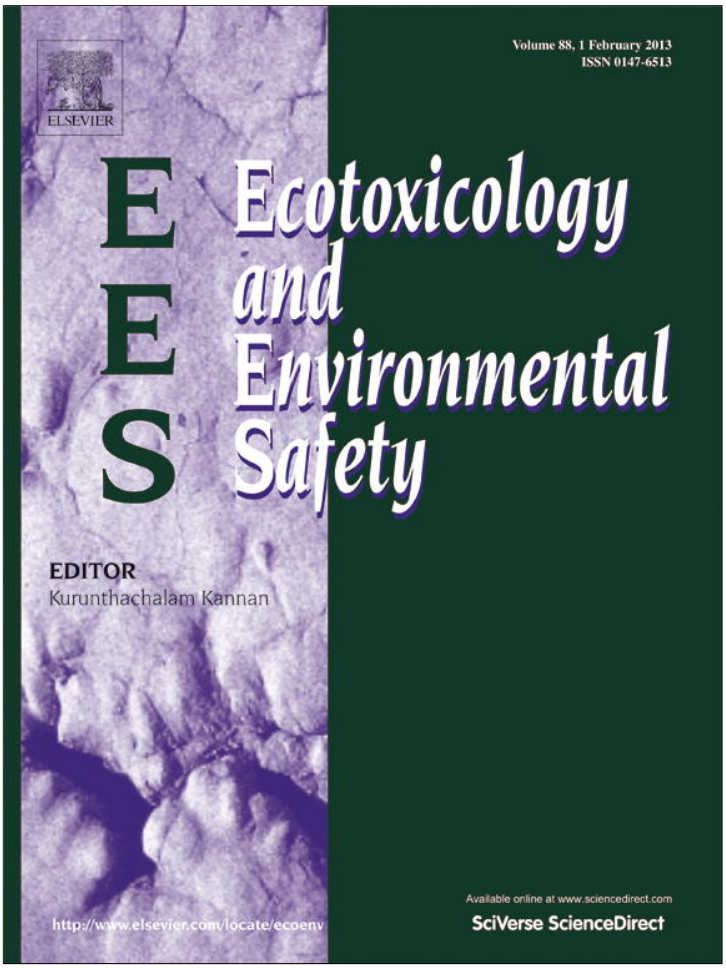

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In situ analysis of cadmium uptake in four sections of the gastro-intestinal tract of rainbow trout (Oncorhynchus mykiss)

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ABSTRACT

This study links results from past *in vitro* and *in vivo* experiments, by implementing an *in situ* experiment in order to determine the relative importance for cadmium (Cd) uptake of different sections of the gastro-intestinal tract (GIT) of rainbow trout. Transport of Cd from four sections of the GIT of adult rainbow trout (~220 g) was individually examined by infusing ligated sections of the GIT in live, free-swimming fish with 50 μM Cd spiked with radiolabelled ^{109}Cd (0.5 μCi ml^{-1}). Fish were exposed for an 8-h period. The percentage of the total injected ^{109}Cd which was internalized from the different segments was only between ~0.1 and ~7%, indicating low uptake efficiency. The stomach is the most important GIT segment for Cd transport into the internal compartment of the animal, while the posterior intestine also plays a significant role. The majority of ^{109}Cd recovered at the end of the flux period was detected within gut material (ranging from 28 to 95%); the portion of Cd which was internalized was largely found in the carcass (32 to 60%). Distribution between the measured organs varied with uptake from the various GIT sections. Our results also confirm that the GIT acts as a protective barrier against Cd uptake from dietary exposure.

1. Introduction

Cadmium(Cd) is a nonessential metal, which is toxic to fish at low concentrations by causing hypocalcaemia, affecting growth, olfactory sensitivity, endocrine disruption and/or other physiological functions (McGeer et al., 2012). It is a ubiquitous metal entering the environment from both natural processes and anthropogenic inputs such as mining and manufacturing, and from agricultural uses in fertilizers and pesticides. Fish take up metals from the water by their gills and/or from contaminated foodstuff along their gastro-intestinal tract (GIT). Current regulatory guidelines in most countries are generally concerned with waterborne concentrations (McGeer et al., 2012) despite evidence that diet-borne exposure often presents a greater danger to aquatic organisms (Dallinger and Kautzky, 1985; Clearwater et al., 2002; Meyer et al., 2005). Most research on the mechanisms of metal uptake in fish in the past has focused on characterizing branchial transport and not on gut transport. The contribution of each pathway is likely to vary greatly depending on water and diet chemistry.

Recently, Klinck and Wood (2011) and Klinck et al. (2012) have provided evidence that Cd and Ca are taken up in part by a common transporter along the GIT which is different from the shared transporter at the gill. They give evidence that these two metals are transported via a mechanosensitive L-type Ca channel, and perhaps to a lesser degree, via a non-voltage gated Ca transporter, and that the stomach likely contributes to the overall uptake. The divalent metal transporter 1 (DMT1) and a Zn transporter also appear to be involved in Cd transport along the intestinal portion of the GIT (Cooper et al., 2006; Kwong and Niyogi, 2009, 2012; Kwong et al., 2010, 2011; Klinck and Wood, 2011). Less is known about basolateral transport of Cd out of enterocytes, but it has been linked to the Na^+/Ca^{2+} exchanger, Ca^{2+}-ATPase and Na^+-ATPase (Schoenmakers et al. 1992; Kwong et al., 2011). Overall, it appears that there are multiple sites and mechanisms of Cd uptake into, and out of, the GIT of rainbow trout. The presence and abundance of the above proposed mechanisms vary depending on the gut section.

