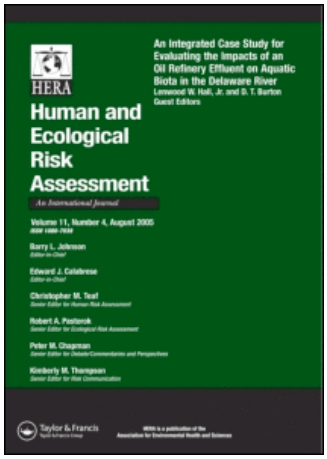

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Radiotracer Studies on Waterborne Copper Uptake, Distribution, and Toxicity in Rainbow Trout and Yellow Perch: A Comparative Analysis

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ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*) are often used to estimate important biotic ligand model (BLM) parameters, such as metal-binding affinity (log *K*) and capacity (B_{max}). However, rainbow trout do not typically occupy metal-contaminated environments, whereas yellow perch (*Perca flavescens*) are ubiquitous throughout most of North America. This study demonstrates that dynamic processes that regulate Cu uptake at the gill differ between rainbow trout and yellow perch. Rainbow trout were more sensitive to acute aqueous Cu than yellow perch, and toxicity was exacerbated in soft water relative to similar exposures in hard water. Whole body Na loss rate could account for acute Cu toxicity in both species, as opposed to new Cu uptake rate that was not as predictive. Time course experiments using radiolabelled Cu (^{64}Cu) revealed that branchial Cu uptake was rather variable within the first 12 h of exposure, and appeared to be a function of Cu concentration, water hardness, and fish species. After 12 h, new branchial Cu concentrations stabilized in both species, suggesting that metal exposures used to estimate BLM parameters should be increased in duration from 3 h to 12+ h. In rainbow trout, 71% of the new Cu bound to the gill was exchangeable (*i.e.*, able to either enter the fish or be released back to the water), as opposed to only 48% in yellow perch. This suggests that at equal exposure concentrations, proportionally more branchial Cu can be taken up by rainbow trout than yellow perch, which can then go on to confer toxicity. These qualitative differences in branchial Cu handling between the two species emphasize the need to develop BLM parameters for each species of interest, rather than the current practice of extrapolating BLM results derived from rainbow trout (or other laboratory-reared species) to other species. Data reported here indicate that a one-size-fits-all approach to predictive modeling, mostly based on rainbow trout studies, may not suffice for making predictions about metal toxicity to yellow perch—that is, a species that inhabits metal-contaminated lakes around northern Canadian industrial operations.

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Key Words: biotic ligand model, copper, species differences, exchangeable fraction, metal–gill binding dynamics, acute toxicity.

INTRODUCTION

Most research examining the mechanisms of metal toxicity to fishes has been conducted on rainbow trout (*Oncorhynchus mykiss*). However, rainbow trout rarely occupy metal-contaminated waters in northern Canada, and questions have been raised about the ecological relevance of rainbow trout toxicity models (Burger 1994; Campbell *et al.* 2006). Yellow perch (*Perca flavescens*) are ubiquitous throughout Canada (Scott and Crossman 1973), are very tolerant to dissolved metals (Taylor *et al.* 2003), and are now well known to inhabit metal-contaminated lakes around northeastern Ontario industrial operations (Pyle *et al.* 2005). Therefore, to improve the ecological relevance of ecological risk assessments (ERA), it is important to understand the differences between rainbow trout and yellow perch with respect to their sensitivity to metals, the mechanism of toxic action, and to determine species differences with respect to how each species handles metals at sensitive sites of metal uptake.

The biotic ligand model (BLM) depends on the relationship between metal bound to fish gills and toxicity (Paquin *et al.* 2002; Niyogi and Wood 2004). An obvious assumption of the BLM is that gill-bound metal is significantly related to observed toxicity. However, it is much more likely that only that fraction of Cu that can actually *enter* the gill (*i.e.*, the exchangeable fraction) will go on to confer toxicity to the fish. Most attempts at estimating BLM parameters, including metal–gill binding affinity (log *K*) and capacity (B_{max}), expose standard laboratory test species, such as rainbow trout or fathead minnows (*Pimephales promelas*), to a series of increasing metal concentrations over a saturable range in simple, soft water for 3–24 h. Under these controlled conditions, the BLM performs well in terms of predicting acute metal toxicity (Playle *et al.* 1992; Playle *et al.* 1993; Santore *et al.* 2001; Di Toro *et al.* 2001; Paquin *et al.* 2002). However, it has been difficult to reconcile toxicological predictions in fish from metal-contaminated environments owing to differences in the standard BLM parameters (log *K* and B_{max}) between laboratory-reared and wild fish (Niyogi *et al.* 2004; Taylor *et al.* 2004; Niyogi *et al.* in press). These differences are likely related to chronic metal exposure and other biotic or abiotic factors inherent in natural systems (*e.g.*, water quality, especially as it pertains to metal speciation, and natural adaptive processes in chronically exposed fish).

Therefore, to improve the ecological relevance of ERAs that use the BLM to make toxicological predictions for natural fish populations inhabiting metal-contaminated environments, we need a better understanding about how metals interact with biotic ligands (*i.e.*, fish gills). It is important to understand species differences in metal–gill interactions to estimate the relevance of BLM parameters derived from one species (*e.g.*, rainbow trout) to that of another (*e.g.*, yellow perch). It is also important to understand the nature of the metal–gill relationship (how much gets taken up into the gills to confer toxicity), and whether or not disparate species handle metals similarly at the gills.

This article describes a series of experiments that was conducted in order to address these questions with respect to waterborne copper exposure. In the first

experiment, time-to-death (ET50) was established for each species (rainbow trout and yellow perch), in both hard and soft water, as a surrogate measure of acute Cu toxicity. Initial ET50 tests allowed us to estimate Cu concentrations that could yield the same level of toxicity for each species. We then exposed both species to these equi-toxic Cu concentrations in hard and soft water to examine the influence of water hardness on Cu uptake and toxicity as a function of tissue and whole body Na loss to determine the extent to which Na flux contributed to the toxic response. Moreover, tissue metal accumulation was also examined to provide some indication about any differences in metal accumulation patterns in various tissues (including gills, liver, and carcass). The second experiment focused on the time-course of Cu uptake to gills of each species in hard and soft water and at low (20 μg/L) and high (60 μg/L) Cu concentrations, to determine whether or not fish accumulate branchial Cu gradually over time, or if metal binding and uptake is a physiologically regulated process. We followed up the time-course experiments with a pulse-chase study, where fish from each species were exposed for either 30 or 360 min to 60 μg/L ^{64}Cu in moderately hard water (*i.e.*, the pulse). Fish were then transferred to water containing the same Cu concentration, but without the radiotracer. Fish were then sampled at regular intervals (*i.e.*, the chase) to examine the proportion of total accumulated ^{64}Cu that was exchangeable and thus capable of entering the fish to confer toxicity.

MATERIALS AND METHODS

Experimental Animals

Juvenile yellow perch (mean ± SD; 2.03 ± 0.99 g, n = 196 total over all experiments) were purchased from Kinmount Fish Farm (Kinmount, ON). Juvenile rainbow trout (mean ± SD; 3.46 ± 1.59 g, n = 196 total over all experiments) were purchased from Humber Springs Fish Farm (Orangeville, ON). Fish were held in circular 600 L polyethylene tanks under flow-through (1 L/min) conditions. Photoperiod was maintained at 12 h. Fish were allowed to acclimate to laboratory conditions for at least two weeks prior to experimentation in moderately hard Hamilton, ON, Canada, municipal water (12–14°C, 0.6 mM Na^+, 1.02 mM Ca^{2+}, total hardness 120 mg/L as $CaCO_3$, pH 7.6–8.0, background Cu concentration, 3 μg/L). Fish were fed daily on a commercial diet (Corey Hatchery Feed, Corey Feed Mills, Ltd., Fredericton, NB) at a rate of approximately 2% total fish mass. Debris was siphoned from each tank daily.

