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Waterborne vs. dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure

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Kamunde, Collins, Cheryl Clayton, and Chris M. Wood. Waterborne vs. dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure. *Am J Physiol Regulatory Integrative Comp Physiol* 283: R69–R78, 2002. First published March 22, 2002; 10.1152/ajpregu.00016.2002.—Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne Cu (22 $\mu\text{g/l}$) in moderately hard water for up to 28 days. Relative to control fish kept at background Cu levels (2 $\mu\text{g/l}$), Cu-preexposed fish displayed decreased uptake rates of waterborne Cu via the gills but not of dietary Cu via the gut during 48-h exposures to ^{64}Cu -radiolabeled water and diet, respectively. At normal dietary and waterborne Cu levels, the uptake rates of dietary Cu into the whole body without the gut were 0.40–0.90 $\text{ng}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$, >10-fold higher than uptake rates of waterborne Cu into the whole body without the gills, which were 0.02–0.07 $\text{ng}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$. Previously Cu-exposed fish showed decreased new Cu accumulation in the gills, liver, and carcass during waterborne ^{64}Cu exposures and in the liver during dietary ^{64}Cu exposures. A 3-h gill Cu-binding assay showed downregulation of the putative high-affinity, low-capacity Cu transporters and upregulation of the low-affinity, high-capacity Cu transporters at the gills in Cu-preexposed fish. Exchangeable Cu pools in all the tissues were higher during dietary than during waterborne ^{64}Cu exposures, and previous Cu exposure reduced waterborne exchangeable Cu pools in gill, liver, and carcass. Overall, these results suggest a quantitatively greater role for the dietary than for the waterborne route of Cu uptake, a key role for the gill in Cu homeostasis, and important roles for the liver and gut in the normal metabolism of Cu in fish.

copper metabolism; copper preexposure; water; diet

COPPER HOMEOSTASIS in animals is tightly regulated, because copper is both essential and toxic to living systems. Mammalian studies have demonstrated that this homeostasis is primarily regulated at the hepatogastrointestinal level (24, 26, 28), but the situation in fish is confounded by the presence of two potential routes of uptake: the gill and the gastrointestinal tract. Although numerous studies have examined the effects of waterborne Cu exposure in fish, most have focused on Cu toxicity, with little emphasis on the possible effects of such exposure on Cu homeostasis (18, 29). Nonetheless, significant progress has been made recently by Grosell et al. (6–11), who investigated key

aspects of Cu metabolism in fish during waterborne exposures, including branchial uptake, plasma clearance, and renal and hepatobiliary excretion. These studies demonstrated that Cu homeostasis in fish entails regulated uptake, transport, and excretion, as is the case for mammals (26). However, to fully understand Cu homeostasis and toxicity in fish, a clear perception of the interactions between dietary and waterborne Cu uptake is necessary. To this end, we recently demonstrated that dietary Cu preexposure downregulates branchial uptake of waterborne Cu (14, 15), whereas deprivation of Cu upregulates branchial uptake (14). However, a key aspect of this interaction that has been ignored is the possible effect of waterborne Cu preexposure on subsequent uptake, distribution, and excretion of dietary Cu.

Acclimation to waterborne Cu is still a matter of controversy. McDonald and Wood (18) defined acclimation as being characterized by increased tolerance to acute doses of metal after chronic exposure to sublethally toxic doses. This acclimation could be attributed to several factors, including changes in branchial cellular morphology and permeability to ions, changes in uptake and accumulation rate of the metal, and increased excretion, storage, and detoxification capacity. With respect to Cu, there are contradictory reports on the effect of acclimation on the subsequent uptake of waterborne Cu. Constant uptake (6, 17), increased uptake (25), and decreased uptake (11) have been reported. Detailed evaluation of Cu uptake after preexposure to waterborne Cu is therefore warranted to elucidate the effects of acclimation on uptake of waterborne Cu. We hypothesize that the reported differences in Cu uptake rates are due to different effects of acclimation on the two putative types of Cu-binding sites/transporters in the gills (9, 25).

The purpose of the present study was therefore, first, to determine the relative quantitative contributions of waterborne and dietary uptake rates for Cu. The second aim of this study was to characterize the effects of waterborne Cu preexposure on Cu homeostasis and unidirectional uptake of waterborne Cu using direct ^{64}Cu flux measurements. Given the dual routes of Cu uptake in fish, the third objective was to evaluate the

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effects of waterborne Cu preexposure on the uptake and distribution of dietary Cu. Our hypothesis was that Cu homeostasis is regulated centrally and that the exposure of fish to Cu via one route would impact Cu uptake via the other route. Finally, 3-h binding of waterborne Cu at different concentrations to the gill determined using ^{64}Cu (cf. Ref. 22) was assessed to illuminate the effects of Cu preexposure on the two putative types of Cu-binding sites/transporters in the gills.

METHODS

Fish. About 900 juvenile rainbow trout (*Oncorhynchus mykiss*, 9–11 g; Humber Springs Trout Farm) were initially maintained for 3 wk in one 500-liter tank supplied with a flow-through of dechlorinated municipal (Hamilton, ON, Canada) tap water (0.6 mM Na^+ , 1.0 mM Ca^{2+} , 1.9 mM HCO_3^- , 0.7 mM Cl^- , pH 7.7, 3 mg/l dissolved organic carbon, and 2 $\mu\text{g/l}$ background Cu at $14 \pm 1^\circ\text{C}$). Fish were fed a 2% daily ration (dry feed/wet body wt) of commercial trout chow (1 point, Martin's Feed Mill, Elmira, ON, Canada) containing 52% (maximum) crude protein, 17% (minimum) crude fat, 2.5% (maximum) crude fiber, 0.4% (actual) Na^+ , 1.4% (actual) Ca^{2+} , 1.0% (actual) phosphorus, and vitamins [10,000 IU/kg (minimum) A, 300 IU/kg (minimum) C, 3,000 IU/kg (minimum) D₃, and 100 IU/kg (minimum) E]. Measured total Cu concentration in the diet was 40 mg/kg.

Time course of acute waterborne and dietary ^{64}Cu uptake. An initial study examined ^{64}Cu uptake from the diet and water over time to determine the appropriate conditions for subsequent exposures. Sixty control fish (30 each for a waterborne and a dietary flux) were exposed to waterborne or dietary ^{64}Cu for 48 h and sampled after 3, 6, 12, 24, 36, and 48 h of exposure. The exposure conditions are outlined below. For both fluxes, gills, plasma, liver, gut, and the rest of the carcass were collected individually and analyzed for ^{64}Cu radioactivity. For the dietary flux, sampling was done beginning at 6 h, because fish retained substantial amounts of food in the esophagus within 3 h of feeding and often regurgitated it during terminal anesthesia, thereby contaminating other tissues, especially the gill.

Exposure to waterborne Cu and depuration. The rest of the fish were divided into four groups each of ~200 fish and placed in 150-liter experimental tanks. Daily rations were increased to 4%. Fish in two of the tanks were exposed for 28 days to a nominal 20 $\mu\text{g/l}$ Cu achieved by delivering a concentrated stock solution of Cu (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, analytic grade) from a Mariotte bottle to a head tank fed with dechlorinated municipal tap water. Experimental tanks were supplied from the head tank at a flow rate of 1.1 l/min. The actual water Cu concentration in the experimental tanks determined by atomic absorption spectrophotometry was 22.2 ± 0.9 (SE) $\mu\text{g/l}$ ($n = 29$). At days 0, 7, 14, and 28, 10 fish were removed for the determination of total Cu and measurement of Cu uptake from the water or diet using ^{64}Cu (see below).

