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KINETIC ANALYSIS OF ZINC ACCUMULATION IN THE GILLS OF JUVENILE RAINBOW TROUT: EFFECTS OF ZINC ACCLIMATION AND IMPLICATIONS FOR BIOTIC LIGAND MODELING

DEREK H. ALSOP* and CHRIS M. WOOD
Bioavailability of Metals Group, Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

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Abstract—Juvenile rainbow trout were acclimated to hard water (Ca^{2+} = 1.0 mM, Mg^{2+} = 0.2mM; hardness = 120 mg $CaCO_3$/L) and hard water plus 250 μg/L Zn (3.8 μM). After 30 d of exposure, there was no difference in the total Zn levels of the gills of Zn-exposed and control fish (~70 μg Zn/g gill). Exposure of both groups to a range of Zn concentrations (0–2,900 μg/L Zn) for up to 7 d also had no effect on the measured total Zn levels in the gills. However, using radiolabeled ^{65}Zn, measurement of new Zn appearance in the gills was possible. Trout were exposed to a range of Zn concentrations (with ^{65}Zn) and the gills were sampled at times ranging from 0.5 to 72 h. The fast turnover pool of Zn in the gills increased with increasing acute Zn exposure concentration, while the maximum size of the fast pool was about ninefold larger in Zn-acclimated fish (4.14 μg Zn/g gill) versus control fish (0.45 μg Zn/g gill). At all sampling times, gill ^{65}Zn accumulation exhibited saturation kinetics, allowing calculation of binding capacity (B_{max}) and affinity (K_d). In both control and Zn-acclimated trout, K_d decreased rapidly (affinity increased) from 0.5 to 3 h and then remained constant up to 72 h. B_{max} increased rapidly from 0.5 to 3 h in both groups, then the rate of increase began to subside but was still increasing from 24 to 72 h. At all times, the K_d of Zn-acclimated fish was higher (i.e., lower affinity) and B_{max} was greater than controls. The stabilized K_ds (>3 h) were approximately 280 μg/L total Zn (log K = 5.6 as Zn^{2+}) and 575 μg/L total Zn (log K = 5.3 as Zn^{2+}) in control and Zn-acclimated fish, respectively. The B_{max} of control fish at 0.5 h was 0.37 μg Zn/g gill and increased to 8.63 μg Zn/g gill by 72 h. The B_{max} of Zn-acclimated fish increased from 0.70 to 11.61 μg Zn/g gill over the same time period. Preexposure to 250 μg/L Zn appeared to have little effect on acute zinc toxicity, though the 96-h LC50s for both groups were relatively high (~3,000 μg/L Zn) in comparison to previous measurements. The relationship between gill binding constants for different metals and relative toxicity is critically assessed with respect to biotic ligand modeling.

Keywords—Rainbow trout Zinc Gills Acclimation Biotic ligand modeling

INTRODUCTION

Zinc (Zn) is an essential micronutrient found at high levels in the tissues of fish [1,2]. Normally, Zn is acquired principally from the diet, and uptake of waterborne Zn by the gills occurs only at a low level, though the latter can increase under conditions of dietary deficiency [3]. However, if waterborne Zn levels are elevated, toxicity can occur due to pathological interactions of Zn with transport functions on the gill surface [4–6]. While normal Zn levels in pristine freshwaters are only a few micrograms per liter or less, concentrations of 50 μg/L are routine in industrialized areas. Maximum Zn concentrations in contaminated surface waters are reported to range from 130 to 1,170 μg/L in different areas of Canada [7]. At these levels, Zn specifically disrupts calcium uptake by the gills [4–6], leading to hypocalcemia, which may end with the death of the fish within a few days, depending on the Zn concentration.

A frequently reported observation that accompanies chronic, sublethal exposure to Zn (and other metals) is physiological acclimation. If a fish survives the metal exposure, then the ionic disturbance that can occur may eventually be corrected [8], as seen with the full recovery of plasma Ca^{2+} levels during a sublethal exposure to Zn [5]. In addition, an increased tolerance (in terms of survival) to the metal may arise upon a threshold exposure. With Zn-exposed trout, this toxicological acclimation or increased tolerance was fully acquired within 5 d, with the tolerance having increased 2.5 times compared to unexposed fish as judged by LC50 tests [9].

Because the gill is the primary target organ of acute metal toxicity and because most metals exert their pathological effects by directly binding to functional groups on or in the gill cells, it has been proposed that the amount of metal accumulating at the gill could be used as a direct indicator of toxicity [10–15]. Indeed, there has been much interest recently in developing a method, based on geochemical modeling applied to the gill, for predicting heavy metal toxicity to aquatic biota in different water chemistries. The gill receptor-loading model (one form of the biotic ligand model) is one such approach. After first experimentally determining binding constants of the gill for metals and other ions, one can then use aquatic geochemical programs such as MINTEQA2 [16] and MINEQL+ [17] to predict metal bound to gills in a range of different water chemistries. This approach has been successful for metals such as copper and cadmium [10,18], silver [19], and cobalt [20]. Attempts have been made to apply these techniques to Zn [21], but the extremely high levels of Zn found in the gills of unexposed fish made detecting any accumulation difficult.

Galvez et al. [21] had some success in determining the affinity (K_d) and binding capacity (B_{max}) of the gills of juvenile rainbow trout by utilizing radiolabeled ^{65}Zn to distinguish newly accumulated Zn from the large pool of native Zn already present. Alsop et al. [15] used a temporal approach with ^{65}Zn exposure for different periods at one concentration as opposed to varying the ^{65}Zn concentration at fixed sample times. From

* To whom correspondence may be addressed (dalsop@uoguelph.ca).

this, they were able to determine that the gills possessed at least two pools of Zn, a fast and a slow turnover pool. The size of the fast pool in control fish was found to be only 0.14% of the total Zn in the gills and increased in size with acclimation to increasing waterborne Zn concentrations. The time to 50% turnover ($t_{1/2}$) of this fast pool, as directly estimated from the hyperbolic loading curve, was 3 to 4 h. In contrast, the slow pool appeared to turn over linearly with time from 24 to 72 h, with an overall $t_{1/2}$ of days to months.

Using Galvez et al. [21] and Alsop et al. [15] as points of departure, the present study was designed to define both the temporal and concentration-dependent patterns of ^{65}Zn accumulation in the rainbow trout gill. In addition, we were interested in determining whether the accumulation patterns were predictive of acute Zn toxicity. The effects of a chronic preexposure to sublethal Zn on toxicity and gill accumulation kinetics were also evaluated. The results will be of use for future development of a biotic ligand model for zinc in freshwater fish.

