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Relationship between exposure duration, tissue residues, growth, and mortality in rainbow trout (*Oncorhynchus mykiss*) juveniles sub-chronically exposed to copper

J.A. Hansen [a,*], J. Lipton [a], P.G. Welsh [a], J. Morris [b], D. Cacela [a], M.J. Suedkamp [b]

[a] *Stratus Consulting Inc., PO Box 4059, Boulder, CO 80306, USA*
[b] *University of Wyoming, PO Box 3166, Laramie, WY 82071, USA*

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Abstract

We conducted a 56-day sub-chronic test on the effects of Cu on rainbow trout (*Oncorhynchus mykiss*) fry at a nominal water hardness of 100 mg l^{-1} (as $CaCO_3$). Response measures were growth, whole body Cu concentrations, and mortality. Significant mortality was observed in fish exposed to 54.1 μg Cu l^{-1} (47.8%) and 35.7 μg Cu l^{-1} (11.7%). Growth was dose-dependent over the range of Cu treatments (0–54 μg Cu l^{-1}), and was modeled as a function of Cu exposure concentration and exposure duration. Calculated inhibition concentrations (based on change in wet weight through a 56-day Cu exposure) were $IC_{50} = 54.0$ μg Cu l^{-1}, $IC_{20} = 21.6$ μg Cu l^{-1}, $IC_{10} = 10.8$ μg Cu l^{-1}, and $IC_{01} = 1.1$ μg Cu l^{-1}. Measured whole body Cu was also dose-dependent, and growth of trout fry was readily modeled as a function of tissue Cu and exposure duration. This model was virtually identical to a model previously developed for rainbow trout exposed to Cu at a hardness of 25 mg l^{-1}. Following the 56-day exposure period, we performed a 96-h acute challenge to Cu and Cd to evaluate the effects of Cu acclimation on acute Cu and Cd toxicity. Sensitivity to Cu was dependent on the 'acclimation dose'; trout previously held in control aquaria (i.e. not acclimated to Cu) suffered over 80% mortality, whereas trout previously exposed to 35.7 μg Cu l^{-1} for 56 day suffered 20% mortality. These fish also showed somewhat reduced sensitivity to Cd, suggesting acclimation to Cu can enhance tolerance to other metals. Finally, the relationship between growth response and hardness (derived from several studies) appeared to have a different slope than the hardness relationship previously observed for lethality responses.

Keywords: Rainbow trout; Copper; Growth; Mortality; Bioaccumulation

1. Introduction

Although much is known about acute toxicity responses to Cu in rainbow trout (*Oncorhynchus mykiss*), less is known about chronic sublethal

* Corresponding author. Tel.: +1-303-381-8000; fax: +1-303-381-8200.
E-mail address: jhansen@stratusconsulting.com (J.A. Hansen).

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(i.e. sub-chronic) responses such as growth effects. To date, only one early life stage chronic study on the effects of Cu on rainbow trout has been published in which exposures were initiated with newly fertilized eggs (Seim et al., 1984). Another study of the long-term effects of Cu on rainbow trout was initiated on eyed eggs (McKim et al., 1978), and thus is considered a partial chronic test (ASTM, 1992). Several other investigators have completed sub-chronic tests in which the growth of fry or juvenile rainbow trout was examined during Cu exposure (Lett et al., 1976; Waiwood and Beamish, 1978; Marr et al., 1996; Taylor et al., 1998).

Due to the relative paucity of chronic tests, the effects of various water quality variables on chronic responses are relatively unknown. Hardness, more specifically the effects of calcium, has been shown to be an important modifier of acute Cu toxicity to rainbow trout (Welsh et al., 2000). However, no early life stage chronic tests have evaluated the effects of hardness on chronic toxicity, and two studies with rainbow trout have been conducted using multiple hardness concentrations, both of which were conducted with juvenile fish greater than 1 g wet weight (Waiwood and Beamish, 1978; Taylor et al., 1998).

Furthermore, there are few studies relating Cu accumulation to adverse effects. Elucidation of this relationship is necessary if tissue residues are used to assess chronic effects in field settings. Marr et al. (1996) found a strong relationship between whole body Cu and growth effects in a 60-day test performed at a hardness of 25 mg l^{-1} (as $CaCO_3$). However, the relationship between tissue Cu and growth was also a function of exposure duration, a variable that is usually unknown in wild fish.

Pre-exposure and acclimation to Cu is also important in evaluating the toxic effects of Cu in acute and chronic exposures. Several investigators have shown that acclimation to Cu can enhance tolerance to acute challenges of Cu (Dixon and Sprague, 1981a; Marr et al., 1995; Taylor et al., 1998). This tolerance has been attributed to synthesis of nonspecific metallothioneins (Dixon and Sprague, 1981b), although the induction of this protein has been suggested to require a metabolic cost that could lead to reduced growth (Marr et al., 1995).

In the present study, we conducted a 56-day (8-week) sub-chronic growth exposure on rainbow trout in water with a hardness of 100 mg l^{-1}. Response measures were mortality, growth, and whole body Cu. In addition, we performed a 96-h acute challenge to a single concentration of Cu or Cd to evaluate whether acclimation to different Cu doses influences subsequent acute sensitivity to metals.

2. Methods

2.1. Experimental fish

Rainbow trout were obtained as eggs from Dubois State Fish Hatchery, Dubois, WY (USA), and hatched in hatching jars with continuous replacement of well water at 7–8 °C. Following hatch, sac fry were maintained in a rectangular holding tank containing well water at 7–8 °C. Following resorption of the yolk sac, fry were fed a vitamin-fortified commercial trout starter diet (BioDiet Starter Crumbles, Bio-Oregon, Inc, Warrenton, OR, USA) at a ration of 3.5% total biomass per day (manufacturer's recommendation at 8 °C).

Fish were acclimated to test water quality conditions (hardness 100 mg l^{-1}; pH 7.9; temperature 8 °C) for 17 days prior to testing. All culture and acclimation water was monitored daily for hardness, alkalinity, pH, temperature, and dissolved oxygen. Ammonia levels were monitored weekly. Before the start of the chronic test, all fish were free of obvious disease or distress from handling and culture conditions.

2.2. Toxicity test methods

Testing was conducted at Red Buttes Environmental Biology Laboratory, University of Wyoming, Laramie, WY (USA). Well water at this laboratory contains an average hardness of 220 mg l^{-1}, 51 mg Ca l^{-1}, 25 mg Mg l^{-1} (Ca:Mg molar ratio of 1.2), 8.2 mg Na l^{-1}, 2.1 mg K l^{-1}, 6.3 mg Cl l^{-1}, and 26 mg SO_4 l^{-1}. Well water

was continuously mixed with deionized water in a 190 l head tank to produce a target hardness of 100 mg l^{-1}. Test water pH was controlled with a Signet pH controller (Signet Scientific, Tustin, CA, USA), which controlled the addition of H_2SO_4 and KOH with metering pumps. Temperature was maintained at 8 °C by chilling deionized water before delivery to the head tank. All fish were maintained and tested under a 16-h light:8-h dark illumination cycle.

