Characterization of dietary Ni uptake in the rainbow trout, *Oncorhynchus mykiss*

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1. Introduction

Ni is found in bodies of water impacted by both natural processes through the erosion and weathering of rocks such as silicates (Bencko, 1983) and industrial processes such as mining, electroplating and smelting (Eisler, 1998; Ptashnyk and Klauserkamp, 2002; Brix et al., 2004). Ni is a transition metal which is considered to be essential micronutrient in most plant species, bacteria, invertebrates and perhaps mammals (Bencko, 1983; Nielsen et al., 1993). Ni essentiality has been well established in terrestrial organisms (see review by Phipps et al., 2002). In microorganisms and plants, Ni-containing enzymes are involved in nitrogen fixation, hydrogen metabolism and carbon cycling (Ragsdale, 2005). In fish, its essentiality status remains uncertain (Muysse et al., 2004), but recent evidence does indicate that it is homeostatically regulated in at least one freshwater fish species, the rainbow trout (*Oncorhynchus mykiss*) (Chowdhury et al., 2008). However, Ni may also be toxic. For example, in mammals Ni has been shown to cause apoptotic damage to cells (Park et al., 2007), allergies (Bocca et al., 2007) and renal disorders (Denkhaus and Salnikow, 2002).

Relative to other metals, waterborne Ni levels causing toxicity to aquatic organisms are relatively high (i.e. toxicity is low), and rates of Ni entry into the organism across the gills are also high (Meyer et al., 1999; Pane et al., 2003a,b, 2004a; Brix et al., 2004; Deleebeeck et al., 2007). Waterborne Ni appears to be primarily a respiratory toxicant with marked inhibitory effects on branchial gas exchange during acute high level exposures (Pane et al., 2003a,b), and more subtle pathological effects during chronic low level exposures (Pane et al., 2004b). The acute effects are due to external Ni, not internal Ni (Pane et al., 2004a). The exact mechanism of these effects is not known, but perhaps may reflect inflammatory swelling of the branchial epithelium associated with allergic reactions.

However there is a growing awareness that metal uptake and toxicity from the diet may be more important in many field situations (Dallinger and Kautzky, 1985; Meyer et al., 2005), and this may well be the case with Ni (reviewed by Ptashnyki et al., 2001). Histo-pathological lesions in livers, kidney, and intestine have been recorded in response to high concentrations of Ni in the
diet (Ptashynski et al., 2001, 2002). Fish readily take up Ni from spiked food (Ptashynski et al., 2001; Ptashynski and Klaverkamp, 2002), and when Ni is infused into the stomach (Chowdhury et al., 2008). In isolated intestinal sac preparations from trout, uptake rates of Ni were greater than those of five other metals (Ojo and Wood, 2007). However, at present, nothing is known about the mechanisms(s) of gastro-intestinal Ni uptake in fish, apart from the fact that it can be homeostatically down-regulated after chronic exposure to elevated waterborne Ni levels (Chowdhury et al., 2008).

Cellular mechanisms of Ni transport have not been completely elucidated. However, some studies suggest that Ni may use Ca and/or Mg channels (see Eisler, 1998 for a review), or the proton-coupled divalent metal transporter variously known as DCT1, DMT1, or Nramp2 (Gunshin et al., 1997). Ni interacts antagonistically with both Ca and Mg in a number of different systems. Ni is an effective blocker of several different types of Ca channels (McFarlane and Gilly, 1998; Todorovic and Lingle, 1998; Lee et al., 1999). Costa (1991) outlined the competitive binding behaviour between Ni and Mg in mammalian studies, and there is similar evidence at many other phylogenetic levels including bacteria (Kaltwasser and Naegle, 1998; Todorovic and Lingle, 1998; Lee et al., 1999).

2. Methods

2.1. Experimental organisms

Adult rainbow trout (200–300 g) of both sexes were obtained from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada. Rainbow trout were acclimated to their 500 L tanks for two weeks prior to the experiments in aerated, flowing, dechlorinated Hamilton (Ontario, Canada) tap water with a water composition of (in mmol l\(^{-1}\)): Na\(^{+}\) = 0.5, Cl\(^-\) = 0.7, Ca = 1.0, hardness = 140 ppm as CaCO\(_3\), pH = 8; 12 ± 2°C. The background Ni concentration in the Hamilton tap water was 20–30 nmol l\(^{-1}\). Fish were fed a 2% of body weight ration daily with Martin’s commercial dried pellet feed (5 point; Martin Mills Inc., Elmira, ON, Canada, containing 41.0% crude protein, 11.0% crude fat, 3.5% crude fiber, 1% Ca, 0.85% total P, 0.45% Na). The measured concentration of Ni in the food was 431 ± 14 nmol g\(^{-1}\).

2.2. In vivo determination of Ni uptake

Stored samples from the in vivo experiments conducted by Bucking and Wood (2006a,b, 2007) were re-analyzed for Ni content in the present study. The experimental procedures are briefly described below.

2.2.1. Diet preparation

Martin’s commercial dried pellet feed (5 point) was minced using a Braun PowerMAX Jug Blender (Gillette Company; Massachusetts, USA). The fine powder was transferred to an automatic pasta maker (Ronco Inventions; California, USA) and 8.5 grade lead–glass ballotini beads (0.40–0.45 mm in diameter; Jencons Scientific, PA, USA Inc.) were added at a density of 4% dry food mass. The beads and food powder were mixed for 30 min, NANO pure-II lead–glass ballotini beads (0.40–0.45 mm in diameter; Jencons Scientific, PA, USA Inc.) were added at a density of 4% dry food mass. The beads and food powder were mixed for 30 min, NANO pure-II water was then added (ratio 2:1) and the wet mixture was mixed for an additional 30 min to ensure even distribution of the beads throughout the wet food mixture. The mixture was then extruded and hand-shaped to resemble the 5 point trout chow the fish previously consumed. The pellets were air-dried and refrigerated at –20°C until further use. Tests by Gregory and Wood (1998) showed that the ballotini beads did not affect the palatability of the food, which was readily consumed to the same extent as the normal food.