Waterborne Cu exposure was terminated after 28 days, and daily feeding was reduced to 2% for the following 30 days. At days 10, 20, and 30 after exposure, 10 control and 10 Cu-acclimated fish were randomly netted out from the tanks and terminally anesthetized with tricaine methanesulfonate (MS-222; 1 g/l), and the gill, liver, and the rest of the carcass were dissected out for total Cu analysis (see below).

Waterborne and dietary ^{64}Cu exposures. Unidirectional Cu uptake from the water via the gills and from the diet via the

gut was measured using 48-h ^{64}Cu exposures at days 0, 7, 14, and 28 of waterborne Cu exposure. Waterborne flux was measured in 30-liter flux chambers under static conditions of dechlorinated aerated municipal tap water. Fish were not fed during the flux to avoid fouling of the water. Water quality parameters including O_2 content, pH, and temperature monitored during the experiment remained close to levels measured in the flow-through tank water in which the fish were maintained. For day 0, the acute waterborne Cu exposure was performed using 10 fish at background water Cu concentration with 1 $\mu\text{g Cu/l}$ added as ^{64}Cu (CuNO_3 , specific activity 3.3 $\mu\text{Ci}/\mu\text{g}$; produced in the McMaster University Nuclear Reactor). Addition of ^{64}Cu brought the measured final water Cu concentration to 2.8 $\mu\text{g/l}$. For days 7, 14, and 28, flux measurements were performed at background water Cu concentration (2–3 $\mu\text{g/l}$) and 20–22 $\mu\text{g/l}$ for the control ($n = 10$ at each time and concentration) and Cu-exposed ($n = 10$) fish to allow direct comparison of the Cu uptake rates. For each concentration, the water was spiked with 1 $\mu\text{g/l}$ ^{64}Cu (3.3 $\mu\text{Ci}/\mu\text{g}$ as CuNO_3). Water samples were taken 15 min after the start of the flux and thereafter every 12 h for 48 h for analysis of ^{64}Cu gamma radioactivity and total Cu.

Similarly, dietary ^{64}Cu flux was measured at waterborne concentrations of 2 and 22 $\mu\text{g/l}$ Cu for control ($n = 10$ at each time and concentration) and waterborne Cu-preexposed ($n = 10$) fish, except for day 0, when the flux measurement was performed only at background waterborne Cu concentration. For each dietary flux measurement day, a fresh ^{64}Cu -labeled diet was made by the method described by Kamunde et al. (15), except that radioactive Cu was used. For the initial acute time-course study described above, 5 mg of ^{64}Cu -labeled CuNO_3 (17 mCi) were introduced into 50 g of commercial trout chow, thus elevating the diet Cu concentration to 165 mg/kg. However, in subsequent experiments, we elected to use a lower level of ^{64}Cu in the diet (5 mg of $^{64}\text{Cu} = 17$ mCi added to 250 g of commercial trout chow), thereby raising measured diet Cu concentration to only 65 mg/kg, closer to the acclimation diet Cu level (40 mg/kg). For all preparations, addition of the ^{64}Cu was followed by 45 min of thorough mixing of the food in a commercial pasta maker. Thereafter, the food was extruded and air-dried in an oven at 70°C for ~1 h. Subsequently, fish were fed a 4% daily ration of the radioactive food and allowed to feed for 1 h. Thereafter, they were transferred to the 30-liter flux tanks for 48 h. Food remaining in the feeding tank was collected, dried, and weighed to estimate the exact amount consumed (typically 60–75% of the ration). Water in the flux tanks was continuously aerated and changed every 12 h to avoid build up of fecal waste.

Sampling. Fish were killed with an overdose (1 g/l) of neutralized tricaine methanesulfonate and rinsed with double-distilled water after the 48-h waterborne and dietary ^{64}Cu exposures. Blood was immediately collected by caudal puncture and centrifuged at 13,000 g to collect plasma. The fish were then dissected, and bile was collected immediately by aspiration with 1-ml syringes. Subsequently, liver, gill, gut (separated into stomach, pyloric caecae + anterior intestine, midintestine, and posterior intestine), and the rest of the carcass were dissected out, rinsed with double-distilled water, and weighed.

Analyses and calculations. The ^{64}Cu gamma radioactivity was determined in water, diet, and all tissues collected using a Canberra-Packard MINAXI Auto-gamma counter with an on-board program for decay correction. Absolute whole body uptake of waterborne and dietary ^{64}Cu was calculated by summing ^{64}Cu activities [counts/min (cpm)] in all tissues plus carcass. Fish weights were determined by summing the



weights of liver, gills, plasma, gut tissue, bile, and carcass for each fish. Whole body new Cu uptake from the water and diet was then calculated as follows

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

where a is the ^{64}Cu in cpm/g fish, b is ^{64}Cu in cpm/l or cpm/g in the water or diet, respectively, and c is the total Cu concentration in the water or diet ($\mu\text{g/l}$ or $\mu\text{g/g}$). Equation 1 has been widely used to calculate unidirectional waterborne (6, 8, 10, 11, 14, 15) and dietary (4) Cu uptake in fish. For the purpose of comparison, the waterborne and diet ^{64}Cu specific activities were very similar. The time-course study showed that the uptake organs themselves (gills for waterborne ^{64}Cu exposure and gut for dietary ^{64}Cu exposure) tended to saturate over the 48-h exposure periods, whereas internal accumulation in the remainder of the body proceeded linearly with time. Therefore, uptake rates were also calculated for the whole body without the gills and for the whole body without the gut for the waterborne and dietary exposures, respectively, to yield the rates of internal accumulation via the two routes.

Newly accumulated Cu into individual tissues or organs, by reference to the specific activity of Cu in the exposure system (water or diet), was calculated using Eq. 1, where a is ^{64}Cu in cpm per gram of tissue or organ.

For total Cu determination, all tissues were digested overnight at 70°C with 6 vol of 1 N nitric acid (Fisher Scientific, trace metal grade), and 1.5-ml aliquots were centrifuged for 4 min at 13,000 g. A subsample of the supernatant was diluted appropriately with 0.5% nitric acid, and total Cu concentration was determined by atomic absorption spectroscopy (Varian AA-1275 with GTA furnace atomizer) using a 10- μl injection volume and operating conditions for Cu specified by the manufacturer. Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range.

For the purpose of comparison (see DISCUSSION), the exchangeable pools of Cu after the 48-h waterborne and dietary ^{64}Cu exposures were calculated at day 28 from the total Cu and newly accumulated Cu for each tissue using two different methods. The first method was by reference to the specific activity of Cu in the exposure system (diet or water) as in Eq. 1. This approach assumed that ^{64}Cu in the exposure medium was equally available for uptake into all the tissues. The second method was by reference to the specific activity of the previous compartment (11, 15), employing the following equation to calculate newly accumulated Cu

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

where a is cpm/g tissue, d is ^{64}Cu (in cpm/l or $\mu\text{g/g}$) in the previous compartment, and e is the total Cu concentration in the previous compartment (in $\mu\text{g/l}$ or $\mu\text{g/g}$). Here it was assumed that ^{64}Cu and total Cu in the previous compartment were in complete equilibrium and that the specific activity of the previous compartment was homogeneous throughout the compartment. For the waterborne exposures, the previous compartments were water for gill, gill for plasma, and plasma for all other tissues. For the dietary exposures, the previous compartments were diet for gut tissue, gut tissue for plasma, and plasma for all other tissues.

