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PHYSIOLOGICAL EFFECTS OF CHRONIC COPPER EXPOSURE TO RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IN HARD AND SOFT WATER: EVALUATION OF CHRONIC INDICATORS

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Abstract—Effects of chronic copper exposure on a suite of indicators were examined: acute toxicity, acclimation, growth, sprint performance, whole-body electrolytes, tissue residues, and gill copper binding characteristics. Juvenile rainbow trout were exposed for 30 d to waterborne copper in hard water (hardness = 120 mg/L as $CaCO_3$, pH = 8.0, Cu = 20 and 60 μg/L) and soft water (hardness = 20 mg/L as $CaCO_3$, pH = 7.2, Cu = 1 and 2 μg/L). Significant acclimation to the metal occurred only in fish exposed to 60 μg/L, as seen by an approx. twofold increase in 96-h LC50 (153 vs 91 μg Cu/L). Chronic copper exposure had little or no effect on survival, growth, or swimming performance in either water hardness, nor was there any initial whole-body electrolyte loss (Na^+ and Cl^-). The present data suggest that the availability of food (3% wet body weight/day, distributed as three 1% meals) prevented growth inhibition and initial ion losses that usually result from Cu exposure. Elevated metal burdens in the gills and livers of exposed fish were measures of chronic copper exposure but not of effect. Initial gill binding experiments revealed the necessity of using radiolabeled Cu (^{64}Cu) to detect newly accumulated Cu against gill background levels. Using this method, we verified the presence of saturable Cu-binding sites in the gills of juvenile rainbow trout and were able to make estimates of copper-binding affinity (log $K_{gill=Cu}$) and capacity (B_{max}). Furthermore, we showed that both chronic exposure to Cu and to low water calcium had important effects on the Cu-binding characteristics of the gills.

Keywords—Copper Rainbow trout Toxicity

INTRODUCTION

The acute toxicity of copper to freshwater fish has been well characterized [1–3]. Published values for 96-h LC50s range from as little as 10 μg/L to 10,000 μg/L total copper. Once species sensitivity differences are accounted for, most of the variation can be attributed to differences in water chemistry (particularly hardness) and secondarily to body size [2]. For example, Howarth and Sprague [4] reported that the concentrations of total dissolved copper that produced 50% mortality in 96 h (96-h LC50) for rainbow trout ranged from 20 μg/L in soft acidic water to 520 μg/L in hard alkaline water. In the same study, 10-g trout were 2.5 times more tolerant to Cu than were 0.7-g trout [4].

The effects of chronic copper exposure in fish have also been well documented in the literature [5–9] and include a whole variety of biochemical and physiological indicators of which growth and ionoregulation are the most prominent. However, there is as yet no consensus as to the ranking of these effects in order of sensitivity. An early ranking by Spear and Pierce [2] in 1979 is now of limited value because it was drawn from several studies that each measured only a few indicators and where there was no standardization of Cu exposure (e.g., age of the organism, water quality, diet/nutritional status, temperature changes). Furthermore, there are at least three newer indicators of copper exposure that are worthy of inclusion in any rank order. These indicators are tissue Cu residues, the presence/absence of acclimation, and gill Cu-binding characteristics. Tissue residues have proven to be a useful indicator of copper exposure in field-collected fish [6,10]. Gill metal-binding characteristics have been used to relate the fractional saturation of high-affinity metal-binding sites on gills directly to acute toxicity—the so called Biotic Ligand Modeling (BLM) approach [11–15]—and may, in turn, be a sensitive indicator of chronic metal exposure. Acclimation can be used as a chronic indicator since it probably reflects copper exposure at a concentration that at least initially was sufficient to induce damage: the damage-repair hypothesis [16].

Thus, the objective of the present study was to document the effects of chronic copper exposure on a larger suite of indicators than has previously been attempted and to propose a new rank order of the effects of copper [2]. The indicators used were acute toxicity, acclimation, growth, sprint performance, whole-body electrolytes, tissue residues, and gill copper-binding characteristics. Exposure concentrations were chosen in relation to initial 48- or 96-h LC50s and were based on the hypothesis that some mortality or physical damage is necessary to elicit compensatory mechanisms [16]. To modify Cu bioavailability, the exposures were conducted in hard and soft water. The hard-water exposure provided an abundance of the necessary cations (Ca^{2+} and Mg^{2+}) to compete with Cu for binding sites on the gill and anions (HCO_3^-) to compete with gill sites for complexation of copper [11]. The exposure in soft water provided an environment low in such ions and thus increased Cu toxicity. Exposure to soft water alone also provided a physiological challenge to fish acclimated to hard water [17].

MATERIALS AND METHODS

Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*, 1–2 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON,

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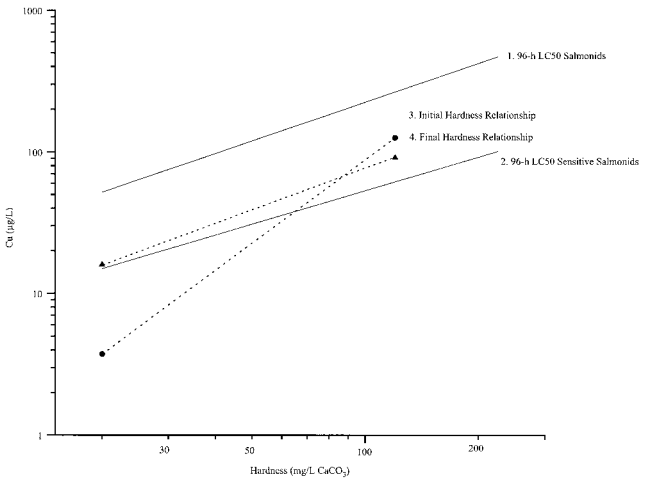

Fig. 1. The effect of hardness on acute toxicity of Cu to rainbow trout (dotted lines, present study) and other salmonids (solid lines, from [2]). Initial hardness relationship (line 3) was determined either in normal hard water (48-h LC50; 126.0 [88.0–167.0] μg/L) or after two weeks of acclimation to soft water (96-h LC50; 3.74 [0.96–5.23] μg/L). The final hardness relationship (line 4) was determined either in normal hard water (96-h LC50; 91 [65.9–107.6] μg/L) or after 12 weeks acclimation to soft water (96-h LC50; 15.9 [13.3–22.0] μg/L).

Canada) and maintained in either dechlorinated Hamilton tap water of moderate hardness (14°C, pH 8, Na^+ 13.8, Cl^- 24.8, Ca^{2+} 40.0, dissolved organic carbon [DOC] 3, hardness 120, alkalinity 95, all in mg/L) or soft water (17°C, pH 7.2, Na^+ 3.0, Cl^- 3.5, Ca^{2+} 5.2, DOC 0.4, hardness 20, alkalinity 15, all in mg/L) for a minimum of two weeks before experimentation. The 3°C temperature difference between the two experiments reflect ambient Lake Ontario water temperatures in winter and summer. Soft water was synthesized by mixing one part hard water to six parts ion-reduced water, the latter produced by reverse osmosis (Anderson Water Systems, Dundas, ON, Canada). Photoperiod was set to a light/dark cycle similar to the natural photoperiod for Western Lake Ontario.

Experimental protocol

Copper exposure in hard water. Fish were exposed to two Cu concentrations (20 and 60 μg/L), with a control (background Cu ~3 μg/L) in a flow-through system for a minimum of 30 d. These concentrations were based on initial rangefinder 48-h median lethal concentration tests (LC50; Fig. 1) using naive fish and were chosen to produce some mortality in the high Cu concentration and no mortality in the lower exposure. The high- and low-exposure concentrations correspond to approximately half and one-sixth of the initial 48-h LC50 for Cu.

Fish were fed a dry ration of commercial trout pellets at a rate of 3% wet body weight/day, distributed as three meals of 1%/day for the entire period of exposure. The feed contained ~3 μg/g Cu dry weight (Martins Feed Mill, Elmira, ON, Canada). Growth was monitored weekly through bulk weighing of fish in individual tanks, and the meal size was adjusted as the fish grew to maintain the 3% ration. Copper stock ($CuSO_4 \cdot 5H_2O$; Fisher Scientific, Toronto, ON, Canada) was metered into diluent water in two mixing head tanks. These tanks, in turn, fed two separate 200-L tanks at a rate of 1500 ml/min to each. Each exposure tank was aerated and initially contained 280 fish. Feces and organic matter were siphoned from the tanks daily. Copper concentrations in the fish tanks were measured 12 times throughout the exposure (Table 1). At days 2, 10, 20, and 30, a subsample ($N = 10$) of fish from each tank (five fish per tank, two tanks per concentration) were sacrificed. Gills were excised and rinsed for 10 s in clean control water to displace any surface-bound Cu and were immediately frozen. Livers were also removed and together with the remaining carcass were frozen for subsequent metal-burden and electrolyte analysis.

