Copper metabolism and gut morphology in rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal dietary copper exposure

Collins N. Kamunde, Martin Grosell, John N. A. Lott, and Chris M. Wood

Abstract: Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to 11 (control), 300 (medium), and 1000 μg $Cu \cdot g^{-1}$ (high) (as $CuSO_4 \cdot 5H_2O$) in the diet for 28 days at a daily ration of 4% wet body weight, with a background waterborne Cu concentration of 3 $\mu g \cdot L^{-1}$. There was no effect of dietary Cu on growth, condition factor, or food conversion efficiency. Whole-body Cu content increased continuously over the exposure period in all groups and was twofold and fourfold higher than controls at day 28 for the medium- and high-Cu diets, respectively. Copper accumulated mainly in liver and gut tissue, with the latter stabilizing by day 14. Accumulation also occurred in gill, kidney, and carcass. Plasma Cu concentration was not different from the controls whereas Cu in bile was greatly elevated, an indication of increased hepatobiliary excretion. Dietary Cu pre-exposure decreased the uptake of waterborne Cu across the gills, providing the first evidence of homeostatic interaction between the two routes of uptake. Electron microscopic observations of the midintestine revealed numerous mitochondria, lysosomes, lamellated bodies, and extensive lamellar processes in the enterocytes. Apoptosis, mitosis, and eosinophilic granule cells were more apparent in Cu-exposed fish.

Résumé : Des jeunes Truites arc-en-ciel (*Onchorhynchus mykiss*) ont été exposées pendant 28 jours à des concentrations de 11 (témoin), de 300 (moyenne) ou de 1000 μg $Cu \cdot g^{-1}$ (élevée), sous forme de $CuSO_4 \cdot 5H_2O$, dans des rations alimentaires équivalant à 4% de leur masse corporelle humide et dans une eau contenant des concentrations d'arrière plan de Cu de 3 $\mu g \cdot L^{-1}$. Le Cu alimentaire est resté sans effet sur la croissance, le coefficient d'embonpoint et l'efficacité de la conversion de la nourriture. Le contenu corporel total en Cu a augmenté de façon constante chez tous les groupes au cours de l'exposition et, au jour 28, il était deux fois plus élevé dans le groupe à exposition moyenne que chez le groupe témoin et de quatre fois plus élevé chez le groupe à exposition élevée. Le Cu s'accumulait principalement dans le foie et dans les tissus du tube digestif, atteignant un palier chez ces derniers au jour 14. Il y avait aussi une accumulation dans les branchies, les reins et la carcasse. Les concentrations plasmatiques de Cu n'ont subi aucune hausse; en revanche, les concentrations dans la bile ont crû fortement, ce qui indique une augmentation de l'excrétion hépatobiliaire. Une exposition préalable au Cu alimentaire diminuait l'incorporation du Cu de l'eau par les branchies, ce qui constitue une première mention d'une interaction homéostatique entre ces deux voies d'incorporation. L'examen du tube digestif moyen au microscope électronique a révélé la présence dans les entérocytes de nombreux lysosomes, mitochondries, corpuscules lamellés et grands processus lamellaires. L'apoptose, la mitose et la présence de granulocytes éosinophiles étaient fréquentes chez les poissons exposés au Cu.

[Traduit par la Rédaction]

Introduction

Fish assimilate Cu from both water and diet (Dallinger et al. 1987). While the uptake and effects of waterborne Cu are fairly well understood (McDonald and Wood 1993), effects of dietary Cu in fish have been less thoroughly investigated (Handy 1996). A few studies have highlighted a micronutrient requirement for Cu (Ogino and Yang 1980; Murai et al. 1981), while others have recognized dietary exposure as a source of Cu intoxication in fish (Woodward et al. 1994, 1995; Handy 1996). Available dietary Cu exposure data indicate that bioavailability is very low (3% in rainbow trout (*Oncorhynchus mykiss*)) and toxic effects occur at much higher exposure levels relative to waterborne exposures (Lanno et al. 1985; Julshamn et al. 1988; Handy 1992). It has been argued that the gastrointestinal tract offers a strong barrier to dietary toxicants and effectively regulates metal uptake (Berntssen et al. 1999). Although the majority of the studies on dietary Cu exposure in fish have assessed growth and tissue-specific accumulation, results have been rather variable (Handy 1992; Mount et al. 1994; Berntssen et al. 1999) and probably reflect the differences in experimental designs, diet variables such as Cu concentrations and bioavailability, and the length of the exposure.

Interactions between dietary and waterborne Cu uptake are likely important in Cu homeostasis in fish. Unfortunately, nothing is known about the effects of dietary Cu preexposure on subsequent waterborne Cu uptake or vice versa. Previous studies that attempted to delineate effects of waterborne and dietary Cu exposure (Miller et al. 1993; Farag et al. 1994) focused mainly on tissue metal accumula-

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tion attending Cu exposure through the two routes. The problem with such studies is distinguishing Cu of dietary uptake from that of waterborne uptake. Application of direct measurements of Cu uptake using radioisotope methodologies (Wood 1992) not only permits this but also distinguishes Cu of recent uptake from Cu accumulated over the long term.

Exposure to elevated dietary metal levels is often accompanied by morphological changes in the gut, but there are only a few studies on Cu. Effects of a dietary metal mixture (including Cu) on rainbow trout gut morphology, as described by Woodward et al. (1995), include gut impaction, swelling, ulceration, and epithelial lifting. Recently, Berntssen et al. (1999) reported increased apoptosis and cell proliferation in the ileum of Atlantic salmon (*Salmo salar*) parr exposed to elevated dietary Cu. Additional morphological studies are desirable to more fully describe the impact of dietary Cu on the fish gut.

In the present study, we evaluated tissue-specific partitioning and bioaccumulation of Cu following dietary exposure at environmentally realistic sublethal levels. Secondly, we assessed the impact of dietary Cu exposure on subsequent uptake and distribution of waterborne Cu using a sensitive radioisotope methodology that distinguishes recently accumulated Cu from that already present in tissues prior to exposure. Finally, we described the morphological changes occurring in the midintestine during chronic elevated dietary Cu exposure.

Materials and methods

Experimental animals

One hundred and twenty juvenile rainbow trout (weight and length (mean ± SEM) = 37.24 ± 1.20 g and 14.86 ± 0.20 cm, respectively) were transferred from laboratory stock (originally from Humber Springs Trout Farm, Ontario) and equally divided into three 80-L experimental tanks supplied with a throughflow of aerated dechlorinated Hamilton city tap water (Na^+, 13.8 mg·L^{-1}; Cl^-, 24.8 mg·L^{-1}; Ca^{2+}, 40 mg·L^{-1}; HCO_3^-, 115.9 mg·L^{-1}; pH, 7.9–8.2; background Cu, 3 μg·L^{-1}) at flow rates of 1.2 L·min^{-1}. Seven days before starting the experiment, all the fish were anesthetized with 1% tricaine methanesulphonate (MS 222) and individually marked by introducing alcian blue dye (0.06 g·mL^{-1} in nanopure water) spots on their undersides with Panjet (Wright Dental Manufacturing Co., Dundee). This facilitated fish identification and determination of specific growth rates.

Diet preparation

Copper-enriched diets were "made" in-house by grinding commercial trout chow (5 point Regular Trout Grower Pellets; composition: 40% crude carbohydrate, 42% crude protein, 1% calcium, 0.75% phosphorus, and 0.35% sodium (Martin's Feed Mill Ltd., Elmira, Ont.)) and mixing it with Cu (as $CuSO_4 \cdot 5H_2O$; Fisher Scientific) to give nominal diet concentrations of 300 (medium) and 1000 μg Cu·g^{-1} (high). The food was then extruded through a pasta maker, air-dried, and broken into small pellets (approximately 8 mm^3) by hand. The actual Cu content of the diets (mean ± SEM) as determined by atomic absorption spectroscopy was 277.8 ± 5.9 (n = 5) and 1041.9 ± 17.5 μg·g^{-1} (n = 5) for the medium- and high-Cu diets, respectively. These dietary levels of exposure were chosen to fall within environmentally realistic levels (Dallinger and Kautzky 1985) and above maximum tolerable levels (Lanno et al. 1985), respectively. Control diet consisted of the same commercial fish chow treated in the same way but not supplemented with Cu. Cu concentration of the control diet was 11.4 ± 0.2 μg·g^{-1} (n = 5).

