aquatic toxicology

In vitro analysis of the bioavailability of six metals via the gastro-intestinal tract of the rainbow trout (*Oncorhynchus mykiss*)

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Abstract

An *in vitro* gut sac technique was used to compare the uptake rates of essential (copper, zinc and nickel) and non-essential metals (silver, cadmium and lead) at 50 μmol L^{-1} each (a typical nutritive level in solution in chyme) in the luminal saline in four sections of the gastro-intestinal tract (stomach, anterior, mid and posterior intestines) of the freshwater rainbow trout. Cu, Zn, Cd and Ag exhibited similar regional patterns: on an area-specific basis, uptake rates for these metals were highest in the anterior intestine, lowest in the stomach, and approximately equal in the mid and posterior intestinal segments. When these rates were converted to a whole animal basis, the predominance of the anterior intestine increased because of its greater area, while the contribution of the stomach rose slightly to approach those of the mid and posterior intestines. However, for Pb and Ni, area-specific and whole organism transport rates were greatest in the mid (Pb) and posterior (Ni) intestines. Surprisingly, total transport rates did not differ appreciably among the essential and non-essential metals, varying only from 0.025 (Ag) to 0.050 nmol g^{-1} h^{-1} (Ni), suggesting that a single rate constant can be applied for risk assessment purposes. These rates were generally comparable to previously reported uptake rates from waterborne exposures conducted at concentrations 1–4 orders of magnitude lower, indicating that both routes are likely important, and that gut transporters operate with much lower affinity than gill transporters. Except for Ni, more metal was bound to mucus and/or trapped in the mucosal epithelium than was transported into the blood space in every compartment except the anterior intestine, where net transport predominated. Overall, mucus binding was a significant predictor of net transport rate for every metal except Cd, and the strongest relationship was seen for Pb.
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1. Introduction

There is increasing interest in the uptake and potential toxicity of metals through the gastro-intestinal tract of fish (see Clearwater et al., 2002; Bury et al., 2003; Meyer et al., 2005, for recent reviews). Essential metals such as copper and zinc are required for the normal growth and physiological function of fish, and the diet can be their main supply. Nickel is similarly thought to be essential, based on evidence in terrestrial vertebrates, though definitive evidence is not yet available for fish and other aquatic animals (see Muyssen et al., 2004, for a recent critical review). Non-essential metals such as silver, cadmium and lead have no known physiological function in fish, but can also be acquired from the diet. Both types of metals can origi-

nate from a variety of sources—for example, from domestic and agricultural/aquacultural sources for copper and zinc; industrial processes for copper, zinc, nickel, cadmium and lead; mining, forestry and waste disposal for cadmium; natural leaching and photographic processing for silver; geological weathering, smelting, coal burning, batteries and paint for lead (e.g. Pratap et al., 1989; Farag et al., 1994; World Health Organisation, 1995; Purcell and Peters, 1998; Wood, 2001).

In fish, some studies have indicated that the anterior intestine is a site of high absorptive capacity for copper, zinc and cadmium uptake compared to other parts of the tract, while the stomach in particular has been considered to exhibit low copper, zinc and cadmium absorption (Pentreath, 1976; Shears and Fletcher, 1983; Hardy et al., 1987; Clearwater et al., 2000, 2002; Chowdhury et al., 2004). However, at least with respect to copper, other studies have reached different conclusions, variously emphasizing the importance of the stomach, the mid and posterior intestines (Handy et al., 2000; Kamunde et al., 2002; Nadella et al., 2006a,b). An early study (Hodson et al., 1978) indicated that dietary lead was not absorbed in trout, though more recent

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studies have demonstrated that lead can be absorbed through the gastro-intestinal tract (Crespo et al., 1986; Mount et al., 1994; Alves et al., 2006). Based on accumulation patterns in dietary studies, the anterior intestine appears to be the most important site for lead uptake in trout (Alves and Wood, 2006) and for nickel uptake in whitefish (Ptashynski and Klaverkamp, 2002). Silver is also absorbed from the diet in trout (Galvez and Wood, 1999; Galvez et al., 2001), though the regional sites of uptake have not been investigated. Overall, while it is clear that the gastro-intestinal tract is an important site of metal uptake in fish, no clear picture has emerged as to the regional distribution of uptake capacity for the different metals in a single species.

At a mechanistic level, metal transport via the gastro-intestinal tract likely involves several steps (Campbell et al., 2005). The first step is binding of the metal to the mucus in the lumen, which may either facilitate or retard its uptake (Part and Lock, 1983; Whitehead et al., 1996). The second step involves the transfer of the metal from the lumen into the mucosal epithelium across the apical membranes of the enterocytes, while the third step involves the export of the metal out through the basolateral membranes of the enterocytes into the blood or extracellular fluid. Alternately or additionally, it is possible that metals may move through paracellular channels, thereby bypassing cellular transport mechanisms (Bronner, 1998; Foulkes, 2000).

In recent years, isolated gut sac techniques have proven to be a very powerful approach for studying transport processes in the fish gastro-intestinal tract (e.g. Grosell and Jensen, 1999; Grosell et al., 1999, 2001, 2005; Bury et al., 2001). The preparations generally absorb Na^+ and Cl^- on a net basis, and this process constrains an accompanying osmotic absorption of water. Treatments which inhibit ion transport result in reduced fluid transport (e.g. Grosell et al., 1999). Recently, Nadella et al. (2006b) have demonstrated that *in vitro* preparations from the gastro-intestinal tract of trout remain viable for at least 4 h, transporting both a metal (copper) and fluid at a steady rate over this duration. The technique allows manipulation of both the luminal (mucosal) and serosal media, and the sampling of mucus, the mucosal epithelium (i.e. the enterocytes), and the blood side (muscle tissue and serosal saline), thereby enabling the study of three steps in the transport process (mucus binding; accumulation in the mucosal epithelium; and transport to the blood side). In the present study, we have used this approach to compare the transport of three essential (copper, zinc, nickel) and three non-essential metals (cadmium, silver, lead) in various segments (stomach, anterior, mid and posterior intestines) of the trout gastro-intestinal tract. All metals were presented individually at the same concentration (50 µmol L^{-1}), and fluid transport rate was monitored as an indicator of possible toxicity to ion transport processes.

Our study was designed as a first step to understanding metal uptake in the fish gastro-intestinal tract under standardized conditions, with the long term goal of future mechanistic analyses and ionic competition studies (e.g. Nadella et al., 2006b, 2007), an approach that has been very successful in modeling water-borne metal toxicity at fish gills (e.g. Playle et al., 1993; Di Toro et al., 2001; Playle, 2004; Niyogi and Wood, 2004). Based on the general belief that nutritive (essential) metal uptake in the fish gut may be facilitated by specific carriers (Bury et al., 2003), we hypothesized that essential metals would be taken up at a higher rate than non-essential metals. We further hypothesized that there would be regional differences in the uptake of various metals, due to differential distribution of these specific carriers or differences in passive permeability. Finally, we hypothesized that the rate of metal accumulation in the various compartments (mucus, mucosal epithelium, blood side) would differ among the various metals. For example, mucus might facilitate the uptake of essential metals and retard the uptake of non-essential metals, while the latter might reduce their own uptake by inhibiting specific transporters. In this regard, cadmium has been shown to have a high affinity for epithelial mucus (Part and Lock, 1983), to increase the rate of mucus secretion (Gardner and Yevich, 1970; Glover and Hogstrand, 2003), and to inhibit basolateral Ca^{2+}-ATPase in fish enterocytes (Schoenmakers et al., 1992).

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*Oncorhynchus mykiss*, $N = 101$), ~250 g (~30 cm) were obtained from Humber Springs Fish Hatchery (Orangeville, Ont.). Fish were maintained in 500 L tanks with flowing aerated and dechlorinated Hamilton city tap water from Lake Ontario (approximate ionic composition in mmol L^{-1}: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8–8.0, dissolved organic carbon ~ 3 mg C L^{-1}, hardness ~140 mg L^{-1} as $CaCO_3$). The fish were fed a maintenance ration of Martin's commercial dried trout pellet feed five times per week at 1% body weight per feeding. Manufacturer's specifications include: crude protein 41%, crude fat 11%, crude fibre 3.5%, calcium 1%, phosphorus 0.85%, sodium 0.45%, vitamin A 6800 IU kg^{-1}, vitamin D2 100 IU kg^{-1}, vitamin E 80 IU kg^{-1} (Martins Mills Inc., Elmira, Ont.). Metal contents of the food were 27 µg g^{-1} dry wt copper, 173 µg g^{-1} zinc, 0.26 µg g^{-1} cadmium, 10 µg g^{-1} lead, 0.05 µg g^{-1} silver and 3.9 µg g^{-1} nickel. Water temperature was maintained between 11 and 13 °C. Fish were starved for three days prior to the experiments.

2.2. Experimental solutions

In vivo dietary studies have recently shown that the normal concentration of the essential metals copper (Nadella et al., 2006a) and nickel (E. Leonard, personal communication) in the fluid phase of the gastro-intestinal chyme of trout fed on the standard diet used here are 5–70 and 3–32 µmol L^{-1}, respectively, the variation reflecting regional heterogeneity in various sections of the digestive tract. Glover and Hogstrand (2003) studied intestinal zinc uptake at 50 µmol L^{-1} in rainbow 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. Therefore, a standard concentration 50 µmol L^{-1}, in the nutritive, non-toxic range and representative of normal gastro-

intestinal fluid levels for the essential metals, was chosen for all the metals (copper, zinc, nickel, silver, cadmium and lead) to compare their uptake rates via the gastro-intestinal tract of rainbow trout.