2.2.2. Feeding and sampling procedures

Rainbow trout were starved for one week prior to experiment to allow for gut clearance and then fed the pelleted food described in Section 2.2.1 until satiation. The mean meal size amounted to 3.06 ± 0.02% of body mass (Bucking and Wood, 2006b, 2007). Six fish were sacrificed with a cephalic blow at each of the seven time intervals (2, 4, 8, 24, 48, 72 h) and weighed, as chemical anesthesia induced vomiting in earlier trials. An incision was made above the pelvic fin at the esophagus to just above the anal fin at the rectum. The gastrointestinal tract was tied off using silk ligature at each boundary between the stomach and anterior (including the caeca), mid and posterior intestine to contain all the gut contents in the appropriate segment. The GI tract was then dissected out and...
2.3. In vitro determinations of Ni uptake

2.3.1. Stomach and intestinal sac preparations

$^{63}$NiCl$_2$ (0.1 μCi); Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA) was used in all in vitro experiments to increase the sensitivity of Ni uptake measurements. General methods followed those outlined in Nadella et al. (2006a) and Ojo and Wood (2007). Fish were starved for one week prior to experiment, similar to the in vivo study. The in vitro gut sac method facilitates the measurement of short term metal uptake and binding by the gastrointestinal tract, and allows independent control of mucosal and serosal solutions. This technique allows separate study of the stomach, anterior, mid, and posterior intestines, and the sampling of Ni which has accumulated in the mucus, the mucosal epithelium (i.e. the enterocytes), and the blood compartment (muscle tissue and serosal saline) in each, thereby enabling the study of three steps in the transport process.

Trot was euthanized by over-dose with MS-222 (0.25 g/L). A ventral incision was made from the gills to the anus of the fish and the G.I. tract was removed from posterior to the common cardinal vein to the anus. The contents of the tract were gently emptied and then the tract was divided into four sections: the stomach (posterior to the esophagus), the anterior, mid and posterior intestine which can be differentiated based on morphological characteristics. The sacs were then flushed with saline (composition below) to displace any remaining intestinal fluid, food or feces. The posterior end of each section was tied off tightly using silk ligature. The anterior end of each gut segment, a heat flared PE-50 tubing was inserted, tied in with silk ligature, used to fill the sac with the test solution to a uniform stretch, and then flame-sealed. The sac was then weighed to obtain its initial weight (0.1 mg accuracy; Sartorius GMBH Gottingen; H110** V40, Germany) and immersed into a fixed volume of Cortland saline (modified from Wolf, 1963); NaCl, 133 mmol l$^{-1}$; KCl, 5 mmol l$^{-1}$; CaCl$_2$, 2.9 mmol l$^{-1}$; MgSO$_4$·7H$_2$O, 1.9 mmol l$^{-1}$; NaHCO$_3$, 1.9 mmol l$^{-1}$; NaH$_2$PO$_4$·H$_2$O, 2.9 mmol l$^{-1}$; glucose, 5.5 mmol l$^{-1}$; pH 7.4 for a 2h flux period (12 ml for the middle and posterior intestine and 40 ml for the anterior intestine and stomach). This saline bath solution, which acted as the serosal saline, was aerated with 99.7% O$_2$ and 0.3% CO$_2$ (i.e. PCO$_2$ = 2.25±0.25 torr) gas mixture to duplicate in vivo conditions. The temperature was maintained at 11–13 °C.

Following the 2-h incubation period, the sacs were removed from the saline solution and re-weighed toallow calculation of fluid transport rate. These data are not reported here because they were very similar to values reported for the various sections in previous gut sac studies (Nadella et al., 2006b, 2007; Ojo and Wood, 2007). However these measurements served as check to detect leakage; data from preparations showing unusually large fluid transport rates were discarded. Samples of the serosal saline and mucosal saline were collected. Sacs were then cut longitudinally, rinsed first 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. As described by Ojo and Wood (2007), the washing solutions plus blotting paper were collected for analysis as the “mucus-bound fraction” of nickel. The mucosal epithelium (i.e. the enterocytes) was then scraped off gently with a glass slide and collected separately, representing Ni 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 “mucosal layer”. The Ni in this fraction, combined with that in the serosal saline, represents metal that had been exported across the basolateral surface of the enterocytes into the blood compartment, and provides a conservative estimate of Ni absorption (Ojo and Wood, 2007). The surface area of the sacs was determined on graph paper as outlined in Grosell and Jensen (1999).
2.3.2. Transepithelial potential (TEP) measurements

TEP measurements across gut sacs of the stomach and mid-intestine were made in a subset of the experimental treatments. The experimental conditions chosen (60 μmol·l⁻¹ Ni in the luminal saline) were based on the highest Ni concentration used in the in vitro experiments of Section 2.3.5. Ag/AgCl electrodes were connected to agar bridges composed of 3 M KCl in 4% agar and TEP was recorded using a Radiometer PHM 82 standard pH meter (Radiometer; Copenhagen) as a high impedance voltmeter. One electrode accessed the mucosal side of the gut sac via the PE-50 tubing inserted into the tied off sac; this side was referenced at 0 mV. The other electrode was placed in the serosal bath. Each condition was measured five times for 5 min each.

