomach (0.01 ± 0.002 nmol/cm^2/h) (Fig. 2-3 A). Thus comparing across metals within segments, the transport rate of lead was generally lower than the other metals in the anterior intestine, and higher than the other metals in the mid and posterior intestine. Nickel transport rate was similar to the other metals in the anterior intestine, but significantly higher than the other metals except lead in the mid intestine and posterior intestine (Fig. 2-3 B).

3.3. Binding of metals to the surface mucus in different segments of the gastrointestinal tract.

Metals collected on blotting paper and wash solutions represent metals that were loosely bound to surface mucus. Cadmium and lead were bound to the surface mucus to a greater extent at the mid-intestine compared to other segments of the gastrointestinal tract (Fig. 2-4 A). The binding rate for lead at mid-intestine (0.51 ± 0.08 nmol/cm^2/h) was about 4- fold higher than anterior intestine (0.14 ± 0.02 nmol/cm^2/h), about 2-fold higher than the posterior intestine (0.28 ± 0.03 nmol/cm^2/h) and about 10-fold higher than the

stomach (0.052 ± 0.01 nmol/cm^2/h). Similarly, for cadmium the binding rate at the mid-intestine (0.42 ± 0.18 nmol/cm^2/h) was about 5-fold higher than at the anterior intestine (0.08 ± 0.02 nmol/cm^2/h), about 3- fold higher than at the posterior intestine (0.13 ± 0.03 nmol/cm^2/h) and about 13- fold higher than at the stomach (0.032 ± 0.01 nmol/cm^2/h) (Fig. 2-4 A). At the stomach, lead, copper, silver and cadmium binding rate to the surface mucus were higher than other metals (Fig. 2-4B). At the anterior intestine, there was a statistically significant difference between lead and nickel, but lead and cadmium binding rates were almost the same as other metals (Fig. 2-4B). At the mid-intestine, lead and cadmium were bound to the surface mucus to a much greater extent (about 4-8 fold) over other metals. At the posterior intestine, lead was bound to the surface mucus by a significantly greater extent of about 2-5 fold over other metals (Fig. 2-4 B).

For copper, the binding rate to the surface mucus was about 2-fold higher at the anterior intestine (0.08 ± 0.02 nmol/cm^2/h) than at the stomach (0.04 ± 0.01 nmol/cm^2/h), but the binding rates were the same in all the three segments of the intestine (approximately 0.07 ± 0.01 nmol/cm^2/h) (Fig. 2-4 A). For zinc, the binding rate to the surface mucus at anterior and posterior intestine were higher than at the stomach, but binding rates were the same in all three segments of the intestine (Fig. 2-4 A). However, silver was bound to the surface mucus at lower rate at the stomach than at the anterior intestine but binding rate were approximately the same in all the 3 segments of the intestine (Fig. 2-4 A). Nickel binding rate was higher at the mid-intestine and posterior intestine than at the stomach and anterior intestine (Fig. 2-4 A).

3.4. Apical metal transport rates in different segments of the gastrointestinal tract: mucosal epithelium.

The calculated rate of accumulation of metals in the mucosal epithelium essentially represents a snapshot of the metal passing through this compartment and may provide an indication of the apical entry step if basolateral export is the rate-limiting step. Cadmium and lead had significantly higher accumulation rates in the mucosal epithelium at the mid-intestine compared to other segments of the gastrointestinal tract (Fig 2-5A). The accumulation rate in the mucosal epithelium for copper was about 4-fold higher at the mid-intestine (0.023 ± 0.01 nmol/cm^2/h) than at the stomach (0.006 ± 0.001 nmol/cm^2/h), but the accumulation rates at the anterior and posterior intestine were similar (approximately 0.016 ± 0.005 nmol/cm^2/h) (Fig. 2-5A). For zinc and silver, the accumulation rate in the mucosal epithelium in all the three segments of the intestine was higher than at the stomach (Fig. 2-5A). Stomach and anterior intestine accumulated less nickel than mid intestine, but accumulation rate at the mid and posterior intestine were the same (Fig. 2-5A).

When comparison among the metals at each segment was made, cadmium accumulated to a greater extent than other metals at the stomach (Fig. 2-5B). But at the anterior intestine, copper, zinc, silver and cadmium were accumulated to a significantly greater extent than lead and nickel in the mucosal epithelium. At the mid-intestine, cadmium was accumulated more than other metals in the mucosal epithelium. At the posterior intestine, the accumulation rates were the same among the metals, but there was a significant difference between cadmium and nickel (Fig. 2-5B). Nickel was consistently accumulated to the lowest extent of the six metals.

3.5. Partitioning among the three compartments for all six metals

Fig. 2-6 provides an overview of metal partitioning among the three measured compartments; note the differences in scale used for the various compartments. The term "absorptive part'' is used to refer to metal transported through the enterocytes into the muscle tissue and serosal layer (i.e. data of Fig. 2-3).

At the stomach, a greater proportion of copper, zinc, silver, cadmium, lead and nickel were bound to the surface mucus compartments than other compartments (Fig. 2-6). At the mucosal epithelium, there was a greater proportion of cadmium than copper, silver, zinc, lead and nickel (Fig. 2-6). At the absorptive part, copper, silver, cadmium, lead and nickel tended to show the same absorptive proportion, but zinc tended to have lower absorptive proportion (Fig. 2-6).

Copper, zinc, silver and cadmium exhibited almost the same pattern at the anterior, mid and posterior intestine, with their greater proportion of uptake into the absorptive part at the anterior intestine and lower, almost the same proportion, at the mid and posterior intestine (Fig. 2-6). However, lead and nickel showed different patterns with a greater proportion of lead transport rate at the mid–intestine, followed by posterior intestine and lowest at the anterior intestine (Fig. 2-6). For nickel, a greater proportion of the absorptive part was at the posterior intestine and almost the same at the mid-intestine and lowest at the anterior intestine (Fig. 2-6).

Copper, zinc, silver and cadmium accumulation rates at the mucosal epithelium demonstrated almost the same proportion at the anterior and posterior intestine, but at the mid intestine cadmium showed a greater proportion than copper, zinc and silver (Fig. 2-6). However, for lead, there appears to be slightly higher accumulation rate at the mid-

intestine compared to other parts of the intestine (Fig. 2-6). For nickel, there appears to be virtually no detectable mucosal accumulation rate in all the three segments of the intestine (Fig. 2-6).

At the surface mucus, all the metals were bound at almost the same rate at the anterior intestine (Fig. 2-6). At the mid- intestine, cadmium and lead were more bound to the surface mucus than all other metals (Fig. 2-6). At the posterior intestine, lead was more bound to the surface mucus than all other metals (Fig. 2-6).

4.0. Discussion

4.1. Fluid transport rates in the presence of all the metals via the gut.

The gut can play an important role in water absorption in fish (House and Green, 1965; Buddington et al., 1997). The anterior intestine tended to be the most active region of the gastrointestinal tract for the fluid transport rates and this was in line with previous studies (Bergman et al., 2003; Nadella et al., unpublished). Water absorption via the gut is accompanied by the absorption of Na^+, titratable base and Cl^-, and indeed may be driven by this ionic transport (Evans, 1993; Loretz, 1995; Bergman et al., 2003; Grosell et al., 2005). Therefore water absorption rates may provide a broad indicator of the integrity of ion transport processes.

There was no effect of any of the six metals at 50 $\mu mol.L^{-1}$ on the fluid transport rates at the intestine. This finding is in accord with the finding that copper uptake had no effect on water transport via the intestine of the African walking catfish (Handy et al., 2000), a conclusion that can now be extended to other metals at the intestine of rainbow trout (Handy et al., 2000). Moreover, copper absorption in the rat intestine that was related to

sodium did not have any effect on water transport (Wapnir and Steil, 1987). Therefore, it seems probable that at the concentration tested (50 μmol. L^{-1}), none of the metals were toxic to the transport mechanisms for Na^+, Cl^- and titratable base at the intestine. This conclusion can also applied at the stomach for all other metals except for copper.

4.2. Basolateral metal transport rates via the gastrointestinal tract: serosal fluid + muscle.

The highest absorptive region for metals via the gastrointestinal tract was as follows:

Copper = Anterior intestine > Mid-intestine > Posterior intestine ≥ Stomach

Zinc and silver = Anterior intestine > Mid-intestine ≥ Posterior intestine ≥ Stomach

Cadmium = Anterior intestine > Mid-intestine ≥ Posterior intestine > Stomach

Lead = Mid-intestine > Posterior intestine > Anterior intestine ≥ Stomach

Nickel = Posterior intestine ≥ Mid-intestine > Anterior intestine > Stomach

Overall, these findings agree with previous *in vivo* studies which showed that copper, zinc and cadmium are absorbed most at the anterior intestine compared to other parts of the gastrointestinal tract (Shears and Fletcher, 1983; Clearwater et al., 2002; Chowdhury et al., 2004). These results indicate that the four regions of the gastrointestinal tract might have different affinities for these metals or there might be regionalisation of carriers and cell types specific for each metal as suggested by Handy et al. (2000). This might also be explained at the molecular level, by knowing the specific type and number of transport proteins for metals and for amino acids (which might bind metals) present in each segment of the gastrointestinal tract.

For example, the possible basolateral exit mechanisms for copper through the gut of

fish can be through a Cu/anion symport that prefers Cl^- or through Cu-ATPase (Handy et al., 2000). The possible basolateral exit mechanism for cadmium and zinc can be through ZnT-1, since it is a possible candidate for the cadmium export mechanism (mammals: Palmiter and Findley, 1995) as well as for the zinc export mechanism (Cousins and McMahon, 2000). Moreover, anterior and midsections of the gut have been shown to be primary sites of amino acid uptake (Dabrowski and Dabrowska, 1981; Ash, 1985). In support of this, intestinal zinc uptake has been shown to occur in part through amino acid transport mechanisms involving Zn-histidine or Zn-cysteine complexes (Glover and Hogstrand, 2002a). Absorption via pinocytosis or proteins can also occur in the posterior regions of the intestine (Georgopoulou et al., 1985, 1986; Clearwater et al., unpublished). Essential metals (copper, zinc and nickel) were taken up at almost the same rate as non-essential metals (silver, cadmium and lead) at their highest absorptive region.

For the absorptive rates among the metals at each segment of the intestine, nickel exhibited the highest capacity in all four segments of the gastrointestinal tract. Hence, nickel exhibited the highest overall rate of net absorption of all metals via the gastrointestinal tract. This might indicate that nickel is diffusing through the gut epithelium via a non-specific pathway at the gut as it does at the gill (Pane et al., 2003). Similarly, nickel has been shown to be rapidly absorbed from the gastrointestinal tract of dogs and cats given $NiSO_4.6H_2O$ or $NiCl_2$ by the diet or by gavage (USPHS, 1993). Lead exhibited the capacity of being transported most at the mid and posterior-intestine (i.e. both segments). This might indicate a separate mechanism for lead uptake via the gastrointestinal tract. Copper, zinc, silver and cadmium exhibited the capacity of being transported most only at the anterior intestine. This might be suggesting a common

mechanism of uptake for copper and silver and zinc and cadmium via the gut because they have the same regional uptake and rates. Thus, the general transport trend for metals transport via the gut is Ni > Pb > Cu = Zn = Cd = Ag.

4.3. Binding of metals to the surface mucus via the gut.

At the stomach, there was a consistently greater or equal proportion of all the metals at the loosely surface mucus-binding fraction than at other compartments of the gastrointestinal tract (Fig. 2-6). This might indicate that the stomach has a more well developed mucus layer to accumulate metals than the intestine (Kapoor et al., 1975), which can facilitate or retard metals absorption. Lead exhibited the highest overall rate of surface mucus binding of all metals in all the four segments of the gastrointestinal tract. Cadmium and lead bound more to the surface mucus at the mid-intestine compared to the copper, zinc, silver and nickel. These differences might indicate that cadmium and lead have the capacity of increasing mucus cell activity when present in the gut (Crespo et al., 1986; Glover et al., 2003; Gardener and Yevich, 1970; Part and Lock, 1983). Nickel had the least capacity of all metals of being bound to the surface mucus. Hence, the general trend for metals binding to the mucus is Pb > Cd > Zn > Ag > Cu > Ni. Studies from Handy et al. (1999) have shown that mucus production can be an important defense mechanism that contributes to the effectiveness of the gut as a barrier to metal uptake. This might be true for cadmium but not for other metals (i.e. copper, zinc, nickel, silver and lead) as shown in (Fig. 2-7 and Fig. 2-8). In which there was a statistical significant correlation between net transport rate and mucus-binding for copper, zinc, nickel, silver and lead. This agrees with the studies from Part and Lock (1983);

Whitehead et al. (1996) and Glover and Hogstarnd (2002b) that showed that the ability of metal ion to reach the mucosal epithelium to depend on their mobility through the mucus layer.

4.4. Apical metal transport rates via the gut: mucosal epithelium.

Accumulation in the mucosal epithelium, can be thought of providing a snapshot of the metal "in transit'' through the enterocytes. Accumulation here will reflect the balance between the rate of apical entry and basolateral exit. Cadmium exhibited the highest overall rates of mucosal accumulation of all metals through the gastrointestinal tract. This might be due to the fact that it blocks the basolateral exit mechanism (Ca-ATPase) at the gut (Schoenmakers et al., 1992) similar to its action at the gill (Verbost et al., 1987; 1988; Wicklund –Glynn, 1996). Nickel exhibited the least overall rates of mucosal accumulation, but the highest net transport rate, suggesting that it moves rapidly through both surfaces (Fig. 2-9). However, the accumulation rates for all other metals (copper, zinc, silver and lead) at the mucosal epithelium were more or less the same. The general trend of mucosal accumulation of all metals is Cd > Cu = Zn = Ag = Pb > Ni. This study shows that there is statistical significance correlation between net transport rate (basolateral export) and mucosal epithelium (apical entry) for nickel, silver and lead uptake via the gastrointestinal tract (Fig. 2-9 and Fig. 2-10). Thus, there might be some well-controlled homeostatic (internal regulation or balance) mechanisms regulating uptake of other metals such as copper, zinc and cadmium beyond the apical entry step.

4.5. Comparison of metal uptake rates at the gut versus the gill.

At least for copper, the gut appears to be a low affinity transport system because of the large μM range of concentration (Nadella et al., unpublished) as compared to the gill which has very high affinity sites which function at the low concentrations typically present in water (Grosell and Wood, 2002; Playle, 2004). This was consistent to the present study, when uptake rate/concentration ratio was taken into consideration for higher concentrations (50:M) at the gut (Table 2-2) and lower concentrations at the gill (Table 2-4), (0.001 to 5:M range), using literature values for gill uptake rates of metals from freshwater by rainbow trout measured in comparable water quality (Table 2-3). This analysis clearly demonstrated that uptake rate at the gill was much higher than at the gut when normalized to concentration. This indicates that the gut is a low affinity absorptive pathway and the gill is a high affinity pathway. This knowledge is important for BLM sites.

4.6. Relative importance of different sections of the gastrointestinal tract in metal uptake

Uptake rate of each metals at different segments of the gastrointestinal tract were normalized to a common surface area for each segment as shown in (Table 2-1 representative of a 250g fish). These uptake rates (in nM/g/h) were as follows: for copper = 0.03, zinc = 0.04, cadmium = 0.03, silver = 0.025, lead = 0.04, nickel = 0.05. The average surface area at the stomach for Cu = 48.1, Zn = 48.1, Ag = 47.7, Cd = 46.75, Pb = 44 and Ni = 45, at the anterior intestine the average surface area for Cu = 29.25, Zn = 33.2, Ag = 30.5, Cd = 30.2, Pb = 44, Ni = 33, at the mid-intestine the average surface

area for Cu = 12, Zn = 11.4, Ag = 11.4, Cd = 12, Pb = 15 and Ni = 12.5 and at the posterior intestine the average surface area for Cu = 18, Zn = 21, Ag = 16.2, Cd = 14, Pb = 22 and Ni = 19.5. The calculations were done by multiplying uptake rates of each metal at different segments of the gastrointestinal tract as shown in Fig 2-3 (but not the average uptake rate) for each preparation by their individual surface areas. The average of each metal at each segment of the gastrointestinal tract was now calculated. This now showed the likely relative importance *in vivo* for each metal uptake at each segment of the gastrointestinal tract as shown in (Table 2-1). Tables 2-5; 2-6; 2-7; 2-8; 2-9 and 2-10 provide the detailed data on which this analysis is based.

5.0. The importance/ relevance of the results:

(i) These results show the relative importance of stomach (which has been ignored by previous workers) for copper, zinc, silver and cadmium uptake in which the uptake rates at the stomach were almost comparable to uptake rates at the mid and posterior intestine. For lead, the uptake rate at the stomach was comparable to the uptake rate at the anterior intestine while for nickel, uptake rate at the posterior intestine was higher than the mid intestine when the relative importance of surface area of each segment of gastrointestinal tract was taken into consideration (Table. 2-5; 2-6; 2-7; 2-8; 2-9; 2-10).

(ii) For absorptive rates at the intestine, copper and silver show similar patterns and values suggesting that they may be transported by a common mechanism. Since they share the same group in the periodic table, they have similar properties in the following areas: crystal field theory, ionic potential, ionic index, and covalent index (Walker et al., 2003). And at the gill they share a same common entry pathway through the sodium

channel (Bury and Wood, 1999; Grosell and Wood, 2002). Cadmium and zinc show similar patterns and rates again suggesting a common mechanism. Again, they share the same group in the periodic table and they share a common pathway at the gill through the calcium channel (Verbost et al., 1987; Hogstrand et al., 1996). Lead and zinc show similar rates and again they share a common pathway at the gill through the calcium channel (Rogers and Wood, 2004).

(iii) These results can be of environmental importance in that the uptake rates of all the metals via the gastrointestinal tract were almost the same when uptake rate to concentration ratio was taken into consideration. This finding is important for risk assessment because it means that the use of a single uptake rate for different metals at a particular concentration would not introduce a large error into exposure models.

(iv) This study shows better correlation between surface mucus-binding and rate of absorption for copper, zinc, nickel, silver and lead (Fig. 2-7 and Fig. 2-8). It also shows correlation between mucosal epithelium and rate of absorption for silver, lead and nickel (Fig. 2-9 and Fig. 2-10). These results will be important for the development of a BLM (Biotic Ligand Model) via the gastrointestinal tract of fish, such that surface binding of copper, zinc, nickel, silver and lead can be used as an index of uptake, and therefore potentially toxicity via the gastrointestinal tract, as commonly suggested for copper and lead in the gill BLM (Meyer et al., 1999; Di Toro et al., 2001).

Fig. 2-1. Diagram of the vertebrate gut wall (Clearwater et al., unpublished). The four principal layers of the gut wall consist of mucosa, submucosa, muscularis externa and serosa. The mucosa consists primarily of epithelial cells, lamina propria and muscularis mucosa. The muscularis externa consists mainly of circular and londitudinal muscle.