The percent exchangeable Cu was then calculated as follows

$$100 (\text{Cu}_{\text{new}} \cdot \text{Cu}_{\text{tot}}^{-1}) \quad (3)$$

where Cu_{new} is newly accumulated Cu and Cu_{tot} is total Cu in the previous compartment, both in $\mu\text{g/g}$ (or $\mu\text{g/ml}$ for plasma).

Gill Cu binding. Waterborne ^{64}Cu flux measurements during the Cu exposure experiment revealed decreased Cu uptake in Cu-exposed fish. Consequently, an additional 28-day exposure of juvenile rainbow trout to $22 \mu\text{g/l}$ Cu ($n = 60$) or background conditions ($2 \mu\text{g/l}$, $n = 60$) was carried out. At the end of the 28-day period, a 3-h gill Cu-binding assay was performed for the control and Cu-exposed fish. To characterize the possible effects of Cu exposure on the high-affinity, low-capacity and low-affinity, high-capacity Cu-binding sites (9, 25), a wide range of waterborne Cu concentrations (2.6 – $84.9 \mu\text{g/l}$) was used. For each concentration, the fish were exposed to the required concentration labeled with ^{64}Cu [total dose as ^{64}Cu (CuNO_3)] for 3 h. The fish were then terminally anesthetized in tricaine methanesulfonate (1 g/l), and the gills were excised, rinsed in double-distilled water, and counted for ^{64}Cu gamma radioactivity as described above. Cu bound to the gills was calculated using an equation analogous to that for whole body Cu uptake (Eq. 1).

Statistics. Values are means \pm SE. Linear and nonlinear regression lines were fitted using SigmaPlot 2000 (SPSS, Chicago, IL). Effects of exposure conditions on tissue Cu concentration and subsequent waterborne and dietary Cu uptake at each sampling point were assessed using two-way analysis of variance with time and waterborne Cu concentration, acclimation, or route of exposure as variables. Tukey's multiple comparison procedure for significant difference or paired Student's t -test (as appropriate) was used for comparisons between measurements. In all cases, differences were considered significant at $P < 0.05$.

RESULTS

Time course of acute waterborne and dietary Cu uptake. Unidirectional Cu uptake over time into the whole body with and without the gill for the waterborne exposure is shown in Fig. 1A. New Cu accumulation into the whole body tended to plateau, whereas "internal" uptake into the whole body without the gills was linear over the 48 h. This observation is consistent with the linear Cu uptake into all the organs over time except the gills, in which there was an initial rapid uptake to $\sim 40 \text{ ng/g}$ newly accumulated Cu followed by a decline and stabilization at $\sim 25 \text{ ng/g}$ (Fig. 2A). After 48 h, newly accumulated Cu concentrations in the tissues were ranked in order as follows: liver > gill > gut > plasma > carcass.

Similarly, for the dietary time course experiment, Cu uptake into the whole body tended to saturate over time, whereas internal uptake remained linear for the whole body without gut over the 48 h (Fig. 1B). Again, the saturable component was attributed to the uptake site, i.e., gut tissues (stomach, pyloric caecae + anterior intestine, midintestine, and posterior intestine; Fig. 2C), while uptake into the other organs was linear with time (Fig. 2B). At 48 h, the highest amount of Cu was found in the posterior intestine followed, in decreasing order, by the pyloric caecae + anterior intestine, midintestine, and stomach. In the other tissues, new Cu accumulation was ranked in the following order: liver > plasma > gill > carcass. Generally, new Cu accumulation into the whole body and internal tissues was much higher during dietary ^{64}Cu exposure than during waterborne ^{64}Cu exposure.

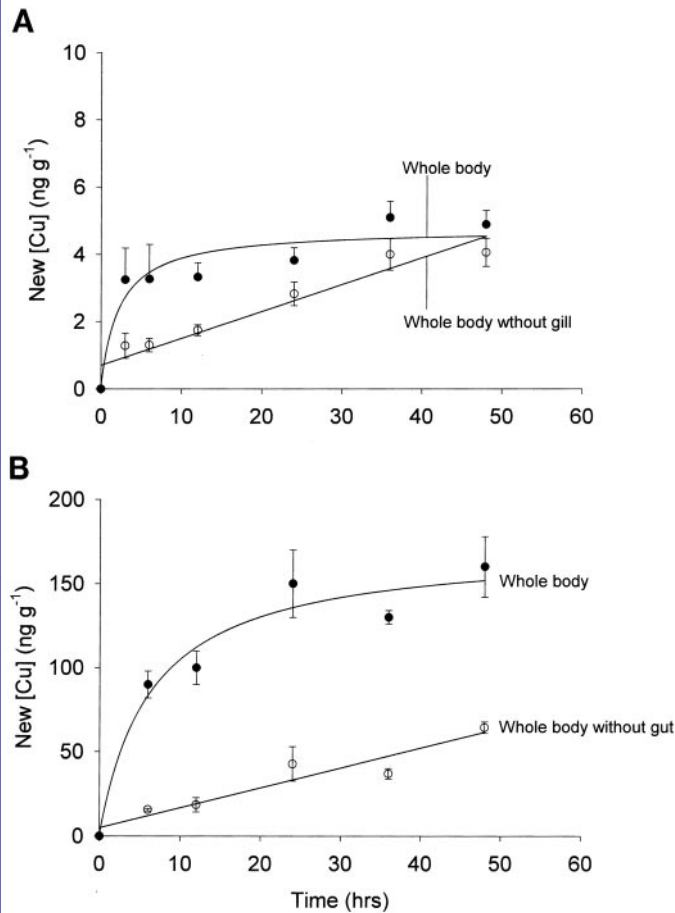


Fig. 1. Time course of unidirectional waterborne (A) and dietary (B) Cu uptake traced with ^{64}Cu in juvenile rainbow trout during acute exposure. Values are means \pm SE ($n = 5$). ●, Whole body newly accumulated Cu for each route of uptake; ○, Cu accumulation in the whole body without gills for waterborne exposure and whole body without the gut for dietary exposure. Uptake into the whole body approached a plateau for both routes of uptake, while uptake into the whole body without the gills or gut remained linear over time. For all regression lines, $r^2 = 0.91\text{--}0.95$. [Cu], Cu concentration.

On the basis of these results, 48-h exposures were employed in subsequent waterborne and dietary ^{64}Cu uptake determinations, and our measurements focused on the linear internal accumulation rates (i.e., whole body minus uptake organ). However, whole body uptake rates were also calculated, yielding the same basic trends.

Growth, Cu accumulation, and Cu depuration. Exposure to $22\ \mu\text{g/l}$ Cu had no effect on growth. All the fish exhibited a twofold increase in weight from 9–11 to 23–24 g over 28 days, and there was negligible mortality (2 deaths in 900 fish). Whole body Cu concentration increased in the Cu-exposed fish from ~ 1.5 to $2.0\ \mu\text{g/g}$. The increase in whole body Cu concentration was due to a small Cu accumulation in the gill and the carcass and a large accumulation in the liver. There were no treatment-related changes in the Cu concentration in the plasma, kidney, gut, and bile. In particular, total plasma Cu remained within narrow margins between 0.65 and $0.86\ \mu\text{g/ml}$ for the control and Cu-exposed fish.

