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Aquatic Toxicology 38 (1997) 257–276

AQUATIC TOXICOLOGY

Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*)

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Accepted 24 October 1996

Abstract

^{64}Cu accumulation and total Cu concentration were measured in plasma, red blood cells, gills, liver, kidney and bile during 65 h of exposure to ^{64}Cu at 20 μg of Cu per liter, in non acclimated and Cu acclimated (28 days of preexposure) rainbow trout (*Oncorhynchus mykiss*) fitted with a dorsal aortic catheter. By measuring both ^{64}Cu accumulation and total Cu concentrations, we were able to analyse the ongoing uptake and turnover of ambient Cu, independent of any Cu already present in the fish.

Plasma accounted for at least 90% of the ^{64}Cu labelled Cu present in the blood and Cu-acclimation clearly involves changes in copper accumulation kinetics in the plasma. The acclimated fish showed a 65% reduced ^{64}Cu accumulation after 65 h and an increased turnover of Cu in the plasma compared to the non-acclimated fish. Total Cu in the plasma increased by 59% after 3 h of exposure in the non-acclimated fish but was recovered during the following 24 h and remained at control levels throughout 65 h; even after 28 days the acclimated fish showed no increase in total plasma [Cu]. Apparently Cu acclimation involves an increased clearing of plasma Cu, primarily to the liver, stimulated during the first 12 h of exposure.

Acclimation did not have an unambiguous effect on branchial Cu uptake and differences in branchial uptake could not explain the reduced accumulation in the plasma. The rapidly exchangeable Cu pools were 54% in the gills and 33% in the liver, suggesting a considerable hepatic Cu elimination. No increase in the total [Cu] in the kidney was observed, but the kidney did show substantial ^{64}Cu accumulation and thus also a potential renal Cu excretion. © 1997 Elsevier Science B.V.

Keywords: Cu-acclimation; Rainbow trout; *Oncorhynchus mykiss*; ^{64}Cu; Cu uptake; Cu metabolism; Metallothionein

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PII S0166-445X(96)00843-0

1. Introduction

Cu is an essential metal in cellular metabolism (Cousins, 1985) but also potentially highly toxic to fish (Chakoumakos, 1979; Cusimano et al., 1986). The toxicity of Cu can be modified by previous sublethal exposure to the element which sometimes leads to a higher tolerance (Dixon and Sprague, 1981). The enhanced lethal resistance has been shown to be the result of a true acclimation (defined by Prosser, 1973) to copper during prolonged exposure to sublethal concentrations (McDonald and Wood, 1993), which involves physiological and biochemical modifications that restore the impaired branchial Na^+ transport (Laurén and McDonald, 1987a,b).

Sublethal Cu exposures can lead to increased hepatic metallothionein (MT) concentrations (McCarter and Roch, 1983, 1984; Laurén and McDonald, 1987b) and a study on atlantic cod has also shown increased branchial MT during Cu exposure (C. Hogstrand and R.M. Stagg, unpublished, 1990). However, Noël-Lambot et al. (1978) and Laurén and McDonald (1987b) did not find any signs of increased branchial MT concentrations during Cu exposure of as well European eels as rainbow trout. Since the gills are the first and most important target of Cu toxicity (Laurén and McDonald, 1985, 1986) and since Cu exposure did not, in all cases, lead to increased branchial MT, the formation of branchial MT can hardly be among the primary mechanisms responsible for increased Cu tolerance.

Acclimation to other metals such as zinc (Bradley et al., 1985; Hogstrand et al., 1994), cadmium (Wicklund-Glynn and Olsson, 1991) and aluminum (Reid et al., 1991) often involves a reduced branchial metal uptake. Cu acclimation, however, does not seem to involve any reduced branchial Cu uptake; McCarter and Roch (1984) reported rather a tendency of increased branchial Cu uptake in coho salmon exposed to 100 μg of Cu per liter for two weeks, and more recently, European eels have shown a constant branchial Cu uptake during 28 days of Cu exposure at 8 and 64 μg of Cu per liter (Grosell et al., 1996). Furthermore, previous Cu exposure leads to a change in the tissue distribution of the Cu entering the fish; the Cu accumulation rates in liver and muscle tissue were significantly reduced in Cu-acclimated eels even at a constant branchial Cu uptake (Grosell et al., 1996).

In the present investigation we have used a very low, but environmentally realistic (Spry et al., 1981), ambient Cu concentration (20 μg of Cu per liter) compared to expected LC50 values in hard water (Chakoumakos, 1979; Laurén and McDonald, 1986). Our aim was to study possible physiological effects, at the lower end of the toxic scale, in hard water chemistry identical to that used by Laurén and McDonald (1987a,b).

Cu can also be found at low concentrations in the organs of teleost fish that have not been exposed to elevated ambient Cu concentrations (Buckley et al., 1982; McCarter and Roch, 1983, 1984; Collvin, 1984a,b; Laurén and McDonald, 1987a,b; Grosell et al., 1996). This makes it necessary to use radiotracers to investigate the ongoing uptake, and possible changes in distribution and excretion, of Cu during prolonged exposure to relatively low waterborne concentrations. By using ^{64}Cu as a radiotracer it is possible to follow the ongoing uptake, accumulation and

turnover of ambient Cu independent of any Cu already present in the fish at the start of the experiment.

In the present investigation, we used ^{64}Cu to pursue three main objectives in rainbow trout preexposed and not preexposed to Cu:

1. We wished to compare the transport of Cu from the gills, as the primary Cu uptake site to the kidney and the liver in non-acclimated and Cu-acclimated rainbow trout during, sublethal exposure (total water [Cu]=20 μg of Cu per liter). To do this we used ^{64}Cu to monitor the accumulation, turnover and further transport of plasma Cu in rainbow trout fitted with dorsal aortic catheters.
2. We wished to determine whether 28 days of sublethal Cu exposure resulted in a changed branchial Cu uptake by rainbow trout.
3. We wished to monitor rates of Cu turnover in liver and kidney, as well as the hepatic Cu excretion, in rainbow trout during Cu exposure. For this purpose, the liver, kidney, and the content of the gall bladder were sampled at the end of each experiment. Cu excretion is described in more detail in a parallel paper (Grosell et al., 1997).

2. Materials and methods

2.1. Fish

Rainbow trout, *Oncorhynchus mykiss* (133–333 g; mean 236 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario. The fish were held in 264-l fiberglass tanks (25 per tank), each supplied with a flow-through of dechlorinated aerated Hamilton city tapwater ($[Na^+]$ 0.6 mmol l^{-1}; $[Cl^-]$ 0.7 mmol l^{-1}; $[Ca^{2+}]$ 1.0 mmol l^{-1}; $[HCO_3^-]$ 1.9 mmol l^{-1}; pH 7.9–8.2; background [Cu] 0.89 μg of Cu per liter) at a rate of 2.0–3.0 l min^{-1}. On arrival, the fish were held at 6°C. After 6 days of acclimation the temperature was increased by approximately 2°C per day to 15 ± 1°C. The fish were held at that temperature at least 14 days prior to the experiments and were fed dry trout pellets (Martin's Feed Mill Ltd, Ontario) at a rate of 1% of their body mass three times a week. The Cu content of the food was 2.9 ± 0.5 μg Cu per gram (mean ± S.E.M., $N = 5$).