MATERIALS AND METHODS

Fish care

Sixteen hundred juvenile rainbow trout (*Oncorhynchus mykiss*; 3–5 g) were purchased from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and were divided equally into two aerated 500-L tanks supplied with 3 L/min of dechlorinated Hamilton tap water (ionic composition: Ca^{2+}, 1.0 mM; Mg^{2+}, 0.2 mM; Na^{+}, 0.6 mM; Cl^{-}, 0.7 mM; hardness, 120 mg $CaCO_3$/L; alkalinity, 95 mg $CaCO_3$/L; dissolved organic matter, 3 mg/L; pH 7.5). Fish were fed to satiation once daily with a commercial ration (fish food composition: crude protein [min], 52%; crude fat [min], 17%; crude fiber [max], 2.5%; water, 12%; Ca^{2+}, 1.4%; Na^{+}, 0.4%; zinc [measured], 0.02% [173 μg/g]). Feces and organic debris were siphoned out of the tanks daily. Photoperiod was set to mimic natural photoperiod. Fish were held under these conditions for 2 weeks prior to the commencement of chronic, sublethal Zn exposure. Water temperature was maintained at 13 ± 1°C during holding and experiments.

Zn acclimation

After the 2 weeks of holding, one of the two tanks was randomly designated as the chronic Zn exposure group (250 μg/L Zn). To the head tank of this group, flow from a Mariotte bottle of concentrated Zn solution ($ZnSO_4 \cdot 7H_2O$, Anachemia with the addition of 1 ml concentrated HNO_3/L deionized water, trace metal analysis grade, BDH Chemicals, Toronto, ON, Canada) was started where it mixed with inflowing fresh water by vigorous aeration. Measured water Zn levels in the exposed group ranged from 204 to 268 μg/L (mean 244 μg/L; 3.76 μM). No mortalities were recorded during the Zn-acclimation period. Trout were exposed for at least 30 d before experiments began, and tests were performed over the subsequent 90-d period.

Elevated Zn exposure and toxicity testing

At the start of these continuous flow tests, 200 Zn-acclimated and 200 nonacclimated trout were removed and divided into six 18-L tanks (40 fish/tank), with each tank receiving a flow of 250 ml/min. Then flow from a Mariotte bottle of concentrated Zn solution ($ZnSO_4 \cdot 7H_2O$ with the addition of 1.0 ml concentrated HNO_3/L deionized water) was started into a head tank where it mixed with inflowing fresh water by vigorous aeration. At the same time, Zn was added to each tank (apart from the control tanks) to immediately bring them up to the chosen Zn concentration. Nominal concentrations were 0, 250, 700, 1,200, 1,900, and 2,900 μg/L Zn. At 24, 48, 72, and 144 h, seven fish from each tank were removed and sacrificed with a blow to the head. Their gills were removed, blotted dry, and weighed to the nearest 0.001 g. Mortalities were recorded over 144 h. Water samples were taken daily and acidified for later analysis of actual Zn concentration. Zn levels in the gills were determined by digestion in five volumes of 1 N HNO_3 (trace metal analysis grade, BDH Chemicals) for 24 h at 60°C. Samples were vortexed and allowed to settle for 24 h. One hundred microliters of supernatant was diluted to 1 ml with deionized water (NANOpure II®, Sybron-Barnstead, Dubuque, IA, USA) and analyzed by atomic absorption spectroscopy (Varian AA-1275, using an air/acetylene flame; [Varian, Walnut Creek, CA, USA]).

Surprisingly, there was insufficient mortality to determine 96- or 144-h LC50 values, so a second 96-h LC50 test was performed 3 weeks later using a higher range of Zn concentrations. In this second exposure, groups of eight fish from both groups were exposed to six Zn concentrations (0, 500, 1,000, 2,000, 3,000, and 4,500 μg/L Zn, nominal values) as in the first exposure for 96 h. Mortalities were recorded and the 96-h LC50s ± 95% confidence limits were calculated by log probit analysis of mortality versus measured waterborne Zn concentration [22].

^{65}Zn exposure

After 30 d of exposure, 250-μg/L Zn-acclimated and nonacclimated trout were exposed to a range of radiolabeled ^{65}Zn levels for 72 h. Twenty-one 250-μg/L Zn-acclimated trout were placed in each of the 25-L tanks containing 50, 100, 150, 350, 850, 1,250, or 1,750 μg/L Zn (by the addition of $ZnSO_4 \cdot 7H_2O$; Anachemia, Toronto, ON, CA) plus 30 μCi ^{65}Zn per tank (as $ZnCl_2$, specific activity = 1.97 mCi/mg; NEN Life Science Products, Boston, MA, USA), an amount that had negligible influence on the total Zn concentration of the water. Similarly, 21 nonacclimated trout were placed in each of the 25-L tanks containing 50, 100, 200, 400, or 800 μg/L Zn plus 30 μCi ^{65}Zn per tank. At the sampling times of approximately 24, 48, and 72 h, water samples were taken and seven fish from each tank were quickly removed and rinsed in a bath containing a lethal amount of anesthetic (0.5 g/L MS222) and 40 mg/L Zn for 1 min. In pilot experiments, this concentration of Zn was shown to be sufficient to displace any ^{65}Zn loosely bound. After rinsing, the gills were excised, blotted dry, and weighed to the nearest 0.001 g. Gills were assayed for ^{65}Zn activity in a γ-counter (MINAXI γ Auto-Gamma 5000 Series, Canberra-Packard, Meriden, CT, USA). Water samples were similarly assayed for ^{65}Zn activity as well as for total Zn by atomic absorption spectrophotometry to allow calculation of the specific activity of the waterborne Zn. All calculations were performed on measured Zn levels, but for ease of reporting, levels noted in the text are nominal, though they were always within 10% of the measured values.