During the depuration phase of the study (data not shown), loss of whole body Cu after 28 days of waterborne Cu exposure was slow and could be attributed to the slow loss from the liver. Liver and whole body Cu concentration returned to control levels only at *day 30* of depuration. Gills exhibited more rapid clearance of the Cu burden and returned to the control levels within 20 days after exposure.

Whole body waterborne and dietary Cu uptake rates. At $2.8\ \mu\text{g/l}$ waterborne Cu, whole body (without gills) unidirectional uptake rates of Cu from the water were $0.02\text{--}0.07\ \text{ng}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ and were significantly lower in the previously Cu-exposed fish at *day 28* (Fig. 3A). The mass-specific uptake rates tended to decrease with time as fish increased in size. At $22\ \mu\text{g/l}$ waterborne

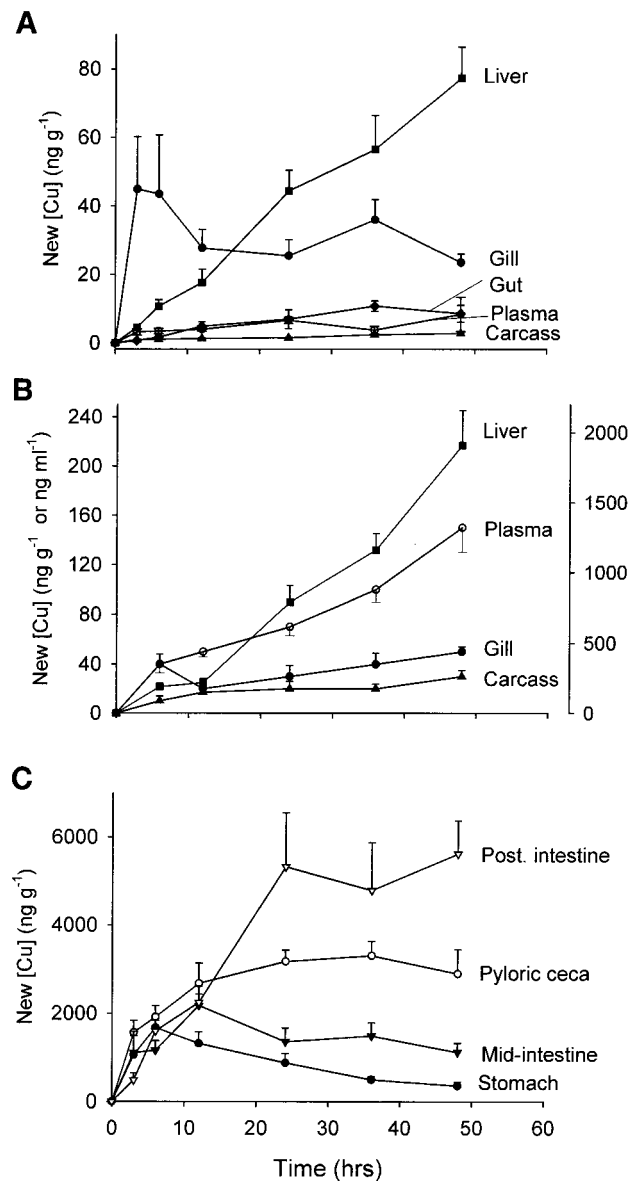


Fig. 2. Distribution of newly accumulated Cu in tissues of juvenile rainbow trout during acute exposure to waterborne (A) and dietary (B and C) ^{64}Cu for 48 h. Values are means \pm SE ($n = 5$). Note difference in scale for liver plot in B.

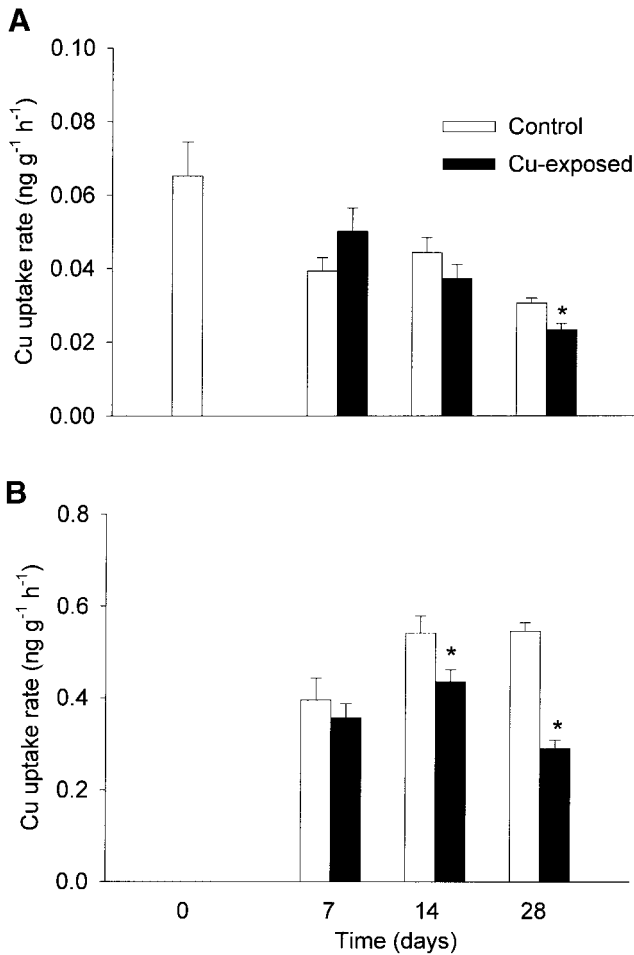


Fig. 3. Whole body (without gills) unidirectional Cu uptake rates traced with ^{64}Cu from water in control and chronic Cu-exposed rainbow trout. Values are means \pm SE ($n = 10$). A: uptake rates at $2.8 \mu\text{g/l}$ waterborne Cu; B: uptake rates at $22 \mu\text{g/l}$ waterborne Cu. *Significantly different from respective control, $P < 0.05$.

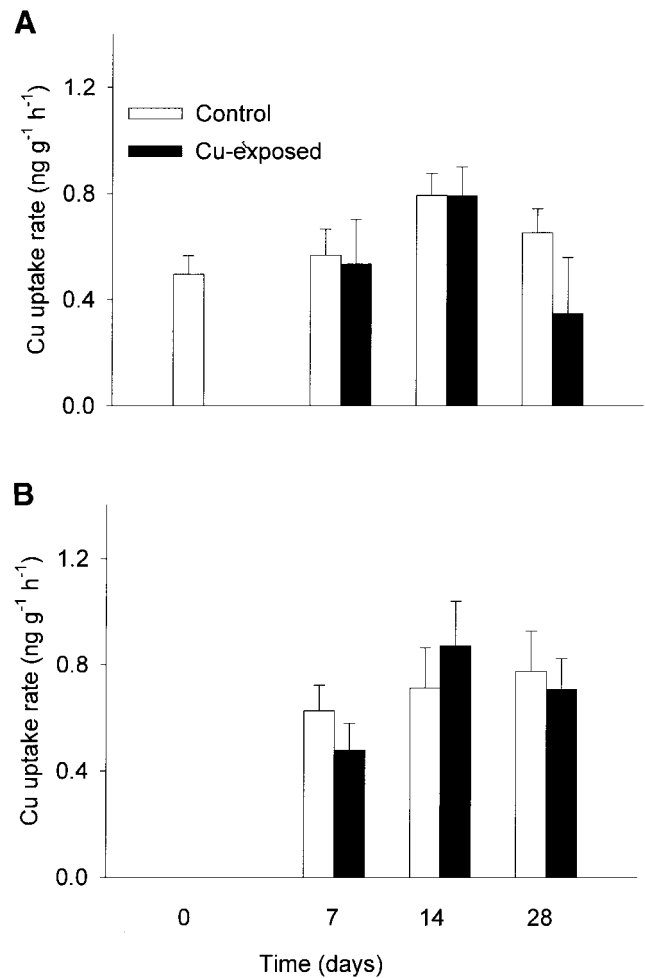


Fig. 4. Whole body (without gut) unidirectional Cu uptake rates traced with ^{64}Cu from diet in control and chronic Cu-exposed rainbow trout. Values are means \pm SE ($n = 10$). A: dietary Cu uptake rate at $2.8 \mu\text{g/l}$ waterborne Cu. B: uptake rates at $22 \mu\text{g/l}$ waterborne Cu.