The importance of each gut segment in terms of Cd uptake has been debated in the past, and there is circumstantial evidence that the stomach is surprisingly important (e.g., Franklin et al., 2005; Wood et al., 2006; Ojo and Wood, 2007; Klinck et al., 2009). Arguments for different orders of importance of each GIT section have been made based on in vivo feeding experiments, or by *in vitro* gut sac experiments. Feeding experiments are helpful in determining overall Cd absorption efficiency, which appears to be very low (reviewed in McGeer et al., 2012), but do not distinguish between low unidirectional uptake rates and high unidirectional efflux rates, or identify the gastrointestinal segments involved. *In vitro* experiments are helpful in determining Cd uptake rates, affinities, and capacities of the various segments. However, they are unrealistic in that the experiments usually occur over short

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time periods, expose gut segments only, and do not account for the influence of the circulatory system *in vivo*, which transports, nutrients, hormones, and oxygen to the GIT, while removing waste material and Cd itself.

This study attempts to link the results found previously in both *in vitro* and *in vivo* experiments by implementing an *in situ* experiment borrowing the 'gut sac' technique from *in vitro* experiments mentioned above. By using this *in situ* technique which maintains an intact enteric circulation in free-swimming fish, we hoped to confirm previous conclusions based on circumstantial evidence from *in vitro* experiments and chronic *in vivo* feeding experiments as to the importance of the stomach in Cd uptake, and the low uptake efficiency of the entire tract. Specifically, we wished to illuminate the relative importance of different sections of GIT to dietary Cd uptake and internalization. Another goal of this study was to determine where Cd is distributed (after 8 h) within the body of the fish after being absorbed across different segments of the gut. Based on the results of previous studies, we hypothesized that the stomach and posterior intestine would be the most important areas for Cd uptake, that the entire GIT would have a low absorptive efficiency of luminal Cd, and that most of the internalized Cd would be found in the kidney and liver.

2. Methods

2.1. Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*) (~220 g, fork length ≈30 cm; $N=20$) from Humber Springs Fish Hatchery (Orangeville, ON) were held for at least 4 weeks in aerated 500-l tanks supplied with free-flowing dechlorinated Hamilton city tap water (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 (DOC) ~3 mg C l^{-1}, hardness ~140 mg l^{-1} as $CaCO_3$, 12–13 °C). Prior to experimentation fish were fed commercial trout pellet feed (composition: crude protein 41%, crude fat 11%, crude fiber 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, ON) at a ration of ~1% body weight per day two times a week for approximately one month before experimentation. This low ration was employed to reduce adipose mass around the GIT, thereby facilitating subsequent surgery. Background concentration of Cd in the food was 0.27 μg g^{-1}. Food was withheld from fish 96 h before experimentation. All experiments were in compliance with regulations set by the Canadian Council on Animal Care.

2.2. In situ gut sac technique

Rainbow trout ($N=5$ per gut section) were anaesthetized and artificially ventilated on an operating table using neutralized MS-222 (0.125 g l^{-1}), and a small incision (~5 cm long) was made on the ventral body wall of these fish which were about 30 cm long. The mortality rate was ~1 fish per gut section studied (~17%). The location of the incision depended on the portion of the gut being studied (i.e., over the stomach, anterior-, mid-, or posterior- intestine). The appropriate intact segment of interest (only one per fish) was carefully manipulated in order to make a ligation with surgical silk (2–0, pre-threaded to a reverse cutting needle) at its anterior and posterior end. Special care was taken with the ligation placement so not to occlude any major blood vessels which run along the length of the intestinal serosal surface by running the thread underneath them. This formed an *in situ* sealed 'gut sac' into which a treatment saline solution (described below) was injected using a 25-gauge needle. Each ligated region was filled to approximately the same tension (~200 mm H_2O of pressure). On average the stomachs were filled with 2.05 ± 0.53 ml mucosal saline, the anterior intestinal sections with 0.78 ± 0.09 ml, mid intestines with 0.23 ± 0.04 ml, and the posterior intestines with 0.28 ± 0.05 ml and therefore contained about 1.0, 0. 4, 0.1, and 0.1 μCi of radioactive ^{109}Cd, and about 100, 40, 10, and 15 pmol total Cd (see Section 2.3). The needle puncture site was blotted with tissue and checked for any leakage (none were observed). The gut sac was then carefully replaced into the body cavity and the incision was tightly stitched closed with surgical silk. The fish was then transferred to a dark 10-l tank which was continuously supplied with air and freshwater, remaining there for 8 h to allow absorption of the radiolabelled Cd.

After the 8-h flux period, fish were quickly killed by an overdose of neutralized MS-222 (~600 mg l^{-1}). Approximately 1 ml of blood was removed immediately by caudal puncture using a 3-ml Hamilton syringe pre-rinsed with lithium heparin (20. i.u. ml^{-1}). The incision was reopened and the entire GIT was removed, separated into its four distinct segments (stomach, anterior-, mid-, and posterior- intestine), and collected into separate vials. Any remaining *mucosal saline* from the ligated segment was collected in a separate container. Only the exposed segment was then rinsed in Cortland saline and blotted dry. The luminal side of this segment was gently scraped using a microscope slide to remove surface mucus and epithelial cells, which were transferred with water to an additional container. Saline rinse plus blotting paper (together containing any ^{109}Cd which was loosely bound to the mucus layer- referred to as the *mucus-bound fraction*) and scrapings (containing ^{109}Cd which was in the surface mucus and epithelial cells- representing partially absorbed Ca- referred to as the *mucosal-epithelium compartment*) were collected and individually analysed for Cd. The muscle tissue of the ligated GIT section, as well as the gallbladder, liver, spleen, gill, and kidney were also collected separately for analysis. The remaining carcass was saved, diced, and analysed for ^{109}Cd.