2.2. Chronic Cu-exposure

Ten non-exposed fish from the holding tanks served as a control group (A) for tissue [Cu] and levels of metallothionein. For the 28 days of Cu acclimation, one tank (supplied at a rate of 2.05 liters of water per minute) was equipped with a dosing system that added Cu, as $CuCl_2 \cdot 2H_2O$ (analytical grade), from a stock solution. The flow rate of the added Cu stock (36.65 mg $CuCl_2 \cdot 2H_2O$ per liter) was maintained at 3 ml min^{-1}, using a Mariotte bottle, to achieve a nominal con-

centration of Cu in the tank of 20 μg of Cu per liter (actual measured Cu concentration; 26.60 ± 1.62, mean ± S.E.M., $N = 15$). The exposure was initiated by adding a sufficient amount of Cu^{2+} stock solution to the water in order to obtain the desired Cu concentration instantly. Water flow and dosing rates were checked daily and adjusted if necessary. No mortality occurred during the Cu acclimation. However there was a tendency to a decreased appetite during the first three days of exposure.

Water samples from the Cu acclimation tank were taken for analysis of Cu levels every second day prior to feeding. Samples were acidified with HNO_3 (trace metal analysis grade, BDH Chemicals, was used in all procedures), and Cu was measured by graphite furnace atomic absorption spectroscopy (AAS; Varian AA-1275 with GTA-95 atomizer) using 20 μl injection volume, N_2 gas, and standard operating conditions as documented by the manufacturer.

2.3. Experimental design: dorsal aortic catheter experiments

Groups of ten non-acclimated (B) and ten acclimated (C) fish were anaesthetized with MS-222 from a stock solution (25 g l^{-1}; pH 7) at a final concentration of 0.1 g l^{-1}, fitted with in-dwelling dorsal aortic catheters (PE 50 tubing) as described by Soivio et al. (1972) and allowed to recover for at least 30 h prior to the experiments. After cannulation each fish was held in an individual experimental chamber with a total volume of 7.1 l. The chambers have been illustrated by McDonald (1983); in brief they consist of a transparent, inner, plexiglass container, which confines the fish, and an outer, black, plexiglass container which contains the bulk of the water volume. An airlift pump at the rear of the inner container recirculates water past the fish at more than 300 ml min^{-1} and together with aeration in the outer container, it ensures the maintenance of air-saturated conditions. Each of the 10 fish boxes were supplied with dechlorinated Hamilton city tapwater at a rate of 200 ml min^{-1} in a recirculated system containing a total of 500 l of water.

In experiments with Cu-acclimated rainbow trout, the water in the recirculated system contained 20 μg of Cu per liter during the recovery period. At the start of each experiment the recirculation was stopped, and 420 l of the water was replaced by fresh dechlorinated Hamilton city tap water (15 ± 1°C). 10.0 mg Cu (as $CuCl_2$); 20 mCi ^{64}Cu, was added to the reservoir of the recirculated system and allowed to mix by aeration for 15 min prior to restarting the recirculation resulting in 20.8 ± 1.7 μg of Cu per liter (mean ± SEM; $n = 12$)

2.4. Sampling

After 3 h of ^{64}Cu exposure, a water sample (in triplicate) was taken as a reference (^{64}Cu specific activity) for the ^{64}Cu measurements in blood and tissue samples. Later sampling demonstrated that the specific activity of the water did not change appreciably over the 65 h experiment (corrected for decay). After 3, 6, 12, 24, 48 and 65 h of ^{64}Cu exposure a blood sample of 500 μl was drawn from each fish via

the catheter into a heparinized Hamilton syringe (1000 I.U. heparin). The blood volume taken was replaced by an equal volume of non-heparinized Cortland saline (Wolf, 1963). The blood sample was treated as two subsamples of 200 and 300 μl. The blood cells and plasma of the 200 μl subsamples were separated by centrifugation (5 min at 14000*g*) and placed in different vials. The 300 μl subsamples were separated into red blood cells (RBC), white blood cells (WBC) and plasma by centrifugation in heparinized hematocrit tubes of a known volume. Each fraction was measured and the volume determined. The plasma and RBC fractions were isolated, by cutting the hematocrit tubes, and placed in different vials. After blood sampling at 65 h, the fish were anaesthetized as described above and killed by a blow to the head. Bile was collected by emptying the gall bladder with a 1 ml syringe, in which the volume was read. Liver, kidney and gill filament samples were taken by dissection and treated as described below.

2.5. Newly accumulated Cu

The ^{64}Cu γ-activity was determined in the reference water, the liver, kidney and gill filaments, the 200 μl blood fraction, the plasma and the RBC from the 300 μl blood fraction and the bile using a γ counter (MINAXI γ Auto-gamma 5000 Series, Canberra-Packard). The recorded cpm were corrected for radioactive decay by an on-board program in the counter. The liver, gill filaments and kidney were freeze dried and the dry weights were determined at room temperature and room humidity. In this study, the term "newly accumulated Cu" is used to designate the part of the Cu present in a compartment which came from the "previous" compartment, during the period of ^{64}Cu exposure. The previous compartment for the gills is assumed to be the water; for the plasma, the gills; for the liver and kidney, the plasma; and for the bile, the liver. The newly accumulated Cu was calculated by the following equation:

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

Where "*a*" is ^{64}Cu cpm per gram of tissue (dry weight) or ^{64}Cu cpm per liter, "*b*" is ^{64}Cu counts in the previous compartment (cpm per gram or per liter), and "*c*" is the total Cu concentration in the previous compartment (μg of Cu per liter or μg Cu per gram). The mean specific activities (bc^{-1}) for the previous compartments used for calculating the excess Cu accumulation are shown in Fig. 1. The calculations were based on the measured individual specific activities.

The newly accumulated Cu in the white blood cells was calculated by subtraction of the concentrations of newly accumulated Cu in plasma and RBC from the newly accumulated Cu in the corresponding full blood sample, hematocrit values taken into consideration.

2.6. Experiments with terminal sampling

32 non-acclimated (group D) and 32 acclimated (group E) rainbow trout were

transferred to a tank containing 250 l of aerated and dechlorinated Hamilton city tap water (15 ± 1°C) and 5.0 mg Cu (as $CuCl_2$); 10 mCi of ^{64}Cu (one group at the time) (24.8 ± 1.6 μg of Cu per liter).