Table 1. Nominal and actual water copper concentrations (mean ± standard evaluation [*N*]) throughout the 30-d exposure period

	Nominal (μg/L)	Actual (μg/L)
Hard Water		
Control	0	2.95 ± 1.05 (12)
Low copper exposure	20	19.89 ± 1.76 (12)
High copper exposure	60	62.60 ± 1.73 (12)
Soft water		
Control	0	0.65 ± 0.24 (9)
Low copper exposure	1	1.05 ± 0.13 (18)
High copper exposure	2	1.73 ± 0.24 (16)

Copper exposure in soft water. This exposure used the same flow-through system, feeding regime, and growth monitoring as outlined previously. The two exposure concentrations of Cu were 1 and 2 μg/L based on an initial 96-h LC50 rangefinder using naive fish acclimated to soft water for two weeks (Fig. 1). The trout had been acclimated to soft water for a total of seven weeks at the start of the exposure. The Cu exposures correspond to approx. one-half and one-quarter of the lethal concentration, respectively. A control (background Cu ~ 0.7 μg/L) was also run simultaneously with the copper-exposed fish. Each tank held 225 fish, and fresh soft water was supplied to the exposure tanks at a rate of 500 ml/min and a temperature of 17°C. Water and tissue sampling was the same as in the hard-water exposure except that three instead of five fish were sampled from each tank per interval ($N = 6$ for each concentration). Also, three intact whole bodies from each tank were sampled and frozen for subsequent metal-burden and electrolyte analysis.

Analytical techniques

Sample analyses. Tissue samples were weighed and digested in five volumes of 1N HNO_3 (Fisher Scientific; trace metal analysis grade) for 3 h at 80°C. Copper was measured in whole bodies, liver, and gills after appropriate dilution of the supernatant with reagent-grade deionized water, using flame and graphite furnace atomic absorption spectrophotometry (Varian AA-1275, Varian, Walnut Creek, CA, USA) against Fisher Scientific–certified copper standards. Whole-body Cu burdens in hard water were calculated by adding the removed tissues and the carcass metal content. Sodium was analyzed from whole-body digests following the same process as Cu. Chloride was determined from whole-body digests without further dilution by titration with a Radiometer-Copenhagen CMT-10 chloridometer (Copenhagen, Denmark). Water samples (15 ml) were acidified (50 μl concentrated HNO_3) and analyzed for Cu by either flame or graphite atomic absorption spectrophotometer, as appropriate.

Sprint performance. Fixed velocity sprint tests were conducted according to procedures described by McDonald et al. [18] using a 100-L swim flume constructed of nontoxic poly-

ethylene vinyl chloride. Fish were not fed on the day of sprint trials and were transferred in batches of 10 to the flume containing either hard or soft control water. Water temperature in the swim flume was identical to the exposure system from which fish were removed. After an initial orientation period of 5 min at 1 body length (BL)/s, water velocity was increased over 2 min to 6 or 8 BL/s in soft and hard water, respectively. Fish were removed when they became exhausted, and time to fatigue, fork length, and fish weight were recorded. Fish were considered exhausted when they became impinged on the back screen and would not swim after being manually reoriented toward the current.

Gill Cu-binding characteristics. Three different approaches were used to measure short-term Cu uptake by the gills in vivo: 3-h exposure to hard-water Cu solutions ranging in concentration from 30 to 1,050 μg/L Cu ($N = 6$ per exposure), 0.5- to 2-h exposures to hard-water Cu solutions ranging from 120 to 430 μg/L Cu ($N = 4$ per exposure and time interval) with radiolabeled ^{64}Cu (specific activity 3.9–16.1 KBq/μg), and 3-h exposures to ^{64}Cu-labeled solutions ranging from 2 to 30 μg/L Cu (specific activity of 79–407 KBq/μg) in hard water ($N = 4$ per exposure) and 1 to 17 μg/L (specific activity of 73–578 KBq/μg) in soft water ($N = 10$ per exposure). McMaster Nuclear Reactor, Hamilton, Ontario, Canada, supplied the ^{64}Cu.

The first approach was derived from the method of Playle et al. [19] for measuring gill surface metal binding in vivo in soft water. An exposure time of 3 h was used because Playle et al. [19] found that gill Cu levels in vivo had reached equilibrium at this time. Relatively high Cu levels were used to allow detection of gill Cu above background levels and also to ensure that sufficient free Cu^{2+} ion was available in the water for binding to the gills in hard water. Background gill Cu (gill Cu before exposure) was subtracted from the amount found on the gill after the 3-h exposure to determine the newly accumulated copper. The second approach was employed to increase the resolution of gill Cu measurement in hard water. The third approach was used because the first two approaches did not reveal saturable gill binding sites on the gills. Radioactivity in gill tissues, whole bodies, and acidified water samples was measured in a well-type gamma counter with a 7.62-cm NaI crystal (Packard Minaxi Auto-Gamma 5000 Series, Packard Instruments, Meridan, CT, USA).

Calculations

Growth. Growth was calculated from periodic measurements of bulk weights. The data were best represented by the exponential curve:

$$\text{wt(g)} = ae^{bt}$$

where t = time (days), a = length (cm), and b = growth coefficient expressed as %/day. The 95% confidence limits for b were calculated using the statistical package SPSS for Windows, Release 8.0.0.

Swimming performance. The following steps were used to calculate fatigue times in the sprint tests according to procedures outlined in McDonald et al. [18]. Sprint times were first sorted in ascending order against rank. Time was converted to log time, and rank was converted to percentage fatigue and then to probit fatigue. A linear regression of probit fatigue (X) versus log time (Y) yielded slope ± 95% CL and intercept ± 95% CL for the line. These data were used to calculate the time to 50% fatigue with 95% confidence limits.

Gill Cu binding. Newly accumulated copper concentrations in the gill were calculated based on the accretion of radioactivity in the gill:

$$\text{Cu}_{\text{gill}} = \frac{^{64}\text{Cu}_{\text{gill}}}{(\text{W} \times \text{SA})}$$

where $^{64}\text{Cu}_{\text{gill}}$ = radioactivity of the gill in KBq/min, W = gill mass (μg), and SA = specific activity of the water in KBq/μg.

Statistics

All data presented are means ± 1 standard error (SE) except for LC50, growth, and swim performance, where means and 95% confidence limits are given. For the exceptions, a Student's *t* test (two-tailed, unpaired) was used to test for significant differences, employing a Bonferroni adjustment for multiple comparisons to the *t* value. The LC50 values were determined by linear functions relating the log concentration of copper to probit transformation of percentage mortality (U.S. Environmental Protection Agency, Washington, DC). The Probit Analysis Program, Version 1.5, was used for calculating lethal concentration/effect concentration values. All other data were analyzed for statistical significance by analysis of variance followed by a Student–Newman–Keuls ranking test for means of equal and unequal sample size. Significance was set at $p < 0.05$.

RESULTS

Acute toxicity

Based on published LC50 data for salmonids (Fig. 1), our rainbow trout population was particularly sensitive to Cu, especially in soft water. Both our hard-water and soft-water LC50s for control fish were well below the incipient lethal concentrations for other rainbow trout (Fig. 1, line 1), and in soft water were even lower than the most sensitive salmonid species (Fig. 1, line 2), as reported by Spear and Pierce [2].

Furthermore, while we confirm that Cu was more toxic in soft water than in hard water, the effect of hardness was much greater in our study than in previous studies. Copper was approximately 20 times more toxic in soft water than in hard water for 1- to 2-g trout, whereas the literature predicts only a fivefold difference (Fig. 1, line 1 or 2). However, after 10 weeks of further acclimation in soft water, trout were only six times more sensitive to Cu in soft water than in hard water.

Chronic toxicity

Mortality. In hard water with 60 μg/L Cu, 3% of the fish died during the exposure period, whereas the controls and the fish exposed to 20 μg/L Cu had exhibited less than 0.5% mortality. Most of the Cu-induced mortalities occurred within 5 d of exposure to copper in hard water. In contrast, in soft water, mortality was 10% after 15 d of exposure in all three treatments (independent of Cu exposure) and continued at a low rate until day 30.

Acclimation. In hard water, only the 60-μg/L-Cu group showed significant acclimation to Cu after 30 d of exposure. These fish were approx. 1.7 times more resistant to Cu than were control fish, with a 96-h LC50 of 153.0 μg/L (95% confidence limits, 147.8–165.0) versus a 96-h LC50 of 91.0 μg/L (65.9–107.6). In contrast, neither exposure to Cu in soft water resulted in a significant increase in copper tolerance: The LC50s for trout in soft water averaged 16 μg/L after 30 d of exposure.