2.3.3. Counting of ⁶³Ni

For fluid samples (mucosal and serosal saline and rinses), 1 ml of sample was added to 4 ml of distilled water plus 10 ml of Aqueous Counting Scintillant (ACS, Amersham Biosciences, Buckinghamshire, England). The muscle tissue, epithelial scrapings and blotting paper were digested in sealed vials using 1N HNO₃ for 48 h at 60°C in an oven, with vortexing at 24h. 1 ml of the digested tissue was added to 5 ml Ultima Gold (Perkin Elmer) as the scintillation fluid. All samples were then read using a Perkin Elmer Tri-carb 2900TR scintillation counter. After subtraction of background, all samples were corrected to the same counting efficiency as the fluid samples, using a quench curve constructed from various amounts of digest from the tissues of interest under the same counting conditions, and the external standard ratio method.

2.3.4. Concentration-dependence of Ni uptake in vitro

To determine the concentration-dependence (“kinetics”) of Ni transport in all four sections of the gastrointestinal tract (and thereby estimates of Km and Jmax), seven concentrations of Ni were employed: 1, 3, 10, 30, 60, 100 and 300 μmol·l⁻¹ with n = 5 preparations per concentration. A known amount of ⁶³Ni (0.1 μCi) was added to “cold” NiCl₂ to achieve the desired Ni concentrations in the mucosal saline. The serosal saline remained Ni-free. Concentrations were verified using graphite furnace atomic absorption spectroscopy (GFAAS; Varian SpectrAA-220 with graphite tube atomizer [GTA-110], Mulgrave, Australia) against certified atomic absorption standards (Fisher Scientific). Measurements were conducted at a wavelength and slit width of 232.0 nm and 0.2 nm, respectively, to obtain an optimal working range of 0.1–20 μg/ml, a sufficient concentration range for this study. Environment Canada certified reference material (a trace element fortified sample) was run with the standard for precision.

2.3.5. Effects of Mg and Ca on Ni uptake in vitro

The possible inhibitory nature of two divalent cations, Mg and Ca, against Ni uptake was tested in the stomach and mid-intestine. The concentration of Ni in the mucosal saline was maintained at 30 μmol·l⁻¹ in all exposures; this value was selected based on Km values obtained from Section 2.3.4 and was at the upper end of measurements obtained in Section 2.2.2 of fluid phase Ni concentrations in the chyme. Elevated concentrations of 50 mmol·l⁻¹ of Mg (as MgSO₄·7H₂O) and 50 mmol·l⁻¹ Ca (as Ca(NO₃)₂·4H₂O) were added to the mucosal saline to provide competition versus 1.9 mmol·l⁻¹ Mg and 1.0 mmol·l⁻¹ Ca in control preparations. These levels were chosen based on measurements of Bucking and Wood (2007); who reported Mg and Ca levels in the fluid phase of the chyme of rainbow trout fed this same diet between 3–45 mmol·l⁻¹ and 10–50 mmol·l⁻¹, respectively. Mannitol was added to the serosal saline to achieve osmotic balance when these Mg and Ca salts were added to the mucosal saline. Osmolality was checked with a Wescor 5100C (Logan, Utah, USA) vapour pressure osmometer.

Based on the results of the preceding experiment, the nature of the inhibition of Ni uptake by Mg (50 mmol·l⁻¹) and Ca (50 mmol·l⁻¹) was further investigated in the stomach and mid-intestine. A range of Ni concentrations (1–60 μmol·l⁻¹) in the mucosal saline at either 1.9 mmol·l⁻¹ or 50 mmol·l⁻¹ Mg and 1.0 mmol·l⁻¹ or 50 mmol·l⁻¹ Ca (N = 5 per combination) were employed to perform “kinetic analysis” (as in Section 2.3.4) so as to ascertain whether the effects were competitive or non-competitive in nature.

2.3.6. Calculations for in vitro series

Ni uptake rates (UR) were calculated as follows, using background- and quench-corrected CPM:

\[
UR = \frac{CPM}{(SA \times GISA \times t)}
\]

where CPM refers to the total counts per minute, SA the measured Ni specific activity (cpm/μmol), GISA the gastrointestinal surface area (cm²) and t the time (h) and UR the uptake rate (μmol cm⁻² h⁻¹).

In the Ni concentration-dependence series (“kinetics”), a modified Michaelis–Menten equation was fitted to the averaged data using non-linear regression analysis in GraphPad InStat (GraphPad Software, Inc., San Diego, CA, USA):

\[
UR = \frac{(J_{max} + Ni)}{(K_m \times [Ni])}
\]

where [Ni] is the substrate concentration (μmol·l⁻¹), Jmax is the maximum uptake for the saturable component (pmol cm⁻² h⁻¹) and Km is the concentration of half saturation (μmol·l⁻¹).

2.4. Statistical analysis

All data passed normality and homogeneity tests, or were transformed as necessary, before statistical analyses were performed (GraphPad InStat, GraphPad Software, Inc., San Diego, CA, USA).

In the in vivo study, the effect of location on Ni concentration was tested using a repeated measures ANOVA with gastrointestinal tract section as the main variable examined at each time point. The effect of time was tested using a one-way ANOVA with time as the main variable, and each tract section was examined individually for Ni concentration. The comparison between phases (fluid and solid) at each time point was evaluated using paired t-tests. Significant effects (p < 0.05) were determined after applying a Tukey's HSD (honest significant difference) post-hoc test or Bonferroni's correction as appropriate.

In the in vitro studies, comparisons between two treatment groups employed Student's two-tailed unpaired t-test, whereas comparisons amongst multiple treatment groups were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical significance was allotted to differences with a p < 0.05. Throughout, data have been reported as means ± SEM (N) where N = number of fish or number of preparations.