A similar experiment was conducted over a shorter time period. Nonacclimated and 250-μg/L Zn-acclimated trout were exposed to a range of radiolabeled ^{65}Zn levels for 3 h. Twenty-one 250-μg/L Zn-acclimated trout were placed in each of five 15-L tanks containing 250, 500, 900, 1,400, or 2,100 μg/L Zn plus 15 μCi ^{65}Zn per tank. Similarly, 21 nonacclimated trout were placed in each of five 15-L tanks containing 75, 150,

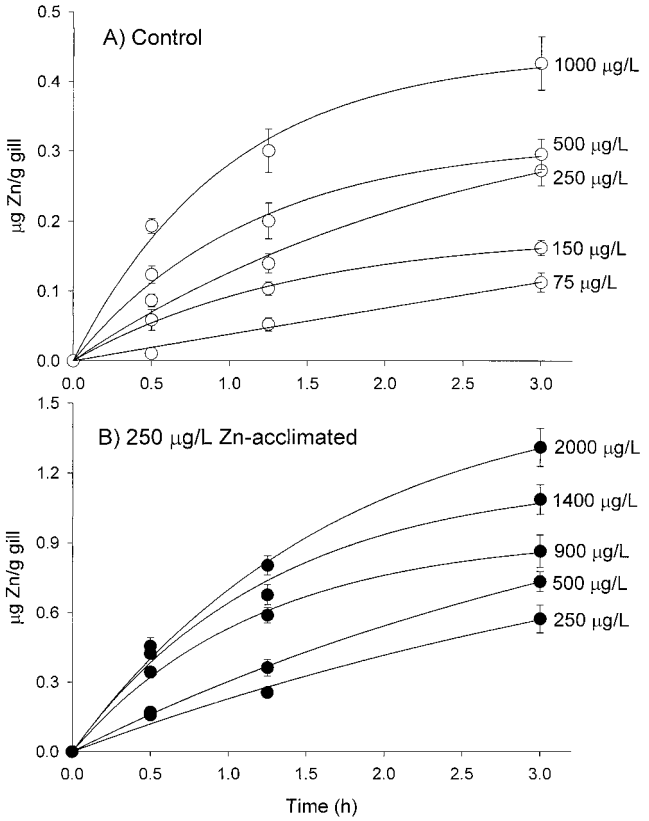

Fig. 1. Radioisotopically (^{65}Zn) determined Zn accumulation in the gills of (**A**) control and (**B**) 250-μg/L Zn-acclimated trout exposed to a range of Zn concentrations up to 3 h. Note the threefold difference in y-axis scales between panels **A** and **B**. Values expressed as means ± SEM (standard error of mean); $N = 7$.

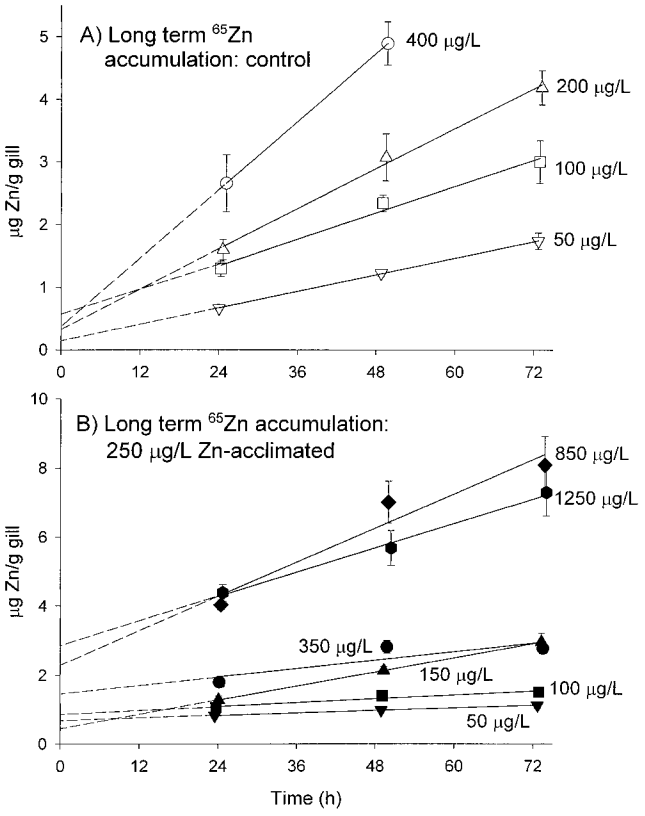

Fig. 2. Radioisotopically (^{65}Zn) determined Zn accumulation in the gills of (**A**) control and (**B**) 250-μg/L Zn-acclimated trout exposed to a range of Zn concentrations up to 72 h. Note the twofold difference in y-axis scales between panels **A** and **B**. Values expressed as means ± SEM (standard error of mean); $N = 6$ to 7. The extrapolations of the linear regressions to 0 h provide estimates of the sizes of the fast turnover pool of Zn in the gills (y-axis intercept), which are displayed in Figure 3.

250, 500, or 1,000 μg/L Zn plus 15 μCi ^{65}Zn per tank. Trout were sampled as above at 0.5, 1.25, and 3 h. A final separate experiment was carried out as above with seven trout per tank and terminal sampling at 7.75 h.

Calculations and statistics

The K_d and B_{max} of the gill ^{65}Zn accumulation were calculated via a nonlinear regression for line of best fit to a Michaelis—Menten equation using SigmaPlot® 4.0 (SPSS, Chicago, IL, USA) against a plot of gill ^{65}Zn load versus water total Zn concentration. The K_d measures the affinity of the gill (the higher the K_d, the lower the affinity) for waterborne Zn, while the B_{max} measures the binding capacity of the gill for waterborne Zn. In a similar fashion, gill Zn accumulation was plotted against the calculated free Zn^{2+} ion concentration at each exposure to determine ionic K values, which were then converted to traditional log K binding constants. Based on measured water chemistry, the free Zn^{2+} ion concentrations in our test water were approximately 60% of the total Zn concentrations using the aquatic geochemistry program MINEQL+ [17].

Data have been expressed as means ± standard error except for the 96-h LC50s, where means ± 95% confidence limits have been reported. A Student's t test (unpaired) was used to test for significant differences in the K_d and B_{max} between control and Zn-acclimated trout. A 5% significance level was employed throughout.

RESULTS

Gill Zn levels

Acclimation to 250 μg/L Zn for at least 30 d had no detectable effect on the total Zn levels of the gills as determined by atomic absorption spectroscopy (68.0 ± 13.6 μg Zn/g gill and 72.0 ± 7.7 μg Zn/g gill for control and Zn-acclimated trout gills, respectively). In addition, there was no detectable increase or trend in total gill Zn levels when both control and Zn-acclimated fish were exposed to a range of water Zn concentrations (from 0 to 2,900 μg/L Zn) for 7 d, illustrating the insensitivity of total Zn analyses.

