

# The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss*

Collins Kamunde\*, Chris M. Wood

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, Canada L8S 4K1

Received 15 November 2001; received in revised form 21 June 2002; accepted 24 June 2002

## Abstract

The influence of ration size on homeostasis and sublethal toxicity of copper (Cu) was assessed in rainbow trout (*Oncorhynchus mykiss*) during dietary Cu exposure in synthetic soft water. A constant dietary dose of 0.24  $\mu\text{mol Cu}$  per g fish per day as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was delivered via diets containing 15.75, 7.87, and 5.24  $\mu\text{mol Cu g}^{-1}$  fed at 1.5, 3.0, and 4.5% wet body weight daily ration, respectively. Juvenile rainbow trout showed clear effects of ration but not Cu on growth suggesting that growth is hardly a sensitive endpoint for detection of sublethal dietary Cu exposure. All Cu-exposed fish accumulated the same total metal load when expressed on a per fish basis. This suggests that differences in tissue and whole-body Cu concentrations among the treatments reflected the differences in the fish size rather than total Cu accumulation, and demonstrate that absorption and accumulation of Cu from the gut during dietary exposure are independent of the food quantity in which the Cu is delivered. Fish fed a high ration exhibited greater mass-specific unidirectional uptake of waterborne Cu than fish fed a low ration indicating an increased need for Cu for growth processes in rapidly growing fish. Stimulated excretion of Cu was indicated by greater Cu accumulation in the bile of the Cu-exposed fish. Branchial  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase was not affected by dietary Cu exposure or ration but gut  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities showed stimulatory effects of increasing ration but not of Cu exposure. The 96-h LC50 for waterborne Cu (range 0.17–0.21  $\mu\text{mol l}^{-1}$  (10.52–13.20  $\mu\text{g l}^{-1}$ ) was the same in all treatment groups indicating that ration size was unimportant and that dietary Cu did not induce an increase in tolerance to waterborne Cu. Taken together these results suggest that the nutritional status, fish size, and growth rates should be considered when comparing whole-body and tissue Cu concentration data for biomonitoring and risk assessment. Moreover, expressing the exposure as total metal dose rather than metal concentration in the diet is more appropriate.

© 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Cu homeostasis; Dietary Cu; Rainbow trout; Ration;  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase; Risk assessment

\* Corresponding author. Tel.: +1-905-525-9140x23237; fax: +1-905-522-6066

## 1. Introduction

Although dietary factors have marked effects on fish physiology and metabolism (Cowey and Sargent, 1979), the possible modifying effect of food-related variables on the toxicity and homeostatic regulation of metals has been largely neglected in aquatic toxicology (Lanno et al., 1989). Most dietary studies carried out on fish have concentrated on growth–ration relationships geared toward establishing nutritional adequacy of diets for fish in aquaculture (Brett and Groves, 1979; Cho et al., 1982; Cowey, 1992). These studies have established that growth of fish is strongly regulated by the quantity of food consumed. However, metal contaminants in the diet may influence fish health negatively by inducing toxicosis or by affecting food utilization. For example some previous studies have reported that environmental pollutants affect appetite of the fish resulting in changes in the dynamics of metal/chemical uptake, metabolism, and depuration (Jiminez et al., 1987; Lanno et al., 1989; Wilson et al., 1994; D’Cruz et al., 1998). Several studies that have specifically assessed the effect of feeding/starvation (Buckley et al., 1982; Collvin, 1985; Segner, 1987) on metal toxicity employed waterborne exposures and reported variable results. However, there are indirect indications that nutritional factors such as high ration may mitigate waterborne (Taylor et al., 2000) and dietary (Kamunde et al., 2001) Cu toxicity in fish. These data highlight the need for a detailed examination of the role of nutritional status on the responses of fish to metals exposure.

There is apparently no agreement on the level of dietary Cu toxic to fish. Although earlier work by Lanno et al. (1989) determined the toxic threshold to be approximately  $730 \mu\text{g Cu g}^{-1}$  diet, recent data have not concurred with this finding (Berntsen et al., 1999; Kamunde et al., 2001). Possible causes of these discrepancies include differences in exposure periods, feeding regimes, fish size, and species. Several studies have investigated the effects of varying dietary metal concentrations in fish at constant ration (see review by Handy, 1996) but none, to our knowledge, have investigated the influence of constant metal load presented in different ration levels.

Impairment of branchial  $\text{Na}^+, \text{K}^+$ -ATPase during acute waterborne Cu exposure has been unambiguously characterized in rainbow trout (Laurén and McDonald, 1987; Li et al., 1998). However, it remains to be determined whether dietary Cu imparts toxicity by affecting  $\text{Na}^+, \text{K}^+$ -ATPase activities in the gastrointestinal tract. Furthermore, since dietary Cu has been shown to accumulate in the gills in some studies (Miller et al., 1993; Kamunde et al., 2001, 2002), it would be interesting to assess if Cu accumulated at the gill from the diet would have a similar inhibitory effect as waterborne Cu.

The aim of the present study was therefore to test the hypothesis that fish maintained on high ration have superior ability to regulate and arrest the deleterious effects of dietary Cu exposure. This was done by offering juvenile rainbow trout the same Cu load in three rations ranging from 1.5 to 4.5% wet body weight per day. We anticipated that sublethal endpoints of chronic metal toxicity such as growth would be influenced by the nutritional status of the animal and subsequently impact metal uptake, distribution, excretion, and accumulation. In addition, exposing fish to metal under different feeding regimes is environmentally realistic because food abundance and feeding indices vary with season in aquatic ecosystems (Segner, 1987; Smith and Griffith, 1994). A second objective was to establish possible connections between tissue Cu accumulation, metal dose, and toxicity for purposes of risk assessment in aquatic toxicology. Previous studies (Miller et al., 1992; Farag et al., 1995; Marr et al., 1996) have associated tissue metal residues with adverse effects and recently Bergman and Dorward-King (1997), proposed the use of tissue metal burdens for biomonitoring, risk assessment, and derivation of water quality criteria. Third, a waterborne Cu toxicity test was performed to determine if dietary Cu induced acclimation to waterborne Cu (McDonald and Wood, 1993) and whether ration size had any role in this process. Finally, we examined the possible effect of chronic sublethal dietary Cu exposure and ration size on branchial and gastrointestinal tract  $\text{Na}^+, \text{K}^+$ -ATPase activities.

## 2. Materials and methods

### 2.1. Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) 9–10 g in weight were obtained from Humber Springs trout farm and acclimated to laboratory conditions for 2 weeks. Laboratory conditions consisted of a flow-through of aerated dechlorinated Hamilton tap water containing:  $\text{Na}^+$  0.6 mmol  $\text{l}^{-1}$ ,  $\text{Cl}^-$  0.7 mmol  $\text{l}^{-1}$ ,  $\text{Ca}^{2+}$  1 mmol  $\text{l}^{-1}$ ,  $\text{Mg}^{2+}$  0.21 mmol  $\text{l}^{-1}$ , hardness 1.4 mmol  $\text{l}^{-1}$  as  $\text{CaCO}_3$ , alkalinity 0.95 mmol  $\text{l}^{-1}$  as  $\text{CaCO}_3$ , and dissolved organic carbon (DOC) 3.0 mg  $\text{l}^{-1}$ . Water pH and temperature were 7.9–8.2, and 14 °C, respectively, and background Cu concentration was about 47.24 nmol  $\text{l}^{-1}$  (3  $\mu\text{g l}^{-1}$ ). Subsequently, the fish were gradually acclimated to synthetic soft water. Soft-water was generated from dechlorinated Hamilton tap water by reverse osmosis and mixed gradually with regular dechlorinated tap water to achieve a final mixture of about 6:1 reverse osmosis–tap water, over a period of 2 weeks. Fish were then maintained in this synthetic water for 2 months before initiation of the experiment. Water composition at the end of acclimation was:  $\text{Na}^+$  0.12 mmol  $\text{l}^{-1}$ ,  $\text{Cl}^-$  0.10 mmol  $\text{l}^{-1}$ ,  $\text{Ca}^{2+}$  0.13 mmol  $\text{l}^{-1}$ ,  $\text{Mg}^{2+}$  0.04 mmol  $\text{l}^{-1}$ , and hardness 0.20 mmol  $\text{l}^{-1}$  as  $\text{CaCO}_3$ , alkalinity 0.15 mmol  $\text{l}^{-1}$  as  $\text{CaCO}_3$ , DOC 0.4 mg  $\text{l}^{-1}$ . Background Cu in soft water was 22.05 nmol  $\text{l}^{-1}$  (1.4  $\mu\text{g l}^{-1}$ ), pH was 7.0, and temperature was 14 °C. During the pre-experimental and acclimation period, fish were fed once daily at 2% wet body weight on commercial trout chow (Corey Feed Mills Ltd., Fredericton, New Brunswick) containing 55% crude protein, 17% crude fat, 2% crude fiber, 1.5%  $\text{Ca}^{2+}$ , 0.6%  $\text{Na}^+$ ). Cu concentration of the diet was 0.31  $\mu\text{mol g}^{-1}$  (20  $\mu\text{g g}^{-1}$ ).

### 2.2. Experimental diets and feeding

Experimental diets were made in-house by supplementing the commercial trout chow with the required amount of Cu calculated to deliver 0.24  $\mu\text{mol}$  (15  $\mu\text{g}$ ) Cu per g fish per day in 1.5, 3.0, and 4.5% daily ration. This was achieved by making diets containing 15.75 (1000), 7.87 (500),

and 5.24 (333)  $\mu\text{mol g}^{-1}$  ( $\mu\text{g g}^{-1}$ ) Cu for the 1.5, 3.0, and 4.5% rations, respectively (Table 1). For all the diets the commercial trout chow was ground in a blender and the appropriate amount of Cu (as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) for each diet dissolved in 10% diet weight of double distilled water and mixed in a pasta maker for 45 min. This ensured homogenous distribution of the Cu throughout the food. Thereafter, further 30% diet weight double distilled water was added (bringing total volume of water added to 40% diet weight) and mixed for a further 15 min. The food was subsequently extruded via a pasta maker, air-dried, and broken into small pellets (approximately 3 mm<sup>3</sup>) by hand. Control diet was treated in the same way except that no Cu was added. All diets were kept at –20 °C till use. The nominal and actual Cu concentrations of the diets are shown in Table 1. During the experiment fish were fed the designated diet at half the designated ration twice a day, once in the morning (08:00–09:00 h) and again in the evening (18:00–19:00 h). The fish were allowed to feed for 1 h, after which fecal material was siphoned and a water sample taken for Cu analysis. Visual observation revealed that all food was ingested. Bulk fish weights obtained weekly for each group were used to calculate the ration for the following week.