Modified Cortland saline (Wolf, 1963) in which chloride was replaced by sulphate was used for all the metals because silver was found to visibly precipitate with chloride (cloudiness) in chloride-based saline and there was potential for other metals to form similar perhaps undetectable precipitates. At least for copper, Nadella et al. (2007) have shown that this saline yields the same uptake rates in intestinal sac preparations as for traditional chloride-based saline. The basic composition of this saline was (in mmol L^{-1}): Na$_2$SO$_4$, 66.5; K$_2$SO$_4$, 2.5; CaSO$_4$, 1; MgSO$_4$·7H$_2$O, 1.9; NaHCO$_3$, 1.9; NaH$_2$PO$_4$·H$_2$O, 2.9; glucose, 5.5, pH 7.4. Osmolality was adjusted to 276 mOsm kg^{-1} by adding mannitol. In all experiments, this saline was used on the serosal surface, while the same saline spiked with the appropriate metal was used on the luminal (mucosal) surface, so transport was studied under symmetrical conditions. The pH of the luminal saline was checked and set back to 7.4 after metal addition using NaOH. With the exception of SO$_4{}^{2-}$, the concentrations of all ions fall within the ranges observed for chyme fluid sampled *in vivo* from trout kept on a comparable diet and in similar water quality (Bucking and Wood, 2006, 2007).

In separate experiments, luminal saline was spiked with 50 µmol L^{-1} (nominal concentration) of six different soluble metal salts (all analytical grade), of which five were radio-labeled: ^{64}Cu(NO$_3$)$_2$ (McMaster Nuclear Reactor, Hamilton, Ont., Canada), ^{65}ZnCl$_2$ (Los Alamos National Laboratory, Los Alamos, NM, USA), ^{110m}AgNO$_3$ (Risф National Laboratory Radiation Research Department, Denmark), ^{109}CdCl$_2$ (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA), ^{63}NiCl$_2$ (Perkin-Elmer Life and Analytical Sciences) and non-labeled Pb(NO$_3$)$_2$. Final radioactivities were >1 µCi mL^{-1} for the radiolabeled solutions. Actual measured total metal concentrations of the luminal solutions used in the tests were: copper 43.6 µmol L^{-1}, zinc 57.4 µmol L^{-1}, silver 48.8 µmol L^{-1}, cadmium 46.8 µmol L^{-1}, nickel 77.2 µmol L^{-1} and lead 37.7 µmol L^{-1}. All measured metal concentrations in the final luminal salines were therefore reasonably close to the nominal target of 50 µmol L^{-1}, and for the purpose of comparison, final calculated transport rates were normalized to 50 µmol L^{-1} by multiplying the measured transport rate by the ratio of 50 µmol L^{-1} to the measured concentration.

2.3. Experimental techniques

An *in vitro* stomach and gut sac technique similar to that used by Bury et al. (2001) and Grosell et al. (1999, 2001) in marine teleost fish and adapted by Nadella et al. (2006b, 2007) for freshwater rainbow trout was followed. This technique allows separate study of the stomach, anterior, mid and posterior intestines, and the sampling of mucus, the mucosal epithelium (i.e. the enterocytes), and the blood side (muscle tissue and serosal saline) in each, thereby enabling the study of three steps in the transport process (see Section 1).

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 gastro-intestinal tract. The tract was then placed immediately in a Petri dish for dissection on ice, and bathed with the modified Cortland saline solution. The liver and gall bladder were removed after tying off the bile duct that enters just posterior to the stomach with a ligature. Gut contents were squeezed gently from the stomach and intestine. Visceral fats were removed from the entire gastro-intestinal tract with forceps. The gastro-intestinal tract was flushed with modified Cortland saline to remove food and faeces and then sectioned into stomach, anterior, mid and posterior intestines. 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 intestinal gut sacs, the regional division of the intestine was made along obvious morphological differences (i.e. the size and colour of each segment) for the posterior section, while the remaining portion between the pyloric aperture and posterior region was split into anterior and mid-intestinal segments based on the presence of cecae in the anterior intestine only. One end of each segment was sealed tightly with surgical silk and into the other end a 5-cm piece of PE-160 (for stomach) or PE-50 (for intestine) polyethylene tubing was inserted and tied with silk ligature to allow for administration and sampling of luminal saline.

To these gut sacs, 1 mL of appropriate luminal saline containing approximately 50 µmol L^{-1} of the metal of interest labeled with one of ^{64}Cu, ^{65}Zn, ^{110}Ag, ^{109}Cd or ^{63}Ni was injected via the syringe into the sac. A sub-sample was taken for initial analysis along with a sample of serosal saline. As there was no readily available radioisotope for lead, "cold" lead (50 µmol L^{-1}) was used. A separate control series with metal-free luminal saline was also performed. The PE tubing was sealed and the sacs were weighed (0.1 mg accuracy) for initial weight. The sacs were then transferred into 40-mL (for the stomach and anterior intestine) or 12-mL Falcon^{TM} tubes (for the mid and posterior intestines) for incubation in serosal saline (i.e. metal-free) which was bubbled constantly with a 99.5% O$_2$, 0.5% CO$_2$ gas mixture because *in vivo* PCO$_2$ levels in the blood are approximately 3.75 Torr. The temperature was maintained at 11–13 °C.

After incubation of stomach gut sacs for 4 h and the intestinal gut sacs for 2 h they were re-weighed for final weight to allow calculation of fluid transport rate (Section 2.6, Eq. (1)). The luminal saline was removed and taken for counting or lead analysis, and a sub-sample of the serosal saline was also analyzed for gamma radioactivity (^{64}Cu, ^{110}Ag, ^{65}Zn, ^{109}Cd) or beta radioactivity (^{63}Ni) or lead concentration. All lead samples were measured by graphite furnace atomic absorption spectrophotometry (GFAAS).

The gut sacs were cut open, washed in 5 mL of modified Cortland saline and then with 5 mL of 1 mmol L^{-1} EDTA disodium salt solution, and then blotted dry with a small piece of paper towel. The washing solutions plus blotting paper were collected for analysis as the "mucus-bound fraction"; this procedure ensured removal of mucus and its associated metal burden, so that the metal incorporated into the gut tissue could be considered to represent the actual metal which had been absorbed.

The mucosal epithelium (i.e. the enterocytes) was then scraped off gently with a glass slide and collected separately, representing metal that had been absorbed across the apical surface of the enterocytes but not exported to the blood. This left behind the submucosa, muscle layers and serosa, collectively referred to here as the "muscle layer". This, combined with the serosal saline, comprises the "blood compartment", representing metal that had been exported across the basolateral surface of the enterocytes. The exposed surface area of each segment 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 attached cecae could not be measured so the surface area measurements for this segment were undoubtedly underestimates. The wash solutions plus blotting paper, the mucosal epithelial scrapings, and the muscle layer were analyzed separately for radioactivity or lead burdens.

For lead, the blotting paper and the muscle layer were digested separately in five volumes of 1N HNO$_3$ (Trace Metal Grade, Fisher Scientific, Toronto, Ont., Canada) and then placed in an oven at 60 °C for 48 h in sealed 40-mL Falcon^{TM} tubes. The tubes were then centrifuged, and the supernatants were assayed for lead on the graphite furnace (GFAAS). Serosal saline, luminal saline, saline rinse plus EDTA rinse, and epithelial scrapings were acidified with 1N HNO$_3$ and similarly assayed by GFAAS.

For nickel, where the radioisotope is a beta-emitter rather than a gamma-emitter, 1-mL sub-samples taken from serosal saline, luminal saline, saline rinse plus EDTA rinse and epithelial scrapings were acidified with 1N HNO$_3$ to solubilize the metal and then vortexed. Five milliliters of the scintillant Ultima Gold (Perkin-Elmer) was added. The blotting paper and muscle layer were digested in five volumes of 1N HNO$_3$ at 60 °C for 48 h as described above for lead. One milliliter of supernatant was added to 5 mL of Ultima Gold. All samples were kept in the dark for 5 h to eliminate chemiluminescence before scintillation counting.

2.4. Radioactivity counting

The radioisotopes ^{64}Cu, ^{65}Zn, ^{109}Cd and ^{110m}Ag are gamma-emitters, while ^{63}Ni is a beta-emitter. The gamma radioactivities were measured on a Minaxi-γ Auto gamma 5530 counter (Canberra Packard, Mississauga, Ont., Canada) using energy windows of 433–2000 keV for ^{64}Cu, 15–2000 keV for ^{65}Zn and 15–150 keV for ^{109}Cd. ^{64}Cu was corrected for decay to a common reference time, because it has a very short half life (12.9 h). For ^{110m}Ag, the gamma radioactivity was counted in a specialized window of 1050–2000 keV 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 of ^{63}Ni was counted in the 8–110 keV window on an LKB Wallac 1217 Rackbeta scintillation counter (Pharmacia-LKB AB, Helsinki, Finland). Scintillation counts for ^{63}Ni were corrected for quenching by using a quench curve constructed for the tissues of interest based on the external standard ratio method.

2.5. Atomic absorption spectrophotometry

The concentrations of copper, nickel, silver, cadmium and lead in the luminal saline (and in all compartments for lead) were measured by graphite furnace atomic absorption spectrophotometry (Varian Spectra AA-20 with graphite tube atomizer [GTA-110], Mulgrave, Australia). Zinc concentration was measured by flame atomic absorption spectrophotometry (FAAS; Varian Spectra-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 (Toronto, Ont., Canada), and for cadmium and lead by Sigma–Aldrich (St. Louis, MO, USA).

2.6. Calculations

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

$$FTR = \frac{\text{Change in weight of the gut sac}}{\text{ISA} \times T} \quad (1)$$

where weight change (in mg) was measured gravimetrically, ISA the intestinal surface area of each gut sac in square centimetres and T is the incubation time in hours. This produced the fluid transport rate expressed as $\mu L \, cm^{-2} \, h^{-1}$.

Copper, zinc, nickel, silver, cadmium and lead uptake rates were calculated using Eqs. (2) and (4). The dilution factors associated with digestion were taken into consideration for lead and nickel.

For each preparation, three compartments of metal fate were measured. Firstly, the rinse (i.e. blotting paper, saline rinse plus EDTA rinse) represents metals that were bound to the mucus. Secondly, epithelial scrapings represent metals in mucosal epithelial cells (i.e. enterocytes). Thirdly, serosal fluid + muscle represent a conservative estimate of true metal transport—i.e. metals that had been transported through the enterocytes into the blood compartment.