3. Results

3.1. Ni uptake in vivo

The measured Ni concentration in the supernatant (fluid phase) of the gut contents collected from different sections of the gastrointestinal tract of trout fed a standard commercial diet ranged between 2 μmol·l⁻¹ and 24 μmol·l⁻¹, with 10 μmol·l⁻¹ (Fig. 1A). These data indicated realistic Ni concentrations available to the mucosal epithelium of the tract in vivo under normal physiological conditions, and provided guidance regarding optimum concentrations in subsequent in vitro experiments. In general, these Ni concentrations in the fluid phase (Fig. 1A) were only about 10% of Ni concentrations in the solid phase (Fig. 1B) throughout the tract.
The measured Ni concentrations in the solid phase (Fig. 1B) and total chyme (Fig. 1C) were significantly reduced in the stomach at all time points relative to those in the ingested food. Ni concentration similarly declined over time in the fluid phase of the stomach chyme, highlighting the potential role of the stomach in Ni absorption (Fig. 1A).

There were no significant changes over time in the measured Ni levels in the chyme (fluid, solid and whole phases) within the anterior intestine (Fig. 1A–C). However, at later time points there were significant increases of Ni in both the fluid phase and the whole chyme of the anterior intestine relative to the stomach, indicating the possible secretion of Ni into the lumen of the anterior intestine. In the mid-intestine, the measured Ni concentration decreased over time in the fluid phase from 7.3 μmol l⁻¹ to 5.1 μmol l⁻¹, but Ni levels in the solid phase and total chyme of this compartment increased from about 0.07 μmol g⁻¹ to 0.20 μmol g⁻¹, indicating the possible precipitation of Ni into the solid phase (Fig. 1C). In the posterior intestine, while there were some small but significant variations, values remained more or less constant in all phases.
Net fluxes of Ni (\(\mu\text{mol Ni kg}^{-1}\)) to or from the total chyme of the digestive tract over various time periods after a meal. Negative values represent net Ni absorption and positive values denote net Ni secretion, calculated with reference to the previous segment by Eq. (1). Values are means ± S.E.M.; \(N=6\) per treatment. Statistical significance was tested using one-way ANOVA. Letters denote significant differences (\(p<0.05\)). *Significant decrease from the previous compartment at the same time period (\(p<0.05\)) and □, significant increase from the previous compartment at the same time point (\(p<0.05\)).

However, concentration data alone cannot be used to assess Ni absorption; trends may be confounded by the complex physiological processes of digestion, which may remove solid material (absorption) and either add (drinking, secretion) or remove water (absorption) at different sites simultaneously. Changes in chyme water content and associated fluid fluxes from this experiment have been reported in Fig. 6 of Bucking and Wood (2006b). Therefore, in order to track real changes in Ni absorption or secretion, Ni concentrations were normalized to the concentrations of ballotini beads, which are non-absorbable markers (Fig. 2).

In the fluid phase of chyme in the stomach, the relative Ni concentration declined by 85% over 72 h (from 0.019 \(\mu\text{mol Ni bead}^{-1}\) at 2 h to 0.003 \(\mu\text{mol Ni bead}^{-1}\) at 72 h; Fig. 2A). The relative Ni concentration in the solid phase of the gastric chyme rapidly declined by 40% within the first 2 h of meal ingestion (from 0.29 \(\mu\text{mol Ni bead}^{-1}\) in the food to 0.17 \(\mu\text{mol Ni bead}^{-1}\) 2 h following feeding), but thereafter remained stable (Fig. 2B and C). A similar, but less marked decline (35%) in the relative Ni concentration was observed in the total chyme where the relative Ni concentration fell from 0.29 \(\mu\text{mol Ni bead}^{-1}\) in the food to 0.19 \(\mu\text{mol Ni bead}^{-1}\) 2 h after feeding, but thereafter remained unchanged (Fig. 2C). Overall, these declines which are referenced to the inert marker to eliminate artifact indicate the importance of the stomach in Ni absorption.
The relative Ni concentration, again referenced to the inert marker to eliminate artifact, increased sharply in the total chyme of the anterior intestine when compared to gastric chyme samples (from 0.19 μmol Ni bead$^{-1}$ to 0.35 μmol Ni bead$^{-1}$), reaching similar levels to those present in food (Fig. 2C). These increases were seen in both fluid (Fig. 2A) and solid phases (Fig. 2B), likely reflecting the secretory environment of the pyloric caeca and biliary system. The relative Ni concentration found in the fluid, solid, and whole chyme phases decreased between the anterior and mid-intestine, however the posterior intestine was similar to the mid-intestine at most time points (Fig. 2B and C). There was also a significant decrease over time in the relative Ni concentration found in the fluid phase in all 3 intestinal sections, particularly in the fluid phases of both the anterior and mid-intestine, where the latter fell from 0.013 μmol Ni bead$^{-1}$ to 0.005 μmol Ni bead$^{-1}$ (Fig. 2A). In contrast, the solid and total chyme phases found in the mid-intestine showed increases in the relative Ni concentrations (Fig. 1B and C).

The Ni-to-bead ratios of the total chyme (Fig. 2C) suggest that the stomach was a major site of Ni absorption and a component of Ni absorption occurred in the mid-intestine. Net secretion of Ni into the chyme was apparent as it entered the anterior intestine. The anterior and posterior intestines did not appear to have a major role in dietary Ni uptake on a net basis. Ni content per bead was reduced about 50% from the food (0.29 μmol Ni bead$^{-1}$) to the posterior intestine at 72 h (0.15 μmol Ni bead$^{-1}$; Fig. 2C), indicating a net absorption efficiency of about 50% of dietary Ni after a single meal.