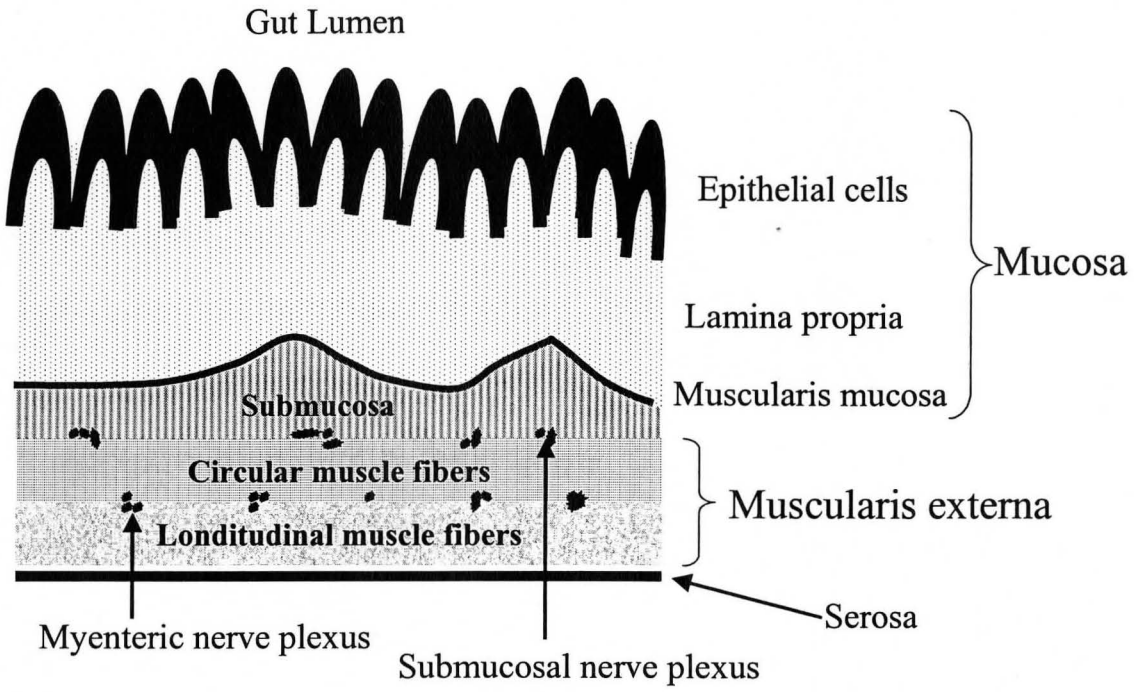

Gut Lumen
Epithelial cells
Lamina propria
Muscularis mucosa
Mucosa
Submucosa
Circular muscle fibers
Londitudinal muscle fibers
Muscularis externa
Serosa
Myenteric nerve plexus
Submucosal nerve plexus

Fig. 2-1

Fig. 2-2. Fluid transport rates in the presence of each of the metals separately (copper, zinc, silver, cadmium, lead and nickel at 50μM each) at the (i) stomach, (ii) anterior (iii) mid and, (iv) posterior intestine following an *in vitro* stomach and gut-sac technique for 4h at the stomach and 2h at the intestine. Plotted values represent the means (± S.E.M), of N = 10 for copper and cadmium, N = 9 for lead and N = 8 for zinc, silver, nickel at the stomach and N = 8 for all at the anterior, mid and posterior intestine. The significance level was tested at $P < 0.05$ using one-way ANOVA analysis with post-hoc LSD analysis. In panel A, a, b and c represent statistically significant differences in the fluid transport rates for each of the metals between the various segments. Means not sharing the same letter are significantly differed. Panel B shows the fluid transport rates in the absence (control) and presence of all the metals at the stomach, anterior, mid and posterior intestine. There were no significant differences associated with the presence of the metal within each segment, except for copper in the stomach.

Fluid Transport Rate

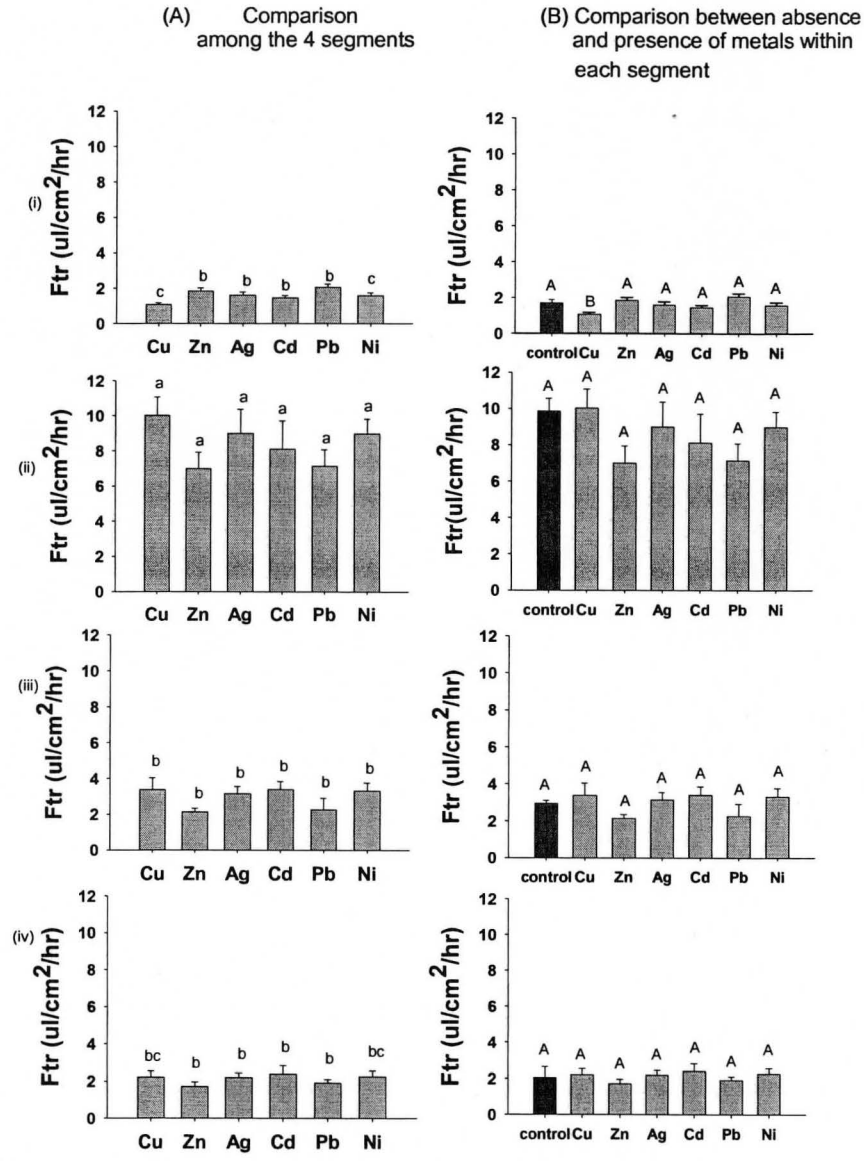


Fig. 2-2

Fig. 2-3. Rates of net absorption for each of the metals separately (copper, zinc, silver, cadmium, lead and nickel at 50μM each) into serosal fluid + muscle at the (i) stomach, (ii) anterior (iii) mid intestine, (iv) posterior intestine following an *in vitro* stomach and gut-sac technique for 4h at the stomach and for 2h at the intestine. Plotted values represent the means (± S.E.M) of N = 10 for copper and cadmium, N = 9 for lead and N = 8 for zinc, silver and nickel at the stomach and N = 8 for all at the anterior, mid and posterior intestine. The significance level was tested at $P < 0.05$ using one-way ANOVA analysis with post–hoc LSD analysis. At panel A, a, b and c represent significant differences between the various segments. At panel B, A and B represent significant differences between copper, zinc, silver, cadmium, nickel and lead absorption within each segment of the intestine. Means not sharing the same letter are significantly different.

Metal Transport Rates

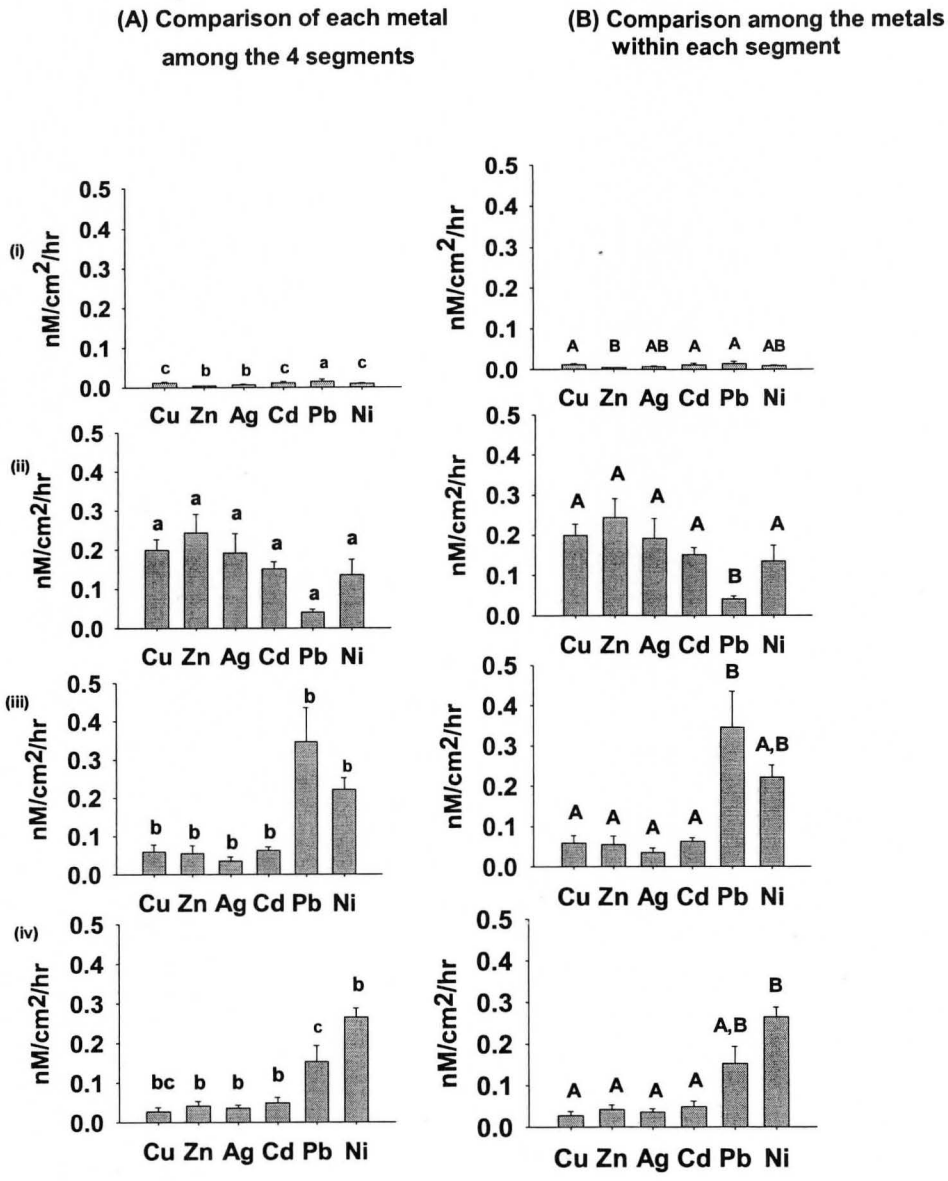


Fig. 2-3

Fig. 2-4. Rates of accumulation of each of the metals separately (copper, zinc, silver, cadmium, lead and nickel at 50μM each) in the loose surface bound fraction (blot plus rinse) at the (i) stomach (ii) anterior (iii) mid and (iv) posterior intestine, following an *in vitro* gut-sac technique for 4h at the stomach and 2h at the intestine. Plotted values represent the means (± S.E.M) of N = 10 for copper and cadmium, N = 9 for lead and N = 8 for zinc, silver and nickel at the stomach and N = 8 for all at the anterior, mid and posterior intestine. The significance levels were tested at $P < 0.05$ using one–way ANOVA with post-hoc LSD analysis. At panel A, a, b and c represent statistically significant differences between the various segments. At panel B, A and B represent significance difference between copper, zinc, silver, cadmium, lead and nickel accumulation within each segment of the intestine. Means not sharing the same letter are significantly different.

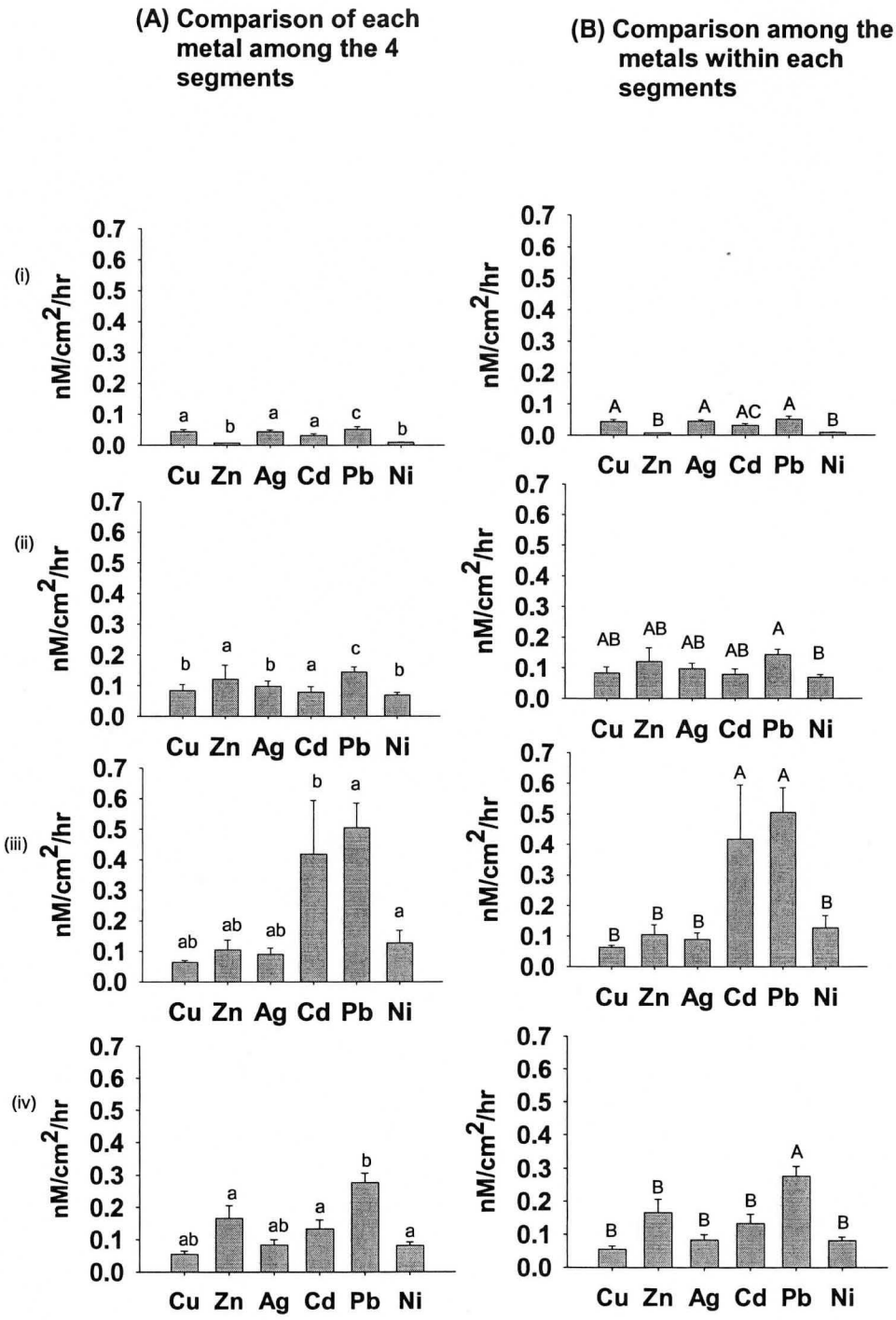

Mucus-binding
(A) Comparison of each metal among the 4 segments
(B) Comparison among the metals within each segments
(i)
(ii)
(iii)
(iv)
nM/cm2/hr
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a b a a c b
A B A AC A B
b a b a c b
AB AB AB AB A B
ab ab ab b a a
B B B A A B
ab a ab a b a
B B B B A B
Cu Zn Ag Cd Pb Ni

Fig. 2-4.

Fig. 2-5. Rates of accumulation of each of the metals separately (copper, zinc, silver, cadmium, lead and nickel at 50μM each) in the mucosal epithelium at the (i) stomach, (ii) anterior, (iii) mid and (iv) posterior intestine, following an *in vitro* stomach and gut-sac technique for 4h at the stomach and 2h at the intestine. Plotted values represent the means (± S.E.M) of N = 10 for copper and cadmium, N = 9 for lead and N = 8 for zinc, silver and nickel at the stomach and N = 8 for all at the anterior, mid and posterior intestine. The significance level was tested at $P < 0.05$ using one-way ANOVA analysis with post-hoc LSD analysis. At panel A, a, b and c represent significant differences between the various segments. At panel B, A and B represent significant differences between copper, zinc, silver, cadmium, lead and nickel accumulation within each segment of the intestine. Means not sharing the same letter are significantly different.

Accumulation in the Mucosal Epithelium

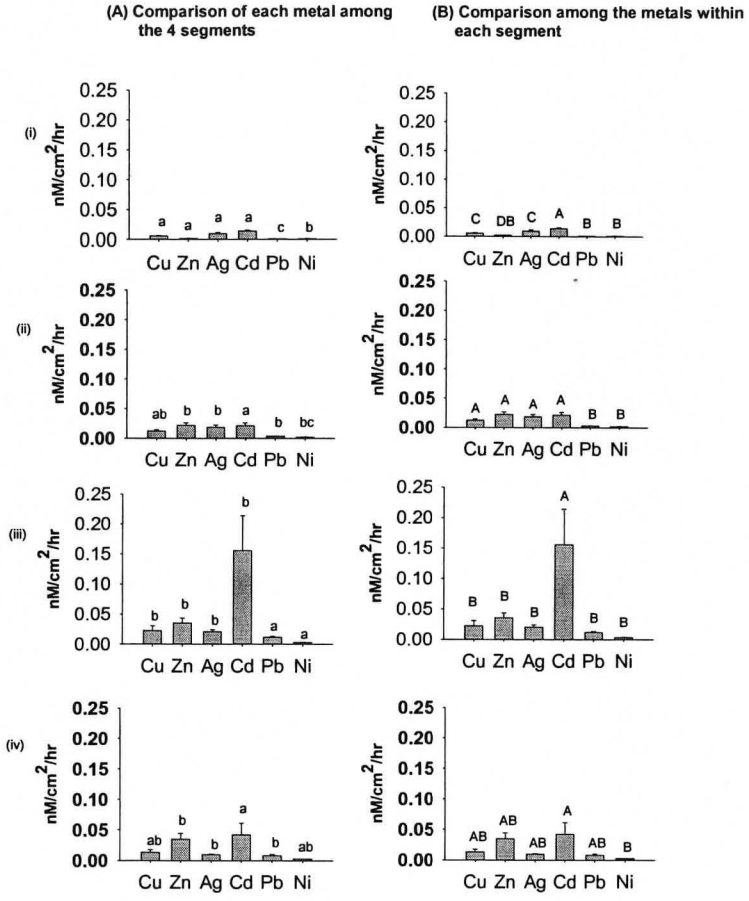
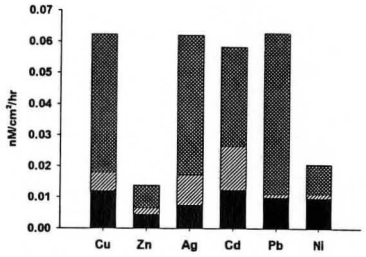


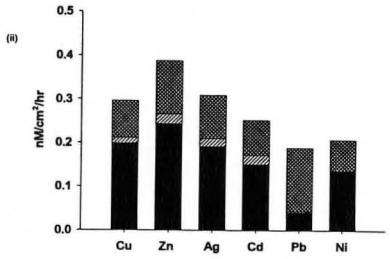
Fig.2-5

Fig. 2-6. Partitioning among the three compartments (absorptive part, mucosal epithelium and surface mucus binding) for all the six metals via the gastrointestinal tract at the (i) stomach (ii) anterior (iii) mid intestine and (iv) posterior intestine. At the stomach, N = 10 for copper and cadmium, N = 9 for lead and N = 8 for zinc, silver and nickel. At the anterior, mid and posterior intestine N = 8 for all the metals.