The exposure system for the sub-chronic test consisted of a continuous-flow proportional diluter and 36 exposure tanks. The diluter was constructed to provide five 35% dilutions of the test metal plus a control. A stock Cu solution ($CuCl_2 \cdot 2H_2O$) was mixed for the entire test, and was metered into the diluter using a Fluid Metering Inc (Syosset, NY, USA) QG20 laboratory metering pump at 2 ml min^{-1}.

Each treatment condition (i.e. nominal Cu concentration) was split into six replicate exposure tanks. The placement of treatments within the bank was by randomized block design. Each exposure tank contained 7.6 l of exposure water with 150 ml min^{-1} exposure water replacement to provide flow rates that far exceed minimum criteria defined by Sprague (1969).

Forty fish were loaded two at a time into each exposure tank to provide random placement of fish. Throughout the study, fish loading into each exposure aquarium was well within loading density and flow-to-density guidelines (ASTM, 1996). Fish were fed 3.5% body weight per day split between two daily feedings. The amount of food fed to each exposure tank was adjusted daily for mortalities, and weekly based on percent growth in the culture tank (i.e. tank containing fish not used in this study). Measured average weight of fish in each exposure tank after 20 and 40 days Cu exposure was used to correct the amount of food to feed to each exposure tank. Exposure tanks were siphoned daily to minimize accumulation of feces and uneaten food.

Copper exposures continued for 56 days. Mortality (i.e. cessation of opercular movement) was monitored at 0, 3, 6, 9, and 12 h through the first day of exposure, then twice daily to the end of the test. Dead fish were removed and discarded after each observation. Mortality caused by tank cleaning procedures was subtracted from total mortality to obtain the mortality caused by Cu exposure.

On day 20, 40, and 56, 10 live fish were collected from each exposure tank and sacrificed for measurement of wet weight and total length. The fish in all exposure tanks were not fed the day before, or the day of, sampling for length and weight. Five of the 10 fish sampled for growth were placed in precleaned vials for measurement of whole body Cu accumulation.

Following the chronic exposure, surviving trout were removed and randomly placed into exposure tanks containing either control (< detect Cd, 0.17 μg Cu l^{-1}), high Cu (91.0 μg Cu l^{-1}, < detect Cd), or high Cd (7.4 μg Cd l^{-1}, 0.19 μg Cu l^{-1}) water for 96 h. Other water parameters were similar to those in the growth test: 8.7 °C, pH 7.9, dissolved oxygen 9.3 mg l^{-1}, hardness 105 mg l^{-1}, and alkalinity 102 mg l^{-1}. Thus, in this acute test, the test fish were acclimated to various Cu concentrations for 56 days before the acute test start. Test Cu and Cd concentrations were selected based on acute LC_{50} concentrations that were determined from previous acute tests conducted with the same fish under similar experimental conditions. Only one replicate exposure of each Cu pre-exposure condition and exposure condition was tested; fish acclimated (i.e. pre-exposed) to the highest Cu concentration (i.e. 54.1 μg Cu l^{-1}) in the 56-day exposure were not used because of relatively high mortality (47.8%) at that concentration.

2.3. Water chemistry

Basic water quality parameters (hardness, alkalinity, pH, temperature, and dissolved oxygen), cations (Ca, Mg, Na, K), anions (Cl, SO_4), metals (Cu, Cd, Zn), total organic carbon (TOC), and total ammonia were analyzed throughout fish culture, acclimation, and test exposures. Ammonia was measured using methods outlined by Verdouw et al. (1978). Copper and Cd were analyzed using graphite furnace atomic absorption spectrophotometry. Zinc and all cations were analyzed by flame atomic absorption spectrophotometry. Anions were analyzed by ion chromatography.

Detection limits for metals were 0.16 μg Cu l^{-1}, 0.013 μg Cd l^{-1}, and 6 μg Zn l^{-1}.

2.4. Tissue analysis

After measurement of total length and wet weight, fish from each exposure tank were individually placed in acid-washed vials and stored in a freezer at −70 °C for whole-body Cu analysis. Fish were later ground to a fine powder using a liquid nitrogen cooled mortar and pestle. The ground tissue from each exposure tank was then placed into a pre-labeled, preweighed scintillation vial, dried in a 70 °C drying oven for 16 h, cooled in a dessicator, and subsequently reweighed to obtain the dry weight of the tissue. The mortar and pestle were washed with laboratory detergent and 10% nitric acid between each set of fish to avoid cross-contamination. Tissues were then digested in 2.5 ml 30% Ultrex II nitric acid (VWR Scientific, San Francisco, CA, USA) and 1 ml of 30% Ultrex-grade hydrogen peroxide for 24 h, and analyzed by graphite furnace atomic absorption spectrophotometry.

2.5. Data analysis and statistics

Significant mortality and time to death (for the mortality figure) were determined using the Kaplan Meier method (Kalbfleisch and Prentice, 1980) to estimate survivorship curves and associated standard errors. In this method, individual live fish that were removed from the tanks to measure growth after 20 and 40 day Cu exposure, fish that were accidentally killed by tank cleaning activities, and fish that survived until the end of the test were designated as censored observations.

Growth data are presented both as the change in weight from the beginning of the test and as total weight measured at each time interval. For the change in weight measure, the mean weight from a subsample of fish at the beginning of the test was subtracted from the weight of each measured fish after 20, 40, and 56 days of Cu exposure. For both measures, significant differences between fish exposed to Cu concentrations and control fish were determined using ANOVA and Dunnett's multiple comparison procedure (one tailed, $\alpha = 0.05$) in Toxstat v3.5 (WEST, 1996). Confidence intervals for graphical representation of data were calculated using the standard deviation of the mean and the corresponding t-statistic (two tailed, $\alpha = 0.05$).

The accumulation of whole body Cu was analyzed using ANOVA ($\alpha = 0.05$). Since a few samples were lost during tissue drying and digestion, an unequal number of replicates were obtained for each exposure concentration. Therefore, differences from control tissue-Cu concentrations were determined using Bonferroni's t-test multiple comparison procedure ($\alpha = 0.05$).

Inhibition concentration (IC) values were estimated using series duration-specific linear models describing fish growth increment [final weight (g) − mean initial weight (g)] as a function of either the Cu concentration in exposure water or the Cu concentration in fish tissue. Regression analyses were conducted using non-transformed weight measurements (i.e. change in weight) from the beginning of the test. Reference values for the relative reduction in weight of unexposed fish were defined as the corresponding linear regression estimates at water exposure concentrations of 0 μg Cu l^{-1} or at tissue concentrations of 4.42 μg Cu g^{-1} dry tissue weight. The value 4.42 μg g^{-1} was the average of observed tissue Cu concentrations in unexposed fish measured at exposure durations of 20, 40, and 56 days. The 95% confidence intervals for IC values were based on fiduciary limits.

Model estimates of fish growth against exposure Cu and time, and against tissue Cu and time, were produced using multiple linear regression. Fish weights were natural log transformed. Each factor in the regression equations was significant ($P < 0.05$), however, the interaction of exposure Cu or tissue Cu and time was not significant ($P > 0.05$).

The instantaneous growth rate (% per day) was calculated as the slope of the regression of ln(weight) versus exposure duration (day) using a method similar to that by Wootton (1990).