2.7. Sampling

After 3, 6, 12 and 24 h of exposure respectively, a group of 8 fish was netted out of the exposure system and anaesthetized as described above. A blood sample of a least 0.5 ml was withdrawn from the caudal vessel, with a heparinized Hamilton syringe, and the fish were killed by a blow to the head. Blood cells and plasma were separated by centrifugation (5 min at 14000*g*). Blood cells, plasma, and gill filaments were placed in separate vials, for ^{64}Cu γ activity determination as described above.

2.8. Levels of metallothionein and total Cu

A blood sample of a least 0.5 ml was withdrawn from the caudal vessel of the controls (group A) as described above, and the fish were killed by a blow to the head. A subsample of the livers from the controls (group A) and the Cu-acclimated fish (group C) was transferred to cryostatic vials, frozen in liquid nitrogen immediately after dissection and then stored at -70°C. The frozen liver samples were later weighed and homogenized individually in 4 volumes of 50 mmol l^{-1} Tris-HCl, pH 8.0, at 0°C, using a glass-Teflon homogenizer. The samples were centrifuged at 10000*g*, 4°C, for 20 min; the supernatant was decanted, frozen in liquid nitrogen, and stored at −70°C. MT levels were analysed with a double antibody radioimmunoassay (RIA) as described by Hogstrand and Haux (1990).

After determination of ^{64}Cu activity, the freeze-dried liver, gill filament and kidney samples from groups (A), (B) and (C) and gill filaments from groups (D) and (E), were digested in acid-washed glass tubes for 1 h with 5 volumes of 70% HNO_3 at 120°C. The samples were then cooled to room temperature and 0.75 volumes of H_2O_2 was added. The digests were evaporated to dryness at 120°C. Finally 5 ml of 1% HNO_3 was added to the digestion vials. [Cu] in the digested tissue samples and in the plasma samples was analysed by atomic absorption spectroscopy as described for the water samples above.

2.9. Statistical methods

Significant differences between the kinetics of new Cu accumulation in blood, plasma and RBC of non-acclimated (B) and acclimated (C) fish, as well as the kinetics of new Cu accumulation in the gills and blood of non-acclimated (D) and acclimated (E) fish in the terminal sampling experiment were compared in a two-factor ANOVA (Statgraphics for Windows version 1.3) with acclimation and ^{64}Cu exposure time as the main variables. Significant differences between the data for total Cu concentration in the investigated tissues in groups (A), (B) and (C), together with the MT values from groups (A) and (C) were evaluated by Student's

t-test (two tailed, unpaired) using Bonferroni corrections for multiple sample comparison of total Cu concentration in the plasma. In all cases, groups were considered significantly different at $P < 0.05$.

3. Results

The newly accumulated Cu in the blood of non-acclimated rainbow trout (group B) was clearly different ($P < 0.0003$) from that of acclimated rainbow trout (C) (Fig. 2). In acclimated fish (group C), the newly accumulated Cu in the blood showed a saturation pattern with maximum values of about 0.04 μg of Cu per ml, obtained after 6 h of ^{64}Cu exposure. The newly accumulated Cu in the blood of non-acclimated fish (group B) did not seem to fully saturate within the 65 h of ^{64}Cu exposure, and reached a newly accumulated Cu level almost three times higher than that of the acclimated fish by the end of the experiment.

It appears from Table 1 that 89.8–98.5% of the Cu newly accumulated by the blood is found in the plasma fraction and that only 2.4–8.0% is found in the RBC fraction. The newly accumulated Cu in the white blood cells during ^{64}Cu exposure could not be determined by the methods described above. From the data on ^{64}Cu distribution between plasma and RBC in Table 1, however, it seems that the white blood cells contained no more than 5.0% of the newly accumulated blood-Cu. Relatively, the portion of the newly accumulated blood-Cu found in plasma (Table 1, percentage of blood Cu) varied only slightly with time. The acclimated fish (group C) show a tendency, though not significant, to have slightly more (0.4–5.2%) than the non-acclimated fish (group B) of the newly accumulated blood-Cu in the plasma fraction. The opposite was true for the RBC measurements; the acclimated fish (group C) tended to have less of the newly accumulated blood-Cu in the RBC fraction ($P < 0.0008$).

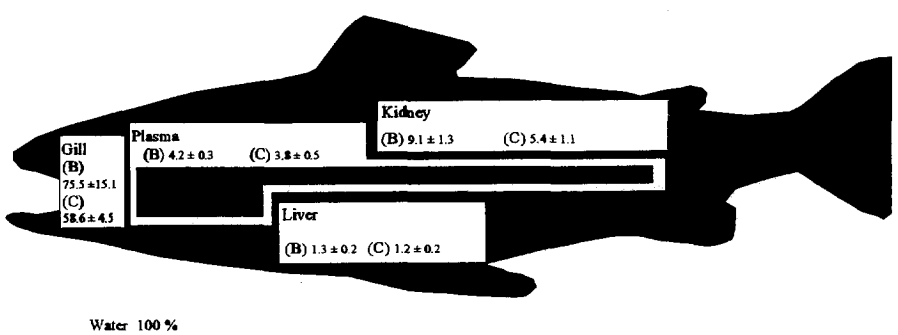


Fig. 1. Relative specific activity of Cu in gill, plasma, kidney and liver of non-acclimated (B) and acclimated (C) rainbow trout expressed as a percentage of water Cu specific activity ($(bc^{-1})100\%$, see Section 2). For the gills, kidney and liver, the values express the specific activities after 65 h of ^{64}Cu exposure at, 20 μg of Cu per liter. For the plasma, the values express the time-weighted mean relative specific activity. Means ± 1 SEM ($N = 10$ for non acclimated and $N = 9$ for acclimated).