Gill ^{65}Zn accumulation

As fish were acutely exposed to ^{65}Zn at a variety of total water Zn concentrations for up to 3 h, accumulation was initially rapid but began to level off by 3 h at the higher Zn exposure concentrations (Fig. 1A and B). The ^{65}Zn accumulation was greater with increasing Zn exposure concentrations. When fish were exposed to a range of Zn concentrations and gills were sampled from 24 to 72 h, accumulation occurred in an approximately linear fashion over time (Fig. 2A and B). In our previous study [15], this linear accumulation was termed the slow turnover of Zn in the gills. Linear regression analyses against time demonstrated that the rate of ^{65}Zn appearance in the slow pool (i.e., slope) was greater with increasing Zn exposure concentration (Table 1). However, at a given Zn ex-

Table 1. ^{65}Zn loading rates into the slow turnover pool of Zn in the rainbow trout gill; values are given as means ± SE (standard error); N = 18 to 21 except for control 400 μg/L Zn, where N = 12

Control: Zn exposure level (μg/L)	^{65}Zn appearance (μg Zn/g gill/h)	Zn-acclimated: Zn exposure level (μg/L)	^{65}Zn appearance (μg Zn/g gill/h)
50	0.0224 ± 0.002	50	0.0064 ± 0.002
100	0.0361 ± 0.006	100	0.0095 ± 0.003
200	0.0542 ± 0.008	150	0.0351 ± 0.005
400	0.0932 ± 0.024	350	0.0211 ± 0.005
		850	0.0849 ± 0.019
		1,250	0.0297 ± 0.017

posure concentration, ^{65}Zn appearance in the slow pool was considerably slower in the Zn-acclimated fish than in the control fish (Table 1). By extrapolating back to 0 h (cf., Fig. 2A and B), the size of the fast turnover pool at different Zn exposure levels could be estimated [15]. This analysis indicated that the fast turnover pool appeared to be saturable in both control and Zn-acclimated fish (Fig. 3A and B). The absolute size of the fast turnover pool of Zn in the gills was clearly much larger in the Zn-acclimated trout. The maximum size of the fast pool was calculated to be 0.45 ± 0.16 μg Zn/g gill

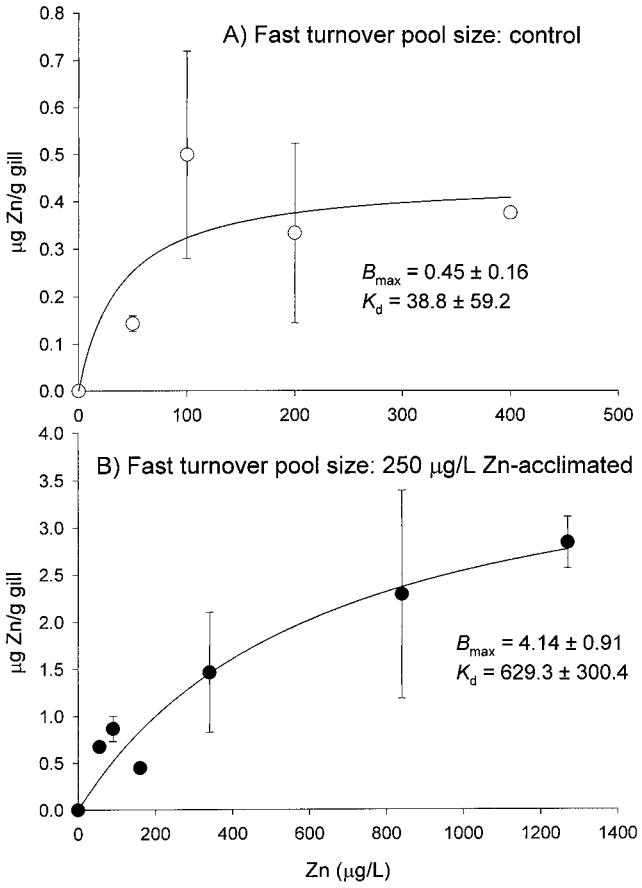

Fig. 3. The calculated sizes of the fast turnover Zn pools as a function of water Zn concentration in the gills of (**A**) control and (**B**) 250-μg/L Zn-acclimated trout over a range of waterborne Zn concentrations. Note the difference in axis scales. Values expressed as means ± SEM (standard error of mean). Values were determined by the extrapolation of the linear regressions in Figure 2 to 0 h (y-axis intercept). The K_d and B_{max} were calculated via a nonlinear regression for line of best fit to a Michaelis–Menten equation.

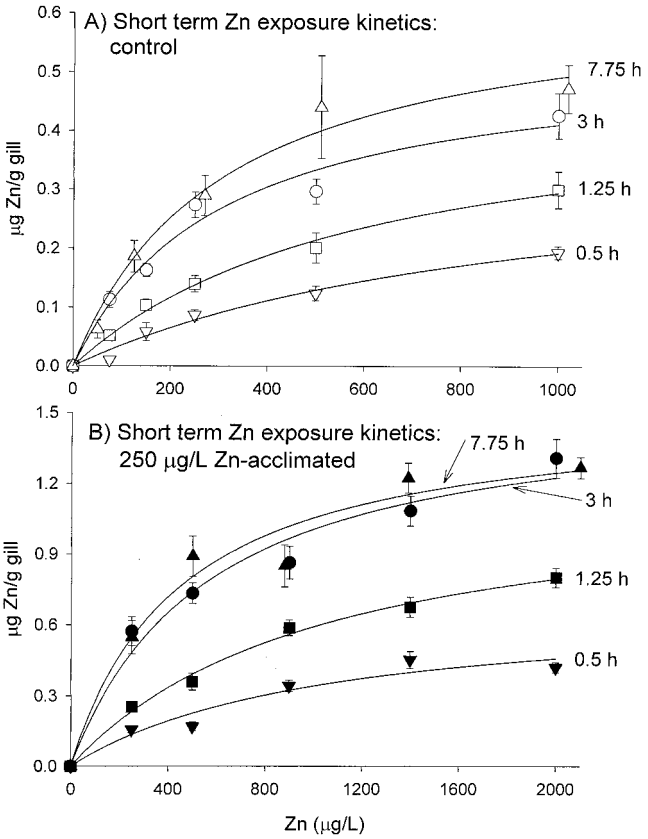

Fig. 4. A kinetic analysis of radioisotopically (^{65}Zn) determined Zn accumulation in the gills of (**A**) control and (**B**) 250-μg/L Zn-acclimated trout exposed to a range of Zn concentrations for 0.5 h (upside-down triangles), 1.25 h (squares), 3 h (circles), and 7.75 h (triangles). Values expressed as means ± SEM (standard error of mean); N = 7. Note the difference in scales of the axes between panels **A** and **B**. The K_d and B_{max} of the gill ^{65}Zn accumulation were calculated via a nonlinear regression for line of best fit to a Michaelis–Menten equation.

and 4.14 ± 0.91 μg Zn/g gill for control and Zn-acclimated fish, respectively. In addition, affinity appeared to be lower (i.e., 16-fold higher K_d) in the Zn-acclimated trout, although the difference was not significant, reflecting variability in the data set.