Cu, whole body Cu uptake rates were ~ 10 -fold higher than the values at background waterborne Cu level ($2.8 \mu\text{g/l}$) and were $0.29\text{--}0.55 \text{ ng}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ (Fig. 3B). Previously Cu-exposed fish had significantly lower rates of uptake than the controls on days 14 and 28. Absolute whole body waterborne unidirectional Cu uptake rates shown in Table 1 followed similar trends but

were quantitatively higher, a reflection of the branchial contribution.

Whole body (without gut) unidirectional Cu uptake rates during the 48-h dietary ^{64}Cu exposure ranged from 0.40 to $0.90 \text{ ng}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ for control and Cu-acclimated fish (Fig. 4). Thus whole body (without gut) dietary Cu uptake rates were >10 -fold higher than

Table 1. Whole body Cu uptake rates in control and Cu-exposed juvenile rainbow trout after 48-h exposure to waterborne or dietary ^{64}Cu

	Waterborne				Dietary			
	Background ($2.8 \mu\text{g/l}$)		High ($22 \mu\text{g/l}$)		Background ($2.8 \mu\text{g/l}$)		High ($22 \mu\text{g/l}$)	
	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed
Day 0	0.08 ± 0.009				$0.80 \pm 0.10^*$			
Day 7	0.04 ± 0.004	0.05 ± 0.004	$0.39 \pm 0.03^*$	$0.38 \pm 0.04^*$	$0.86 \pm 0.12^*$	$0.70 \pm 0.20^*$	$0.87 \pm 0.12^*$	$0.65 \pm 0.12^*$
Day 14	0.06 ± 0.004	0.05 ± 0.008	$0.58 \pm 0.04^*$	$0.47 \pm 0.03^*\dagger$	$1.25 \pm 0.13^*$	$1.31 \pm 0.16^*$	$0.94 \pm 0.20^*$	$1.11 \pm 0.20^*$
Day 28	0.05 ± 0.007	$0.03 \pm 0.001\dagger$	$0.59 \pm 0.02^*$	$0.32 \pm 0.02^*\dagger$	$1.62 \pm 0.48^*$	$0.78 \pm 0.47^*$	$1.84 \pm 0.26^*$	$1.50 \pm 0.23^*$

Values are means \pm SE in $\text{ng}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ($n = 10$). *Significantly different from control waterborne fish at background Cu level at the same time, \dagger significantly different from control fish for the same exposure route and water Cu level.

those of the whole body without the gills under background conditions (2–3 $\mu\text{g Cu/l}$) but comparable to waterborne uptake rates in fish exposed to an elevated waterborne level (22 $\mu\text{g/l}$). Unlike waterborne Cu uptake, neither the ambient water Cu concentration nor the fish size affected the rate of Cu uptake from the diet. Absolute whole body unidirectional Cu uptake rates (Table 1) followed the same trend but were up to twofold higher, reflecting a major contribution of gut tissue to whole body ^{64}Cu accumulation during dietary exposures.

Newly accumulated Cu in tissues. Newly accumulated waterborne Cu in tissues of control and previously Cu-exposed fish are shown in Fig. 5. Newly accumulated Cu (acquired over 48 h of exposure to waterborne ^{64}Cu) in the gills was 6–16 ng/g in the fish exposed to background Cu level (2.8 $\mu\text{g/l}$) and was

significantly lower in previously Cu-exposed fish on *days 14* and *28* (Fig. 5, 1A). At 22 $\mu\text{g/l}$ waterborne Cu (Fig. 5, 1B), newly accumulated gill Cu was 36–90 ng/g and was therefore about six times higher than in fish exposed to background (2.8 $\mu\text{g/l}$) waterborne Cu. Again, previously Cu-exposed fish had significantly lower values on *day 28*. In the liver, newly accumulated waterborne Cu was between 19 and 65 ng/g in the 2.8 $\mu\text{g/l}$ exposure (Fig. 5, 2A), whereas the value increased to 240–860 ng/g at the high waterborne Cu (22 $\mu\text{g/l}$; Fig. 5, 2B). Previously Cu-exposed fish had significantly lower new hepatic Cu accumulation than the controls on *day 28* for the background Cu level and on *days 14* and *28* for the elevated Cu level. For plasma (Fig. 5, 3A and 3B) and gut (Fig. 5, 4A and 4B), Cu preexposure had no effect on the newly accumulated waterborne Cu. Plasma newly accumulated Cu was,

