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. —Juvenile rainbow trout (Oncorhynchus mykiss) were exposed to waterborne Cu (22 μg/l) in moderately hard water for up to 28 days. Relative to control fish kept at background Cu levels (2 μg/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 64Cu-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 ng·g⁻¹·h⁻¹, >10-fold higher than uptake rates of waterborne Cu into the whole body without the gills, which were 0.02–0.07 ng·g⁻¹·h⁻¹. Previously Cu-exposed fish showed decreased new Cu accumulation in the gills, liver, and carcass during waterborne 64Cu exposures and in the liver during dietary 64Cu 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 64Cu 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 64Cu flux measurements. Given the dual routes of Cu uptake in fish, the third objective was to evaluate the
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 Ca²⁺, 1.9 mM HCO₃⁻, 0.7 mM Cl⁻, pH 7.7, 3 mg/l dissolved organic carbon, and 2 μg/l background Cu at 14 ± 1°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) Ca²⁺, 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 via the gills and from the diet via the gut 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.

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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:

\[ a(bc^{-1})^{-1} \]  

where \( a \) is the \(^{64}\text{Cu}\) in cpm/g fish, \( b \) is \(^{64}\text{Cu}\) in cpm/l or cpm/g in the water or diet, respectively, and \( c \) is the total Cu concentration in the water or diet (\( \mu \text{g}/\text{l} \) or \( \mu \text{g}/\text{g} \)). 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 \(^{64}\text{Cu}\) specific activities were very similar. The time-course study showed that the uptake organs themselves (gills for waterborne \(^{64}\text{Cu}\) exposure and gut for dietary \(^{64}\text{Cu}\) 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 \(^{64}\text{Cu}\) 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 \(^{64}\text{Cu}\) 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 \(^{64}\text{Cu}\) 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

\[ a(de^{-1})^{-1} \]  

where \( a \) is cpm/g tissue, \( d \) is \(^{64}\text{Cu}\) (in cpm/l or \( \mu \text{g}/\text{g} \)) in the previous compartment, and \( e \) is the total Cu concentration in the previous compartment (in \( \mu \text{g}/\text{l} \) or \( \mu \text{g}/\text{g} \)). Here it was assumed that \(^{64}\text{Cu}\) 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

\[ 100 \left( \frac{\text{Cu}_{\text{new}} - \text{Cu}_{\text{tot}}}{\text{Cu}_{\text{tot}}} \right) \]  

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

Gill Cu binding. Waterborne \(^{64}\text{Cu}\) 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 \( \mu \text{g}/\text{l} \) Cu (\( n = 60 \)) and background conditions (2 \( \mu \text{g}/\text{l} \), \( n = 60 \)) 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 \( \mu \text{g}/\text{l} \)) was used. For each concentration, the fish were exposed to the required concentration labeled with \(^{64}\text{Cu}\) [total dose as \(^{64}\text{Cu}\) (Cu(NO\(_3\))\(_2\))] 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 \(^{64}\text{Cu}\) 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 ± 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 ~40 ng/g newly accumulated Cu followed by a decline and stabilization at ~25 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 cecae + anterior intestine, mid intestine, 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 cecae + 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 \(^{64}\text{Cu}\) exposure than during waterborne \(^{64}\text{Cu}\) exposure.
On the basis of these results, 48-h exposures were employed in subsequent waterborne and dietary $^{64}\text{Cu}$ 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 $\mu$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 1.1 to 2.0 $\mu$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 $\mu$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 $\mu$g/l waterborne Cu, whole body (without gills) unidirectional uptake rates of Cu from the water were 0.02–0.07 ng.g$^{-1}$.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 $\mu$g/l waterborne

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**Fig. 2.** Distribution of newly accumulated Cu in tissues of juvenile rainbow trout during acute exposure to waterborne (A) and dietary (B and C) $^{64}\text{Cu}$ for 48 h. Values are means ± SE ($n = 5$). Note difference in scale for liver plot in B.
Cu, whole body Cu uptake rates were ~10-fold higher than the values at background waterborne Cu level (2.8 μg/l) and were 0.29–0.55 ng·g⁻¹·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 ⁶⁴Cu exposure ranged from 0.40 to 0.90 ng·g⁻¹·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 ⁶⁴Cu