Net Ni uptake calculations with reference to the previous compartment (Eq. (1)) confirmed that absorption occurs in the stomach and mid-intestine, and to a very slight extent in the posterior intestine, whereas Ni secretion occurs in the anterior intestine (Fig. 3). The stomach appears to be the largest site of Ni absorption accounting for 78.5% of the total absorptive flux, with the majority of this absorption occurring in the first 4 h following feeding. In contrast, the mid-intestine absorbed Ni at a low rate over all time points, accounting for 18.9% of the total.

### 3.3. In vitro concentration-dependent Ni uptake

Ni uptake into the stomach, mid and posterior intestine of rainbow trout was biphasic over the range of luminal [Ni] from 1 μmol l$^{-1}$ to 300 μmol l$^{-1}$. At lower Ni concentrations of 1–30 μmol l$^{-1}$, Ni uptake was saturable displaying Michaelis–Menten kinetic parameters, whereas at higher concentrations, a linear relationship was seen. While this was true for all three compartments (mucus-bound, mucosal epithelium, and blood space), Fig. 6 reports data from only the latter. Representative data for Michaelis–Menten kinetic parameters (K_m, J_max) for the other compartments are summarized for the stomach and mid-intestine (the intestinal segment exhibiting greatest uptake) in Table 1. Within a segment, there were no significant differences in either K_m or J_max values, although the latter reflected the partitioning seen in Fig. 5—i.e. a high mucus-bound J_max in the stomach, and a high blood compartment J_max in the mid-intestine. Ni uptake into the anterior intestine was linear over the full range of concentrations tested.

For uptake into the blood compartment, the stomach exhibited the lowest K_m value (i.e. highest affinity) of 11 μmol l$^{-1}$ with a J_max of 53 pmol cm$^{-2}$ h$^{-1}$ (Fig. 6A), whereas the mid and posterior intestine displayed higher K_m values of 42 μmol l$^{-1}$ and 31 μmol l$^{-1}$ with J_max values of 215 pmol cm$^{-2}$ h$^{-1}$ and 510 pmol cm$^{-2}$ h$^{-1}$, respectively (Fig. 6C and D). The anterior intestine (Fig. 6B) exhibited a linear slope of 5.7 ± 0.1 pmol cm$^{-2}$ h$^{-1}$ per μmol l$^{-1}$. In the segments with biphasic uptake, the second phase of Ni uptake at 60–300 μmol l$^{-1}$ was linear in nature with slopes of 1.1 ± 0.2 pmol cm$^{-2}$ h$^{-1}$ per μmol l$^{-1}$, 6.5 ± 1.1 pmol cm$^{-2}$ h$^{-1}$ per μmol l$^{-1}$ and 6.6 ± 1.1 pmol cm$^{-2}$ h$^{-1}$ per μmol l$^{-1}$ for the stomach (Fig. 6A) and mid and posterior intestine (Fig. 6C and D) respectively.

To verify if Ni uptake in the G.I. tract corresponds more strongly to a linear relationship over the concentrations tested, the relative strengths of the linear and saturable relationships were assessed. Whereas the anterior intestine exhibited a better linear fit over the entire concentration range, the stomach and mid and posterior intestine exhibited stronger saturable r$^2$ values at low concentration in comparison to linear fits. The linear r$^2$ values at higher

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**Table 1**

Michaelis–Menten kinetic constants (J_max, K_m) for saturable Ni uptake in the three compartments of both the stomach and mid-intestine. Means ± 1 SEM.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>J_max (pmol cm$^{-2}$ h$^{-1}$)</th>
<th>K_m (μmol l$^{-1}$)</th>
<th>r$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood compartment</td>
<td>53 ± 21</td>
<td>11 ± 11</td>
<td>0.955</td>
</tr>
<tr>
<td>Mucosal epithelium</td>
<td>20 ± 9</td>
<td>22 ± 19</td>
<td>0.947</td>
</tr>
<tr>
<td>Mucus-bound</td>
<td>168 ± 115</td>
<td>93 ± 82</td>
<td>0.991</td>
</tr>
<tr>
<td>Mid-intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood compartment</td>
<td>215 ± 52</td>
<td>42 ± 9</td>
<td>0.930</td>
</tr>
<tr>
<td>Mucosal epithelium</td>
<td>10 ± 6</td>
<td>7 ± 11</td>
<td>0.527</td>
</tr>
<tr>
<td>Mucus-bound</td>
<td>15 ± 1</td>
<td>46 ± 7</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Note: Within each compartment, there were no significant differences. Constants were calculated from lines fitted to data points where N=5 per point, as in Fig. 6.
concentration (60–300 μmol l⁻¹) were 0.94, 0.95 and 0.97 for the stomach, mid and posterior intestines, respectively.

3.4. Cation competition with Ni

A luminal Ni concentration of 30 μmol l⁻¹ was employed in this series. In the stomach, elevated levels of Mg in the luminal saline (50 mmol l⁻¹ versus 1.9 mmol l⁻¹) significantly decreased Ni uptake into the blood compartment by 50%, and in the mid-intestine by 36% (Fig. 7). Interestingly, Mg had the opposite effect in the posterior intestine where it significantly increased Ni uptake by 34% (Fig. 7).

Elevated concentrations of Ca (50 mmol l⁻¹ versus 1.0 mmol l⁻¹) inhibited Ni uptake in the mid-intestine by 39% from 88 pmol cm⁻² h⁻¹ in controls to 54 pmol cm⁻² h⁻¹ (Fig. 7). Ni uptake remained uniform in all other gut segments with respect to the control.

Kinetic analysis (i.e. Ni concentration-dependence) was employed in the subsequent series so as to elucidate the nature of the inhibitory effects in the stomach and mid-intestine.