2.3. Experimental salines

Cortland saline (Wolf, 1963) was used for the rinse solution and was modified for the mucosal treatment saline. To avoid precipitation of Cd, modifications described by Ojo and Wood (2008) were used, where $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ were eliminated and $Ca(NO_3)_2$ replaced $CaCl_2$. The mucosal saline therefore had a

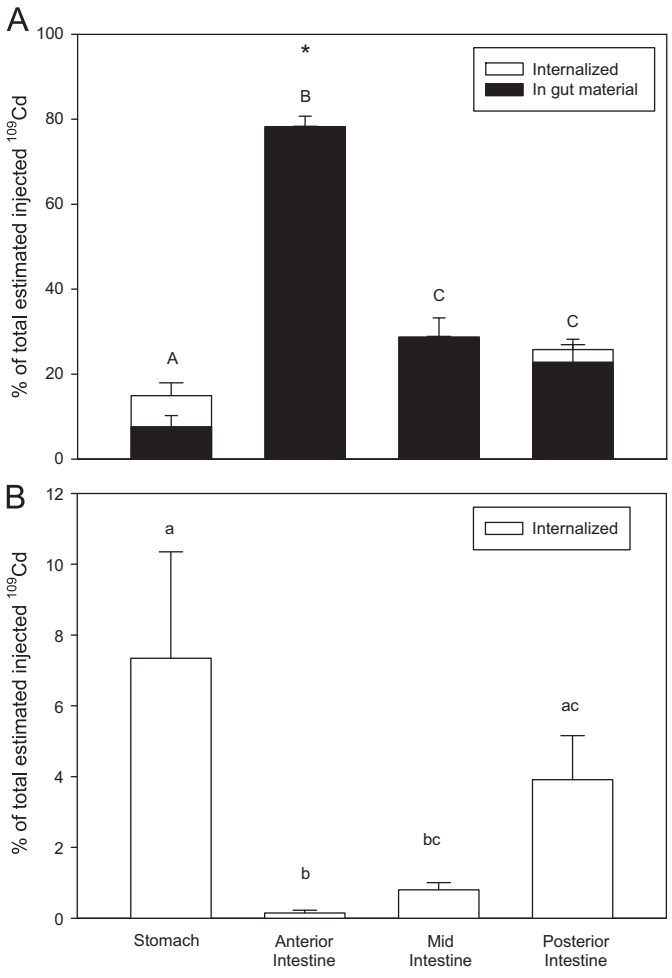

Fig. 1. (A) Percentages (±SEM) of the total injected ^{109}Cd (estimated) which was bound to, or taken up into gut material (combined and presented as black bars; narrow error bars), or internalized by fish (white bars; wider error bars) ($N=5$) via four different sections of the GIT. The combined heights of the black and white bars represent the total fraction of injected ^{109}Cd which was found in the fish. (B) Percentages (±SEM) of the total injected ^{109}Cd (estimated) which was internalized by fish ($N=5$) from four different sections of the GIT (same as white bars in Panel A, but using a different *y*-axis scale). Significant differences between groups in Panels A and B were determined by One-way ANOVA followed by Tukey's Multiple Comparison *post hoc* tests ($P>0.05$) after data had been arcsine transformed. The asterisk indicates that the total tissue bound or internalized ^{109}Cd (internalized+in gut material) in fish with ligated anterior intestine is significantly higher in comparison to fish with other ligated gut sections. Bars with differing capital letters indicated significant differences between percentages of injected ^{109}Cd bound to, or absorbed in, gut material; differing lower-case letters indicate significant differences between internalized ^{109}Cd.

composition (in mmol l^{-1}) of: 133 NaCl; 5 KCl; 1 $Ca(NO_3)_2 \cdot 4H_2O$; 1.9 $MgSO_4 \cdot 7H_2O$; and 5.5 glucose, and pH balanced to 7.4 by adding NaOH. 50 μM Cd (as Cd $(NO_3)_2 \cdot 4H_2O$) as well as 0.5 μCi ml^{-1} radioactive ^{109}Cd (as Cd Cl_2, specific activity = 3.65 Ci μg^{-1} (IICH, Kansas, USA)) were added to the mucosal saline.

2.4. Analytical techniques and calculations

The concentration of Cd in the mucosal treatment saline was measured via flame atomic absorption spectrophotometry (FAAS; Varian Spectra- 220 FS, Mulgrave, Australia) using prepared standards from Fisher Scientific (Toronto, ON, Canada). Certified analytical standards (TM24, National Water Research Institute, Environment Canada, Burlington, Canada) were measured before and after saline samples were analysed to ensure accuracy (all measured concentrations fell within the specified allowable range of ± 2 standard deviations).

The radioactivity of ^{109}Cd in all fluids and tissue samples were analysed individually by measuring gamma-emissions using a 1480 Wallac Wizard 3″ Automatic Gamma counter (Perkin Elmer, Turku, Finland). The total amount of radioactive Cd injected was estimated gravimetrically. The Hamilton syringe used to infuse the gut sac was first filled, weighed, and then reweighed following the injection, the difference in weight equaling the volume infused (assuming 1 mg ≈ 1 μl of mucosal saline). Using this information it was also possible to calculate the recovery percentage of ^{109}Cd at the end of the flux period by summing the measured activity from each collected sample.

The total amount of ^{109}Cd in fish blood was estimated based on the concentration measured in the blood sample, and multiplying this concentration by an estimate of the total blood within each fish (~0.55 ml per 100 g, from Gingerich et al., 1987). This amount was then subtracted from the ^{109}Cd amounts measured in the entire carcass.