3. Results

3.1. Water quality

The water quality parameters analyzed during the study are summarized in Table 1. For both acclimation and exposure water, mean pH was within 0.1 U of the nominal pH 7.9, mean hardness was within 10% of nominal, and mean alkalinity values were approximately 90% of the hardness concentration. Mean dissolved oxygen was approximately 79% of saturation during acclimation and exceeded 95% of saturation during exposure. The maximum measured unionized ammonia during acclimation and exposure was 0.0018 mg NH_3-N l^{-1}. Analyzed cation (Ca, Mg, Na, and K), anion (Cl and SO_4), and background metals (Cu, Cd, and Zn in acclimation; Cd and Zn in exposure) concentrations were similar between acclimation and exposure with low standard deviations (Table 1). Sulfate concentrations fluctuated because of additions of H_2SO_4 for pH control.

The continuous-flow proportional diluters provided consistent 35% dilutions of the Cu stock solution (Table 2). Throughout the exposure, minimal differences were observed between dissolved (i.e. 0.45 μm filtered) and total (i.e., unfiltered) metal concentrations; the fraction of total metal in Cu exposures in the dissolved state (i.e. < 0.45 μm) ranged from 96.4 to 109% (Table 2). Due to minimal differences in Cu concentration were observed between filtered and unfiltered Cu concentrations, and more unfiltered samples were collected during tests, analysis of data was based on mean unfiltered Cu concentrations.

3.2. Test fish

The fish used in the test had a mean (S.D., n) initial wet weight of 244 mg (55, 40) and a mean initial total length of 30.8 mm (2.16, 40). Throughout the 56-day test, fish behavior was observed qualitatively at the time of feeding. Fish actively fed throughout the test, and no differences in feeding activity levels were observed between exposure concentrations.

3.3. Mortality

Significant mortality ($P < 0.05$) was observed in fish exposed to 35.7 μg Cu l^{-1} (11.7%), and 54.1 μg Cu l^{-1} exposures (47.8%) over the 56-day test period (Fig. 1). By the end of the 56-day test, mortality in fish exposed to all lower Cu treatments (i.e. æ22 μg Cu l^{-1}) was less than 5%.

Table 1
Mean (S.D., n) water quality parameters, cations, anions, and background metals in acclimation and exposure water

Parameter	Detection limit	Acclimation water	Exposure water
Temperature (°C)	NA	7.80 (0.22, 17)	8.08 (0.38, 348)
pH (units)	NA	7.85 (0.10, 17)	7.87 (0.11, 348)
Dissolved oxygen (mg l^{-1})	NA	7.10 (0.41, 8)	9.56 (0.43, 98)
Hardness (mg l^{-1} as $CaCO_3$)	NA	109 (13.7, 8)	102 (8.5, 174)
Alkalinity (mg l^{-1} as $CaCO_3$)	NA	100 (13.0, 8)	89.5 (8.6, 174)
Calcium (mg l^{-1})	0.1	23.6 (3.15, 5)	22.7 (3.28, 17)
Potassium (mg l^{-1})	0.2	4.02 (1.43, 5)	0.91 (0.13, 17)
Magnesium (mg l^{-1})	0.1	11.9 (1.51, 5)	11.3 (1.62, 17)
Sodium (mg l^{-1})	0.2	3.86 (0.32, 5)	3.48 (0.54, 17)
Chloride (mg l^{-1})	0.02	3.01 (0.20, 5)	2.97 (0.33, 18)
Sulfate (mg l^{-1})	0.02	11.9 (0.77, 5)	16.6 (3.73, 18)
Cadmium (μg l^{-1})	0.013	<dl (−, 3)	<dl (−, 17)
Copper (μg l^{-1})	0.157	0.22 (0.02, 3)	NA
Zinc (μg l^{-1})	6	<dl (−, 3)	<dl (−, 17)

NA-Not applicable; <dl, Mean values were less than the detection limit.

Table 2
Mean (S.D., n) copper concentrations (in μg l^{-1}) in exposure waters used in testing

Treatment	Total copper (unfiltered)	Dissolved copper (0.45 μm filtered)	% dissolved
Control	0.19 (0.10, 17)	0.24 (0.11, 9)	126%
1	9.47 (0.43, 17)	9.21(0.23, 9)	97.3%
2	14.5 (0.73, 17)	15.8 (5.27, 9)	109%
3	22.2 (1.80, 17)	21.7 (1.45, 9)	97.7%
4	35.7 (4.12, 17)	34.4 (3.46, 9)	96.4%
5	54.1 (5.46, 17)	52.5 (4.87, 9)	97.0%

Rapid (i.e. acute) mortality occurred within the first 10 days of exposure in the highest exposure concentration, followed by continued, gradual mortality through the end of the test (Fig. 1).

3.4. Growth

Growth data are presented as the relative change in weight during the test exposure (Table 3) and as the average weight (Fig. 2). Growth of test fish was dose-dependent, with fish exposed to higher Cu concentrations growing slower than fish exposed to the control and lower Cu concentrations (Fig. 2). Based on the relative change in weight, fish exposed to 9.47, 14.5, 35.7, and 54.1 μg Cu l^{-1} had grown significantly less than controls by day 20 ($P < 0.05$); fish exposed to 22.2 μg Cu l^{-1} were not different from controls (Table 3). On day 40, only fish exposed to 35.7 and 54.1 μg Cu l^{-1} were significantly smaller than controls ($P < 0.05$), and by day 56, all fish but those exposed to the lowest Cu concentration (9.49 μg Cu l^{-1}) showed significantly lower growth than controls ($P < 0.05$) as measured by relative change in weight.

Total growth (based on weight change at day 56) was modeled as a function of Cu exposure by linear regression (Fig. 3). This model [change in weight $= 0.4779 - 0.0044$ (Cu); $r^2 = 0.55$] was used to calculate growth inhibition concentration (IC) values for different percent growth reductions relative to growth rates of control fish (Table 4). Calculated IC values for 50, 20, 10, and 1% growth inhibition (based on change in weight from the beginning of the test) were 54.0, 21.6, 10.8, and 1.1 μg Cu l^{-1}, respectively.

3.5. Whole body Cu accumulation

Fish from all Cu treatments and all sampling periods (20, 40, and 56 days Cu exposure) contained significantly higher ($P < 0.05$) tissue Cu concentrations than controls; Cu accumulation was related to both Cu treatment and exposure duration (Fig. 4). Whole body Cu concentrations in control fish remained relatively constant throughout the test. After 20 days Cu exposure, fish exposed to 54 μg Cu l^{-1} contained more than twice the Cu concentration as control fish and after 56 days Cu exposure, these fish contained almost four times the Cu concentration of control fish.