Uptake rates into the rinse, the epithelial scrapings, and the serosal fluid plus muscle compartments were calculated for the radiolabeled metals (i.e. copper, zinc, nickel, silver and cadmium) in a very similar manner to that used by Glover et al. (2003a,b) for zinc uptake as follows:

$$\text{Metal uptake rate} = \frac{\text{Compartment cpm}}{\text{SA} \times \text{ISA} \times T} \quad (2)$$

Compartment cpm represents the total ^{64}Cu, ^{110m}Ag, ^{109}Cd, ^{65}Zn or ^{63}Ni activity of the relevant compartment measured on the gamma or scintillation counter, taking all volumes into account. SA is the initial specific activity of the luminal saline (cpm nmol^{-1}) calculated as:

$$SA = \frac{\text{Activity}}{[M]} \quad (3)$$

where activity is in cpm mL^{-1} as measured on the gamma or scintillation counter and [M] is the concentration of each metal

(copper, zinc, nickel, silver and cadmium) in nmol mL^{-1} as measured by GFAAS or FAAS. This produced copper, zinc, nickel, silver and cadmium uptake rates as nmol cm^{-2} h^{-1} in Eq. (2).

Lead uptake rate measured by GFAAS was calculated in an analogous fashion as:

$$\text{Pb uptake rate} = \frac{\text{Compartment Pb}}{\text{ISA} \times T} \quad (4)$$

where Compartment Pb is the total lead burden in each compartment (in nmol) taking volumes and dilution and digestion factors into account. This produced lead uptake rate expressed as nmol cm^{-2} h^{-1} in Eq. (4).

2.7. Statistical analysis

Plotted values represent the means (±S.E.M.) with $N=10$ for copper and cadmium, $N=9$ for lead, $N=8$ for zinc, silver and nickel and the control series at the stomach and $N=8$ for each treatment at anterior, mid and posterior intestines. The significance of differences for each metal among the four segments of the gastro-intestinal tract was assessed using one way analysis of variance (ANOVA) followed by a post hoc least significant differences (LSD) test to identify individual differences. For this comparison on figures, means sharing the same lower case letters are not significantly different. Similarly, the significance of differences among the six metals within each of the four segments of the gastro-intestinal tract was assessed using the same approach. For this comparison, means sharing the same upper case letter are not significantly different. Regression lines were fitted by the method of least squares, and the significance of the correlation coefficient assessed. Analysis was performed using SPSS software, and a significance level of $P<0.05$ was used throughout.

3. Results

3.1. Fluid transport rates

Fluid transport rates were always in the direction of net fluid absorption, and were higher in the anterior intestine than in the other three segments of the gastro-intestinal tract, regardless of the presence or absence of any of the six metals (Fig. 1). Overall control mean values were $1.72 \pm 0.18 \, \mu L^{-1} \, cm^{-2} \, h^{-1}$ at the stomach, $9.88 \pm 0.68 \, \mu L \, cm^{-2} \, h^{-1}$ at the anterior intestine, $2.95 \pm 0.15 \, \mu L \, cm^{-2} \, h^{-1}$ at the mid-intestine and $2.07 \pm 0.59 \, \mu L \, cm^{-2} \, h^{-1}$ at the posterior intestine, respectively. Values were very similar when each of the metals was present. Indeed the only significant effect associated with the presence of any of the metals in any segment was for copper at the stomach, which caused a mild inhibition of fluid absorption, as indicated by capital letters in Fig. 1A. For copper and nickel, the fluid transport rate was higher at the mid-intestine than at the stomach, as indicated by the small letters in Fig. 1B, but for other metals, the fluid transport rates were the same at the stomach and mid-intestine.
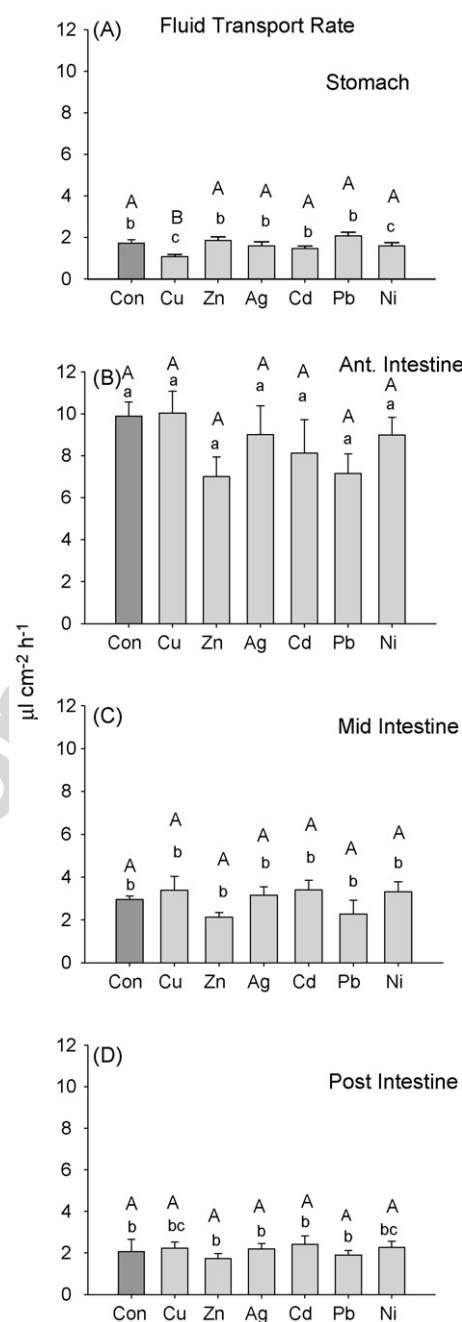

Fig. 1. Fluid transport rates ($\mu L \, cm^{-2} \, h^{-1}$) in the presence of each metal at nominally 50 μmol L^{-1} in the luminal compartment at: (A) stomach, (B) anterior intestine, (C) mid-intestine and (D) posterior intestine. Plotted values represent the means (±S.E.M.) of $N=10$ for copper and cadmium, $N=9$ for lead and $N=8$ for zinc, silver, nickel and the control series at the stomach and $N=8$ for all treatments at the anterior, mid and posterior intestines. The significance level was tested at $P<0.05$ using one-way ANOVA analysis with post hoc LSD analysis using SPSS software. The small letters represent a comparison of each metal treatment among the four regions of gastro-intestinal tract. The capital letters represent a comparison among the various metal treatments within an individual region of the tract. Means sharing the same small letters within a treatment are not significantly different among regions. Means sharing the same capital letters within a region are not significantly different among metal treatments.

3.2. Transport rates of metals into the blood compartment

The rate of penetration 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 metal transport (i.e. all the way through the enterocytes into the blood compartment; see Section 2).

For copper, zinc, silver and cadmium there was a common pattern with highest rate of absorption at the anterior intestine, lower and approximately equal rates of absorption at the mid and posterior intestines and lowest rates of absorption at the stomach, though not all of the differences were significant (as indicated by the small letters in Fig. 2). Typical transport rates for copper, zinc, silver and cadmium at the stomach were 0.012 ± 0.002, 0.004 ± 0.001, 0.008 ± 0.001 and 0.012 ± 0.003 nmol cm^{-2} h^{-1}, respectively (Fig. 2). In the intestine, the typical transport rates for these four metals were about 0.20 ± 0.04 nmol cm^{-2} h^{-1} at the anterior segment, 0.05 ± 0.01 nmol cm^{-2} h^{-1} at the mid-segment and 0.03 ± 0.01 nmol cm^{-2} h^{-1} at the posterior segment (Fig. 2).

Lead, on the other hand, exhibited the highest absorptive rate at the mid-intestine (0.35 ± 0.09 nmol cm^{-2} h^{-1}), 8-fold higher than in the anterior intestine (0.04 ± 0.01 nmol cm^{-2} h^{-1}), 2-fold higher than in the posterior intestine (0.15 ± 0.04 nmol cm^{-2} h^{-1}) and about 35-fold higher than in the stomach (0.01 ± 0.001 nmol cm^{-2} h^{-1}) as indicated by the small letters in Fig. 2.

Finally, for nickel, net transport rate was highest at the posterior intestine (0.27 ± 0.02 nmol cm^{-2} h^{-1}), lower at the mid-intestine (0.22 ± 0.03 nmol cm^{-2} h^{-1}), still lower at the anterior intestine (0.13 ± 0.04 nmol cm^{-2} h^{-1}) and lowest at the stomach (0.01 ± 0.002 nmol cm^{-2} h^{-1}) as indicated by the small letters in Fig. 2. Thus, comparing across metals within each segment, the transport rate of lead was generally lower than the other metals at the anterior intestine and higher than the other metals at the mid and posterior intestines (except for nickel at the posterior intestine), as indicated by capital letters in Fig. 2. Nickel transport rate was similar to the other metals at the anterior intestine, but significantly higher than the other metals (except for lead) at the mid and posterior intestines, as indicated by the capital letters in Fig. 2.

3.3. Binding of metals to the surface mucus

Metals collected on the blotting paper and wash solutions (i.e. saline plus EDTA rinse) represent metals that were bound to surface mucus.

Among the four segments of the gastro-intestinal tract, copper, zinc and silver all bound to mucus to a greater extent at the anterior intestine compared to the stomach, as indicated by small letters in Fig. 3. However, for cadmium and lead, the mid-intestine was the highest region of mucus binding. Nickel exhibited a higher mucus binding at the mid and posterior intestines compared to the stomach and anterior intestine.