Feeding, presampling procedure, and sampling regime

Fish were fed twice daily in the morning (08:00–09:00) and evening (18:00–19:00) at 2% wet body weight, totaling 4%·day^{-1}, and water temperature was maintained at 14 ± 1°C throughout the experimental period. Visual examination during feeding revealed that all fish readily ingested the diets; there was no avoidance of the high-Cu diet. Fecal material was siphoned within 1 h of feeding, and the flow-through system continuously flushed away any fecal matter deposited between the feeding times. Copper concentrations in the exposure tanks before and after feeding ranged from 2.89 to 3.21 μg·L^{-1} in all groups; there were no differences before and after feeding. Sampling was done at the start of the exposure (day 0) and at days 14 and 28. Prior to sampling, all the fish were anesthetized with 1% MS 222, identified, and their weights and lengths taken individually. After recovery from the anesthesia, the fish were returned into their respective experimental tanks and starved for 24 h to clear the gut of ingesta. During the starvation period, fecal material was siphoned from the tanks twice every 12 h to minimize any chance of coprophagy.

Waterborne ^{64}Cu exposure

Measurement of waterborne Cu uptake, by means of ^{64}Cu flux determination, preceded terminal sampling at all sampling times. The radioisotope ^{64}Cu (as $Cu(NO_3)_2$, $t_{1/2}$ = 12. 65 h) was prepared at the McMaster Nuclear Reactor, Hamilton, Ont. Fish (n = 10) from the control group and the two dietary Cu exposed groups were moved into separate tanks containing 20 L of static aerated dechlorinated Hamilton city tap water and allowed to settle for 1 h. Subsequently, 0.7 μCi ^{64}Cu·L^{-1} (specific activity 0.35 μCi·g^{-1}) (1 μCi = 37 kBq) was introduced into each tank and the exposure carried out for 12 h. A time course study demonstrated that 12 h of exposure was adequate to allow measurable uptake of the Cu into the target organs and whole body. Furthermore, whole-body uptake was linear over the 12-h period, demonstrating that a 1-h settling period prior to exposure was adequate and that there was no deterioration of conditions that affected Cu uptake over the 12 h. The radioisotope dosage administered added a total concentration of 0.2 μg Cu·L^{-1} in the water and was chosen to ensure that the total water Cu was not substantially elevated above the ambient level of 3 μg·L^{-1}.

During the 12-h static ^{64}Cu flux, excretory Cu significantly elevated the water Cu levels above the ambient concentration. To overcome this problem, a separate ^{64}Cu flux experiment was performed on fish that had been exposed to either control or high-Cu diet for 14 days. In this experiment, 50 individually marked fish (25 control and 25 high dietary Cu exposed) were exposed to ^{64}Cu for 12 h in the same flux chambers so that total Cu concentrations in the water would be identical for the two groups. Five different water Cu concentrations (1.3, 2.5, 5.0, 6.2, and 12.0 μg·L^{-1}) were tested with five fish from each treatment in each flux chamber.

When it became evident that waterborne Cu uptake was strongly influenced by body size, an additional experiment was performed with 144 juvenile rainbow trout ranging in body mass from 0.5 to 77 g. ^{64}Cu flux was measured under control conditions (control diet, water Cu approximately 3 μg·L^{-1}) using methods identical to those outlined above.

Sampling

Fifteen minutes after introduction of ^{64}Cu to the flux tanks, a 10-mL water sample was taken from each tank. After 12 h of exposure to ^{64}Cu, a second 10-mL water sample from each tank was taken. Subsequently, the fish were anesthetized with 1% MS 222 and a blood sample obtained by caudal puncture into heparinized 1-mL syringes fitted with 23-gauge needles. An aliquot of 50 μL of

each blood sample was immediately centrifuged for 4 min at 13 000 × *g* to separate plasma. The fish were then killed by a blow to the head and the entire gill baskets, liver, gut, kidney, muscle, and the rest of the carcass dissected out and collected into separate preweighed scintillation vials or Eppendorf tubes. Before dissecting out the liver, bile was collected by aspiration from the gallbladder into a 1-mL syringe fitted with a 23-gauge needle. In addition, the carcass and gills were rinsed in nanopure water to remove surface-bound radioactivity.

Analysis

^{64}Cu activity in the tissue and water samples was measured on a Canberra-Packard Minaxi gamma counter with an onboard program for correcting for decay. Subsequently, the tissues were digested overnight at 70°C with six volumes of 1 N nitric acid (Fisher Scientific, trace metal grade) and then centrifuged for 4 min at 13 000 × *g*. A subsample of the supernatant was diluted suitably with 0.5% nitric acid. Total tissue Cu concentration was determined by atomic absorption spectrophotometry (Varian AA-1275 with GTA furnace atomizer) using a 10-µL injection volume and the operating conditions specified for Cu by the manufacturer. Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range. Under the conditions of the analyses (taking dilution factors into account), the detection limits for water and tissue were 0.3 and 0.6 $\mu g \cdot g^{-1}$, respectively, far below experimental values measured in this study.

Calculations

Whole-body total Cu concentration was calculated by dividing the sum of Cu contents (concentration multiplied by weight or volume) of all the tissues plus the carcass by the sum of weights of all the tissues plus the carcass. Fish Cu content was calculated by multiplying whole-body (fish) Cu concentration by the fish weight.

The ^{64}Cu uptake of the tissues was calculated on individual fish basis using the equation

$$(1) \qquad a(bc^{-1})^{-1}$$

where a is the ^{64}Cu counts per minute per gram of tissue, b is the ^{64}Cu counts per minute per litre of water, and c is the total Cu concentration in water in micrograms per litre. Proportional distribution was calculated from the product of eq. 1 for each tissue and that tissue weight divided by the sum of these values for all tissues plus carcass.

"Previous compartment analysis," as outlined by Grosell et al. (1997), was used to trace the movement of Cu between different body compartments. Newly accumulated Cu in each tissue or organ was calculated on an individual fish basis using the equation

$$(2) \qquad a(de^{-1})^{-1}$$

where a is ^{64}Cu counts per minute per gram of tissue, d is ^{64}Cu counts in the previous compartment (counts per minute per litre), and e is the total Cu concentration in the previous compartment (micrograms per gram or micrograms per litre, respectively). Previous compartments, based on the criteria of Grosell et al. (1997), were water for gill, gill for plasma, plasma for liver, gut tissue, kidney, muscle, and carcass, and liver for bile.

Specific growth rate (SGR) was calculated for each 2-week period (0–14 and 15–28 days) using the formula

$$(3) \qquad \mathrm{SGR} = 100(\ln(\mathrm{wt}_2) - \ln(\mathrm{wt}_1))t^{-1}$$

where wt_1 and wt_2 are individual fish weights at the start and end of the each growth period, respectively, and t is the time interval in days. Fish were identified by their distinctive marks and weighed individually.