^{65}Zn accumulation kinetics

At each sampling time from 0.5 to 72 h, ^{65}Zn accumulation showed saturation kinetic trends (Figs. 4 and 5). The B_{max} increased with time of ^{65}Zn exposure. The rate of increase was rapid at first, after which it began to subside (Fig. 6A). The B_{max} of Zn-acclimated fish was always higher than that of unexposed controls. For both Zn-acclimated and unexposed fish, the K_d decreased rapidly from 0.5 to 3 h, where it remained constant up to the last sampling time of 72 h. In all cases, K_d was greater (decreased affinity) in Zn-acclimated fish in comparison with unexposed controls (Fig. 6B).

Zn toxicity

Significant mortality occurred in the long-term ^{65}Zn exposures. One third of the control fish did not survive at the 400-μg/L Zn exposure, and all died at exposures greater than 400 μg/L Zn. All Zn-acclimated trout died at exposures greater than 1,250 μg/L Zn. However, classic 96-h LC50 tests performed at a later date showed the trout to be much less sen-

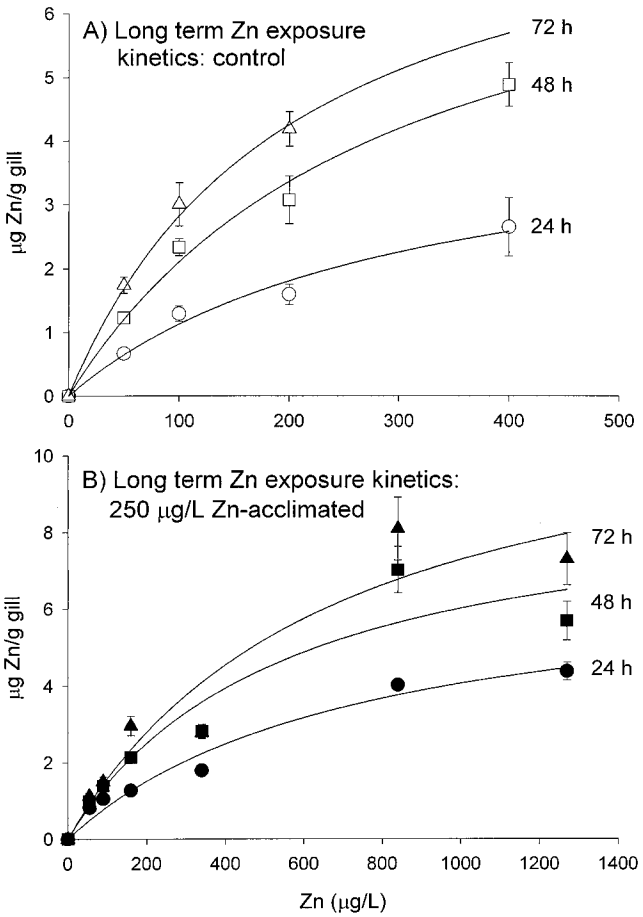

Fig. 5. A kinetic analysis of radioisotopically (^{65}Zn) determined Zn accumulation in the gills of (**A**) control and (**B**)) 250-µg/L Zn-acclimated trout exposed to a range of Zn concentrations at 24, 48, and 72 h. Values expressed as means ± SEM (standard error of mean); $N = 6$ to 7. Note the differences in scales of the axes between panels **A** and **B**. The K_d and B_{max} of the gill ^{65}Zn accumulation were calculated via a nonlinear regression for line of best fit to a Michaelis–Menten equation.

sitive. The LC50s were 2,615 µg/L Zn (2,083–3,326 µg/L, 95% confidence limits) for control trout and slightly higher for Zn-acclimated fish at 3,340 µg/L Zn (2,758–4,141 µg/L, 95% confidence limits), though this difference was not significant.

DISCUSSION

Gill Zn levels

Acclimation to 250 µg/L Zn for at least 30 d had no detectable effect on the total Zn levels of the gills, measured by atomic absorption spectrophotometry, compared with unexposed controls (~70 µg/g gill). In addition, exposure of both Zn-exposed and unexposed groups to up to 2,900 µg/L Zn for 7 d had no detectable effect on the gill Zn levels. A similar result was found previously [15], where juvenile rainbow trout were exposed to 150 µg/L Zn and 450 µg/L Zn in the same water chemistry as the present study and did not accumulate any Zn over 30 d. These data reconfirm the difficulties encountered when attempting to look at tissue accumulation of a nutrient metal already present in high concentrations in unexposed fish and reinforce the conclusion that the use of radiotracer (^{65}Zn) is essential to detect gill Zn uptake.