In addition to calculating the IC values based on water concentration, we calculated growth in-

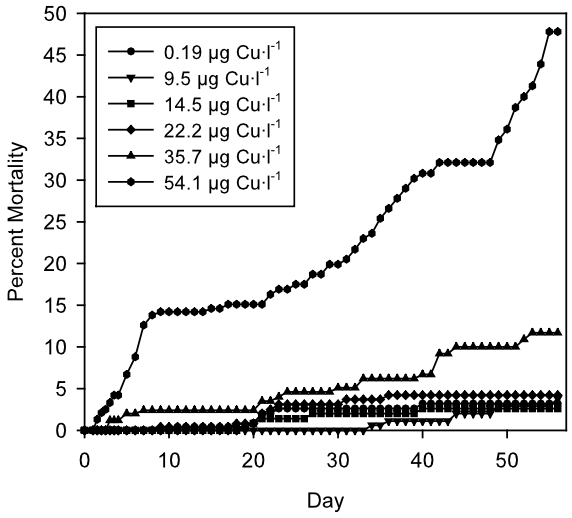

Fig. 1. Percent mortality in rainbow trout through 56 day of the sub-chronic Cu exposure. Lines and symbols indicate the mean percent mortality at each exposure concentration. Significant mortality was observed in the 35.7 and 54.1 μg Cu l^{-1} treatments.

Table 3
Mean (S.D., n) change in weight (mg) in fish at d 0 and after 20, 40, and 56 day of copper exposure

Treatment	Cu concentration ($\mu g\ l^{-1}$)	Day 20	% of control	Day 40	% of control	Day 56	% of control
Control	0.19	117 (15, 6)		278 (55, 6)		504 (74, 6)	
1	9.47	78 (25, 6)*	−33.9%	248 (78, 6)	−10.7%	438 (53, 6)	−13.1%
2	14.5	74 (39, 6)*	−36.8%	228 (68, 6)	−18.0%	395 (91, 6)*	−21.6%
3	22.2	89 (41, 6)	−24.1%	233 (38, 6)	−16.2%	355 (65, 6)*	−29.5%
4	35.7	39 (27, 6)*	−66.7%	167 (56, 6)*	−40.0%	315 (73, 6)*	−37.5%
5	54.1	24 (20, 6)*	−79.8%	95 (31, 6)*	−65.6%	255 (86, 6)*	−49.4%

Note: Mean change in weight was the mean weight of fish at d 0 was subtracted from each measured weight after 20, 40, and 56 day Cu exposure.*, Weight changes were significantly different from control ($P<0.05$) using Dunnett's procedure.

hibition concentrations based on whole body Cu (Table 4). This model [change in weight = $0.604 - 0.022$ * Tissue Cu ($\mu g\ g^{-1}$); $r^2 = 0.63$] was used to calculate growth inhibition concentration (IC_x) values based on tissue Cu concentrations. Since fish contain background levels of Cu, changes in tissue Cu concentrations were corrected for the mean analyzed tissue Cu in control fish (4.42 $\mu g\ g^{-1}$ dw). Based on change in weight, the IC_{01} was 4.6 $\mu g\ g^{-1}$ (dw), the IC_{10} was 6.7 $\mu g\ g^{-1}$ (dw), the IC_{20} was 9.0 $\mu g\ g^{-1}$ (dw), and IC_{50} was 15.9 $\mu g\ g^{-1}$ (dw) (Table 4).

3.6. Acute Cu and Cd challenge

Following the chronic exposure, surviving trout were removed and randomly placed into control exposure (< detect Cd, 0.17 $\mu g\ Cu\ l^{-1}$), high Cu exposure (91.0 $\mu g\ Cu\ l^{-1}$, < detect Cd), or high Cd exposure (7.4 $\mu g\ Cd\ l^{-1}$, 0.19 $\mu g\ Cu\ l^{-1}$) for 96 h. No mortality was observed in fish in the control exposure tanks regardless of pre-exposure conditions. Pre-exposure to Cu had a strong influence on acute toxicity in the Cu challenge (Fig. 5). Fish that had not been pre-exposed (i.e. control fish) suffered 86% mortality in the 96-h acute test, whereas fish pre-exposed to 35.7 $\mu g\ Cu\ l^{-1}$ showed only 20% mortality. Hence, Cu pre-exposure protected fish from subsequent Cu insult; the degree of protective effect of Cu pre-exposure was related to the 'acclimation dose'.

Cu pre-exposure also slightly protected fish from subsequent Cd exposure. Only fish pre-exposed to 35.7 $\mu g\ Cu\ l^{-1}$ in the growth study (i.e. highest acclimation concentration tested) showed an increased tolerance to acute Cd concentrations (Fig. 5). However, this absence of dose-response appears to have been related to the relatively high toxicity associated with the Cd challenge concentration.

4. Discussion

4.1. Mortality

The highest two Cu concentrations tested, 36 and 54 $\mu g\ Cu\ l^{-1}$, caused 11.7 and 47.8% mortal-

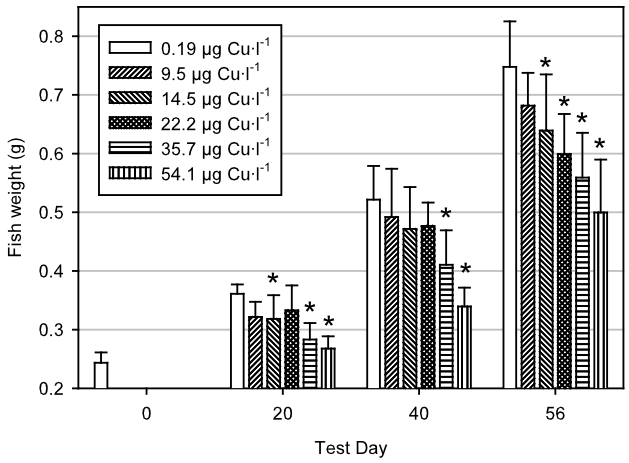

Fig. 2. Mean (+95% confidence interval) weight of rainbow trout collected from each concentration after 0, 20, 40, and 56 day Cu exposure. Asterisks (*) indicate differences from control weight based on Dunnett's procedure ($P<0.05$).

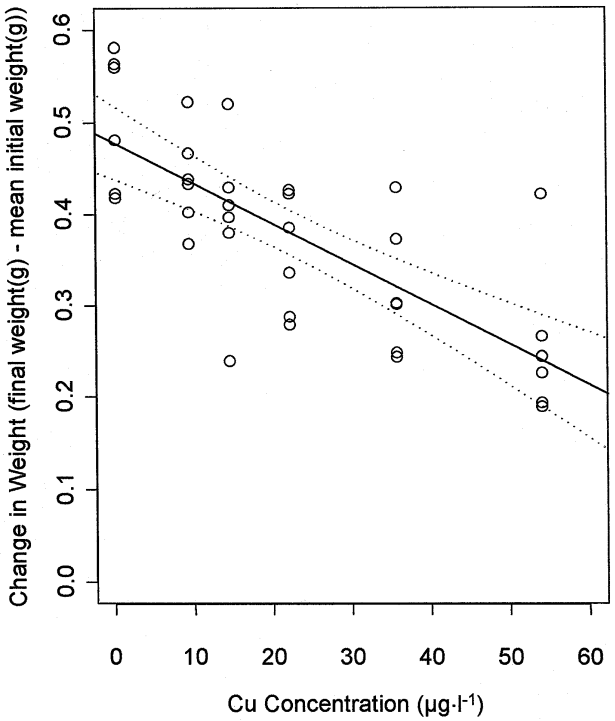

Fig. 3. Linear regression of the mean change in weight for each replicate tank (open circles) against Cu exposure concentration. The solid line represents the regression, and dotted lines represent the 95% confidence interval about that regression line.

ity, respectively, whereas lower concentrations (9.5–22 µg Cu l^{-1}) did not cause significantly greater mortality than the control treatment (Fig.