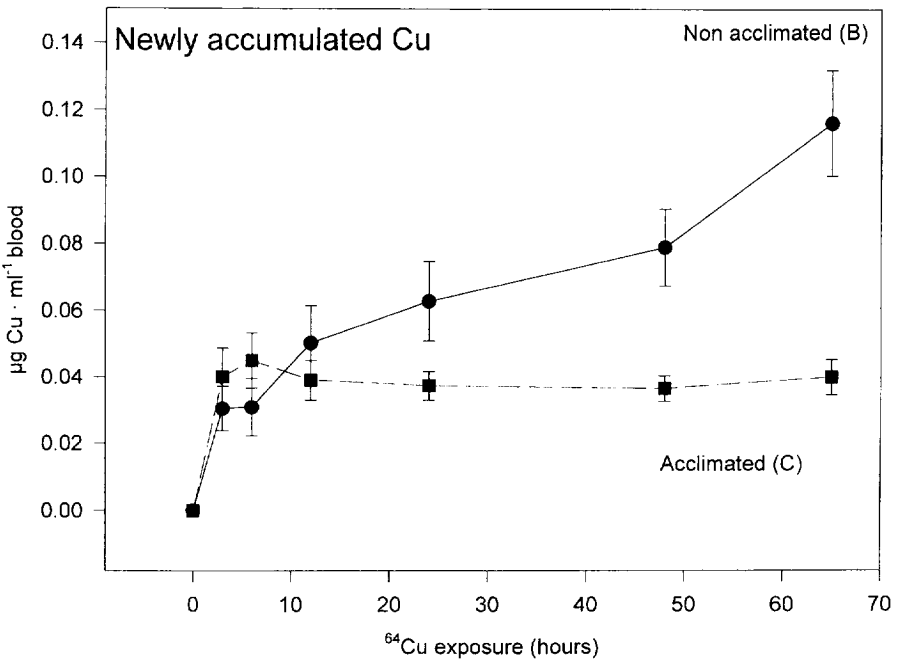


Fig. 2. Newly accumulated Cu in the blood of non-acclimated (●) and Cu acclimated (■) rainbow trout during 65 h of ^{64}Cu exposure at, 20 μg of Cu per liter. Means ± 1 SEM ($N = 10$ for non acclimated and $N = 9$ for acclimated). Data from groups B and C were compared by an ANOVA and found to be significantly different at $P < 0.0003$.

The new accumulation of Cu in the plasma (Fig. 3a) follows the same general pattern as that seen in the blood (Fig. 2). The difference between the two experimental groups (B) and (C) is significant at $P < 0.0001$.

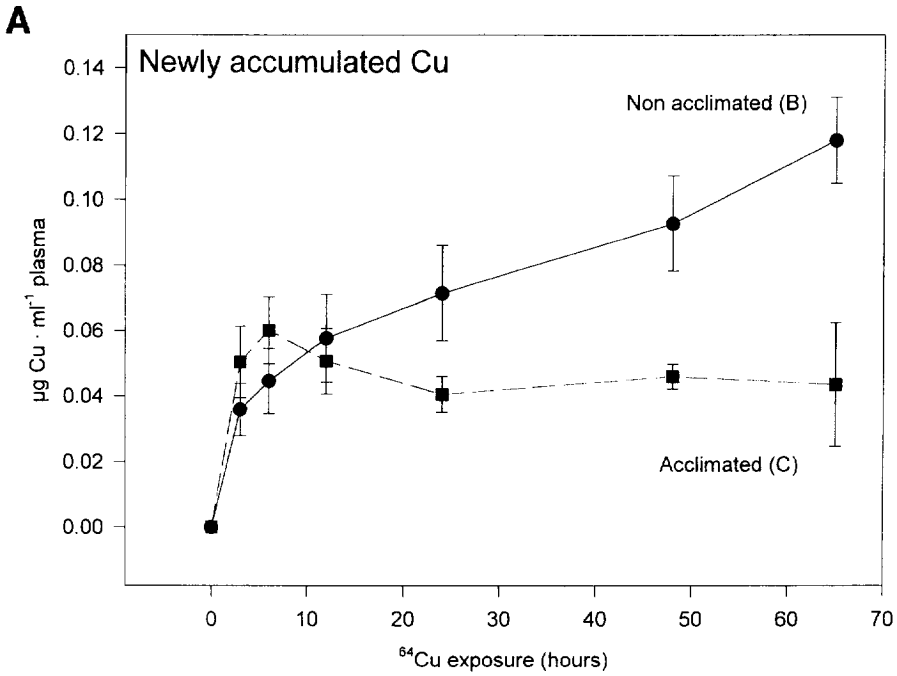
The total Cu concentrations in the plasma (Fig. 3b) of the non-acclimated and the acclimated fish were also very different; the non-acclimated fish (B) exhibited a marked increase in plasma Cu concentration already after 3 h of Cu exposure, about 0.44 μg of Cu per ml higher than that of the controls ($P < 0.05$). Total plasma [Cu] remained elevated at 6 and 12 h. After 24 h of exposure, however, the Cu concentration returned to the control levels and did not significantly exceed the control levels for the rest of the 65 h of Cu exposure. The acclimated fish (C) showed no increase in plasma Cu concentration at the first sampling point (3 h), but they showed a tendency of reduced Cu-concentration relative to the controls after 6 and 12 h of exposure. Thereafter, the Cu concentrations in the acclimated fish (C) did not differ significantly from the controls.

The newly accumulated Cu in the livers of the non-acclimated fish (B) during the 65 h of Cu exposure was calculated to be 134.4 ± 27.7 (Fig. 4a) The total Cu concentration in the liver increased from 234.8 ± 42.2 (mean ± SEM) in the controls (A) to 419.9 ± 36.3 μg of Cu per gram (dry weight) in the same non-acclimated fish (B) (Fig. 4b; $P < 0.032$), an absolute gain of 185.1 μg of Cu per gram (dry weight), i.e.

Table 1
Newly accumulated Cu and distribution measured in plasma and red blood cells (RBC), of non-acclimated (B) and acclimated (C) rainbow trout during 65 h of ^{64}Cu exposure at, 20 μg l^{-1}

	^{64}Cu exposure time (h)						
	3	6	12	24	48	65	*P*
Plasma Cu (% of blood Cu),							
non acclimated (B)	93.4 ± 1.7	92.8 ± 2.2	94.8 ± 1.5	89.8 ± 4.5	96.5 ± 2.9	90.5 ± 5.7	
Acclimated (C)	96.5 ± 1.5	97.1 ± 1.1	96.0 ± 2.3	90.2 ± 3.5	98.5 ± 3.7	95.7 ± 5.0	n.s.
RBC Cu (μg Cu per ml)							
non acclimated (B)	0.005 ± 0.001	0.008 ± 0.002	0.016 ± 0.003	0.031 ± 0.001	0.034 ± 0.010	N.D.	
Acclimated (C)	0.004 ± 0.001	0.006 ± 0.001	0.001 ± 0.002	0.013 ± 0.003	N.D.	N.D.	< 0.0089
RBC Cu (% of blood Cu)							
non acclimated (B)	4.1 ± 0.6	5.6 ± 1.4	6.9 ± 1.6	8.0 ± 1.8	5.5 ± 1.3	N.D.	
Acclimated (C)	2.4 ± 0.3	2.5 ± 0.2	4.0 ± 0.7	4.8 ± 0.7	N.D.	N.D.	< 0.0008

Mean ± SEM ($N = 10$ for non acclimated and $N = 9$ for acclimated).
N.D.: Non detectable.
All data sets were compared by ANOVA's, *P*: level of statistical significance


A
Newly accumulated Cu
Non acclimated (B)
Acclimated (C)
µg Cu · ml-1 plasma
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60
70
64Cu exposure (hours)