### 2.3. Exposure set-up

The exposure set-up was a complete factorial design composed of six 150-l tanks partitioned in half by dividers giving a total of 12 experimental chambers for three treatments and respective controls, in duplicates. Water flow rate into the tanks was 1.2  $\text{l min}^{-1}$  providing 50% replacement of the water in 1.4 h. Each partition of the tank was provided with gentle aeration. For each ration the control and treatment group were kept together in one tank (but in separate partitions), so that any changes in waterborne Cu concentration would affect both the control and the treatment group in the same way. A previous study (Kamunde et al., 2001) had reported elevated waterborne Cu concentration, probably from excretion, during static waterborne Cu flux experiments in fish exposed to dietary Cu. However, since the fish

Table 1  
Ration size, dietary Cu concentrations, and total Cu dose delivered per g fish per day during the experiments

Ration (% wet body weight)	Diet Cu concentration [ $\mu\text{mol g}^{-1}$ , nominal (actual)]		Total Cu dose ( $\mu\text{mol per g fish per day}$ )	
	Control	Treatment	Control	Treatment
1.5	0.31 (0.35 $\pm$ 0.03)	15.75 (15.94 $\pm$ 0.54)	$4.72 \times 10^{-3}$	0.24
3.0	0.31 (0.35 $\pm$ 0.03)	7.87 (8.0 $\pm$ 0.35)	$11.03 \times 10^{-3}$	0.24
4.5	0.31 (0.35 $\pm$ 0.03)	5.24 (5.32 $\pm$ 0.30)	$15.74 \times 10^{-3}$	0.24

Actual dietary Cu concentrations are means  $\pm$  SEM,  $n = 9$ .

were maintained in a flow-through system in the present study, measurement of water Cu concentration soon after feeding (range 18.9–25.2 nmol  $l^{-1}$ ) (1.2–1.6  $\mu\text{g l}^{-1}$ ) showed no significant changes.

#### 2.4. Sampling

Fish were bulk weighed in a 20-l bucket lined with a plastic sieve and then starved for 36 h before sampling on days 0, 9, 21, and 35. For each treatment and its associated control, 10 fish (five from each replicate) were randomly removed, initially for determination of unidirectional waterborne Cu uptake as described below. Subsequently, the fish were killed with an overdose of neutralized tricaine methanesulfonate (MS-222; 1 g  $l^{-1}$  containing 0.25 g  $l^{-1}$   $\text{NaHCO}_3$ ) and organs [gills, liver, muscle, gut (stomach, pyloric caecae + anterior intestine, mid intestine and posterior intestine), plasma, bile, and rest of carcass] were collected into separate pre-weighed scintillation vials or bullet tubes, weighed, and then frozen for subsequent analyses.

#### 2.5. Unidirectional waterborne Cu uptake

The effect of the dietary exposure conditions on unidirectional waterborne Cu uptake by gills was assessed at each sampling time. For each feeding regime 10 control and 10 Cu-treated fish were moved into partitioned 20 l flux containers and exposed to waterborne  $^{64}\text{Cu}$  at ambient water Cu concentration. The radioisotope  $^{64}\text{Cu}$  (as  $\text{CuNO}_3$ ) was prepared at the McMaster University Nuclear

Reactor. The radioisotope dosage administered ( $0.7 \mu\text{Ci l}^{-1}$ ) added a total concentration of 3.15 nmol ( $0.2 \mu\text{g l}^{-1}$ ) Cu into the water, and therefore did not substantially elevate the nominal water Cu concentration or alter the body or tissue burdens accumulated in the preceding weeks. The fish were exposed to the  $^{64}\text{Cu}$  for 12 h under static water conditions and continuous gentle aeration similar to the protocol of Kamunde et al. (2001, 2002). The tanks used for the flux measurements were pre-incubated overnight with soft water of the same chemical characteristics (i.e. same Cu concentrations) as the water used during the flux at all sampling times. A 10-ml water sample was taken from each tank 15 min after introduction of  $^{64}\text{Cu}$  and again after the 12-h flux period. Water  $^{64}\text{Cu}$  and total Cu concentration did not change by more than 5% during the 12 h period. Unidirectional Cu uptake was determined as outlined in calculations (below).

#### 2.6. $\text{Na}^+, \text{K}^+$ -ATPase activities

$\text{Na}^+, \text{K}^+$ -ATPase activities were determined for the entire gill baskets and specific sections of the gut (stomach, pyloric caecae [includes anterior intestine], mid intestine, and posterior intestine) after 35 days of exposure to dietary Cu employing the microplate UV detection method (McCormick, 1993). Six fish from each group were sacrificed as described above. Gut and gill samples were rinsed in double distilled water upon dissection, frozen immediately in liquid nitrogen, and subsequently stored at  $-70^\circ\text{C}$  until they could be analyzed for  $\text{Na}^+, \text{K}^+$ -ATPase activity. The tissues were initi-

ally thawed and subsequently homogenized on ice in  $10 \times$  volumes of 4:1 SEI–SEID buffer for 45 s; SEI = 250 mM sucrose, 10 mM  $\text{Na}_2\text{EDTA}$ , 50 mM imidazole, pH 7.3; SEID = 0.5% sodium deoxycholic acid in SEI. The homogenized tissues were then centrifuged at  $5000 \times g$  and the supernatant immediately analyzed for  $\text{Na}^+, \text{K}^+$ -ATPase activity. For all the tissues 10  $\mu\text{l}$  of the supernatant was used in a reaction mixture containing 50  $\mu\text{l}$  salt solution (50 mM imidazole, 189 mM NaCl, 10.5 mM  $\text{MgCl}_2 \cdot 5\text{H}_2\text{O}$ , 42 mM KCl, pH 7.5) and 150  $\mu\text{l}$  assay solution A or B, where assay solution A contains 50 mM imidazole, 2.8 mM phosphoenolpyruvate, 0.22 nicotinamide adenosine dinucleotide, 0.7 mM adenosine triphosphate, 4  $\text{U ml}^{-1}$  lactate dehydrogenase, 5  $\text{U ml}^{-1}$  pyruvate kinase, pH 7.5, while assay solution B is solution A + 0.5 mM ouabain. Each sample was read in quadruplicates, two with solution A and two with solution B. In this assay the rate of hydrolysis of ATP to ADP in the presence and absence of ouabain is coupled to the oxidation of NADH to  $\text{NAD}^+$ . Changes in absorbance of the reaction mixture due to NADH oxidation were measured at 340 nm over 15-s intervals for 10 min.  $\text{Na}^+, \text{K}^+$ -ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of ouabain, and normalized to total protein of the respective sample as determined according to Bradford (1976).

### 2.7. Toxicity test

Tolerance to acute waterborne Cu exposure was assessed using a 96-h acute toxicity test carried out at the end of the 35-day experiment. To allow direct comparison between treatments and controls, the containers (30-l capacity) used for the test were partitioned in the same way as the experimental tanks to ensure exposure to the same Cu concentration for both treatment and control. Ten fish for each treatment and respective control were tested in five Cu concentrations (nominally 0, 0.08, 0.16, 0.32, and  $0.63 \mu\text{mol l}^{-1}$ ) according to the protocol of Taylor et al. (2000). The test was carried in a flow-through system at a flow rate of  $150 \text{ ml min}^{-1}$  allowing 50% replacement of water in about 2.3 h. The water Cu

concentration was checked three times daily by atomic absorption analysis and maintained within  $\pm 5\%$  of the nominal value. Fish were not fed during the toxicity test. Mortality was monitored and dead fish were removed and treatment and time of death recorded. The 96-h LC50 was calculated by Probit analysis (SPSS version 10.0, SPSS Inc., Chicago, IL) using the measured mean total water Cu concentrations.

### 2.8. Analysis

At all sampling times the tissues and water samples were first measured for  $^{64}\text{Cu}$  activity by means of a Canberra-Packard MINAXI Gamma counter with an on-board program for decay correction. Subsequently, the tissues were reweighed and digested overnight at  $70^\circ\text{C}$  with six volumes of 1N nitric acid (Fisher Scientific, trace metal grade), and then centrifuged for 4 min at  $13000 \times g$ . A sub-sample of the supernatant was diluted appropriately with 0.5% nitric acid and total tissue Cu concentrations were determined by atomic absorption spectroscopy (AAS; Varian AA-1275 with GTA-95 atomizer) using a 10- $\mu\text{l}$  injection volume and the operating conditions specified for Cu by the manufacturer (McKenzie, 1982). Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range. Water total Cu concentrations from the samples taken after feeding and from the waterborne Cu uptake and waterborne Cu toxicity tests were analyzed by comparable furnace atomic absorption methods using acidified samples without digestion.

### 2.9. Calculations

Specific growth rate (SGR) was calculated on a per tank basis ( $n = 2$  per treatment and respective control) for three growth periods using the formula:

$$\text{SGR} = 100[(\ln(\text{wt}2) - \ln(\text{wt}1))t^{-1}],$$

where 'wt1' and 'wt2' are tank biomass at the start and end of each growth period and 't' is the interval in days.

Food conversion efficiency (FCE) was calculated on a per tank basis ( $n = 2$  per treatment and respective control) for three growth periods using the formula:

$$\text{FCE (\%)} = 100(\text{weight gain per tank} \times \text{per food eaten}).$$

Whole-body total Cu concentration was calculated by dividing the sum of Cu contents (concentration multiplied by weight) of all the tissues plus the carcass by the sum of weights of all the tissues plus carcass.

Retention efficiency (RE) of Cu was calculated as:

$$\text{RE (\%)} = 100[(C2 - C1) \times \text{per Cu eaten}],$$

where 'C1' and 'C2' are total Cu content per fish ( $\mu\text{mol}$  per fish) at the beginning and at the end of the experiment, respectively.

The unidirectional uptake of waterborne Cu by the whole fish over 12 h was calculated by adding up  $^{64}\text{Cu}$  activities (cpm) in all tissues and carcass. Fish weights were determined by summing up the weights of all tissues and rest of carcass for each fish. Whole-body Cu uptake was then calculated using the equation:

$$a(bc^{-1})^{-1},$$

where 'a' is the mean  $^{64}\text{Cu}$  cpm per gram fish, 'b' is the  $^{64}\text{Cu}$  cpm  $\text{l}^{-1}$  of water and 'c' is the mean total Cu concentration in water in  $\mu\text{mol l}^{-1}$ . The uptake was then expressed as a rate by dividing by the time of exposure (12 h).

### 2.10. Statistical analysis

Data are presented as means  $\pm$  SEM. Treatment and replicate effects were analyzed using three-way factorial analysis of variance (ANOVA) (Statistica 5.0, StatSoft Inc., Tulsa, OK) with time, ration, and diet Cu exposure as variables. Subsequently a posteriori Tukey's honest significant difference (HSD) test was used to make comparisons between means of the measurements. Chi-square, goodness-of-fit and Bartlett tests performed on all data sets revealed that assumptions of normality of distribution and homogeneity of variance were met. All data from replicate tanks did not show

any statistical difference when the means were tested against each other using Tukey's HSD test. Hence data from the replicates were pooled. Interaction terms among the three factors were not consistently significant at any instance. Growth data (cumulative weight gains, SGR, and FCE) were compared with a paired *t*-test. LC50 values with 95% confidence limits (95% C.L.) were computed using Probit analysis on SPSS 10.0 (SPSS Inc. Chicago, IL). Proportional data were arcsine transformed before statistical analyses.