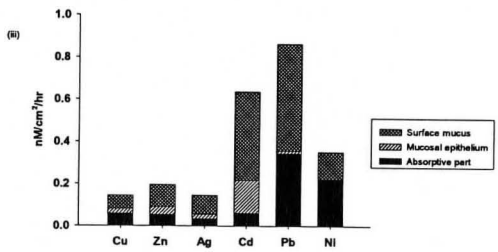
Stomach



Anterior intestine



Mid-intestine



Posterior intestine

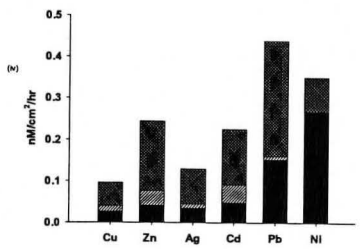
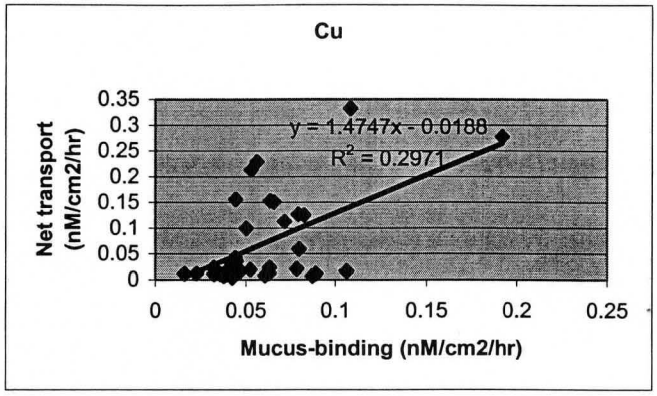
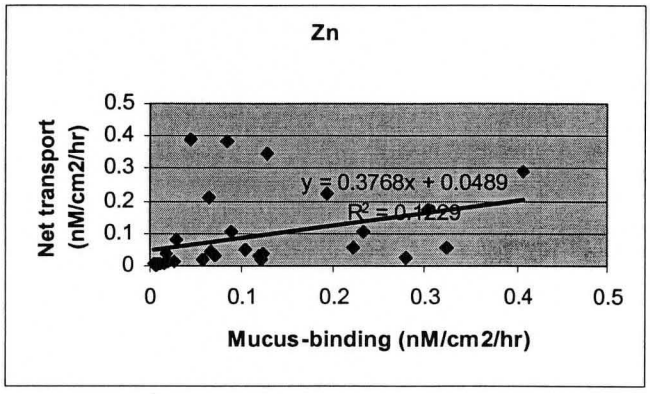


Fig. 2-6

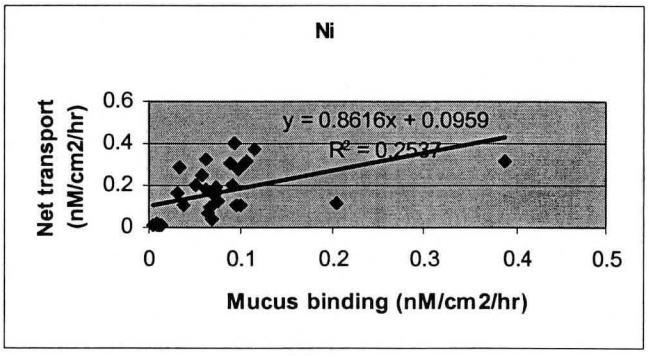
Fig. 2-7. Linear regression relationships between net transport rates and mucus-binding for essential metals (i.e. copper, zinc and nickel) via the gastrointestinal tract of trout. For copper $N = 34$, for zinc and nickel $N = 32$ in all four segments of the gastrointestinal tract. The equation of the regression line and the significance of the correlation (unidirectional probability, $P < 0.05$) is reported for each metal relationship. The significance level was assessed using Vassar stat.



Significance level for copper = 0.0004



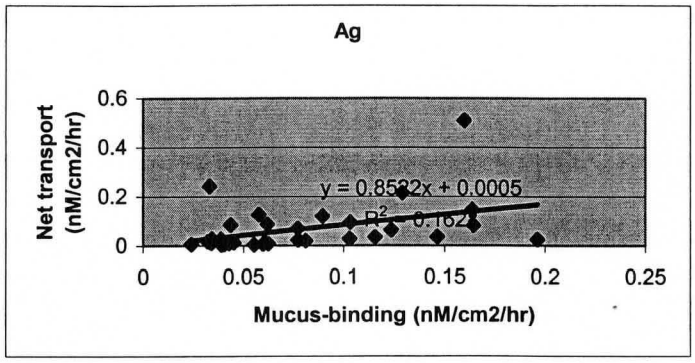
Significance level for zinc = 0.025



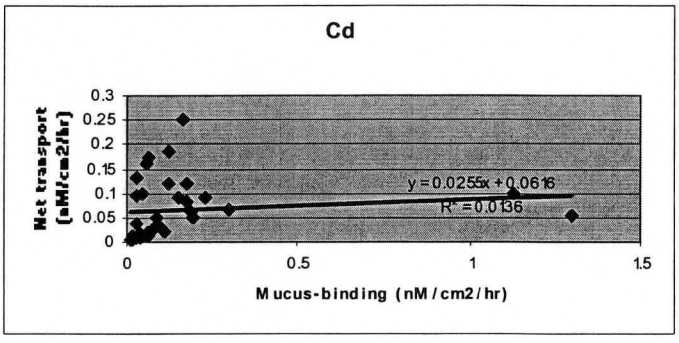
Significance level for nickel = 0.002

Fig. 2-7.

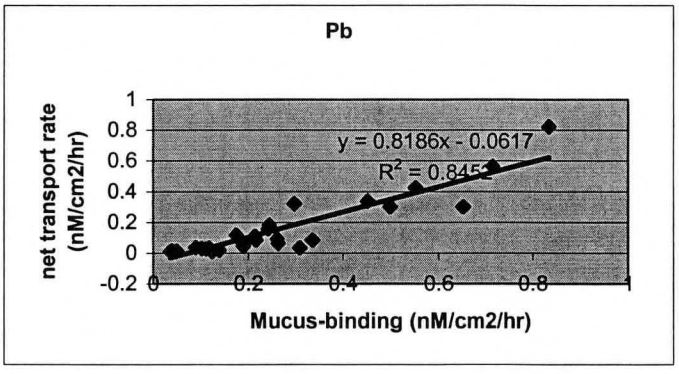
Fig. 2-8. Linear regression relationships between net transport rates and mucus-binding for non-essential metals (silver, cadmium and lead) via the gastrointestinal tract of trout. For silver N = 32, for cadmium N = 34 and for lead N = 33 in all four segments of the gastrointestinal tract. The equation of the regression line and the significance of the correlation (i.e. unidirectional probability, $P < 0.05$) is reported for each metal relationship. The significance level was assessed using Vassar stat.



Significance level of silver = 0.011



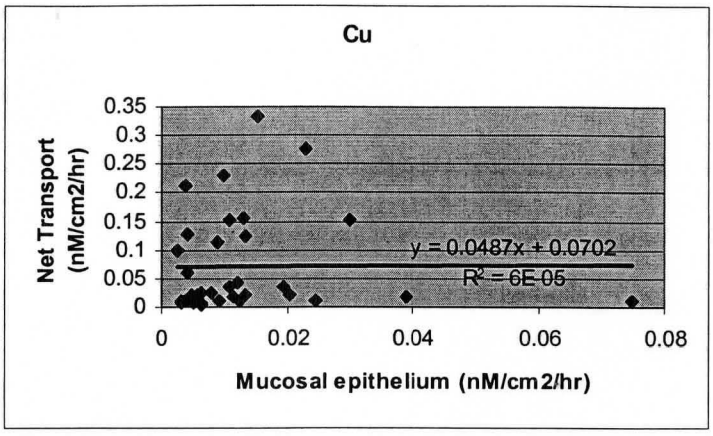
Significance level of cadmium = 0.26



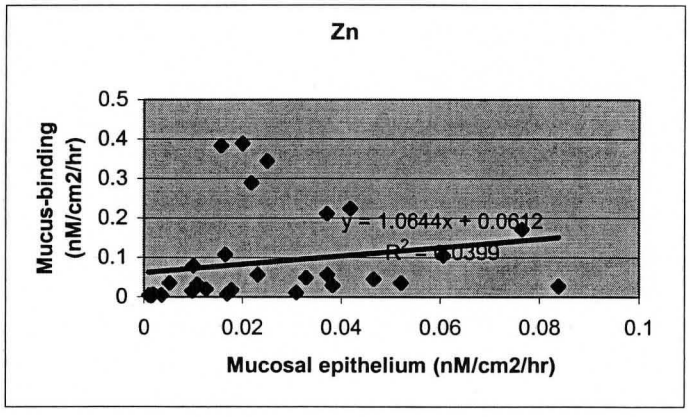
Significance level for lead = <0.001

Fig. 2-8

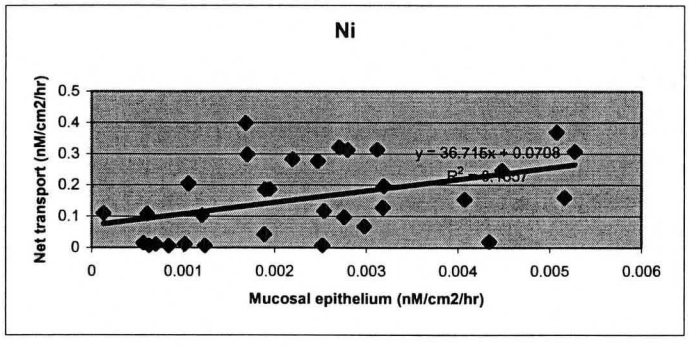
Fig. 2-9. Linear regression relationships between net transport rates and mucosal epithelium for essential metals (i.e. copper, zinc and nickel) via the gastrointestinal tract of trout. For copper $N = 34$, for zinc and nickel $N = 32$ in all four segments of the gastrointestinal tract. The equation of the regression line and the significance of the correlation (unidirectional probability, $P < 0.05$) is reported for each metal relationship. The significance level was assessed using Vassar stat.



Significance level for copper = 0.484



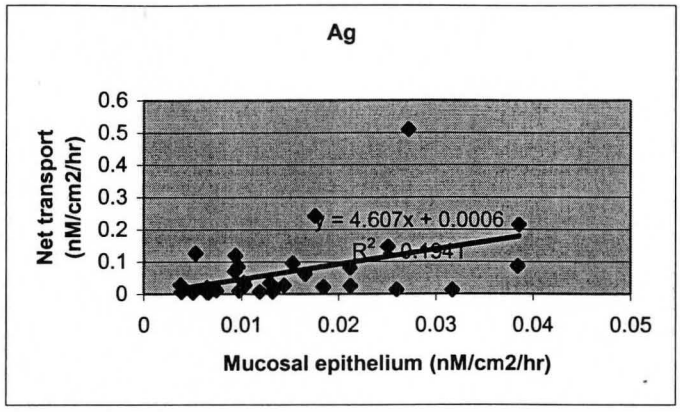
Significance level for zinc = 0.136



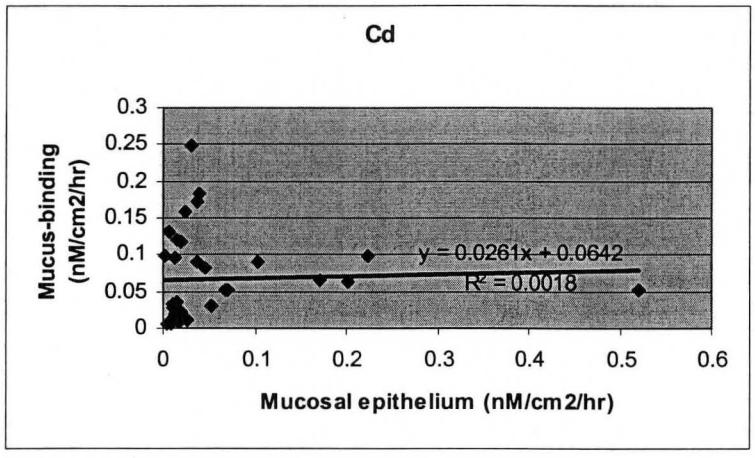
Significance level for nickel = 0.035

Fig. 2-9

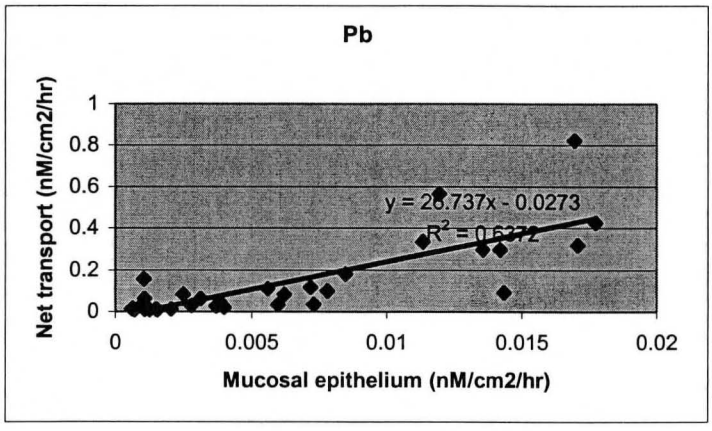
Fig. 2-10. Linear regression relationships between net transport rates and mucosal epithelium for non- essential metals (silver, cadmium and lead) via gastrointestinal tract of trout. For silver N = 32, for cadmium N = 34 and for lead N = 33 in all four segments of the gastrointestinal tract. The equation of the regression line and the significance of the correlation (unidirectional probability $P < 0.05$) is reported for each metal relationship. The significance level was assessed using Vassar stat.



The significance level for silver = 0.006



The significance level for cadmium = 0.401



The significance level for lead = < 0.0001

Fig. 2-10.

Table. 2-1: Unidirectional metals uptake rate in intestinal segments of rainbow trout at the serosal fluid + muscle

Region	Copper nM/h	Zinc nM/h	Cadmium nM/h	Silver nM/h	Lead nM/h	Nickel nM/h
Stomach	0.56	0.21	0.55	0.38	0.44	0.44
Anterior intestine	5.36	7.92	4.70	4.89	1.77	4.0
Mid intestine	0.57	0.57	0.75	0.33	4.74	2.68
Posterior intestine	0.42	0.90	0.67	0.55	3.19	5.26
Total in all the segments	6.91 nM/h	9.6 nM/h	6.67 nM/h	6.15 nM/h	10.14 nM/h	12.38nM/h
Uptake in 250g fish	0.03 nM/g/h	0.04 nM/g/h	0.03 nM/g/h	0.025 nM/g/h	0.04 nM/g/h	0.05 nM/g/h

Table 2-2: Metals uptake rate/concentration ratio via the intestinal segment of rainbow trout

Metal	Uptake rate nM/g/h	Concentration μM	Uptake rate/concentration (m/g/h)
Copper	0.030	50	0.0006
Zinc	0.040	50	0.0008
Cadmium	0.030	50	0.0006
Silver	0.025	50	0.0005
Lead	0.040	50	0.0008
Nickel	0.050	50	0.0010

Table. 2-3: Literature values for metal uptake rates via the gills of rainbow trout under comparable water quality conditions.

Metals	Concentration	Uptake rates	References
Copper	0.04 μM	0.002 nM/g/h	Kamunde et al. (2002)
Zinc	2.3 μM	0.41 nM/g/h	Hogstrand et al. (1998)
Silver	0.02 μM	0.005 nM/g/ h	Morgan et al. (2004)
Cadmium	0.001 μM	0.012 nM/g/h	Hollis et al. (2000)
Lead	5 μM	0.05 nM/g/h	Rogers et al. (2004)
Nickel	0.07 μM	0.014 nM/g/h	Pane et al. (2003)

Table 2.4: Metals uptake rate/concentration ratio at the gill of rainbow trout

Metal	Uptake rate nM/g/h	Concentration μM	Uptake rate/concentration (m/g/h)
Copper	0.002	0.040	0.05
Zinc	0.410	2.300	0.18
Silver	0.005	0.020	0.25
Cadmium	0.012	0.001	12.00
Lead	0.050	5.000	0.01
Nickel	0.014	0.070	0.20

Sample calculations about relative importance of different segments of the gastrointestinal tract

Table 2-5: Copper

Region	Uptake rate (nM/cm^2/h)	Uptake rate (nM/h)
Stomach (N = 10)	0.012 ± 0.002	0.56 ± 0.085
Anterior intestine (N = 8)	0.20 ± 0.03	5.36 ± 0.33
Mid-intestine (N = 8)	0.06 ± 0.02	0.57 ± 0.14
Posterior intestine (N = 8)	0.03 ± 0.01	0.42 ± 0.12

Table 2-6: Zinc

Region	Uptake rate (nM/cm^2/h)	Uptake rate (nM/h)
Stomach (N = 8)	0.004 ± 0.0005	0.21 ± 0.02
Anterior intestine (N = 8)	0.24 ± 0.05	7.92 ± 1.43
Mid-intestine (N = 8)	0.06 ± 0.02	0.57 ± 0.18
Posterior intestine (N = 8)	0.042 ± 0.01	0.90 ± 0.26

Table 2-7: Silver

Regions	Uptake rate (nM/cm^2/h)	Uptake rate (nM/h)
Stomach (N = 8)	0.01 ± 0.001	0.38 ± 0.058
Anterior intestine (N = 8)	0.2 ± 0.05	4.89 ± 0.63
Mid intestine (N = 8)	0.04 ± 0.01	0.33 ± 0.08
Posterior intestine (N = 8)	0.04 ± 0.01	0.55 ± 0.10

Table 2-8: Cadmium

Regions	Uptake rate (nM/cm^2/h)	Uptake rate (nM/h)
Stomach (N = 10)	0.012 ± 0.003	0.55 ± 0.11
Anterior intestine (N = 8)	0.15 ± 0.02	4.70 ± 0.91
Mid intestine (N = 8)	0.06 ± 0.01	0.75 ± 0.11
Posterior intestine (N = 8)	0.05 ± 0.013	0.67 ± 0.19

Table 2.9: Lead

Region	Uptake rate (nM/cm^2/h)	Uptake rate (nM/h)
Stomach (N = 9)	0.02 ± 0.001	0.44 ± 0.04
Anterior intestine (N = 8)	0.04 ± 0.007	1.77 ± 0.33
Mid intestine (N = 8)	0.35 ± 0.09	4.74 ± 1.16
Posterior intestine (N = 8)	0.15 ± 0.04	3.19 ± 0.75

Table 2-10: Nickel

Region	Uptake rate (nM/cm^2/h)	Uptake rate (nM/h)
Stomach (N = 8)	0.01 ± 0.002	0.44 ± 0.072
Anterior intestine (N = 8)	0.14 ± 0.04	4.0 ± 0.81
Mid intestine (N = 8)	0.22 ± 0.03	2.68 ± 0.38
Posterior intestine (N = 8)	0.27 ± 0.02	5.26 ± 0.66

Chapter 3: Study 2

Metal Interactions via the Gastro-intestinal Tract of Rainbow Trout (*Oncorhynchus mykiss*)

Abstract

Interactions were investigated among essential and non-essential metals and between essential metals for uptake via the gastrointestinal tract. There was an antagonistic effect of calcium on cadmium uptake at the stomach but not at the intestine, which indicates that there is some cadmium transport at the stomach but not at the intestine that is sensitive to calcium transport. There was a synergistic effect of calcium on zinc transport at the stomach, in contrast to no interaction at the intestine, indicating that zinc and calcium interaction was not exerted at the level of a calcium channel in any of the segments. There was an antagonistic effect of zinc on cadmium uptake and vice-versa at the apical entry step at the intestine in contrast to the stomach where there was no interaction, indicating that uptake of cadmium can be through DMT1. There was a synergistic effect of copper on zinc uptake and vice-versa at the apical entry step in the stomach, indicating that zinc and copper uptake can be through hZip 1 at the stomach. In contrast, to the stomach, there appears to be only an antagonistic effect of zinc on copper uptake at the apical entry-step at the intestine, indicating that uptake of copper might be through DMT1 but via a different mechanism for zinc. Competitive interaction between copper and zinc at the basolateral side can be explained based on competition for cellular

protein such as metallothionein. These results can be of nutritional and toxicological importance, and they can be used to develop a BLM at the intestinal tract of fish.