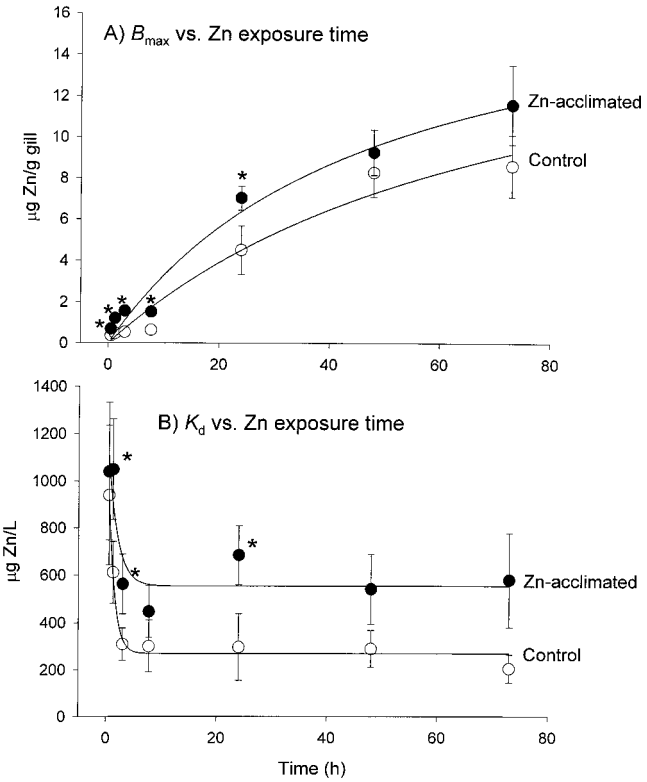

Fig. 6. (**A**) B_{max} (binding capacity) and (**B**) K_d (affinity) of radioisotopically (^{65}Zn) determined Zn accumulation in the gills of control (open circles) and 250-µg/L Zn-acclimated trout (filled circles) up to 72 h. An asterisk (*) indicates a significant difference between the control and Zn-acclimated trout at each time. Overall, the K_d and B_{max} of Zn-acclimated trout were both significantly different from the control trout ($p < 0.05$).

Temporal patterns of gill ^{65}Zn accumulation

The ^{65}Zn appearance was rapid during the first 3 h of exposure (Fig. 1A and B). However, at the higher Zn exposure concentrations, the rate of appearance had begun to curtail by 3 h. This rapid appearance occurred in what has been termed the fast turnover pool of Zn in the gills [15]. In addition, from 24 to 72 h, ^{65}Zn appearance in the gills was linear over time (Fig. 2A and B), and this appearance occurred in the slow turnover pool of Zn in the gill [15]. Within a treatment group, the loading rate into the slow turnover pool increased with increasing Zn exposure concentration. However, at a given Zn concentration, the loading rate into the slow turnover pool was consistently faster in control fish gills than 250-µg/L Zn-acclimated fish gills (Table 1). This is contrary to our first study [15], where we found the loading rate was faster in 250-µg/L Zn-acclimated fish than in control fish when both were exposed to 1,125 µg/L Zn. The apparent discrepancy could be due to the fact that, in Alsop et al. [15], only a single concentration was tested, while the present results at a range of concentrations (Table 1) indicate that the rates can be quite variable in Zn-acclimated fish, especially at higher concentrations.

When the linear regressions of the gill ^{65}Zn levels from 24 to 72 h are extrapolated back to 0 h, an estimate of the size of the fast turnover pool at different exposure concentrations can be determined [15]. With increasing Zn exposure concentration, the size of the fast pool increased but could only expand to a limit, leveling off at higher concentrations (Fig. 3A

and B). The maximum size of the fast pool estimated by this approach was approximately nine times larger in the Zn-exposed trout than in the control trout (0.45 versus 4.14 μg Zn/g gill). The maximum size of the fast pool amounted to 0.66 and 5.75% of the total Zn present in the gills of control and 250-μg/L Zn-acclimated fish, respectively. These results confirm and extend our previous study [15], where pool size measurements by this approach were made only at the Zn exposure concentration to which the fish had been acclimated and not at a range of concentrations as in the present study.

^{65}Zn accumulation kinetics

At every point in time when the gills were sampled, the accumulation of ^{65}Zn showed saturation kinetics as a function of exposure concentration and the affinity (K_d) and binding capacity (B_{max}) of the gill for Zn could be calculated (Figs. 4 and 5). The B_{max} values determined by this approach up to 7.75 h exhibited good quantitative agreement with the maximum size of the fast pool by the linear regression extrapolation approach (Fig. 3) in the control group and at least qualitative accord in the Zn-acclimated group (Fig. 6A).

The K_ds decreased rapidly from 0.5 to 3 h in both control and 250-μg/L Zn-acclimated trout whereas from 3 to 72 h they remained constant (Fig. 6B). It appears that, at first, Zn binds to lower affinity sites at the gill while it takes more time for Zn to access and bind to the higher affinity sites (3 h). At times greater than 3 h, the K_ds have stabilized and Zn is continuously able to bind to a set of sites with higher affinities than those at the onset of the exposure.

Unlike K_d, the B_{max} increased continually with ^{65}Zn exposure time in both groups (Fig. 6A). The rapid increase at first would represent mainly ^{65}Zn appearance in the fast turnover pool, whereas from 24 to 72 h, the slower increase would represent entry into the slow pool. The B_{max} (binding capacity) of Zn-acclimated fish was always greater than that of control fish (Fig. 6A). This is also seen in the size of the fast turnover pools of Zn in the gill (Fig. 3). In addition, Zn-acclimated fish exhibited a higher K_d (decreased affinity) than control fish at all the times that were tested (Fig. 6B), again in accord with the trends seen in the fast turnover pool analysis (Fig. 3).

Hogstrand et al. [23] similarly found a decrease in the branchial affinity for Zn transport (K_m) with sublethal Zn acclimation, which was apparent by the first day of exposure. Note that Hogstrand et al. [23] determined these Zn transport values by uptake of ^{65}Zn into the whole body over 24 h whereas we determined Zn binding to the gills by branchial accumulation of ^{65}Zn from 0.5 to 72 h. The absolute values of Hogstrand et al. [23] for K_m were very close to the longer term (i.e., 3–72 h) K_d values for Zn gill binding in the present study, i.e., control fish ~350 μg/L Zn [23] versus ~280 μg/L Zn (present study); 150-μg/L Zn-acclimated fish ~700 μg/L Zn [23] versus 250-μg/L Zn-acclimated fish ~550 μg/L Zn (present study). Our branchial K_d is also in good agreement with Spry and Wood [24], who found the K_m for Zn transport in control fish to be 240 μg/L Zn. The average K_d determined by Galvez et al. [21], with 3-h exposures in the same manner as the present study, was more than twofold greater (650 μg/L Zn) than our measurement (280 μg/L Zn). However, the K_d value in Galvez et al. varied greatly over the 15-d period during which the measurements were made. Perhaps as a result of this variability, Galvez et al. found that acclimation to 150 μg/L Zn had no effect on the K_d or B_{max}, contrary to Hogstrand et al. [23] and the present study. All studies were performed in the same water quality (dechlorinated Hamilton tap water from Lake Ontario, Canada).