Time to Death: Acute Copper Toxicity in Hard and Soft Water

To establish the relative toxicity of Cu to rainbow trout (mean ± SD: 3.08 ± 1.61 g; n = 30) and yellow perch (2.03 ± 0.97 g; n = 30), we conducted preliminary toxicity tests evaluating time-to-death upon exposure to acute aqueous Cu exposure in hard and soft water. The hard water used in this test was simply dechlorinated Hamilton, ON, municipal water (120 mg/L as $CaCO_3$). Soft water was prepared by adding one part hard water to 10 parts nanopure water (18 mΩ Nanopure II, Sybron/Barnstead, Boston, MA, USA), resulting in exposure water having a total hardness of 11 mg/L

as $CaCO_3$. Copper solutions were prepared from a 70 mg Cu/L stock solution, prepared from $CuSO_4$ in nanopure water. Appropriate volumes of Cu stock solution were added to hard and soft exposure waters to yield exposure concentrations of 160, 400, and 1000 μg Cu/L (nominal). Five fish were placed in 5 L of exposure water in each treatment for up to 660 min (*i.e.*, 11 h), which corresponded to when at least half of all the test fish had died. Surviving fish were enumerated on 30 min intervals over the course of the exposure.

This analysis allowed us to estimate approximately equi-toxic Cu concentrations for each species exposed to Cu in soft water. Here, we wanted to expose both rainbow trout and yellow perch to concentrations of Cu that would yield 50% mortality in 180 min. in soft water. Establishing equi-toxic Cu concentrations in soft water allowed us to examine the effect of hardness on each species' sensitivity to acute Cu exposure. Extrapolating from a regression analysis on ET50 data (determined from the exposures described earlier) for rainbow trout, that concentration was 250 μg Cu/L and for yellow perch it was 550 μg Cu/L. These exposure concentrations were used in the next experiment.

Copper Uptake and Sodium Loss

Based on the preceding analysis, we exposed 10 rainbow trout and yellow perch to each of Cu (*i.e.*, 550 μg/L for yellow perch, 250 μg/L for rainbow trout) or no Cu in hard or soft water (mean $\pm$ SD: rainbow trout, 3.22 $\pm$ 1.33 g; yellow perch, 1.16 $\pm$ 0.42 g; n = 40 for each species). Hard and soft water in this analysis was prepared in the same way as described earlier. Fish were held under these conditions for up to 33 h (*i.e.*, 1980 min) or until at least half the fish died in a particular treatment. Surviving fish were enumerated every 30 min during exposures. The median time-to-death (ET50), as defined earlier, was estimated for each exposure treatment.

To each Cu-exposure medium, we added 20 μCi/L of ^{64}Cu. Copper isotope was prepared from 0.1 mL $CuNO_3$ dissolved in 1N HNO_3 that was allowed to evaporate to dryness. The crystals were then irradiated for 870 min via neutron activation (McMaster University) to a final activity of 388.3 μCi. The neutron-activated Cu was redissolved in 200 μL of concentrated HNO_3 (trace metals grade, Fisher Scientific, Nepean, ON) and brought up to a final volume of 10 mL with nanopure water. To each 5 L Cu exposure treatment replicate, 2 mL of this ^{64}Cu stock was added (*i.e.*, approximately 20 μCi/L). This amount of Cu isotope was sufficient to provide a useful radiotracer level yet had little influence on the exposure Cu concentration. Water samples in all experimental treatments were taken at the beginning and end of each exposure and analyzed for total and radioactive Cu (see Analytical Details).

Once fish either died or became moribund (or the duration of the exposure elapsed), they were immediately removed from exposure vessels to minimize the influence of post-mortem autolysis on tissue ion or metal concentrations. Sampled fish were dissected to remove gills and livers. Carcasses were defined as the remaining fish body after gills and livers were removed. Gills, livers, and carcasses were rinsed in nanopure water to remove any surface bound metal and counted for γ emissions or analyzed for Cu or Na (see Analytical Details).

Time Course for Branchial and Whole Body Cu Uptake

In the third experiment, we examined Cu–gill binding and uptake dynamics in hard and soft water to rainbow trout (mean ± SD: 3.53 ± 1.75 g; n = 84) and yellow perch (2.26 ± 1.05; n = 84). Fish were randomly assigned to 5 L exposure chambers in groups of three in hard or soft water (as described earlier). Fish of each species were exposed to either 20 or 60 μg Cu/L for a maximum of 24 h. A Cu concentration of 20 μg/L was selected for its ecological relevance in metal-contaminated soft-water systems in northern Canadian lakes (Pyle *et al.* 2005), and 60 μg/L, although still ecologically relevant, is known to induce toxicological effects in rainbow trout (Taylor *et al.* 2000). As in the previous experiment, approximately 20 μCi/L of ^{64}Cu was added to each exposure chamber at the beginning of the test. Subsamples of three fish of each species in each experimental treatment were collected randomly from the experimental animals at 0.5, 1, 3, 6, 9, 12, and 24 h after the initiation of exposure. Whole gill baskets were removed from subsampled fish. Gill baskets and carcasses (*i.e.*, whole bodies without gill baskets) were counted for γ emissions and analyzed for total Cu concentration (GFAAS).

Pulse-Chase Experiment

In the final experiment, we studied qualitative differences in metal handling capabilities in rainbow trout and yellow perch as a possible way to account for species differences in Cu sensitivity. Fish were acquired from the same sources as listed earlier, but weights were slightly different (mean ± SD: rainbow trout, 3.84 ± 1.84 g; yellow perch, 2.41 ± 0.79 g, n = 42 for each species). This experiment was conducted in two trials, a long-pulse and a short-pulse trial. In each case, fish of each species were initially exposed to 60 μg ^{64}Cu/L (*i.e.*, "hot" Cu, the total Cu concentration in the initial exposure medium was entirely derived from ^{64}Cu) for either 0.5 or 6 h in hard water (as aforementioned), depending on whether the particular trial was a short-pulse or long-pulse, respectively. In this experiment, we wanted to examine differences in metal-handling strategies between the two species at a concentration known to induce a toxicological response in both hard and soft waters. Therefore, 60 μg/L was selected as the exposure concentration based on results of the previous experiments. At the end of this radioactive "pulse," all of the fish were briefly (30 s) rinsed in isotope-free, clean hard water. Half of the fish were then transferred to the same hard exposure water containing 60 μg Cu/L (*i.e.*, "cold" Cu, not radioactive) and held for a maximum of 1 h for fish exposed to the short-term pulse, or 2 h for fish exposed to the long-term pulse. This subsequent exposure to cold Cu was the "chase" phase. The other half were transferred back into the hot Cu exposure water to serve as a control, providing an estimate of the total new gill Cu burden at the end of the 1 h or 2 h period in the presence of continued exposure to the radiolabelled Cu solution. During the short-pulse trial, three fish of each species were sampled at 0, 15, 30, and 60 min. in the chase phase. During the long-pulse trial, three fish of each species were sampled at 0, 30, 60, and 120 min. in the chase phase. Control fish were also sampled during the same sampling events. Gill baskets were removed from sampled fish. Gills and carcasses were analysed for total Cu by GFAAS and Na by FAAS. Water samples were collected at each sampling time and analysed for Cu (GFAAS), Na, and Ca (FAAS).

Analytical Details

Radioactive Cu was used in this study to allow for the discrimination between newly accumulated Cu in experimental treatments from background Cu concentrations. Radioactive Cu (^{64}Cu), a γ-emitting isotope of Cu, was analysed in tissues and water samples on a Canberra-Packard Minaxi Auto-Gamma 5000 series gamma counter with on-board automatic decay correction for ^{64}Cu (Canberra-Packard Instruments, Meriden, CT, USA). Newly accumulated Cu in fish tissues was calculated following the approach of Grosell *et al.* (1997). Briefly, new Cu is estimated from the following equation:

$$M_{New} = \frac{a}{\left(\frac{b}{c}\right)} \tag{1}$$

where, M_{New} is newly accumulated Cu (ng/g), a is the number of γ-emissions per minute (cpm) per gram of tissue, b is the cpm per litre of water, and c is the total measured Cu concentration in the water. To establish new Cu uptake rates, M_{New} was simply divided by the exposure time. In the pulse-chase experiment, M_{New} was calculated in a slightly different manner. Because fish were transferred into a cold-Cu exposure after the radioactive pulse phase, the maximum amount of newly accumulated Cu that could be detected in a particular tissue using the radiotracer approach was that which was measured at t = 0 (*i.e.*, the time when fish were transferred to the cold-Cu chase phase). Therefore, the denominator of equation (1) is the specific activity of the water immediately prior to fish being transferred to the cold-Cu chase phase (*i.e.*, t = 0).