Comparing the six metals within each segment, copper, silver, cadmium and lead binding rates to the surface mucus of the stomach were higher than those of zinc and nickel, as indicated
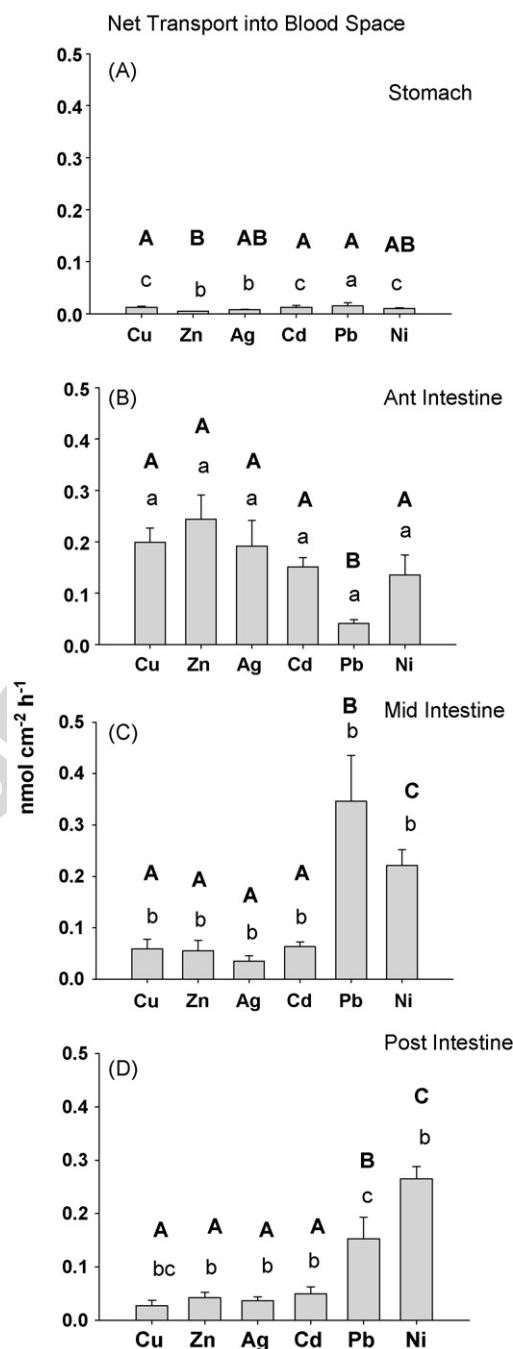

Net Transport into Blood Space

Fig. 2. Rates of net transport (nmol cm^{-2} h^{-1}) into the blood space (i.e. serosal fluid plus muscle) for each of the metals at nominally 50μmol L^{-1} at: (A) stomach, (B) anterior intestine, (C) mid-intestine and (D) posterior intestine. Values represent the means ($\pm$S.E.M.). Other details and statistical conventions as in legend of Fig. 1.

by capital letters in Fig. 3. 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 for the other metals (Fig. 3). At the mid-intestine, lead and cadmium were bound to the mucus to a much greater extent (about four- to eight-fold) over other metals, as indicated by the capital letters in Fig. 3. At the posterior intestine, lead was bound to the surface mucus by a significantly greater extent, two- to five-fold over other metals.
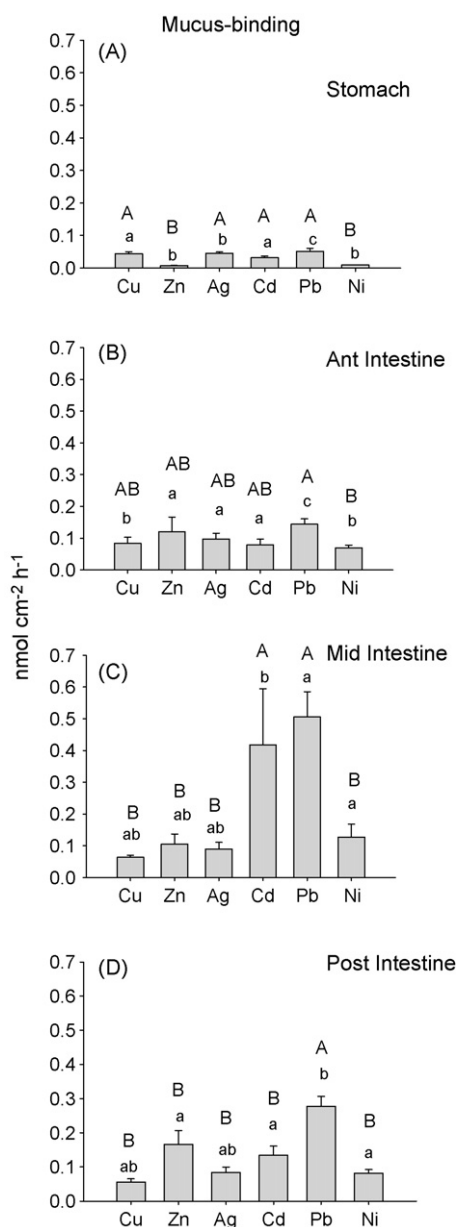
Fig. 3. Rates of binding (nmol cm^{-2} h^{-1}) to the mucus (i.e. blotting paper plus rinse solutions) for each of the metals at nominally 50 μmol L^{-1} at: (A) stomach, (B) anterior intestine, (C) mid-intestine and (D) posterior intestine. Values represent the means (±S.E.M.). Other details and statistical conventions as in legend of Fig. 1.

Fig. 4. Rates of accumulation (nmol cm^{-2} h^{-1}) in the mucosal epithelium for each of the metals at nominally 50 μmol L^{-1} at: (A) stomach, (B) anterior intestine, (C) mid-intestine and (D) posterior intestine. Values represent the means (±S.E.M.). Other details and statistical conventions as in legend of Fig. 1.

3.4. Transport rates of metals into the mucosal epithelium.

The rate of accumulation of metals at the mucosal epithelium (i.e. enterocytes) essentially represents a snapshot of the metal passing through this compartment and may provide an indication of the rate of the apical entry step if basolateral export is the rate-limiting step.

Among the four segments of the gastro-intestinal tract, copper, zinc and silver exhibited similar mucosal accumulation rates at the anterior, mid and posterior intestines, and these tended to be higher than in the stomach, as indicated by small letters in Fig. 4. For cadmium especially, and also for lead, the highest
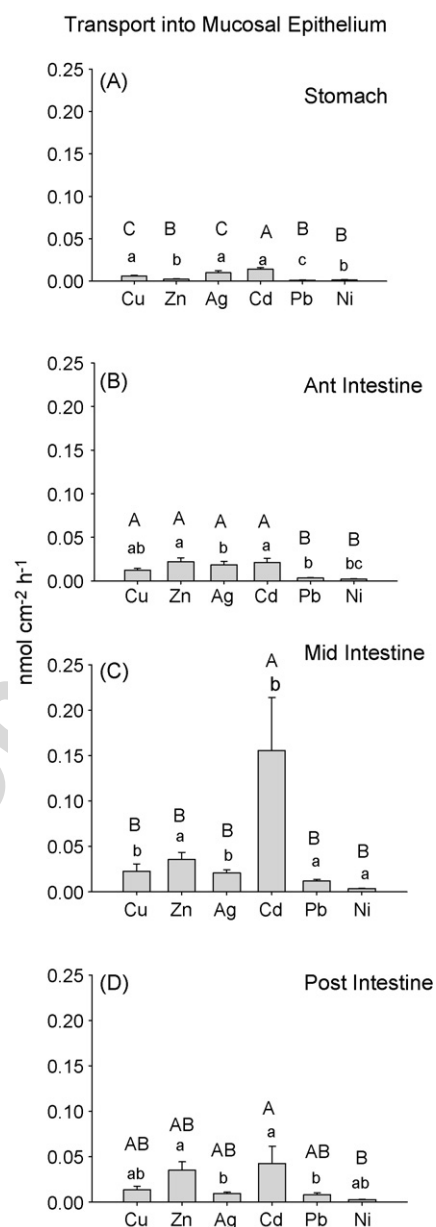
mucosal accumulation rate was at the mid-intestine compared to other parts of the gastro-intestinal tract. For nickel, there appeared to be more or less the same mucosal accumulation rates in all the four segments of the gastro-intestinal tract, as indicated by small letters in Fig. 4. Mucosal accumulation rates were particularly low for lead and zinc in the stomach.

For comparison of all the metals within each segment, cadmium accumulated to a greater extent than other metals at the stomach, as indicated by the capital letters in Fig. 4. But at the anterior intestine, copper, zinc, silver and cadmium were accumulated to a significantly greater extent than lead and nickel. At the mid-intestine, cadmium had by far the highest mucosal accumulation compared to other metals, as indicated by capi-
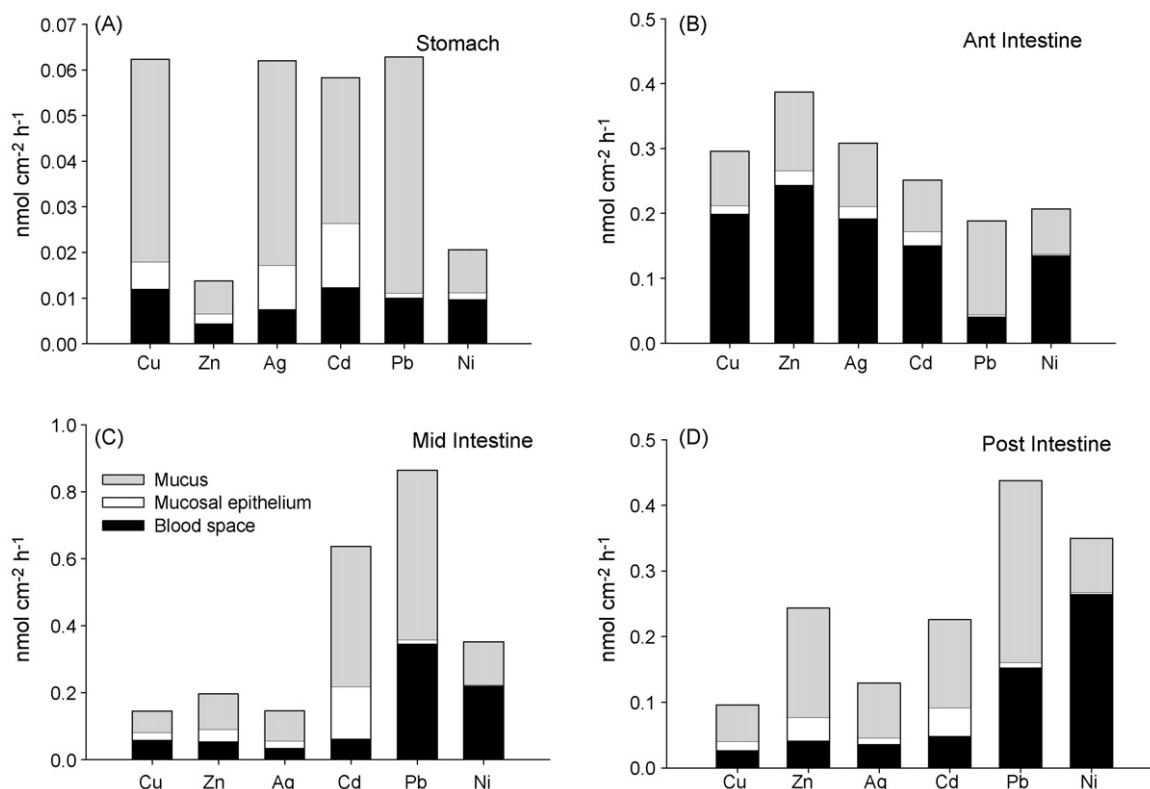
Fig. 5. Mean partitioning among the three compartments (mucus, mucosal epithelium and blood space) for each of the six metals at: (A) stomach, (B) anterior intestine, (C) mid-intestine and (D) posterior intestine. At the stomach, $N = 10$ for copper and cadmium, $N = 9$ for lead and $N = 8$ for zinc, silver and nickel. At the anterior, mid and posterior intestines, $N = 8$ for all the metals.

tal letters in Fig. 4. At the posterior intestine, the accumulation rates were generally the same among the metals, but there was a significant difference between cadmium and nickel.