The toxic action of Zn is a specific disruption of branchial calcium uptake [4–6], which leads to hypocalcemia. It has been proposed that the changes in binding affinity (increased K_m or K_d) that occur with Zn exposure are adaptive to help minimize Zn uptake while protecting calcium uptake [23]. The increase of B_{max} (binding capacity) that occurs with Zn exposure may be due to the induction of detoxification or temporary storage mechanisms in the gills such as Zn-binding proteins (e.g., metallothionein). Metallothionein levels in the gills of 150-μg/L Zn-exposed trout were elevated after 30 d of exposure in one study [5], though metallothionein was not elevated after 60 d of exposure to the same Zn concentration in another study [25].

Zn kinetics and toxicity

Although the gill accumulation characteristics were different between control and 250-μg/L Zn-exposed trout, there was no significant difference between their respective LC50s. However, the sensitivity to Zn appears to change with time. In the first test (which also yielded the [lack of] changes in total 'cold' gill Zn levels with exposure to different Zn concentrations), resistance was high in both groups (survived up to 2,900 μg/L Zn for 144 h) and 96-h LC50s could not be determined. In the second test 3 weeks later, both 96-h LC50s were in the range of 3,000 μg/L Zn, although slightly higher in the 250-μg/L Zn-acclimated trout. However, the same batch of fish appeared to be much more sensitive to Zn during the long-term ^{65}Zn exposures, where control fish died at exposures greater than 400 μg/L Zn and Zn-acclimated fish died at exposures greater than 1,250 μg/L Zn, suggesting the fish had acquired increased tolerance to Zn. The absolute levels of the 96-h LC50s in the present study (~3,000 μg/L Zn) were relatively high in comparison to our previous work when toxicological acclimation had occurred (869 μg/L Zn for control trout versus ~2,200 μg/L Zn for Zn-exposed; [15]). It appears that fish may not show increased tolerance with sublethal exposure at times of low sensitivity to Zn. Sensitivity and toxicological acclimation to Zn may be lower during periods of reduced Ca^{2+} uptake in the cycles described by Wagner et al. [26] and greater during periods of high Ca^{2+} uptake.

Previous studies in our lab [15] have shown that rainbow trout exhibited toxicological acclimation when chronically exposed to Zn levels of either 150 μg/L Zn or 450 μg/L Zn for 30 d in the same water chemistry as the present study (96-h LC50s increased 2.5-fold). The 2.5-fold increase in tolerance is very similar to the 2.1-fold increase in K_d (decrease in affinity) determined in the present study. This decrease in affinity for Zn may be the basis of waterborne Zn acclimation. Alternatively or additionally, the larger increase in maximum binding capacity (B_{max}) may be involved as a detoxification mechanism.

Implications for the biotic ligand model

The gill receptor loading model is one form of the biotic ligand model and has been successfully employed to predict metal levels in the gills of fish using experimentally determined binding constants (affinities and capacities) of the gill for the metal and for other ions [13]. This method has worked for metals such as copper and cadmium [10,14,18], silver [19], and cobalt [20].

The binding constants determined by Playle et al. [10],

Table 2. Binding constants (log K) and number of binding sites (B_{max}) for five heavy metals binding to control (previously unexposed) fish gills; all experiments were conducted in a similar manner with 2 to 3-h metal exposures; in addition, approximate LC50 values (96–144 h) determined previously with juvenile rainbow trout in our laboratory are reported, which were all performed in the same water chemistry; the cobalt LC50, however, is estimated based on toxicity tests performed by Diamond et al. [34] in water of similar chemistry

Metal	log K	Binding sites (B_{max}; nmol/g gill)	LC50 in Hamilton tapwater (μM)
Silver	10.0 [19]	6.1 [19]	0.083 [32]
Cadmium	8.6 [10]	2 [10]	0.196 [14]
Copper	7.4 [10]	30 [10]	1.10 [33]
Zinc	5.6[a]	8.3[a]	13.29 [15]
Cobalt	5.1 [20]	88 [20]	>50 [34]

[a] Present study.

Janes and Playle [19], and Richards and Playle [20], using methods for determination of K_d and B_{max} comparable to those employed here, are summarized in Table 2 and were derived based on 2- to 3-h exposures to a range of metal concentrations. Playle et al. [10] concluded that this was an appropriate time, based on cadmium levels in the gills of minnows having saturated by 2.5 h during exposure to a single concentration of cadmium. The present study, however, is the first to critically assess the influence of exposure time on the gill binding kinetics (K_d and B_{max}) of a heavy metal (i.e., at a range of concentrations).

It appears that, with Zn, a 3-h exposure would be the minimum time required to obtain a stable K_d and hence log K (Fig. 6B, Table 3). Our stable log K (>3 h) for Zn^{2+} binding to control trout gills of 5.6 is slightly higher than the log K of Galvez et al. [21], who determined a value of 5.1 using a 3-h exposure. It is also similar to that of Cusimano et al. [27], who determined a theoretical gill binding constant of 5.4 based on 96- and 168-h Zn LC50 studies rather than gill metal accumulation. Although acclimation to 250 μg/L Zn increased the stable K_d more than twofold (i.e., decreased affinity; Fig. 6B), our stable log K value only dropped from 5.6 to 5.3, reflecting the fact that log K values are expressed on a logarithmic scale. Again, the decrease in affinity of the gill for Zn may be a basis of the reduced toxicity of acute Zn exposure to preexposed fish.

The biotic ligand model assumes that there is a set number of binding sites at the gill. Depending on the time of Zn exposure, this number can change greatly. For example, at 0.5 h, B_{max} was 0.37 μg Zn/g gill, or 5.7 nmol Zn binding sites/g gill (Fig. 6A). By 72 h, this number had grown to 8.63 μg Zn/g gill, or 132.0 nmol Zn binding sites/g gill, an increase of 23-fold. In addition, prior Zn exposure increased the number of Zn binding sites in comparison to control at every time point (Fig. 6A). The difference was more apparent at the shorter Zn exposure times; Zn acclimation increased the number of binding sites by 150% when the gills were sampled at 1.25 h, while at 72 h, the number of sites was increased by only 35%. Our 3-h binding site number for control trout gills (8.3 nmol/g gill; Fig. 6A) is similar to that of Galvez et al. [21], who reported 8.6 nmol/g gill at 3 h.