Table 4
Mean (Å 95% confidence interval) ICx (x% inhibition concentration) values for Cu exposure concentrations that result in reduced weight after 56 d Cu exposure. Also shown are ICx values calculated based on tissue concentrations associated with reduced growth

Icx	Water concentration (µg Cu l^{-1})	Tissue concentration exposure (µg Cu g^{-1} tissue, dry weight)
	change in weight	Change in weight
IC_{01}	1.1 (0.0, 8.3)	4.6 (2.4, 6.3)
IC_{10}	10.8 (2.4, 16.7)	6.7 (5.0, 7.9)
IC_{20}	21.6 (15.7, 27.4)	9.0 (7.8, 10.2)
IC_{50}	54.0 (45.2, NA)	15.9 (14.6, 18.3)

NA-The range of the confidence interval was beyond the range of the data.

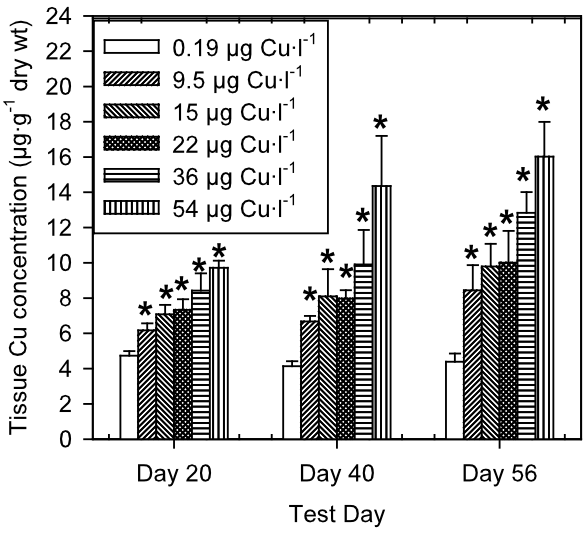

Fig. 4. Mean (+ 95% confidence interval) whole body copper concentrations (based on dry weight) from rainbow trout after 20, 40, and 56 day Cu exposure. Asterisks (*) indicate differences from control based on Bonferroni t-Test ($P < 0.05$).

1). This mortality was more protracted than in companion acute lethality tests (Hansen et al., 2001), in which mortality was essentially complete within 120 h. In this sub-chronic study, we observed an initial rapid onset of 'acute' mortality during the initial 144 h of exposure followed by a lower rate of mortality from 20 day Cu exposure

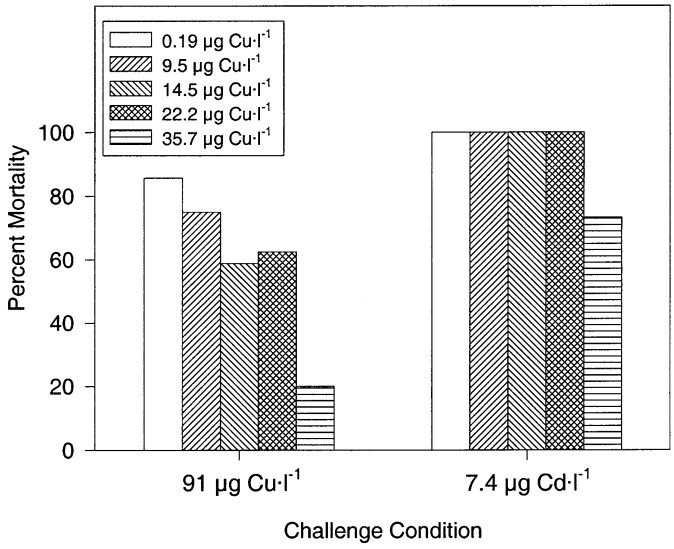

Fig. 5. Percent mortality of rainbow trout pre-exposed to several copper concentrations for 56 days, and acutely challenged to either 91 µg Cu l^{-1} or 7.4 µg Cd l^{-1} for 96 h. The figure legend indicates the pre-exposure copper concentration.

until the end of the test (Fig. 1). This delayed mortality response continued at a relatively constant rate to the end of the test. The two-stage mortality response may point to differential mechanisms of mortality, with the acute period related to gill-mediated ion loss and the chronic phase associated with slower metal uptake and adverse effects on internal tissues (McDonald and Wood, 1993).

4.2. Growth

Exposure to Cu concentrations as low as 14.5 μg l^{-1} resulted in reduced growth (i.e. a lower wet weight) in fish even after 20 day Cu exposure (Fig. 2). By day 56 (the end of the test), all fish exposed to Cu except those in the lowest Cu concentration tested (i.e. 9.5 μg l^{-1}) were significantly smaller than control fish. For example, fish exposed to 22.2 μg Cu l^{-1} were about 20% smaller than controls, and fish exposed to 54.1 μg Cu l^{-1} were about 33% smaller than controls. When expressed as a change in weight from the start of the test, (i.e. mean day 0 weight subtracted from each measured weight after 20, 40, and 56 day Cu exposure), differences between control fish and Cu-exposed fish were greater. Compared with control fish at d 56, the change in mean weight was 21.6% less in fish exposed to 14.5 μg Cu l^{-1}, and 49.4% less in fish exposed to 54.1 μg Cu l^{-1} (Table 3).

By regressing change in weight against exposure Cu, the modeled IC_{20} at day 56 was 21.6 μg Cu l^{-1}. Our results were similar to those of other studies that were conducted at similar water hardness conditions. Waiwood and Beamish (1978) observed a 20% reduction in growth rate in rainbow trout exposed to 23 μg Cu l^{-1} over a 30-day test. Similarly, Seim et al. (1984) observed that fish exposed to 31 μg Cu l^{-1} were approximately 20% smaller than controls. From the data in Seim et al. (1984), Chapman (1999) estimated a chronic effect threshold value of 22.3 μg Cu l^{-1}.

We found that the effect of Cu on rainbow trout growth was best expressed by a model that included both magnitude and duration of Cu exposure:

$$\ln(\text{fish weight in grams}) = -1.3336 - 0.0051(\mu\text{g Cu l}^{-1}) + 0.0165(\text{d}) \quad [r^2 = 0.91]$$

Although growth was readily expressed as a function of Cu exposure and exposure duration, we caution that strict application of model predictions to field conditions is inappropriate. Growth responses in wild fish are likely to differ from those observed in controlled laboratory tests because of increased energy expenditures and more limited food availability in field settings. Additionally, Cu exposure conditions and other water quality variables such as temperature are typically not constant in the field; and intermittent Cu exposure concentrations can produce more dramatic effects on growth (Seim et al., 1984). Therefore, our modeled growth responses in laboratory fish exposed to Cu illustrate growth effects only under constant exposure concentrations and optimum growth conditions.