Condition factor (k) for individual fish was calculated using the formula

$$(4) \qquad k = 100(\mathrm{weight} \times \mathrm{length}^{-3}).$$

Food conversion efficiency (FCE) on a per tank basis was calculated as

$$(5) \qquad \mathrm{FCE}\ (\%) = 100(\mathrm{weight\ gain} \times \mathrm{food\ eaten}^{-1}).$$

Transmission electron microscopy

At each sampling time (0, 14, and 28 days), three additional fish per treatment (27 in total) were killed with an overdose of MS 222 and the gastrointestinal tract exposed. Control and experimental fish were processed simultaneously. The midintestine was identified as the section of the intestine between the last pyloric cecum and the start of the distal intestine (darker region with larger diameter and annular rings), dissected out, immediately flushed with Cortland saline (Wolf 1963), and fixed by immersion for 2 h in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, osmolarity, 230 mosmol. Subsequently, it was cut into four equal regions, which were further subdivided into four medium-sized blocks. Each block was then diced into small pieces, approximately 1 mm^3 in size. These pieces were then fixed further overnight in greater than 10 times volume of the same solution. Ten such pieces per fish were selected randomly and postfixed in 1% buffered osmium tetraoxide for 4 h at 4°C. The tissues were subsequently dehydrated in a series of ethanol solutions from 50 through 100% and embedded in Spurr's resin. Five-micrometre-thick semithin monitor sections were obtained and stained with toluidine blue for light microscopic observation. Thin sections (80 nm thick) were then obtained and mounted on Cu grids, stained with uranyl acetate and lead citrate, and viewed under a JEM–1200EXII transmission electron microscope at 80 kV.

Statistical analysis

All data except the qualitative microscopic observations and food conversion efficiency are presented as means ± SEM. Effects of different oral exposure of Cu on growth, tissue Cu concentration, and subsequent waterborne Cu uptake at each sampling point were assessed using a two-way analysis of variance with time and diet Cu concentration as variables. Percentage data were subjected to arcsine transformation prior to statistical testing. Significance was set at $p < 0.05$. The Student–Newman–Keuls pairwise multiple comparison procedure was used to make comparisons between measurements.

Results

Growth and mortality

All the fish maintained high rates of growth during the exposure. Mean weights rose from about 37 g at the start of the exposure to about 70 g, while length increased from 15 to 18 cm (Table 1). Under our conditions of exposure, dietary Cu concentration as high as 1042 $\mu g \cdot g^{-1}$ had no significant effect on specific growth rate, condition factor, and food conversion efficiency. However, nonsignificant growth stimulation and inhibition were observed with medium- and high-Cu diets, respectively. A similar trend was evident for condition factor and food conversion efficiency (Table 1). No mortality occurred during the exposure.

Copper bioaccumulation and partitioning

When expressed on an individual fish basis, Cu content increased continuously with normal growth throughout the experiment in the control fish as well as in the Cu-exposed fish (Fig. 1). However, by day 28, total Cu content per fish was about twofold higher in the medium-Cu diet group and

Table 1. Means ± SEM (*n*) starting and final wet weights and lengths, specific growth rate (SGR), condition factor (*k*), and food conversion efficiency (FCE) for control and Cu-exposed rainbow trout.

	Diet Cu content		
Biological index	Control (11 μg·g^{-1})	Medium (300 μg·g^{-1})	High (1000 μg·g^{-1})
Starting weight (g)	36.99±1.93 (40)	37.68±2.16 (41)	37.04±2.219 (40)
Final weight (g)	63.32±5.73 (17)*	75.99±4.98 (18)*	65.69±5.15 (17)*
Starting length (cm)	14.67±0.40 (41)	14.94±0.33 (41)	14.99±0.33 (40)
Final length (cm)	17.57±0.49 (17)*	18.72±0.34 (18)*	18.53±0.84 (17)*
SGR (%·day^{-1})			
Days 0–14	2.44±0.12 (25)	2.69±0.14 (27)	2.16±0.14 (27)
Days 15–28	2.41±0.12 (14)	2.50±0.23 (13)	2.40±0.16 (13)
k			
Day 0	1.14±0.06 (40)	1.11±0.04 (41)	1.07±0.03 (40)
Day 14	1.10±0.02 (30)	1.15±0.02 (31)	1.09±0.01 (30)
Day 28	1.11±0.01 (17)	1.13±0.02 (18)	1.06±0.06 (17)
FCE (%)			
Days 0–14	72.8 (25)	81.1 (27)	64.2 (27)
Days 15–28	69.0 (14)	69.9 (13)	66.7 (13)

*Significant difference ($p < 0.05$) from starting values.

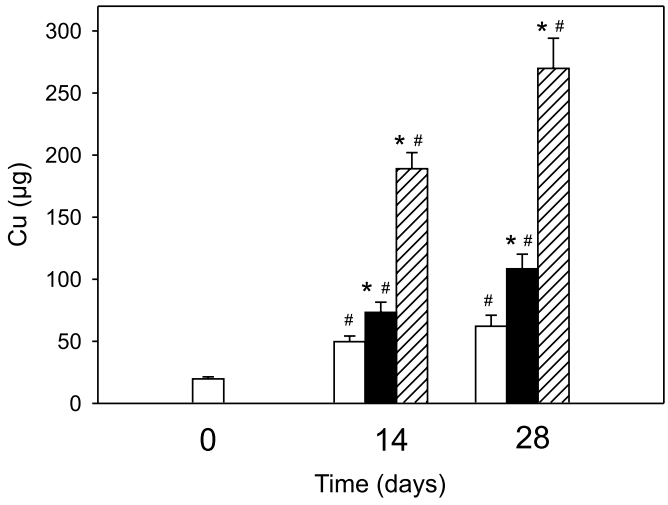
fourfold higher in the high-Cu diet group relative to the simultaneous controls. Since growth rates were unaffected by these treatments, whole-body Cu concentrations exhibited similar trends (Table 2). However, Cu concentrations in the gut tissue rose to a much greater extent, from about 1 μg·g^{-1} in controls to approximately 5 μg·g^{-1} for the medium-Cu diet and 25 μg·g^{-1} for the high-Cu diet on days 14 and 28. Uptake and transport of Cu to internal organs occurred with significant accumulation in the liver, bile, kidney, gills, muscle, and carcass. Only in the gut tissue and bile did Cu accumulation appear to reach steady state. Despite this pattern of widespread accumulation, plasma Cu remained constant between 0.5 and 0.7 μg·mL^{-1} throughout the exposure.

The proportional distribution of total Cu in the organs and tissues sampled is shown in Table 3 based on measurements of the absolute amounts of Cu in the different organs and in the whole animal. The key organs and tissues for Cu accumulation were the liver, gut, and carcass (which includes muscle). In control animals, the liver and carcass together contained 75–85% of the Cu, while all the other organs together contained 15–25% of the Cu. Following dietary Cu exposure, the contribution of the carcass decreased and Cu was distributed mainly in the liver and gut tissue. The contribution of these two tissues to total body Cu was 65–75% in fish exposed to the medium-Cu diet and 85% in fish exposed to the high-Cu diet. Gills contributed less than 3.5% of the total Cu in control fish, and fish exposed to dietary Cu had a significantly lower proportion of the Cu in gills. The kidney accounted for less than 2% of the total Cu in all the groups.

Waterborne ^{64}Cu uptake

Exposure to waterborne ^{64}Cu for 12 h resulted in detectable levels of radioactivity in all the body tissues sampled (Fig. 2A). In the early stages of the exposure, gills showed high levels of radioactivity, but with time, greater radioactivity was registered in internal organs, especially the liver. By the end of the 12 h, the ^{64}Cu concentrations were highest in the liver followed in decreasing order by the gill, gut, and carcass. Uptake into the whole body (sum of activities in all the tissues over total weight of all the tissues) increased linearly with time over the 12-h period (Fig. 2B).

Fig. 1. Total Cu content per fish in control and dietary Cu exposed rainbow trout at days 0, 14, and 28. Open bars, control; solid bars, 300 μg Cu·g diet^{-1}; hatched bars, 1000 μg Cu·g diet^{-1}. Values are means ± SEM, $n = 10$ per treatment per time. An asterisk indicates a significant difference from the corresponding control; a pound sign indicates a significant difference from the day 0 control.



Two complications arose in the measurement of ^{64}Cu uptake from the water. Firstly, weight-specific uptake clearly declined with body mass. Secondly, levels of Cu in the water varied among different trials due to differential Cu excretion by the fish, as outlined below. Therefore, two additional experiments were performed. In the first, analysis of Cu uptake measurements taken from 144 unexposed fish of different sizes ranging from 0.5 to 77 g showed a clear nonlinear dependence of mass-specific uptake (*y*, nanograms per gram) on body mass (*x*, grams) that was best described by a negative exponential relationship (Fig. 3A): $y = 0.179 \exp(-0.0743x)$ ($r^2 = 0.45$, $p < 0.0001$). This relationship was used to correct

Table 2. Total Cu concentration (means ± SEM, $\mu g \cdot g$ wet weight^{-1} or $\mu g \cdot mL^{-1}$, n = 10 per treatment) in tissues and whole body of control and dietary Cu exposed rainbow trout after 0, 14, and 28 days of exposure.