Copper was measured in water and tissue samples using GFAAS (Varian 1275 AA with a GTA95 atomizer, Mississauga, ON) using 10 μL injections and settings as recommended by the manufacturer. All fish tissues were prepared for Cu analysis following previously published methodologies (*e.g.*, see Pyle *et al.* 2003). Briefly, tissues were digested at 70°C for 24 h in 5 volumes of 1 N trace metals grade (TMG) HNO_3 (Fisher Scientific, Nepean, ON), and centrifuged at 10,000 *g* for 5 min to remove any remaining solids. The supernatant was diluted to within the analytical range of the instrument using 0.5% trace metals grade HNO_3. Water samples were unfiltered and acidified to pH 2 with concentrated TMG HNO_3. Preliminary measurements on water samples determined whether or not samples had to be diluted to within the analytical range of the instrument. When dilution was necessary, samples were diluted with 0.5% TMG HNO_3 in nanopure water. Samples prepared for Na analysis in tissues and water were prepared in the same way as for Cu analysis. However, Na measurements were conducted using FAAS (Varian 1275). New standard curves were established after every 10 samples in both GFAAS and FAAS analyses. Certified reference materials (including SLRS and DOLT, National Research Council of Canada) were run with each set of samples, and were always well within the specified range.

Statistical Treatment

To evaluate relative Cu toxicity, time-to-death data were analyzed using a parametric survival analysis by fitting a Weibull distribution to the number of surviving fish during each 30 min interval of the exposure. The median time required to kill 50% of the test animals (ET50, and 95% confidence interval) was estimated

from the Weibull model. In this preliminary experiment, we estimated equi-toxic Cu concentrations to both yellow perch and rainbow trout in soft water from the results of this ET50 analysis by plotting simple linear regressions of ET50 results, and back-calculated subsequent exposure concentrations from the resulting regression equations. ET50 analysis in the final (using the two different Cu concentrations for each species) time-to-death experiment was conducted in the same way as the first. Significant species, hardness, and Cu effects were determined using a modified log-likelihood ratio analysis. Comparative analyses were conducted by analysis of variance (ANOVA) or Student's *t*-test, as appropriate. Any observed mean differences were considered significant when $p \leq .05$. All statistical analyses were performed on JMP statistical software, version 5.1.

RESULTS

Relative Copper Toxicity

Time-to-death, estimated as ET50, varied significantly by species ($p < .0001$), hardness of the exposure water ($p < .0001$), and Cu concentration ($p < .0001$; Figure 1a). In general, yellow perch were significantly more tolerant to Cu than rainbow trout. Fish held in hard water lived longer at a specific Cu concentration than those in soft water. Time-to-death was significantly reduced at higher Cu concentrations relative to at lower concentrations for both species.

In the second ET50 analysis, we exposed rainbow trout and yellow perch to equitoxic Cu concentrations for soft water exposures (250 μg Cu/L for rainbow trout and 550 μg Cu/L for yellow perch) in both hard and soft water. Measured Cu concentrations in the two exposures were slightly lower than nominal concentrations: for the rainbow trout exposures (mean $\pm$ SD; n = 6), 200.0 $\pm$ 37.8 μg/L in soft water and 231.8 $\pm$ 37.8 μg/L in hard water, and for the yellow perch exposures, 483.7 $\pm$ 36.6 μg/L in soft water and 477.3 $\pm$ 29.3 μg/L in hard water. Copper concentrations measured in the control water (nominal 0 μg Cu/L; n = 6), were 2.4 $\pm$ 0.9 μg/L. Despite the lower-than-nominal metal concentrations in exposure treatments, yellow perch were still significantly more tolerant to Cu than rainbow trout ($p = .005$), and elevated water hardness also caused a significant reduction in Cu toxicity in both species ($p < .0001$; Figure 1b). The different Cu sensitivities between the preliminary exposure and this one probably reflect differences in exposure Cu concentrations in the two. The preliminary exposure was based on nominal Cu concentrations, whereas in this exposure, metal concentrations were slightly lower than nominal concentrations.

Copper Uptake and Sodium Loss

We monitored Cu uptake and Na loss in the fish involved in the previous ET50 exposure. Yellow perch held in soft water had significantly lower total Cu in gills and livers, but higher Cu in carcasses relative to those in hard water in the 0 μg Cu/L exposures (*i.e.*, hardness effect; Figure 2a). Yellow perch exposed to 550 μg Cu/L showed significantly higher total Cu in gills and livers, but not carcasses, in soft water relative to hard water. All tissues (gills, livers, and carcasses) had significantly

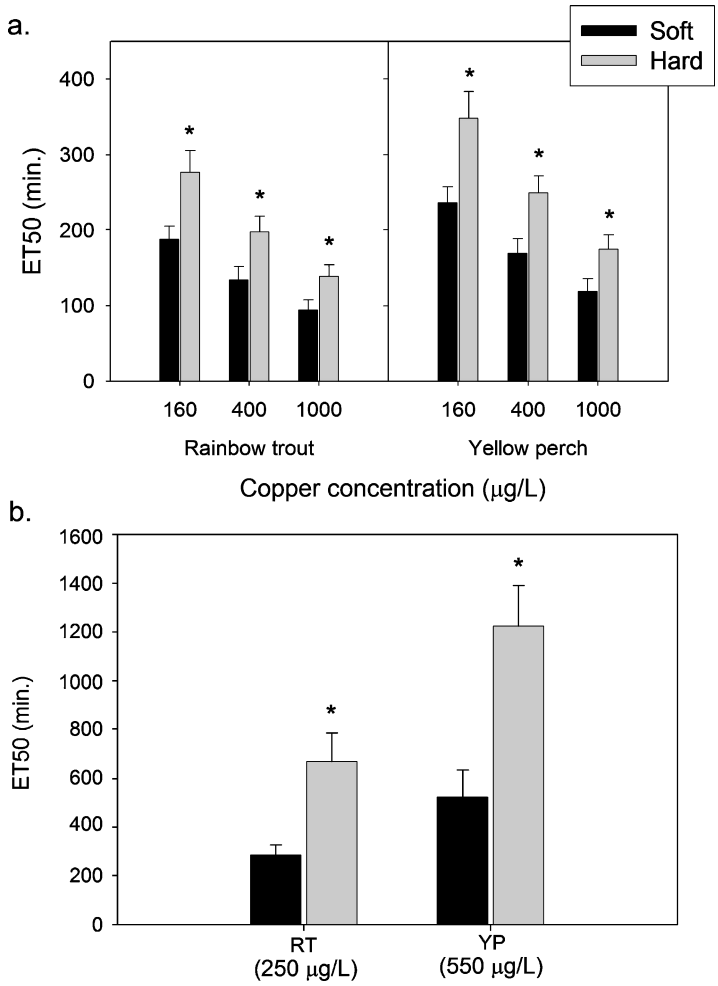

Figure 1. (a) Median time to death (ET50; n = 5) for rainbow trout and yellow perch exposed to aqueous Cu in hard (120 mg/L as $CaCO_3$) and soft (20 mg/L as $CaCO_3$) water. (b) ET50s for rainbow trout and yellow perch exposed to equi-toxic Cu concentrations in hard and soft water, as estimated from (a). Asterisks (*) indicate a significant hardness effect ($p < .05$). Error bars represent the upper 95% confidence interval.

higher Cu concentrations in the Cu exposure treatments relative to controls. In rainbow trout, no hardness effect in any of the three tissue-types was evident in control treatments. In the Cu treatments, however, only liver showed a significant hardness effect, such that fish held in soft water had significantly *lower* total liver Cu relative to those held in hard water. In all tissues (except in carcasses of fish held in soft water), total Cu was higher in the Cu treatment relative to controls. Interestingly, for both species, the highest tissue Cu concentrations were measured in livers (even