All data are presented as percentages (± SEM) of either the total estimated ^{109}Cd injected into each ligated segment of the GIT (Fig. 1), or as a percentage of the total recovered ^{109}Cd (Figs. 2–5). This allowed comparisons of data between fish with different ligated gut sections and pooling of data within the groups which had the same ligated GIT segment. Absolute rates are not presented due to complications which arise from each fish being injected with slightly different amounts of mucosal saline, and each ligated segment having different surface areas exposed to the mucosal saline. Approximate surface areas of the various segments for trout of the current size are reported by Klinck and Wood (2011), and relative compartment sizes for tissue accumulation are reported by Hogstrand et al. (2003). Significant differences between groups were determined by One-way ANOVA followed by Tukey's Multiple Comparison *post hoc* tests ($P < 0.05$) after data had been arcsine transformed.

3. Results

3.1. Summary of differences between gut segments

The recovery rate of ^{109}Cd from the stomach was 26 ± 8%, while rates were much higher in the anterior-, mid-, and posterior intestines (82 ± 1, 88 ± 2, 73 ± 10%, respectively). Fish with uptake from the anterior intestine yielded the greatest percentage (78 ± 2%) of the total estimated injected ^{109}Cd in, or bound to, its tissues (internalized + gut material) compared to the fish with other segments of the GIT studied (stomach: 15 ± 5%; mid intestine: 29 ± 7%; posterior intestine: 26 ± 7%). Anterior intestine treated fish also had the highest percentage of the injected ^{109}Cd in gut material (mucus bound + mucosal epithelium + muscle tissue) (Fig. 1A). Fish with uptake from the mid- and posterior- intestine had lower fractions of the total injected Cd in their gut material than the anterior intestine gut sac fish, but higher than fish with uptake via the stomach. However, fish with uptake via the stomach, internalized a significantly greater percentage of the total injected ^{109}Cd internalized (7 ± 3%) compared to all the other fish with different gut sacs (Fig. 1B). Uptake of ^{109}Cd which was internalized from the posterior intestine (3 ± 1%) was also significantly greater than that of the anterior intestine (which was only 0.15 ± 0.08%), but not significantly different from uptake from the mid intestine (0.8 ± 0.2%).

3.2. Uptake from stomach

The collected radioactivity was either internalized by the fish (26 ± 4%); found in, or loosely bound to the stomach (28 ± 6%); or remained unabsorbed in the mucosal fluid (45 ± 6%) (Fig. 2). Three fractions of the stomach were examined separately for ^{109}Cd, and gave the following absorption (or bound) percentages: the muscle tissue (47 ± 9%), the mucus-bound fraction (46 ± 5%),

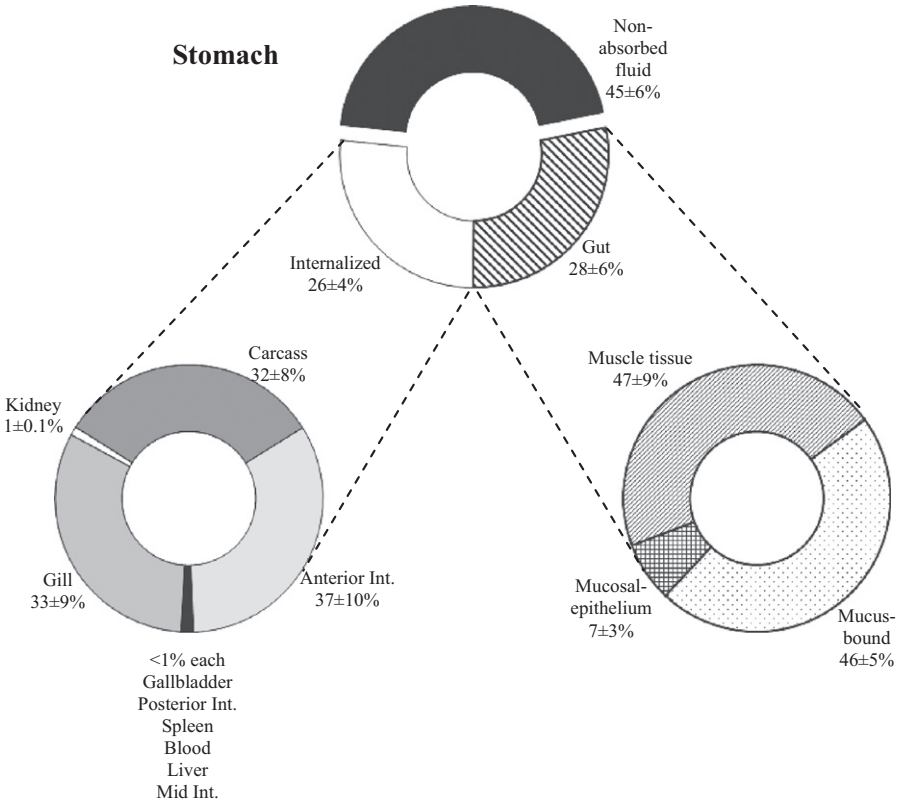

Fig. 2. Percent distribution (± SEM) of radioactivity recovered from fish whose stomach was ligated and infused with 50 μM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the stomach gut material.

and the mucosal epithelium contained the remaining 7 ± 3%. The majority of the internalized Cd was evenly distributed between the anterior intestine, gill, and carcass (~37, ~33, and ~32%, respectively). Only ~1% of the total internalized Cd was found in the kidney. The remaining five measured samples: gallbladder, posterior intestine, spleen, blood, liver, and mid intestine each