	Gill	Liver	Gut	Kidney	Muscle	Carcass	Bile	Plasma	Whole body
Day 0, control	0.69±0.04	16.85±1.89	1.26±0.13	1.57±0.09	0.36±0.02	0.48±0.05	13.94±1.11	0.70±0.07	0.63±0.06
Day 14									
Control	0.54±0.02	18.57±2.12	2.29±0.23	2.77±0.25	0.31±0.02	0.34±0.04	7.04±0.76	0.56±0.03	0.93±0.06
Medium	0.65±0.06*	29.91±3.71*	3.20±0.62	4.48±1.09	0.27±0.01	0.51±0.07*	16.36±1.7*	0.52±0.03	1.84±0.32*
High	0.94±0.16*	62.67±12.28*	25.96±2.42*	5.31±1.12*	0.37±0.04	0.48±0.03*	28.54±3.01*	0.52±0.02	3.74±0.16*
Day 28									
Control	0.91±0.07	38.40±6.43	2.19±0.73	1.70±0.32	0.26±0.02	0.36±0.04	10.75±0.99	0.50±0.04	1.46±0.24
Medium	1.22±0.04*	45.18±6.87	5.41±1.03*	1.73±0.16	0.32±0.04	0.45±0.03	15.39±1.7*	0.52±0.01	1.72±0.15
High	1.37±0.09*	100.27±22.51*	23.58±2.44*	9.98±3.3*	0.32±0.01*	0.70±0.12*	27.73±2.89*	0.50±0.01	5.24±0.53*

*Significant difference ($p < 0.05$) relative to the respective control.

the data for all fish in the second experiment to a common weight of 10 g.

In this experiment, control and high dietary Cu exposed fish were exposed to ^{64}Cu in the same flux chambers at a range of identical water Cu concentrations. The results (Fig. 3B) demonstrate that dietary Cu pre-exposure significantly reduced subsequent branchial ^{64}Cu uptake. In addition, Cu uptake increased linearly with water Cu concentration over the range of water Cu tested in both control and experimental groups.

During the initial 12-h flux experiments where the control and Cu-treated fish were kept in separate chambers, net excretion of Cu from the medium and high dietary Cu exposed groups significantly elevated the water Cu concentration above the ambient level of 3 $\mu g \cdot L^{-1}$. Furthermore, in different trials, the extents of these elevations were different. Thus, absolute rates of Cu uptake from the water could not be validly compared between treatments, and the ^{64}Cu data could only be used to compare relative internal distribution of ^{64}Cu with that of total Cu. Treatment-related differences for ^{64}Cu distribution at day 28 were relatively minor, but there were major differences in comparison with total Cu distribution (Fig. 4). In particular, the gills contained approximately 20% of ^{64}Cu but only 2% of the total Cu. Furthermore, the carcass contained about 60% of the ^{64}Cu but only 20% of the total Cu. The other major differences were in gut and liver, which each contained about 5–10% of the ^{64}Cu whereas about 50% of total Cu was in the liver and 25% in the gut. Similar patterns were seen at day 14 (data not shown).

Newly accumulated Cu

Newly accumulated Cu in each of the tissues sampled calculated using the previous compartment analysis of Grosell et al. (1997) is shown in Table 4. In both the medium and high dietary Cu exposed groups, all the tissues analyzed exhibited newly accumulated Cu concentrations that were similar to those of the respective controls, except for the liver of the high dietary Cu group at day 28 and the gut of the medium dietary Cu group at day 14. In all cases, the level of newly accumulated Cu was one to two orders of magnitude lower than the corresponding total Cu concentration (note the different units in Tables 2 and 4) except for bile and plasma. For bile and plasma, the calculated values of newly accumulated Cu were in excess of the corresponding total Cu concentrations, indicating the dynamic turnover in these two compartments.

Midintestinal morphology

Representative electron micrographs of midintestine from control (n = 15) and Cu-exposed (n = 12) rainbow trout are shown in Fig. 5. Normal midintestinal epithelium comprised absorptive cells, the enterocytes, and mucous cells (Figs. 5A and 5B). Enterocytes possessed numerous closely packed microvilli (brush border) and were joined by typical epithelial cell junctional complexes, while the mucous cells were densely packed with secretory granules of variable size and electron density.

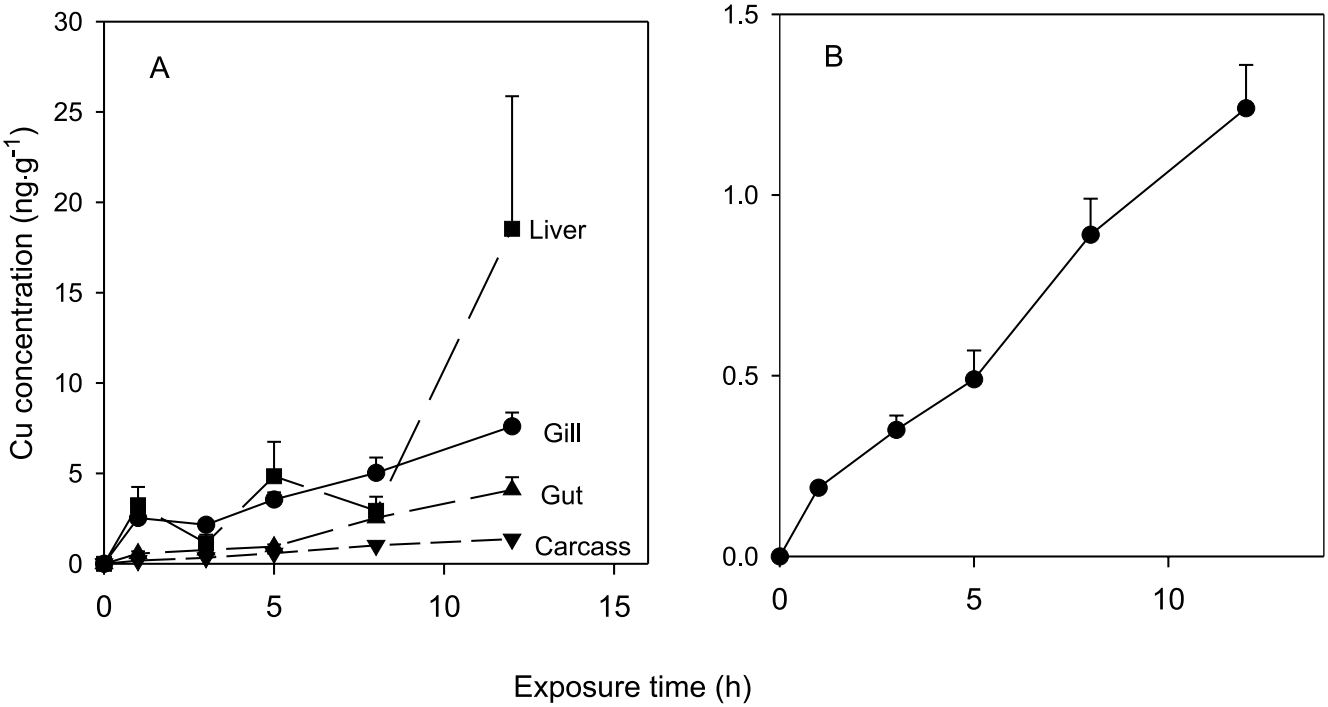
No differences were noted in midintestinal structure between the medium and high dietary Cu exposed groups or between day 14 and day 28. The most prominent effect of

Table 3. Proportional distribution (%, means ± SEM) of total body Cu in control and dietary Cu exposed rainbow trout.