## 3. Results

### 3.1. Growth

At the start of the experiment mean fish weight per tank was between 9 and 10 g for all the treatments. Growth occurred in all the treatments as shown by the cumulative weight gain curves (Fig. 1). There was a clear, statistically significant, effect of ration but not Cu on growth for all the treatments and the growth–ration relationship was curvilinear (Fig. 1, insert). Fish on 1.5% ration with or without  $0.24 \mu\text{mol Cu g}$  per fish per day showed the lowest growth gaining only 5 g per fish in 35 days to reach a final weight of 14–15 g. Fish on the 3.0 and 4.5% daily ration gained 14 g per fish to 23–24 g, and 16 g per fish to 25–26 g by day 35, respectively. The differences in weight gains were reflected in per tank SGRs which were highest in fish on 4.5% ration (Fig. 2A). However, food conversion was highest in fish on low ration and decreased in the highest ration (Fig. 2B).

The weights of liver, gastrointestinal tract, and gill relative to whole-body weight (somatic indices) are shown in Fig. 3. Hepatosomatic index ranged from 1 to 2.3%, being low in the fish on lower ration and higher in the fish on high ration, but was not affected by dietary Cu exposure. Gastro-intestinosomatic index ranged between 3 and 5%, tended to increase with ration and was slightly decreased with dietary Cu exposure. Branchiosomatic index tended to be lower in fish on intermediate ration, though the effect was not

consistent over time (Fig. 3C). Dietary Cu exposure had no effect on the branchiosomatic index.

### 3.2. Whole-body Cu status

Exposure of juvenile rainbow trout to the same Cu quantity resulted in the same total Cu content per fish (Fig. 4A) regardless of the ration in which the Cu was delivered. Whole fish Cu content increased linearly over time. For the control fish, Cu content increased from an initial value of about 75 to 180, 335, and 340 nmol per fish at day 35 in the groups on 1.5, 3.0, and 4.5% body weight ration per day, respectively. All Cu-exposed fish had the same Cu content per fish reaching a high of about 630 nmol per fish at day 35. However, when expressed as a concentration (per wet body weight) there were striking differences among the treatment groups. While all the controls had Cu concentrations of between 12 and 21 nmol g<sup>-1</sup>,

fish exposed to 0.24 μmol Cu g per fish per day delivered in 1.5% body weight ration per day had fourfold higher whole-body Cu concentration than the respective control group. For fish in which the Cu load was delivered in 3.0 or 4.5% body weight ration per day, only about 1.5-fold increase in whole-body Cu concentration occurred relative to the respective controls (Fig. 4B).

RE of Cu was decreased strongly by Cu-exposure, and to a lesser extent by ration (Table 2). For all the Cu-exposed treatments RE was the same regardless of ration, but much lower than for the control fish, and ranged from 0.7 to 0.8% for all the feeding regimes. Among the control groups RE decreased from about 9% in the fish on 1.5% body weight ration per day to 5.6% in fish on 4.5% body weight ration per day.

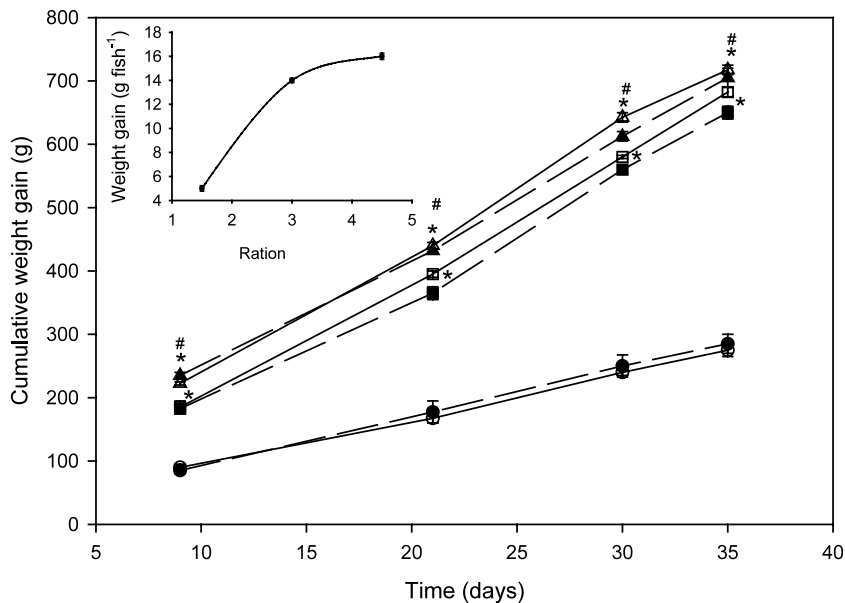


Fig. 1. Effect of exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight per day ration on growth in juvenile rainbow trout. Values are means ± SEM for cumulative mean weight gains per tank,  $n = 2$  per treatment. For all the groups solid lines represent control fish and broken lines represent fish exposed to dietary Cu. ○, 1.5% body weight per day control; ●, 1.5% body weight per day + 0.24 μmol g<sup>-1</sup> per day Cu; □, 3.0% body weight per day control; ■, 3.0% body weight per day + 0.24 μmol g<sup>-1</sup> per day Cu; △, 4.5% body weight per day control; ▲, 4.5% body weight per day + 0.24 μmol g<sup>-1</sup> per day Cu. # Indicates significant difference relative to 3.0% body weight per day ration and \* indicates significant difference relative to 1.5% body weight per day ration ( $t$ -test,  $P < 0.05$ ). There was no effect of Cu exposure on growth at any ration. Insert shows the growth–ration relationship under the conditions of exposure.

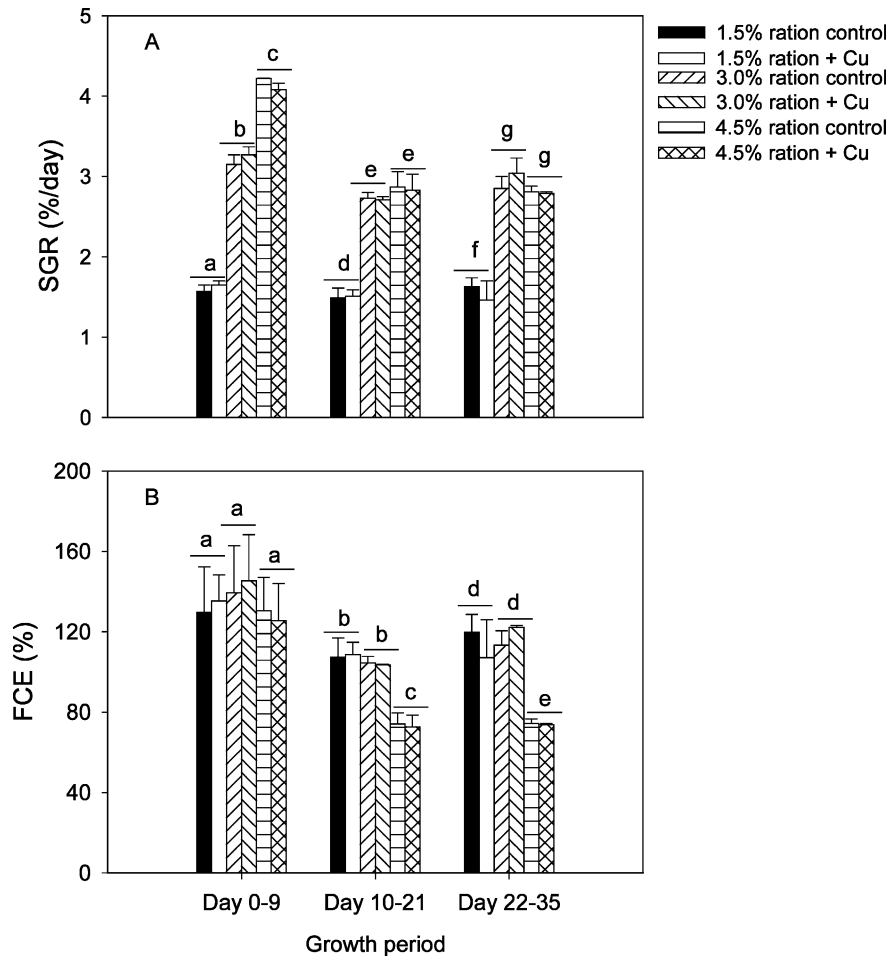


Fig. 2. Effect of exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, and 4.5% body weight per day ration on SGRs [A] and food conversion efficiencies (FCEs) [B] in juvenile rainbow trout. Values are means  $\pm$  SEM on per tank basis ( $n = 2$ ). Bars with different letters within the same sampling time are significantly different ( $t$ -test,  $P < 0.05$ ).

### 3.3. Tissue partitioning of Cu

Accumulated Cu partitioned into most body tissues sampled, with the liver showing the highest concentrations. For the control fish, Cu content in liver increased significantly from an initial value of about 30 to 80, 170, and 190 nmol per liver at day 35 in the group on 1.5, 3.0, and 4.5% body weight ration per day, respectively (Fig. 5A). Moreover, the liver Cu content was significantly lower at day 35 in the fish on 1.5% body weight ration per day compared to both 3.5 and 4.5 body weight ration

per day. However, as with whole-body, the total Cu content (nmol) per liver was the same for all the Cu-exposed groups independent of ration, and rose linearly from about 30 nmol per liver at the beginning of the experiment to about 400 nmol Cu per liver at the end of the experiment. Liver Cu concentration also increased linearly over time in all the treatments (Fig. 5B). Livers of the group fed the 1.5% body weight ration per day ( $15.75 \mu\text{mol g}^{-1}$  Cu diet) showed the highest liver Cu concentration of  $3.62 \mu\text{mol g}^{-1}$  followed by the group on 3.0% ( $7.87 \mu\text{mol g}^{-1}$  Cu diet) with  $1.26 \mu\text{mol g}^{-1}$ ,