4.3. Relationship between whole body Cu concentrations and trout growth

Whole body Cu concentrations from all Cu exposures (i.e. ≥ 9.5 μg Cu l^{-1}) were all significantly greater ($P < 0.05$) than controls (Fig. 4). Moreover, Cu accumulation did not appear to have reached equilibrium by the end of the test. In fact, fish exposed to 35.7 and 54.1 μg Cu l^{-1} accumulated Cu at a faster rate than did fish exposed to lower Cu concentrations. A similar dose-response relationship was observed by Seim et al. (1984), Marr et al. (1996).

Fish weight following Cu exposure is a function of exposure duration and whole body Cu concentration (Fig. 6):

$$\ln(\text{Fish weight in g}) = -1.3328 + 0.0220(\text{Exp. time in days}) - 0.0394(\text{Tissue Cu in } \mu\text{g g}^{-1}\text{(dw)}) \quad [r^2 = 0.88]$$

Interestingly, this model has virtually identical slope functions as a model previously produced by Marr et al. (1996):

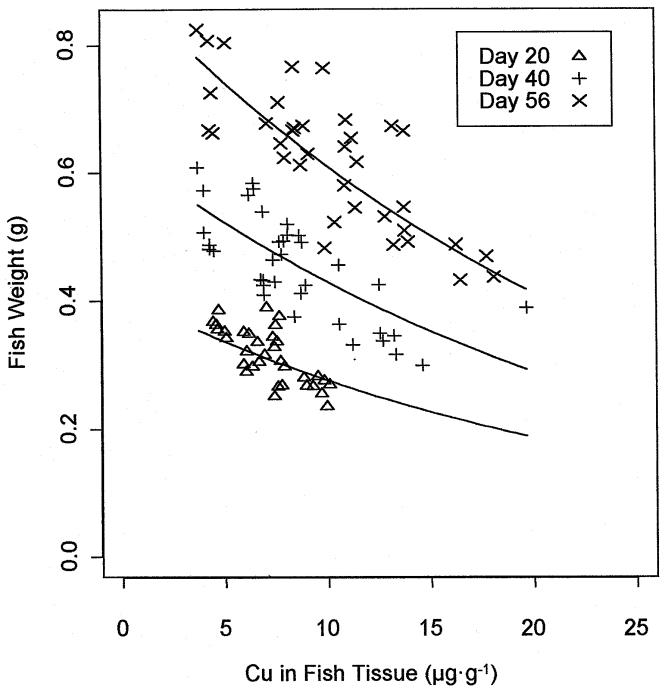

Fig. 6. Weight of rainbow trout expressed as a function of whole body Cu concentration and exposure duration. Fitted lines are from different sampling days. Different symbols indicate observed fish weights and tissue Cu concentrations from different sampling days.

$$\ln(\text{Fish weight in g}) = 4.799 + 0.028(\text{Exp. time in days}) - 0.0384(\text{Tissue Cu in } \mu\text{g g}^{-1}\text{(dw)}) \quad [r^2 = 0.94]$$

Even though the present study and Marr et al. (1996) found similar relationships between tissue Cu concentrations, Cu exposure durations, and fish growth, there are several differences between the two studies. Marr et al. (1996) exposed 0.12 g fish to Cu in 25 mg l^{-1} hardness water at a temperature of 9.9 °C, whereas the present study exposed 0.244 g fish to Cu in 100 mg l^{-1} hardness water at a temperature of 8.1 °C. These differences in fish size, water hardness, and test temperature had little effect on regression slope functions, but did affect the regression intercept. However, when both sets of growth data are expressed as instantaneous growth rate (% per day) standardized to test temperature, a relatively constant relationship between tissue Cu and growth responses is observed (Fig. 7). This relatively constant relationship between tissue burden and growth in two tests performed at different water hardness levels suggests that tissue burdens reflect the internally regulated dose, and that this critical body dose is associated with toxicity (expressed as growth reductions) across a range of water quality exposure conditions. As noted in our discussion of the water-based growth model (above), application of this tissue residue-based model to field conditions is inappropriate because of unknown exposure durations and dynamics.

4.4. Relationship between hardness and Cu induced growth reductions

Growth can be expressed as differences in size of fish between treatments, change in size in fish from each treatment compared with control fish, or as differences in growth rate between treatments, so studies on fish growth are not always comparable. However, data from several chronic and sub-chronic tests show the effects of Cu on growth are dependent on water hardness (Fig. 8). Waiwood and Beamish (1978) showed that 4, 23, and 168 µg Cu l^{-1} produced a 20% reduction in growth rate at water hardness values of 30, 100, and 360 mg l^{-1} respectively. Using the difference in dry weight, Seim et al. (1984), as analyzed by

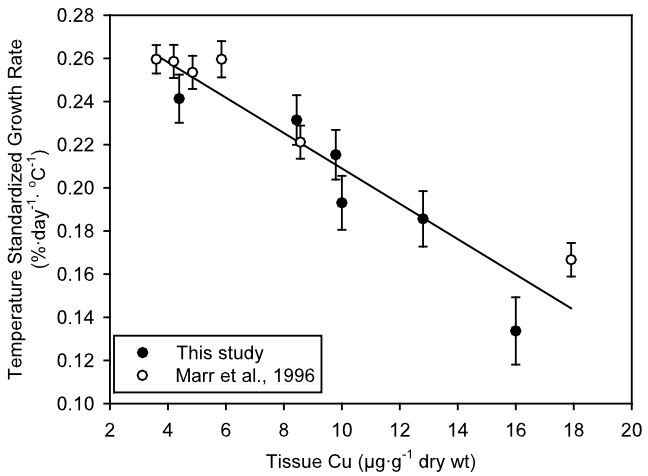

Fig. 7. Temperature standardized growth rate (% per d per °C) in rainbow trout as functions of tissue copper concentration (µg Cu g^{-1} dry weight). Data points are means for each study and the vertical lines on each data point are ±1 S.E. The solid line represents the regression through all data.

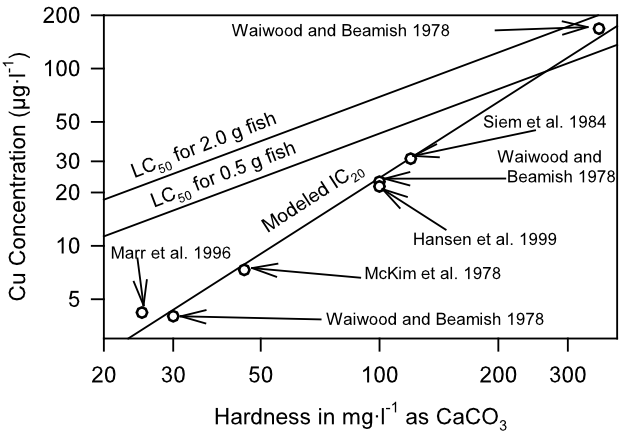

Fig. 8. Comparison of predicted 20% inhibition concentration (IC_{20}) with hardness between our study and other published studies on rainbow trout. For reference, predicted LC_{50} concentrations for 0.5 g and 2.0 g rainbow trout are also presented. Predicted LC_{50}s are based on calculations by Chapman (1999).