3.5. Kinetic analysis of cation competition on Ni uptake

In the stomach, this series demonstrated that $K_m$ values remained the same in the control and Mg treated sections (11.1 μmol l⁻¹ and 10.7 μmol l⁻¹ respectively); however, $J_{\text{max}}$ values were significantly reduced by 50% from 53 pmol cm⁻² h⁻¹ in controls to 25 pmol cm⁻² h⁻¹ (Fig. 8A). The inhibition of Ni uptake occurred only over the apparent range of high-affinity carrier-mediated transport, at or below 30 μmol l⁻¹. If anything, the opposite trend (though not significant) was observed in the linear phase above 30 μmol l⁻¹ where Ni uptake was slightly enhanced in the presence of elevated Mg (Fig. 8A). There was no significant inhibition of $K_m$ or $J_{\text{max}}$ values in the Ca treated stomach sacs (Fig. 8A), in agreement with the results of the preceding series (cf. Fig. 7).

A similar trend was observed in the elevated Mg treatment in the mid-intestine where uptake of Ni in the carrier-mediated phase caused a significant 65% reduction in $J_{\text{max}}$ from 215 pmol cm⁻² h⁻¹ to 79 pmol cm⁻² h⁻¹ (Fig. 8B). Although not significant, the elevated Mg treatment caused a decrease in $K_m$ as well. In the linear phase, above 30 μmol l⁻¹, Ni uptake was not significantly inhibited by Mg. A similar, less marked relationship was observed in the elevated Ca condition, where $K_m$ remained the same and $J_{\text{max}}$ values significantly decreased from 215 pmol cm⁻² h⁻¹ to 127 pmol cm⁻² h⁻¹, a 40% decline (Fig. 8B), again in agreement with the results of the preceding series (cf. Fig. 7).

3.6. Transepithelial potential (TEP)

Under normal physiological conditions, the TEP in the stomach was +7.5 ± 0.5 mV on the serosal side with reference to a mucosal (luminal) zero (Fig. 9A). The presence of 60 μmol l⁻¹ Ni in the mucosal saline caused a significant TEP drop to 0.4 ± 0.5 mV. Neither this trend nor the absolute values of TEP were significantly altered with the addition of 50 mmol l⁻¹ Ca to the mucosal saline.
Fig. 8. Influence of elevated concentrations of Mg and Ca on the concentration-dependent uptake kinetics of Ni in (A) the stomach and (B) the mid intestinal segments over a Ni concentration range of 1–60 μmol l$^{-1}$. In the experimental conditions, the Mg and Ca concentrations were maintained at 50 mmol l$^{-1}$. Values are means ± S.E.M.; $N=5$ per treatment. *Significant difference between the control and experimental condition ($p<0.05$).

However, the addition of 50 mmol l$^{-1}$ Mg lowered the TEP in the absence of Ni, and blunted the drop in TEP caused by the addition of 60 μmol l$^{-1}$ Ni (Fig. 9A).

The mid-intestine exhibited a lower TEP (+1.2 ± 0.4 mV) in comparison to the stomach under normal physiological conditions (Fig. 9B). Addition of 60 μmol l$^{-1}$ Ni to the mucosal side of the epithelium did not cause any significant change (Fig. 9B). There was no significant change in TEP with the addition of 50 mmol l$^{-1}$ Mg (0.6 ± 0.5 mV), and the lack of response to Ni remained unaltered. However, the presence of 50 mmol l$^{-1}$ Ca alone significantly decreased the TEP to −5.5 ± 1.3 mV; this negative TEP was not significantly altered (−4.0 ± 0.8 mV) by the addition of 60 μmol l$^{-1}$ Ni (Fig. 9B).

4. Discussion

4.1. Ni uptake—in vivo

We evaluated the passage of chyme and the net transport of Ni along the G.I. tract in the presence of an inert marker, using a validated protocol from earlier digestion studies (Bucking and Wood, 2006a,b; Nadella et al., 2006a). The use of the inert marker is essential because both the solid and fluid content of the chyme change continually as it moves along the digestive tract due to digestive, absorptive, and secretory processes. Tracking the disappearance of Ni from a single meal we measured 50% absorption efficiency (A.E.) for Ni in the gastro-intestinal tract (Fig. 2C). In mammalian studies, Nielsen et al. (1993) reported a 1.7–10% intestinal absorption of Ni in mice and 10–25% absorption of Ni in humans (as reviewed by Denkhaus and Salnikow, 2002). The A.E. determined in the present study is considerably higher than mammalian studies, potentially because the latter studies are measuring net retention of Ni rather than absorption via the gut (cf. Nadella et al., 2006a). As well, we note that fish in the present study were starved prior to experimentation, a condition shown to increase Ni absorption in mice (Nielsen et al., 1993). The high Ni absorption found in the tract is in contrast to some studies which indicated that Ni remains relatively unabsorbed through the gut in mammals and its uptake is only increased during periods of Fe-deficiency (Tallkvist and Tjalve, 1998), pregnancy or lactation (Nielsen, 1993). To our knowledge there has been no comparable study to provide a comparison for dietary Ni absorption in fish. In trout, Nadella et al. (2006a) reported a similar A.E. of about 50% for Cu, an essential nutrient. Ni essentiality in fish has been argued for based on evidence of homeostatic regulation through the gut of trout (Chowdhury et al., 2008) but remains conjectural (Muyssen et al., 2004).
Estimating net Ni absorbed in each segment in relation to Ni content of the previous compartment by Eq. (1), we provide clear evidence of the stomach and the mid-intestine as major sites of net Ni absorption in vivo (Fig. 3). A small fraction of net Ni absorption occurred in the posterior intestine as well (Fig. 3). Mean Ni concentrations in the fluid phase of the gastro-intestinal tract ranged from 2 μmol l⁻¹ to 24 μmol l⁻¹ (Fig. 1A), indicating that this is a biologically important concentration range for trout consuming a non-contaminated diet. Approximately 90% of Ni in the food was associated with the solid phase and 10% with the fluid phase in the stomach (Fig. 1A and B). Consequently, Ni content in the fluid phase was 2-fold higher in the stomach compared to the intestinal segments (Fig. 1A). Gastric acid is known to facilitate the release of metals and boost bioavailability (Whitehead et al., 1996). The acidic environment in this region is believed to release metalsbound to food conjugates, thereby increasing bioavailability to facilitate digestion (Gollan, 1975). The stomach has recently emerged as an important site associated with nutrient ion absorption (Bucking and Wood, 2006b, 2007) and accumulation of other metals (Franklin et al., 2005; Wood et al., 2006; Ojo and Wood, 2007) in trout. Our observations are in accordance with these studies; quantitatively, the stomach was the primary site of Ni absorption, accounting for 78.5% of the absorptive flux (Fig. 3). Our data also clearly demonstrate that the anterior intestine is not engaged in net Ni absorption but is a site of Ni secretion/addition. Bucking and Wood (2006b) postulated that the majority of fluid secretions into the anterior intestine were a result of gall bladder bile secretions. Elevated Ni concentrations have been reported in gall bladder (39 μmol kg⁻¹) and bile (238 μmol kg⁻¹) of lake white fish fed a high Ni diet (Ptashynski and Klaverkamp, 2002). It is conceivable therefore that biliary secretion is the source of the observed net addition in the anterior intestine (Fig. 3).