3.5. Partitioning of metals among the three compartments

The distribution among the three measured compartments for each of the metals is illustrated in Fig. 5; note the differences in scale used for the various compartments. The term "blood compartment" is used to refer to metal transported through the enterocytes into the muscle tissue and serosal fluid (i.e. the data of Fig. 2).

At the stomach, much greater amounts of copper, silver, cadmium and lead were bound to the surface mucus than were transported to other compartments (Fig. 5). At the mucosal epithelium, cadmium, silver and copper showed higher accumulation than the other metals. For transport into the blood space, copper, cadmium, silver, lead and nickel exhibited similar values, but zinc tended to be lower.

At the anterior intestine, all the metals were bound to the mucus by almost the same proportion, which was much greater than in the mucosal epithelium, but less than one-third of the fraction transported into the blood space (Fig. 5). The one exception was lead, where the fraction in the blood space was only about 25% of the fraction bound to mucus.

At the mid-intestine, cadmium and lead showed much greater portions that were bound to the mucus than for copper, zinc, silver and nickel, and this fraction was relatively greater for

all metals than it had been in the anterior intestine (Fig. 5). Thus, the fraction transported into the blood space was less than 50% of the total, except in the case of nickel, where it predominated. Notably, accumulation in the mucosal epithelium was again relatively small, except in the case of cadmium.

At the posterior intestine, fractional distribution patterns were similar to those in the mid-intestine, though the mucus-bound fraction was larger for zinc. Again, uptake into the blood compartment was less than 50% of the total, except in the case of nickel where it predominated. Accumulation in the mucosal epithelium was a generally small percentage of the total, except for cadmium and zinc.

Several notable trends in all gut segments were that the mucosal epithelium fraction was always very small for nickel, and was always greater for cadmium than for the other metals. For nickel, transport into the blood space was always the dominant fraction. For the other metals, binding to mucus generally predominated in the stomach, mid and posterior intestines, while transport into the blood space predominated in the anterior intestine. In general, accumulation in the mucosal epithelium was small relative to the other two compartments for all six metals.

4. Discussion

4.1. Fluid transport rates

Fluid absorption by the fish gastro-intestinal tract is driven by active ion transport (House and Green, 1965; Loretz, 1995),

and the anterior intestine is usually the most active area in this regard due to the presence of the cecae (Buddington and Diamond, 1986, 1987; Buddington et al., 1997; Bergman et al., 2003). The present results (Fig. 1) agree with this pattern. For the essential metals (copper, zinc and nickel), the concentrations used in our experiments (50 μmol L^{-1}) were in the typical nutritive range normally found in the dissolved phase of chyme (see Section 2.2). The fact that none of the metals at this concentration, whether essential or non-essential (lead, cadmium, silver) altered fluid transport rates to any great extent (the one exception was copper in the stomach) indicates that there were likely few if any toxic effects on ion transport processes, and that differences in metal transport rates were not due to differences in fluid transport rates (e.g. by solvent drag) or confounded by pathological responses.

4.2. Comparison of metal uptake rates among different regions of the gastro-intestinal tract

Using transport into the blood space as a conservative measure of net uptake rate (Fig. 2), it is clear that the stomach always exhibited the lowest rates for all six metals on an area-specific basis, but that there were three different patterns in the intestine. For copper, zinc, silver and cadmium, area-specific rates were several-fold higher in the anterior intestine relative to either the mid or posterior intestinal segments, where rates were approximately equal. However, this was not true for lead or nickel, where the mid (particularly for lead) and posterior (particularly for nickel) sections dominated on an area-specific basis. Thus, our original hypothesis that there would be regional differences in the uptake rates of various metals was substantiated. Area measurements are undoubtedly underestimates as they do not take into account cecae (a particular problem for the anterior intestine) or villi and micro-villi (problems for all segments). From the point of view of the whole animal, a more realistic measure is the uptake rate through the whole of each segment, and this has been estimated in Table 1 using the actual area measurements for these ~250 g trout, and then summed and converted to a mass-specific basis for the total uptake rate. Clearly the pattern of dominance for the anterior intestine with respect to copper, zinc, cadmium and silver uptake becomes even more pronounced because this has the greatest area, the contribution of the stomach increases slightly, while the relative contributions of the mid and posterior intestinal segments remain about equal to one another, and generally comparable to or greater than the stomach (Table 1). For lead and nickel, the mid and posterior segments,

respectively, still dominate, but the contribution of the anterior intestine increases in importance because of its greater area.

The overwhelming dominance of the anterior intestine for the transport of copper, zinc, cadmium and silver is not surprising, in view of its size and the general belief that the activities of most nutrient and ion transport mechanisms are greatest in this segment, though some exceptions have been reported (see Section 1). No attempt was made to characterize the transporters involved either kinetically, pharmacologically, or by competition experiments in the present study. However, based on knowledge of uptake mechanisms at the freshwater teleost gill (reviewed by Wood, 2001; Bury et al., 2003), we might anticipate copper and silver to behave similarly and to be transported by a common mechanism (the "sodium pathway"), and by the same token, we might anticipate zinc and cadmium to behave similarly and to be transported by a separate mechanism (the "calcium pathway"). However, recent studies on the trout intestine which have examined the interactions of these four metals suggest that the situation may be very different from, and far more complex than at the gill (Glover and Hogstrand, 2003; Nadella et al., 2006b; Wood et al., 2006). The reader is referred to these and other recent studies on trout (Clearwater et al., 2000, 2002, 2005; Glover and Hogstrand, 2002a,b; Glover et al., 2003a,b; Burke and Handy, 2005; Nadella et al., 2006a, 2007) and other species (Handy et al., 2000; Hogstrand et al., 2002; Glover et al., 2003a,b) for recent progress on the mechanisms by which these metals might be absorbed. Suffice it to say that as in the mammalian gastro-intestinal tract, uptake appears to be mediated by specific channels and carriers at both apical and basolateral surfaces of the enterocytes, and some but not all of these mechanisms appear to be homologous to mechanisms reported in higher vertebrates. Notably however, even in mammals, many mechanisms still remain in dispute or unresolved.

More surprising was the importance of the mid and posterior intestines for the transport of lead and nickel. Based on lead and nickel accumulation patterns during chronic feeding experiments (Ptashynski and Klaverkamp, 2002; Alves and Wood, 2006), the anterior intestine was expected to dominate, though at least for lead, actual concentrations were fairly similar in all tissues of the tract (Alves and Wood, 2006). Nothing is known about the mechanisms of uptake of these two metals in fish, but in mammals, the proton-coupled divalent metal transporter variously known as DCT1, DMT1 or Nramp2 has been implicated in the transport of both metals (Gunshin et al., 1997). Absorption by pinocytotic processes occurs in the posterior intestine in trout (Georgopoulou et al., 1985, 1986) and offers another possibility.

Table 1
Total net transport of metals into the blood space in each segment of the gastro-intestinal tract for ~250 g rainbow trout

	Copper	Zinc	Cadmium	Silver	Lead	Nickel
Stomach (nmol h^{-1})	0.56 ± 0.08	0.21 ± 0.02	0.55 ± 0.11	0.38 ± 0.06	0.44 ± 0.04	0.44 ± 0.07
Anterior intestine (nmol h^{-1})	5.36 ± 0.33	7.92 ± 1.43	4.70 ± 0.91	4.89 ± 0.63	1.77 ± 0.33	4.00 ± 0.81
Mid-intestine (nmol h^{-1})	0.57 ± 0.14	0.57 ± 0.18	0.75 ± 0.11	0.33 ± 0.08	4.74 ± 1.16	2.68 ± 0.38
Posterior intestine (nmol h^{-1})	0.42 ± 0.12	0.90 ± 0.26	0.67 ± 0.19	0.55 ± 0.10	3.19 ± 0.75	5.26 ± 0.66
Total for 250 g trout (nmol h^{-1})	6.91 ± 0.68	9.60 ± 1.89	6.67 ± 1.32	6.15 ± 0.87	10.14 ± 2.28	12.38 ± 1.92
Mass-specific total (nmol g^{-1} h^{-1})	0.028 ± 0.003	0.038 ± 0.008	0.027 ± 0.005	0.025 ± 0.003	0.041 ± 0.009	0.050 ± 0.008

Values represent the means (±S.E.M.); N = 8–10.

An important *caveat* is that exposure conditions *in vivo* may be very different than in gut sacs *in vitro* where an effort was purposely made to standardize them. One of the biggest differences will be the presence of partially digested food in the chyme. This organic matter will undoubtedly alter metal speciation greatly from the simple ionic solutions used in the present study (see Meyer et al., 2005). Complexation of metals by proteins, amino acids, and other organic molecules will occur. On the one hand, this may reduce bioavailability by removing ionic metal from solution; on the other hand, it may increase bioavailability by facilitating the uptake of metals complexed to organic molecules (e.g. the dipeptide transport system characterized in the intestine of the lobster—Conrad and Ahearn, 2005). Furthermore, conditions may vary greatly from one part of the tract to another. For example, in recent studies on trout, dissolved copper (Nadella et al., 2006a), cadmium (Baldisserotto et al., 2005) and nickel concentrations (E. Leonard, personal communication) were all several-fold higher in stomach chyme than in chyme sampled from various parts of the intestine. This in turn may relate to the much lower pH in the stomach, and the changing chemistry of the chyme as it moves down an increasingly alkaline intestine (Shehadeh and Gordon, 1969). Thus, despite relatively low transport rates under standardized conditions *in vitro*, it is possible that the stomach may play a much greater role in metal absorption *in vivo*. The same appears to be true for nutrient ions such as Na^+, Mg^{2+} and Ca^{2+}, for which recent *in vivo* studies have identified both high chyme concentrations and high absorption rates in the stomach of trout (Bucking and Wood, 2006, 2007).