	Gill	Liver	Gut	Kidney	Carcass
Day 0, control	3.16±0.24	27.16±2.24	12.28±1.47	1.30±0.08	56.10±2.18
Day 14					
Control	1.90±0.13	46.86±2.03	19.14±2.12	1.77±0.19	28.50±1.64
Medium	1.37±0.15*	50.70±5.47	14.10±2.44	1.87±0.54	28.82±4.59
High	0.77±0.13*	37.99±4.48	47.78±4.19*	0.74±0.16*	11.07±0.72*
Day 28					
Control	2.26±0.45	59.35±5.32	13.73±3.36	0.79±0.15	23.87±3.33
Medium	2.03±0.19	49.66±3.56	23.11±3.39	0.64±0.07	24.56±2.16
High	0.83±0.09*	51.00±3.91	35.05±3.89*	1.10±0.28	12.01±1.95*

Note: Statistical analysis was performed on arcsine-transformed proportional data.
*Significant difference ($p < 0.05$) from the respective control; $n = 30$ for day 0 control and $n = 10$ per tissue in all other treatments.

Fig. 2. Time course of waterborne ^{64}Cu uptake (means ± SEM) in previously unexposed rainbow trout on the control diet into the (A) gill, liver, gut, and carcass and (B) whole body ($n = 5$ per data point). Water Cu concentrations were 2.64 ± 0.21 ($n = 4$) and 2.81 ± 0.14 $\mu g \cdot L^{-1}$ ($n = 4$) before and after the flux, respectively.



high dietary Cu was the appearance of numerous mitochondria in the apical region of the enterocytes (Fig. 5C). A number of cells showed characteristics of apoptosis (Fig. 5D). Apoptotic cells were shrunken and possessed many electron-dense bodies and very few microvilli and mitochondria. The plasma membrane appeared to be intact and showed no signs of rupture as would occur in necrotic cells. In addition, the midintestine of fish exposed to elevated dietary Cu showed dramatic organellar changes marked by formation of numerous lamellated bodies (Figs. 6A and 6B). The lamellated bodies were composed of a whorl of circular membranous lamellae surrounding electron-dense granules and occurred in the cytoplasm, or within mitochondria and lysosomes. As well, cell renewal was evidenced by observation of mitosis and immature cells (Fig. 6C). Immature mucous cells contained extensive rough endoplasmic reticulum, a well-developed Golgi apparatus, and secretory granules at various stages of formation (Fig. 6C). In the lamina propria and the stratum compactum, there was an apparent increase in the number of eosinophilic granule cells (Fig 6D). The eosinophilic granule cells contained electron-dense granules of different sizes and occasional myelin bodies.

Discussion

Growth

Within the range of concentrations tested here, inhibitory growth effects of dietary Cu have been reported in rainbow trout (Lanno et al. 1985) and channel catfish (*Ictalurus punctatus*) (Murai et al. 1981), while lack of effect has been reported in channel catfish (Gatlin and Wilson 1986), rainbow trout (Mount et al. 1994; Handy et al. 1999), and Atlan-

Fig. 3. (A) Relationship between body mass (x) and waterborne ^{64}Cu uptake (y) across the gills in previously unexposed rainbow trout on the control diet (n = 144). The relationship is best described by the negative exponential equation $y = 0.179 \exp(-0.0743x)$ (r^2 = 0.45, $p < 0.0001$). (B) Cu uptake rate measured with ^{64}Cu at different water Cu concentrations in unexposed fish (solid circles) and fish exposed to a high-Cu diet for 14 days (open circles). Values are means ± SEM, n = 5 per group per sampling time. The equation in Fig. 3A was used to correct for size differences; rates are expressed for 10-g fish. An asterisk indicates a significant difference.

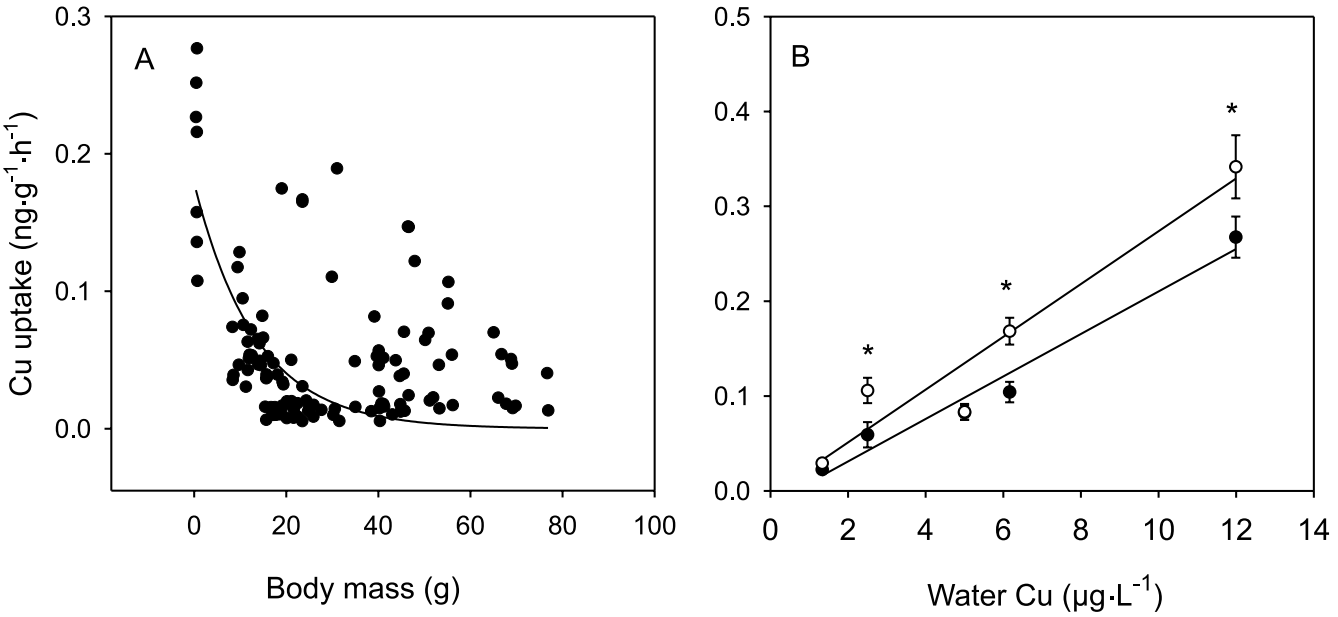
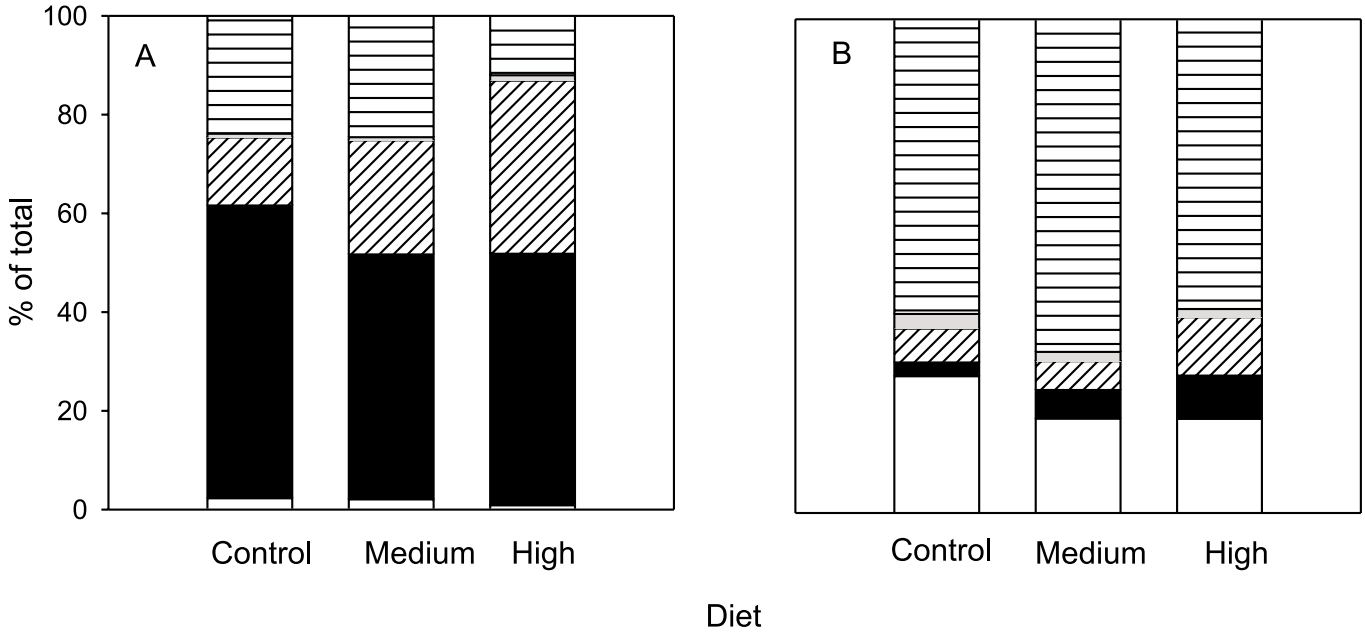