Table 2. Growth (specific growth rate, %/day) and swimming performance (time to 50% fatigue, s) after 30-d exposure to Cu. Specific growth rates (SGR) were calculated from weekly bulk weight measurements (hard water $N = 4$, soft water $N = 7$). Sprint performance was measured at 8 and 6 body lengths per second, which corresponded to 57 cm/s in hard water and 60 cm/s in soft water ($N = 20$). Average fish weight and length in soft water was 14.35 ± 0.84 g and 10.68 ± 0.19 cm (mean ± SE, $N = 60$). In hard water, average weight and length was 3.73 ± 0.15 g and 6.67 ± 0.09 cm (mean ± SE, $N = 60$). Included in parentheses are the 95% confidence limits. NS = SGR values of Cu-exposed fish statistically similar ($p > 0.05$) to control value

Exposure	Specific growth rate (%/d)	Time to 50% fatigue (s)
In hard water		
Control	2.88 (2.4–3.4)	224 (196–256)
20 μg/L	2.85 (2.4–3.3) NS	282 (236–337)
60 μg/L	3.07 (2.7–3.4) NS	330 (278–392)
In soft water		
Control	3.08 (2.5–3.6)	134 (113–159)
1 μg/L	3.22 (3.0–3.5) NS	201 (169–239)
2 μg/L	3.36 (3.1–3.6) NS	271 (215–340)

Performance measures

Growth. Even though our fish were particularly sensitive to copper in terms of acute toxicity, overall growth of the survivors was not affected throughout the metal exposure in either hard or soft water (Table 2). There was also no evidence of an initial growth reduction during the first 10 d of Cu exposure. Growth rates in hard water ranged from 2.9 to 3.1%/day from a starting weight of 1 to 2 g and from 3.1 to 3.4%/day in soft water from a larger starting weight of 5 to 6 g. The slightly higher growth rate in soft water was probably due to the 3°C higher temperature.

Sprint performance. Sprint performance was not impaired by Cu exposure in either hard or soft water (Table 2). In fact, fish exposed to Cu sprinted for longer times than unexposed fish.

Condition measures

Whole-body electrolytes. Immediately before Cu exposure, whole-body Na^+ and Cl^- concentrations were identical between hard- and soft-water fish (Na^+ and $Cl^- \sim 50$ and 40 μM/g, respectively). Upon Cu exposure, there was no initial or subsequent decline in whole-body electrolytes in either hard or soft water.

Tissue residues. Before Cu exposure, the body Cu content and its distribution were similar in both hard- and soft-water fish. The liver had the highest levels of Cu (up to 15 μg Cu/g wet tissue in controls) of any tissue measured, which represents 10 and 22% of the whole-body content (0.78 μg Cu/g) in hard and soft water, respectively. Gill Cu was lower than liver Cu and was virtually the same in hard- and soft-water fish (1.4% of the whole-body content) despite the fact that background gill Cu in hard water was sixfold higher than in soft water (3.0 vs 0.5 μg/L).

Gills of fish exposed to 60 μg/L copper in hard water had significantly elevated Cu at all times starting at day 2 (Fig. 2). In contrast, gill Cu of fish exposed to 2 μg/L in soft water were only significantly higher than controls at day 30. In general, gill Cu burdens reflect the exposure Cu concentrations (fourfold higher Cu burden in hard water vs soft water at the highest Cu exposure level; Fig. 2).

In contrast, the livers did not show this difference between hard and soft water, with ~35 μg Cu/g wet tissue at the highest Cu exposure concentrations (Fig. 3). Nonetheless, the liver burdens did increase over time with Cu exposure within each water chemistry. In hard water, liver Cu of the fish exposed to 60 μg/L was significantly elevated at days 2, 20, and 30. Livers in the 2-μg/L soft-water treatment showed significantly higher Cu content only at days 20 and 30. Although the gills and livers accumulated Cu, the amounts were small enough to have no detectable effect on whole-body Cu above the increase seen with growth. In hard and soft water at day 30, the whole-body Cu burden in all fish averaged 1.1 and 1.7 μg Cu/g wet tissue, respectively.

Gill Cu-binding characteristics

Naive fish. Hard-water-acclimated fish, naive to Cu and exposed for 3 h to cold copper (30–1,050 μg/L, no ^{64}Cu), showed no detectable accumulation of Cu on their gills (beyond background levels) with exposures up to 185 μg Cu/L (Fig. 4A). Only at 1,050 μg Cu/L was there significant accumulation (~ sixfold vs background of 0.5 μg/g). The second experiment using ^{64}Cu (120–430 μg/L; Fig. 4B) was able to detect the accumulation of new Cu at 120 μg Cu/L and showed that Cu accumulation was approximately linear over the range of Cu employed at each of the exposure times tested (0.5, 1, and 2 h). However, the amount bound to the gills at each concentration also increased with time (Fig. 4B), suggesting either that the loading of Cu on surface sites was slow or, more likely, that Cu was going beyond surface sites to enter the gill tissue.

Only in the third experiment (2–25 μg Cu/L) did we see clear evidence of saturable binding of copper to the gills in both hard and soft water (Fig. 4C). The binding sites were high affinity and low capacity (but higher capacity in soft water compared to hard water) and saturated at ~15 μg/L Cu. Indeed, at only slightly higher Cu (~23 μg/L) in hard water was there a sharp increase in gill Cu, suggesting the appearance of a second type of binding on (or in) the gills. In fact, the value at 23 μg/L fits directly on the line established in the second experiment for gill binding by 2 h (Fig. 4B).

The binding characteristics of the high-affinity sites (binding capacity, B_{max} and affinity, $K_{gill=Cu}$) were approximated as follows. First, the B_{max} values in hard and soft water were estimated visually to be 0.038 and 0.12 μg Cu/g, respectively (the point on the y-axis where the concentration of Cu on the gill does not increase when the water Cu concentration is increased; Fig. 4C). Second, a Langmuir adsorption isotherm,

$$\text{Gill Cu} = \frac{B_{max} \times [\text{Cu}]}{(K_{gill=Cu} + [\text{Cu}])}$$

was fitted to the data using the estimated values for B_{max} (μg/g), [Cu] = total dissolved copper (μg/L), and gill Cu (μg/g). Finally, the binding affinity ($K_{gill=Cu}$) was determined from the isotherms as the concentration of total [Cu] needed to produce half-saturation of binding sites. This method yielded $K_{gill=Cu}$ of 2 and 1 μg/L Cu for hard and soft water, respectively.

Effects of chronic Cu exposure. Chronic Cu exposure in hard water had distinct effects on gill Cu dynamics. Chronic gills accumulated more Cu than naive gills when gills were challenged with Cu concentrations substantially above acutely toxic levels (an increase in gill Cu binding capacity; Fig. 5A). For example, at an acute Cu of 185 μg/L, the chronic gills (chronically exposed to 20 and 60 μg Cu/L) exhibited net Cu accumulation of 0.63 ± 0.18 and 0.86 ±0.26 μg/g, respectively

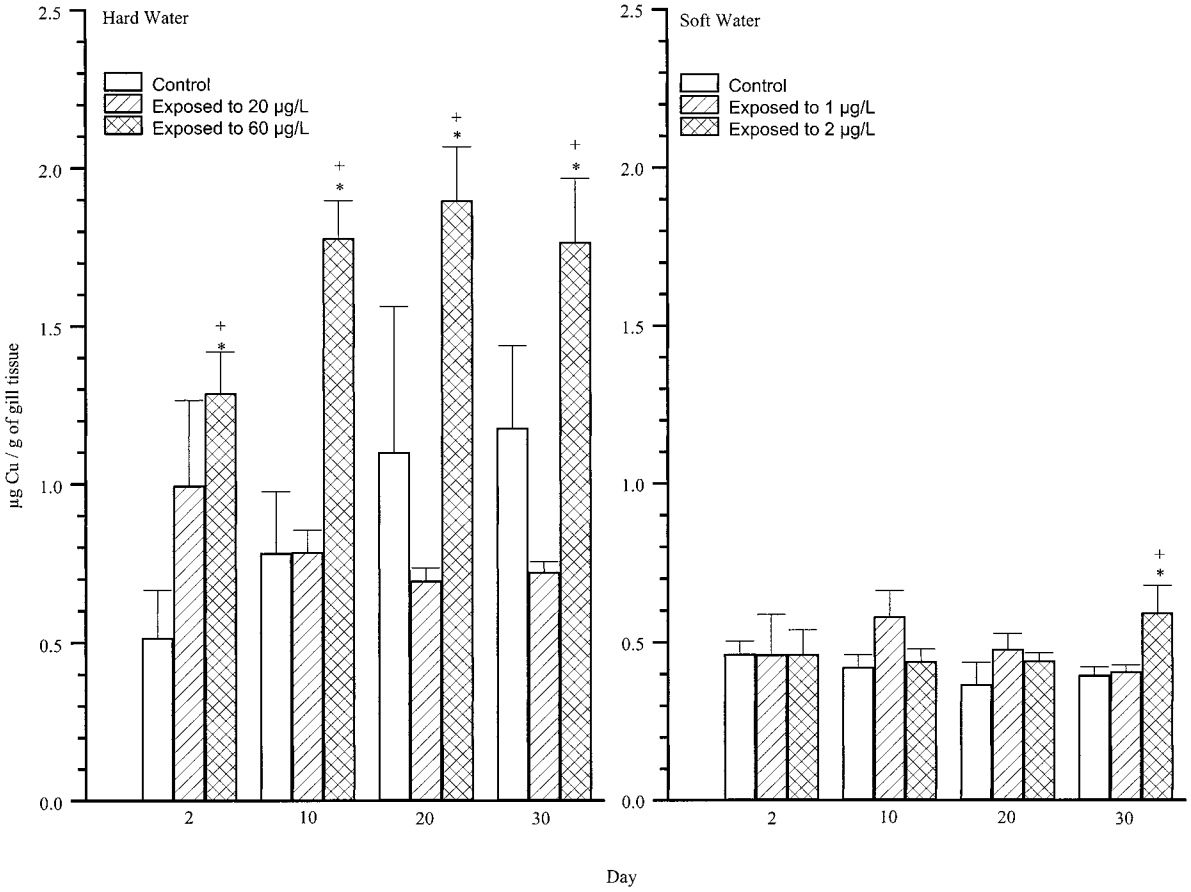

Fig. 2. Gill Cu accumulation (µg/g wet tissue, means ± one standard error) in hard water (N = 10 fish/day) and soft water (N = 6) over 30 d of exposure. * = significant Cu accumulation when compared to respective controls at day 2 ($p < 0.05$). + = significant accumulation when compared to other treatments on the same day ($p < 0.05$).