4.3. Comparison of uptake rates among different metals

A particularly surprising finding was that despite the regional differences noted in the preceding section, overall metal transport rates were very similar among the six different metals. Again using transport into the blood space as the measure of interest, on a mass-specific basis, the difference was only about two-fold, from a low of 0.025 nmol kg^{-1} h^{-1} for silver to a high of 0.050 nmol kg^{-1} h^{-1} for nickel, when all metals were presented at the same concentration of 50 μmol L^{-1} (Table 1). This contrasts with our original hypothesis that essential metals (copper, zinc, nickel) would be taken up at much higher rates than non-essential metals (cadmium, lead, silver). Of course, under most natural situations *in vivo*, it is likely that actual metal concentrations in the food will be higher for the essential metals than for the non-essential metals, favouring the uptake of the former. Nevertheless, this finding has important implications for risk assessment (e.g. Chapman et al., 2003), because it means that as a first approximation, one can apply the same uptake rate constant to any of the six metals to predict bioaccumulation rates from the diet.

4.4. Comparison of uptake rates of metals into the mucus and mucosal epithelium compartments

The general predominance of the anterior intestine seen for net metal uptake did not apply to either metal binding to mucus (Fig. 3), or metal trapped in the enterocytes of the mucosal epithelium (Fig. 4). Indeed with a few exceptions, these compartments, particularly mucus binding, generally predominated in the stomach, mid and posterior intestines (Fig. 5), so it could be argued that the overall effect was to retard metal absorption in these segments. Nickel was the most marked exception to this generalization, with much less of the total accumulation being held up in the mucus and mucosal epithelium, and the largest fraction being transported into the blood space, which again suggests that the nickel uptake mechanism, which gave the highest overall transport rates, is rather different from the other metals. However, only for cadmium and lead in the mid-intestine, was there clear evidence in support of our original hypothesis that greater mucus binding would occur for non-essential metals (Figs. 3 and 5), and only for cadmium in the mid-intestine and the stomach was there evidence of selective accumulation in the epithelium (Figs. 4 and 5), as might occur if a basolateral transport mechanism were selectively blocked. This could be explained based on the ability of cadmium to inhibit basolateral Ca^{2+}-ATPase (Schoenmakers et al., 1992) at the gut, similar to its action at the gill (Wicklund-Glynn, 1996; Verbost et al., 1987, 1988). Possibly, the concentrations of non-essential metals used (50 μmol L^{-1}) were below those necessary to elicit abundant mucus secretion or cause frank toxic effects on other transporters.

4.5. Relationships between metal binding and metal transport in the gastro-intestinal tract

Current modeling approaches to predicting waterborne metal toxicity such as the Biotic Ligand Model relate short-term gill binding to acute or chronic toxic effect (e.g. Playle et al., 1993; Di Toro et al., 2001; Playle, 2004; Niyogi and Wood, 2004). For dietary metal toxicity, it is unlikely that there will ever be acute toxic effects, but rather that chronic toxicity will relate to bioaccumulation (Meyer et al., 2005). Therefore, we have examined the current data set to see if short-term metal accumulation in either of the "binding" compartments (the mucus, or the mucosal epithelium) can serve as a predictor of bioaccumulation rate (i.e. transport into the blood compartment).

The results of this analysis (Table 2) demonstrated significant positive linear relationships between short-term metal accumulation in the mucus and net transport rate into the blood space for all metals except cadmium. There were also significant relationships for three metals only (nickel, silver and lead) between accumulation rate in the mucosal epithelium and net transport into the blood space (Table 2). Overall, the relationships were by far the strongest for lead, as illustrated in Fig. 6. Inasmuch as the absolute values of "metal binding" were greater in the mucus for most metals (and therefore easier to measure) than in the mucosal epithelium, and the former relationships were generally stronger, these may present a first step for future predictive models of gastro-intestinal metal uptake and toxicity, and a guide for the design of competition experiments.

Table 2
Regression analyses relating either (a) mucus binding rate (nmol cm^{-2} h^{-1}) or (b) rate of accumulation in mucosal epithelium (nmol cm^{-2} h^{-1}) to net transport rate (nmol cm^{-2} h^{-1}) of the metal into the blood space

Metal		r	N	P
(a) Mucus binding rate (x) vs. net transport rate (y)				
Copper	$Y = 1.4747x - 0.0188$	0.545	34	<0.001
Zinc	$Y = 0.3768x + 0.0489$	0.350	32	<0.05
Nickel	$Y = 0.8616x + 0.0959$	0.504	32	<0.005
Silver	$Y = 0.8522x + 0.0005$	0.404	32	<0.02
Cadmium	$Y = 0.0255x + 0.0616$	0.117	34	N.S.
Lead	$Y = 0.8186x + 0.0617$	0.919	33	<0.0001
(b) Mucosal epithelium accumulation rate (x) vs. net transport rate (y)				
Copper	$Y = 0.0487x + 0.0702$	0.010	34	N.S.
Zinc	$Y = 1.0644x + 0.0612$	0.199	32	N.S.
Nickel	$Y = 36.715x + 0.0708$	0.431	32	<0.02
Silver	$Y = 4.607x + 0.0006$	0.441	32	<0.02
Cadmium	$Y = 0.0261x + 0.0642$	0.040	34	N.S.
Lead	$Y = 26.731x - 0.0273$	0.798	33	<0.0001

4.6. Comparison of metal uptake rates via the gastro-intestinal tract versus the gills

In Table 3, we have compared the current measurements of total gastro-intestinal metal uptake rates into the blood space at an exposure concentration of 50 μmol L^{-1} with total uptake rates across the gills from the waterborne phase in previous studies on rainbow trout in comparable water quality. Despite the fact that the waterborne exposures were performed at much lower concentrations so as to avoid acute toxicity (by 1–4 orders of magnitude), the uptake rates across the gills and across the gut were generally in the same range (Table 3). There are several important implications. Firstly, for risk assessment of bioaccumulation (Chapman et al., 2003), this emphasizes the importance of considering both waterborne and dietary routes of metal exposure. Secondly, it illustrates that the gastro-intestinal uptake mechanisms must operate with much lower affinities for the metals than the gill uptake mechanisms. This is underlined by calculation of the uptake rate:concentration ratios which are many-fold higher at the gills than at the gut (Table 3). Clearly, freshwater fish have both the benefits and costs of operating two

transporting epithelia (gills and gut), each of which can be an important site of acquisition of nutritive metals, but also a site of acquisition of toxic metals. The gut is designed to operate at, and tolerate normal metal levels in the chyme (e.g. 50 μmol L^{-1}) which would kill the fish within a few hours if the same exposure concentration occurred in the waterborne phase.

4.7. Future directions

The present study has laid the methodological groundwork for a variety of future investigations on gastro-intestinal metal uptake. Of particular interest will be studies examining the concentration-dependence of uptake (e.g. Nadella et al., 2006b), and the possible interactive effects of the same metals when tested in combination, a topic which has been modeled recently at the gills (Playle, 2004). Similarly, the possible effects of factors such as pH, competing cations, and complexation by organic matter on rates of uptake should be determined, since these have proven to be so important in modifying metal uptake in the gill Biotic Ligand Model (Playle et al., 1993; Di Toro et al., 2001; Niyogi and Wood, 2004). In particular, the effects

Table 3
A comparison of metal uptake rates via the gut and gill of rainbow trout

Metals	Uptake rate (nmol g^{-1} h^{-1})		Uptake rate:concentration ratio ((nmol g^{-1} h^{-1})/(μmol L^{-1}))	
	Gut	Gill	Gut	Gill
Copper	0.028	0.002[a]	0.0006	0.05
Zinc	0.038	0.410[b]	0.0008	0.18
Nickel	0.050	0.014[c]	0.0010	0.20
Cadmium	0.027	0.012[d]	0.0005	12.00
Lead	0.041	0.050[e]	0.0008	0.01
Silver	0.025	0.005[f]	0.0005	0.25

Note: At the gut, uptake rates for all metals were at an exposure concentration of 50 μmol L^{-1} each.

[a] At the gill representative data are from Kamunde et al. (2002) at waterborne [Cu] = 0.040 μmol L^{-1}.

[b] At the gill representative data are from Hogstrand et al. (1996) at waterborne [Zn] = 2.3 μmol L^{-1}.

[c] At the gill representative data are from Pane et al. (2003) at waterborne [Ni] = 0.070 μmol L^{-1}.

[d] At the gill representative data are from Hollis et al. (2000) at waterborne [Cd] = 0.001 μmol L^{-1}.

[e] At the gill representative data are from Rogers and Wood (2004) at waterborne [Pb] = 5 μmol L^{-1}.