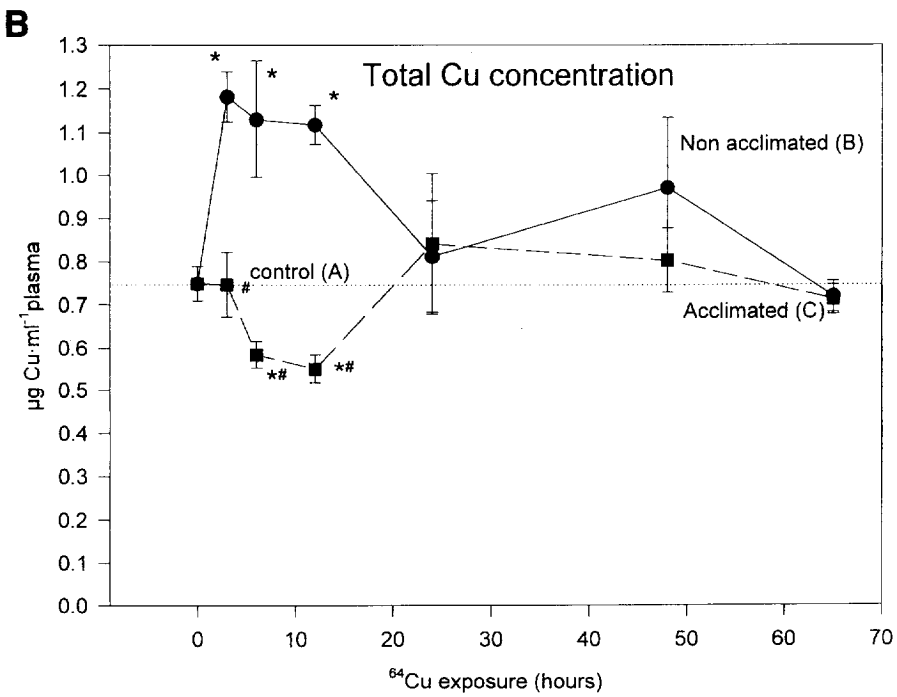

B
Total Cu concentration
Non acclimated (B)
control (A)
Acclimated (C)
µg Cu·ml-1plasma
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
64Cu exposure (hours)

Fig. 3. a. Newly accumulated Cu in the plasma of non-acclimated (●) and Cu-acclimated (■) rainbow trout during 65 h of ^{64}Cu exposure at, 20 μg Cu per liter. Means ± 1 SEM ($N = 10$ for non-acclimated and $N = 9$ for acclimated). Data from group B and C were compared by an ANOVA and found to be significantly different at $P < 0.0001$. b. Total Cu concentration in plasma of non-acclimated (●) and Cu-acclimated (■) rainbow trout during 65 h of ^{64}Cu exposure at, 20 μg of Cu per liter. Means ± 1 SEM ($N = 10$ for controls and for non acclimated and $N = 9$ for acclimated). Values for the acclimated fish (C) were tested statistically against the corresponding values for non-acclimated fish (B) by a unpaired *t*-test, using Bonferroni correction for multiple sample comparison. A significant difference between B and C, at $P < 0.05$, is indicated by #. Values from both (B) and (C) were tested against the controls by unpaired *t*-tests. A significant difference from the controls (A), at $P < 0.05$, is indicated by *.
←

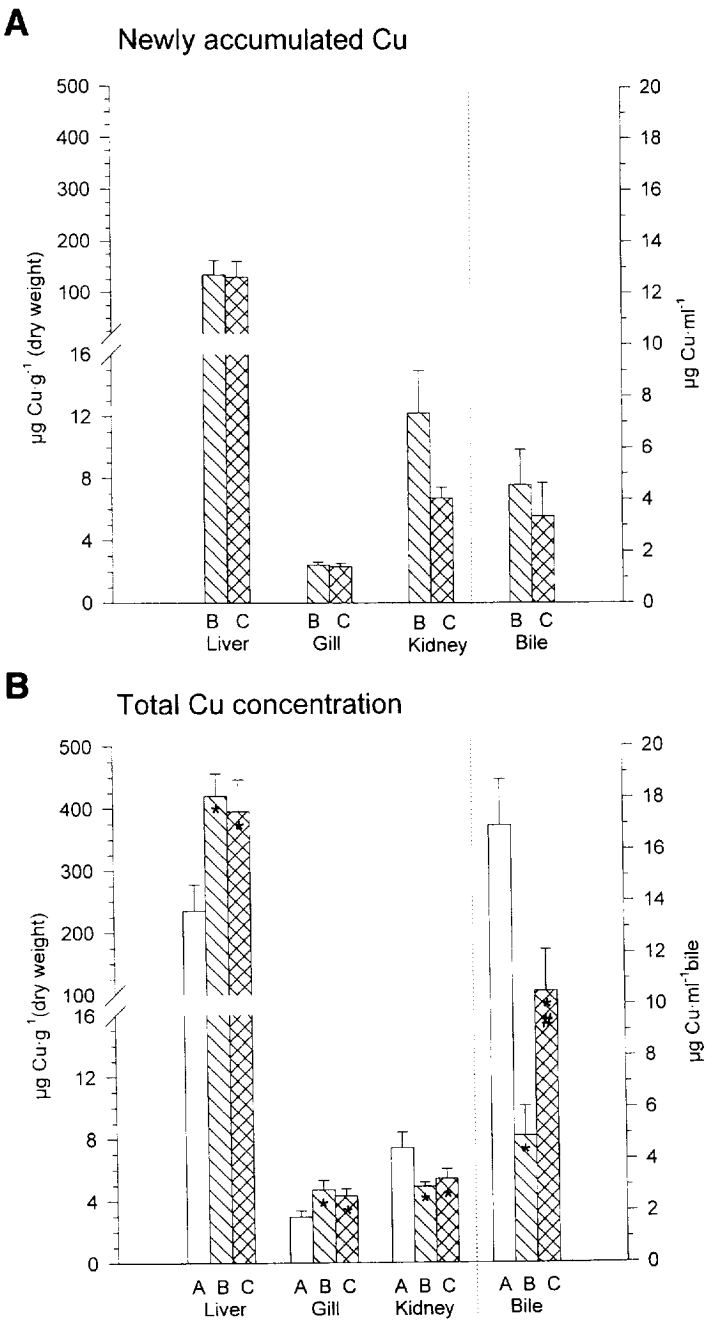
in relatively close agreement. In the acclimated fish (C) there is no further increase in the total Cu concentration in the liver (Fig. 4b) compared to in the non-acclimated fish (B). The newly accumulated Cu during the ^{64}Cu exposure did not differ between the two experimental groups (B) and (C), (Fig. 4a). The acclimated fish showed only a statistically non-significant increase in the amount of hepatic metallothionein from control values; 97.5 ± 13.3–141.9 ± 22.1 μg g^{-1} (wet weight) after 31 days (28 days+65 h) of Cu exposure at 20 μg of Cu per liter.