(a doubling of Cu over background levels), while the naive gills showed no net accumulation (Fig. 5A). However, when challenged at near chronic levels, this effect was less dramatic (more so at 60 than at 20 µg/L; Fig. 5B). For example, at 16 µg/L the chronic gills (60 µg/L) accumulated about 1.5 times the amount accumulated by naive gills (an increase in binding sites; Fig. 5B). In contrast, the 20-µg/L exposure group accumulated the same amount as naive gills at 16 µg/L (no increase in binding sites, although the data suggest a lower affinity).

In soft water, a similar pattern was seen, particularly at the 2-µg/L chronic level (Fig. 5C). Here, there was a substantial alteration in gill Cu-binding properties from saturable to apparently linear (R^2 = 0.940) over the range of [Cu] tested. This change is consistent with a lowering of affinity and an increase in capacity or number of binding sites on or in the gills. The reduction of affinity is well illustrated at the 7-µg/L exposure concentration, where there was a ~40% reduction in newly accumulated Cu in fish preexposed to 2 µg Cu/L relative to naive fish.

DISCUSSION

Because we measured a suite of different indicators in the same study, we can now propose a new rank order for the chronic effects of copper, one substantially different from that proposed originally by Spear and Pierce [2]. We rank the sensitivity of each indicator by considering the number of statistical differences between Cu-exposed and control fish in the four exposure regimes considered (two Cu concentrations and two water hardness levels). By this criterion, the least sensitive indicators are growth, sprint performance, and initial electrolyte loss (no effect in any treatment group). The others, in order of increasing sensitivity are acclimation (increased lethal tolerance, one out of four, difference being statistically significant), increased gill and liver Cu burdens (two out of four), and changes in gill copper-binding characteristics (three out of four). However, this new rank order must be considered conditional because of two unusual responses exhibited by our rainbow trout compared to previous studies on the same species: a much greater sensitivity to acutely toxic copper (Fig. 1) but a lower chronic sensitivity to copper.

Acute sensitivity to copper

In their review of the acute toxicity literature extant in 1979, Spear and Pierce [2] concluded that the Cu sensitivity of most salmonids, including rainbow trout, could be captured in a single equation relating 96-h LC50 to water hardness (Fig. 1, line 1). Our results, in contrast, deviated significantly from this line in two respects (Fig. 1, line 3): a threefold greater sensitivity in typical hard waters (120 mg/L as $CaCO_3$) and 20-fold greater sensitivity in very dilute soft waters (20 mg/L). We attribute the higher acute sensitivity in hard water to strain variance [20]. In any case, the acute sensitivity that we observed in hard water is at least comparable to that of a second group of salmonids identified by Spear and Pierce [2] (sensitive salmonids, line 2). The much greater sensitivity of our fish in

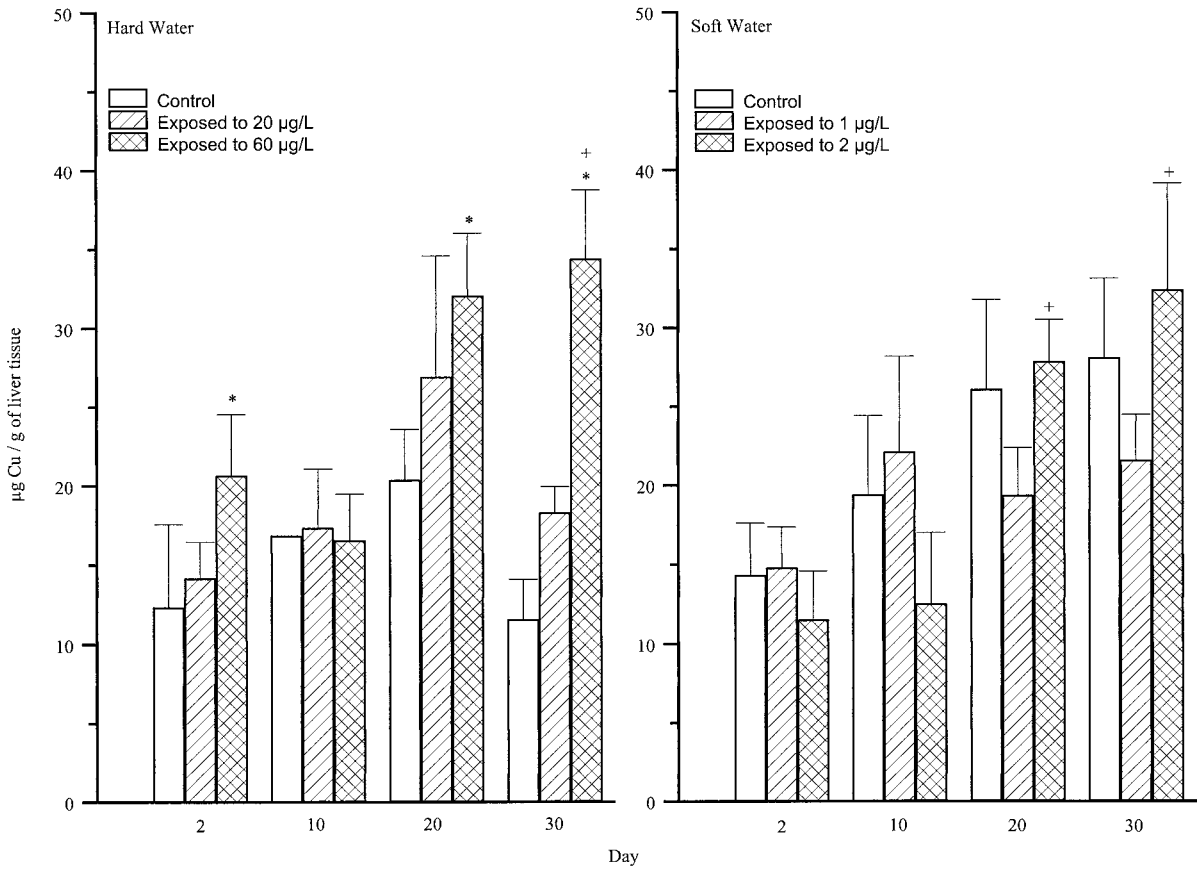

Fig. 3. Liver Cu accumulation (µg/g wet tissue, means ± one standard error) in hard water (N = 10 fish/day) and soft water (N = 6) over 30 d of Cu exposure. * = significant Cu accumulation when compared to respective controls at day 2 ($p < 0.05$). + = significant accumulation when compared to other treatments on the same day ($p < 0.05$).

soft water we attribute mainly to incomplete soft-water acclimation, as illustrated (line 3).

Sensitivity to soft water

Previous studies, including our own, have shown that acute exposure of hard-water–acclimated trout to soft water by itself produces physiological disturbances that consist of depression in whole-body electrolytes followed by gradual recovery [17]. More important, acute soft-water exposure can substantially amplify the effects of toxicant exposure, especially if the toxicant targets ionoregulation [21]. For this reason, prior acclimation to soft water is usually recommended in toxicity studies. There seems to be a consensus that a two-week period of acclimation is long enough [7,22], but no study has yet documented how much time is required for acclimation to be complete. Indeed, Erickson et al. [23] found that two weeks of acclimation to soft water had no effect on the relationship between hardness and acute (96-h LC50) Cu toxicity in fathead minnows, suggesting that soft-water acclimation either was unnecessary in this species or had not even begun. In any case, the soft water used in their study had almost six times the hardness of our soft water (~120 vs 20 mg/L as $CaCO_3$), and we believe that the time for soft-water acclimation lengthens with decreasing hardness.