[f] At the gill representative data are from Morgan et al. (2004) at waterborne [Ag] = 0.02 μmol L^{-1}.
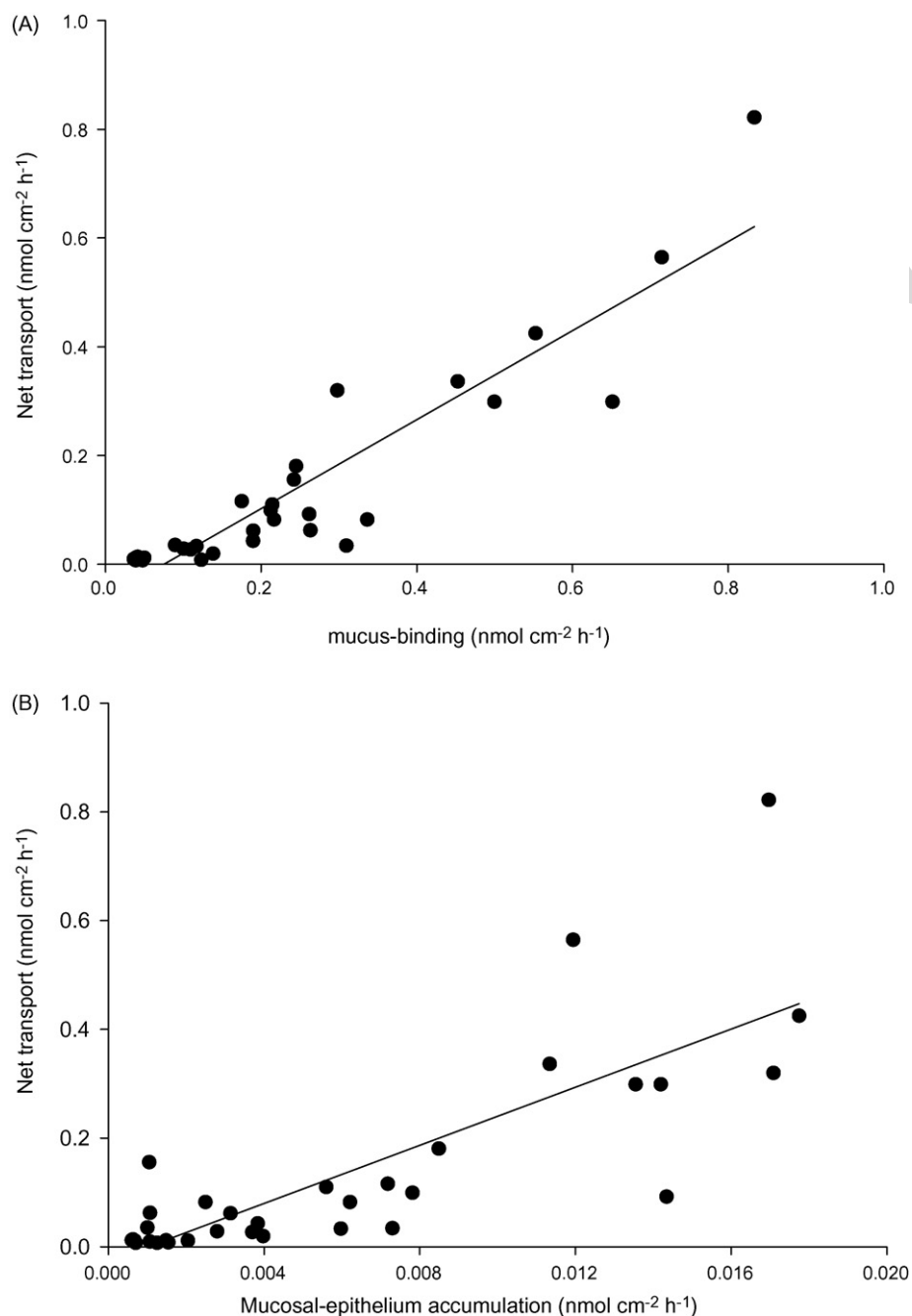
Fig. 6. Relationships between net transport rate of lead into the blood space and (A) mucus binding and (B) accumulation in the mucosal epithelium. Equations of the regression lines and statistical analyses are summarized in Table 2.

on bioavailability of metal complexation by proteins, amino acids and other organic molecules in the chyme should be examined (Conrad and Ahearn, 2005; Meyer et al., 2005). A useful first step in this regard might be to compare uptake rates from real chyme (sampled *in vivo*) with uptake rates from simple solutions as used in the present study. Furthermore, the time is ripe to integrate such physiological studies with new molecular approaches targeted at identifying piscine homologues of metal transport mechanisms known to be important in the mammalian gastro-intestinal tract (Bury et al., 2003).

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References

Alves, L., Wood, C.M., 2006. The chronic effects of dietary lead in freshwater juvenile rainbow trout (*Oncorhynchus mykiss*) fed elevated calcium diets. Aquat. Toxicol. 78, 217–232.

Alves, L., Glover, C.N., Wood, C.M., 2006. Dietary Pb accumulation in juvenile freshwater rainbow trout (*Oncorhynchus mykiss*). Arch. Environ. Contam. Toxicol. 51, 615–625.

Baldisserotto, B., Chowdhury, M.J., Wood, C.M., 2005. Effects of dietary calcium and cadmium on cadmium accumulation, calcium and cadmium uptake from the water, and their interactions in juvenile rainbow trout. Aquat. Toxicol. 72, 99–117.

Burke, J., Handy, R.D., 2005. Sodium-sensitive and -insensitive copper accumulation by isolated intestinal cells of rainbow trout *Oncorhynchus mykiss*. J. Exp. Biol. 208, 391–407.

Bergman, A.N., Laurent, P., Otianga-Owiti, G., Bergman, H.L., Walsh, P.J., Wilson, P., Wood, C.M., 2003. Physiological adaptations of the gut in the Lake Magadi, *Alcolapia grahami*, an alkaline- and saline-adapted teleost fish. Comp. Biochem. Physiol. 136A, 701–715.

Bronner, F., 1998. Calcium absorption—a paradigm for mineral absorption. J. Nutr. 128, 917–920.

Buddington, R.K., Diamond, J.M., 1986. Aristotle revisited: the function of pyloric ceca in fish. Proc. Natl. Acad. Sci. U.S.A. 83, 8012–8014.

Buddington, R.K., Diamond, J.M., 1987. Pyloric ceca of fish: a "new" absorptive organ. Am. J. Physiol. 252, G65–G76.

Buddington, R.K., Krogdahl, A., Bakke-Mckellep, A.M., 1997. The intestines of carnivorous fish: structure and functions and the relations with diet. Acta Physiol. Scand. 161, 67–80.

Bucking, C., Wood, C.M., 2006. Gastrointestinal processing of monovalent ions (Na^+, Cl^-, K^+) during digestion: implications for homeostatic balance in freshwater rainbow trout. Am. J. Physiol. R 291, 1764–1772.

Bucking, C., Wood, C.M., 2007. Gastrointestinal transport of Ca^{2+} and Mg^{2+} during the digestion of a single meal in the freshwater rainbow trout. J. Comp. Physiol. B [Epub print ahead].

Bury, N.R., Grosell, M., Wood, C.M., Hogstrand, C., Wilson, R.W., Rankin, J.C., Busk, M., Hecklin, T., Jensen, F.B., 2001. Intestinal iron uptake in the European flounder (*Platichthys flesus*). J. Exp. Biol. 204, 3779–3787.

Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. 206, 11–23.

Campbell, P.G.C., Clearwater, S.J., Brown, P.B., Fisher, N.S., Hogstrand, C., Lopez, G.R., Mayer, L.M., Meyer, J.S., 2005. Digestive physiology, chemistry, and nutrition. In: Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M. (Eds.), Toxicity of Dietborne Metals to Aquatic Organisms. SETAC Press, Pensacola, pp. 13–57 (Chapter 2).

Chapman, P.M., Wang, F., Janssen, C.R., Goulet, R.R., Kamunde, C.N., 2003. Conducting ecological risk assessments of inorganic metals and metalloids: current status. Hum. Ecol. Risk Assess 9–4, 641–697.

Chowdhury, M.J., McDonald, D.G., Wood, C.M., 2004. Gastrointestinal uptake and fate of cadmium in rainbow trout acclimated to sublethal dietary cadmium. Aquat. Toxicol. 69, 149–163.

Clearwater, S.J., Baskin, S.J., Wood, C.M., McDonald, D.G., 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. J. Exp. Biol. 203, 2455–2466.

Clearwater, S.J., Farag, A.M., Meyer, J.S., 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp. Biochem. Physiol. C 132, 269–313.

Clearwater, S.J., Handy, R.D., Hogstrand, C., 2005. Appendix A: interactions of dietborne metals with digestive processes of fishes. In: Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M. (Eds.), Toxicity of Dietborne Metals to Aquatic Organisms. SETAC Press, Pensacola, pp. 205–225.

Conrad, E.M., Ahearn, G.A., 2005. ^{3}H-L-histidine and ^{65}Zn^{2+} are cotransported by a dipeptide transport system in intestine of lobster *Homarus americanus*. J. Exp. Biol. 208, 287–296.

Crespo, S., Nonnotte, G., Colin, D.A., Leray, C., Nonnotte, L., Aubree, A., 1986. Morphological and functional alterations induced in trout intestine by dietary cadmium and lead. J. Fish. Biol. 28, 69–80.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic Ligand Model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383–2396.

Farag, A.M., Boese, C.J., Woodward, D.F., Bergman, H., 1994. Physiological changes and tissue metal accumulation in rainbow trout exposed to foodborne and waterborne metals. Environ. Toxicol. Chem. 13, 2021–2029.

Foulkes, E.C., 2000. Transport of toxic heavy metals across cell membranes. Proc. Soc. Exp. Biol. Med. 223, 234–240.

Galvez, F., Hogstrand, C., McGeer, J.C., Wood, C.M., 2001. The physiological effects of a biologically incorporated silver diet in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 55, 95–112.

Galvez, F., Wood, C.M., 1999. The physiological effects of dietary silver sulphide exposure in rainbow trout. Environ. Toxicol. Chem. 18, 84–88.

Gardner, G.R., Yevich, P.P., 1970. Histological and haematological responses of an estuarine teleost to cadmium. J. Fish. Res. Bd. Can. 27, 2185–2196.

Georgopoulou, U., Sire, M.F., Vernier, J.M., 1985. Macromolecular absorption of proteins by epithelial cells of the posterior intestinal segment and their intracellular digestion in the rainbow trout. Ultrastructural and biochemical study. Biol. Cell 53, 269–282.

Georgopoulou, U., Sire, M.F., Vernier, J.M., 1986. Immunological demonstration of intestinal absorption and digestion of protein macromolecules in the rainbow trout (*Salmo gairdneri*). Cell Tissue Res. 245, 387–395.

Glover, C.N., Hogstrand, C., 2002a. *In vivo* characterization of intestinal zinc uptake in freshwater rainbow trout. J. Exp. Biol. 205, 141–150.

Glover, C.N., Hogstrand, C., 2002b. Amino acid modulation of *in vivo* intestinal Zn absorption in freshwater rainbow trout. J. Exp. Biol. 205, 151–158.