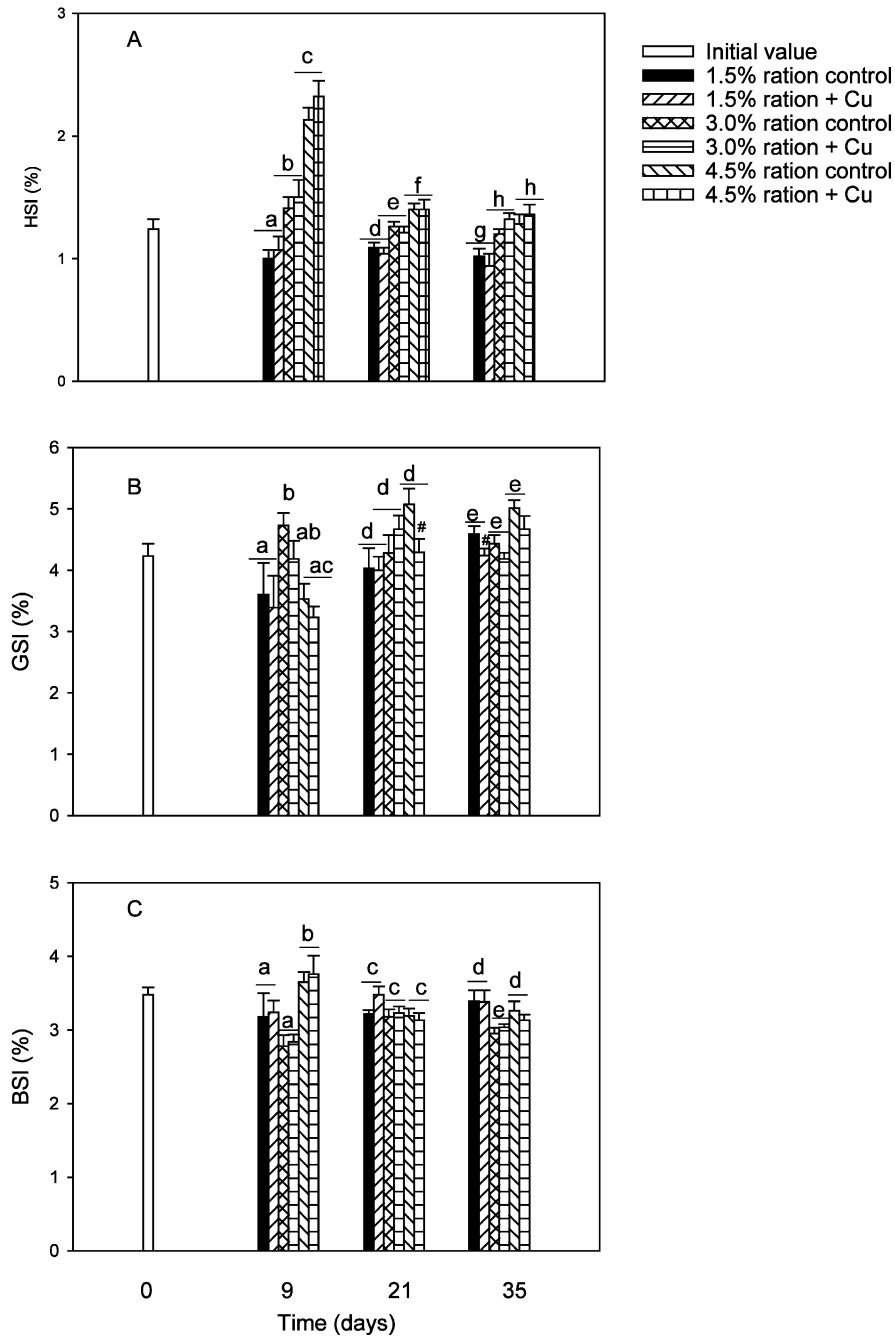


Fig. 3. Somatic indices for liver (A), gastrointestinal tract (B), and gill (C) in juvenile rainbow trout following exposure to 0.24  $\mu\text{mol}$  Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight per day ration. HSI, hepatosomatic index; GSI, gastrointestinosomatic index; BSI, branchiosomatic index. Values are means  $\pm$  SEM,  $n = 10$  per data point. Bars with different letters within the same sampling time are significantly different, and # indicates significant difference from control for same ration at that sampling time, Tukey's HSD,  $P < 0.05$ .

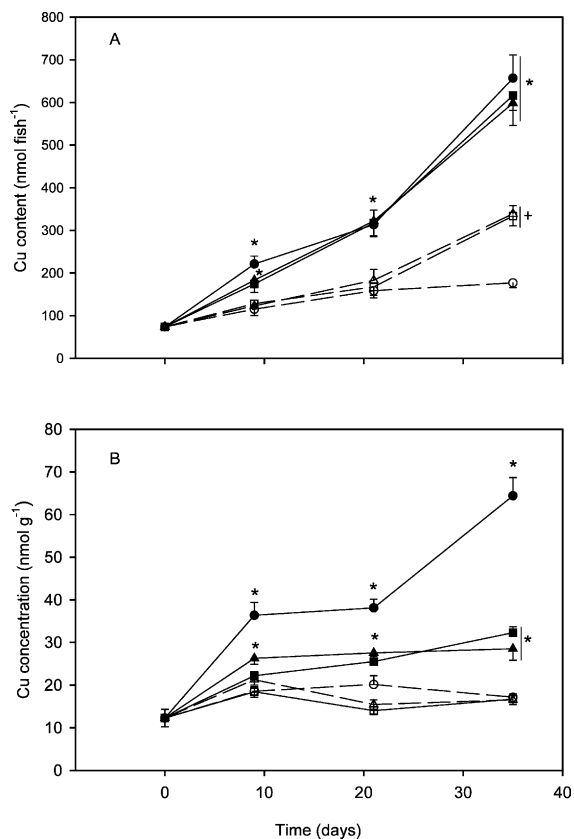


Fig. 4. Whole fish Cu content (A) and whole-body Cu concentration (B) in juvenile rainbow trout following exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, or 4.5% body weight per day ration. Values are means  $\pm$  SEM,  $n = 10$  per treatment per data point except day 0 controls where  $n = 24$ . ○, 1.5% body weight per day ration control; ●, 1.5% body weight per day +  $0.24 \mu\text{mol g}^{-1}$  per day Cu; □, 3.0% body weight per day ration control; ■, 3.0% body weight per day ration +  $0.24 \mu\text{mol g}^{-1}$  per day Cu; △, 4.5% body weight per day ration control; ▲, 4.5% body weight per day +  $0.24 \mu\text{mol g}^{-1}$  per day Cu. \* Indicates significant difference from controls and + indicates significant difference from 1.5% body weight per day ration, Tukey's HSD,  $P < 0.05$ . There were no significant differences amongst the experimental groups in A. In B all points for the 1.5% body weight per day ration were significantly higher than for the 3.0 and 4.5% body weight per day rations.

4.5% body weight ration per day ( $5.24 \mu\text{mol g}^{-1}$  Cu diet) with  $0.94 \mu\text{mol g}^{-1}$ , and controls ( $0.32 \mu\text{mol g}^{-1}$  Cu diet) with  $0.40$ – $0.63 \mu\text{mol g}^{-1}$ . In direct correlation with liver Cu concentration, bile Cu concentration was highest in the group

Table 2

Copper retention efficiencies (%) in juvenile rainbow trout following exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, and 4.5% daily ration for 35 days

Treatment	Retention efficiency (%)
1.5% ration control	8.92
1.5% ration + Cu	0.84
3.0% ration control	7.10
3.0% ration + Cu	0.77
4.5% ration control	5.68
4.5% ration + Cu	0.73

which Cu was delivered in 1.5% body weight ration per day followed in descending order by the 3.0, 4.5% body weight ration per day, and the control groups in decreasing order (Fig. 6A). In addition, bile concentration appeared to be more or less in steady state in all Cu-exposed fish from day 21 onwards.

Significant Cu accumulation occurred in the kidney, carcass, and to a very small extent in the muscle (day 35 only), in the groups fed high Cu diets (Fig. 6B–D). For all the organs and tissues, the group on the 1.5% body weight ration per day + Cu had the highest Cu concentration. However, plasma and gill Cu concentration remained unchanged (Fig. 6E and F), although plasma values for day 35 were significantly higher than for the rest of the period in all the treatments.

Cu accumulation in segments of the gut is shown in Fig. 7A–D. In all the treatment groups, the posterior intestine had the highest Cu concentration followed in descending order by the pyloric caecae + anterior intestine, stomach, and mid-intestine. As with whole-body, fish on 1.5% body weight ration per day had higher Cu concentrations in the gut segments followed in decreasing order by the fish on 3.0 and 4.5% body weight ration per day. Cu concentration in the control group followed a similar pattern except that the concentration in the mid-intestine was slightly higher than in the stomach. Cu concentrations in all regions of the gut appeared to be more or less in steady state from day 9 onwards.

Proportional distribution of Cu in the major tissues and organs (liver, carcass, entire gut, gill,

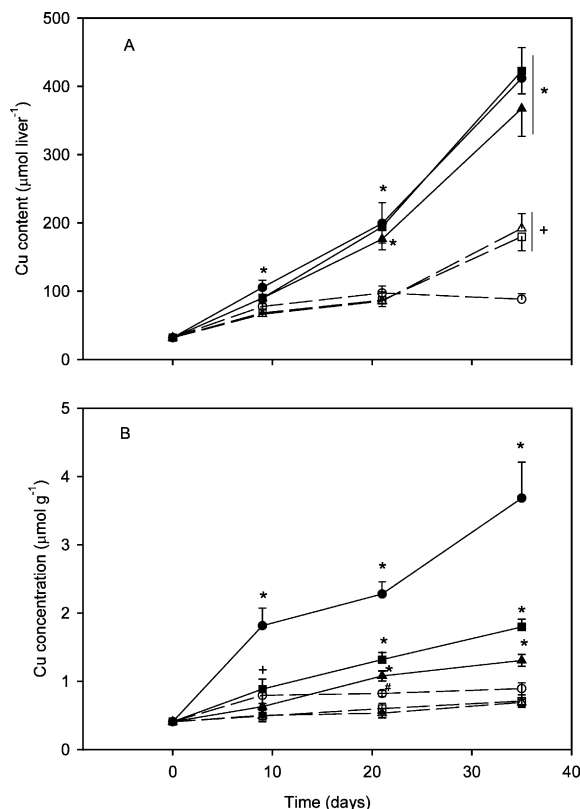


Fig. 5. Liver Cu content (A) and liver total Cu concentration (B) in juvenile rainbow trout following exposure to  $0.24 \mu\text{mol Cu}$  per g fish per day delivered in 1.5, 3.0, or 4.5% ration. Values are means  $\pm$  SEM,  $n = 10$  per treatment per data point 0 controls where  $n = 24$ .  $\circ$ , 1.5% body weight per day ration control;  $\bullet$ , 1.5% body weight per day +  $0.24 \mu\text{mol g}^{-1}$  per day Cu;  $\square$ , 3.0% body weight per day ration control;  $\blacksquare$ , 3.0% body weight per day ration +  $0.24 \mu\text{mol g}^{-1}$  per day Cu;  $\nabla$ , 4.5% body weight per day ration control;  $\blacktriangledown$ , 4.5% body weight per day ration +  $0.24 \mu\text{mol g}^{-1}$  per day Cu. \* Indicates significant difference from controls, and + indicates significant difference from 1.5% body weight per day ration, Tukey's HSD,  $P < 0.05$ . There were no significant differences amongst the experimental groups in A. In B all points for the 1.5% body weight per day ration were significantly higher than for the 3.0 and 4.5% body weight per day rations.

and kidney) in control and exposed animals at the end of the 35-day exposure is shown in Fig. 8. About 50–59% of the Cu in control animals were contained in the liver and this proportion increased to 61–69% in Cu exposed fish. The carcass contained about 30–40% of the Cu in control animals and this proportion decreased to 15–26%

in Cu-exposed animals. Gut contained 7–10% of the Cu in control fish and fish on the 3.0 and 4.5% body weight ration per day + Cu, and 22% in the 1.5% body weight ration per day Cu-exposed group. The gills and kidneys each contained less than 2% of the total body Cu burden and this proportion decreased to less than 1% in the Cu-exposed animals at all rations.