Fig. 4. Proportional distribution of (A) total Cu and (B) waterborne ^{64}Cu in the carcass (horizontal hatch), kidney (gray), gut (diagonal hatch), liver (solid), and gill (open) of control and dietary Cu exposed rainbow at day 28 (n = 10 per treatment).



tic salmon (Berntssen et al. 1999). Interestingly, Mount et al. (1994) reported mortalities in the absence of growth effect. In the present study, no significant growth effects or mortalities were manifest even in fish at a dietary Cu exposure as high as 1042 µg·g^{-1}. According to Lanno et al. (1985), dietary Cu concentrations of 664–730 µg·g^{-1} and above would cause poor food conversion and retarded growth in rainbow trout. Although the discrepancy among these data sets could be due to the different exposure periods, it is also possible that dietary Cu levels necessary to cause toxicity are much higher than previously thought. Furthermore, the amount of Cu absorbed, and hence effects, may depend on the bioavailability of the Cu in different food formulations.

Bioaccumulation and partitioning of Cu

Gut tissue Cu accumulation exhibited saturation with time and dose dependence. Seemingly, elevated dietary Cu level within the nontoxic range was well regulated. On a local ba-

Table 4. Newly accumulated Cu (means ± SEM, ng·g wet weight^{-1} or μg·mL^{-1}, n = 30 for day 0 samples and n = 10 for day 14 and 28 samples).

	Gill (ng·g^{-1})	Liver (ng·g^{-1})	Gut (ng·g^{-1})	Kidney (ng·g^{-1})	Muscle (ng·g^{-1})	Carcass (ng·g^{-1})	Bile (μg·mL^{-1})	Plasma (μg·mL^{-1})
Day 0, control	8.14±0.43	284.33±78.60	89.14±49.79	35.25±13.96	82.11±40.83	15.37±5.06	33.34±9.74	1.48±0.59
Day 14								
Control	2.73±0.27	198.71±46.67	52.18±20.90	177.83±61.68	142.99±59.52	31.00±7.16	56.67±14.15	0.48±0.09
Medium	2.82±0.42	485.20±152.17	403.12±260.54	376.20±103.21	261.57±102.78	57.73±20.99	215.54±141.62	0.61±0.12
High	2.22±0.18	419.72±129.55	190.93±54.51*	233.05±58.52	183.76±62.25	37.81±11.05	139.23±39.47	1.10±0.35
Day 28								
Control	1.86±0.10	61.76±9.05	167.64±24.90	36.85±6.38	53.18±8.67	30.01±5.50	262.82±104.85	1.18±0.29
Medium	1.96±0.30	272.41±134.11	135.28±19.12	38.40±7.71	34.59±7.44	33.66±5.08	52.65±14.20	1.75±0.37
High	2.41±0.27	325.11±71.73*	177.07±31.50	170.74±88.98	32.44±5.55	45.77±6.71	132.06±45.54	1.59±0.26

*Significant difference ($p < 0.05$) from respective control.

Fig. 5. Representative electron micrographs of the apical region of the midintestine in control and dietary Cu exposed rainbow trout. (A) Control. E, enterocyte; L, lumen; Mv, microvilli; Ld, lipid droplet; m, mitochondrion; small arrowhead, tight junction; large arrowhead, desmosome. (B) Control. mc, mucous cell; sg, secretory granule. (C) Cu exposed. Arrows, mitochondria; arrowhead, multivesicular body. (D) Cu exposed. AE, apoptotic enterocyte; E, normal enterocytes; arrows, electron-dense granules in apoptotic cell; small arrowheads, multivesicular body; large arrowhead, denuded mirovillus.

sis, small but significant Cu accumulation occurred in gut tissue during exposure to the medium-Cu diet, and internally, increases in most tissue compartments were modest. Therefore, 300 μg Cu·g^{-1} in the diet appears to be well tolerated and may well be in the nutritional range, since it stimulated growth slightly and did not cause severe accumulation of Cu. In contrast, the high-Cu diet, representing a threefold increase in dietary Cu concentration relative to the medium-Cu diet, resulted in a dramatic 20-fold increase (relative to day 0) in gut tissue Cu concentration, a marked increase in Cu concentration in most internal compartments (especially the liver), and a nonsignificant tendency for reduced growth, suggesting that it was beyond the nutritional range.

The general trend at both levels of exposure was that during the early stage of exposure (14 days), a greater proportion of the Cu was retained in the gut tissue than at the later stage (day 28). This is probably due to the mobilization of the Cu from the primary site of uptake to other tissues, especially the liver. Furthermore, dietary Cu exposure resulted in continuous Cu accumulation in the whole body in contrast with waterborne Cu exposure where stable whole-body Cu was reported in rainbow trout (Marr et al. 1996; Taylor et al. 2000) following 1–2 months of waterborne Cu exposure. This probably indicates that dietary Cu uptake is less tightly controlled than waterborne Cu uptake.

Liver Cu accumulation was linear over time and dose dependent and was higher than that of all the other tissues, in agreement with previous studies (Julshamn et al. 1988; Handy 1993). The response of the liver to the 300 μg·g^{-1} diet is important with regard to the threshold of dietary Cu causing significant accumulation in the liver. Handy (1992) reported that dietary Cu of 200 μg·g^{-1} did not cause significant accumulation in rainbow trout liver. Results from this study show that 300 μg Cu·g^{-1} caused significant accumulation in the liver early in the exposure but not later in the exposure, indicating that regulatory mechanisms had checked further accumulation. It can therefore be deduced that the maximum dietary level of Cu causing sustained significant accumulation in juvenile rainbow trout liver at 4% body weight ration is greater than 300 μg·g^{-1}.