1.0. Introduction

The results of the previous chapter demonstrated that essential metals such as copper and zinc were absorbed at almost the same rate as non-essential metals such as cadmium at their highest absorptive regions in the trout gastrointestinal tract, and that these three metals in particular had the same highest absorptive regions at the anterior intestine. Furthermore, transport rates at the stomach, mid and posterior intestine were nearly similar for copper, zinc and cadmium when relative importance of surface area was taken into consideration. Notably, the uptake rates at the stomach, which had been previously overlooked as a potential site of metal transport were small but significant. This now led me to investigate possible interactions between these metals via all sections of the gastrointestinal tract.

Interactions between metals taken up through the gastrointestinal tract might help gain insight into nutritional requirements and transport mechanisms of metals in fish. Interactions between metals can result in additive, antagonistic or synergistic effects (Newman and Unger, 2003) upon lumen to tissue metal movement. Recently, Playle (2004) has developed multiple-metal modeling at the gill, using the classic toxic unit concept and a single binding site model. This approach might also help to develop the BLM at the intestinal tract of fish, once the natures of the sites have been worked out.

Verbost et al. (1987, 1988 and 1989) have shown interactions between calcium and cadmium transport through the gill at the level of chloride cell. At the intestinal tract of fish, an *in vivo* dietary study from Franklin et al. 2005 (in press) showed that dietary calcium inhibited dietary cadmium accumulation in the tissue of the stomach, but not at the intestine. This suggests that the stomach, but not the intestine, may behave like the

gill with respect to cadmium versus calcium interactions. It also indicates that the stomach, which has been largely ignored as a site of metal uptake until now, may be an important site for metal interactions.

For zinc, apical zinc uptake at the gill epithelium in rainbow trout has also been shown to be through a calcium channel (Hogstrand et al., 1996b). Bentley (1992) suggested that the hypocalcemic effects of zinc and calcium indicate that the two ions share, at least partially, a common uptake pathway via the chloride cell in the gills. Wicklund-Glynn (2001) reported that waterborne zinc competitively inhibits apical cadmium uptake through the gill in zebrafish (*Danio rerio*). Foulkes (1985) also suggested a similar mechanism for the inhibitory interaction between zinc and cadmium in rat jejunum. However, this might not be the case in the intestine of rainbow trout, in which there was lack of inhibitory action of lanthanum and cadmium on zinc movement through the apical membrane of intestinal epithelia in rainbow trout examined using right-side-out brush border membrane vesicles (BBMV's) (Glover et al., 2004). However, Shears and Fletcher (1983) using an *in situ* bolus technique showed that cadmium, as well as copper, cobalt, chromium, nickel, iron, manganese and mercury all inhibited intestinal zinc uptake in winter flounder (*Pseudoplueronectes americanus*). Competitive interaction between zinc and copper was explained based on competition for cellular (cytosolic) protein, probably metallothionein (Shears and Fletcher, 1979). Dietary studies from Knox et al. (1984) have shown antagonistic effects between copper and zinc in trout. Moreover, an *in vitro* study in rat small intestine has shown that copper significantly reduces the influx of zinc, in a concentration-dependent manner (Condomina et al., 2002). In mammals, high zinc content has been shown to cause reduction in copper uptake in the

mucosal cells (Cousins, 1985). However, zinc and copper interactions at the stomach have not been documented in fish.

The results of the previous Chapter also indicated that there was no significant correlation between mucosal epithelium accumulation (i.e. apical entry step) and rate of absorption (i.e. basolateral export step) for copper, zinc and cadmium via the gut. One possible interpretation is that there might be different transporters involved in uptake of these metal at the apical surface versus the basolateral export side as also suggested by Clearwater et al. (unpublished).

In mammals, several transporters such as the DMT1 (Divalent Metal Transporter 1), ZIP (Zinc Importer Protein) and CDF (Cation Diffusion Facilitator) families have been implicated in metal transport (Bury et al., 2003). ZIP transporters (such as hZIP 1, hZIP 2 and hZTL) and DMT1 can be involved in apical metal transport across the intestinal epithelium or through other cells (Gunshin et al., 1997; Gaither and Eide, 2000 and 2001b; Elisma and Jumarie, 2001; Cragg et al., 2002). One of the major pharmacological characteristics for the DMT1 transporter (which has broad specificity for many metals) is the competitive interaction between the metals (Gunshin et al., 1997). CDF (such as ZnT-1) can be involved in the basolateral transport of zinc into the blood (Cousins and McMahon, 2000; Gaither and Eide, 2001). This is also supported from the findings from the mammalian literature that there might be interactions between dietary metals at the level of brush border membrane transport (Bertolo et al., 2001), independent of interactions at the lumen with other nutrients. Metal interactions could also be at the level of cellular binding protein such as metallothionein, which can play both nutritional

and toxicological roles and has been shown to have a strong affinity and capacity for zinc (Hogstrand and Wood, 1996).

Based on this rather unclear situation in the present literature, the focus of this study, is to use an *in vitro* stomach and gut sac techniques to investigate:

(i) The effect of calcium on cadmium and zinc uptake via the gastrointestinal tract to see whether there will be antagonistic effects of calcium on cadmium and zinc uptake via the gastrointestinal tract as compared to the gill.

(ii) The possible interaction between an essential metal (zinc) and a non-essential metal (cadmium) via the gastrointestinal tract to see whether there will be competition between zinc and cadmium uptake via the gastrointestinal tract as compared to the gill.

(iii) The possible interaction between two essential metals (zinc and copper) via the gastrointestinal tract, to see whether there will be competitive or synergistic interaction between zinc and copper uptake via the gastrointestinal tract.

2. 0. Materials and Methods.

2. 1. Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*; N = 183 for all the experiments), approximately 250g (30cm) in size, were obtained from Humber Springs Fish Hatchery (Orangeville, ON). Fish were maintained in a 500L tanks with flowing aerated, and dechlorinated

Hamilton city tap water. Water temperature was maintained between 11-13°C. Fish were starved for three days prior to the experiment.

2. 2. Experimental Technique.

An in vitro gut sac technique: *In vitro* stomach and gut sac techniques were employed using the methods described in Chapter 2.

2.3. Experimental Protocol.

2.3.1. Effect of calcium on cadmium and zinc uptake.

The gut fluid of the freshwater rainbow trout has been shown to contain 2mM calcium, under starved conditions (Shehadeh and Gordon, 1969) while Baldisserotto et al. (2005) have also shown that the calcium concentration in the supernatant chyme of fed fish is about 3mM. The background calcium concentration in the basic Cortland saline used in the present study was 1mM. In light of these data, 10-fold and 100-fold (i.e.10mM and 100mM) increases in calcium concentration were chosen to test their effects on cadmium uptake and 100mM calcium for possible effects on zinc uptake via the gastrointestinal tract.

(i) Effect of 10mM $CaSO_4$ on cadmium uptake in sulphate-based saline.

Preparation of luminal saline containing cadmium with 10mM $CaSO_4$: Modified Cortland saline ($mmol.l^{-1}$): Na_2SO_4, 60.5; K_2SO_4, 2.5; $CaSO_4$, 10; $MgSO_4.7H_2O$, 1.9; $NaHCO_3$, 1.9; $NaH_2PO_4.H_2O$, 2.9; Glucose, 5.5. pH 7.4. Osmolality of the saline was

272 mOsm. 7.5μci of radioactive $^{109}CdCl_2$ was added to a 50μM solution of $CdNO_3.H_2O$ in the saline and the pH was 7.4.

Preparation of serosal saline and control luminal saline: Modified Cortland saline (mmol.l^{-1}): Na_2SO_4, 66.5; K_2SO_4, 2.5; $CaSO_4$, 1; $MgSO_4.7H_2O$, 1.9; $NaHCO_3$, 1.9; $NaH_2PO_4.H_2O$, 2.9; Glucose, 5.5 pH 7.4. Osmolality was adjusted to 272 mOsm by adding mannitol. This also served as the composition of luminal saline for the control experiment (cadmium alone) with 7.5μci of radioactive $^{109}CdCl_2$ added to 50μM solution of $CdNO_3.H_2O$ in the saline with pH 7.4.

(ii) Effect of 10mM Ca $(NO_3)_2$ on cadmium uptake in chloride-based saline.

I changed from sulphate-based saline to chloride-based saline so as to test the effect of calcium nitrate on cadmium uptake at higher calcium concentration (100mM) because calcium sulphate was not soluble at higher concentration (100mM). In order to test if this will cause any change in the result, I repeated the same experiment in chloride-based saline.

Preparation of luminal saline containing cadmium with 10mM $CaNO_3$: Cortland saline (mmol.l^{-1}): NaCl, 133; KCl, 5; Ca $(NO_3)_2$. $4H_2O$, 10; $MgSO_4.7H_2O$, 1.9; Glucose, 5.5. pH 7.4. There was no $NaHCO_3$ and $NaH_2PO_4.H_2O$ in the saline because they were precipitating with $Ca(NO_3)_2$ at higher concentration in the saline. Osmolality of the saline was 297 mOsm. 7.5μci of radioactive $^{109}CdCl_2$ was added to a 50μM solution of $CdNO_3.H_2O$ in the saline and the pH was 7.4.

Preparation of serosal saline and control luminal saline: Cortland saline (mmol.l^{-1}): NaCl, 133; KCl, 5; Ca $(NO_3)_2$. $4H_2O$, 1; $MgSO_4.7H_2O$, 1.9; Glucose, 5.5 pH 7.4. Osmolality was adjusted to 297 mOsm by adding mannitol. This also served as the

composition of luminal saline for the control experiment (cadmium alone) with 7.5μci of radioactive $^{109}CdCl_2$ added to 50μM solution of $CdNO_3.H_2O$ in the saline with pH 7.4.

(iii) Effect of 100mM $Ca(NO_3)_2$ on cadmium and zinc uptake in chloride-based saline.

Preparation of luminal saline containing cadmium and zinc with 100mM $CaNO_3$: Cortland saline (mmol.l^{-1}): NaCl, 133; KCl, 5; $Ca(NO_3)_2$. $4H_2O$, 100; $MgSO_4.7H_2O$, 1.9; Glucose, 5.5 pH. 7.4. Osmolality of the saline was 490 mOsm. 7.5μci of radioactive $^{109}CdCl_2$ or $^{65}ZnCl_2$ was added to a 50μM solution of $CdNO_3.H_2O$ or $ZnSO_4.H_2O$ respectively in the saline and the pH was 7.4.

Preparation of serosal saline and control luminal saline: Cortland saline (mmol.l^{-1}): NaCl, 133; KCl, 5; Ca $(NO_3)_2$. $4H_2O$, 1; $MgSO_4.7H_2O$, 1.9; Glucose, 5.5 pH 7.4. Osmolality was adjusted to 490 mOsm by adding mannitol to balance the osmotic pressure on the sides (i.e. luminal side and serosal side). This also served as the composition of luminal saline for the control experiment (cadmium alone or zinc alone) with 7.5μci of radioactive $^{109}CdCl_2$ or $^{65}ZnCl_2$ added to a 50μM solution of $CdNO_3.H_2O$ or $ZnSO_4.H_2O$ respectively in the saline. The pH was 7.4.

2.3.2. Interactions between cadmium and zinc in chloride-based saline

Wicklund Glynn (2001) had tested the effect of 2, 4 and 16μM of zinc on 30nM of cadmium uptake through the apical membrane of the gill epithelium in zebra fish (*Danio rerio*). Therefore, in his trials, the zinc concentration was in about 66.7, 133.3 and 530-fold molar excess of the cadmium concentration. Based on this, 10mM of zinc or cadmium were chosen to test their effects on either 50μM cadmium or 50μM zinc uptake

respectively. Therefore zinc or cadmium were in about 200-fold molar excess of the respective cadmium or zinc concentrations.

Preparation of luminal saline containing zinc or cadmium: Cortland saline ($mmol.l^{-1}$): NaCl, 133; KCl, 5; $Ca(NO_3)_2.4H_2O$, 1; $MgSO_4.7H_2O$, 1.9; Glucose, 5.5 pH 7.4. Osmolality of the saline was 268 mOsm. 7.5μci of radioactive $^{109}CdCl_2$ or $^{65}ZnCl_2$ was added to a 50μM solution of $CdNO_3.H_2O$ or $ZnSO_4.H_2O$ respectively in the saline and the pH was 7.4. When cadmium uptake was being measured, 10mM $ZnSO_4.H_2O$ was present. When zinc uptake was being measured, 10mM $CdNO_3.H_2O$ was present.

Preparation of serosal saline and control luminal saline: Cortland saline ($mmol.l^{-1}$): NaCl, 133; KCl, 5; $Ca(NO_3)_2.4H_2O$, 1; $MgSO_4.7H_2O$, 1.9; Glucose, 5.5 pH 7.4. Osmolality of the saline was 268 mOsm. This also served as the composition of luminal saline for the control experiment (cadmium alone or zinc alone) with 7.5μci of radioactive $^{109}CdCl_2$ or $^{65}ZnCl_2$ added to a 50μM solution of $CdNO_3.H_2O$ or $ZnSO_4.H_2O$ respectively in the saline and the pH was 7.4.

2.3.3. Interactions between copper and zinc in sulphate-based saline

Nadella et al. (unpublished) have recently shown that 500μM zinc inhibits 50μM copper uptake at the trout intestine. Based on this observation, interactions between 50μM zinc and 500μM copper, and *vice-versa* were tested via the gastrointestinal tract.

Preparation of luminal saline containing zinc and copper: Cortland saline ($mmol.l^{-1}$): Na_2SO_4, 66.5; K_2SO_4, 2.5; $CaSO_4$, 1; $MgSO_4.7H_2O$, 1.9; $NaHCO_3$, 1.9; $NaH_2PO_4.H_2O$, 2.9; Glucose, 5.5. pH 7.4. Osmolality of the saline was adjusted to 276 mOsm by adding mannitol. 7.5μci of a radioactive $^{65}ZnCl_2$ with 500μM salts of $CuSO_4.5H_2O$ was added to

a 50μM solution of $ZnSO_4.H_2O$ in the saline and the pH was 7.4. Effects of 500μM zinc on 50μM radioactive ^{64}Cu were similarly tested.

Preparation of serosal saline and control saline: Cortland saline ($mmol.l^{-1}$): Na_2SO_4, 66.5; K_2SO_4, 2.5; $CaSO_4$, 1; $MgSO_4.7H_2O$, 1.9; $NaHCO_3$, 1.9; $NaH_2PO_4.H_2O$, 2.9; Glucose, 5.5. pH 7.4. Osmolality of the saline was adjusted to 276 mOsm by adding mannitol.

^{64}Cu was prepared in the McMaster Nuclear Reactor exactly as in Chapter 2.

2. 4. Sample calculations

The same calculation procedures as outlined in Chapter 2 were employed for cadmium, copper and zinc uptake rates via the gastrointestinal tract.

2.5. Statistical Analysis

Plotted values represent the means (± SEM) with appropriate N values. At the stomach and intestine, the difference between control and experimental means was assessed at significance level of $P < 0.05$ using simple unpaired t-tests.

3.0. Results

In all the experiments in this Chapter 3, rinse (i.e. mucus-binding) data have not been reported because there were negligible interactions of metals on mucus-binding, while substantial effects were seen on transport into the serosal fluid + muscle and mucosal epithelium compartments.

3.1. Effects of calcium on cadmium and zinc uptake.

The presence of 10mM calcium in the luminal saline significantly inhibited 50μM cadmium uptake rate into the serosal fluid + muscle of the stomach by 60% with the rate falling to 0.005 ± 0.001 nmol/cm^2/h from the control value of 0.012 ± 0.003 nmol/cm^2/h. A similar inhibitory action on uptake into the mucosal epithelium in the stomach was seen with the rate falling to 0.005 ± 0.001 nmol/cm^2/h from the control value of 0.014 ± 0.002 nmol/cm^2/h (Figs. 3-1 A and B). However, in marked contrast to the stomach, there was no inhibitory effect of 10mM calcium in the luminal saline on 50μM cadmium uptake rates into serosal fluid + muscle and mucosal epithelium at the anterior, mid or posterior intestine in sulphate-based saline (Fig. 3-2 A and B).

Changing from sulphate-based to chloride-based saline, to further test the effect of 10mM and 100mM calcium in the luminal saline on 50μM cadmium uptake rates at the anterior, mid and posterior intestine again demonstrated no inhibitory effect on cadmium uptake rates into serosal fluid + muscle and mucosal epithelium (Figs. 3-3 A and B; Fig. 3-4 A and B).

There was an insignificant stimulatory effect of 100mM calcium in the luminal saline on zinc uptake into serosal fluid + muscle by about 3- fold from control value of 0.02 ± 0.01 nmol/cm^2/h to 0.06 ± 0.02 nmol/cm^2/h (Fig. 3-5A) and a significant stimulatory effect at the mucosal epithelium by 3-fold from control value of 0.003 ± 0.002 nmol/cm^2/h to 0.01 ± 0.003 nmol/cm^2/h at the stomach (Fig. 3-5B). In contrast, there was no effect of 100mM calcium in the luminal saline on zinc uptake rates (50μM) into serosal fluid + muscle (Fig. 3-6A) and mucosal epithelium (Fig. 3-6B) at the anterior, mid and posterior intestine in chloride-based saline.

3.2. Interactions between non-essential and essential metal via the gastrointestinal tract: cadmium and zinc

Interactions between zinc and cadmium were tested in a reciprocal-manner. At the stomach there was no significant effect of 10mM zinc in the luminal saline on 50μM cadmium (and vice-versa) at the stomach (Fig. 3-7A and B; 3-9A and B). Also at the intestine, 10mM zinc in the luminal saline did not inhibit 50μM cadmium uptake rates into serosal fluid + muscle at the anterior, mid and posterior intestine (Fig. 3-8A) but there was a significant stimulatory effect at the mid-intestine by about 2-fold from 0.063 ± 0.021nmol/cm^2/h to 0.13 ± 0.02 nmol/cm^2/h. However, at the same time 10mM zinc significantly inhibited 50μM cadmium accumulation rates in the mucosal epithelium at the mid-intestine by 94% with rate falling to 0.008 ± 0.002 nmol/cm^2/h from control value 0.113 ± 0.029 nmol/cm^2/h and at the posterior intestine by 73% with rate falling to 0.007 ± 0.002 nmol/cm^2/h from control value 0.026 ± 0.005 nmol/cm^2/h (Fig. 3-8B). In contrast, at the anterior intestine, 10mM zinc had no significant effect on 50μM cadmium accumulation rate at the mucosal epithelium (Fig. 3-8B).

Doing the reciprocal test at the intestine, 10mM cadmium in the luminal saline did not inhibit 50μM zinc uptake rates into serosal fluid + muscle at the anterior, mid and posterior intestine (Fig. 3-10A) though there was an insignificant stimulatory effect at the mid intestine. But again at the mucosal epithelium, 10mM cadmium in the luminal saline significantly inhibited 50μM zinc accumulation rate at the mid-intestine by 91% with the rate falling to 0.0037 ± 0.0004 nmol/cm^2/h from the control value of 0.041 ± 0.014 nmol/cm^2/h (Fig. 3-10B). At the posterior intestine, 10mM cadmium insignificantly

inhibited 50μM zinc accumulation rates in the mucosal epithelium and there was no inhibition at the anterior intestine (Fig. 3-10B).