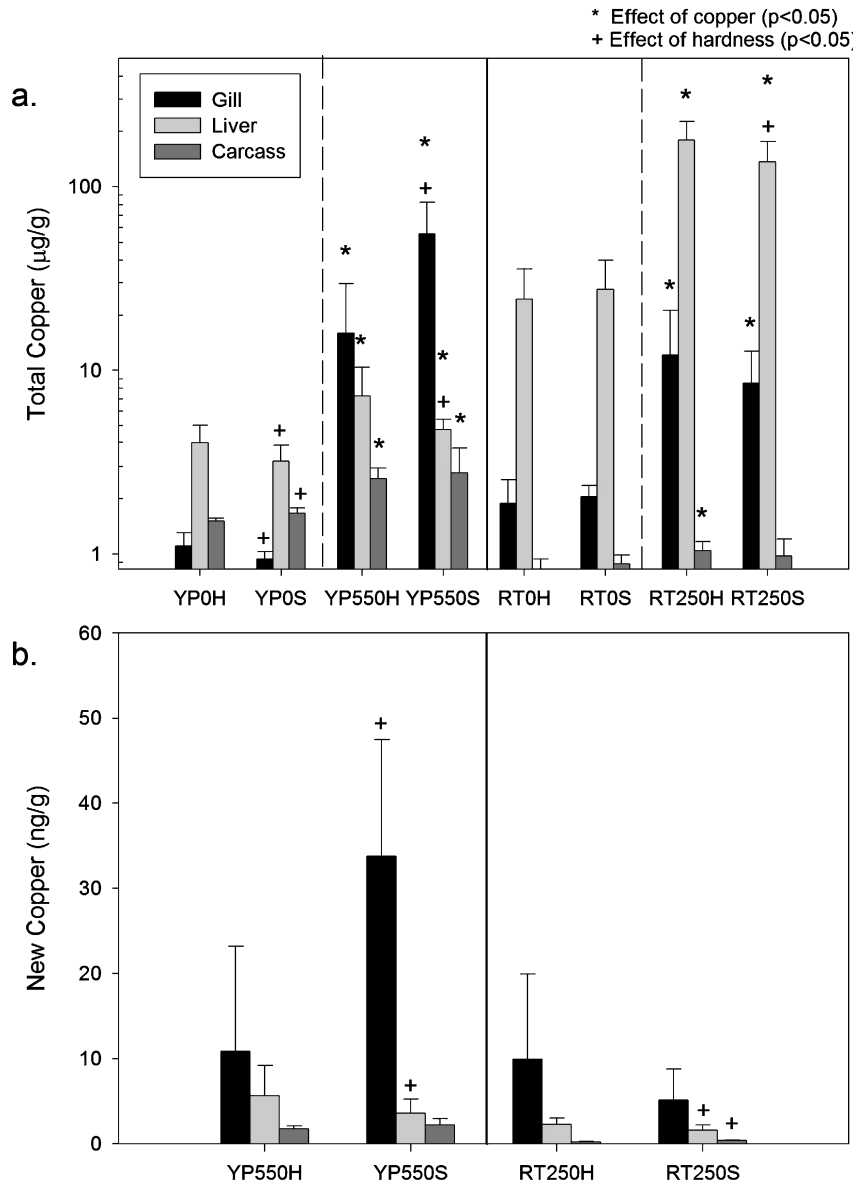

Figure 2. Total (a) and new (b) Cu accumulation in gills, livers, and carcasses of rainbow trout (RT) and yellow perch (YP) in hard (H) and soft (S) water. Both species were exposed to equi-toxic Cu concentrations for soft-water exposures, estimated from the ET50 data reported in Figure 1. Yellow perch were exposed to 550 μg Cu/L, and rainbow trout to 250 μg Cu/L. New Cu could only be estimated for fish exposed to ^{64}Cu (*i.e.*, not in the controls). Data are means + SD (n = 10). Note the log scale in (a).

in control fish), except in yellow perch held in either hard or soft water and exposed to Cu. In those fish, the highest total Cu concentrations were measured in gills.

Yellow perch in soft water yielded significantly higher new gill Cu, and lower liver Cu, than those held under hard water conditions (Figure 2b). New Cu in gills was not affected by hardness in rainbow trout. However, rainbow trout in soft water showed significantly higher new carcass Cu and significantly lower new liver Cu relative to rainbow trout held under hard water conditions (Figure 2b).

Tissue Na concentrations were affected by Cu concentration and hardness in both species (Figure 3a). Yellow perch controls demonstrated significantly lower carcass Na in soft water than in hard water. All yellow perch exposed to elevated Cu showed significantly lower tissue Na concentrations relative to those in the Cu controls. Yellow perch gills and carcasses, but not livers, had lower Na concentrations in soft water than in hard water. Rainbow trout held under soft water conditions in the Cu controls (0 μg Cu/L) had significantly lower tissue Na than those held under hard water conditions. Rainbow trout held in hard or soft water with Cu had significantly lower tissue Na concentrations relative to those held in clean water—except for livers of fish held in soft water. Sodium concentrations were significantly lower in Cu-exposed rainbow trout gills, but neither livers nor carcasses showed any effect of hardness in Cu-exposed animals.

Whole body Na concentrations as a function of Cu-exposure and hardness yielded similar effects between the two species (Figure 3b). Fish of either species held in clean, soft water had significantly lower whole body Na concentrations than those held in clean, hard water. Moreover, Cu-exposed fish of either species held in hard or soft water showed significantly lower whole body Na concentrations to those held in clean water. Finally, among the Cu-exposed fish, those held under soft water conditions had significantly lower whole body Na than those held in hard water.

New Cu uptake rates were significantly higher in all three tissues of yellow perch held in soft water relative to those held in hard water (Figure 4a). New Cu uptake to yellow perch gills was more than 7 times higher in soft than in hard water. However, in rainbow trout, the only significantly higher new Cu uptake rate was in carcasses of fish held in soft water relative to those in hard water.

Despite the fact that yellow perch had much higher new Cu uptake rates in the gills, rainbow trout appeared to have a higher rate of whole body Na loss (Figure 4b). As with whole body Na concentrations (above), whole body Na flux rates yielded the same basic patterns of Cu and hardness effects between the two species. In each case, fish held in the Cu treatments showed a significantly higher Na loss rate than those in clean water, and fish held under soft water conditions lost significantly more Na than those held under hard water conditions. Figure 5 shows whole body Na flux rates for individual fish as a function of time-to-death. Individual fish showing high Na loss rates died considerably faster than those with lower Na loss rates. Fish that did not die as the result of acute Cu exposure (all yellow perch in hard water) showed the lowest rate of Na loss.

Time Course for Branchial and Whole Body Cu Uptake

In the time course study, measured metal concentrations were slightly different from nominal concentrations. The 20 μg Cu/L exposure was measured at