### 3.4. Waterborne Cu uptake

Whole-body unidirectional Cu uptake from the water was determined by  $^{64}\text{Cu}$  appearance in the fish at various times during the 35-day experiment (Fig. 9). Waterborne Cu uptake decreased over time as the fish increased in size and was significantly lower at days 21 and 35 relative to day 0 for all the groups. High ration appeared to stimulate Cu uptake, with the uptake rates being significantly higher for the fish on 4.5% body weight ration per day on days 21 and 35. However, there was no significant influence of dietary Cu pre-exposure on waterborne Cu uptake at any time. In the flux experiments, Cu speciation analysis by the MINEQL+ aquatic geochemical model (Schecher and McAvoy, 1994) indicated that Cu was present as 25%  $\text{Cu}^{2+}$ , 7.3%  $\text{CuOH}^+$ , 3% aqueous  $\text{CuCO}_3$ , and 64.4% Cu-humate. There was no  $\text{CuCl}_2$  species indicating that under conditions of our exposure complexation of Cu with Cl was unimportant.

### 3.5. Acute toxicity test

A 96-h acute waterborne Cu toxicity test carried out at the end of the experiment showed no effect of ration or dietary Cu on Cu tolerance. The LC50s (means  $\pm$  95% C.L.) for the three control groups ranged from  $0.17 \pm 0.07$  to  $0.19 \pm 0.08 \mu\text{mol l}^{-1}$  while the LC50s in the Cu-fed groups ranged from  $0.17 \pm 0.07$  to  $0.21 \pm 0.08 \mu\text{mol l}^{-1}$ . There were no significant differences amongst the groups.

### 3.6. $\text{Na}^+, \text{K}^+$ -ATPase activities

$\text{Na}^+, \text{K}^+$ -ATPase activities (Fig. 10) in various gastrointestinal tissues and the gill were deter-

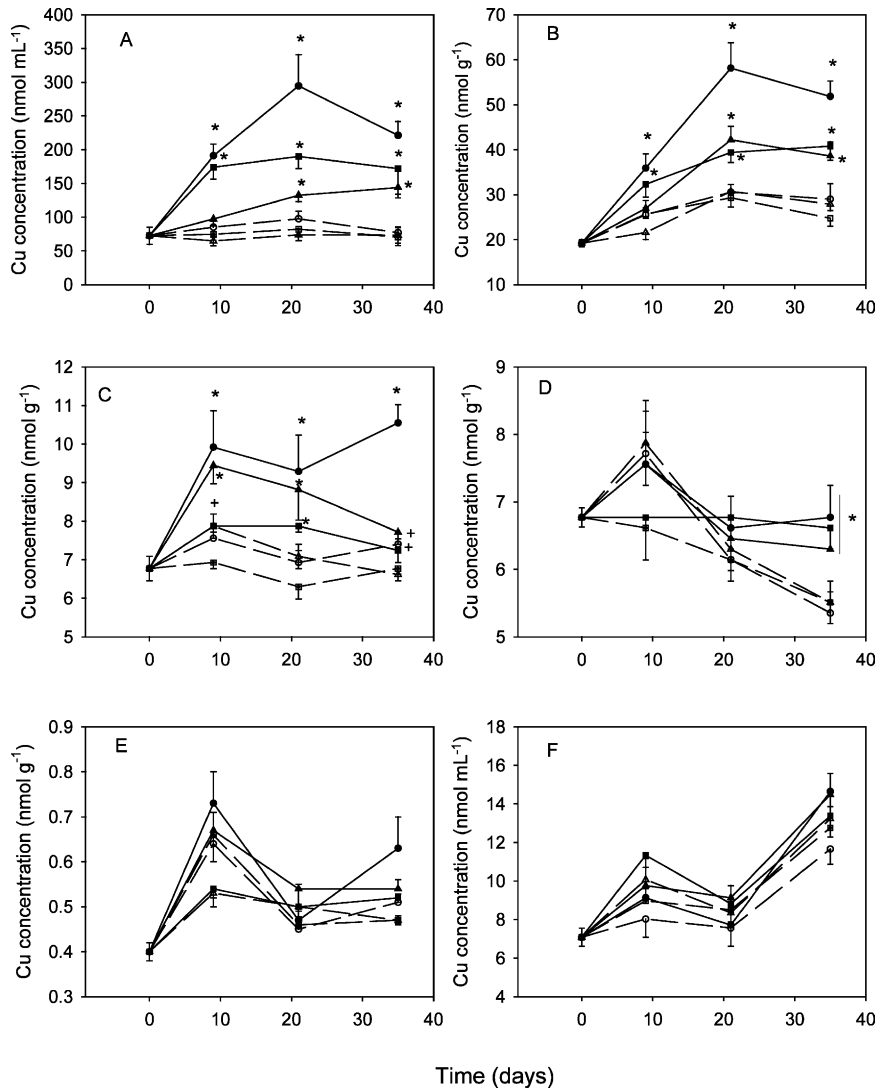


Fig. 6. Cu concentration in tissues of juvenile rainbow trout following exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, or 4.5% body weight per day ration. A, bile; B, kidney; C, carcass; D, muscle; E, gill; F, plasma. Note difference in scale in different panels. Values are means  $\pm$  SEM,  $n = 10$  per treatment per data point for all the organs/tissues except day 0 controls where  $n = 24$ .  $\circ$ , 1.5% body weight per day ration control;  $\bullet$ , 1.5% body weight per day +  $0.24 \mu\text{mol g}^{-1}$  per day Cu;  $\square$ , 3.0% body weight per day ration control;  $\blacksquare$ , 3.0% body weight per day ration +  $0.24 \mu\text{mol g}^{-1}$  per day Cu;  $\triangle$ , 4.5% body weight per day ration control;  $\blacktriangle$ , 4.5% body weight per day ration +  $0.24 \mu\text{mol g}^{-1}$  per day Cu. \* Indicates significant difference from controls for each tissue, and + indicates significant difference from 3.0% body weight per day ration control, Tukey's HSD,  $P < 0.05$ .

mined at day 35. Except for the stomach in the fish on 1.5% body weight ration per day, chronic Cu exposure had no effect on the  $\text{Na}^+, \text{K}^+$ -ATPase in the gut tissue. However, high ration significantly

increased the  $\text{Na}^+, \text{K}^+$ -ATPase activity in the mid-intestine, pyloric caecae (includes anterior intestine) and posterior intestine. For the stomach, though the activity increased with ration, this was

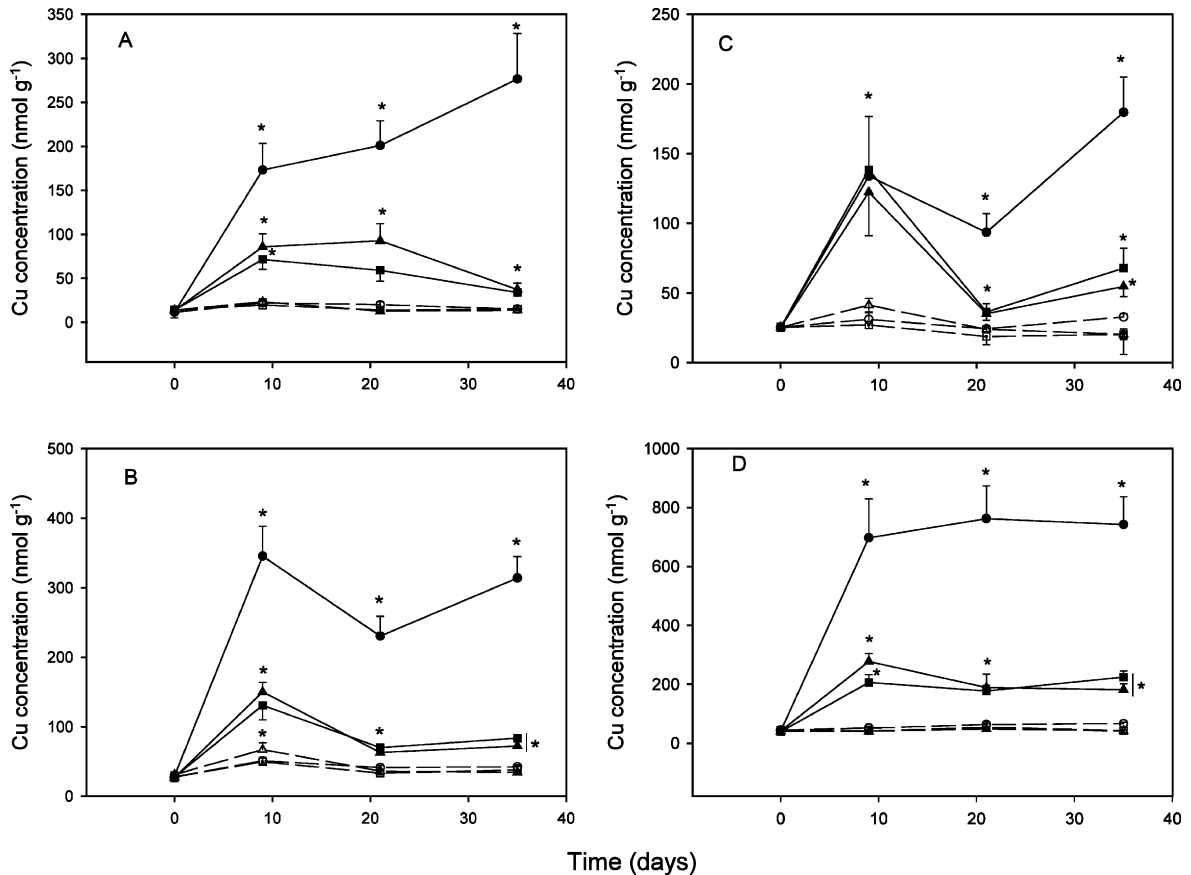


Fig. 7. Cu concentration in various segments of the gut in juvenile rainbow trout following exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, and 4.5% body weight per day rations. A, stomach; B, pyloric caecae+anterior intestine; C, mid-intestine; D, posterior intestine. Note difference in scale in different panels. Values are means  $\pm$  SEM,  $n = 10$  per treatment per data point for all the organs/tissues except day 0 controls where  $n = 24$ .  $\circ$ , 1.5% body weight ration control;  $\bullet$ , 1.5% body weight per day +  $0.24 \mu\text{mol g}^{-1}$  per day Cu;  $\square$ , 3.0% body weight per day ration control;  $\blacksquare$ , 3.0% body weight per day ration +  $0.24 \mu\text{mol g}^{-1}$  per day Cu;  $\triangle$ , 4.5% body weight per day ration control;  $\blacktriangle$ , 4.5% body weight per day ration +  $0.24 \mu\text{mol g}^{-1}$  per day Cu. \* Indicates significant difference from controls, Tukey's HSD,  $P < 0.05$ .

not statistically significant. In the gill (Fig. 10E) neither dietary Cu exposure nor ration affected the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity.