3.3. Interactions between two essential metals via the gastrointestinal tract: zinc and copper

Interactions between zinc and copper were tested in a reciprocal manner. 500μM zinc in the luminal saline did not have any effect on 50μM copper uptake rates into serosal fluid + muscle at the stomach (Fig. 3-11A). However, there was a significant stimulatory effect of 500μM zinc in the luminal saline on 50μM copper uptake at the mucosal epithelium by about 1.5 fold from 0.0059 ± 0.0008 nmol/cm^2/h to 0.0092 ± 0.0012 nmol/cm^2/h at the stomach (Fig. 3-11B).

But at the intestine, 500μM zinc in the luminal saline caused significant inhibition in copper uptake rates (50μM) into serosal fluid + muscle by 67% at the mid-intestine with rate falling to 0.010 ± 0.002 nmol/cm^2/h from the control value of 0.030 ± 0.005 nmol/cm^2/h and by 33% at the posterior intestine with rate falling to 0.024 ± 0.004 nmol/cm^2/h from the control value of 0.036 ± 0.006 nmol/cm^2/h. However, there was a stimulatory effect at the anterior intestine (Fig. 3-12A). Also, at the mucosal epithelium, 500μM zinc in the luminal saline significantly inhibited 50μM copper accumulation rate by 60% at the mid-intestine with rate falling to 0.007 ± 0.001 nmol/cm^2/h from the control value of 0.017 ± 0.004 nmol/cm^2/h (Fig. 3-12B), but caused no inhibition of copper uptake at the anterior and posterior intestine (Fig. 3-12B).

However doing the reciprocal test (i.e. effect of 500μM copper on 50μM zinc uptake), there was again a significant stimulatory effect of 500μM copper in the luminal saline on

50μM zinc uptake by 1.7 fold from 0.004 ± 0.0004 nmol/cm^2/h to 0.007 ± 0.001nmol/cm^2/h into serosal fluid + muscle at the stomach (Fig. 3-13A). Also, at the mucosal epithelium, there was a significant stimulatory effect of 500μM copper in the luminal saline on 50μM zinc by about 2 fold from 0.002 ± 0.0003 nmol/cm^2/h to 0.004 ± 0.001 nmol/cm^2/h at the stomach (Fig. 3-13B).

But again at the intestine, 500μM copper in the luminal saline significantly inhibited 50μM zinc uptake rates into serosal fluid + muscle by 54% at the mid-intestine with the rate falling to 0.097 ± 0.02 nmol/cm^2/h from the control value of 0.213 ± 0.027 nmol/cm^2/h and by about 78% at the posterior intestine with the rate falling to 0.042 ± 0.008 nmol/cm^2/h from the control value of 0.188 ± 0.03 nmol/cm^2/h. However, there was no effect at the anterior intestine (Fig. 3-14A). There were also no significant effects in any of the three intestinal segments at the mucosal epithelium (Fig. 3-14B).

4. 0. Discussion.

For this discussion, metal uptake rates at the both the apical entry step and basolateral exit side were examined and compared with each other. A model is proposed about copper, zinc and cadmium uptake, which postulates that these metals might have different mechanisms of uptake in various segments of the gastrointestinal tract (Fig. 3-15).

4.1. Effects of calcium on cadmium and zinc uptake.

A clear antagonistic effect of calcium on cadmium uptake occurred at the stomach (at the serosal fluid + muscle (basolateral side) and the mucosal epithelium (apical entry step) (Fig. 3-1A and B)) but there was no effect of calcium on cadmium uptake in any of

the three segments of the intestine (Fig. 3-2A and B) in contrast to what happened at the stomach. This was strongly supported by an *in vivo* study from Franklin et al. (In Press) who found that dietary calcium inhibited dietary cadmium accumulation at the stomach but not at the intestine. It was also supported by in *vitro* dietary studies at the intestine (Baldisserotto et al., submitted; Baskin, 1999). These authors found out that calcium did not have an effect cadmium uptake via the intestine. Thus, there is at least a portion of cadmium uptake at the stomach but not at the intestine that is sensitive to calcium. This can be interpreted that cadmium transport occurs at least in part through a calcium channel at the stomach similar to the situation at the gill (Verbost et al., 1987, 1988, 1989) but not at the intestine, suggesting another transporter (probably through DMT1) for cadmium transport via the intestine.

Surprinsingly, there was synergistic effect of calcium on zinc uptake at the stomach (Fig. 3-5A and B), but in contrast there was no effect of calcium on zinc uptake in any of the three segments of the intestine (Fig. 3-6A and B). This was in line with *in vivo* work by Glover et al. (2004) that showed zinc and calcium interaction at the intestine was not exerted at the level of calcium channel. This indicates that zinc uptake via the intestine, similar to cadmium uptake via the intestine, is not through calcium channels in contrast to the situation at the gill (Hogstrand et al., 1996). This was also supported by studies on zinc uptake in mammals (Lonnerdal, 2000) in which there was a lack of antagonistic effect of calcium on zinc uptake, and indicating that other transporters, probably hZTL1 transporter (Cragg et al. 2002), can account for zinc uptake via the gastrointestinal tract of fish, as in mammals.

4.2. Inhibitory studies: Effect of zinc on cadmium transport and vice-versa at the intestine.

This now leads me to investigate interactions between cadmium and zinc via the gastrointestinal tract, since there was no competitive interaction between them and calcium at the intestine.

At the stomach, there was no significant interaction between zinc and cadmium into serosal fluid + muscle and mucosal epithelium (Figs. 3-7A and B; Fig. 3-9A and B), confirming the fact that zinc transport at stomach was not via a calcium channel. At the intestine, there was no inhibitory effect of zinc on cadmium transport (and vice-versa) in all the three segments of the intestine at the basolateral side (i.e. serosal fluid + muscle) (Fig 3-8 A; Fig 3-10 A), but there appears to be a reciprocal stimulatory effect at the mid-intestine, indicating separate mechanisms at the basolateral side from the apical side. The basolateral export mechanism for cadmium and zinc at the intestine of fish can be through ZnT-1, since it is a possible candidate for the cadmium (mammals: Palmiter and Findley, 1995) and zinc export mechanism (Cousins and McMahon, 2000), and since the same homologs have been cloned in fish (Balesaria and Hogstrand, 2001). This indicates a different basolateral export mechanism from the apical entry mechanism, which was also supported by the findings in Chapter 2, and work from Clearwater et al. (unpublished).

However, a substantial interaction was seen at the mucosal epithelium (i.e. apical entry mechanism), where there was a large inhibitory effect of zinc on cadmium transport (and vice-versa) at the mid intestine and probably at the posterior intestine (Fig 3-8B; Fig 3-10B). This was also consistent at least in part with the findings from Wicklund-Glynn

(2001) in which there was a competitive interaction between cadmium and zinc at the apical transport site at the gill. This was further supported by the work from Bentley (1992), which showed that cadmium decreased zinc uptake at the gill. This result was also consistent with *in vivo* studies in the intestine of fish (Shears and Fletcher, 1983) and in mammals (Foulkes, 1985; Blakeborough and Salter, 1987; Tacnet et al., 1990; Tacnet et al., 1991; Bertolo et al., 2001; Elisma and Jumarie, 2001). In mammals, transporters such as DMT1 (Gunshin et al., 1997; Gaither and Eide, 2000; Park et al., 2002; Bressler et al. 2004) can mediate cadmium uptake. At least one of the three homologs to DMT1 exists in the intestine of fish (Bury et al., 2003). Zinc has not been shown to be transported through DMT1, but the competitive interaction between cadmium and zinc might also be explained by the fact that zinc can influence the function and expression of DMT1 even though it might not be transported through it (Yamaji et al., 2001). So it is possible that cadmium uptake at apical transport site occur through DMT1 at the mid and posterior intestine but via a different mechanism at the anterior intestine.

4.3. Stimulatory studies: Effect of copper on zinc transport and vice-versa at the stomach.

At the basolateral side (i.e. serosal fluid + muscle) in the stomach, there was no effect of 500μM zinc on copper transport (Fig 3-11A). But by doing the reciprocal (i.e. 500μM copper on 50μM zinc), a synergistic interaction of copper on zinc uptake at the stomach was revealed (Fig 3-13A) (i.e. a stimulatory effect at higher copper concentration on zinc uptake into serosal fluid + muscle). At the apical entry side (i.e. mucosal epithelium) in the stomach, there was synergistic interaction (i.e. a stimulatory effect of zinc on copper

uptake) and vice-versa (Fig 3-11B; Fig 3-13B). This suggests a specific transport mechanism for zinc and copper uptake at the level of apical entry at the stomach. In mammals, the apical transporter hZip2 has been identified to transport zinc and it can also transport other substrates such as copper (Gaither and Eide, 2000). The mechanism of zinc transport by this protein can be driven by the concentration gradient of the metal ion substrate as suggested by Gaither and Eide (2001a). This may mean that the high concentration of copper can enhance zinc transport and vice-versa across the epithelium in the stomach of fish as suggested in mammalian work from Gaither and Eide (2001a). This kind of mechanism can be possible at the stomach but not intestine because, there is low zinc uptake at the stomach compared to the intestine i.e. the intestine can serve as the bulk acquisition mechanism for zinc uptake rather than the stomach. The basolateral exit mechanism for zinc export at the stomach can be through ZnT-1 (Cousins and McMahon, 2000) since there was synergistic effect of copper on zinc uptake at the serosal fluid + muscle.

4.4. Inhibitory studies: Effect of copper on zinc transport and vice-versa at the intestine.

For copper and zinc uptake at the basolateral side (i.e. serosal fluid + muscle), there was an antagonistic effect of copper on zinc uptake and vice-versa (Fig 3-12A; Fig 3-14A), which was very consistent with the findings from *in vitro* and *in vivo* studies in rats (Condomina et al., 2002) and in fish (Shears and Fletcher, 1983) respectively. The competitive interaction between copper and zinc in an *in vivo* investigation by Shears and Fletcher (1979) was explained based on competition for a cytosolic protein, presumably

metallothionein. So there is possibility for zinc and copper to compete for intracellular metallothionein in fish. At the mucosal epithelium (i.e. apical entry step), there was an antagonistic effect of zinc on copper uptake at the mid intestine (Fig 3-12B), indicating a specific copper transport mechanism, possibly through DMT1 (Gunshin et al., 1997). Evidence has recently been provided that this transporter (DMT1) may mediate copper uptake at the intestine of fish (Nadella et al., unpublished). In support of this, 50% of copper uptake had been shown to be mediated through DMT1 in human intestinal Caco-2 cells (Arredondo et al., 2003). This present study supports DMT1 as the transporter for copper uptake via the mid-intestine of fish but there might be different mechanisms at the anterior intestine probably through an amino acid transporter as has been indicated for copper (Nadella et al. unpublished). However, there was no effect of copper on zinc uptake at the mucosal epithelium (Fig 3-14B), which was consistent with the *in vitro* studies of Glover et al. (2004), in which there was no inhibitory effect of copper on zinc uptake at the level of the brush border membrane in the intestine of fish. However, there is a possibility for a zinc-specific transporter to mediate zinc uptake at the intestine that might not depend on the influence of copper, such as mZip 4 (mouse Zip 4) that had no effect on copper transport (Kim et al., 2004). Another zinc transporter hZTL1 (human ZnT-Like transporter 1) has been identified at the human brush border membrane (Cragg et al., 2002), but transporting properties of these proteins have not been fully described.

5. 0. Relevance/Importance of the Results

These results can be of environmental importance in that they help in the development of an intestinal BLM at the intestinal tract of fish. These results can also help to gain

insight into transport mechanisms for copper, zinc and cadmium via the gastrointestinal tract. This can be useful in gaining insight into homeostatic regulations of these metals at both the cellular and organismal level. These results also bring to our attention the need to consider the stomach as an important site for metals absorption and interactions in fish. They also suggest that analogs (or homologs) of mammalian transporters can also be present in fish. But there is need to understand all of these at the molecular level to further investigate the role of DMT1 in copper and cadmium uptake and Zip transporters for zinc uptake via the gastrointestinal tract.

The significance of these findings from a human health and risk assessment perspective lies in the indication that an excess of dietary calcium (an essential metal) at high concentration in an aquatic environment can prevent the toxic effect of dietary cadmium uptake (a non-essential metal) at lower concentration via the gut of fish. This concept can also be applied for two essential metals (i.e. copper and zinc) via the intestine, in which copper or zinc at higher concentration can prevent the uptake of one another at lower concentration via the gut. However, the findings from this study were the first to show that zinc and calcium and zinc and copper interactions at the stomach were different from those at the intestine. These indicate separate mechanisms for these two essential metals (copper and zinc) via the gastrointestinal tract of fish.

The implications of these findings for copper, cadmium and zinc BLM sites lie in the perspective from Chapter 2 that the zinc uptake rate at the gill differs from the uptake rate at the gut which shows the reason why zinc and calcium interactions differ between the gill and gut. Notably, zinc-binding affinity to these two different sites differ, so the effect of calcium on zinc uptake at these two different sites also differ. Cadmium uptake at the

gill also differs from uptake at the gut and this can give the reason why calcium and cadmium interactions differ between the gill and intestine. These conclusions are important in understanding the nature of potential BLM sites at the gut.

A conceptual model for copper, zinc and cadmium uptake via the gastrointestinal tract is proposed in Fig 3-15. At the stomach, copper and zinc uptake could occur through an apical Zip or hZTL1 transporter, because of the synergistic interaction between calcium and zinc and between zinc and copper at the mucosal epithelium and the basolateral exit pathway for these metals can be different. Basolateral zinc export at stomach can be through ZnT-1. Cadmium uptake at the stomach could be through epithelial calcium channels (ECaC), because of antagonistic interaction between cadmium and calcium. At the anterior intestine, copper uptake at the apical side could be through an amino acid transporter because of synergistic effect of zinc on copper uptake but basolateral exit is unknown. The pathway for zinc and cadmium uptake at the anterior intestine is unknown at both the apical and basolateral side since there was no significant interaction between these two metals at both sides. This could be due to the presence of ceca at the anterior intestine. At the mid and posterior intestine, copper and cadmium uptake could be through DMT1 transporter, since there was antagonistic interaction between copper and zinc and also between cadmium and zinc at the mucosal surface. The basolateral exit mechanism for copper can be through Cu-ATPase and for cadmium it can be through ZnT-1. Zinc uptake at the mid and posterior intestine could be through Zip or hZTL1 since there was no interaction between copper and zinc at the mucosal surface. And basolateral exit mechanism for zinc at the mid and posterior intestine could be through

ZnT-1. While these proposals are tentative based on the limited evidence discovered in thesis, they provide a useful basis for future experimentation.

Figures

Fig. 3-1. Influence of 1mM calcium and 10mM calcium on the rate of absorption of 50μM cadmium into (A) serosal fluid + muscle and (B) the mucosal epithelium at the stomach, following an *in vitro* stomach gut-sac technique for 4h. Plotted values represent the means (± S.E.M) of N = 10 for control and 10 experimental means. The difference between treatment and control (*) was tested at $P < 0.05$ significance level using a simple unpaired t-test.

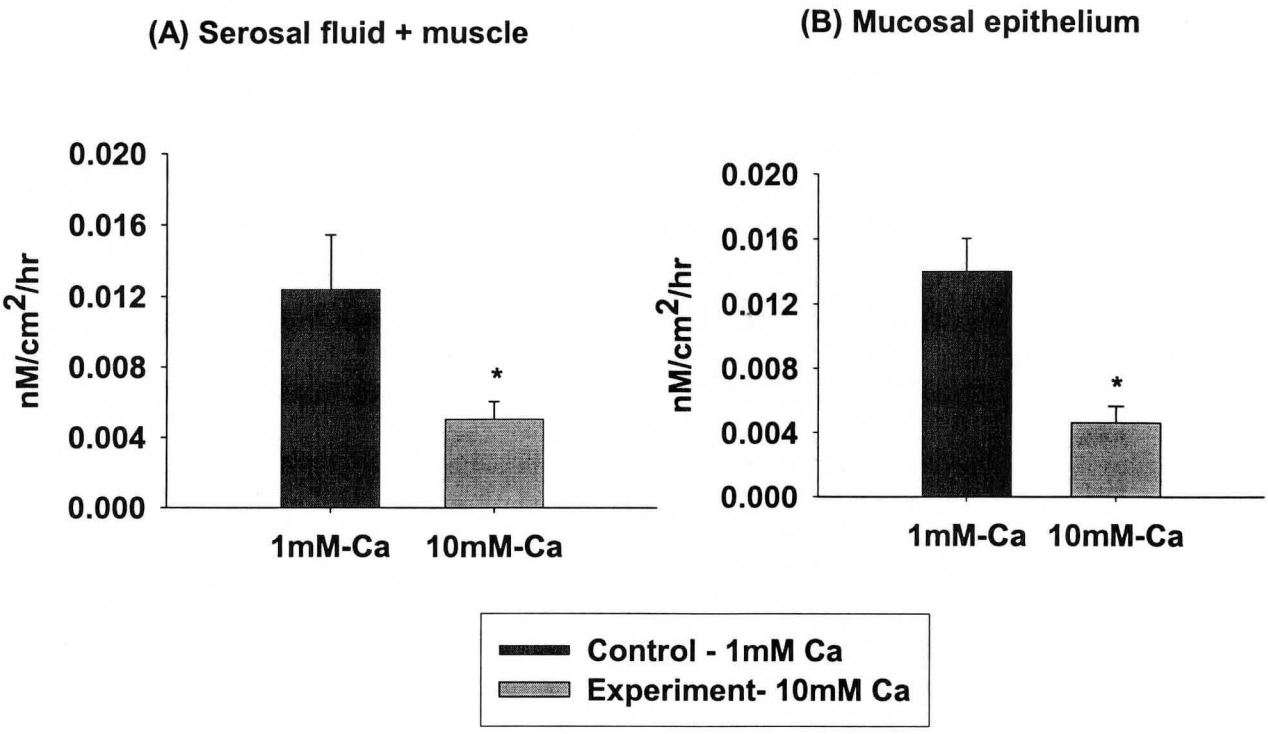

(A) Serosal fluid + muscle
(B) Mucosal epithelium
nM/cm2/hr
0.020
0.016
0.012
0.008
0.004
0.000
*
1mM-Ca
10mM-Ca
Control - 1mM Ca
Experiment- 10mM Ca

Fig. 3-1

Fig. 3-2. Influence of 1mM calcium and 10mM calcium on the rate of absorption of cadmium 50μM into (A) serosal fluid + muscle and (B) mucosal epithelium (in sulphate-based saline) at the (a) anterior (b) mid and (c) posterior intestine, following an *in vitro* gut-sac technique for 2h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means. There were no significant differences within any of the three sections.