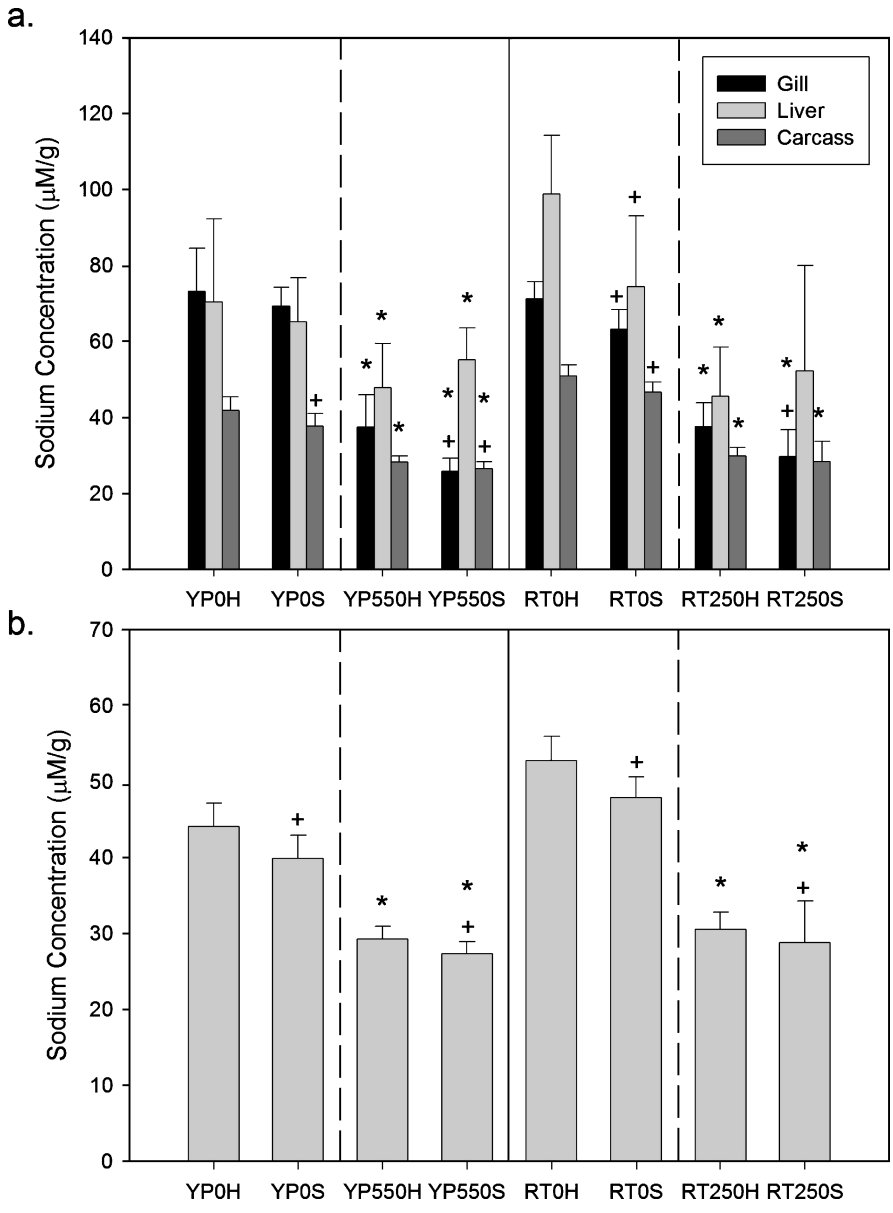

Figure 3. Gill, liver, and carcass (a) and whole body (b) sodium concentrations in rainbow trout (RT) and yellow perch (YP) exposed to Cu (250 μg Cu/L for RT, 550 μg Cu/L for YP) in hard and soft water. Bars represent means + SD (n = 10). Asterisks (*) indicate a significant effect of Cu concentration, plus signs (+) indicate a significant effect of hardness ($p < .05$).

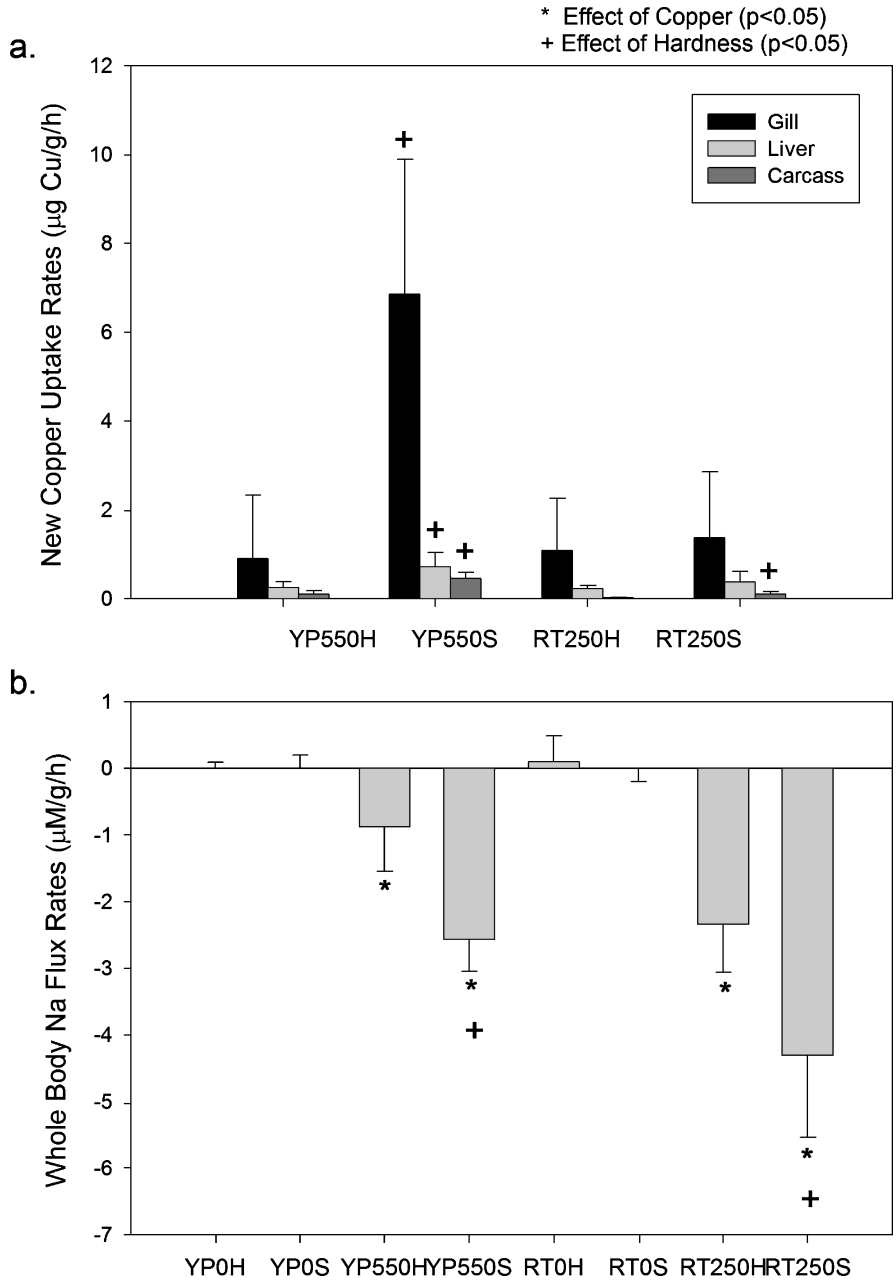

Figure 4. Gill, liver, and carcass new Cu uptake rates (a) and whole body sodium flux rates (b) in rainbow trout (RT) and yellow perch (YP) exposed to 250 μg Cu/L or 550 μg Cu/L, respectively, in hard (H) or soft (S) water. Bars represent means + SD (n = 10).

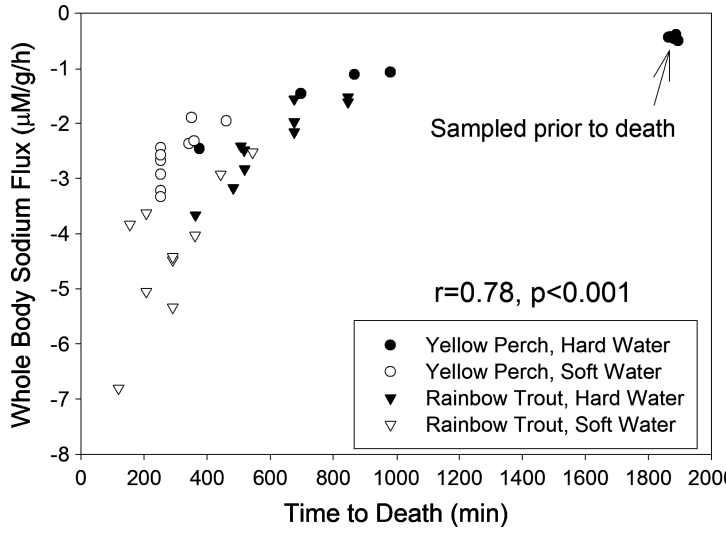

Figure 5. Relationship between whole body Na flux rate and time-to-death in rainbow trout and yellow perch exposed to 250 μg Cu/L or 550 μg Cu/L, respectively, in hard or soft water. Only Cu-exposed fish are presented.

28.3 ± 6.9 μg/L, and the 60 μg Cu/L exposure was measured at 49.6 ± 2.9 μg/L (means ± SD, n = 32). Therefore, the low Cu exposure solution was slightly higher and the high Cu exposure solution was slightly lower than nominal concentrations.