After two weeks of soft-water acclimation, the initial hardness relationship (Fig. 1, line 3) was much more pronounced (slope of line 3 was 6.1 vs 2.0 for line 1), suggesting that soft-water acclimation was incomplete in our fish but was apparently complete by 12 weeks (line 4, slope 3.7) by the same criterion. However, in the 10 weeks between the initial and the final LC50, the fish had grown from 1.5 to 19 g. Although Cu sensitivity is known to decrease with increasing size, this increase in mass can probably explain only about one-half of the reduced sensitivity based on the size–toxicity regression of Howarth and Sprague [4] for juvenile rainbow trout exposed to copper.

Sublethal Cu exposures in soft water started after seven weeks of soft water acclimation. Whether soft-water acclimation was complete as this point is uncertain. On the one hand, whole-body electrolyte levels at this time were the same as those for hard-water–acclimated fish, which is one criterion for acclimation [17]. On the other hand, the presence of residual mortality in all soft-water groups (including controls) suggests a continuation of soft-water stress in this size of rainbow trout. Thus, we conclude that rainbow trout (of this size range, <20 g), require a much longer period of acclimation to soft water than two weeks, particularly at very low hardness levels.

Analyses of chronic effects (from least to most sensitive)

Growth inhibition. Sublethal toxic effects of Cu and subsequent acclimation (increased tolerance) to Cu are consistent with a damage-repair model [16]. This model includes an initial damage phase during which the pathophysiological effects of the metal are expressed and reach maximal values, followed by a repair phase with attendant bioenergetic cost during which the effects diminish and may even disappear. The repair phase is commonly accompanied by an increase in lethal tolerance

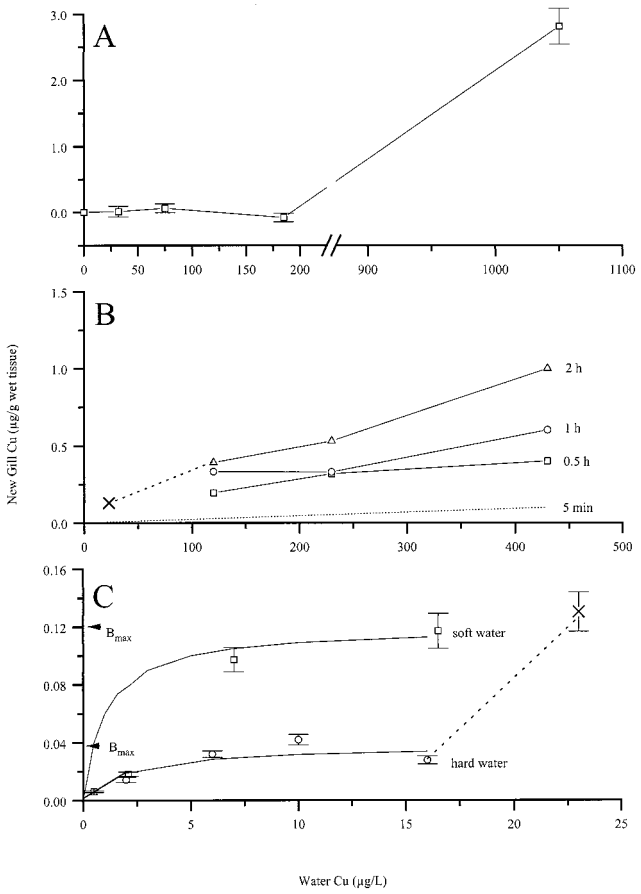

Fig. 4. Short-term (0.5–3 h) gill Cu binding in juvenile rainbow trout. Trout had not been previously exposed to Cu. (**A**) Net uptake of Cu in hard water over 3 h. Net uptake was determined by subtracting a background level of 1.2 μg/g (determined on control gills at 0 Cu) from gill total Cu measurements. Data are means ± 1 SE (N = 6). (**B**) Net uptake of ^{64}Cu in hard water from 0.5 to 2 h. X = gill uptake value from Figure 4C. The lower dotted line is gill Cu binding in hard water determined on trout gills in vitro over 5 min [36]. Data are means (N = 4). (**C**) Net uptake of ^{64}Cu in hard (N = 4) and soft (N = 10) water at low range of [Cu]. Note difference in x- and y-axes from panels A, B, and C.

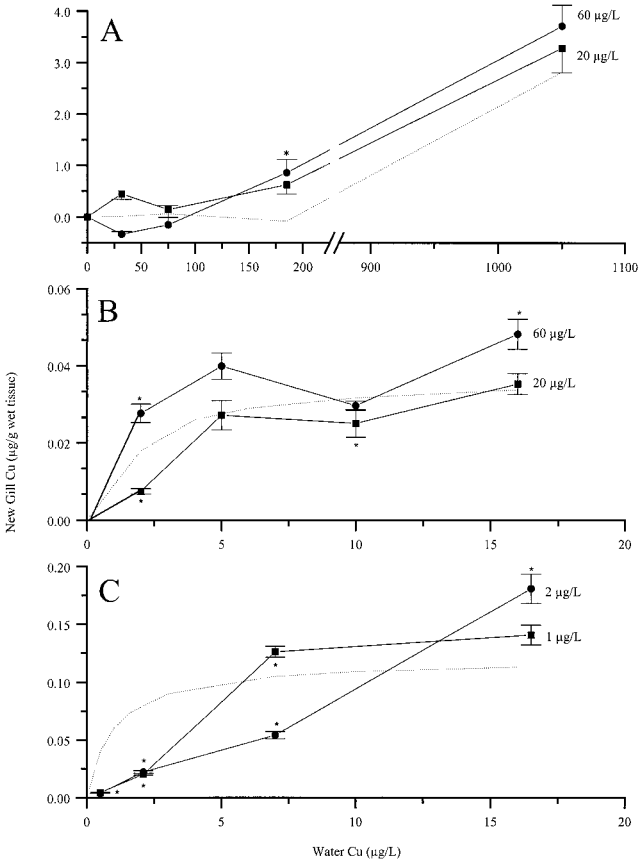

Fig. 5. Short-term (3 h) gill Cu binding in juvenile rainbow trout. Trout had been previously exposed to Cu for 30 d in hard and soft water. (**A**) Net uptake of Cu in hard water over 3 h. Net uptake was determined by subtracting background levels of 1.2 μg/g (control gills at 0 Cu) from gill total Cu measurements. Data are means ± 1 SE (N = 6). The dotted line represents the actual Cu accumulation in control fish from Figure 4A. (**B**) Net uptake of ^{64}Cu in hard water (N = 4) at a low range of [Cu]. The dotted line represents the estimated Cu accumulation in control fish from Figure 4C. Note difference in x- and y-axes from panel A. (**C**) Net uptake of ^{64}Cu in soft water (N = 10) at low range of [Cu]. The dotted line represents the Cu accumulation estimated in control fish from Figure 4C. In all panels, * = mean values significantly different from control ($p < 0.05$).

to the metal. One of the strongest supports for this concept is the consistent observation of initial growth reduction followed by recovery in juvenile salmonids exposed to Cu (at least seven studies since 1976: [5,7–9,24–26]). The same effect has also been noted in perch [27] and carp [28].

In marked contrast to these studies, we found no evidence of an overall reduction, or even an initial reduction in growth, in any of our exposures. Similarly, we found no significant initial reduction in whole-body Na^+ and Cl^-. Our previous studies on rainbow trout indicate that under similar exposure conditions (but with unfed fish), we should expect at least 20% loss of sodium and chloride by 48 h [22]. The key difference between the present and previous studies [5,7–9,24–26] is that we fed fish a higher ration (3% vs 1–2%) that was administered in three daily feedings rather than one. Interestingly, Miller et al. [29], who similarly employed three daily feedings, also reported an absence of Cu effect on growth in rainbow trout. If more frequent feeding is the key variable, then it could be masking the costs of Cu damage repair by overcoming appetite suppression [25], by accelerating repair of the damage responsible for ion losses centered at the gills [30], and/or by providing elevated levels of much-needed electrolytes. Recently, DCruz et al. [31] and DCruz and Wood [32] provided direct evidence for these three hypotheses by demonstrating that satiation feeding (twice per day) completely prevented the electrolyte disturbances in rainbow trout exposed to low pH compared to fish exposed to the same low pH but fed a limited ration. Furthermore, they showed that the salt content of the diet was more important than the energy content in preventing the electrolyte effects. Moreover, satiation-fed, acid-exposed fish exhibited greater appetites and growth rates than their comparable satiation-fed controls. The effect of the diet as an important modifier of toxicity has usually been overlooked (see synthesis by Lanno et al. [33]), even though we have observed critical intralaboratory exposure differences. This would have vast implications on the chronic toxicity to Cu in wild fish, where the quantity and quality of the diet is much more variable.