Chapman, 1999) produced an IC_{20} of 31 μg Cu l^{-1} in a hardness of 120 mg l^{-1}. From raw wet weight data from Marr et al. (1996), fish exposed to 4.6 μg Cu l^{-1} in 25 mg l^{-1} hardness water were 23.5% smaller than controls, and the extrapolated IC_{20} from the data is 4.2 μg Cu l^{-1}. In 45 mg l^{-1} hardness water, McKim et al. (1978) as analyzed by Chapman, 1999) observed an IC_{20} for rainbow trout growth of 7.3 μg Cu l^{-1}. Although there were differences in exposure times and experimental methods between the studies, a linear relationship (log–log) between IC_{20} and hardness is apparent. The equation for this relationship is:

$$\log(IC_{20}) = 1.423[\log(\text{hardness in mg } l^{-1})] - 1.463$$

In contrast, Chapman (1999) modeled data from several acute Cu toxicity tests and observed a different relationship between hardness and lethality. The relationship between LC_{50} concentrations and hardness that also includes a constant to normalize for fish size was:

$$\log(LC_{50}) = (0.0766 + 0.831*\log(\text{hardness in mg } l^{-1})) + \log(\text{Fish weight(g)}^{0.344})$$

The difference between these two relationships is that the slope of hardness to Cu effects on growth is steeper than the slope of hardness to Cu LC_{50}. This slope difference suggests that hardness may have a greater role in protecting against chronic growth effects than against acute lethality. Hardness is believed to influence acute Cu toxicity by reducing gill uptake of Cu through competition for binding sites with Ca (Wood et al., 1997), by reducing ion loss through Ca-mediated influences on gill permeability (Laurén and McDonald, 1986), or by reducing aqueous Cu bioavailability through inorganic complexation with carbonate (Wood et al., 1997). A stronger influence of Ca on chronic growth effects compared with acute lethality suggests a different mechanism of long-term Ca–Cu interaction, possibly related to the Cu uptake rate and the regulation of Cu in internal compartments.

4.5. Acute-to-chronic ratios

The acute-to-chronic ratio (ACR) has been used to estimate chronic effects concentrations when tests of chronic effects are not available (Wood et al., 1997). ACRs are the ratio of an acute endpoint (e.g. LC_{50}) to a chronic endpoint (e.g. EC_{20} or EC_{50}). The choice of chronic endpoints can influence the ACR value. However, to date, no guidelines have been set to define which chronic endpoints should be used to derive ACRs. In this study, we calculated ACRs using the EC_{20} for growth as a suitable chronic endpoint because the EC_{20} Cu concentration consistently reduces growth in fish but typically produces little mortality. The EC_{50} endpoint is usually at a Cu concentration that also produces significant mortality; since much of this mortality is most likely due to acute mechanisms, the EC_{50} is an unsuitable chronic endpoint to use in deriving ACR values (Fig. 1, Table 4).

Estimation of one ACR from our studies is difficult. In two companion acute tests conducted using similar water quality conditions, 120-h LC_{50} concentrations were estimated to be 35.1 and 76.6 μg Cu l^{-1} (Hansen et al., 2001), yielding an ACR of either 1.6 or 3.5, depending on which acute test was used in the comparison. The acute test that produced the ACR of 1.6 used the same group of fish as the fish used in the sub-chronic study presented here. Moreover, the acute test began on d 24 of this study. In contrast, the acute test that

produced the ACR of 3.5 used the same strain of rainbow trout as used in the sub-chronic study presented here, but the fish were obtained from different hatcheries, and the test was conducted several weeks before the start of this sub-chronic study. Although the ACR of 1.6 may be more appropriate, the range in ACRs obtained from two different bioassays illustrates the practical difficulty of any precise estimations of ACRs.

An alternative, and potentially more robust, approach to deriving an ACR involves comparison of pooled responses of rainbow trout across a range of tests. Chapman (1999) developed a relationship between hardness and LC_{50} for rainbow trout across a number of different bioassays. This relationship was found to be a function of fish size. Chapman (1999) relationship for 0.5 and 2.0 g trout is presented in Fig. 8 along with the modeled relationship we observed for 20% growth inhibition. Using these models, an ACR can be calculated as the ratio of the LC_{50} and IC_{20} concentrations as a function of hardness (Fig. 9). A clear relationship was observed between ACRs and water hardness, with greater ACRs at lower hardness levels. For example, the ACR for a 0.5-g fish was roughly 2.5 at a hardness of 50 mg l^{-1} and approximately 1.5 at a hardness of 150 mg l^{-1}. The ACR appears to reach an asymptote of 1.0 at a hardness of approximately 250 mg l^{-1}, indicating no difference between Cu concentrations causing acute and chronic effects.

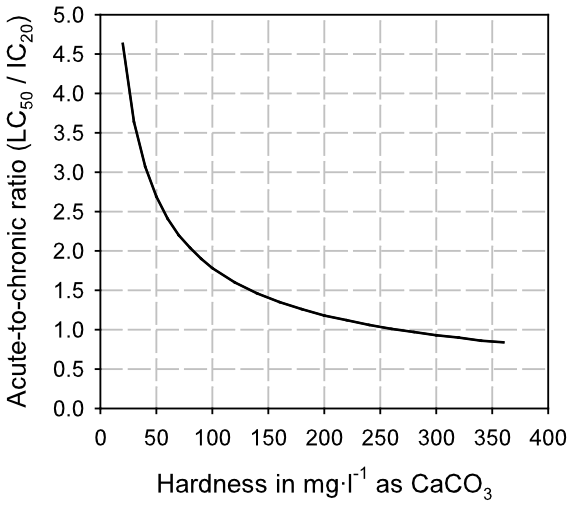

Fig. 9. Influence of increased hardness on acute-to-chronic ratios based on modeled LC_{50} and IC_{20} concentrations. Modeled LC_{50} concentrations were calculated using the equation produced by Chapman (1999) for a 0.5 g rainbow trout. Modeled IC_{20} concentrations were calculated using the regression for IC_{20} concentrations in Fig. 8.

We also evaluated the relationship between aqueous Ca concentrations and ACRs because Ca mitigates Cu toxicity to a much larger extent than Mg (Welsh et al., 2000). This evaluation was performed to ascertain whether the relationship between ACR and water hardness was influenced by possible differences in the ratio of Ca to Mg in the water hardness in the studies used in the evaluation of ACRs. We found virtually the same relationship between Ca and ACRs as between hardness and ACRs; the ACR for a 0.5-g fish was approximately 2.5 in water with 10 mg Ca l^{-1} and approximately 1.0 in water with 40 mg Ca l^{-1}. Therefore, the hardness-dependency of ACRs was not related to differences in Ca concentration between the chronic and acute studies used to develop the relationship.

4.6. Acute Cu and Cd challenge

We observed roughly a dose-dependent relationship between the Cu concentration to which fish had been pre-exposed during the sub-chronic test and percent mortality subsequently observed in a 96-h acute Cu challenge (Fig. 5). In our sub-chronic study, fish exposed to all Cu concentrations accumulated significantly more Cu than controls ($P < 0.05$). Copper exposure and accumulation may have induced metalloprotein synthesis in fish; metalloproteins have been shown to detoxify metals by reducing the bioavailability of intracellular metals (Hogstrand et al., 1995). Our data support, but do not provide independent proof, that higher Cu acclimation concentrations reduce acute Cu sensitivity. Moreover, our results suggest that fish pre-exposed to Cu may have a reduced sensitivity to Cd.