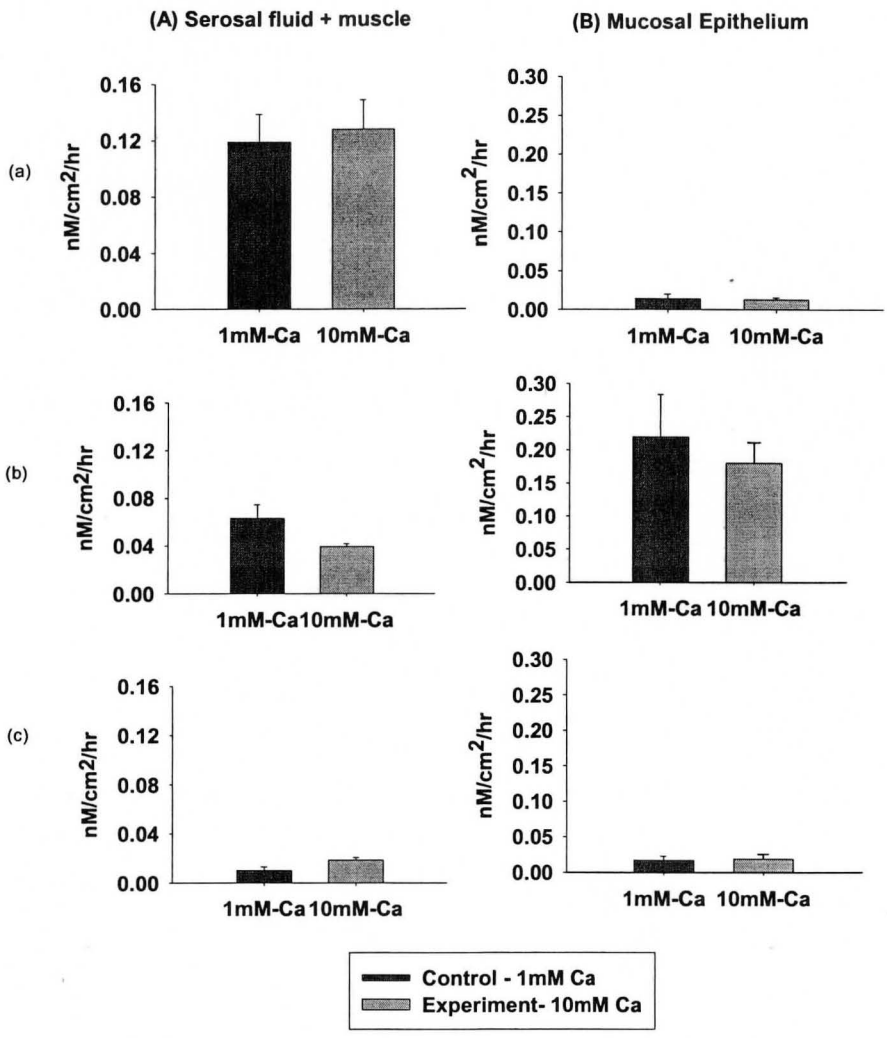

(A) Serosal fluid + muscle
(B) Mucosal Epithelium
(a)
(b)
(c)
nM/cm2/hr
nM/cm2/hr
0.16
0.12
0.08
0.04
0.00
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1mM-Ca
10mM-Ca
Control - 1mM Ca
Experiment- 10mM Ca

Fig. 3-2

Fig. 3-3. Influence of 1mM and 10mM calcium on the rate of absorption of cadmium 50μM into (A) serosal fluid + muscle and (B) mucosal epithelium (in chloride-based saline) at the (a) anterior (b) mid and (c) posterior intestine, following an *in vitro* gut-sac technique for 2h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means. There were no significant differences within mid and posterior intestine using a simple unpaired t-test, but the difference in the anterior intestine at the serosal fluid + muscle (*) was significant.

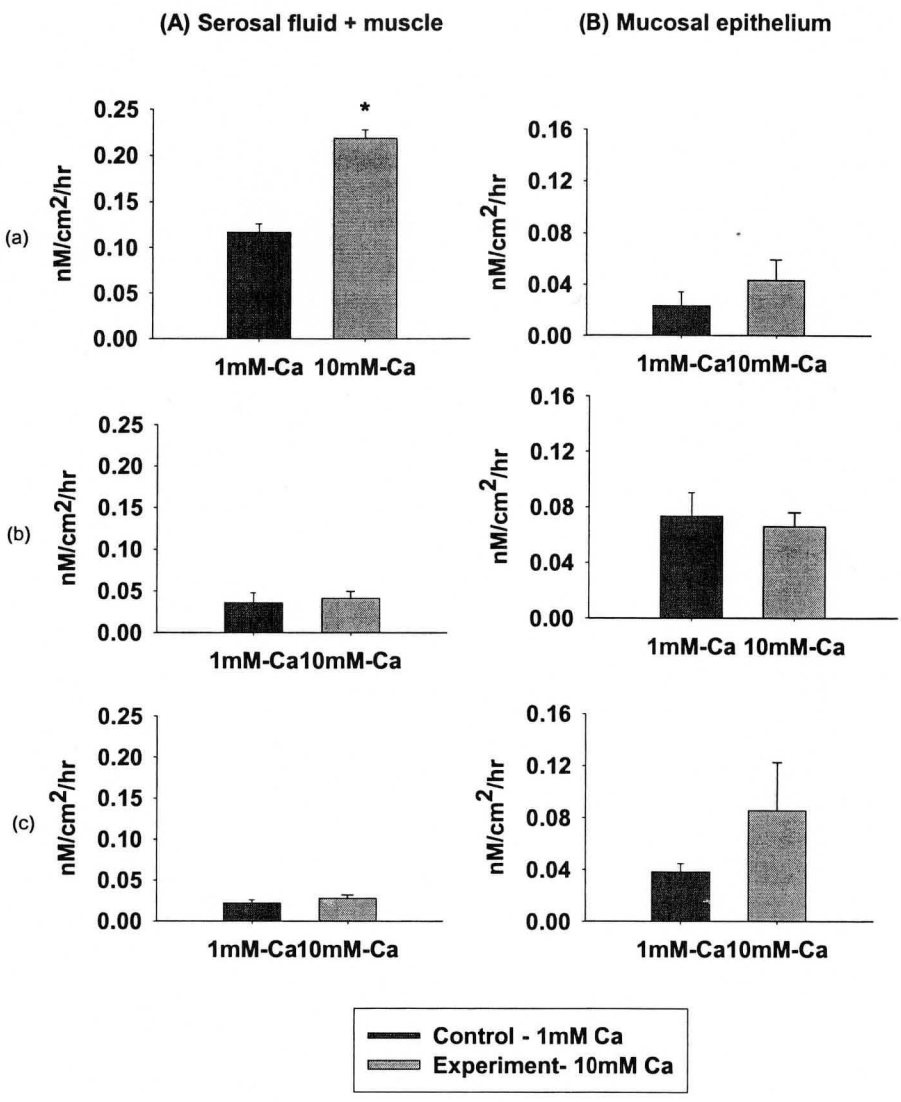

(A) Serosal fluid + muscle
(B) Mucosal epithelium
(a)
(b)
(c)
nM/cm2/hr
0.25
0.20
0.15
0.10
0.05
0.00
0.16
0.12
0.08
0.04
*
1mM-Ca 10mM-Ca
1mM-Ca10mM-Ca
Control - 1mM Ca
Experiment- 10mM Ca

Fig. 3-3

Fig. 3-4. Influence of 1mM and 100mM calcium on the rate of absorption of cadmium 50μM into (A) serosal fluid + muscle and (B) mucosal epithelium (in chloride-based saline) at the (a) anterior (b) mid and (c) posterior intestine, following an *in vitro* gut-sac technique for 2h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means for anterior and posterior intestine and 4 for mid-intestine. There were no significant differences within any of the three sections.

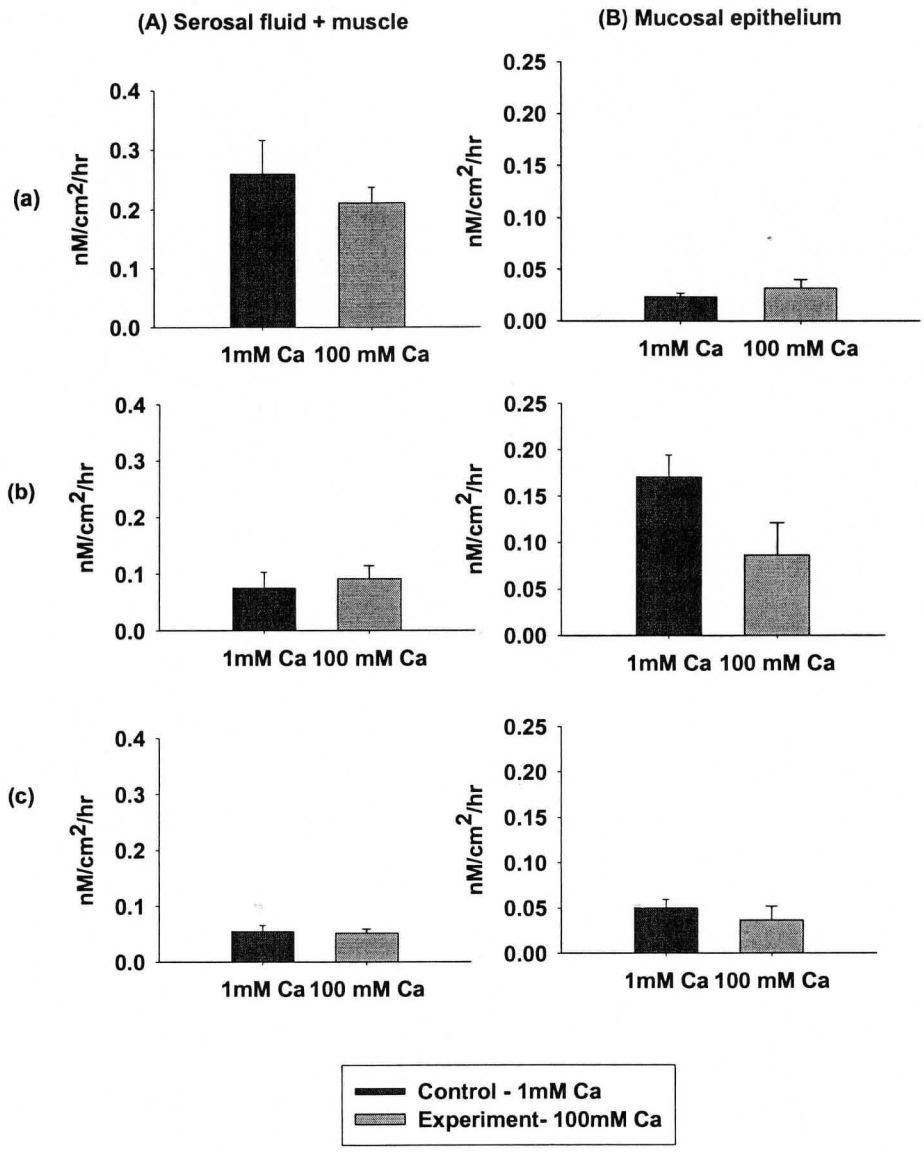

(A) Serosal fluid + muscle
(B) Mucosal epithelium
(a)
(b)
(c)
nM/cm2/hr
0.4
0.3
0.2
0.1
0.0
0.25
0.20
0.15
0.10
0.05
0.00
1mM Ca
100 mM Ca
Control - 1mM Ca
Experiment- 100mM Ca

Fig. 3-4

Fig. 3-5. Influence of 1mM calcium and 100mM calcium on the rate of absorption of 50μM zinc into (A) serosal fluid + muscle and (B) the mucosal epithelium at the stomach, following an *in vitro* stomach gut-sac technique for 4h. Plotted values represent the means (± S. E. M) of N = 5 for control and 5 experimental means. The difference between treatment and control (*) was tested at $P < 0.05$ significance level using simple unpaired t-test.

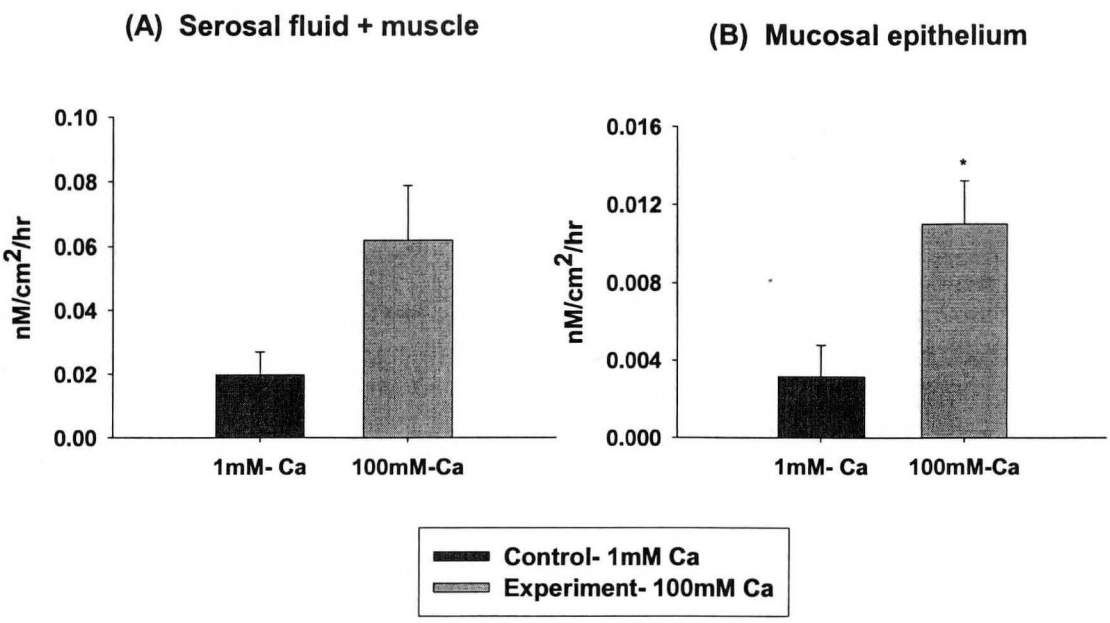

(A) Serosal fluid + muscle
(B) Mucosal epithelium
nM/cm2/hr
0.10
0.08
0.06
0.04
0.02
0.00
1mM- Ca
100mM-Ca
0.016
0.012
0.008
0.004
0.000
*
Control- 1mM Ca
Experiment- 100mM Ca

Fig. 3-5

Fig. 3-6. Influence of 1mM and 100mM calcium on the rate of absorption of zinc 50μM into (A) serosal fluid + muscle and (B) mucosal epithelium (in chloride-based saline) at the (a) anterior (b) mid and (c) posterior intestine, following an *in vitro* gut-sac technique for 2h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means. There were no significant differences within any of the three sections.

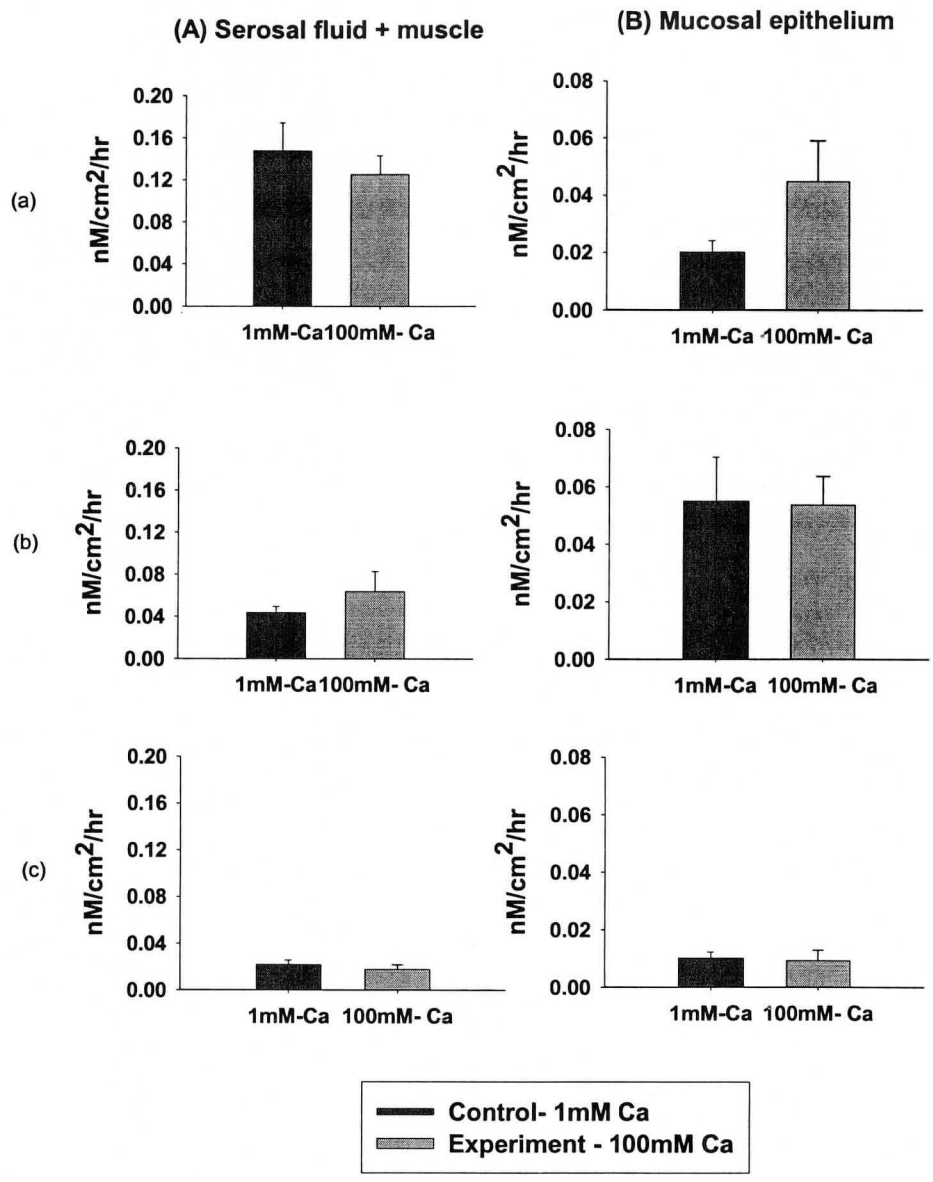

(A) Serosal fluid + muscle
(B) Mucosal epithelium
(a)
(b)
(c)
nM/cm2/hr
0.20
0.16
0.12
0.08
0.04
0.00
0.08
0.06
0.04
0.02
0.00
1mM-Ca
100mM- Ca
Control- 1mM Ca
Experiment - 100mM Ca

Fig. 3-6

Fig. 3-7. Influence of 10mM zinc on the rate of absorption of 50μM cadmium into (A) serosal fluid + muscle and (B) mucosal epithelium (in chloride-based saline) at the stomach, following an *in vitro* stomach gut-sac technique for 4h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means. There were no significant differences at the serosal fluid + muscle and mucosal epithelium at the stomach.

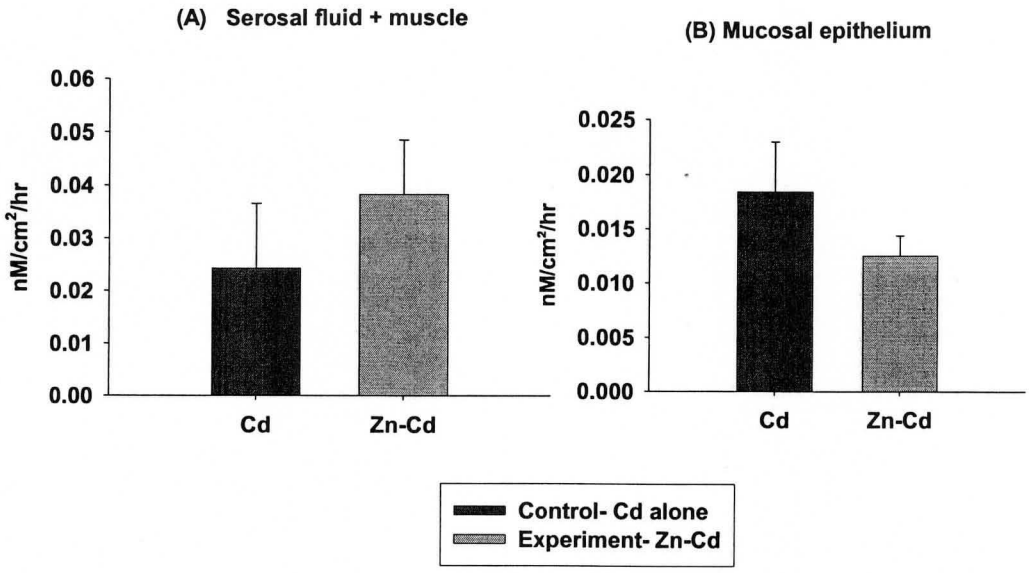

(A) Serosal fluid + muscle
(B) Mucosal epithelium
nM/cm²/hr
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.025
0.020
0.015
0.010
0.005
0.000
Cd
Zn-Cd
Control- Cd alone
Experiment- Zn-Cd

Fig. 3-7

Fig. 3-8. Influence of 10mM zinc on the rate of absorption of 50μM cadmium into (A) serosal fluid + muscle and (B) mucosal epithelium (in chloride-based saline) at the (a) anterior (b) mid and (c) posterior intestine, following an *in vitro* gut-sac technique for 2h. Plotted values represent the means (± S.E.M) of N = 10 for control and 10 experimental means. Differences between treatment and control (*) were tested at $P < 0.05$ significance level using simple unpaired t-test at the serosal fluid + muscle and mucosal epithelium.