Swim performance. Another absent effect of Cu exposure was diminished swim performance. Like growth inhibition, swim performance has previously been shown to be a sensitive indicator of Cu exposure [34], especially in soft water [35], the latter study documenting effects at Cu concentrations as

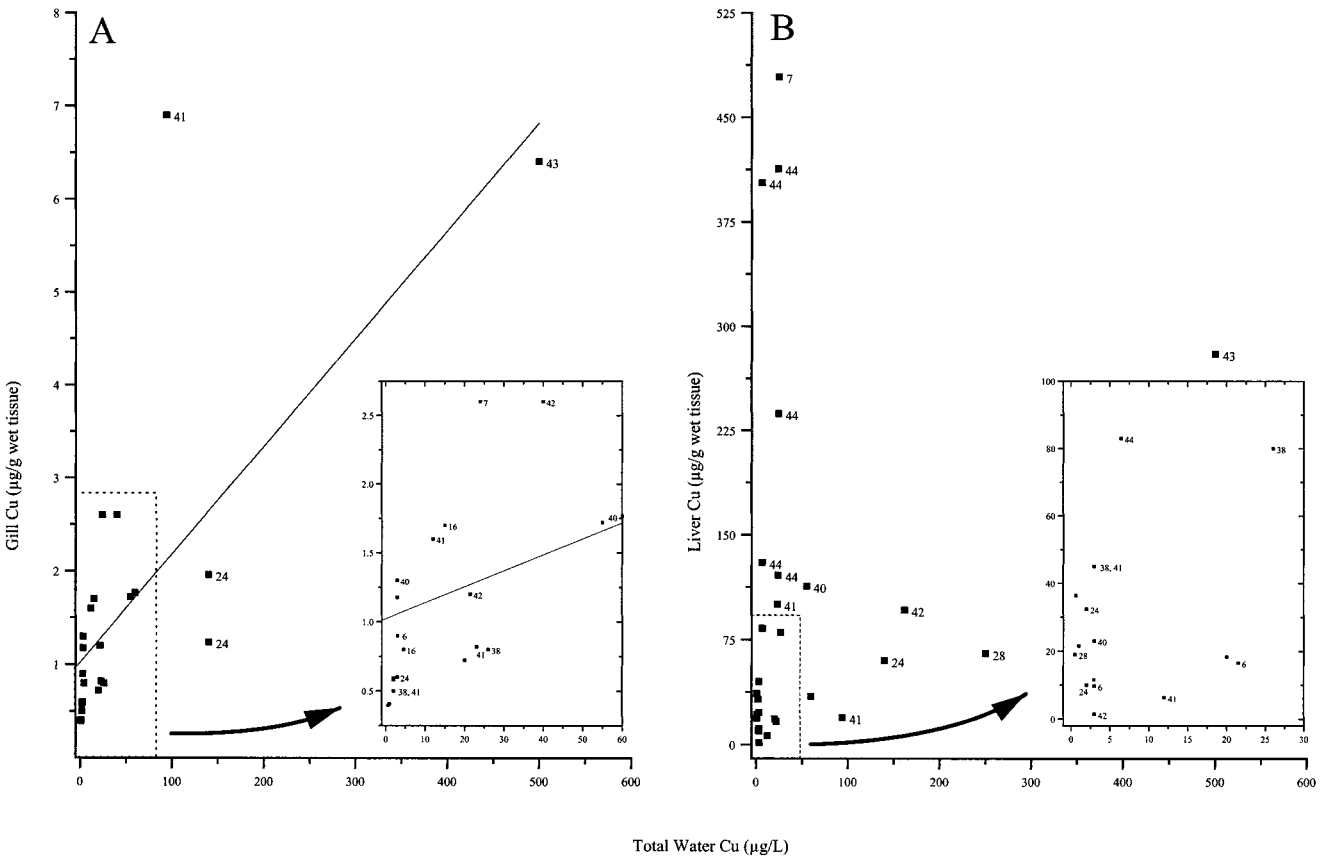

Fig. 6. Summary of literature values (including the present study) for total Cu levels (μg/g wet tissue) in (**A**) gills [6,16,24,38,40–42] and (**B**) liver [6,24,28,38,40–44] over a range of water Cu concentrations. Data are from 10 and 11 laboratory or field studies between 1975 and 1998 and represent a range of fish species, fish age, size, length of exposure, and water chemistry. The line in (**A**) represents a linear relationship between gill Cu accumulation and water Cu ($R^2 = 0.72$, $p < 0.01$). There was no significant relationship between liver Cu accumulation and water Cu ($R^2 = 0.21$, $p > 0.25$). Areas outlined by boxes are expanded in insets.

low as 12% of the LC50. However, Waiwood and Beamish [34] showed that swim performance fully recovered during chronic exposure, suggesting that it was impacted only during the damage phase. Since we did not test swim performance during the damage phase, we do not know whether swim performance was affected while growth was not, but we can confirm that swimming performance was not impaired after 30 d of Cu exposure in any of our treatments.

Acclimation to copper. An increase in lethal tolerance to Cu arising from chronic Cu exposure has been well established, at least for salmonids [5,9]. The increase in lethal tolerance is proportional to the exposure concentration [5] and is complete in about two weeks, and the maximum response is about a twofold increase in 96-h LC50. This is similar to the acclimation response achieved in the present study in the one exposure where it occurred (60 μg/L Cu in hard water). Although water hardness has not been evaluated as a variable affecting acclimation, by comparing existing studies, each done at a single hardness, there is a trend suggesting that the threshold (in terms of relative toxicity) required for acclimation increases with declining hardness. At hardnesses of 374 and 276 mg/L $CaCO_3$ ([5,9], respectively) acclimation occurred at 25 to 30% of the Cu LC50 without accompanying mortality, whereas it occurred at 50% of the Cu LC50 at the lower hardness of the present study (120 mg/L as $CaCO_3$) and was accompanied by additional mortality. If this trend holds, then the acclimation threshold would be expected to increase toward the incipient lethal threshold (96-h LC50) with declining hardness, that is, narrowing the window for acclimation. Acclimation to Cu may thus be a phenomenon confined to relatively hard water, which limits its effectiveness as a indicator of chronic Cu effect.

Tissue accumulation. A number of laboratory studies have examined the relationship between Cu accumulation in tissues and water Cu concentrations. Liver and gill are the tissues most commonly used as indicators of Cu exposure. The general pattern that emerges from these studies is that tissue Cu levels gradually increase in response to exposure at a constant Cu level until a steady state is reached. This may take from two to six weeks, depending on the Cu exposure level [7]. As yet, no comprehensive laboratory model to relate tissue Cu burdens with environmental exposure has been developed, with the exception of Marr et al. [7], who studied whole-body Cu only. Nonetheless, a survey of laboratory and field studies reveals an essentially linear relationship between gill Cu concentrations and environmental Cu (Fig. 6A). In contrast, there is no relationship between liver Cu concentrations and water Cu concentrations despite there being a diversity of fish species in this plot and differences in exposure duration and water chemistry (particularly hardness; Fig. 6B). Miller et al. [10] came to essentially the same conclusion in their field study, finding the liver and gill of white suckers to be correlated to water Cu concentrations. One of the key conclusions suggested by this analysis is that Cu accumulation may be largely dependent on total dissolved Cu, whereas toxicity is not only dependent on total dissolved Cu but is reliant on water chemistry as well.

To this framework, we can now add two new conclusions. First, Cu concentrations in the water must exceed a certain threshold value before significant tissue accumulation will occur. Only the high Cu concentrations in soft and hard water (2 and 60 μg/L, respectively) produced elevated gill or liver tissue levels. Since we know from our ^{64}Cu experiments that Cu was absorbed from the water into the blood in all exposures (L.N. Taylor, unpublished results), the absence of net accumulation in two of the four exposures suggests that the fish were precisely regulating Cu balance. The threshold thus represents the point at which Cu homeostasis begins to fail. This, by itself, suggests that tissue accumulation is not likely to be an especially sensitive indicator of low-level Cu exposure, which is not surprising since Cu is an essential metal. Second, gill Cu appears to be a more sensitive tissue indicator for waterborne Cu exposure than the liver for at least three reasons: The high background Cu levels in the liver (30 times greater than the gills) means that it is more difficult to detect a net increase in liver Cu (Fig. 2 vs. Fig. 3), when the gills accumulate Cu they appear to reach steady state more rapidly than does the liver (Figs. 2 and 3), and gill Cu tends to more accurately reflect the water Cu concentration, as represented by a fourfold higher Cu level in gills between the highest water Cu levels in hard and soft water but with virtually no difference in liver Cu content.

Copper binding by the gills. In this study, we used short-term (3-h) Cu exposures to assay the Cu-binding characteristics of the gills in hard and soft water. We found that the only practical way to perform this assay uniformly in hard and soft water was to use ^{64}Cu. Water Cu concentrations had to be elevated to acutely toxic levels otherwise, at least in hard water (Fig. 4A), in order to detect an increase in gill Cu above background. The use of ^{64}Cu permitted the detection of small increases in new Cu on or in the gills within a 3-h period. Furthermore, this approach yielded important insights into gill Cu dynamics (relevant to biotic ligand modeling of Cu toxicity; see the following discussion) and how binding characteristics change with soft water acclimation and with chronic sublethal Cu exposure. Indeed, these were the measurements in the present study that were able to detect effects resulting from three out of four chronic copper exposures.