Rainbow trout and yellow perch responded differently to Cu exposures at 20 or 60 μg Cu/L (nominal) under hard or soft water exposure conditions (Figure 6). In soft water, both rainbow trout and yellow perch took up more new Cu to their gills during exposure to 60 μg Cu/L than at 20 μg Cu/L. However, this was not the case when fish were exposed to Cu under hard water conditions, where Cu uptake at the high Cu concentration was very low. Both species showed highly variable new gill Cu accumulation patterns early in each exposure scenario, which tended to stabilize from 12 h onward (except for rainbow trout exposed to 60 μg Cu/L in soft water). Fish exposed to 60 μg Cu/L in soft water showed a massive increase in new Cu accumulation, followed by a precipitous drop. In yellow perch, this new gill Cu spike occurred after 6 h of exposure, whereas in rainbow trout it occurred after 12 h. Rainbow trout showed a similar new gill Cu accumulation spike when exposed to 20 μg Cu/L in hard water after only 1 h (Figure 6b). This spike represented rainbow trout new gill Cu concentrations that were nearly double those measured in rainbow trout exposed to the same Cu concentration in soft water. Although yellow perch also showed a Cu accumulation spike under the same exposure conditions (*i.e.*, 20 μg Cu/L in hard water), which was equivalent to the spike observed in soft water, new gill Cu concentrations were only about half those observed in rainbow trout and it occurred after 3 h (rather than after 1 h). At the end of the exposure period, fish of both species accumulated more new gill Cu under soft water conditions (not withstanding the spike just described) than under hard water conditions. This was particularly apparent in gills of fish exposed to 60 μg Cu/L (Figs. 6c and d).

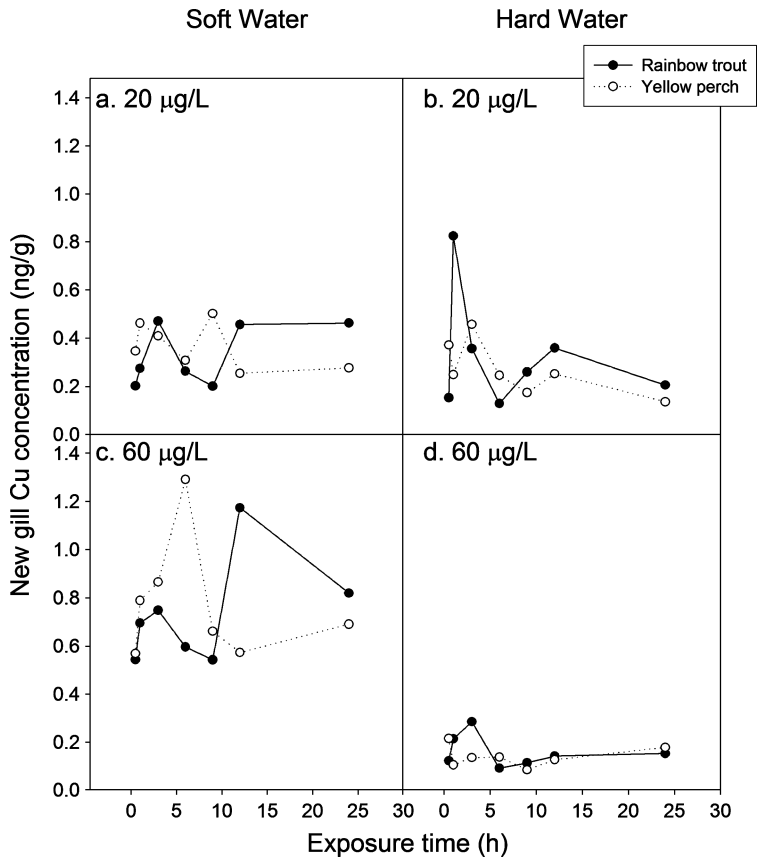

Figure 6. Time course of new Cu uptake to gills of rainbow trout or yellow perch exposed to 20 (a and b) or 60 (c and d) μg Cu/L in soft (a and c) or hard (b and d) water during a 24 h exposure. Each point represents a mean (n = 3). Error bars were omitted for the sake of clarity.

Pulse-Chase Experiment and Exchangeable Branchial Copper

Measured total Cu in the pulse-chase exposures was close to the nominal exposure concentration of 60 μg Cu/L, and was 62.6 $\pm$ 3.1 μg/L (mean $\pm$ SD; n = 30). This concentration did not vary when considering exposure concentrations for rainbow trout or yellow perch separately.

As in the previous experiment, new branchial Cu uptake patterns varied between the two species (Figure 7). In the short-term pulse exposure (30 min in radioactive Cu), new Cu in the gills declined immediately upon transferring fish into the cold-Cu exposure. In rainbow trout, measurable declines in branchial Cu did not occur until approximately 30 min after transfer. However, there were no significant differences in new Cu uptake between the two species during the short-term pulse exposure ($p > .05$). During the long-term pulse exposure, rainbow trout took up approximately twice as much new Cu as yellow perch (Figure 7b). From 30 min until the end of the exposure, rainbow trout demonstrated significantly higher new Cu in the gills

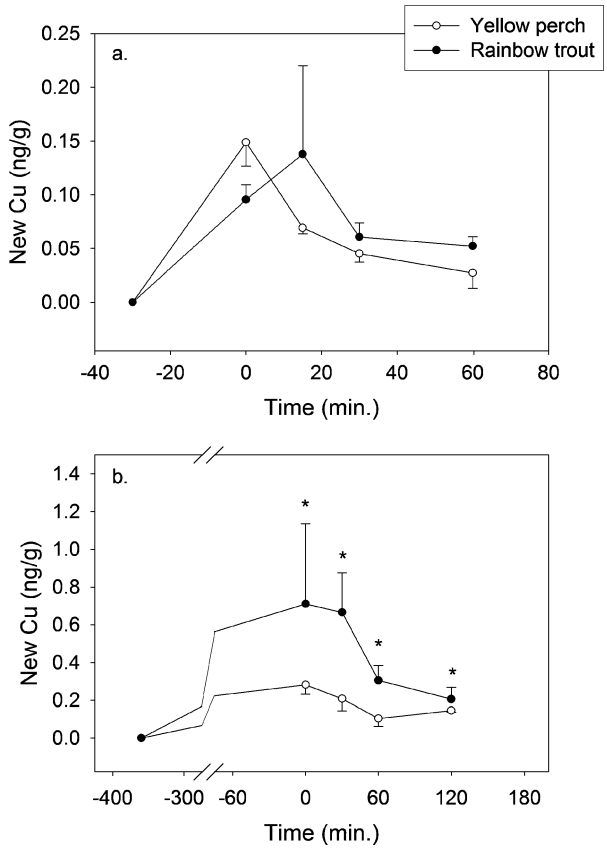

Figure 7. New gill Cu uptake and depuration in rainbow trout and yellow perch exposed to a short-term (30 min) pulse (a) or long-term (360 min) pulse (b) of 60 μg ^{64}Cu/L in hard water, followed by a "cold" chase at the same Cu concentration. Time t = 0 represents when fish were transferred from "hot" to "cold" Cu exposure solutions. Points represent means + (or −) SD (n = 3) at each sampling event; asterisks (*) represent significant differences between rainbow trout and yellow perch ($p < .05$).

relative to yellow perch, despite an approximately 50% decline in new Cu between 30 and 60 min in cold Cu. Therefore, even after 120 min in cold Cu, rainbow trout retained significantly higher branchial Cu concentrations than yellow perch.

New Cu uptake to carcasses also varied by species (Figure 8). During the short-term pulse exposure, rainbow trout took up significantly more new carcass Cu than yellow perch at t = 0. However, this high carcass Cu concentration fell almost immediately, such that at and beyond 15 min in the cold-Cu chase phase there was no significant difference detected in carcass new Cu measured between the two species. Yellow perch showed only a modest increase in carcass new Cu after the short-term pulse, which declined gradually over the duration of the experiment.