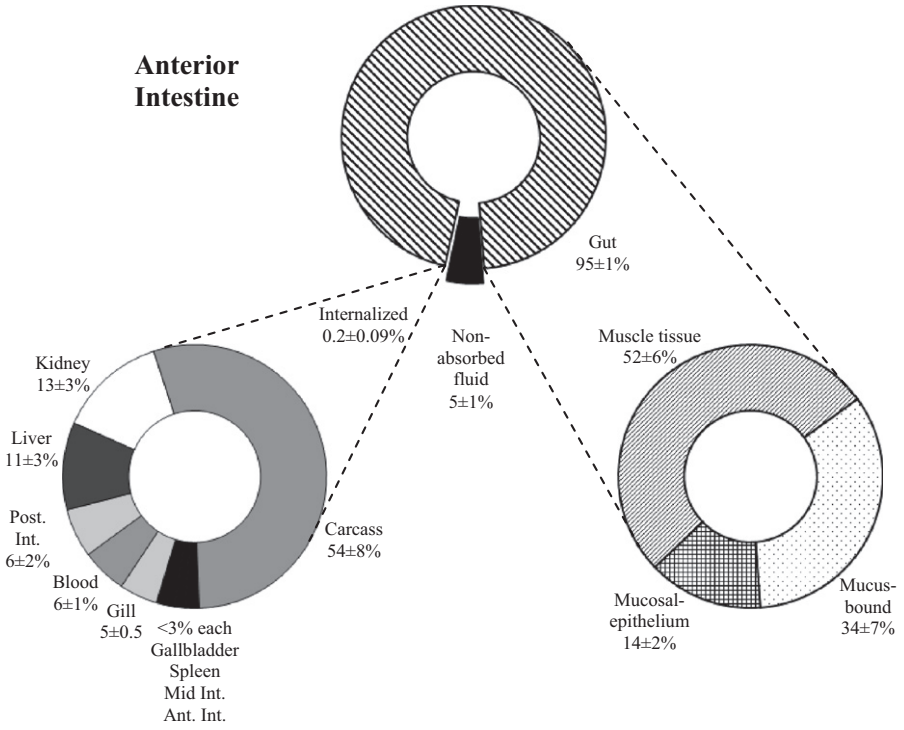

Fig. 3. Percent distribution (± SEM) of radioactivity recovered from fish whose anterior intestine was ligated and infused with 50 μM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the anterior intestine gut material.

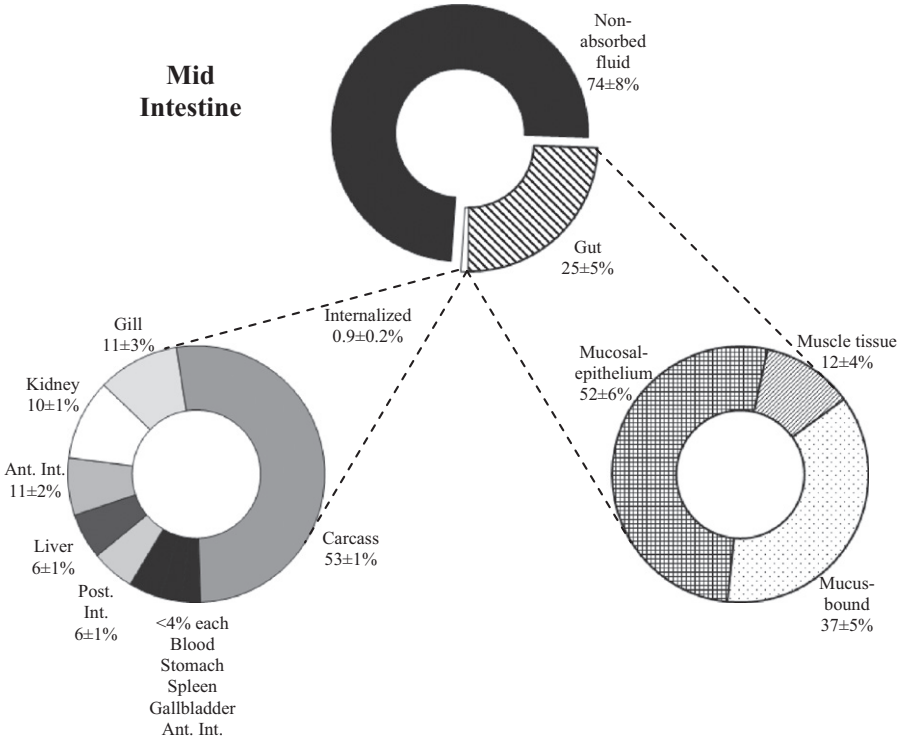

Fig. 4. Percent distribution (± SEM) of radioactivity recovered from fish whose mid intestine was ligated and infused with 50 μM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the mid intestine gut material.