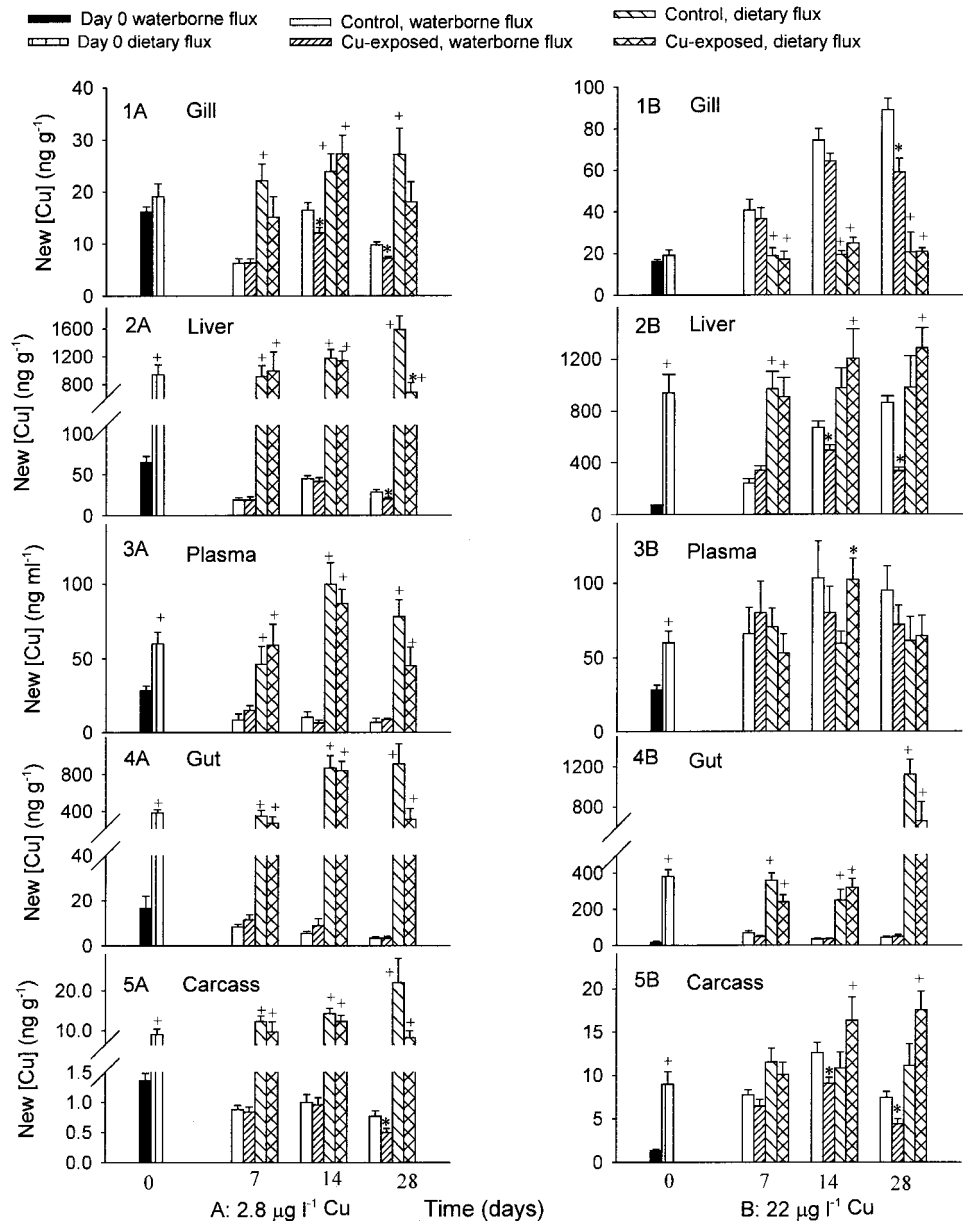


Fig. 5. Newly accumulated Cu calculated by reference to specific activity of Cu in exposure system (water or diet) in tissues of control and chronic Cu-exposed fish 48 h after waterborne and dietary ^{64}Cu exposures. Values are means \pm SE ($n = 10$). 1A–5A: exposures performed at 2.8 $\mu\text{g/l}$ waterborne Cu; 1B–5B: exposures performed at 22 $\mu\text{g/l}$ waterborne Cu. +Significantly different from newly accumulated waterborne Cu at background Cu level; *significantly different from control fish within an exposure condition at a particular sampling time, $P < 0.05$.

however, strongly influenced by the water Cu concentration during the exposure, rising from 6–28 ng/g during background exposures (2.8 $\mu\text{g/l}$) to 66–103 ng/g during high waterborne Cu exposures (22 $\mu\text{g/l}$). In the carcass, newly accumulated Cu was 0.5–1.4 ng/g under background conditions (2.8 $\mu\text{g/l}$ Cu; Fig. 5, 5A) and was significantly elevated to 4–13 ng/g at high ambient water Cu concentration (22 $\mu\text{g/l}$; Fig. 5, 5B). Newly accumulated Cu was lower in the carcass of Cu-preexposed fish on *day 28* under background Cu levels (2.8 $\mu\text{g/l}$) and on *days 14* and *28* under elevated Cu levels (22 $\mu\text{g/l}$).

For the 48-h dietary ^{64}Cu exposures, new Cu accumulation in the gills was 15–27 ng/g and was higher on all days than that accumulated during waterborne ^{64}Cu exposures at background water Cu level (Fig. 5, 1A). However, at 22 $\mu\text{g/l}$ water Cu level (Fig. 5, 1B), the newly accumulated dietary gill Cu was lower than during the comparable waterborne ^{64}Cu exposures on all days. Newly accumulated Cu in the liver during dietary exposures was 680–1,590 ng/g and, therefore, much higher than waterborne exposures (Fig. 5, 2A and 2B). Previously Cu-exposed fish had significantly lower new Cu accumulation in the liver on *day 28* in the exposures done at 2.8 $\mu\text{g/l}$ waterborne Cu. Plasma accumulated 45–100 ng/ml new Cu, and Cu preexposure and ambient water Cu concentration during the dietary exposures had no effect, except on *day 14*, when acclimated fish had higher levels (Fig. 5, 3A and 3B). Newly accumulated Cu in plasma was higher for all the dietary exposures than for the waterborne exposures carried out at 2.8 $\mu\text{g/l}$ waterborne Cu. In the gut, newly accumulated Cu was 400–1,200 ng/g, up to 200-fold higher than during waterborne exposures (Fig. 5, 4A and 4B). Waterborne Cu preexposure had no effect on new dietary Cu accumulation in the gut. New dietary Cu accumulation in the carcass was 8–22 ng/g and was not affected by preexposure to waterborne Cu (Fig. 5, 5A and 5B). However, these values were generally higher than those of the waterborne ^{64}Cu exposures.

Gill Cu-binding characteristics. The short-term (3-h) gill Cu-binding assay revealed a biphasic effect of previous waterborne Cu exposure on gill Cu-binding kinetics (Fig. 6). At waterborne Cu <10 $\mu\text{g/l}$, preexposed fish exhibited lower Cu binding than the controls (Fig. 6, *inset*). However, at higher waterborne Cu, the reverse occurred: control fish exhibited much lower gill Cu binding than the acclimated fish.

DISCUSSION

Dietary vs. waterborne Cu uptake. Under the conditions of background levels of Cu in the water and diet, unidirectional Cu uptake rates into internal tissues, as well as into the whole body, were >10-fold higher during dietary than during waterborne ^{64}Cu exposure, suggesting that diet is a much more important source of Cu to fish than water. This finding is based on direct measurements but considers only unidirectional uptake at the two sites, not net fluxes. Nevertheless, the finding is consistent with our recent conclusion, based

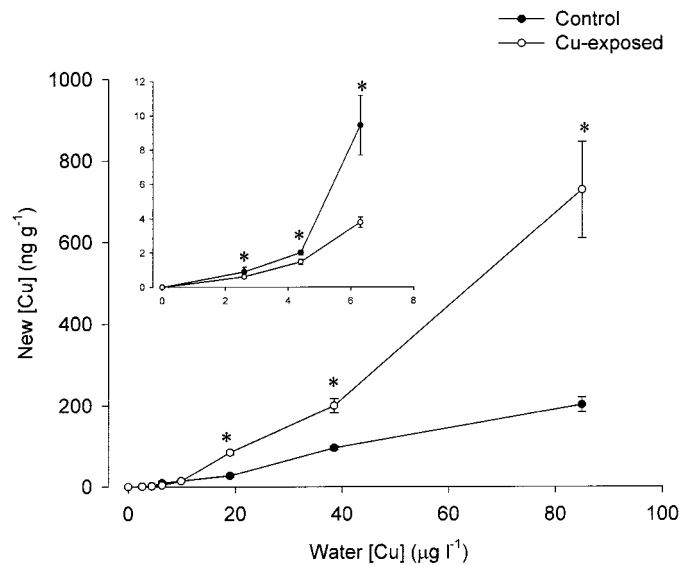


Fig. 6. Gill Cu binding in control and acclimated (chronic Cu exposed) rainbow trout at *day 28*. Values are means \pm SE ($n = 8$). *Inset*: gill Cu binding at water Cu <10 $\mu\text{g/l}$. *Significantly different from Cu-exposed fish, $P < 0.05$.

on indirect calculations, that dietary uptake contributes >90% of body Cu content under normal waterborne and dietary Cu conditions (14). Interestingly, both uptake sites, i.e., gills for waterborne exposure and gut for dietary exposure, saturated over time, but the rest of the organs/tissues exhibited linear Cu accumulation via either route of exposure (Fig. 1). This suggests the presence of homeostatic mechanisms at both tissues that operate to bring rates of uptake and export (to the internal tissues or to the medium) into equilibrium. The present study also demonstrates that Cu absorption from water and diet and transport across the gill and gut epithelial cells are rapid processes, with Cu appearing in plasma within 3 h of exposure to ^{64}Cu via either route. For dietary uptake, this probably means that partial Cu absorption occurred in the fish stomach, as is the case in mammals (28). The appearance of ^{64}Cu radioactivity in all segments of the gut within 3–6 h of feeding (when all the ingested food was still in the stomach) suggests that Cu movement and absorption along the gut are independent of diet movement and absorption.