Immediately following the long-term pulse exposure, there was no significant difference in new carcass Cu between the two species (t = 0). However, rainbow trout

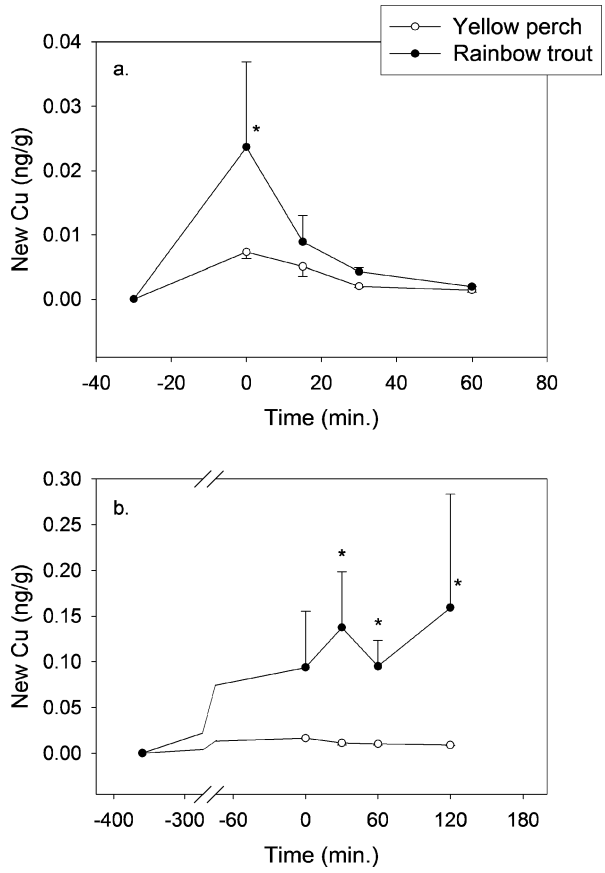

Figure 8. New Cu uptake to carcasses of rainbow trout or yellow perch exposed for 30 min (a) or 360 min (b) to 60 μg ^{64}Cu/L in hard water, followed by a "cold" chase at the same Cu concentration. Formatting is the same as in Figure 7.

demonstrated significantly higher new carcass Cu at every subsequent sampling time than yellow perch. In fact, yellow perch demonstrated only very minor increases in new carcass Cu for the duration of the experiment, such that the plot in Figure 8b for yellow perch appears to remain very close to no new Cu throughout the experiment.

In the pulse-chase experiment, it was reasonable to assume that the maximum amount of new branchial Cu that a fish would take up from the water occurred immediately prior to transferring that fish into the cold-Cu chase exposure ($t = 0$). The new, radiolabelled Cu bound to (or taken up by) the gills could only decrease over time, as surface-bound Cu was taken up by the gills and distributed around the body or displaced from the gills and redissolved back into the water. The amount of radiolabelled Cu remaining after the chase period was considered "unexchangeable," whereas the difference between the maximum new branchial Cu ($t = 0$) and the unexchangeable fraction was considered the "exchangeable" fraction.

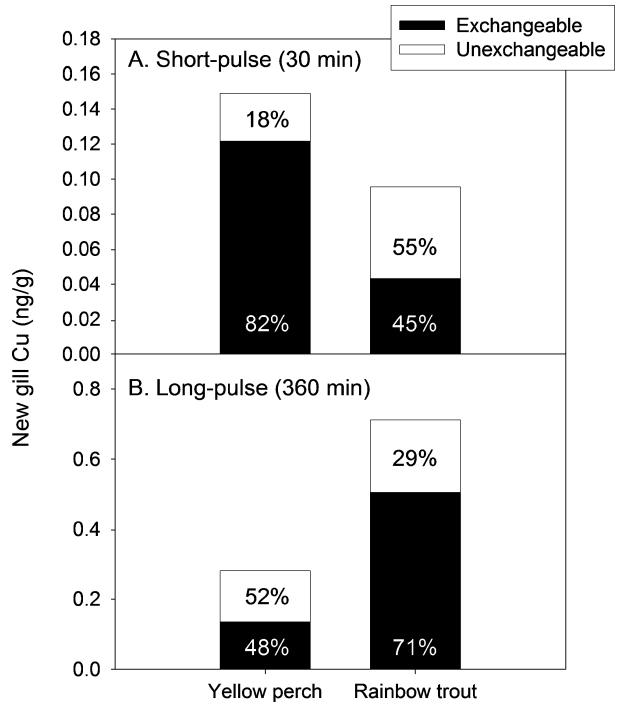

Figure 9. New Cu accumulation in gills of rainbow trout and yellow perch exposed to 60 μg ^{64}Cu/L for (A) 30 min (short-pulse) or (B) 360 min (long-pulse). Note the difference in scale between panels A and B. Total new Cu was the concentration of radiolabelled Cu in fish gills after the pulse-phase (*i.e.*, 30 or 360 min) exposure. The exchangeable Cu fraction was estimated from the amount of radiolabelled Cu remaining after 60 min (in the short-pulse experiment) or 120 min (in the long-pulse experiment) in a cold-Cu rinse. The exchangeable fraction was estimated as the proportional difference between the maximum new Cu accumulated on the gills at the end of the pulse phase and that which remained after the chase phase in cold Cu.

In the short-pulse experiment, 82% of the Cu bound to yellow perch gills was exchangeable (18% was unexchangeable) compared to only 45% (55% was unexchangeable) in rainbow trout (Figure 9a), despite the fact that both yellow perch and rainbow trout took up similar amounts of branchial Cu. In the long-pulse experiment, rainbow trout took up approximately two-thirds more gill Cu than yellow perch. Of the new gill Cu measured in rainbow trout gills in the long-pulse experiment (Figure 9b), 71% was exchangeable (29% was unexchangeable), compared to 48% exchangeable in yellow perch (or 52% unexchangeable).

DISCUSSION

This study demonstrates the different branchial Cu-handling capabilities between two common freshwater fish species, rainbow trout and yellow perch. These

different handling strategies probably account for species differences in their sensitivities to aqueous Cu exposures. Results reported here demonstrate that a significant toxicological factor leading to acute Cu toxicity is whole body Na loss, which is probably mediated through Cu-uptake strategies at the gills. Some 71% of Cu bound to rainbow trout gills, compared to only 48% for yellow perch gills, is exchangeable and able to enter the fish and induce a toxicological response, or be displaced from the gill by cold Cu back into the external exposure water. Consequently, the more Cu entering the fish at toxic levels leads to increased whole body Na loss and subsequent toxicity.

Whole body Na loss has already been suggested as a mechanism of Cu toxicity in freshwater fish (Laurén and McDonald 1985; Laurén and McDonald 1986; Laurén and McDonald 1987b; Grosell *et al.* 2002). This effect appears to be centered at the gill, and results from a reduction of branchial Na uptake at low Cu concentrations and an increase in Na loss at much higher Cu concentrations (Laurén and McDonald 1985). At low aqueous Cu concentrations and over longer exposure times, reduced Na uptake from the water is associated with Na^+/K^+-ATPase inhibition through a reduction in branchial chloride cells (Li *et al.* 1998) and non-competitive inhibitory processes (Laurén and McDonald 1987b). At higher Cu concentrations and shorter exposure times, whole body Na loss is associated with the opening of tight junctions (*i.e.*, paracellular loss pathways) and physical damage at the gill epithelium leading to a significant increase in Na efflux (Wilson and Taylor 1993), non-competitive antagonism of Na^+/K^+-ATPase (Laurén and McDonald 1987a), and competitive antagonism at the apical Na channel (Mallatt 1985; Laurén and McDonald 1987b; Sola *et al.* 1995; Grosell and Wood 2002). Consequently, acute Cu toxicity results from an acute ionoregulatory disturbance resulting from decreased aqueous Na influx and increased Na efflux causing an increase in blood viscosity, tachycardia, cardiovascular collapse, and eventually death (Wilson and Taylor 1993). Results reported here appear to corroborate this view of acute Cu toxicity for both species tested, despite the different sensitivities to Cu between the two species as reflected in our ET50 data (Figure 1).

At approximately equi-toxic Cu concentrations, total tissue Cu accumulation patterns were different between the two species (Figure 2a). Yellow perch exposed to Cu showed the highest total Cu concentrations in gill tissue, whereas rainbow trout accumulated most Cu in liver tissue. De Boeck *et al.* (2004) demonstrated that liver Cu concentrations were highest in the Cu-sensitive rainbow trout, but significantly lower in two Cu-tolerant carp species that showed significantly higher Cu concentrations in kidney. It may be that resistant species like yellow perch or these Cu-tolerant carp species divert Cu away from their livers but toward elimination structures like gills (yellow perch) or kidneys (carp). Taylor *et al.* (2003) demonstrated that branchial Cu binding capacity (B_{max}) in yellow perch was approximately the same as that for rainbow trout when exposures were conducted in soft water. In hard water, however, yellow perch binding capacity was more than double that of rainbow trout (yellow perch $B_{max} = 9.0$ nmol/g, rainbow trout $B_{max} = 3.6$ nmol/g). Although this result could account for higher Cu concentrations in yellow perch relative to rainbow trout under hard water exposure conditions, our tissue Cu accumulation data suggest that the opposite is true—although the discrepancy may be related to the different exposure scenarios; Taylor *et al.* (2003) used the same concentration to expose both

species, while we used equi-toxic concentrations. Yellow perch accumulated significantly more new branchial Cu (Figure 2b) in soft water relative to that in hard water, whereas water hardness had no effect on new Cu accumulation to rainbow trout gills. This effect was also reflected in our total gill Cu data (Figure 2a), and demonstrates different branchial Cu handling strategies between the two species.