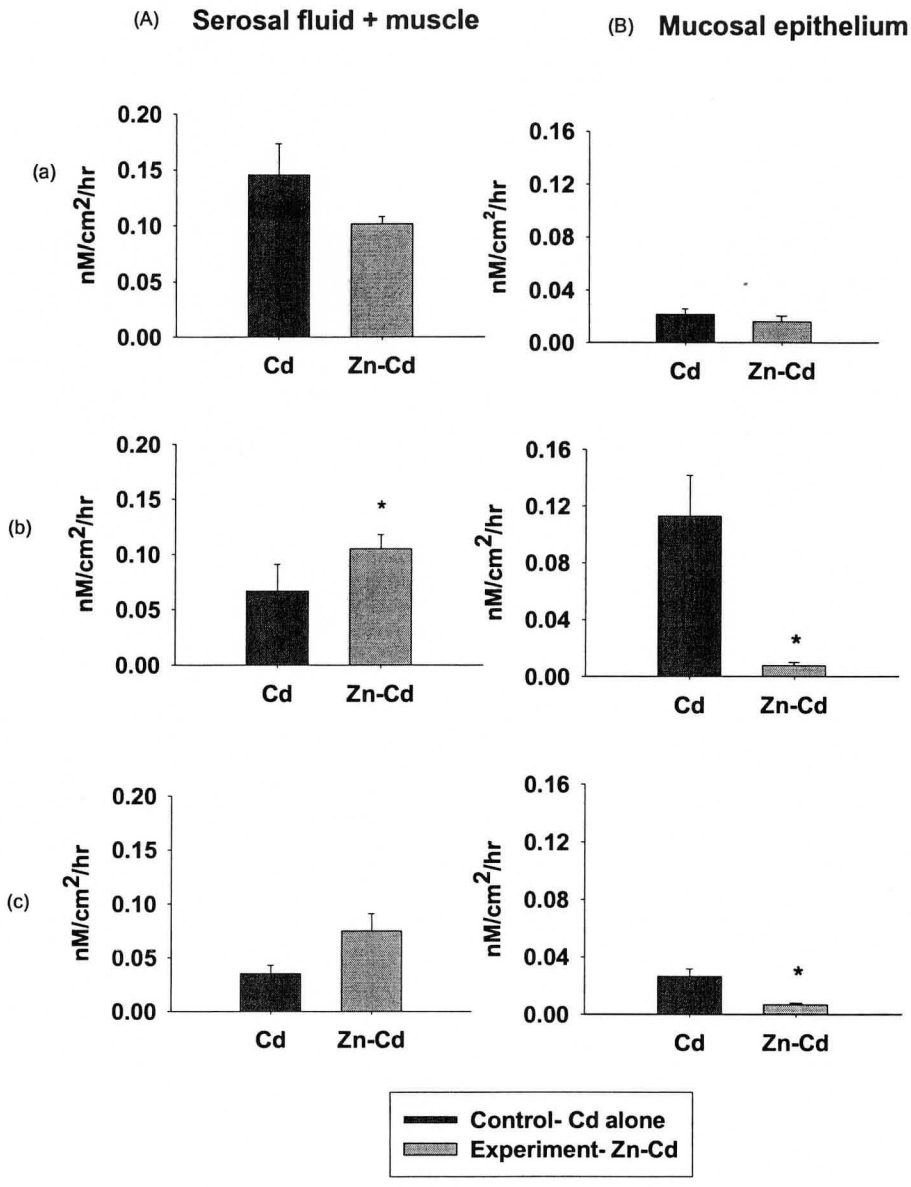

(A) Serosal fluid + muscle
(B) Mucosal epithelium
(a)
(b)
(c)
nM/cm2/hr
0.20
0.15
0.10
0.05
0.00
0.16
0.12
0.08
0.04
Cd
Zn-Cd
*
Control- Cd alone
Experiment- Zn-Cd

Fig. 3-8

Fig. 3-9. Influence of 10mM cadmium on the rate of absorption of 50μM zinc into (A) serosal fluid + muscle and (B) mucosal epithelium (in chloride-based saline) at the stomach, following an *in vitro* stomach gut sac technique for 4h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means. There were no significant differences at the serosal fluid + muscle and mucosal epithelium at the stomach.

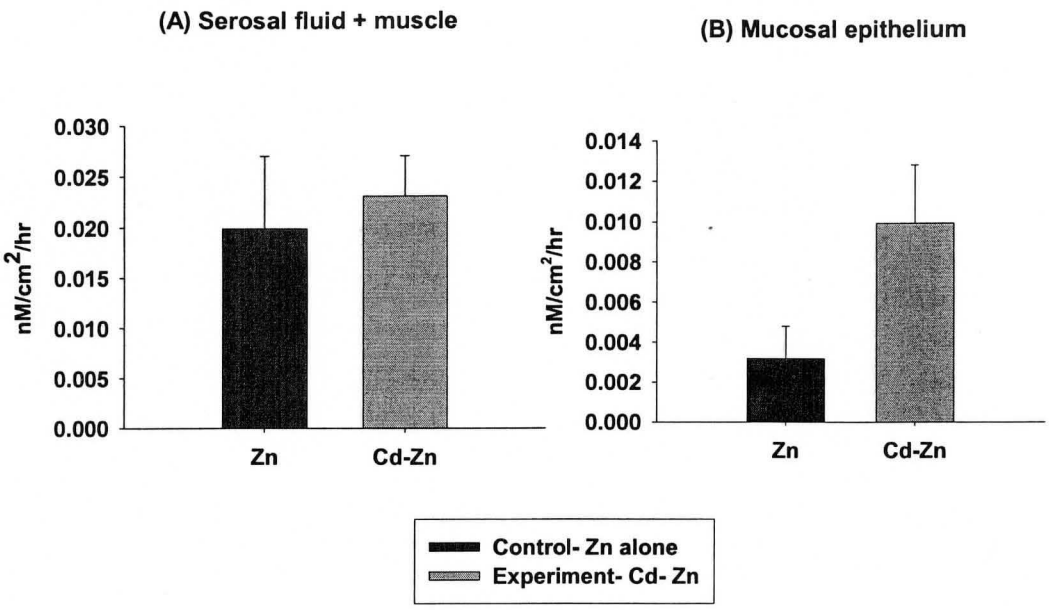

(A) Serosal fluid + muscle
(B) Mucosal epithelium
nM/cm2/hr
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Zn
Cd-Zn
nM/cm2/hr
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Zn
Cd-Zn
Control- Zn alone
Experiment- Cd- Zn

Fig. 3-9

Fig. 3-10. Influence of 10mM cadmium on the rate of absorption of 50μM zinc into serosal fluid + muscle and mucosal epithelium (in chloride-based saline) at the (a) anterior (b) mid and (c) posterior intestine, following an *in vitro* gut-sac technique for 2h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means. There were no significant difference within any of the three sections at the serosal fluid + muscle. Differences between treatment and control (*) were tested at $P < 0.05$ significance level using simple unpaired t-test at the mucosal epithelium.

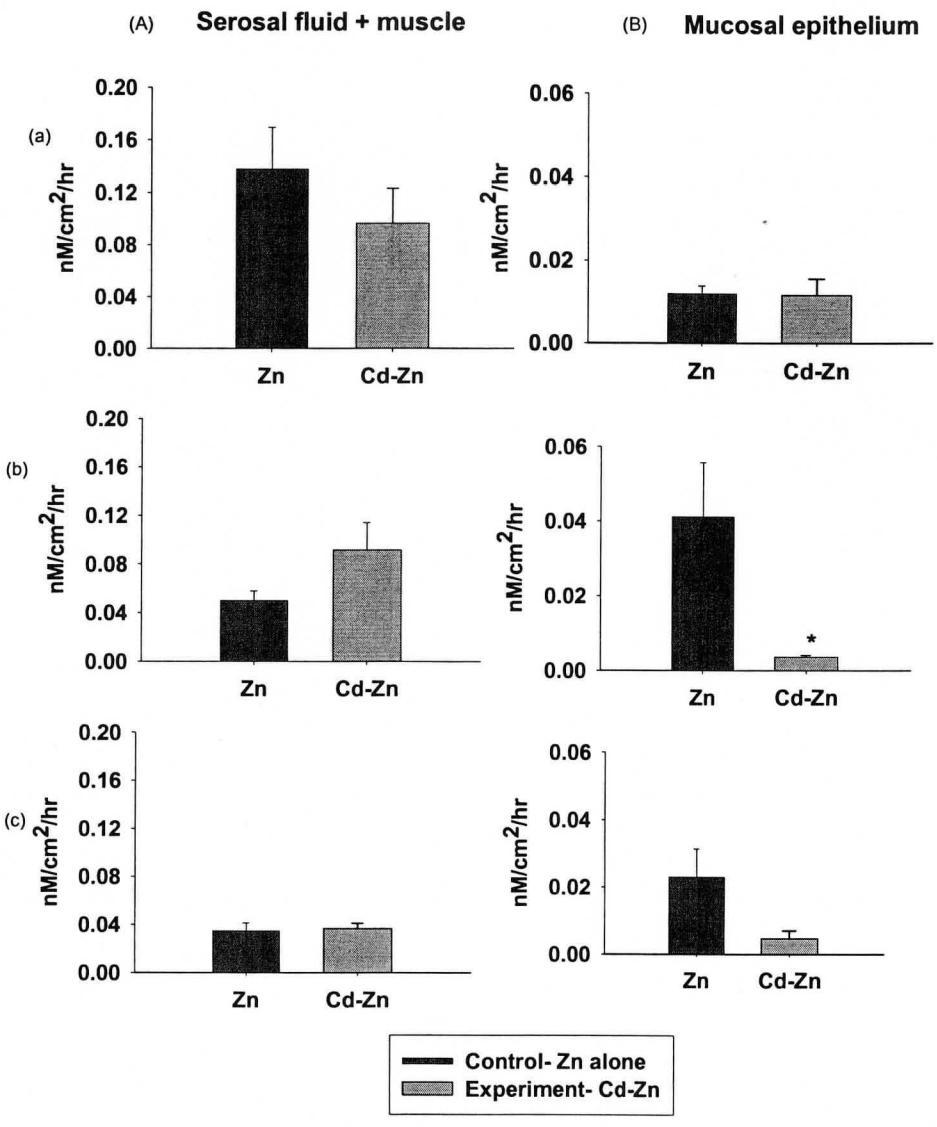

(A) Serosal fluid + muscle
(B) Mucosal epithelium
(a)
(b)
(c)
nM/cm2/hr
0.20
0.16
0.12
0.08
0.04
0.00
0.06
0.04
0.02
0.00
Zn
Cd-Zn
*
Control- Zn alone
Experiment- Cd-Zn

Fig. 3-10

Fig. 3-11. Influence of 500μM zinc on the rate of absorption of copper 50μM into (A) serosal fluid + muscle and (B) the mucosal epithelium at the stomach, following an *in vitro* stomach gut-sac technique for 4h. Plotted values represent the means (± S.E.M) of N = 9 for control and 9 experimental means at the serosal fluid + muscle. The differences between treatment and control (*) were tested at $P < 0.05$ significance level using a simple unpaired t-test.

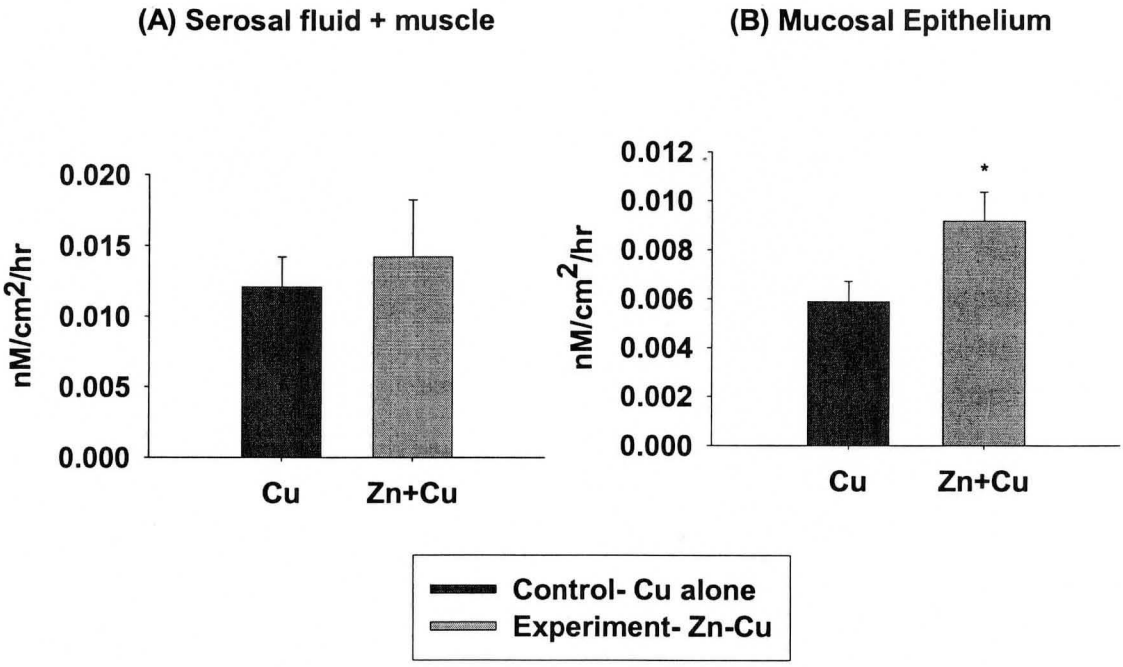

(A) Serosal fluid + muscle
(B) Mucosal Epithelium
nM/cm2/hr
0.020
0.015
0.010
0.005
0.000
Cu
Zn+Cu
nM/cm2/hr
0.012
0.010
0.008
0.006
0.004
0.002
0.000
*
Cu
Zn+Cu
Control- Cu alone
Experiment- Zn-Cu

Fig. 3-11

Fig. 3-12. Influence of 500μM zinc on the rate of absorption of 50μM copper into serosal fluid + muscle and mucosal epithelium (in sulphate-based saline) at the (a) anterior (b) mid and (c) posterior intestine, following an *in vitro* gut-sac technique for 4h and 2h. Plotted values represent the means (± S.E.M) of N = 10 control at the anterior and posterior intestine, 9 at the mid intestine and 10 experimental means at the anterior, mid and posterior intestine at the serosal fluid + muscle. But at the mucosal epithelium, N = 9 for control at the anterior intestine, 10 at the mid and posterior intestine and 9 experimental means at the anterior intestine, 10 at the mid and posterior intestine. Differences between treatment and control (*) were tested at $P < 0.05$ significance level using simple unpaired t-test at the serosal fluid + muscle and mucosal epithelium.

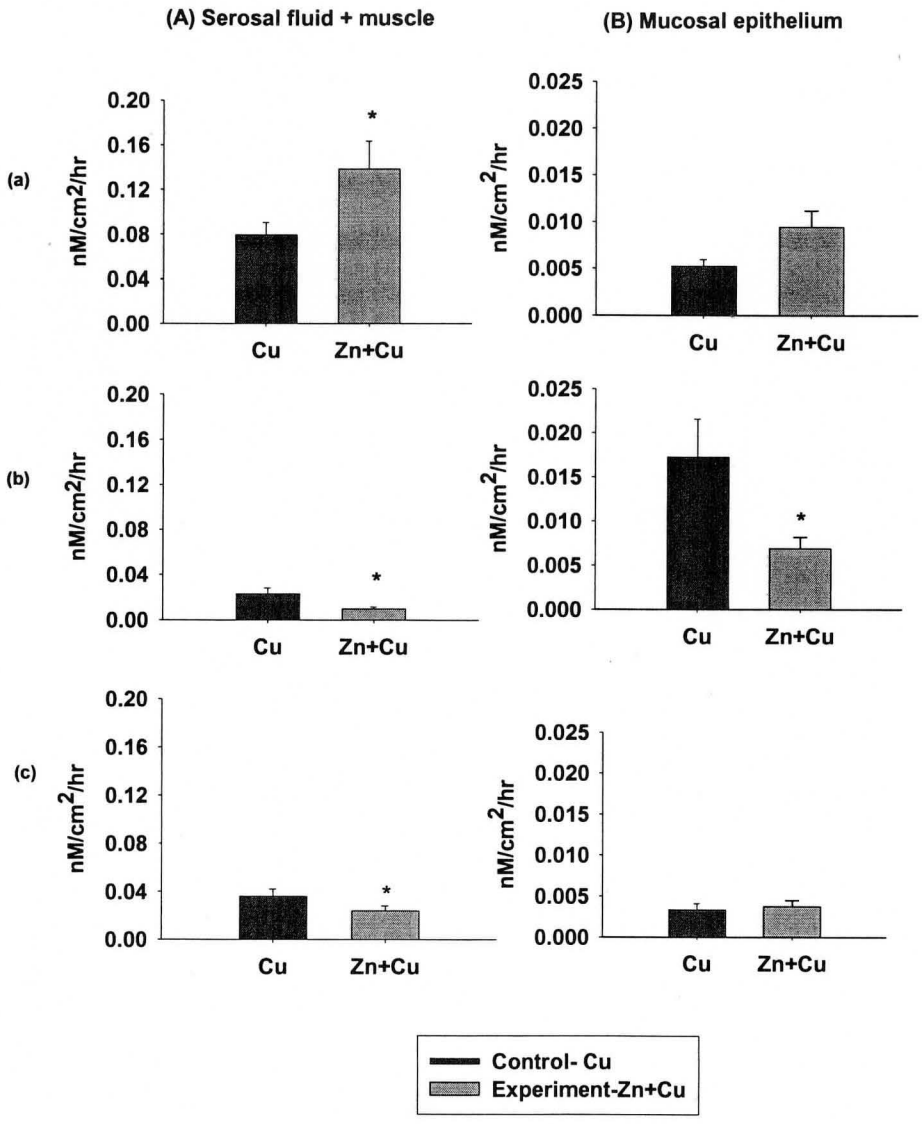

(A) Serosal fluid + muscle
(B) Mucosal epithelium
(a)
(b)
(c)
nM/cm2/hr
0.20
0.16
0.12
0.08
0.04
0.00
0.025
0.020
0.015
0.010
0.005
0.000
*
Cu
Zn+Cu
Control- Cu
Experiment-Zn+Cu

Fig. 3-12

Fig. 3-13. Influence of 500μM copper on the rate of absorption of zinc 50μM into serosal fluid + muscle at the stomach, following an *in vitro* stomach gut-sac technique for 4h. Plotted values represent the means (± S.E.M) of N = 8 for control and 8 experimental means. The differences between treatment and control (*) were tested at $P < 0.05$ significance level using a simple unpaired t-test.