Copper concentration in the gills was significantly elevated at both dietary levels, in agreement with previous reports on exposure to sublethal dietary Cu for 32 days (Handy 1992) and 42 days (Miller et al. 1993). Accumulation of dietary Cu in the gill underscores the unique anatomical location of fish gills and their potential importance in Cu metabolism and toxicity. The gill Cu levels obtained in this study are comparable with those associated with death from waterborne Cu exposure (MacRae et al. 1999). Al-

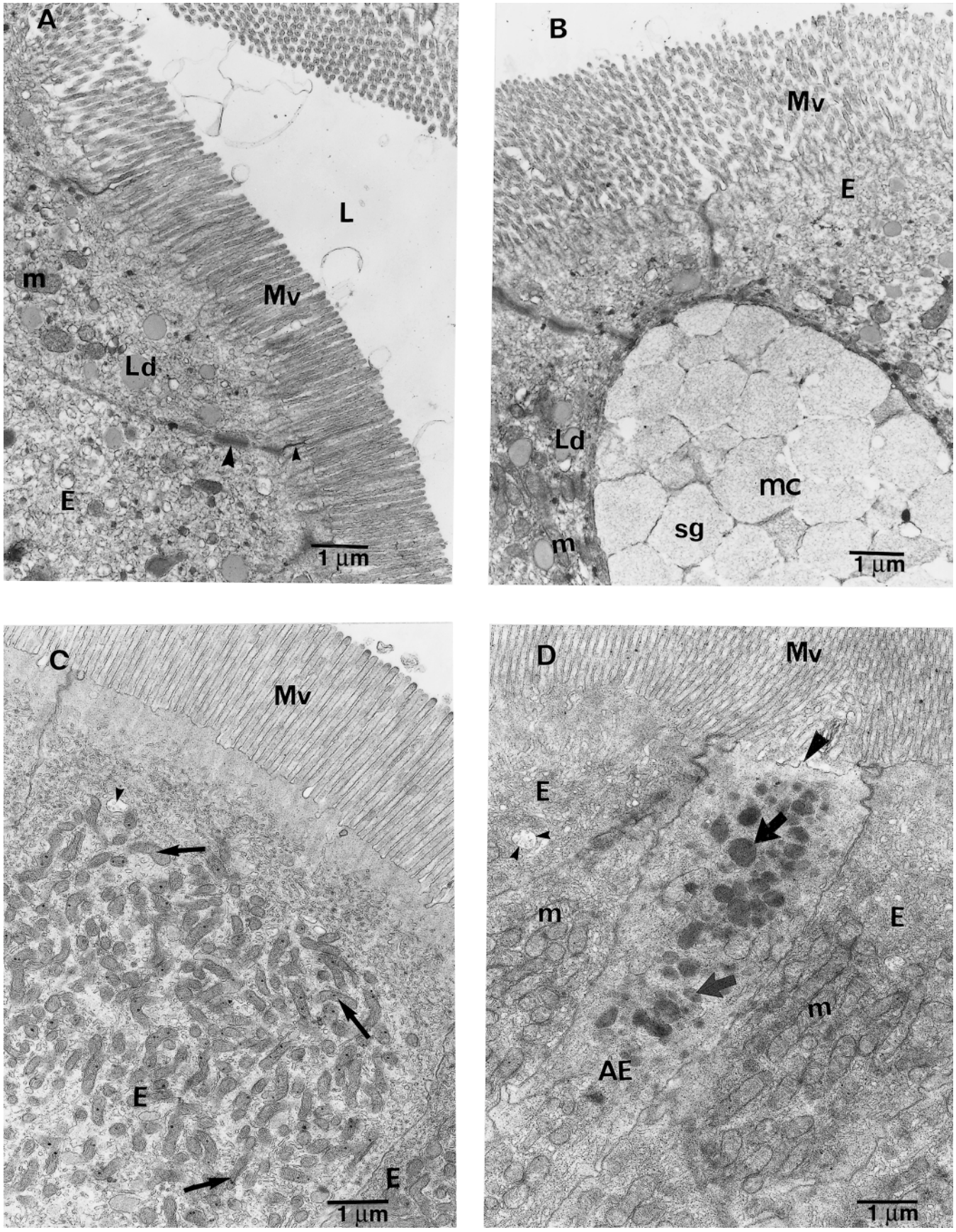
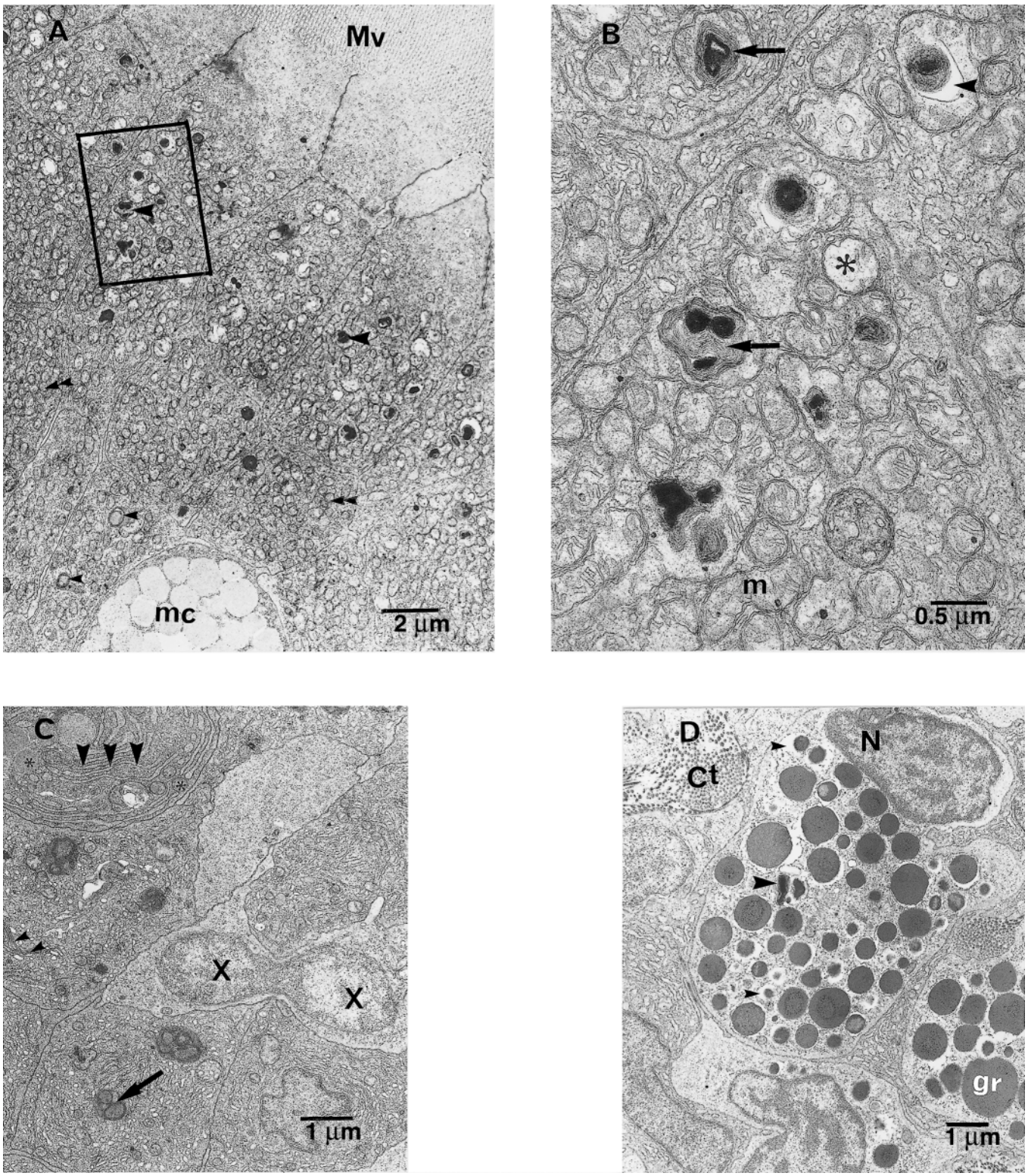


Fig. 6. Representative electron micrographs of enterocytes in a part of the midintestine of rainbow trout exposed to elevated dietary Cu. (A) Changes within enterocytes. Mv, microvilli on the apical side of an enterocyte; mc, mucous cell; large arrowheads, lamellated bodies; small arrowheads, secondary lysosomes. Note the numerous mitochondria (double arrowheads). (B) Closeup of the inset in Fig. 6A. Arrows, lamellated bodies with electron-dense granules; m, mitochondrion; asterisk, vacuole; arrowhead, halo. (C) Mitotic activity. Small arrowheads, Golgi apparatus; asterisk, newly formed secretory granule; large arrowheads, rough endoplasmic reticulum; X, dividing cell nucleus; arrow, secondary lysosome. (D) Eosinophilic granule cell. N, nucleus; gr, granule; large arrowhead, myelin body; small arrowheads, halo; Ct, connective tissue.



though these authors reported that 1.4 μg Cu·g gill tissue^{-1} accumulated in 12 h caused 50% mortality in rainbow trout within 5 days, 1.37 μg Cu·g gill tissue^{-1} accumulated in 28 days of exposure to the high-Cu diet resulted in no mortality. It is possible that the majority of the Cu binding to gills during waterborne Cu exposure is the toxic Cu^{2+} species, while that accumulated in gills after dietary exposure is protein bound (after passage through the liver and basolateral membranes of the gill cells) and probably nontoxic.