In the gills of the control fish, we found 3.0 ± 0.4 μg of Cu per gram of gill filaments (dry weight) and after 65 h of ^{64}Cu exposure a significantly increased Cu-concentration: 4.7 ± 0.6 μg of Cu per gram of gill filaments (dry weight) (Fig. 4b; $P < 0.036$). During the same period the newly accumulated Cu (Fig. 4a) was 2.4 ± 0.2 μg of Cu per gram of gill filaments (dry weight). As in the liver, there was no increase in the total and newly accumulated Cu concentrations in the gills of the acclimated fish (C) relative to the non-acclimated fish (B) (Figs. 4a and 4b).

The total Cu concentrations in the gills (Table 2) indicate that the gills reached a steady state, with respect to Cu concentration, at approximately 5 μg of Cu per gram within the first 3 h of Cu exposure. The Cu concentrations in the gills did not differ between the two experimental groups (D and E). The newly accumulated Cu in the gills (Fig. 5) showed an initial peak during the first 12 h of ^{64}Cu exposure in both the non-acclimated (D) and acclimated fish (E); the non-acclimated (D) showed a slightly higher level of newly accumulated Cu than the acclimated fish (E) during the whole 24 h period of exposure ($P < 0.0050$). The newly accumulated Cu in both experimental groups levelled off after 12 h of ^{64}Cu exposure (Fig. 5) at about 1 and 0.7 μg of Cu per gram (dry weight) in the non-acclimated and acclimated fish, respectively.

In the kidneys of both non-acclimated (B) and acclimated fish (C), we found a significantly reduced ($P < 0.0082$) total Cu concentration (Fig. 4b) in response to Cu exposure for both 65 h and 31 days (28 days+65 h). Despite the shown lack of total Cu accumulation (Fig. 4b), both experimental groups showed newly accumulated Cu (Fig. 4a) during the 65 h of ^{64}Cu exposure; apparently the non-acclimated fish (B) accumulated about twice as much new Cu as the acclimated (C) but this was not statistically significant.

In the bile, we found a reduced total Cu concentration in both experimental



groups (B) and (C) ($P < 0.00002$ and 0.0165, respectively) as described for the kidney (Fig. 4b). The bile of the acclimated fish (C) had a higher Cu concentration than that of the non-acclimated fish (B) ($P < 0.01$). As in the kidney, the non-

Fig. 4. a. Newly accumulated Cu in liver, gill filaments, kidney and bile of non-acclimated (B) and Cu-acclimated (C) rainbow trout after 65 h of ^{64}Cu exposure at, 20 μg of Cu per liter. Means ± SEM ($N = 10$ for non acclimated and $N = 9$ for acclimated). Values for the acclimated fish (C) were tested statistically against values for non acclimated fish (B) by a unpaired t-test. No significant difference between B and C, at $P < 0.05$, was found. b. Total Cu concentration in the same samples as in Fig. 4a. Means ± SEM ($N = 10$ for controls and non acclimated and $N = 9$ for acclimated). Values for the acclimated fish (C) were tested statistically against the values for non-acclimated fish (B) by a unpaired t-test. A significant difference between B and C, at $P < 0.05$, is indicated by #. Values from both (B) and (C) were tested against controls by unpaired t-tests. A significant difference from the controls (A), at $P < 0.05$, is indicated by *.
←

acclimated and acclimated fish showed a new accumulation of Cu in the bile after the 65 h of ^{64}Cu exposure (Fig. 4a) in spite of the reduced total Cu (Fig. 4b). The amount of newly accumulated Cu did not differ statistically between the two experimental groups (B) and (C).

The non-cannulated fish from the terminal sampling experiment (Fig. 6) accumulated only about half as much new Cu in the blood as did the fish of the dorsal aortic catheter experiment (Fig. 2). The acclimated fish (E) had a total Cu concentration in the plasma (Table 2) that did not significantly exceed the control values. The non-acclimated fish (D) (Table 2), however, exhibited increased total Cu concentrations in the plasma after 12 h ($P < 0.0070$) and a reduced (but still slightly higher than the control values) Cu concentration after 24 h of Cu exposure (Table 2).

4. Discussion

Cu exposure caused a transient increase in the total plasma Cu concentrations, but after 24 h (and previously 28–31 days of Cu exposure in the acclimated fish) the

Table 2
Total Cu concentrations in gills and plasma of non-acclimated and acclimated rainbow trout during 24 h of ^{64}Cu exposure at 20 μg Cu per liter

	^{64}Cu exposure (h)				
	Control	3	6	12	24
Gills (μg Cu per gram)					
non acclimated (D)	3.0 ± 0.4	5.5 ± 0.2	4.6 ± 0.5	5.2 ± 0.6	4.5 ± 0.3
Acclimated (E)	—	5.9 ± 0.4	5.6 ± 0.4	4.2 ± 1.0	5.1 ± 0.4
Plasma (μg Cu per ml)					
non acclimated (D)	0.75 ± 0.04	0.75 ± 0.04	0.59 ± 0.03	0.97 ± 0.06*	0.85 ± 0.04
Acclimated (E)	—	0.72 ± 0.05	0.82 ± 0.04	0.80 ± 0.04	0.89 ± 0.06

Mean ± SEM ($N = 8$).
*Indicates significant difference from control, no significant difference was found between groups (D) and (E) (Students t-test, $P < 0.05$)

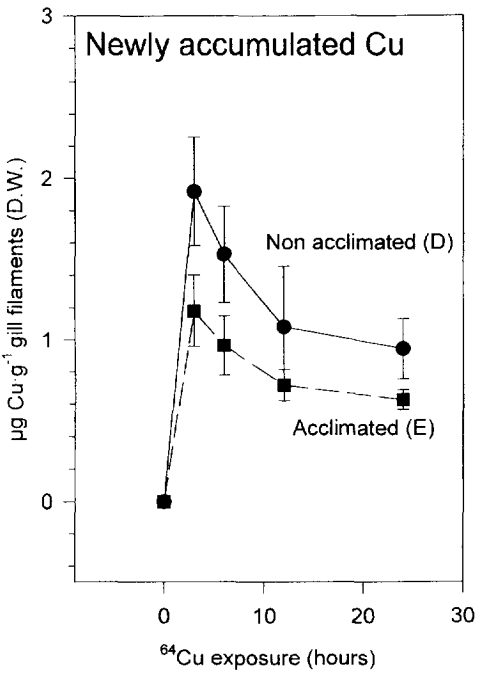


Fig. 5. Newly accumulated Cu in gill filaments of non acclimated (●) and Cu-acclimated (■) rainbow trout, from terminal sampling experiment, during 24 h of ^{64}Cu exposure at, 20 μg of Cu per liter. Means ± SEM ($N=8$). Data from group (D) and (E) were compared by an ANOVA and found to be significantly different at $P<0.05$.

plasma Cu concentrations were back to the control values (Fig. 3b). This latter restoration of normal total plasma Cu concentrations cannot be explained by reduced levels of newly accumulated Cu in the plasma, because it continues to accumulate in the plasma of non-acclimated rainbow trout (Fig. 3a) during the entire 65 h of ^{64}Cu exposure. It is more likely that the return of total plasma Cu concentrations (Fig. 3b and Table 2) to control values and the reduced newly accumulated Cu in plasma of the acclimated fish (Fig. 3a) involved an increased clearance of plasma Cu, initiated during the first 12 h of Cu exposure, because not even the up-to 34–39% reduction of the newly accumulated Cu seen in the gills of acclimated fish (E) (Fig. 5) can fully explain the up-to 63% reduction in newly accumulated plasma Cu (Fig. 3a).