Nature of gill Cu accumulation. The radiotracer experiments (Figs. 4B and C) identified two different types of binding sites on the gills for copper: low-affinity, high-capacity sites (nonsaturating over the range of Cu levels used; Fig. 4B) and high-affinity, low-capacity sites (saturating at very low Cu concentration; Fig. 4C). The former are, in fact, similar to sites characterized by 5-min Cu exposure in vitro ([36]; Table 3) as indicated by the dashed line in Figure 4B. Reid and McDonald [36] demonstrated that these sites do, in fact, saturate, but at very high Cu concentrations (>1,500 μg/L). Their low affinity suggests that they are not very specific to copper and may, in fact, reflect a mixture of different binding sites. Furthermore, they are not likely to accumulate Cu at environmentally realistic exposure levels. Consequently, recent investigations have focused on the high-affinity Cu-binding sites for the purpose of modeling the effects of Cu on the gills, specifically the interaction between water chemistry, gill metal burden, and toxicity. This approach [12–15] has recently been termed Biotic Ligand Modeling (BLM; D. Di Toro et al., unpublished data) and is seen as providing a more mechanistic basis for predicting toxicity of Cu to aquatic biota than the use of only hardness-adjusted relationships between toxicity and total dissolved Cu (Fig. 1). The BLM approach assumes that the gill ligands have their highest affinity for the free metal ion (e.g., Cu^{2+} versus $CuOH^{+}$) and that binding is competitive so that cations that compete with Cu^{2+} (e.g., Ca^{2+}) or anions that complex Cu^{2+} (e.g., DOC, OH^{-}) will reduce the amount of metal bound to the gill ligand. Thus, once the binding capacity and affinity of all the ligands, including the gill, have been determined, these values can be used to calculate gill metal burden in relation to the concentration of free Cu^{2+}. Free Cu^{2+} ion can be calculated with a geochemical modeling program such as MINEQL+ [37].

Table 3. Summary of copper-binding affinities (log $K_{gill=Cu}$; log of the inverse free Cu ion concentration, in moles/L, required to produce half saturation of binding sites) and capacities (B_{max}, nmol/g) determined in soft water (Ca^{2+} ~10 mg/L) in this and other studies using a range of total dissolved Cu from 0 to <30 μg/L. In the present study, the assumptions for the calculation of binding affinities were that there were 0.05 μmol binding sites per mg DOC/L [10] and that pH of the gill microenvironment was 7.5 and 6.2 in hard and soft water, respectively [39]

Species	log $K_{gill=Cu}$	B_{max} (nmol/g)	Duration (h)	Reference
Brook trout	7.1	63[a]	24	[15]
Rainbow trout	7.6	30[a]	24	[15]
Fathead minnow	7.4	30[a]	3	[13]
Rainbow trout	7.9	1.9	3	This study (soft water)
Rainbow trout	9.2	0.6	3	This study (hard water)
Rainbow trout	2.4	930	0.08	[36]

[a] Gill Cu background levels were 8 to 12 nmol/g.

Previous estimates for the Cu-binding properties of the high-affinity ligands are shown in Table 3. To simplify the making of these estimates, previous investigators have used slightly acidic and very soft water (Ca^{2+} of ≤ 10 mg/L). These conditions ensure that most of the Cu is in the free-ion form (≥80% at pH < 7.0) and that competitive and complexing compounds are kept to a minimum. Parenthetically, this approach also ensures that sufficient Cu is accumulated so that it can be detected against background levels in the gills (8–12 nmol/g) without the use of ^{64}Cu. Note that the binding characteristics (log $K_{gill=Cu}$ and B_{max}) for fathead minnow gill [13] are very similar to those of the rainbow trout and brook trout [15], although different durations of exposure were employed. In contrast, we estimated a much lower value for B_{max} in the present study; 1.9 versus 30 nmol/g. Partly this is because the latter value includes a background Cu of 8 to 12 nmol/g, whereas ours does not, but even then there is a 10-fold difference between the two estimates. In addition, Grosell and co-workers [38] reported a naive gill filament saturation level of ~1 μg Cu/g dry weight in the same hard water used in this study. This value converts to ~3.2 nmol/g wet weight, which is a fivefold difference from our B_{max} in hard water (0.6 nmol/g). Our log $K_{gill=Cu}$ estimate for rainbow trout is higher than the previous estimate by MacRae et al. [15] (7.9 vs 7.6), but this estimate is subject to the most error. In fact, ours need have been only 1 μg/L total dissolved copper greater than the estimated value of 1 μg/L (Fig. 4C) to yield an identical log *K* to MacRae et al. [15]. Furthermore, the acute toxicity of Cu (measured under similar water chemistry conditions) was similar between the two studies. After 24 h at 20 μg Cu/L, we obtained 50% mortality versus 75% mortality in the MacRae et al. study [15]. This comparison of toxicity and ligand-bind-

ing properties suggests that it is log $K_{gill=Cu}$ rather than B_{max} that is the key determinant of toxicity, that is, the percentage saturation of binding sites rather than the absolute total number of sites.

To date, no other studies have attempted measurement of Cu binding to the gills in more complex water chemistry to test the validity of the BLM. We have, and we show that in hard water the estimated B_{max} is much lower but that the affinity is much higher (Fig. 4C and Table 3). The lower B_{max} is not surprising because the binding curve in hard water incorporates the competitive effects of Ca^{2+} and complexation due to DOC. The higher affinity is surprising, especially in the light of the reduced toxicity of Cu in hard water (Fig. 1). One possible explanation is that calcium, which is known to be important for regulating membrane permeability and increasing the stability of membrane proteins, may actually regulate both the number and the affinity of binding sites on the gill surface. Whether this effect is actually occurring in the gill epithelium and whether it is acute or chronic is unknown. However, the fact that the acute toxicity to Cu decreased dramatically over 10 weeks of acclimation to soft water (Fig. 1) does suggest that chronic exposure to low Ca^{2+} modified gill binding properties for Cu and that the rate of this change was fairly slow since the first test of toxicity was not conducted until two weeks of soft-water exposure. Moreover, if the response to Ca^{2+} reduction were a gradual reduction of the affinity of gill surface ligands for divalent cations, then such a modification would be protective against an exposure to Cu. In any case, the present biotic ligand model lacks any mathematical way of incorporating modulating effects (as opposed to competitive effects) of environmental Ca^{2+} on gill surface ligands and thus, at least implicitly, must assume that such regulation by Ca^{2+} does not occur.

Another way of testing the validity of the BLM approach is to test whether, in fact, any changes in acute Cu tolerance due to chronic Cu exposure are accompanied by changes in ligand binding. We confirmed that there was an alteration in gill binding in the group that showed increased lethal resistance after chronic exposure to 60 μg Cu/L. The alteration was characterized by an apparent increase in capacity in both the low- and the high-affinity binding sites (Figs. 5A and B, respectively). However, the most substantial, and arguably most protective, change in gill Cu binding (a large decrease in affinity) was seen in the 2-μg/L group in soft water (Fig. 5C), for which there was no increase in lethal resistance. Thus, while we show that gill ligand-binding properties are sensitive to modification by chronic Cu exposure (an increase in binding capacity or decrease in affinity), the relationship of these responses to the presence or absence of acclimation remains unclear.

CONCLUSIONS

The present study leads to four conclusions that are directly relevant to the task of assessing the risk of chronic copper exposure to wild fish populations. First, we confirm that gill Cu burdens are a reliable indicator of chronic Cu exposure (superior in this respect than liver burdens) but that other physiological indicators, such as growth, ion loss, and swim performance, may be less reliable because of the important effect of ration quantity (and possibly quality) on their expression. Second, the presence of increased resistance to Cu in a wild population may also be a reliable indicator of chronic exposure, but only in hard-water environments. In soft waters, the combined effect of increasing Cu toxicity and the added stress associated with soft-water exposure appear to favor lethality over acclimation. Third, the protracted period of soft-water acclimation seen in the present study suggests that, at the very least, laboratory studies of the effects of water hardness on Cu toxicity may need to be re-evaluated (Fig. 1). It is entirely possible, for example, that the hardness relationship would be quite different if tested on fish native to soft-water environments compared to fish native to hard-water environments; that is, the effects of increasing hardness might be quite different from the effects of decreasing hardness. Finally, although changes in gill Cu-binding characteristics offer the most promise for assessing the effect of chronic copper exposure, there is as yet no clear-cut relationship between water chemistry, gill Cu binding, and toxicity. Clearly, more careful and detailed studies are required to resolve these apparently contradictory findings and to derive a compromise set of constants that best predicts both metal accumulation by the gills and resulting toxicity over a wide range of different water chemistries.