Muscle Cu concentration was not affected by dietary exposure except on day 28 when fish exposed to the high dietary Cu showed a significant accumulation. Thus, muscle Cu concentration appears to be homeostatically regulated. Total plasma Cu levels also remained remarkably constant throughout the exposure, in agreement with previous studies (Miller et al. 1993; Berntssen et al. 1999). In one study where elevated plasma Cu was reported (Handy 1992), sampling was not preceded by a substantial period of starvation.

One of the regulatory responses associated with acclimation to waterborne Cu exposure in rainbow trout is increased hepatobiliary Cu excretion (Grosell et al. 1997, 1998). In the present study, increased biliary Cu concentration was observed at day 14, which remained elevated at the same level at day 28, indicating that a balance between Cu uptake into the liver and excretion into bile had been established. It would appear that biliary excretion of Cu, perhaps acting in concert with other regulatory mechanisms not assessed in this study, prevents serious changes in Cu distribution at a dietary Cu concentration of 300 μg·g^{-1}. However, the regulatory capacity is overcome at the high exposure dose (1000 μg·g^{-1}), resulting in Cu accumulation in the liver and other tissues.

Waterborne Cu uptake

Branchial uptake of waterborne Cu increased with fish mass, but when plotted on a per unit body mass basis, a negative exponential relationship was evident. When the allometric equation of Hughes (1984) was used to calculate gill surface area of the fish, it was clear that a difference in surface area explained only a small portion of the relationship. While Cu uptake per unit body weight changed approximately eightfold over the weight range, gill surface area changed by less than 1.5-fold. Thus, other factors are probably also involved. For example, variations in Cu transporter activities and metabolic rates among fish of different size could affect Cu uptake independent of gill surface area.

Absolute rates of Cu uptake will probably be affected by the speciation and therefore the bioavailability of Cu in the water. In the moderately hard, moderately alkaline water of our experiments, Cu largely exists as $Cu(OH)_x$ and $CuCO_3$, which are less bioavailable than Cu^{2+}. For example, Taylor et al. (2000) reported that Cu was more bioavailable to rainbow trout in moderately acidic soft water than in the present water quality.

In a previous study on the interaction between dietary and waterborne Cu, Miller et al. (1993) reported that the uptakes of dietary and waterborne Cu were independent. Our observations using a sensitive radioisotope methodology demonstrate that pre-exposure of Cu through the diet decreased uptake of waterborne Cu. Thus, we provide the first evidence of a homeostatic interaction between the two routes of uptake. Elevated body (including gill) Cu burden negatively influences Cu uptake, perhaps by occupying potential new Cu binding sites or causing down-regulation of Cu transport proteins, to check further increase in tissue Cu concentrations. The presence of a Cu ATPase in rainbow trout gill has recently been suggested (Campbell et al. 1999) based on inhibition of Cu uptake by serosal vanadate in perfused rainbow trout head preparations. Although the mechanisms of Cu uptake across the gills are yet to be clearly explained, previous Cu exposure appears to play some role in the regulatory mechanisms.

The newly accumulated Cu data indicate that the short-term exchangeable pool of Cu within the fish is very small relative to the total body Cu. In all the tissues sampled, except for the bile and plasma, the newly accumulated Cu was one to two orders of magnitude lower than the total Cu. However, in bile and plasma, the calculated values were higher than the total Cu. The apparent overestimation of newly accumulated Cu in the plasma and bile can be explained by high total Cu levels coupled with low specific activities in the previous compartments (gill and liver for plasma and bile, respectively) and lack of equilibrium between ^{64}Cu and total Cu pools. Such a scenario means that the ^{64}Cu is more available than total Cu for exchange in the plasma and bile. In this study, although ^{64}Cu activity was reduced in fish pre-exposed to dietary Cu, newly accumulated Cu in the tissues sampled (taking into account the dilution of ^{64}Cu within the branchial, plasma, and hepatic compartments) was not different among the treatment groups.

However, the distribution pattern of ^{64}Cu among various tissues differed greatly from that of total Cu. While the higher gill and lower gut incorporations were not surprising considering that ^{64}Cu uptake was measured from waterborne exposure, the very high carcass incorporation was remarkable. The carcass is mainly composed of white muscle, and Grosell et al. (2000) have recently shown that white muscle serves as an important short-term buffer compartment when trout are infused with radiolabeled Cu.

Midintestinal morphology

The morphology of the midintestine in control fish was similar to that described previously for normal rainbow trout (Ezeasor and Stokoe 1980; Nonnotte et al. 1986). The impact of dietary Cu on midintestinal structure could be categorized broadly into cellular and subcellular responses. The cellular response was characterized by an increase in apoptosis and cell division in the intestinal epithelium, similar to that reported in previous studies on Pb and Cd (Crespo et al. 1986) as well as on Cu (Berntssen et al. 1999). These changes are therefore not specific to Cu. Assuming that the dying epithelial cells have the same or higher Cu content than the normal ones, evacuation of these dead cells in feces or intestinal mucus would provide a significant route for excretion of dietary Cu. In addition, many of the immature cells were mucous cells, suggesting an increased need for mucus and its attendant protective role. Although metal-binding properties of gastrointestinal mucus have not been assessed in fish, mammalian studies have shown that intestinal mucus will strongly bind divalent ions (Colman and Young 1979).

The appearance of numerous lamellated bodies and multivesicular bodies in intestinal epithelium has not been previ-

ously reported for dietary Cu exposure in fish. In mammalian studies, lamellated bodies are associated with regressive changes affecting cell organelles, e.g., in lysosomes during treatment with drugs and in mitochondria during exposure to toxic agents such as chloroform (Lüllmann-Rauch 1979; Guastadisegni et al. 1999). It is conceivable that abnormally high intracellular Cu causes damage and rearrangement of organelle membranes. The lysosomes observed in the enterocytes would possibly play a role in intestinal Cu sequestration and detoxification similar to their role in the liver during exposure to high Cu levels (Weiss et al. 1986; Lanno et al. 1987). The apparent increase in the number of mitochondria in midintestinal enterocytes suggests increased energy demand. It is possible that the uptake and (or) local regulation of Cu occur by energy-requiring processes and mitochondria supply the necessary energy to drive these processes. Conversely, Cu may impair energy-requiring activities in the enterocytes and the animal responds by increasing the number of mitochondria as a compensatory measure. Finally, dietary Cu stimulated an increase in eosinophilic granule cell infiltration in the lamina propria and stratum compactum. Eosinophilic granule cells have been hypothesized to constitute a composite defense mechanism, both humoral and mechanical, that develops in response to environmental demand (Ezeasor and Stokoe 1980).

The structural changes observed in the midintestine might be expected to have a metabolic cost, although only a marginal decrease in growth was evident at the high exposure dosage. More sensitive parameters, e.g., oxygen consumption or protein synthesis rates, might be more appropriate for assessing the cost of Cu exposure. Nonetheless, it is noteworthy that dietary Cu only modestly affected gut morphology. The bioaccumulation data indicate that, unlike the other tissues, gut tissue Cu was regulated and concentration stabilized by day 14. The absence of growth effect suggests that nutrient absorption in the gut was not significantly compromised, a persuasive indication that dietary Cu did not severely impact gut structure and function.

In conclusion, rainbow trout regulated dietary Cu at the level of the gut by increasing clearance to other tissues, at the liver by increasing biliary Cu excretion, and at the gill by reducing waterborne Cu uptake in response to dietary exposure. The modest morphological changes in the intestinal tract suggested high cell and organelle turnover and local regulation of Cu. In spite of possible increased energy demand for regulation and tissue repair, there was no significant growth inhibitory effect.

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