4.2. Ni uptake—in vitro

Using a constant Ni concentration of 30 μmol l⁻¹ (based on the high end of Ni concentrations measured in the fluid phase of chyme in vivo), uptake along the four sections of the G.I. tract was determined in vitro. Unidirectional Ni uptake per unit area was significantly higher in the three intestinal segments compared to the stomach (Fig. 4). However, by using measurements of surface area of the various sections for a typical 250 g trout of the present study, it was possible to estimate total uptake rates for each segment (Table 2). When the considerably greater surface area of the stomach was taken into account, unidirectional Ni uptake was comparable between the stomach, mid and posterior intestinal segments (Table 2). Surprisingly unidirectional uptake in the anterior segment was higher compared to the mid and posterior segments (Fig. 4). However, in vivo we found the anterior segment was a site of net secretion and not absorption (Fig. 3). It has been shown previously that the absorption of ions occurring in the anterior intestine is generally masked against the large background of net secretion (Bucking and Wood, 2006b, 2007). Studies measuring dietary Ni accumulation in fish (Ptashynski and Klaverkamp, 2002; Chowdhury et al., 2008) have similarly reported highest Ni concentrations in the intestinal tissue. Indeed, Ni transport rates in rainbow trout were higher in the intestinal segments than in the stomach in an in vitro study (Ojo and Wood, 2007) using an approach similar to ours. In contrast, our observations in vivo indicate the stomach to be the predominant site of Ni uptake.

Several factors likely account for this discrepancy. Firstly, in vivo, the stomach is exposed to generally higher dissolved Ni levels than the other segments during the 0–4 h period of high uptake (Fig. 1A), and more importantly it has access to the most bioavailable form of Ni, the free ion, at acidic in vivo pHs. At the higher pHs in the intestine, even dissolved Ni may be complexed with organic molecules released from the food. One major difference between the in vivo and in vitro protocols was that the pH of the luminal contents in vitro was set to 7.4 at the start of the flux to standardize conditions between the segments. We have found that the pH in the intestinal sacs stays close to this value throughout the flux period, whereas that in the stomach tends to fall, but never reaches the low levels reported in vivo. Very recently, in trout, Bucking and Wood (2009) have shown a pre-prandial pH of 2.72 in the stomach chyme which rises to 4.9 after feeding. Under these in vivo conditions, Ni will likely speciate into its ionic form and become more bioavailable for uptake (Shehadeh and Gordon, 1969). Another difference could be that Ni binding to surface mucus in vitro is higher in the stomach relative to the intestine (Fig. 5A, Table 1), and this might impede stomach uptake (Fig. 4). Thus, the role of the stomach in Ni absorption may have been underestimated in these in vitro studies.

4.3. In vitro concentration-dependent Ni uptake

To the best of our knowledge, the present study is the first to characterize the kinetics of gastrointestinal Ni uptake. Ni uptake in the stomach and mid and posterior intestines appears to occur via two mechanisms: a carrier-mediated component (from 1 μmol l⁻¹ to 30 μmol l⁻¹) and a diffusional component (from 60 μmol l⁻¹ to 300 μmol l⁻¹). Michaelis–Menten kinetic analysis for the carrier-mediated component reveals a high-affinity, low capacity transport mechanism in the stomach with $K_{m} = 11 \mu\text{mol} l^{-1}$ and $J_{max} = 53 \mu\text{mol cm}^{-2} h^{-1}$. In comparison the intestinal segments (mid and posterior) exhibit a somewhat lower affinity but much high capacity transport system with $K_{m}$ values ranging from 31 μmol l⁻¹ to 42 μmol l⁻¹ and $J_{max}$ values of 215–510 μmol cm⁻² h⁻¹. Ni uptake by the anterior intestine appears to occur via passive diffusion (Fig. 6). We are not aware of any studies identifying the kinetics of gill Ni uptake in fish. However, a similar saturable trend of Ni uptake was described by Pane et al. (2006a) in the renal brush border membrane vesicles of trout where the $K_{m}$ for renal Ni transport was 18 μmol l⁻¹, comparable to the stomach in the present study. Biphasic Ni uptake has also been demonstrated in the BBMV from the small intestine of rabbit (Knopf et al., 2000) and the ileal and jejunal segments of rats (Tallkvist and Tjalve, 1994). $K_{m}$ values ranging from 20 μmol l⁻¹ to 38 μmol l⁻¹ for Ni uptake in the jejunal segment of rats (Foulkes and McMullen, 1986; Stangl et al., 1998; Muller-Fassbender et al., 2003) are similar to our findings in the trout intestine.