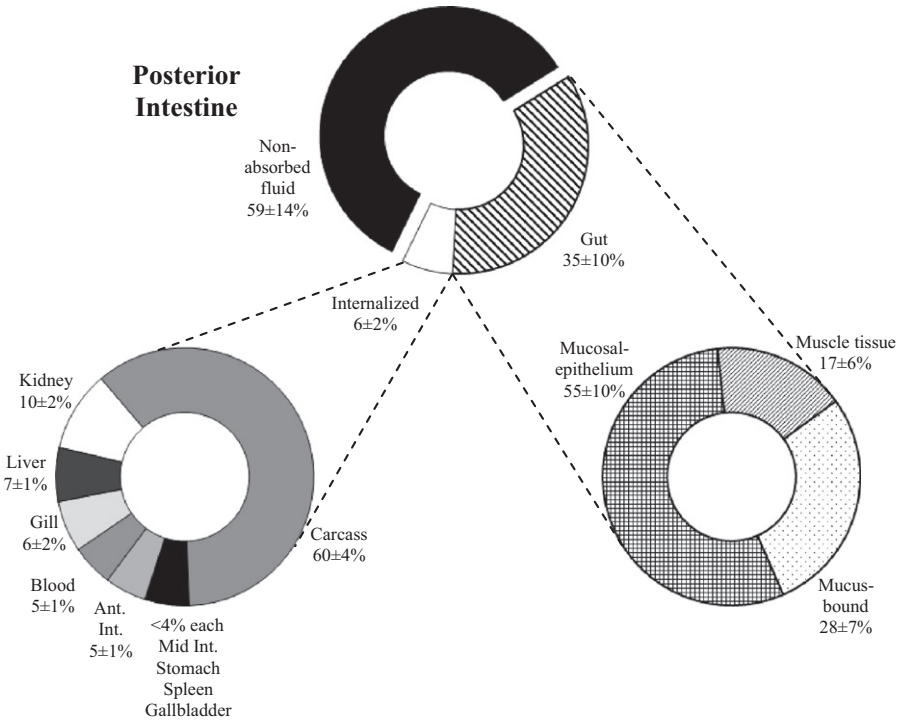

Fig. 5. Percent distribution (± SEM) of radioactivity recovered from fish whose posterior intestine was ligated and infused with 50 μM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the posterior intestine gut material.

contained less than 1% of the total recovered internalized radioactivity (listed in order of decreasing amounts).

3.3. Uptake from anterior intestine

The distribution of the recovered Cd was very different from that of the stomach-ligated fish. The majority of the collected radioactivity was found in, or bound to, the anterior gut material ($95 \pm 1\%$) (Fig. 3). Of the ^{109}Cd measured in the anterior intestine tissues, just over half was found in the muscle tissue, about a third was found in the mucus-bound compartment, and the remaining activity was found in the mucosal epithelium. A relatively small amount remained unabsorbed in mucosal fluid (~5%), and only a small fraction was actually internalized by the fish (making up only $0.2 \pm 0.09\%$). This small portion which was internalized was largely detected in the carcass ($54 \pm 8\%$), kidney ($13 \pm 3\%$), and liver ($11 \pm 3\%$). Lesser amounts were found in the other sampled internal tissues ($\leq 6\%$).

3.4. Uptake from mid intestine

Most of the injected Cd in the mid intestine remained in the mucosal solution after 8 h ($74 \pm 8\%$) (Fig. 4). Of the ^{109}Cd measured in the mid intestine, most was loosely bound to the mucus layer ($52 \pm 6\%$), or in the mucosal epithelium ($37 \pm 5\%$), and only $12 \pm 4\%$ had been absorbed into the muscle tissue, and therefore had a fairly similar distribution to the stomach tissues described earlier. Less than 1% of the recovered ^{109}Cd was found outside the gut sac in the fish. The distribution of this percentage was similar to that of the anterior intestine, with the most measured in the carcass ($53 \pm 1\%$). The gills, kidney, and anterior intestine all had about 11% of the internalized activity, with lesser amount in the liver and posterior intestine (~6%). Small amounts ($< 4\%$) of radioactivity were detected in the blood, stomach, spleen, and gallbladder (listed in order of greatest amounts to lesser amounts).

3.5. Uptake from posterior intestine

Of the total recovered ^{109}Cd, $59 \pm 14\%$ was not absorbed and was therefore collected in the mucosal saline at the end of the 8-h flux (Fig. 5). Similar to the mid intestine, the majority of the Cd within the posterior intestine was loosely bound to the mucosal epithelium, followed by the mucus-bound sample, while the least was in the muscle tissue (~55, 28, and 17%, respectively). Fish from this group had the greatest percentage of internalized Cd ($6 \pm 2\%$) compared to the other fish with different intestinal sections ligated, but not as much as those with the stomach ligated ($26 \pm 4\%$). Distribution of the internalized Cd was fairly similar to the other two intestinal segments, with most found in the carcass ($60 \pm 4\%$). Lesser amounts were found in the kidney, liver, gill, blood, and anterior intestine (~10, 7, 6, 5, and 5%, respectively. Only a small amount of the ^{109}Cd was detected in the posterior intestine, stomach, spleen, and gallbladder (all $< 4\%$ of the internalized activity, listed in order of decreasing amounts). Of the total recovered ^{109}Cd, $35 \pm 10\%$ was found in, or on, the gut tissue.

4. Discussion

4.1. Context

Results of this study have confirmed two of the three initial hypotheses, but challenged the third. Specifically, the stomach and posterior intestine proved to be most important areas for Cd uptake in terms of the percentage (~26% and 6%, respectively) of the recovered ^{109}Cd dose that was transferred to the internal tissues of the fish. Our previous *in vitro* experiments have shown that the stomach absorbs Cd, and our previous *in vivo* chronic feeding experiments have shown that Cd preferentially accumulates in the stomach wall, but the more important mechanistic information-that this translates to preferential internal Cd accumulation in the

fish-was lacking up until now. The present study demonstrates this important consequence, thereby confirming the first hypothesis. Similarly, long term *in vivo* feeding experiments had indicated low absorption efficiency, but had not shown whether this was due to low uptake, or high simultaneous efflux. The present experiments, where unidirectional uptake was measured by radiolabelled ^{109}Cd absorption, show clearly that uptake is low (~0.1–7%, but variable across different sections of the tract), thereby confirming the second hypothesis. However, contrary to third hypothesis, most of the internalized ^{109}Cd was not found in either the kidney or the liver. These points will be elaborated subsequently.