Glover, C.N., Hogstrand, C., 2003. Effects of dissolved metals and other hydrominerals on *in vivo* intestinal zinc uptake in freshwater rainbow trout. Aquat. Toxicol. 62, 281–293.

Glover, C.N., Bury, N.R., Hogstrand, C., 2003a. Zinc uptake across the apical membrane of freshwater rainbow trout intestine is mediated by high-affinity, low-affinity and histidine-facilitated pathways. Biochim. Biophys. Acta 1614, 211–219.

Glover, C.N., Balesaria, S., Mayer, G.D., Thompson, E.D., Walsh, P.J., Hogstrand, C., 2003b. Intestinal zinc uptake in two marine teleosts: squirrel fish (*Holocentrus adscensions*) and gulf toadfish (*Opsanus beta*). Physiol. Biochem. Zool. 76, 321–330.

Grosell, M., Jensen, F.B., 1999. NO_2^- uptake and HCO_3^- excretion in the intestine of the European flounder *Platicthys flesus*. J. Exp. Biol. 202, 2103–2110.

Grosell, M., DeBoeck, G., Johannson, O., Wood, C.M., 1999. The effects of silver on intestinal ion and acid-base regulation in the marine teleost fish *Parophrys vetulus*. Comp. Biochem. Physiol. 124C, 259–270.

Grosell, M., Laliberte, C.N., Wood, S., Jensen, F.B., Wood, C.M., 2001. Intestinal HCO_3^- secretion in marine teleost fish: evidence for an apical rather than a basolateral Cl^-/HCO_3^- exchanger. Fish. Physiol. Biochem. 24, 81–95.

Grosell, M., Wood, C.M., Wilson, R.W., Bury, N.R., Hogstrand, C., Rankin, C., Jensen, F.B., 2005. Bicarbonate secretion plays a role in chloride and water absorption of the European flounder (*Platicthys flesus*) intestine. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288, R936–R946.

Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., Hediger, M.A., 1997. Cloning and characterization of a proton-coupled mammalian metal ion transporter. Nature 388, 482–488.

Handy, R.D., Musonda, M.M., Phillips, K., Falla, S.J., 2000. Mechanisms of gastrointestinal copper absorption in the African walking catfish: copper dose–effects and a novel anion-dependent pathway in the intestine. J. Exp. Biol. 203, 2365–2377.

Hansen, H.J.M., Grosell, M., Jacobsen, U., Jorgensen, J.C., Hogstrand, C., Wood, C.M., 2002. Precautions in the use of ^{110m}Ag as a tracer of silver metabolism in ecotoxicology: preferential bioconcentration of ^{109}Cd by trout gills after ^{110m}Ag exposure. Environ. Toxicol. Chem. 21, 1004–1008.

Hardy, R.W., Sullivan, C.V., Koziol, A.M., 1987. Absorption, body distribution, and excretion of dietary zinc by rainbow trout (*Salmo gairdneri*). Fish. Physiol. Biochem. 3, 133–143.

Hodson, P.V., Blunt, B.R., Spry, D.J., 1978. Chronic toxicity of waterborne and dietary lead to rainbow trout (*Salmo gairdneri*) in Lake Ontario water. Water Res. 12, 869–878.

Hogstrand, C., Verbost, P.M., Wendelaar Bonga, S.E., Wood, C.M., 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am. J. Physiol. 270, R1141–R1147.

Hogstrand, C., Wood, C.M., Bury, N.R., Wilson, R.W., Rankin, J.C., Grosell, M., 2002. Binding and movement of silver in the intestinal epithelium of a marine teleost fish, the European flounder (*Platichthys flesus*). Comp. Biochem. Physiol. C 133, 125–135.

Hollis, L., McGeer, C.J., McDonald, D.G., Wood, C.M., 2000. Protective effects of calcium against chronic waterborne cadmium exposure to juvenile rainbow trout. Environ. Toxicol. Chem. 19, 2725–2734.

House, C.R., Green, K., 1965. Ion and water transport in isolated intestine of the marine teleost, *Cottus scorpius*. J. Exp. Biol. 42, 177–189.

Kamunde, C., Clayton, C., Wood, C.M., 2002. Waterborne vs. dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure. Am. J. Physiol. 283, R69–R78.

Loretz, C.A., 1995. Electrophysiology of ion transport in teleost intestinal cells. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation, vol. 14. Academic Press, San Diego, CA, pp. 25–56.

Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M., 2005. Toxicity of Dietborne Metals to Aquatic Organisms. SETAC Press, Pensacola.

Mount, D.R., Barth, A.K., Garrison, T.A., Barten, K.A., Hockett, J.R., 1994. Dietary and waterborne exposure of rainbow trout (*Oncorhynchus mykiss*) to copper, cadmium, lead and zinc using a live diet. Environ. Toxicol. Chem. 13, 2031–2041.

Morgan, T.P., Grosell, M., Playle, R.C., Wood, C.M., 2004. The time course of silver accumulation in rainbow trout during static exposure to silver nitrate: physiological regulation or an artifact of the exposure conditions. Aquat. Toxicol. 68, 55–72.

Muyssen, B.T.A., Brix, K.V., DeForest, D.K., Janssen, C.R., 2004. Nickel essentiality and homeostasis in aquatic organisms. Environ. Rev. 12, 113–131.

Nadella, S.R., Bucking, C., Grosell, M., Wood, C.M., 2006a. Gastrointestinal assimilation of Cu during digestion of a single meal in the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. C 143, 394–401.

Nadella, S.R., Grosell, M., Wood, C.M., 2006b. Physical characterization of high-affinity gastrointestinal Cu transport *in vitro* in freshwater rainbow trout *Oncorhynchus mykiss*. J. Comp. Physiol. 176, 793–806.

Nadella, S.R., Grosell, M., Wood, C.M., 2007. Mechanisms of dietary Cu uptake in freshwater rainbow trout: evidence for Na-assisted Cu transport and a specific metal carrier in the intestine. J. Comp. Physiol. [Epub print ahead].

Niyogi, S., Wood, C.M., 2004. Biotic Ligand Model: a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.

Pane, E.F., Richards, J.G., Wood, C.M., 2003. Acute waterborne nickel toxicity in the rainbow trout (*Oncorhynchus mykiss*) occurs by a respiratory rather than ionoregulatory mechanism. Aquat. Toxicol. 63, 65–82.

Part, P., Lock, R.A.C., 1983. Diffusion of calcium, cadmium and mercury in a mucus solution from rainbow trout. Comp. Biochem. Physiol. 76, 259–263.

Pentreath, R.J., 1976. Some further studies on the accumulation and retention of ^{65}Zn and ^{54}Mn by the plaice *Pleuronectes platessa* L. J. Exp. Mar. Ecol. 21, 179–189.

Playle, R.C., 2004. Using multiple-metal gill binding models and the toxic unit concept to help reconcile multiple-metal toxicity results. Aquat. Toxicol. 67, 359–370.

Playle, R.C., Dixon, D.G., Burnison, 1993. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modeling of metal accumulation. Can. J. Fish. Aquat. Sci. 50, 2678–2687.

Pratap, H.B., Fu, H., Lock, R.A.C., Wendelaar Bonga, S.E., 1989. Effect of waterborne and dietary cadmium on plasma ions of the teleost *Orechromis mossambicus* in relation to water calcium levels. Arch. Environ. Contam. Toxicol. 18, 568–575.

Ptashynski, M.D., Klaverkamp, J.F., 2002. Accumulation and distribution of dietary nickel in lake whitefish (*Coregonus clupeaformis*). Aquat. Toxicol. 58, 249–264.

Purcell, T.W., Peters, J.J., 1998. Sources of silver in the environment. Environ. Toxicol. Chem. 4, 539–546.

Rogers, J.T., Wood, C.M., 2004. Characterization of branchial lead-calcium interaction in the freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 207, 813–825.

Schoenmakers, T.J.M., Klaren, T.P.H.M., Flick, G., Lock, R.A.C., Pang, P.K.T., Wendelaar Bonga, S.E., 1992. Actions of cadmium on basolateral plasma membrane proteins involved in calcium uptake by fish intestine. J. Membr. Biol. 127, 161–172.

Shears, M.A., Fletcher, G.L., 1983. Regulation of Zn^{2+} uptake from the gastrointestinal tract of a marine teleost, the winter flounder (*Pseudopleuronectes americanus*). Can. J. Fish. Aquat. Sci. 40, 197–205.

Shehadeh, Z.H., Gordon, M.S., 1969. The role of the intestine in salinity adaptation of the rainbow trout, *Salmo gairdneri*. Comp. Biochem. Physiol. 30, 397–418.

Turner, A., Olsen, Y.S., 2000. Chemical versus enzymatic digestion of contaminated estuarine sediment: relative importance of iron and manganese oxides in controlling trace metal bioavailability. Estuar. Coast. Shelf. Sci. 51, 717–728.

Verbost, P.M., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1987. Cadmium inhibition of Ca^{2+} uptake in rainbow trout gills. Am. J. Physiol. 253, R216–R221.

Verbost, P.M., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1988. Cadmium inhibits plasma membrane calcium transport. J. Membr. Biol. 102, 97–104.

Whitehead, M.W., Thompson, R.P.A., Powell, J.J., 1996. Regulation of metal absorption in the gastrointestinal tract. Gut 39, 625–628.

Wicklund-Glynn, A., 1996. The concentration dependency of branchial intracellular cadmium distribution and influx in the zebrafish (*Brachydanio rerio*). Aquat. Toxicol. 35, 47–58.

Wolf, K., 1963. Physiological salines for freshwater teleosts. Prog. Fish Cult. 25, 135–140.

Wood, C.M., 2001. Toxic responses of the gill. In: Schlenk, D.W., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, Organs, vol. 1. Taylor and Francis, Washington, DC, USA, pp. 1–89.

Wood, C.M., Franklin, N., Niyogi, S., 2006. The protective role of dietary calcium against cadmium uptake and toxicity in freshwater fish: an important role for the stomach. Environ. Chem. 3, 389–394.

World Health and Organisation, 1995. Environmental Health Criteria 165 International Programme on Chemical Safety. World Health Organisation, Geneva.