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REFERENCES

1. Alabaster JS, Lloyd R. 1982. *Water Quality Criteria for Freshwater Fish*, 2nd ed. Butterworth, London, UK.
2. Spear PA, Pierce RC. 1979. Copper in the aquatic environment: Chemistry, distribution and toxicology. NRCC 16454. National Research Council of Canada, Ottawa, ON.
3. Sorenson EM. 1991. *Metal Poisoning in Fish.* CRC, Boca Raton, FL, USA.
4. Howarth RS, Sprague JB. 1978. Copper lethality to rainbow trout in waters of various hardness and pH. *Water Res* 12:455–462.
5. Dixon DG, Sprague JB. 1981. Acclimation to copper by rainbow trout (*Salmo gairdneri*): A modifying factor in toxicity. *Can J Fish Aquat Sci* 38:880–888.
6. Farag AM, Stansbury MA, Hogstrand C, MacConnell E, Bergman HL. 1995. The physiological impairment of free-ranging brown trout exposed to metals in the Clark Fork River, Montana. *Can J Fish Aquat Sci* 52:2038–2050.
7. Marr JCA, Lipton J, Cacela D, Hansen JA, Bergman HL, Meyer JS, Hogstrand C. 1996. Relationship between copper exposure duration, tissue copper concentration, and rainbow trout growth. *Aquat Toxicol* 36:17–30.
8. Lett PF, Farmer GJ, Beamish FWH. 1976. Effects of copper on some aspects of the bioenergetics of rainbow trout (*Salmo gairdneri*). *J Fish Res Board Can* 33:1335–1342.
9. Buckley JT, Roch M, McCarter JA, Rendell CA, Matheson AT. 1982. Chronic exposure of coho salmon to sublethal concentrations of copper: I. Effect on growth, on accumulation and distribution of copper, and on copper tolerance. *Comp Biochem Physiol C* 72:15–19.
10. Miller PA, Munkittrick KR, Dixon DG. 1992. Relationship between concentrations of copper and zinc in water, sediment, benthic invertebrates, and tissues of white sucker (*Catostomus commersoni*) at metal-contaminated sites. *Can J Fish Aquat Sci* 49: 978–984.
11. Pagenkopf GK. 1983. Gill surface interaction model for trace-metal toxicity to fishes: Role of complexation, pH and water hardness. *Environ Sci Technol* 17:342–347.
12. Playle RC, Dixon DG, Burnison K. 1993. Copper and cadmium binding to fish gills: Modifications by dissolved organic carbon and synthetic ligands. *Can J Fish Aquat Sci* 50:2667–2677.
13. Playle RC, Dixon DG, Burnison K. 1993. Copper and cadmium binding to fish gills: Estimates of metal-gill stability constants and modeling of metal accumulation. *Can J Fish Aquat Sci* 50: 2678–2687.
14. Bergman HL, Dorward-King EJ, eds. 1997. *Reassessment of Met-*

als Criteria for Aquatic Life Protection: Priorities for Research and Implementation. Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.
15. MacRae RK, Smith DE, Swoboda-Colberg N, Meyer JS, Bergman HL. 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) gills: Implications for assessing bioavailable metal. *Environ Toxicol Chem* 18:1180–1189.
16. McDonald DG, Wood CM. 1993. Branchial mechanisms of acclimation to metals in freshwater fish. In Rankin JC, Jensen FB, eds, *Fish Ecophysiology*. Chapman & Hall, London, UK, pp 297–321.
17. McDonald DG, Rogano MS. 1986. Ion regulation by the rainbow trout, *Salmo gairdneri*, in ion-poor water. *Physiol Zool* 59:318–331.
18. McDonald DG, McFarlane WJ, Milligan CL. 1998. Anaerobic capacity and swim performance of juvenile salmonids. *Can J Fish Aquat Sci* 55:1198–1207.
19. Playle RC, Gensemer RW, Dixon DG. 1992. Copper accumulation on gills of fathead minnows: Influence of water hardness, complexation and pH of the gill micro-environment. *Environ Toxicol Chem* 11:381–391.
20. Grande M. 1967. Effect of copper and zinc on salmonid fishes. *Adv Water Pollut Res* 1:97–111.
21. McDonald DG, Milligan CL. 1997. Ionic, osmotic and acid-base regulation in stress. In Iwama GK, Sumpter J, Pickering A, Schreck CB, eds, *Fish Stress and Health in Aquaculture*, Vol 62. Cambridge University Press, Cambridge, UK, pp 119–145.
22. Laurén DJ, McDonald DG. 1986. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. *Can J Fish Aquat Sci* 43: 1488–1496.
23. Erickson RJ, Kleiner CF, Fiandt JT, Highland TL. 1997. Effect of acclimation period on the relationship of acute copper toxicity to water hardness for fathead minnow. *Environ Toxicol Chem* 16: 813–815.
24. Drummond RA, Spoor WA, Olson GF. 1973. Some short-term indicators of sublethal effects of copper on brook trout, *Salvelinus fontinalis*. *J Fish Res Board Can* 30:698–701.
25. Waiwood KG, Beamish FWH. 1978a. The effect of copper, hardness and pH on the growth of rainbow trout, *Salmo gairdneri*. *J Fish Biol* 13:591–598.
26. Seim WK, Curtis LR, Glenn SW, Chapman GA. 1984. Growth and survival of developing steelhead trout (*Salmo gairdneri*) continuously or intermittently exposed to copper. *Can J Fish Aquat Sci* 41:433–438.
27. Collvin L. 1985. The effect of copper on growth, food consumption and food conversion of perch *Perca fluviatilis* L. offered maximal food rations. *Aquat Toxicol* 6:105–113.
28. DeBoeck G, Vlaeminck A, Blust R. 1997. Effects of sublethal copper exposure on copper accumulation, food consumption, growth, energy stores, and nucleic acid content in common carp. *Arch Environ Contam Toxicol* 33:415–422.
29. Miller PA, Lanno RP, McMaster ME, Dixon DG. 1993. Relative contributions of dietary and waterborne copper to tissue copper burdens and waterborne-copper tolerance in rainbow trout (*Oncorhynchus mykiss*). *Can J Fish Aquat Sci* 50:1683–1689.
30. Laurén DJ, McDonald DG. 1985. Effects of copper on branchial ion regulation in the rainbow trout, *Salmo gairdneri* Richardson. *J Comp Physiol B* 155:635–644.
31. DCruz LM, Dockray JJ, Morgan IJ, Wood CM. 1998. Physiological effects of sublethal acid exposure in juvenile rainbow trout on a limited or unlimited ration during a simulated global warming scenario. *Physiol Zool* 71:359–376.
32. DCruz LM, Wood CM. 1998. The influence of dietary salt and energy on the response to low pH in juvenile rainbow trout. *Physiol Zool* 71:642–657.
33. Lanno RP, Hickie BE, Dixon DG. 1989. Feeding and nutritional considerations in aquatic toxicology. *Hydrobiologia* 188/189: 525–531.
34. Waiwood KG, Beamish FWH. 1978b. Effects of copper, pH and hardness on the critical swimming performance of rainbow trout (*Salmo gairdneri* Richardson). *Water Res* 12:611–619.
35. Beaumont MW, Butler PJ, Taylor EW. 1995. Exposure of brown trout, *Salmotrutta*, to sub-lethal copper concentrations in soft acidic water and its effect upon sustained swimming performance. *Aquat Toxicol* 33:45–63.
36. Reid SD, McDonald DG. 1991. Metal binding activity of the gills of rainbow trout (*Oncorhynchus mykiss*). *Can J Fish Aquat Sci* 48:1061–1068.
37. Schecher W, McAvoy DC. 1994. *MINEQL*$^{+}$, Ver 3.01b. Environmental Research Software, Hallowell, ME, USA.
38. Grosell MH, Hogstrand C, Wood CM. 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* 38:257–276.
39. Playle RC, Wood CM. 1989. Water chemistry changes in the gill micro-environment of rainbow trout: Experimental observations and theory. *J Comp Physiol B* 159:527–537.
40. Laurén DJ, McDonald DG. 1987. Acclimation to copper by rainbow trout, *Salmo gairdneri*: Biochemistry. *Can J Fish Aquat Sci* 44:105–111.
41. Grosell MH, Hansen HLM, Rosenkilde P. 1998. Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne Cu exposure. *Comp Biochem Physiol C* 120:295–305.
42. Benoit DA. 1975. Chronic effects of copper on survival, growth and reproduction of the bluegill (*Lepomis macrochirus*). *Trans Am Fish Soc* 2:353–358.
43. Pilgaard L, Malte H, Jensen FB. 1994. Physiological effects and tissue accumulation of copper in freshwater rainbow trout (*Oncorhynchus mykiss*) under normoxic and hypoxic conditions. *Aquat Toxicol* 29:197–212.
44. Marr JCA, Bergman HL, Lipton J, Hogstrand C. 1995. Differences in relative sensitivity of naïve and metals-acclimated brown and rainbow trout exposed to metals representative of the Clark Fork River, Montana. *Can J Fish Aquat Sci* 52:2016–2030.