## 4. Discussion

### 4.1. Growth

Over the range of ration sizes used in the present study, SGR increased with ration size (Fig. 2A) in

agreement with many previous studies (Brett and Groves, 1979). The curvilinear growth–ration relationship seen in the present study (Fig. 1, insert) is fairly standard and results from the inability of fish to convert food materials into body tissues at high rations effectively so that excess undigested food is passed out in feces. The latter was reflected in the lower food conversion efficiencies in the fish on 4.5% body weight ration per day (Fig. 2B). Furthermore, for juvenile rainbow trout (9–26 g) maintenance ration must be

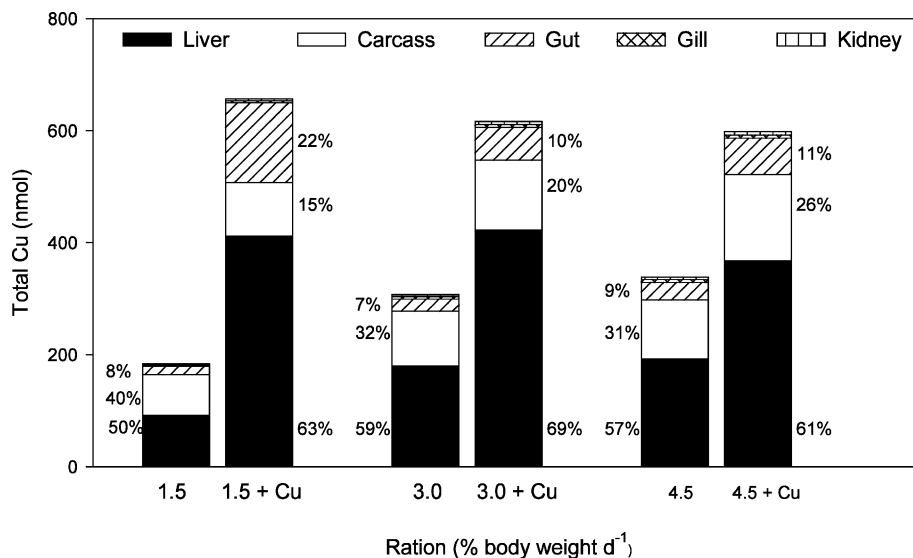


Fig. 8. Distribution of total body Cu in the main organs/tissues at the end of the exposure (35 days) in juvenile rainbow trout following exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, and 4.5% body weight per day rations. Percentage values for liver, carcass, and gut are shown alongside the bars. Values for gill and kidney are 2.0% or less and are not shown. For all tissues at all rations,  $n = 10$ .

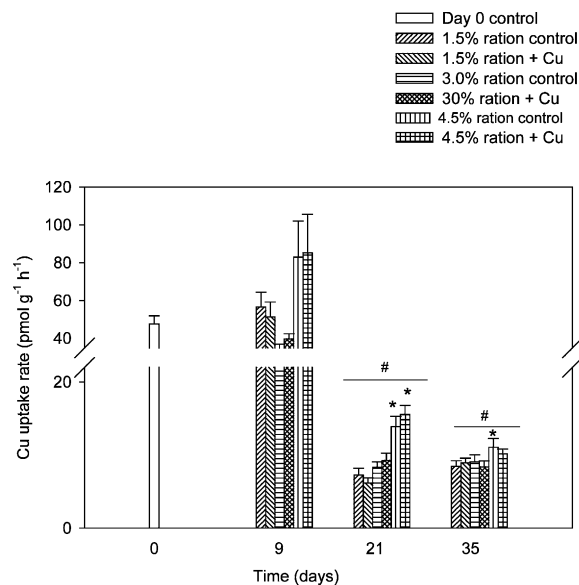


Fig. 9. Whole-body unidirectional waterborne Cu uptake rates measured using  $^{64}\text{Cu}$  in juvenile rainbow trout following exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, and 4.5% body weight per day rations. Values are means  $\pm$  SEM,  $n = 10$  per treatment per data point except day 0 control where  $n = 24$ . \* Indicates significant difference from 1.5% body weight per day ration, and # indicates significant difference from day 0 value, Tukey's HSD,  $P < 0.05$ .

less than 1.5%, since fish maintained on this ration showed positive growth characterized by high FCE.

Contrary to our original hypothesis, dietary Cu ( $0.24 \mu\text{mol Cu per g fish per day}$ ) did not affect growth at the feeding regimes used. This suggests that even at the lowest ration of 1.5%, the possible energy requirement for Cu regulation or detoxification was accommodated within the normal energy budget, with excess energy used in growth. Several other studies have also reported lack of growth inhibition by dietary Cu (Berntssen et al., 1999; Kamunde et al., 2001, 2002) even in the face of mortality (Mount et al., 1994). These previous observations and current data suggest that growth is not a sensitive endpoint for detection of sublethal dietary Cu exposure.

#### 4.2. Whole-body Cu status

In the present study where total metal intake was kept constant while the ration was varied, whole-body Cu concentration varied inversely

with ration (Fig. 4B). However, the absolute Cu content was the same in all the Cu-exposed groups irrespective of the ration size (Fig. 4A), indicating that differences in whole-body Cu concentrations were a reflection of differences in fish body size. Therefore, growth dilution rather than variations in Cu uptake and retention explained the differences in whole-body Cu concentrations. Recently, Kamunde et al. (2002) similarly demonstrated that rapidly growing rainbow trout exhibited decreased whole body Cu concentration due to growth dilution. Thus in employing body metal residues for risk assessment (Bergman and Dorward-King, 1997), nutritional status of the animal is an important consideration because it has direct bearing on metal concentrations in animal tissues. Furthermore, for dietary metal, the present study suggests that expressing the exposure as total metal dose rather than metal concentration in the diet is more appropriate.

That the absolute amount of Cu retained from a similar dose delivered in different rations was the same suggests that the absorption of Cu was independent of feeding regime and depended mainly on the amount of Cu offered. Studies on nutrient bioavailability have generally showed that the efficiency of absorption decreases with increasing food intake (Brett and Groves, 1979) due to increases in the gut evacuation time and passage of undigested food material. Our data on the RE of Cu in fish fed control diets, but not in fish fed Cu-loaded diets (Table 2), are consistent with this dogma. The control observations are consistent with the notion that absorption efficiency of Cu decreases with dose (Turnlund et al., 1998; Linder, 1991; Clearwater et al., 2000; Kamunde et al., 2002). Even small changes in total metal dose (Table 1) that occurred in fish fed control diet containing the same metal concentration ( $0.35 \mu\text{mol g}^{-1}$ ) at variable ration were reflected in the RE of Cu and Cu content of the fish (Table 1, Fig. 4A). Interestingly, although decreased FCE at high ration was evident (Fig. 2B); RE of Cu in fish exposed to the same total Cu dose did not decrease (Table 2). This suggests that Cu absorption efficiency tends to increase as Cu concentration in the diet decreases.

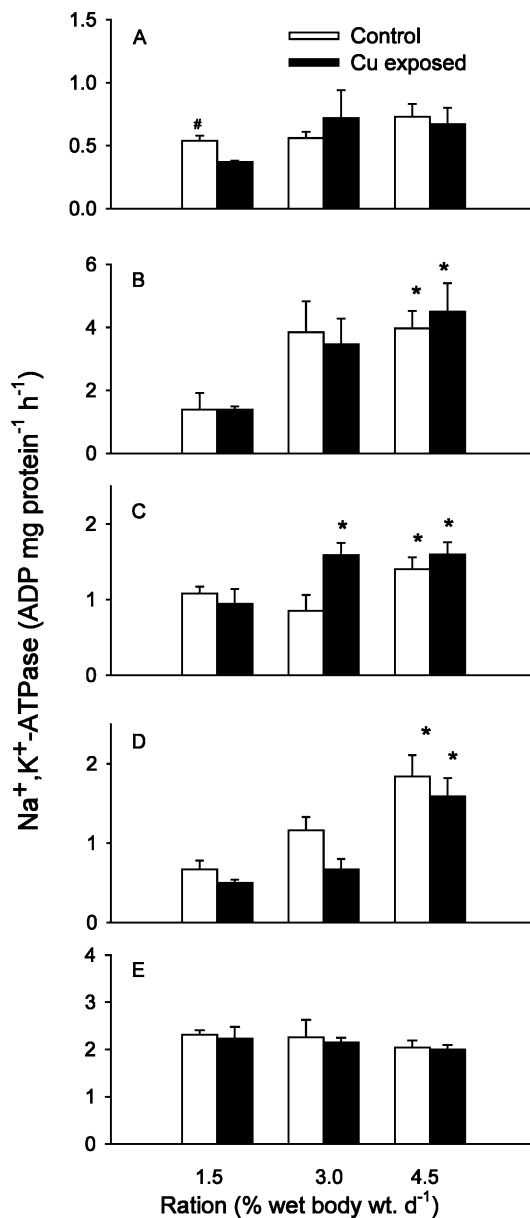


Fig. 10.  $\text{Na}^+, \text{K}^+$ -ATPase activities in various segments of the gut in juvenile rainbow trout following 35 days of exposure to  $0.24 \mu\text{mol Cu per g fish per day}$  delivered in 1.5, 3.0, and 4.5% body weight per day ration. A, stomach; B, pyloric caecae (includes anterior intestine); C, mid-intestine; D, posterior intestine; E, gill. Values are means  $\pm$  SEM,  $n = 6$  per treatment per organ/tissue. \* Indicates significant difference from 1.5% body weight per day ration and # indicates significant difference from Cu-exposed fish at the same ration level, Tukey's HSD,  $P < 0.05$ .

### 4.3. Copper partitioning

Food ration size significantly influenced the concentration of Cu in the organs analyzed except gill and plasma (Figs. 5–7). Previous studies have reported highest Cu concentration in livers of starved versus fed fish exposed to waterborne Cu (Segner, 1987). In addition, Saari et al. (1993) found that food restriction improved the Cu status in rats livers during Cu deficiency and argued that the relatively slower growth of the liver due to food restriction left Cu more concentrated. Our observations following dietary Cu exposure at different rations show that a low feeding regime results in higher Cu concentrations in tissues. We attribute higher metal concentration in animals on lower ration to the smaller size of the organs in the face of the same gastrointestinal Cu uptake. Note also the hepatosomatic and gastrointestinosomatic indices were lower in fish on low ration (Fig. 3). The smaller size of these organs likely contributed to the higher concentrations of Cu in the fish on 1.5% body weight ration per day.

Proportional distribution analysis (Fig. 8) revealed that in control fish, the liver and the carcass were the main Cu reservoirs but following exposure to dietary Cu, the gut became an important tissue for Cu deposition. These observations are in agreement with Kamunde et al. (2001, 2002). Moreover, the group on the lower ration retained a much higher proportion of Cu in the gut than the groups on higher ration suggesting that these animals were not as efficient in clearing Cu from the gut tissue to internal organs. During waterborne Cu exposures, much less of the total body Cu burden accumulates in gut tissue (Miller et al., 1993; Kamunde et al., 2001). So clearly the proportion of total metal burden in the gut tissue is a useful risk assessment tool for diagnosing a dietary versus a waterborne route of uptake in contaminated fish collected from the wild.