<table>
<thead>
<tr>
<th></th>
<th>Waterborne</th>
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<th>Dietary</th>
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<tr>
<td></td>
<td>Background (2.8 μg/l)</td>
<td>High (22 μg/l)</td>
<td>Background (2.8 μg/l)</td>
<td>High (22 μg/l)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Cu-exposed</td>
<td>Control</td>
<td>Cu-exposed</td>
</tr>
<tr>
<td>Day 0</td>
<td>0.08 ± 0.009</td>
<td>0.05 ± 0.004</td>
<td>0.49 ± 0.03*</td>
<td>0.38 ± 0.04*</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.04 ± 0.004</td>
<td>0.05 ± 0.004</td>
<td>0.58 ± 0.04*</td>
<td>0.47 ± 0.03*</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.06 ± 0.004</td>
<td>0.05 ± 0.008</td>
<td>0.59 ± 0.02*</td>
<td>0.32 ± 0.02*</td>
</tr>
<tr>
<td>Day 28</td>
<td>0.05 ± 0.007</td>
<td>0.03 ± 0.001†</td>
<td>0.59 ± 0.02*</td>
<td>0.32 ± 0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SE in ng·g⁻¹·h⁻¹ (n = 10). *Significantly different from control waterborne fish at background Cu level at the same time, †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 μg Cu/l) but comparable to waterborne uptake rates in fish exposed to an elevated waterborne level (22 μg/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 64Cu 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 64Cu) in the gills was 6–16 ng/g in the fish exposed to background Cu level (2.8 μg/l) and was significantly lower in previously Cu-exposed fish on days 14 and 28 (Fig. 5, 1A). At 22 μg/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 μg/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 μg/l exposure (Fig. 5, 2A), whereas the value increased to 240–860 ng/g at the high waterborne Cu (22 μg/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,
however, strongly influenced by the water Cu concentration during the exposure, rising from 6–28 ng/g during background exposures (2.8 μg/l) to 66–103 ng/g during high waterborne Cu exposures (22 μg/l). In the carcass, newly accumulated Cu was 0.5–1.4 ng/g under background conditions (2.8 μg/l Cu; Fig. 5, 5A) and was significantly elevated to 4–13 ng/g at high ambient water Cu concentration (22 μg/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 μg/l) and on days 14 and 28 under elevated Cu levels (22 μg/l).

For the 48-h dietary 64Cu exposures, new Cu accumulation in the gills was 15–27 ng/g and was higher on all days than that accumulated during waterborne 64Cu exposures at background water Cu level (Fig. 5, 1A). However, at 22 μg/l water Cu level (Fig. 5, 1B), the newly accumulated dietary gill Cu was lower than during the comparable waterborne 64Cu 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 μg/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 μg/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 64Cu exposures.

**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 64Cu 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 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 64Cu 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 64Cu 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.

**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 64Cu 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...
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

<table>
<thead>
<tr>
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<th>Waterborne 64Cu Exposure</th>
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<th>Dietary 64Cu Exposure</th>
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<tr>
<td></td>
<td>Background (2.8 μg/l)</td>
<td></td>
<td>High (22 μg/l)</td>
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<tr>
<td></td>
<td>Control</td>
<td>Cu exposed</td>
<td>Control</td>
<td>Cu exposed</td>
</tr>
<tr>
<td>Gill</td>
<td>1.99 ± 0.14</td>
<td>1.60 ± 0.20</td>
<td>15.99 ± 1.35*</td>
<td>11.58 ± 1.90†</td>
</tr>
<tr>
<td>Liver</td>
<td>0.034 ± 0.003</td>
<td>0.020 ± 0.002†</td>
<td>1.01 ± 0.05*</td>
<td>0.34 ± 0.04†</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.78 ± 0.10</td>
<td>1.03 ± 0.11</td>
<td>10.72 ± 1.86*</td>
<td>8.77 ± 1.18†</td>
</tr>
<tr>
<td>Gut</td>
<td>0.18 ± 0.03</td>
<td>0.22 ± 0.05</td>
<td>2.55 ± 0.42*</td>
<td>2.11 ± 0.67*</td>
</tr>
<tr>
<td>Carcass</td>
<td>0.19 ± 0.02</td>
<td>0.12 ± 0.02†</td>
<td>1.70 ± 0.19*</td>
<td>1.00 ± 0.13†</td>
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**System (water or diet) specific activities**

|                | Background (2.8 μg/l)    |    | High (22 μg/l)        |    |
|                | Control                  | Cu exposed | Control                  | Cu exposed |
| Gill           | 1.99 ± 0.14              | 1.60 ± 0.20 | 15.99 ± 1.35*          | 11.58 ± 1.90† |
| Liver          | 6.44 ± 2.47              | 3.06 ± 0.37 | 17.43 ± 5.48*          | 10.39 ± 2.98 |
| Plasma         | 56.98 ± 9.52             | 53.28 ± 6.68 | 50.07 ± 5.22          | 78.05 ± 14.36 |
| Gut            | 34.90 ± 8.11             | 24.43 ± 6.56 | 33.29 ± 9.06          | 16.89 ± 4.11 |
| Carcass        | 27.65 ± 3.64             | 16.75 ± 3.02† | 22.98 ± 4.69          | 26.47 ± 6.32 |

Values are means ± SE expressed as percentage of total Cu content exchangeable in 48 h (n = 10). Percentages were converted to arc sine 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 µg/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 µg/l) vs. 55 µg/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 µg/l, Cu binding to the gill was decreased, while at waterborne Cu >10 µg/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 µg Cu/l and low-affinity, high-capacity sites becoming important above this level of water Cu. More recently, Grosell and Wood (9) identified an Na⁺-sensitive and an 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 ~10 µg/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 µg/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 clon ing of a putative Cu-ATPase in gill tissue (7). The recent identification of a phenamil-sensitive, bafloxicin-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
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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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