The high accumulation of new Cu in the posterior intestine is consistent with recent findings by Clearwater et al. (4) and suggests that this region of the fish intestine is the most important for Cu absorption. Intestinal uptake of Cu in fish is thought to occur via simple diffusion for apical entry and biologically mediated transport for basolateral exit (4, 12). Recently, Bury et al. (2) demonstrated that Fe^{2+} was also preferentially transported in the posterior intestine. The apparent saturation of the gut tissues observed in the present study (Fig. 2C) is consistent with earlier reports of a strong Cu regulatory capacity of gut tissue (1, 4, 15).

Effect of previous Cu exposure on waterborne and dietary Cu uptake. Studies that have assessed the effect of waterborne Cu exposure on subsequent uptake of waterborne Cu focused primarily on uptake into the gills and reported variable results (6, 10, 11, 17, 25). In the present study, we also evaluated the unidirectional uptake of Cu into the whole body and demonstrated a substantial reduction in the uptake rates of waterborne Cu after 2 wk of continuous exposure to environmentally realistic waterborne Cu concentrations (e.g., acclimation). A comparison of waterborne and dietary Cu uptake rates into the whole body excluding the gills or gut (Figs. 3 and 4) revealed a 10-fold higher rate for dietary Cu uptake. Including the gill for the waterborne exposures increased whole body waterborne Cu uptake rate by only ~10%, while including the gut for dietary exposures doubled the dietary Cu uptake rate (Table 2). Thus, in absolute terms, whole body dietary Cu uptake rates were up to 30 times higher than waterborne Cu uptake rates.

Contrary to our original hypothesis, previous exposure to waterborne Cu had no effect on the uptake rates of dietary Cu, even though preexposure to high dietary Cu decreases waterborne Cu uptake and preexposure to low dietary Cu increases waterborne Cu uptake in this species (14, 15). In mammals, which have only one route of uptake, the rates and efficiency of dietary Cu absorption change in response to whole body Cu status and level of dietary Cu exposure (26). This effect has been explained on the basis that Cu regulates the proteins involved in Cu homeostasis, e.g., Cu-ATPases (13, 24). In fish, where there are two significant routes of Cu uptake, it is possible that the gut serves for bulk acquisition, while the gill performs homeostatic fine tuning via adjustment of branchial Cu transport proteins.

Newly accumulated Cu in tissues. Previous waterborne Cu exposure decreased the accumulation of new

Cu in the gills (Fig. 6), contrary to several previous reports (10, 17, 25). Furthermore, the differential gill Cu-binding response dependent on the ambient water Cu concentration (see *Gill Cu binding*) points to the existence of at least two Cu uptake/transport mechanisms at the gills.

Waterborne Cu preexposure decreased new Cu accumulation from the water into the liver, suggesting downregulation of branchial export of Cu into the liver or occupation of potential Cu-binding/deposition sites in the liver by Cu accumulated during the acclimation. Interestingly, new Cu accumulation into the liver during 48-h dietary ⁶⁴Cu exposure was also decreased by previous exposure to waterborne Cu. However, even after differences in the rates of uptake by the two routes were taken into account, newly accumulated liver Cu after dietary ⁶⁴Cu exposures was much higher than during waterborne exposures, indicating that dietary Cu was more readily available for hepatic uptake. Anatomic considerations may play an important role here, since the liver is immediately downstream of the gastrointestinal tract and receives materials absorbed from the gut via the venous hepatic portal system. In contrast, Cu absorbed from the water via the gills is likely transported via arterial circulation and deposited in other peripheral tissues before reaching the liver. The significant decrease in newly accumulated Cu in the carcass during 48-h waterborne ⁶⁴Cu exposures in Cu-preexposed fish underlines the fact that Cu preexposure and the attendant elevation in tissue Cu concentration result in widespread decline in the demand for new Cu uptake in rainbow trout. Together, these data suggest that cellular Cu transport mechanisms respond to body Cu status during chronic waterborne Cu exposure in the same manner as during dietary exposures in fish (14, 15) and humans (26).

Cu turnover and exchangeable Cu pools. The exchangeable Cu pools were estimated for the control and

Table 2. *Exchangeable Cu pools in tissues of control and Cu-exposed rainbow trout on day 28 after 48-h exposure to waterborne and dietary ⁶⁴Cu*

	Waterborne ⁶⁴ Cu Exposure				Dietary ⁶⁴ Cu Exposure			
	Background (2.8 µg/l)		High (22 µg/l)		Background (2.8 µg/l)		High (22 µg/l)	
	Control	Cu exposed	Control	Cu exposed	Control	Cu exposed	Control	Cu exposed
<i>System (water or diet) specific activities</i>								
Gill	1.99 ± 0.14	1.60 ± 0.20	15.99 ± 1.35*	11.58 ± 1.90*†	4.48 ± 1.00*	1.97 ± 0.04	2.64 ± 0.60	3.66 ± 0.59*
Liver	0.034 ± 0.003	0.020 ± 0.002†	1.01 ± 0.05*	0.34 ± 0.04*†	2.27 ± 0.53*	0.56 ± 0.21*†	0.90 ± 0.27*	1.05 ± 0.21*
Plasma	0.78 ± 0.10	1.03 ± 0.11	10.72 ± 1.86*	8.77 ± 1.18*	8.40 ± 1.55*	4.54 ± 1.37*	5.46 ± 1.72*	6.34 ± 1.18*
Gut	0.18 ± 0.03	0.22 ± 0.05	2.55 ± 0.42*	2.11 ± 0.67*	42.92 ± 9.96*	21.61 ± 6.93*	36.50 ± 7.62*	42.46 ± 6.30*
Carcass	0.19 ± 0.02	0.12 ± 0.02†	1.70 ± 0.19*	1.00 ± 0.13*†	3.41 ± 0.93*	1.77 ± 0.52*	2.21 ± 0.63*	3.63 ± 0.63*
<i>Previous compartment analysis</i>								
Gill	1.99 ± 0.14	1.60 ± 0.20	15.99 ± 1.35*	11.58 ± 1.90*	82.93 ± 7.81*	91.02 ± 12.03*	93.25 ± 14.24*	62.50 ± 6.12*
Liver	6.44 ± 2.47	3.06 ± 0.37	17.43 ± 5.48*	10.39 ± 2.98	29.57 ± 4.11*	19.94 ± 4.18*	38.45 ± 5.76*	30.08 ± 5.33*
Plasma	56.98 ± 9.52	53.28 ± 6.68	50.07 ± 5.22	78.05 ± 14.36	13.12 ± 1.50*	15.18 ± 2.95*	9.62 ± 2.11*	12.33 ± 2.31*
Gut	34.90 ± 8.11	24.43 ± 6.56	33.29 ± 9.06	16.89 ± 4.11	42.92 ± 9.96	21.61 ± 6.93	36.50 ± 7.62	42.46 ± 6.30
Carcass	27.65 ± 3.64	16.75 ± 3.02†	22.98 ± 4.69	26.47 ± 6.32	69.40 ± 9.99*	52.52 ± 8.19*	86.23 ± 40.54*	61.10 ± 11.09*

Values are means ± SE expressed as percentage of total Cu content exchangeable in 48 h (*n* = 10). Percentages were converted to arcsine for statistical analysis. *Significantly different from control waterborne exposure pools at background Cu level; †significantly different from control fish at the same water, Cu concentration, and exposure route.