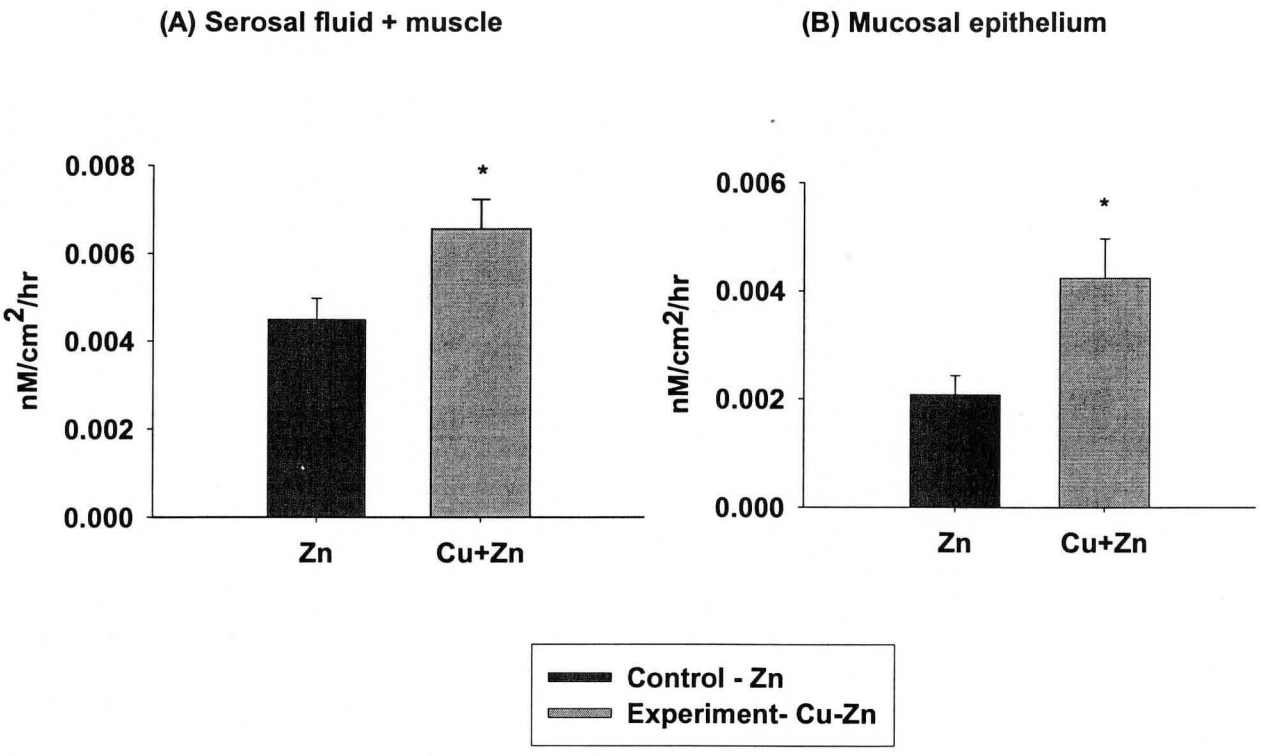

(A) Serosal fluid + muscle
(B) Mucosal epithelium
nM/cm2/hr
0.008
0.006
0.004
0.002
0.000
*
Zn
Cu+Zn
nM/cm2/hr
0.006
0.004
0.002
0.000
*
Zn
Cu+Zn
Control - Zn
Experiment- Cu-Zn

Fig. 3-13

Fig. 3-14. Influence of 500μM copper on the rate of absorption of 50μM zinc into (A) serosal fluid + muscle and (B) mucosal epithelium (in sulphate-based saline) at the (a) anterior (b) mid (c) posterior intestine, following an *in vitro* gut-sac technique for 4h. Plotted values represent the means (± S.E.M) of N = 5 for control and 5 experimental means. Differences between treatment and control (*) were tested at $P < 0.05$ significance level using simple unpaired t-test.

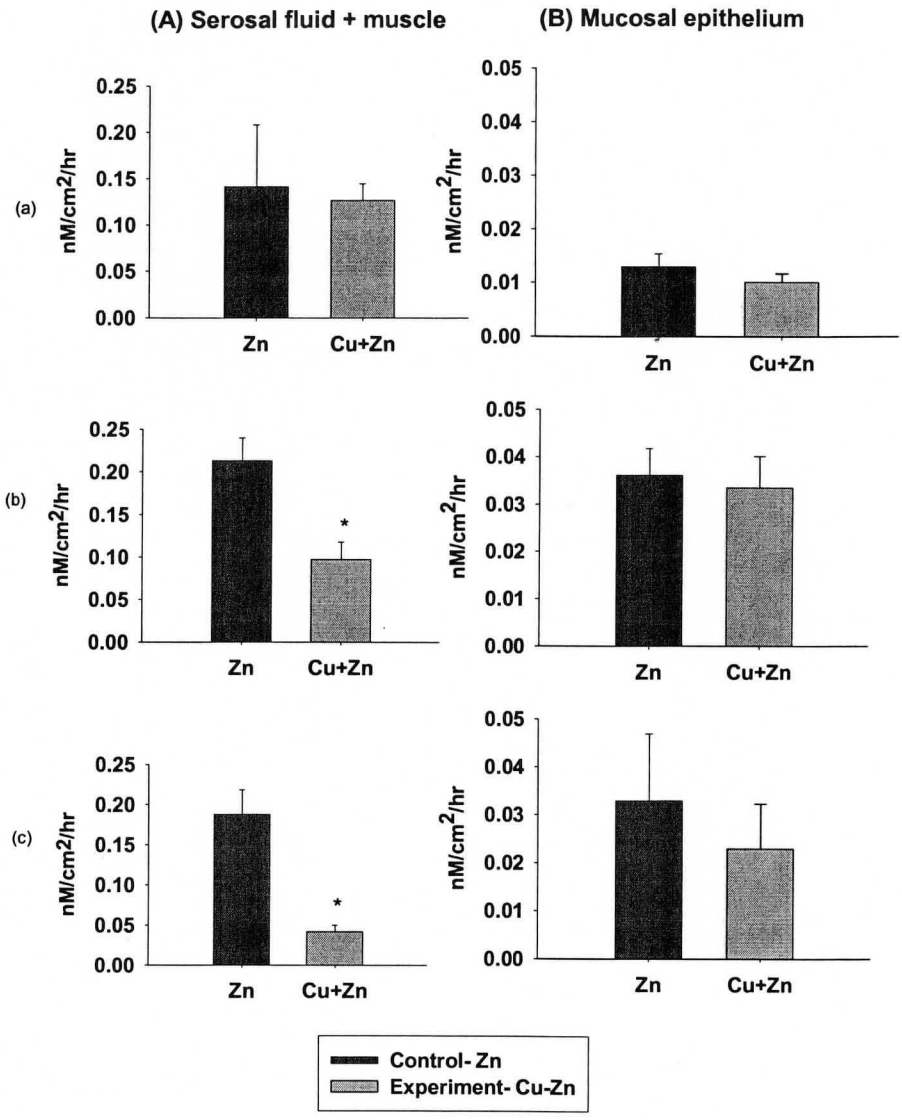
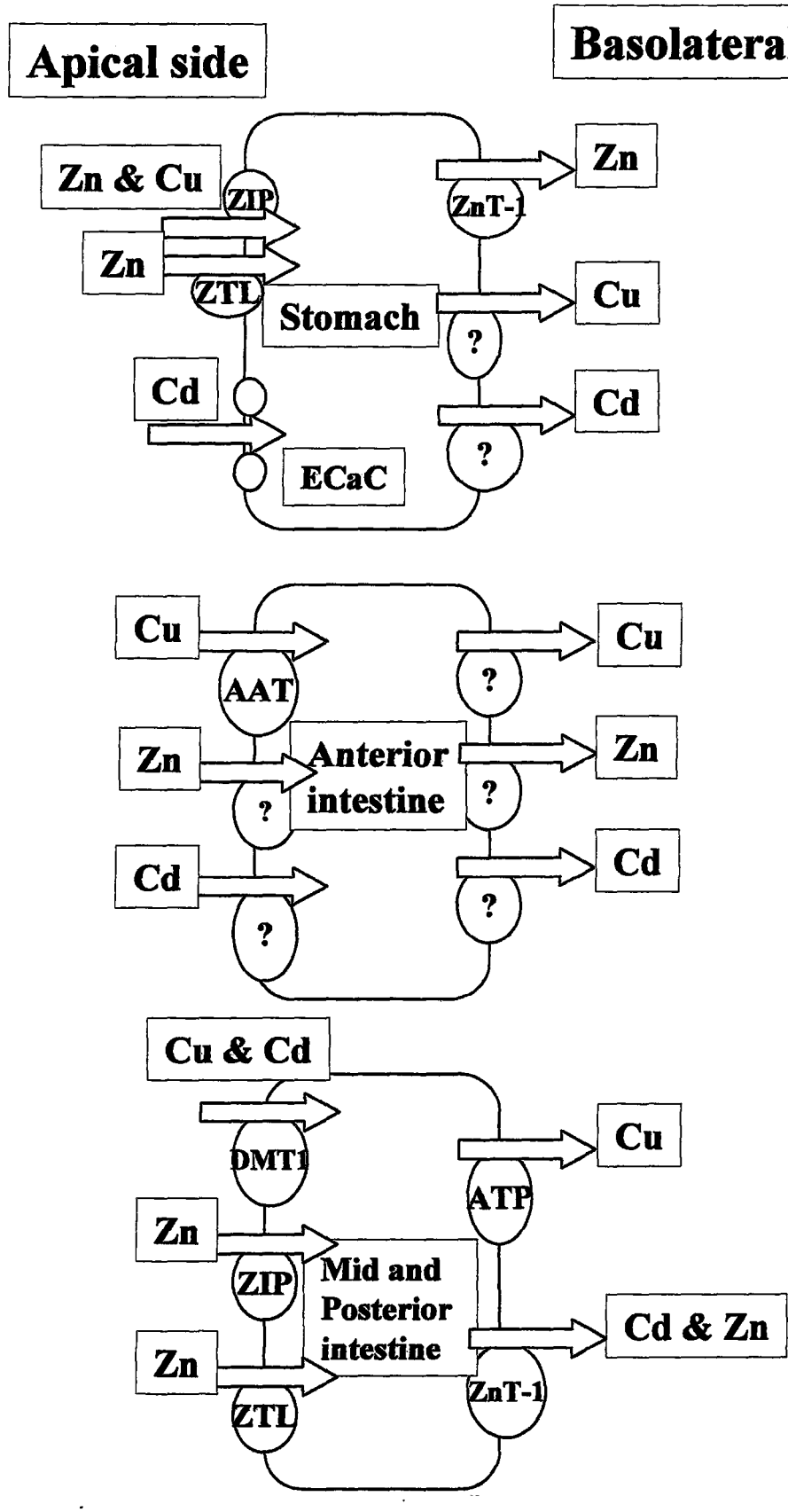

(A) Serosal fluid + muscle
(B) Mucosal epithelium
(a)
(b)
(c)
nM/cm2/hr
0.25
0.20
0.15
0.10
0.05
0.00
0.05
0.04
0.03
0.02
0.01
0.00
Zn
Cu+Zn
*
Control- Zn
Experiment- Cu-Zn

Fig. 3-14

3-15. Schematic diagram of a conceptual model for copper, zinc and cadmium uptake in the trout gastrointestinal tract. At the stomach, copper uptake can be through ZIP while the basolateral export mechanism is unknown, zinc uptake can be through ZIP or ZTL and basolateral export can be through ZnT-1, while cadmium uptake can be through a calcium channel and the basolateral export mechanism is unknown. At the anterior intestine, copper uptake can be through an amino acid transporter and the basolateral export mechanism is unknown. Transport mechanism for both zinc and cadmium uptake and basolateral export are unknown at the anterior intestine. At the mid and posterior intestine, copper and cadmium uptake can be through DMT1 and basolateral export for copper can be through a Cu-ATPase, while cadmium export can be through ZnT-1. Zinc uptake can be through ZIP or ZTL and basolateral export can be through ZnT-1.


Apical side
Basolateral side
Zn & Cu
ZIP
Zn
ZnT-1
Zn
ZTL
Stomach
Cu
?
Cd
Cd
?
ECaC
Cu
Cu
AAT
?
Zn
Anterior
intestine
Zn
?
?
Cd
Cd
?
?
Cu & Cd
Cu
DMT1
ATP
Zn
Mid and
Posterior
intestine
Cd & Zn
ZIP
Zn
ZnT-1
ZTL

Chapter 4

Summary of Results and Conclusions

1.0. Metals uptake via the gastrointestinal tract

The anterior intestine seems to be the most active region of the gastrointestinal tract for water absorption, which agrees well with previous studies from Bergman et al., (2003) and Nadella et al (unpublished). No effects of any of the six studied metals were seen on the fluid transport rates in all the segments of the intestine, which agrees well with studies from Handy et al. (2000) in the African walking catfish and from Wapnir and Steil (1987) in rat. This can also be true at the stomach for all the metals except for copper because there was a significant difference in the fluid transport rate when copper was absent or present. The stomach emerged as a small but important route for copper, zinc, silver and cadmium uptake. There were three different patterns for highest absorptive regions of metals via the gastrointestinal tract.

For Cu, Zn, Ag and Cd – Anterior intestine

Pb - Mid-intestine

Ni - Posterior intestine ≥ Mid-intestine.

Essential metals (copper, zinc and nickel) were absorbed at approximately the same rates as non-essential metals (silver, cadmium and lead) at their highest absorptive region when uptake rate to concentration ratio was taken into consideration.

Nickel exhibited the highest overall rate of net absorption of all metals. Lead exhibited the highest surface mucus binding at the mid-intestine compared to other parts of the intestine in fish, which agrees well with studies from Crespo et al. (1986). Lead

exhibited the highest overall rate of surface mucus-binding while nickel exhibited the least overall rate of surface mucus-binding of all metals. Cadmium exhibited the highest overall rate of accumulation in the mucosal epithelium while nickel exhibited the least overall rate of mucosal accumulation of all metals which signifies that cadmium can block the basolateral exit mechanism Ca-ATPase at the gut (Schoenmakers et al., 1992) similar to its action at the gill (Verbost et al., 1987; 1988; Wicklund-Glynn, 1996). This implied that there is no correlation between rate of absorption and mucosal accumulation rate for cadmium at the mucosal epithelium (Fig. 2-10). But nickel accumulated to a lesser extent at the mucosal epithelium than cadmium, which implied that the rate of movement of nickel at the mucosal epithelium can have an influence on its movement into serosal fluid + muscle (i.e. the absorptive part) (Fig. 2-9).

2.0. Interactions of calcium with cadmium and zinc via the gastrointestinal tract

Calcium inhibited cadmium uptake at the stomach but not at the intestine, suggesting that cadmium uptake at the stomach can be through a calcium-channel but through a different transport mechanism at the intestine. It might also indicate that there is some cadmium transport at the stomach but not at intestine that is sensitive to calcium uptake which agrees well with studies from Franklin et al. (in Press). There was synergistic effect of calcium on zinc uptake at stomach but no effect at the intestine, indicating that zinc transport via the gastrointestinal tract can be through hZTL-1 (Cragg. et al. 2002).

3. 0. Interactions between the essential and non-essential metals (zinc and cadmium) and between two essential metals (zinc and copper) via the gastrointestinal tract.

There was no interaction between zinc and cadmium at the stomach. This indicates that zinc uptake was not exerted at the level of calcium channel at the stomach in constrast to the gill (Hogstand et al., 1996). At the intestine there was antagonistic interaction between cadmium and zinc at the mucosal epithelium (apical entry step) but not at the serosal fluid + muscle (basolateral side), which agrees well with previous studies from Shears and Fletcher, (1983) at the intestine of winter flounder. This finding is also in agreement, at least in part, with demonstrated cadmium and zinc interactions at the apical membrane at the gill (Wicklund-Glynn, 2001; Bentley, 1992) indicating a separate apical mechanism (probably through DMT1 for cadmium) from the basolateral mechanism (which can be through ZnT-1 for both cadmium and zinc) at the intestine. There was a synergistic effect of copper on zinc uptake and vice-versa at the apical entry step in the stomach. This suggests that the same apical transport mechanism is responsible for both zinc and copper uptake at the stomach probably through hZip 2 (Gaither and Eide, 2001a), as in mammals. Antagonistic interaction between copper and zinc at the serosal fluid + muscle (basolateral side), can be explained based on competition for intracellular cytosolic proteins such as metallothionein at the intestine of fish. But at the mucosal epithelium (apical entry step), zinc inhibited copper uptake at the mid-intestine but the reciprocal was not true. This suggests that the transport mechanism for copper is through DMT1 as already shown by Arredondo (et al., 2003) in mammals and suggested by Nadella et al., (unpublished) in trout. This also indicates that zinc

uptake at the intestine is different, perhaps through zinc transporters (such as mZip 4 or ZTL1) as in mammals (Cragg et al., 2002; Kim et al., 2004). A conceptual model for copper, zinc and cadmium via the gastrointestinal tract was presented in Fig. 3-15.

4.0. Nutritional relevance/importance of the findings

These results were of physiological importance in fish in that the in *vitro* technique exploited in this study agrees well with previous *in vivo* study. These results show that at a common level of 50μM tested for each metal via the gastrointestinal tract, essential metals were taken up by almost the same rate as non-essential metals. This finding can be important for environmental risk assessment, in that it can be assumed that, at least in this concentration range, all metals will be taken up at comparable rates regardless of their essentiality or non-essentiality. Thus there will not be large errors in exposure models if a single uptake rate constant is used. Furthermore, at this concentration tested separately for each metal they have no adverse effect on water transport, (and by inference, on ionic transport by the intestine) which indicates that it has no pathological impact at the intestine of fish, which can also be true at the stomach, except for copper. This can be very important for toxicological studies. There is the possibility for dietary metal uptake through the gut to be different from that at the gill.

5.0. Implications for environmental toxicology

Another significance of these findings from a human health and risk assessment perspective lies in the indication that dietary calcium excess may be a significant factor ameliorating risk in cadmium homeostasis at the stomach but not at the intestine. At a

high concentration of dietary calcium and a low concentration of dietary cadmium in the aquatic environment, excess dietary calcium can protect against possible toxicity resulting from cadmium via the gut. This can also be true for the uptake of two essential metals (i.e. copper and zinc) via the gut, in which excess of copper or zinc at higher concentration can prevent the uptake of one another at lower concentration. The present findings helps to show that stomach can be an important site for metal interactions such as for calcium, cadmium and zinc as well as for zinc and copper in fish.

These results were the first to show correlation between surface mucus binding and the rate of absorption for copper, zinc, nickel, silver and lead via the gastrointestinal tract. This information can be used to develop a BLM at the intestinal tract of fish. This can also indicate that mucus can play role in promoting copper, zinc, nickel, silver and lead uptake via the gastrointestinal tract. Moreover it can imply that surface mucus-binding could be a site of toxic action to predict toxic effects of metals via the gastrointestinal tract, which can be implicated in BLM binding sites. This can occur through electrostatic force of attraction between mucus and the essential metals.

6.0. Overview

These results show possible mechanisms for copper, zinc and cadmium transport via the gastrointestinal tract, which in turn can help to gain knowledge into the homeostatic regulations of these metals at both the cellular and organismal levels. And this can also enhance information that can help to protect aquatic organisms. It also helps to bring into our attention that mammalian transporters can also be present in fish. These results help

to show the importance of metals bioavailability and interactions via the gastrointestinal tract of fish at the physiological level.

7.0. Future directions

There is need for further research on the following areas:

(i) Molecular characterization of metals uptake and interaction via the gastrointestinal tract.

(ii) Metals uptake and interactions via the gastrointestinal tract in a concentration dependent manner.

(iii) The role of mucus on metals uptake via the gastrointestinal tract.

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