Approximately one third of the reduction in the total Cu concentration in the plasma of the acclimated fish (C), observed between 3 and 6 h of ^{64}Cu exposure (Fig. 3b), could be explained by the replacement of 0.5 ml blood (containing Cu), equal to approximately 5% of the blood volume, with Cu-free Cortland saline at each sampling. The factor(s) responsible for the other two thirds of the reduction have not been identified. The same "dilution" of the total plasma Cu concentration must likewise occur in the non-acclimated fish (B), which means that the observed initial peak in the total plasma Cu concentration is, in fact, slightly underestimated.

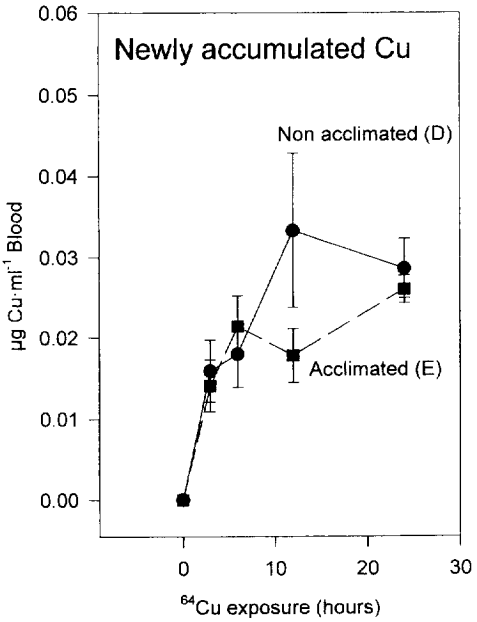


Fig. 6. Newly accumulated Cu in the plasma of non-acclimated (●) and Cu-acclimated (■) rainbow trout, from the terminal sampling experiment, during 24 h of ^{64}Cu exposure at, 20 µg of Cu per liter. Means ± 1 SEM ($N = 8$). Data from groups (D) and (E) were compared by an ANOVA and found not to be significantly different at $P < 0.05$.

The newly accumulated Cu in the blood was primarily found in the plasma (Table 1). At any time, the newly accumulated Cu in the blood represents a balance of two processes: uptake at the gills, and clearance to other tissues, primarily the liver (Fig. 4a). The highly elevated total Cu concentrations in the plasma of the non-acclimated fish (Fig. 3b) cannot fully be explained by the newly accumulated plasma Cu. The total plasma Cu concentrations increase by as much as 0.43 µg of Cu per ml during the first 3 h of exposure, of which only 0.04 µg of Cu per ml can be accounted for by the newly accumulated Cu. Even after 65 h of exposure, the newly accumulated Cu can still only account for 0.12 µg of Cu per ml. This means that some of the initial total Cu increase in the plasma must be derived from a mobilization of readily exchangeable internal Cu pools. Releasing detoxified Cu from internal pools, and thereby opening up available Cu storage and/or detoxifying capacity, could be a protective measure.

In the acclimated fish (C), both the newly accumulated Cu (Fig. 3a) and the total Cu (Fig. 3b) in the plasma seem to be at steady states and the exchangeable pool (newly accumulated [Cu] per total [Cu]) can be calculated to be 6% throughout the ^{64}Cu exposure. The newly accumulated Cu in the plasma of the acclimated fish (C) (Fig. 3a) reached steady state within the first 3 h of ^{64}Cu exposure, which means that the exchangeable Cu pool in the plasma is turned over at a relatively high rate. In the non-acclimated fish (B and D), the total Cu concentration is at steady state

after 24 h of Cu exposure (Fig. 3b) (Table 2); the newly accumulated Cu, however, does not reach steady state within the first 65 h of exposure (Fig. 3a), which means that this pool is turned over at a much slower rate than in the acclimated fish. The exchangeable plasma Cu pool (within 65 h) in the non-acclimated fish (C) can be calculated to be at least 16%. The lower values of newly accumulated Cu in the plasma of fish from the terminal sampling experiment (Table 2) compared to the Cu accumulation in the plasma from fish fitted with catheters (Fig. 3a) could be explained by the different sampling methods. The dorsal aorta catheter draws arterial blood samples immediately distal to the primary uptake site, the gills, however, the blood sampled by caudal puncture in the terminal sampling experiment may well have been venous and therefore could have lost (to other tissues) part of the Cu derived from a recent branchial uptake. The lower levels of newly accumulated Cu in the plasma of the fish from the terminal sampling experiment could also reflect the relatively low newly branchial Cu accumulation observed in these fish (Table 2) compared to in the fish fitted with catheters (Fig. 4a).

Plasma Cu clearance after intravenous injection has been described for non-acclimated rainbow trout, with an elimination half-life of 195.5 min (Carbonell and Tarazona, 1994).

The Cu concentrations in the plasma of control (A) and acclimated rainbow trout (C) (Fig. 3b and Table 2) agrees well with control values reported for tilapia, *Oreochromis mossambicus* (Pelgrom et al., 1995), freshwater adapted flounder *Platichtys flesus* (Stagg and Shuttleworth, 1982) and coho salmon (Buckley et al., 1982). Initially increased and then normalised total plasma Cu concentrations in response to Cu exposure have also been reported for coho salmon exposed to a much higher (140 μg of Cu per liter) concentration (Buckley et al., 1982). In other species, however, there seems to be no complete recovery to normal plasma Cu concentrations during Cu exposure; the flounder was found to have continuously increased plasma Cu concentrations after 37 days of exposure to 15 μg of Cu per liter and tilapia had elevated plasma Cu concentrations after 6 days of exposure to 50–200 μg of Cu per liter (Pelgrom et al., 1995).