5. Conclusion

Reductions in growth can be caused by physiological or behavioral stress during exposure to toxicants. Physiological or behavioral stress can

result from a reduction in food consumption or food assimilation (Lett et al., 1976; Waiwood and Beamish, 1978), and from increased metabolic costs associated with detoxification and homeostasis during chronic, sublethal exposures (Dixon and Sprague, 1981b; Hogstrand et al., 1995; Marr et al., 1995). In our test, we did not observe a reduction in feeding activity with higher Cu exposures. We did, however, observe a dose-response relationship between Cu exposure concentration and Cu accumulation. When waterborne Cu was used to predict growth impairment, hardness was found to strongly influence IC_{20} values, with the relationship more pronounced (i.e. steeper slope) between hardness and chronic growth effects than between hardness and acute lethality. In addition, we found that whole body Cu appeared to be a constant predictor of growth impairment across two studies conducted at different water hardness, suggesting that internal dose is associated with adverse effects. However, critical body residue approaches to biomonitoring do not appear to be useful for Cu because the critical residue was dependent on exposure duration, a variable that typically is unknown in field settings.

Our results indicate that at a water hardness of 100 mg l^{-1}, pH 7.9, and very low TOC concentrations, significantly reduced growth can be observed at Cu concentrations as low as 9.5 μg l^{-1}. The effects of higher TOC concentrations, as observed in many field conditions, on growth responses to Cu exposure are unknown. However, our results are relevant to many high elevation streams with low TOC and many other streams near mine effluents where the kinetics of Cu–TOC binding are slow (Ma et al., 1999). Additional research should focus on mechanisms of chronic growth effects and the relationship between these effects and water quality variables.

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References

ASTM. 1992. Standard guide for conducting early life-stage toxicity tests with fishes. E1241-92. In 1992 Annual Book of ASTM Standards, Section II, Water and Environmental Technology, Volume 11.05 Biological Effects and Environmental Fate. American Society for Testing and Materials, Philadelphia, PA, USA.

ASTM. 1996. Standard guide for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. E729-88a^{e1}. In 1996 Annual Book of ASTM Standards, Section 11, Water and Environmental Technology, Volume 11.05 Biological Effects and Environmental Fate; Biotechnology; Pesticides. American Society for Testing and Materials, Philadelphia, PA, USA.

Chapman, G.A., 1999. An Acute TRV for Rainbow Trout and Bull Trout. Report prepared for the Montana Natural Resources Damage Program, Helena, MT, USA. 16 April.

Dixon, D.G., Sprague, J.B., 1981a. Acclimation to copper by rainbow trout (*Salmo gairdneri*)—a modifying factor in toxicity. Can. J. Fish. Aquat. Sci. 38, 880–888.

Dixon, D.G., Sprague, J.B., 1981b. Copper bioaccumulation and hepatoprotein synthesis during acclimation to copper by juvenile rainbow trout. Aquat. Toxicol. 1, 69–81.

Hansen, J.A., Lipton, J., Welsh, P.G., 2001. Relative sensitivity of bull trout (*Salvelinus confluentus*) and rainbow trout (*Oncorhynchus mykiss*) to acute copper toxicity. Environmental Toxicology and Chemistry, in press.

Hogstrand, C., Reid, S.D., Wood, C.M., 1995. Ca^{2+} versus Zn^{2+} transport in the gills of freshwater rainbow trout and the cost of adaptation to waterborne Zn^{2+}. J. Exp. Biol. 198, 337–348.

Kalbfleisch, J.D., Prentice, R.L., 1980. The Statistical Analysis of Failure Time Data. Wiley, New York.

Laurén, D.J., McDonald, D.G., 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.

Lett, P.F., Farmer, G.J., Beamish, F.W.H., 1976. Effect of copper on some aspects of the bioenergetics of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 33, 1335–1342.

Ma, H., Kim, S.D., Cha, D.K., Allen, H.E., 1999. Effect of kinetics of complexation by humic acid on toxicity of

copper to *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 18, 828–837.

Marr, J.C.A., Bergman, H.L., Lipton, J., Hogstrand, C., 1995. Differences in relative sensitivity of native 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.

Marr, J.C.A., Lipton, J., Cacela, D., Hansen, J.A., Bergman, H.L., Meyer, J.S., Hogstrand, C., 1996. Relationship between copper exposure duration, tissue copper concentration, and rainbow trout growth. Aquat. Toxicol. 36, 17–30.

McDonald, D.G., Wood, C.M., 1993. Branchial mechanisms of acclimation to metals in freshwater fish. In: Rankin, J.C., Jensen, F.B. (Eds.), Fish Ecophysiology. Chapman & Hall, London, pp. 297–321.

McKim, J.M., Eaton, J.G., Holcome, G.W., 1978. Metal toxicity to embryos and larvae of eight species of freshwater fish-II: Copper. Bull. Environ. Contam. Toxicol. 19, 608–616.

Seim, W.K., Curtis, L.R., Glenn, S.W., Chapman, G.A., 1984. Growth and survival of developing steelhead trout (*Salmo gairdneri*) continuously or intermittently exposed to copper. Can. J. Fish. Aquat. Sci. 41, 433–438.

Sprague, J.B., 1969. Measurement of pollutant toxicity to fish: I. Bioassay methods for acute toxicity. Water Res. 3, 793–821.

Taylor, L.N., McGeer, J.C., Wood, C.M., McDonald, D.G., 1998. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: Evaluation of chronic indicators. Environ. Toxicol. Chem. 19, 2298–2308.

Verdouw, H., van Echteld, C.J.A., Dekkers, E.M.J., 1978. Ammonia determination based on indophenol formulation with sodium salicylate. Water Res. 12, 399–402.

Waiwood, K.G., Beamish, F.W.H., 1978. The effects of copper, hardness and pH on the growth of rainbow trout, *Salmo gairdneri*. J. Fish Biol. 13, 591–598.

Welsh, P.G., Lipton, J., Podrabsky, T.L., Chapman, G.A., 2000. Relative importance of calcium and magnesium in hardness-based modification of copper toxicity. Environ. Toxicol. Chem. 19, 1624–1631.

WEST, 1996. Toxstat v3.5 documentation. Western EcoSystems Technology, Cheyenne, WY, USA

Wood, C.M., Adams, W.J., Ankley, G.T., DiBona, D.R., Luoma, S.N., Playle, R.C., Stubblefield, W.A., Bergman, H.L., Erickson, R.J., Mattice, J.S., Schlekat, C.E., 1997. Environmental toxicology of metals. In: Reassessment of Metals Criteria for Aquatic Life Protection: Priorities for Research and Implementation. SETAC Pellston Workshop on Reassessment of Metals Criteria for Aquatic Life Protection. Bergman, H.L., Dorward-King, E.J. (eds.). 10–14 February 1996, Pensacola, FL, USA.

Wootton, R.J., 1990. Ecology of Teleost Fishes. Chapman and Hall, London.