### 4.4. Copper absorption and distribution in the gut tissue

All the segments of the gut showed significant accumulation of Cu following the exposure, suggesting that the whole gut participated in Cu

absorption. However, dietary Cu also induces metallothionein (MT) in the gut (Handy et al., 1999) and most of the metal accumulation in the gut tissues may be MT-bound to limit cytotoxicity. Our data suggest that the posterior intestine is the most active site for Cu absorption and/or sequestration, followed in decreasing order by the pyloric caecae + anterior intestine, stomach, and mid-intestine. Proportionally, however, the majority of the Cu was in the pyloric caecae + anterior intestine (50%) followed in decreasing order by posterior intestine (20%), stomach (20%) and mid-intestine (10%). These findings are in close agreement with a recent study (Clearwater et al., 2000) that reported, 44% in pyloric caecae, 23% in posterior intestine, and 12% in mid-intestine 24 h after administration of  $^{64}\text{Cu}$  by an esophageal catheter. However, these authors reported that over 99% of a single  $^{64}\text{Cu}$  dose had left the stomach in 24 h.

In humans it is generally thought that the acidic environment in the stomach contributes to the freeing of Cu bound to food and facilitates peptic digestion and release of Cu from organic complexes (Gollan, 1975). This is followed by partial absorption of Cu in the stomach with most of the absorption occurring in the intestine. Copper is thought to enter the intestinal mucosal cells by simple diffusion (Linder and Hazegh-Azam, 1996) and/or via a high affinity uptake mechanism (Lee et al., 2001) and to exit the basolateral membrane by a different mechanism (Linder, 1991), possibly a divalent cation transporter (Rolfs and Hediger, 1999). In addition, studies on Menke's disease suggest the presence of a basolateral Cu-ATPase which discharges Cu into the serosal blood capillaries where Cu binds to albumin and amino acids for transport to the liver (Camakaris et al., 1995; Harrison and Dameron, 1999).

Although the situation in fish is less clear, similar mechanisms of Cu absorption are likely. The acidic fish stomach (Fange and Groves, 1979) may free Cu from the food with subsequent partial gastric absorption as in mammals. Indeed our Cu accumulation data suggest a significant role of fish stomach in Cu absorption. In addition,  $Q_{10}$  analysis (Clearwater et al., 2000) suggested both simple diffusion (apical) and biologically mediated (active) transport (basolateral) components of gut



Cu uptake in trout whereas Handy et al. (2000) reported a basolateral limiting step in Cu uptake in African walking catfish via a Cu/anion symport and possibly a Cu-ATPase.

#### 4.5. Waterborne Cu uptake

Assessment of unidirectional uptake of waterborne Cu via gills following dietary Cu exposure at different rations revealed three interesting findings (Fig. 9). First, Cu uptake rates per unit body weight decreased over time with increasing fish size. This agrees with previous findings by Kamunde et al. (2001) that described a non-linear decrease in Cu uptake with body weight in rainbow trout. Second, Cu uptake rates from the water were much higher than in several previous studies (Kamunde et al., 2001, 2002), possibly explained by the fact that the experiment was done in soft water in which rainbow trout generally exhibit higher Cu uptake rates due to fewer competing and complexing ions (Taylor et al., 2000). Third, fish on high ration exhibited greater mass specific Cu uptake rate than fish on low ration probably because there is an increased need for Cu for growth processes in rapidly growing fish since Cu is essential for fish growth (Kamunde et al., 2002). The current data together with a previous study that showed greater uptake of Cu in fed fish compared to starved fish (Segner, 1987) suggest a modifying effect of an animal's nutritional status (energy balance) on Cu metabolism. The maintenance of Cu homeostasis thus involved changes in branchial Cu uptake rates related to ration and fish size but not dietary Cu exposure. The latter observation is contrary to our previous observation (Kamunde et al., 2001) in which exposure to  $15.75 \mu\text{mol g}^{-1}$  Cu diet at 4% wet body weight ration per day in hard water caused significant accumulation of Cu in the gills and reduced subsequent waterborne Cu uptake rate. However, under the conditions of the Kamunde et al. (2001) study, the fish received  $0.63 \mu\text{mol Cu per g fish per day}$ , almost threefold higher than in the present study.

#### 4.6. Tolerance to waterborne Cu

Pre-exposure to sublethal waterborne Cu is generally associated with classical acclimation (McDonald and Wood, 1993), i.e. increased tolerance characterized by increased LC50 (Dixon and Sprague, 1981; Buckley et al., 1982). However, few studies have assessed the effect of dietary Cu on waterborne Cu tolerance. Miller et al. (1993) reported increased tolerance to waterborne Cu following exposure to  $10.77 \mu\text{mol g}^{-1}$  Cu in the diet presented ad libitum for 42 days. In the present study, a dietary dose of  $0.24 \mu\text{mol Cu per g fish per day}$  presented in various rations did not induce tolerance to waterborne Cu. Although the diet Cu concentrations used in this study ( $5.24$ – $17.75 \mu\text{mol g}^{-1}$ ) encompass the diet Cu concentration used by Miller et al. (1993), it is possible that fish in the latter study received more total Cu since they were fed ad libitum. Moreover, there was significant accumulation of Cu in the gills and elevation of waterborne Cu (from food-derived Cu) during the exposure by Miller et al. (1993) that did not occur in the present study. Other studies have reported that exposure to waterborne and dietary metal results in enhanced detoxification and excretory mechanisms and induction of resistance (Lanno et al., 1987; Hogstrand and Haux, 1991; Marr et al., 1995; Szebedinszky et al., 2000). We did not find increased tolerance to acute waterborne Cu exposure despite increased biliary Cu concentration, which suggests augmented biliary excretory capacity (Grosell et al., 2001). Furthermore, it is striking that in *Oncorhynchus kisutch* the increase in tolerance to Cu preceded induction of MT, a metal binding protein in the liver (McCarter and Roch, 1984). Overall these results suggest that changes in gill physiology may be more important than internal detoxification or excretion mechanisms in the development of tolerance to waterborne Cu.

#### 4.7. $\text{Na}^+, \text{K}^+$ -ATPase

The higher  $\text{Na}^+, \text{K}^+$ -ATPase activity in the gut of fish on higher rations compared to fish on low ration, is probably a reflection of the overall

metabolic demand put upon the gastrointestinal tissues. Indeed the higher gastrointestinosomatic index in fish on high ration (Fig. 3) is likely an indication of the greater need of gut tissues for digestive and absorptive purposes. Nutrient absorption (e.g. glucose and amino acids) in mammalian and piscine intestine is an active process coupled to  $\text{Na}^+$  transport (Schultz et al., 1966; Kimmich, 1973; Karasov and Diamond, 1983; Buddington et al., 1986). Iturri and Wolf (1982) have provided evidence of the involvement of  $\text{Na}^+, \text{K}^+$ -ATPase (which generates the  $\text{Na}^+$  gradient in intestinal cells) in nutrient absorption. Thus the greater need for nutrient absorption in fish on high ration may explain the higher  $\text{Na}^+, \text{K}^+$ -ATPase activities in the gut tissues. Compared to the gills, gut tissue  $\text{Na}^+, \text{K}^+$ -ATPase appears to be more than 100 times less sensitive to the inhibitory effects of Cu. While enzyme inhibition occurred in the trout gill following accumulation of less than  $6.30 \text{ nmol g}^{-1}$  in a waterborne exposure (Laurén and McDonald, 1987), up to  $0.63 \text{ } \mu\text{mol g}^{-1}$  Cu above control levels in a dietary exposure had no effect on gut  $\text{Na}^+, \text{K}^+$ -ATPase (Fig. 7). The factors responsible for this apparent insensitivity of gut  $\text{Na}^+, \text{K}^+$ -ATPase to Cu remain unclear but we speculate greater induction of MT concurrent with greater sequestration of Cu in gut than in gill tissue. MT is induced in the gut tissue (Handy et al., 1999) but not in gill tissue (Laurén and McDonald, 1987; Grosell et al., 1997). However, the recent demonstration of MT in fish gill tissue following Cu exposure (Dang et al., 1999, 2000) casts doubts on MT being a major contributor to this insensitivity. Other possible explanations are that the Cu species in the gastrointestinal environment are less toxic than those in a water column, or that there are real tissue-specific differences in  $\text{Na}^+, \text{K}^+$ -ATPase enzyme kinetics. In addition, the stimulatory effect of feeding could have masked the inhibitory effect of Cu.

The lack of effect of dietary Cu exposure on branchial  $\text{Na}^+, \text{K}^+$ -ATPase is consistent with absence of Cu accumulation in gill tissue (Fig. 6E). Moreover, during chronic waterborne Cu exposures, branchial  $\text{Na}^+, \text{K}^+$ -ATPase activities in Cu-exposed fish recover and even exceed the

control levels (Laurén and McDonald, 1987; McGeer et al., 2000). Since the gill samples in our study were collected at day 35, enzyme activities are likely to have recovered from any impact incurred early in the exposure.

#### 4.8. Concluding remarks

The nutritional status of the fish influences whole-body and tissue Cu concentration as well as the uptake and elimination rates of waterborne Cu during sublethal dietary Cu exposure. Absorption and accumulation of Cu from the gut during dietary exposure appear to be independent of the food quantity in which it is delivered. Thus using body metal burdens for biomonitoring and risk assessment is relevant only if the dietary metal intake and the feeding regimes are taken into consideration since regimes that cause higher growth rates can result in lower whole-body and tissue metal concentrations, even when exposure dose is the same. Moreover, expressing exposure as a dose rather than concentration appears to be more appropriate for dietary metal toxicity assessment.

#### Acknowledgements

Financial support for this work was provided by the NSERC Metals in the Environment Research Network (MITE-RN) program. We thank Drs Peter Chapman, Bernard Vigneault, and Kath Sloman for helpful comments on the manuscript. C.M. Wood is supported by the Canada Research Chair Program.

#### References

- Bergman, H.L., Dorward-King, E.J., 1997. Reassessment of metals criteria for aquatic life protection. SETAC Tech. Pub. Series. SETAC Press, Pensacola, USA.
- Berntssen, M.H.G., Lundebye, A.-K., Maage, A., 1999. Effects of elevated dietary copper concentrations on growth, feed utilization and nutritional status of Atlantic salmon (*Salmo salar* L.) fry. *Aquaculture* 174, 167–181.