Which GIT segment of rainbow trout contributes to the greatest internalization of Cd after the ingestion of a contaminated meal has been hypothesized over past recent years, but remains unresolved. The *in vitro* 'gut sac' technique used by numerous researchers (Nadella et al., 2006, 2007; Ojo and Wood, 2007, 2008; Klinck and Wood, 2011) has given insight into relative capacities of GIT segments and has helped identify mechanisms of metal uptake in these different segments. However, past *in vitro* research has not replicated natural *in vivo* conditions. After the ingestion of a meal, each gut segment is subjected to different exposure concentrations of Cd and other potential complexing molecules, as well as different pH, enzyme concentrations, and waste, all for various lengths of time. *In vivo*, the GIT is affected by circulating blood levels of hormones, gases, energy and nutrient levels, and other important physiological molecules. The current study adds more information to this ongoing debate by blending the benefits of the *in vitro* gut sac methodology to those of an *in situ* gut sac in a living, free-swimming fish with an intact circulatory system. The approach is subject to obvious limitations (see below and Section 4.8), but moves the methodology closer to physiological reality and natural *in vivo* conditions. Most importantly, the circulation to the gut is intact, and endogenous neural and hormonal mechanisms are functional.

The selection of a 50 μM concentration of Cd in the mucosal saline for all segments was a compromise. Previous studies on trout fed Cd-enriched diets have found variable differences in Cd concentrations in the chyme in different sections of the tract (Franklin et al., 2005; Baldisserotto et al., 2005; Klinck et al., 2009), but only the latter study employed an environmentally relevant concentration of dietary Cd (12 μg Cd g^{-1} dry wt). In that study (Klinck et al., 2009), chronically fed trout had gut chyme concentrations similar to those used in our experiment (~30 μM) at 24 h after ingesting the contaminated food. Additionally, 50 μM was chosen so that the results could be more easily interpreted and compared to previous experiments done in our lab which used a similar metal concentrations (Nadella et al., 2006; Nadella et al., 2007; Ojo and Wood, 2007, 2008; Klinck, and Wood 2011).

Using Visual MINTEQ ver. 3.0, beta (Gustafsson, 2010; a chemical equilibrium model) 81% of the 50 μM Cd existed as Cd^{2+}. This was determined using the initial chemistry of the mucosal saline. The luminal environment likely changed over the 8 h exposure time as the fish absorbed/excreted various molecules. It would have been impossible to maintain complete consistency between the gut sections over the entire exposure period, but this was not the purpose of the study.

4.2. Relative importance of the stomach

From our *in situ* gut sac results, the stomach appears to be the greatest contributor to internalized Cd. Cd uptake from the stomach, as measured by absorption into internal organs and carcass, was more than 7% of the total Cd to which the stomach had been exposed. Only recently has the stomach been identified as an important site of absorption for some metals and ions. For example some *in vivo* experimentation on freshwater rainbow trout has suggested that the stomach is the most important segment of the GIT for Ca^{2+}, Na^{+}, and K^{+} transport (Bucking and Wood, 2006, 2007) and there is speculation that this may also be the case for Cd, since it is thought to share a common transporter with Ca^{2+} (Wood et al., 2006). The stomach environment has a lower pH compared to the intestinal segments, therefore free Cd^{2+} (the most bioavailable form) concentrations are likely higher (Baldisserotto et al., 2005). Also the stomach has a large surface area and is the first to encounter ingested food so it may be exposed to the highest peak concentrations. Further supporting evidence is given by Chowdhury et al. (2004) who found rapid absorption (within the first 0.5 h) of Cd into blood plasma of rainbow trout after a gastric infusion of ^{109}Cd. The stomach tissues are also known to accumulate Cd after being chronically exposed to Cd-contaminated diets (Baldisserotto et al., 2005; Franklin et al., 2005).

However, some caution may be warranted in the interpretation of our stomach data. Only about a quarter of the estimated radioactivity injected into the stomach was recovered at the end of the experiment. In initial trials, we were satisfied that our ligation techniques were secure and patent. Nevertheless, it remains possible that some ^{109}Cd could have leaked through the anterior ligation, as the tissue here is relatively thick, making it harder to form a tight seal. This could have been compounded by the fish attempting to regurgitate, as rainbow trout are known to do after ingesting a highly contaminated meal (Handy, 1993). Low recovery rates (~50%) of gastrically injected ^{109}Cd were also reported by Chowdhury et al. (2004), although they did not attempt to seal off part of the gut. Because fish were housed in a flow-through container over 8 h, it was impossible to measure ^{109}Cd in the surrounding water, therefore no evidence of leakage was actually observed. Another explanation for the low recovery percentage could be that ^{109}Cd was excreted in the urine or across the gills after being absorbed, but lack of presence of ^{109}Cd in the kidney, and the need for very high excretion rates to sustain this explanation, make this unlikely.

4.3. Relative importance of the anterior intestine

Based strictly on the total percentage of ^{109}Cd bound to, or in tissues, the anterior intestine could be considered the most important segment of the GIT in terms of uptake. Total uptake from the anterior intestine represented more than three quarters of the total estimated injected ^{109}Cd. This fits well with previous findings that the anterior intestine with its pyloric caecae accumulated the most Cd after being chronically fed Cd contaminated diets (Franklin et al., 2005; Klinck et al., 2009; Klinck and Wood, in press). However, this may be misleading since nearly all of the ^{109}Cd was detected within the gut tissue and was not internalized, despite the known role of the anterior intestine as a major site of sugars, amino acids, and dipeptide uptake (more than the other GIT segments combined) (Buddington and Diamond, 1986). As Franklin et al. (2005) suggested, the gut may act as a protective barrier against Cd uptake despite its relatively high accumulation rates.