Table 2

<table>
<thead>
<tr>
<th>G.I. segment</th>
<th>Ni uptake rate (pmol cm⁻² h⁻¹)</th>
<th>Surface area (cm²)</th>
<th>Total Ni uptake rate per G.I. segment (pmol h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>34</td>
<td>35</td>
<td>1190</td>
</tr>
<tr>
<td>Anterior intestine</td>
<td>107</td>
<td>30</td>
<td>3210</td>
</tr>
<tr>
<td>Mid-intestine</td>
<td>88</td>
<td>12</td>
<td>1056</td>
</tr>
<tr>
<td>Posterior intestine</td>
<td>107</td>
<td>15</td>
<td>1605</td>
</tr>
</tbody>
</table>
mediated transport at concentrations less than 100 μmol l\(^{-1}\). At 100 μmol l\(^{-1}\) and higher, simple physical diffusion appeared to contribute to Ni uptake by BBMVs. It can be argued that this passive diffusion was aided by the diminished electrical potential opposing uptake with increasing Ni levels. TEP measured at a Ni concentration of 60 μmol l\(^{-1}\) in both the stomach and mid-intestine was considerably less positive on the serosal side (Fig. 9A). Although the kinetic nature of Ni transport in many species has not yet been characterized, it was noted by Tallkvist and Tjalve (1998) that above 200 μmol l\(^{-1}\) Ni concentrations, the cellular integrity of monolayers are greatly disturbed causing an influx of Ni through the Caco-2 cells. Other studies by Tallkvist et al. (2003) outlined the possibility that the influx of Ni in the presence of metabolic inhibition may be caused by the loosening of junctions between the human Caco-2 cells of the epithelium.

4.4. Cation competition with Ni

Ni uptake was significantly inhibited by Mg in the stomach and by Mg and Ca in the mid-intestine (Fig. 7), at concentrations (50 mmol l\(^{-1}\)) which are known to occur in the fluid phase of the chyme in trout fed normal commercial diets (Buckingham and Wood, 2007). These effects cannot be explained by changes in TEP, because TEP either did not change (Fig. 9A) or became more serosal-negative (with elevated Ca in the mid-intestine; Fig. 9B) in the presence of the inhibiting ions. Kinetic analysis indicated that the mechanism involved non-competitive inhibition (i.e. reduced \(J_{\text{max}}\), unchanged \(K_m\)) for the effect of Mg in the stomach, and both Ca and Mg in the mid-intestine (Fig. 8). In part, this may be due to the ability of Mg and Ca to alter membrane permeability and tighten paracellular junctions (Hunn, 1985), decreasing the uptake and/or binding of metal ions such as Ni (Meyer et al., 1999; Deleebeeck et al., 2007). In bacteria, high Ca conditions may decrease the transcription rate of Mg/Ni transport systems, indirectly causing a non-competitive decrease in Ni uptake (Snavely et al., 1991).

Historically, both Mg and Ca are recognized as specific Ni antagonists in physiological as well as toxicological studies (see Section 1). The reciprocal effect, the blockade of Ca channels by Ni, may have both competitive and non-competitive components (McFarlane and Gilly, 1998; Todorovic and Lingle, 1998; Lee et al., 1999). The existence of a shared uptake pathway for Mg and Ni is also supported by the fact that Ni and Mg have similar dehydrated ionic radii (Weast, 1973). Pane et al. (2006a) reported inhibition of Ni uptake into intestinal BBMVs of trout by Mg at a 100:1 Mg to Ni molar ratio, while Ca inhibited uptake at a 1000:1 molar ratio. The Mg effect was attributed to a non-specific transport of Ni by a low affinity, high capacity Mg transport system (Pane et al., 2006b). A similar electro-diffusive Mg pathway was described by Freire et al. (1996) in trout renal BBMVs. Chronic Ni exposure is also reported to reduce whole-body Mg concentration and unidirectional Mg uptake rate in Daphnia magna (Pane et al., 2003a,b). In microorganisms, transmembrane transport of Ni occurs non-specifically via transport systems for other cations, such as that for Mg in the fission yeast, Schizosaccharomyces pombe (Eitingier et al., 2000). As well, Ni has been implicated as a competitive inhibitor of Mg uptake via three different types of Mg transporters in the prokaryote, Salmonella typhimurium (Snavely et al., 1991).

4.5. Conclusions

We have demonstrated that trout exhibit a high absorption efficiency for Ni (50%), similar to that for the essential metal Cu, from a non-contaminated diet, which adds to the growing evidence for the essentiality of Ni in fish diets (cf. Muyssen et al., 2004; Chowdhury et al., 2008). Our data emphasize specifically the stomach and mid-intestine as being critical sites of dietary Ni absorption both in vivo and in vitro. Characterization of Ni uptake comfortably fits into a Michaelis–Menten type framework in these segments, suggesting carrier- or channel-mediated transport. We provide clear evidence for the interaction between Ni transport and traditionally competitive ions Mg and Ca in the digestive tract of rainbow trout. If Ni does eventually prove to be essential in fish, then it is apparent that the Mg and Ca content of the food will be an important consideration affecting the availability of Ni. It will also be of interest to test whether one or both of these dietary ions ameliorates the toxicity of Ni, just as dietary Na does for Cu (Kamunde et al., 2005), and dietary Ca does for Cd (Wood et al., 2006).

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