Cu-preexposed fish on the basis of new Cu uptake into the tissues directly from the water or diet or from the previous compartment at 2.8 and 22 $\mu\text{g/l}$ water Cu (Table 2). These calculations report the short-term exchangeable pool size, i.e., that percentage of the total tissue Cu content that is exchangeable in a 48-h period, using two different assumptions as outlined in METHODS. Use of the system specific activity (i.e., a calculation that gives the fraction that is exchangeable with Cu in the exposure medium) revealed that ambient water Cu concentration was directly correlated with the exchangeable Cu pools during waterborne exposures: a 10-fold increase in the ambient Cu concentration caused a similar increase in the exchangeable Cu pools. Waterborne Cu preexposure significantly reduced the exchangeable waterborne Cu pools in gills, liver, and carcass, consistent with increased total Cu concentration and reduced newly accumulated Cu in these tissues. Previous studies have reported increased synthesis of Cu-binding proteins, e.g., metallothionein, acid-soluble thiols, and glutathione, in fish tissues during chronic exposure to waterborne Cu (5, 16, 17, 20). It is likely that binding of Cu to such proteins in the Cu-preexposed fish affected the movement of Cu among the tissues. Indeed, the slow loss of whole body and hepatic Cu observed during depuration suggests that the Cu in fish tissues existed in slowly exchangeable form after chronic waterborne Cu exposure. The exchangeable Cu pools were higher for the dietary than for the waterborne exposures in all tissues except the gills. In particular, the gut exchangeable Cu pool from the diet was up to 200 times higher than the gut exchangeable Cu pool from the water, underlining the importance of this organ in dietary, but not waterborne, Cu uptake. Thus Cu turnover in fish is much higher during dietary exposure.

Clearwater et al. (4) and Laurén and McDonald (16) calculated exchangeable Cu pools in trout tissues using a similar method for dietary and waterborne Cu exposure, respectively. Our results are generally in agreement with those of Clearwater et al. for dietary exposure but are lower than those reported by Laurén and McDonald for waterborne exposure. The discrepancy with the latter study can be explained on the basis of the ambient Cu levels used in the present study (2.8 and 22 $\mu\text{g/l}$) vs. 55 $\mu\text{g/l}$ in the study of Laurén and McDonald.

Using previous compartment analysis (i.e., a calculation that gives that fraction that is exchangeable with the previous compartment, as defined in METHODS) resulted in much higher exchangeable Cu pools in all the organs for both exposure routes, except, of course, the gills for the waterborne exposures and gut tissue for the dietary exposures (Table 2). Except for the gills and the liver during waterborne exposures, ambient Cu concentration had no effect on the exchangeable pools calculated in this manner. Another noteworthy observation is the much lower plasma exchangeable Cu pool during the dietary exposures, again perhaps reflecting the fact that much of the newly absorbed Cu was directly shunted to the liver by the venous hepatic

portal system, rather than mixing freely with the entire plasma volume. Overall, the present data demonstrate a greater accessibility of plasma (as well as gill and gut) Cu for turnover with tissue pools than dietary or waterborne Cu.

Gill Cu binding. The gill Cu-binding assay was developed to evaluate adsorption of Cu on or in the gill surface at equilibrium (22). Our data show that 3-h values actually represent a peak (Fig. 2A), and a modest decline may ensue thereafter, which is in agreement with the findings of Grosell et al. (10, 11) and Grosell and Wood (9). Furthermore, significant Cu internalization also occurs within 3 h (Fig. 1A). Waterborne Cu preexposure had a stimulatory and an inhibitory effect on the 3-h gill Cu-binding dependent on the ambient Cu (Fig. 6). At waterborne Cu $<10 \mu\text{g/l}$, Cu binding to the gill was decreased, while at waterborne Cu $>10 \mu\text{g/l}$, Cu binding to the gill was increased in acclimated fish. Several different types of Cu-binding sites have now been identified in trout gills. Taylor et al. (25) described high-affinity, low-capacity Cu-binding sites predominantly at $<15 \mu\text{g Cu/l}$ and low-affinity, high-capacity sites becoming important above this level of water Cu. More recently, Grosell and Wood (9) identified a Na^+ -sensitive and a Na^+ -insensitive component of the high-affinity gill Cu-binding sites. Here we demonstrate a diametrically opposite effect of waterborne Cu preexposure on gill Cu binding dependent on test concentration. The waterborne Cu concentration at which this reversal occurs is $\sim 10 \mu\text{g/l}$ and suggests a different effect of waterborne Cu preexposure on the high-affinity, low-capacity sites and low-affinity, high-capacity sites, as described by Taylor et al. In agreement with the proposal by Reid and McDonald (23), the high-affinity sites appear to be a small proportion of total sites. The present data also offer a credible explanation for the variable reports on the effects of waterborne Cu preexposure on subsequent uptake of Cu into the gills (6, 10, 11, 17, 25). In these studies, assessment of Cu uptake over a wide range of waterborne Cu levels (1–100 $\mu\text{g/l}$) produced different results possibly due to different effects of Cu preexposure on the two gill Cu-binding sites.

Cu uptake at the gills is thought to occur at least partly through Cu-ATPase, on the basis of vanadate sensitivity of branchial Cu uptake (3) and partial cloning of a putative Cu-ATPase in gill tissue (7). The recent identification of a phenamil-sensitive, bafilomycin-sensitive, high-affinity Cu uptake pathway in trout gills (9) suggests the involvement of the apical Na^+ channel/ H^+ -ATPase in part of the high-affinity Cu uptake. Taking results from Taylor et al. (25) and Grosell and Wood (9) into consideration, we interpret this differential gill Cu binding to mean that preexposure to waterborne Cu downregulates high-affinity Cu uptake sites (Na^+ sensitive and Na^+ insensitive) but stimulates low-affinity, high-capacity Cu uptake sites in trout gills. The adaptive significance of the latter is not obvious, but clearly it did not translate into greater



internal or whole body uptake at the exposure concentration.

Perspectives

This study demonstrates greater uptake and turnover of dietary Cu relative to waterborne Cu in fish and a marked reduction in the uptake rates of waterborne, but not dietary, Cu after chronic waterborne Cu exposure. Clearly, waterborne Cu preexposure modifies gill, but not gut, Cu uptake mechanisms. The binding and transport kinetics of Cu in the gut epithelium in fish and the effect of Cu preexposure on dietary and waterborne Cu uptake kinetic parameters require further attention. Although recent studies suggest the presence of Cu transport proteins at the gill (3, 7) and gut (4, 12) and the involvement of Na⁺ uptake pathways (9, 28), characterization of these transport mechanisms using pharmacological and molecular biological tools remains incomplete. Furthermore, illumination of the relationship and interactions between Cu transport at the gill, gut, and hepatobiliary interface would greatly contribute to the understanding of Cu regulation and metabolism in fish.

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