4.4. Relative importance of the mid intestine

The mid intestine does appear to have some role in Cd uptake, but is relatively minor compared to the other GIT segments. Using an *in vitro* gut sac technique Klinck and Wood (2011) also identified it as the least important site of Cd uptake. This is likely in part due to its relatively low surface area—~5.6 cm^2 compared to ~19.4 cm^2 in the stomach, as estimated by Klinck and Wood (2011) using fish with same mass as in the present study. This segment is known to have transport mechanisms for Cd (e.g., lanthanum-sensitive and L-type Ca channels and ZIP-transporters

(Klinck and Wood, 2011), but their abundance appears to be lower compared to the other sections of the GIT.

4.5. Relative importance of the posterior intestine

The posterior intestine has also been targeted as an important area of Cd uptake in rainbow trout. Klinck and Wood (in press) found greatest accumulation in the posterior intestine using a different strain of freshwater rainbow trout (*Oncorhynchus mykiss irideus*). The results of Klinck and Wood (2011) agree that the posterior intestine is a major player in Cd uptake, showing it to have the highest transport rates *in vitro* (when all segments are exposed to equal concentrations of Cd). The posterior intestine has also been implicated as an important site of uptake for Ni and Pb (Ojo and Wood, 2007). Our results offer some support for this hypothesis as we found that a higher percentage of the total exposure content was internalized via the posterior intestine compared to the other intestinal segments (but still not as much as via the stomach). Increased blood supply may also play a role in greater internalization of Cd in this GIT segment. Although all segments are vascularized by the coeliacomesenteric artery (Seth et al., 2011), the posterior intestine benefits from additional blood supply provided by two unpaired arteries coming from the posterior dorsal aorta (Thorarensen et al., 1991).

4.6. The gut- an important barrier against Cd internalization

A large portion of the ^{109}Cd found in the gut material from all GIT segments was mucus-bound or in the mucosal-epithelium, since mucus is produced in large quantities to protect enterocytes from damage caused by digestive processes (Ezeasor, 1981). Also, Crespo et al. (1986) observed increases in the size and number of mucus-releasing goblet cells after dietary exposure to Cd. Perhaps in our experiments mucus was produced as a defensive reaction to Cd in order to form an additional barrier against its uptake, as Cd can bind to the mucus and later be sloughed off.

Overall, percentages of internalized ^{109}Cd from the total injected amounts were quite low, but fit well with the findings of other studies. For example, Chowdhury et al. (2004) found that rainbow trout gastrically infused with ^{109}Cd internalized only 2.4% of the total amount, and Klinck and Wood (in press) found absorption efficiencies of Cd to be ~1% after feeding trout for 21 days a Cd-contaminated diet. Depending on the GIT segment, we found absorption efficiency rates between ~7% and ~0.1%.

4.7. Distribution of internalized Cd

It is possible that internalized Cd from the stomach was greater than the intestinal portions because gastric filling could have induced a greater arterial blood flow, and thereby greater transport of Cd by the circulatory system to the internal organs of the fish. When the stomach is stretched, the aortic blood pressure can increase by ~30%, and venous blood pressure can increase ~500% (Seth et al., 2008). These changes in blood pressure may redistribute blood flow causing greater uptake from the stomach. It was surprising to find very little Cd in the liver and kidney since these organs have been found to accumulate the greatest amounts of internalized Cd in previous long term feeding studies (Szebedinszky et al., 2001; Chowdhury et al., 2004; Franklin et al., 2005; Ng et al., 2009). Exposure time therefore appears to be important; the 8 h incubation time used in our study may not have been long enough to show where Cd is stored in the long term. Cd uptake from the stomach which accumulated in the gill made up a third of the total internalized ^{109}Cd. It is possible that ^{109}Cd could have been transported there via the circulatory system, as Ng et al. (2009) and Klinck and Wood (in press) both found that the gills are an important site of Cd accumulation after ingesting Cd contaminated diets, though they did report that the kidney and liver were of greater importance than the gill on a per g basis.

Very little ^{109}Cd was internalized from the anterior- and mid-intestine, and the majority of what was detected ended up in the carcass. Most of the internalized ^{109}Cd from the stomach and posterior intestine was also found in the carcass; this could be partially explained by the sheer bulk of the carcass material since we did not express uptake on a per g basis. These results fit well with the findings of Klinck and Wood (in press) who also found the highest percentage of internalized Cd in the carcass (~44% in freshwater trout, 64% in seawater trout) after being fed a Cd diet (552 μg Cd g^{-1} food) for 21 days. Unlike the stomach, a substantial portion of the Cd uptake from the posterior intestine was measured in the kidney and liver. Ng et al. (2009) and Klinck and Wood (in press) also found high amounts of Cd accumulation in these two organs. Once Cd is absorbed across the GIT of trout it enters the blood stream and is believed to be transported directly to the liver by the hepatic portal system (Franklin et al., 2005), bound to transferrin (De Smetet al., 2001; Kwong et al., 2011). From there, Cd is transported throughout the body by the circulatory system, accumulating in the kidney, bound to metallothionein-like proteins which are present there in high concentrations (Nordberg and Nordberg, 1987).

4.8. Perspectives

We conclude from our results that the stomach plays the greatest role in the internalization of Cd compared to the other GIT segments. The posterior intestine also appears to contribute a substantial route of Cd entry into the body of fish. Our findings also confirm that the GIT acts as an important barrier against Cd uptake. We believe that the *in situ* gut sac methodology used in this experiment is an improvement over the *in vitro* gut-sac technique, but also realize that it has limitations and there is a need to further understanding of how stress, mucosal saline/chyme composition, exposure time, and other factors affect Cd uptake along the gastro-intestinal tract of fish.

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