Although fish of both species demonstrated a significant increase in total gill Cu in the Cu exposure treatments relative to controls, rainbow trout showed most Cu accumulation after this short-term exposure in liver, which was about an order of magnitude higher than that observed in yellow perch liver. This probably reflects the fact that rainbow trout had higher background liver Cu (by about an order of magnitude) in the control treatments (0 μg Cu/L) relative to yellow perch. Interestingly, fish of both species exposed to elevated Cu in soft water yielded *lower* total liver Cu concentrations relative to similar exposures in hard water. It may be that ionoregulatory disturbances at the gill and associated cardiovascular problems (Wood, 1989) result in less Cu being transported to livers resulting in lower liver Cu concentrations. This idea requires further research for confirmation.

Tissue Na concentrations were clearly reduced in tissues of Cu-exposed animals of each species (Figure 3a). The largest effects on tissue Na concentrations were associated with Cu exposure, not water hardness, particularly in gill tissues of each species. Exposure to Cu in soft water had no effect on liver Na concentrations in either species relative to Na concentrations measured in livers of fish exposed to Cu in hard water. Although tissue-specific patterns were generally similar between the two species (*i.e.*, elevated Cu exposure was associated with lower tissue-specific Na concentrations), there were subtle species differences when Na concentrations were considered in individual tissues. However, when data were expressed in terms of whole body Na concentrations, the effects of Cu and water hardness were very consistent between the two species (Figure 3b). Exposure to Cu led to a significant decrease in whole body Na concentrations, and this effect was magnified in soft water relative to that in hard water. This effect is consistent with other studies reporting on the effects of acute aqueous Cu exposure (Laurén and McDonald 1985; Laurén and McDonald 1986; Laurén and McDonald 1987b; McGeer *et al.* 2000; Taylor *et al.* 2000; Taylor *et al.* 2003; Matsuo *et al.* 2004).

New Cu uptake rates into fish gills were only affected by hardness in yellow perch (Figure 4a). Yellow perch exposed to aqueous Cu showed a significantly higher new Cu accumulation rate in gills than those exposed to the same Cu concentration in soft water. However, hardness did not affect new Cu uptake rates to rainbow trout gills. Despite that, whole body Na loss rates were almost 50% higher in rainbow trout exposed to Cu in soft water than in yellow perch. Wood (1989) determined that Na loss rate was a more reliable indicator of acid sensitivity in fish than absolute whole body Na loss. Our data support this view. Rainbow trout showed greater toxicity to approximately equi-toxic exposure conditions than yellow perch (Figure 1b), which corresponds to a greater rate of whole body Na loss (Figure 4b), relative to yellow perch and regardless of water hardness. That whole body Na loss rate is more important than whole body Na loss is particularly evident in the strong relationship between whole body Na loss rate and time-to-death (Figure 5). Therefore, like the mechanism of acid toxicity (Wood 1989), the mechanism of Cu toxicity is closely related to the *rate* of whole body Na loss. Fish in natural waters suffering from Cu

toxicity may ameliorate whole body Na loss by selecting food items high in Na content (Pyle *et al.* 2003; Kamunde *et al.* 2003; Niyogi *et al.* 2006). Whether or not fish do select high-Na diets in natural, Cu-contaminated environments requires further research.

The BLM approach to predicting metal toxicity examines metal binding affinity (log *K*) and capacity (B_{max}) to the so-called biotic ligand (*i.e.*, gills in the current context). This is most often accomplished by exposing fish to increasing metal concentrations resulting in metal saturation of available binding sites on the gill after a 3 h exposure (Santore *et al.* 2001). Although there is ample evidence to demonstrate metal–gill saturation to increasing aqueous Cu concentrations after a 3 h exposure, new metal uptake dynamics at the gill are highly variable within (approximately) the first 12 h (Figure 6). These new Cu uptake patterns over time appear to vary as a function of Cu concentration, water hardness, and fish species. Fish of both species examined here demonstrated an initial peak in new Cu uptake, followed by a precipitous drop until finally stabilizing at some plateau concentration (Figure 6). A similar result was observed by Grosell *et al.* (1997) in rainbow trout exposed to waterborne Cu and Wood *et al.* (2002) for rainbow trout exposed to waterborne Ag. These peaks were observed earlier in hard water exposures relative to those in soft water. This 12 h instability in new metal uptake dynamics to gills has been observed for silver (Ag) binding dynamics to rainbow trout gills (Morgan *et al.* 2004). Therefore, it may be worthwhile to develop BLM binding constants in fish exposed to metals for longer than 3 h—preferably, for 12 h or longer—to avoid the complications of physiological adjustments to exposure conditions at the gill. This may improve BLM predictions, and ultimately enhance ecological risk assessments that use BLM predictions.

In order for BLM predictions to be broadly applicable across a range of species, differential species sensitivities to metals (*e.g.*, Cu in the current context) must be taken into account. At present, the BLM has been developed using only a few species (including rainbow trout and fathead minnows), yet other species (often more likely to inhabit metal-contaminated lakes), such as yellow perch, have been largely ignored. In our pulse-chase experiment, rainbow trout took up more new Cu at the gills, which resulted in more new Cu being deposited to the carcass (tissues other than gills) than yellow perch under the same exposure conditions (Figures 7 and 8).

Although both species took up similar amounts of branchial Cu during the short-pulse experiment, 82% was exchangeable in yellow perch compared to only 45% in rainbow trout (Figure 9). However, when exposure time increased during the long-pulse experiment, the reverse was true; only 48% of branchial Cu was exchangeable in yellow perch compared to 71% in rainbow trout. This result is in good agreement with literature values. Tao *et al.* (2006) estimated that the exchangeable Cu fraction on common carp (*Cyprinus carpio*) gills was 43%—close to what we measured in yellow perch in the long-pulse experiment—and appeared to be closely associated with the amount of mucus available in the gill microenvironment.

These results suggest that yellow perch induce a rapid physiological adjustment to elevated aqueous Cu concentrations that reduces the exchangeable pool of branchial Cu. This rapid adjustment probably serves to regulate the amount of Cu that can be taken up by the fish to confer toxicity. In contrast, rainbow trout are not as effective at regulating branchial Cu uptake, which is apparent by the increased exchangeable Cu fraction in the gills with increasing exposure time. McDonald *et al.* (1991) found

important structural differences between rainbow trout and yellow perch gills that could account for ion losses associated with acid exposure between the two species. It may be that these same structural differences account for the different branchial Cu-handling strategies and Cu sensitivities observed between the two species.

Taylor *et al.* (2003) examined Cu–gill binding characteristics as a function of water quality and toxicity and determined that Cu-binding affinity (log K) to the gill was not significantly different between yellow perch and rainbow trout in either soft (log $K = 8.4$, both species) or hard (log $K = 9.7$, both species) water. The same authors found that Cu-binding capacity was the same in gills of both species in soft water ($B_{max} = 1.9$ nmol/g), but showed considerable difference in hard water (for rainbow trout, $B_{max} = 3.6$ nmol/g; for yellow perch, $B_{max} = 9.0$ nmol/g), suggesting that yellow perch had a much higher capacity for branchial Cu binding than rainbow trout when exposed to aqueous Cu in hard water. Taylor *et al.* (2003) determined that yellow perch had to accumulate some nine times more branchial Cu than rainbow trout in order to induce 50% mortality in test fish (*i.e.*, LA50, or medial lethal accumulation), suggesting that much of the Cu bound to yellow perch gills was not "biologically reactive." This result can be explained by the relatively low exchangeable fraction of gill-bound Cu, which we report here.

Taken together, these results suggest that qualitative differences between rainbow trout and yellow perch gills can affect not only the amount of new Cu that can bind to the gill and be taken up to other body tissues of the animals, but also for Na loss, which is integral to the mechanism of acute Cu toxicity to freshwater fish. Consequently, to improve toxicological predictions of the BLM, and ultimately the ecological risk assessments that make use of BLM predictions, BLM parameters such as log K and B_{max} need to be established separately for each species of interest.

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