In the dorsal aortic catheter experiment, the new Cu accumulation in the gills of acclimated rainbow trout (B) was the same as in the non-acclimated (C) (Fig. 4a). This agrees with findings for the European eel, which takes up ^{64}Cu in the gills at a constant rate during 28 days of Cu exposure (Grosell et al., 1996). In the terminal sampling experiment (Fig. 5), the acclimated fish (E) had 34–39% less newly accumulated Cu in the gills than did the non-acclimated fish (D). In both these cases, the up-to 63% reduction in newly accumulated Cu in the plasma must also be the result of an increased plasma Cu clearance rate. The gills reach steady state with respect to Cu concentration within the first 3 h of exposure (Table 2), with an initial peak in the new accumulation of Cu. At steady state, the net Cu-transport from the gills across the basolateral membrane to the blood must equal the net Cu-uptake at the apical membrane. This means that the new Cu accumulation rates in the gills prior to steady state (assumed to be equal to uptake rate) can express the Cu uptake by the fish at steady state (Grosell et al., 1996). Steady-state conditions in the gills of

fish exposed to Cu have been reported previously (Buckley et al., 1982; Laurén and McDonald, 1987a; Grosell et al., 1996).

During the first 3 h of exposure, the new accumulation of Cu in the gills is assumed to be equal to the total branchial Cu influx which, at steady state is equal to the further Cu transport from the gills to the rest of the fish. Fig. 5 shows an apparently higher branchial Cu uptake in the non-acclimated fish (D) relative to the acclimated fish (E). This higher uptake could be due to a binding of ^{64}Cu to binding sites in the gills of non-acclimated fish (D), that are not available in the acclimated fish (E) because they are occupied by slowly exchangeable stable ^{63}Cu. Such a mechanism would lead to overestimation of the branchial Cu uptake in the non-acclimated fish (D).

More detailed studies of the gill-Cu binding kinetics in non-acclimated and Cu-acclimated fish are needed in the future to obtain a more detailed picture of the role of branchial Cu uptake in Cu acclimation.

The increase in total Cu concentration of the gills of non-acclimated (B) fish during 65 h of exposure (Fig. 4b) can be explained by the level of newly accumulated Cu obtained in the same period (within the variation indicated by the error bars; Fig. 4a). The newly accumulated Cu in the gills of the acclimated fish (C), however, is not associated with any further increase in the total Cu concentrations. The exchangeable Cu pool (newly accumulated [Cu] per total [Cu]) in the gills of the acclimated fish can thus be calculated to be 54% after 65 h of ^{64}Cu exposure, which is in close agreement with the value found by Laurén and McDonald (1987b) for juvenile rainbow trout adapted to 55 μg of Cu per liter for 28 days (52.8% after 24 h of ^{64}Cu exposure).

The increase in the total Cu concentrations in the livers of the non-acclimated fish (B) (Fig. 4b) can almost be explained by the newly accumulated Cu (within the indicated variation) (Fig. 4a), which shows that the method used to calculate newly accumulated Cu is realistic in this case. In the acclimated fish (C), however, as in the gills, the newly accumulated Cu in the liver is not reflected by an increased total Cu concentration, which means that there is a high turnover of hepatic Cu in the acclimated fish (C). In this case, 33% of the hepatic Cu pool is exchangeable within 65 h (newly accumulated [Cu] per total [Cu]) and potentially available for excretion. This is in contrast to the values of only 1.4% within 24 h reported by Laurén and McDonald (1987b) for juvenile rainbow trout exposed to 55 μg of Cu per liter for 28 days. The values reported by Laurén and McDonald (1987b), however, are based on calculations using the specific activity of water Cu and do not account for the dilution of the external ^{64}Cu as it enters the gills and the plasma Cu pools. They thus underestimate the exchangeable hepatic Cu pool and the potential Cu excretion.

The hepatic metallothionein concentration is shown to be 46% higher in the acclimated fish than in the controls. Using a factor of four to correct for the difference between wet and dry weight and 10:1 for maximum copper content of MT (Kille et al., 1992), we can state that, at most, 14–17% of the hepatic Cu can possibly be bound to hepatic MT in the two groups (A) and (C). In a previous study, the percentage of hepatic Cu bound to MT in both non-exposed and Cu-

exposed rainbow trout varied between 20 and 30% (Hogstrand, 1991). The differences in the results can partly be accounted for by the lower hepatic MT levels found in the fish from the present study. In any case, in the present investigation, MT seems to be of minor importance for the Cu handling during adaptation to waterborne Cu.

Hepatic Cu elimination is evident since there were considerable amounts of Cu present in the bile (Figs. 4a and 4b). The highest total Cu concentration in the bile was found in the controls (A) (Fig. 4a) and the total Cu concentration in the non-acclimated fish (B) (Fig. 4b) was only 30% of the control value. The total Cu concentration in the bile of the non-acclimated fish (B) can be completely accounted for by the newly accumulated Cu (Fig. 4a). This was not the case with the acclimated fish (C) where only 32% of the total Cu concentration in the bile can be explained by the newly accumulated Cu (Figs. 4a and 4b). This difference means that in the acclimated fish (C) the Cu present in the liver prior to ^{64}Cu exposure was excreted to a higher degree than the newly accumulated Cu. The total Cu concentration, and thereby, the potential Cu excretion via the bile of the acclimated fish (C) was twice as high as in the non-acclimated (B), but in both cases lower than in the controls (Fig. 4b).

In the kidney, there was no increase in the total Cu concentration, either in the non-acclimated (B) or the acclimated fish (C). On the contrary, there was a significantly reduced total Cu concentration in both groups compared to the value found in the control (A) (Fig. 4b). Surprisingly, the kidneys of both groups (B) and (C) showed a relatively high new accumulation of Cu (Fig. 4a), actually exceeding the total Cu concentration found in the very same samples (Fig. 4b). This evident overestimation can be explained by the way the newly accumulated Cu is calculated, based on the assumption that the plasma Cu pool is in total equilibrium and that the plasma ^{64}Cu activity represents all plasma Cu equally. These assumptions are evidently not the case for the new Cu accumulation in the kidney, although it seemed to be so for the liver. It means that only a fraction of the Cu found in plasma was available to the kidney and that this fraction comprised a relatively large part of the newly accumulated Cu found in the plasma. Regardless of the overestimation of newly accumulated Cu in the kidney, it is evident that the newly accumulated Cu entering the kidney replaced some of the Cu already present in the kidney, since there is no increase in total Cu concentration. This means that there could, in fact, be substantial renal Cu excretion.

The conclusion that can be drawn from these results is that the acclimation of rainbow trout to elevated ambient [Cu] involves an increased clearing and turnover of plasma Cu which leads to rapidly normalised concentrations even during Cu exposure.

The branchial Cu accumulation results were not unambiguous and do not conclusively show that acclimation has any effect on the further Cu-uptake

In addition to this, acclimated rainbow trout have a high turnover of both hepatic and renal Cu suggesting both hepatic Cu elimination and renal Cu excretion.

Acknowledgements

This study was supported by a Danish research Academy grant to M.H.G. and by an NSERC Canada strategy grant in environmental quality to C.M.W.

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