- Bradford, M.M., 1976. A refined and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* 72, 248.
- Brett, J.R., Groves, T.D.D., 1979. Physiological energetics. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), *Fish Physiology Bioenergetics and Growth*, vol. VIII. Academic Press, New York, San Francisco, London, pp. 280–344.
- Buckley, J.T., Roch, M., McCarter, J.A., Rendell, C.A., Matheson, A.T., 1982. Chronic exposure of coho salmon to sublethal concentrations of copper-I. Effect on growth, on acclimation and distribution of copper, and on copper tolerance. *Comp. Biochem. Physiol.* 73C, 15–19.
- Buddington, R.K., Chen, J.N., Diamond, J., 1986. Genetic and phenotypic adaptation of intestinal nutrient transport to diet in fish. *J. Physiol.* 39, 261–281.
- Camakaris, J., Petris, M., Baily, L., Shen, P., Lockhart, P., Glover, T.W., Barcroft, C., Patton, J., Mercer, J.F., 1995. Gene amplification of the Menkes (MNK; ATP7A) P-type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. *Hum. Mol. Genet.* 4, 2117–2123.
- Cho, C.Y., Slinger, S.J., Bayley, H.S., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. *Comp. Biochem. Physiol.* 73B, 25–41.
- 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.
- Collvin, L., 1985. The effects of copper on growth, food consumption and conversion of perch, *Perca fluviatilis* L. offered maximal food rations. *Aquat. Toxicol.* 6, 105–113.
- Cowey, C.B., 1992. Nutrition: estimating requirements of rainbow trout. *Aquaculture* 100, 177–189.
- Cowey, C.B., Sargent, J.R., 1979. Nutrition. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), *Fish Physiology Bioenergetics and Growth*, vol. VIII. Academic Press, New York, San Francisco, London, pp. 1–58.
- Dang, Z.C., Flik, G., DuCourret, B., Hogstrand, C., Wendelaar Bonga, S.E., Lock, R.A.C., 2000. Effects of copper on cortisol receptor and metallothionein expression in gills of *Oncorhynchus mykiss*. *Aquat. Toxicol.* 51, 45–54.
- Dang, Z., Lock, R.A.C., Flik, G., Wendelaar Bonga, S.E., 1999. Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water. *Am. J. Physiol.* 277, R320–R331.
- D’Cruz, L.M., Dockray, J.J., Morgan, I.J., Wood, C.M., 1998. Physiological effects of sublethal acid exposure in juvenile rainbow trout on a limited and unlimited ration during a simulated global warming scenario. *Physiol. Zool.* 71, 359–376.
- Dixon, D.G., Sprague, J.B., 1981. Acclimation to copper by rainbow trout (*Salmo gairdneri*)—a modifying factor in toxicity. *Can. J. Fish. Aquat. Sci.* 38, 880–888.
- Fange, R., Groves, D., 1979. Digestion. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), *Fish Physiology Bioenergetics and Growth*, vol. VIII. Academic Press, New York, San Francisco, London, pp. 161–260.
- Farag, A.M., Stansbury, M.A., Hogstrand, C., MacConnell, E., Bergman, H.L., 1995. The physiological impairment of free-ranging brown trout exposed to metals in the Clark Fork River, Montana. *Can. J. Fish. Aquat. Sci.* 52, 2038–2050.
- Gollan, J.L., 1975. Studies on the nature of complexes formed by copper with human alimentary canal secretions and their influence on copper absorption. *Clin. Sci. Mol. Med.* 49, 237–245.
- Grosell, M.H., Hogstrand, C., Wood, C.M., 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 38, 257–276.
- Grosell, M., McGeer, J.C., Wood, C.M., 2001. Plasma clearance and biliary copper excretion are stimulated in copper acclimated trout. *Am. J. Physiol.* 280, R797–R806.
- Handy, R.D., 1996. Dietary exposure to toxic metals in fish. In: Taylor, E.W. (Ed.), *Toxicology of Aquatic Pollution: Physiological, Cellular and Molecular Approaches*. Cambridge University Press, UK, pp. 29–60.
- Handy, R.D., Sims, D.W., Giles, A., Campell, H.A., Musonda, M.M., 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. *Aquat. Toxicol.* 38, 257–276.
- Handy, R.D., Musonda, M.M., Philips, C., Fella, 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.
- Harrison, M.D., Dameron, C.T., 1999. Molecular mechanisms of copper metabolism and the role of Menkes disease protein. *J. Biochem. Mol. Toxicol.* 13, 93–106.
- Hogstrand, C., Haux, C., 1991. Binding and detoxification of heavy metals in lower vertebrates with reference to metallothionein. *Comp. Physiol.* 100C, 137–141.
- Iturri, S.J., Wolf, D., 1982. Inhibition of the active transport of D-glucose and L-tyrosine by DDT and DDE in the rat small intestine. *Comp. Biochem. Physiol.* 71C, 131–134.
- Jimenez, B.D., Crimo, C.P., McCarthy, J.F., 1987. Effects of feeding and temperature on uptake, elimination and metabolism of benzo(a)pyrene in bluegill sunfish (*Lepomis macrochirus*). *Aquat. Toxicol.* 10, 41–57.
- Kamunde, C., Grosell, M., Lott, J.N.A., Wood, C.M., 2001. Effects of dietary copper exposure on copper metabolism and gut morphology in rainbow trout *Oncorhynchus mykiss*. *Can. J. Fish. Aquat. Sci.* 58, 293–305.
- Kamunde, C., Grosell, M., Higgs, D., Wood, C.M., 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. *J. Exp. Biol.* 205, 279–290.
- Karasov, W.H., Diamond, J.D., 1983. Adaptive regulation of sugar and amino acid transport by vertebrate intestine. *Am. J. Physiol.* 245, G443–G462.
- Kimmich, G.A., 1973. Coupling between Na<sup>+</sup> and sugar transport in small intestine. *Biochim. Biophys.* 300, 31–78.

- Lanno, R.P., Hickie, B.E., Dixon, D.G., 1989. Feeding and nutritional considerations in aquatic toxicology. *Hydrobiologia* 188/189, 525–531.
- Lanno, R.P., Hicks, B., Hilton, J.W., 1987. Histological observations on intrahepatocytic copper-containing granules in rainbow trout reared on diets containing elevated copper levels. *Aquat. Toxicol.* 10, 251–263.
- Laurén, D.J., McDonald, D.G., 1987. Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. *Can. J. Fish. Aquat. Sci.* 44, 105–111.
- Lee, J., Prohaska, J.R., Thiele, D.J., 2001. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. *Proc. Natl. Acad. Sci.* 98, 6842–6847.
- Li, J., Quabius, E.S., Wendelaar Bonga, S.E., Flik, G., Lock, R.A.C., 1998. Effects of water-borne copper on branchial  $\text{Na}^+/\text{K}^+$ -ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). *Aquat. Toxicol.* 43, 1–11.
- Linder, M.C., 1991. Absorption of copper from the digestive tract. In: Frieden, E. (Ed.), *Biochemistry of Copper*. Plenum Press, New York, pp. 15–40.
- Linder, M.C., Hazegh-Azam, M., 1996. Copper biochemistry and molecular biology. *Am. J. Clin. Nutr.* 63, 797S–811S.
- Marr, J.C.A., Bergman, H.L., Lipton, J., Hogstrand, C., 1995. Differences in relative sensitivity of naïve and metals-acclimated brown trout and rainbow trout exposed to metals representative of the Clark Fork River, Montana. *Can. J. Fish. Aquat. Sci.* 52, 2016–2030.
- Marr, J.C.A., Lipton, J., Cacula, D., Hansen, J.A., Meyer, J.S., Bergman, H.L., Hogstrand, C., 1996. Relationship between copper exposure duration, tissue copper concentration, and rainbow trout growth. *Aquat. Toxicol.* 36, 17–30.
- McCarter, J.A., Roch, M., 1984. Chronic exposure of coho salmon to sublethal concentrations of Cu-III. Kinetics of metabolism of metallothionein. *Comp. Biochem. Physiol.* 77, 83–87.
- McCormick, S.D., 1993. Methods for non-lethal gill biopsy and measurement of  $\text{Na}^+/\text{K}^+$ -ATPase activity. *Can. J. Fish. Aquat. Sci.* 50, 656–658.
- McDonald, D.G., Wood, C.M., 1993. Branchial mechanisms of acclimation to metals in freshwater fish. In: Rankin, J.C., Jensen, F. (Eds.), *Fish Ecophysiology*. Chapman and Hall, London, pp. 297–321.
- McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. *Aquat. Toxicol.* 50, 233–244.
- McKenzie, T., 1982. Analytical data for the GTA-95. In: Rothery, E. (Ed.), *Analytical Methods for Graphite Tube Atomizers*. Varian Techtron Pty. Ltd, Mulgrave, Victoria, Australia, p. 33.
- Miller, P.A., Munkittrick, K.R., Dixon, D.G., 1992. Relationship between concentrations of copper and zinc in water, sediment, benthic invertebrates, and tissues of white sucker (*Catostomus commersoni*) at metal-contaminated sites. *Can. J. Fish. Aquat. Sci.* 49, 978–984.
- Miller, P.A., Lanno, R.P., McMaster, M.E., Dixon, D.G., 1993. Relative contribution of dietary and waterborne copper to tissue copper burdens and waterborne Cu uptake in rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* 50, 1683–1689.
- Mount, D.R., Barth, A.K., Garrison, T.D., 1994. Dietary and waterborne exposure of rainbow trout (*Oncorhynchus mykiss*) to copper, cadmium, lead, and zinc using live diet. *Environ. Toxicol. Chem.* 13, 2031–2041.
- Rolfs, A., Hediger, M.A., 1999. Metal ion transporters in mammals: structure, function and pathological implications. *J. Physiol.* 518, 1–12.
- Saari, J.T., Johnson, W.T., Reeves, P.G., Johnson, L., 1993. Amelioration of effects of severe dietary copper deficiency by food restriction in rats. *Am. J. Clin. Nutr.* 58, 891–896.
- Schecher, W.D., McAvoy, D.C., 1994. MINEQL+, User's Manual. Environmental Research Software, Hallowell, ME.
- Schultz, S.G., Fuisz, R.E., Curran, P.F., 1966. Amino acid and sugar transport in rabbit ileum. *J. Gen. Comp. Physiol.* 49, 849–855.
- Segner, H., 1987. Response of fed and starved roach, *Rutilus rutilus*, to sublethal copper contamination. *J. Fish Biol.* 30, 423–437.
- Smith, R.W., Griffith, J.S., 1994. Survival of rainbow trout during their first winter in Henry's Fork of the Snake River, Idaho. *Trans. Am. Fish. Soc.* 123, 747–756.
- Szebedinszky, C., McGeer, J.C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic Cd exposure via the diet or water on internal organ-specific distribution and subsequent gill Cd uptake kinetics in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* 20, 597–607.
- Taylor, L.N., McGeer, J.C., Wood, C.M., McDonald, D.G., 2000. The physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: an evaluation of chronic indicators. *Environ. Toxicol. Chem.* 19, 2298–2308.
- Turnlund, J.R., Keyes, W.R., Peiffer, G.L., Scott, K.C., 1998. Copper absorption, excretion, and retention by young men consuming low dietary Cu determined using the stable isotope  $^{65}\text{Cu}$ . *Am. J. Clin. Nutr.* 67, 1219S–1225S.
- Wilson, R.W., Bergman, H.L., Wood, C.M., 1994. Metabolic costs and physiological consequences of acclimation to aluminum in juvenile rainbow trout (*Oncorhynchus mykiss*). 1. Acclimation specificity, resting physiology, feeding, and growth. *Can. J. Fish. Aquat. Sci.* 51, 527–535.