Table 3. Log K binding constants for Zn in control and 250-μg/L Zn-acclimated rainbow trout gills over 72 h; values are calculated as the free Zn ion (Zn^{2+}) concentrations (see text for details)

Time (h)	Control log K	Zn-acclimated log K
0.5	5.06	5.02
1.25	5.25	5.02
3	5.55	5.29
7.75	5.54	5.38
24	5.56	5.17
48	5.57	5.30
72	5.72	5.21

The reported binding constants (log K) and number of binding sites (B_{max}) of five different heavy metals binding to fish gills are compiled in Table 2; all values were determined with 2- to 3-h exposures. In addition, acute LC50 concentrations determined in previous studies in our lab are shown. Consistent with chronic prior exposure reducing the affinity and toxicity of Zn, it is obvious that those metals that have a greater affinity for fish gills are also those that are much more toxic. At first glance, the toxicity data are less consistent with the number of metal binding sites in the gills. However, the number of binding sites appears to decrease with metals of greater toxicity when they are grouped by mechanism of toxic action. For example, both silver and copper disrupt Na^+ uptake [28,29], but silver is more toxic and has fewer binding sites. Similarly, cadmium [30], zinc [5], and cobalt [31] all disrupt Ca^{2+} uptake. Cadmium is the most toxic and has the fewest binding sites, whereas cobalt is least toxic and has the greatest number of binding sites. Thus, both the affinity (log K) and capacity (B_{max}) of the gill to bind metals appear to be directly related to metal toxicity.

The gill binding constants of the present study for Zn were derived in hard water, i.e., in the presence of potentially competing cations such as Ca^{2+}, Mg^{2+}, and Na^+. An important goal of future research should be to determine if the constants are the same in soft water, where the availability of these potential competitors is reduced. A priori, higher log K values might be expected in soft water for this reason.

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REFERENCES

1. Vallee BL, Falchuk KH. 1993. The biochemical basis of zinc physiology. *Physiol Rev* 73:79–118.
2. Hogstrand C, Wood CM. 1996. The physiology and toxicology of zinc in fish. In Taylor EW, ed, *Aquatic Toxicology*. Cambridge University Press, Cambridge, UK, pp 61–84.
3. Spry DJ, Hodson PV, Wood CM. 1988. Relative contributions of dietary and waterborne zinc in the rainbow trout, *Salmo gairdneri*. *Can J Fish Aquat Sci* 45:32–41.
4. Spry DJ, Wood CM. 1985. Ion flux rates, acid-base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft water. *Can J Fish Aquat Sci* 42:1332–1341.
5. Hogstrand C, Reid SD, Wood CM. 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.
6. Hogstrand C, Verbost PM, Wendelaar Bonga SE, Wood CM. 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: Interplay with calcium transport. *Americal Journal of Physiology* 270:1141–1147.
7. Canadian Council of Ministers of the Environment. 1995. Canadian Council of Resource and Environment Ministries, Appendix XVIII. In *Canadian Water Quality Guidelines*. Winnipeg, MB.

8. McDonald DG, Wood CM. 1993. Branchial mechanisms of acclimation to metals in freshwater fish. In Rankin JC, Jensen FB, eds, *Fish Ecophysiology*. Chapman & Hall, London, UK, pp 297–321.
9. Bradley RW, DuQuesnay C, Sprague JB. 1985. Acclimation of rainbow trout, *Salmo gairdneri* Richardson, to zinc: Kinetics and mechanism of enhanced tolerance induction. *J Fish Biol* 27:367–379.
10. Playle RC, Dixon DG, Burnison K. 1993. Copper and cadmium binding to fish gills: Estimates of metal-gill stability constants and modelling of metal accumulation. *Can J Fish Aquat Sci* 50: 2678–2687.
11. Wood CM, et al. 1997. Reassessment of metals criteria for aquatic life protection: Priorities for research and implementation. In Bergman HL, Doward-King EJ, eds, *Reassessment of Metals Criteria for Aquatic Life Protection.* Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA, pp 33–40.
12. Renner R. 1997. A better understanding of how natural waters inhibit metal uptake may change current regulatory limits. *Environ Sci Technol* 31:466–468.
13. Playle RC. 1998. Modelling metal interactions at fish gills. *Sci Total Environ* 219:147–163.
14. Hollis L, McGeer JC, McDonald DG, Wood CM. 1999. Cadmium accumulation, gill Cd binding, acclimation, and physiological effects during long term sublethal Cd exposure in rainbow trout. *Aquat Toxicol* 40:101–119.
15. Alsop DH, McGeer JC, McDonald DG, Wood CM. 1999. The costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water. *Environ Toxicol Chem* 18:1014–1025.
16. Allison JD, Brown DS, Novo-Gradac KJ. 1991. *MINTEQA2/ PRODEFA2, A Geochemical Assessment Model for Environmental Systems, User's Manual,* Ver 3.0. U.S. Environmental Protection Agency, Washington, DC.
17. Schecher WD, McAvoy DC. 1994. *MINEQL+ User's Manual.* Environmental Research Software, Hallowell, ME, USA.
18. Hollis L, Muench L, Playle RC. 1997. Influence of dissolved organic matter on copper binding, and calcium on cadmium binding, by gills of rainbow trout. *J Fish Biol* 50:703–720.
19. Janes N, Playle RC. 1995. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 14: 1847–1858.
20. Richards JG, Playle RC. 1998. Cobalt binding to gills of rainbow trout (*Oncorhynchus mykiss*): An equilibrium model. *Comp Biochem Physiol C* 119:185–197.
21. Galvez F, Webb N, Hogstrand C, Wood CM. 1998. Zinc binding to the gills of rainbow trout: The effect of long-term exposure to sublethal zinc. *J Fish Biol* 52:1089–1104.
22. Finney DJ. 1971. *Probit Analysis,* 3rd ed. Cambridge University Press, New York, NY, USA.
23. Hogstrand C, Webb N, Wood CM. 1998. Covariation in regulation of affinity for branchial zinc and calcium uptake in freshwater rainbow trout. *J Exp Biol* 201:1809–1815.
24. Spry DJ, Wood CM. 1989. A kinetic method for the measurement of zinc influx in vivo in the rainbow trout, and the effects of waterborne calcium on flux rates. *J Exp Biol* 142:425–446.
25. Hogstrand C, Wilson RW, Polgar D, Wood CM. 1994. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. *J Exp Biol* 186:55–73.
26. Wagner GF, Hampong M, Copp DH. 1985. A cycle for ^{45}calcium uptake in the rainbow trout, *Salmo gairdneri*. *Can J Zool* 63: 2778–2779.
27. Cusimano RF, Brakke DF, Chapman GA. 1986. Effects of pH on the toxicities of cadmium, copper, and zinc to steelhead trout (*Salmo gairdneri*). *Can J Fish Aquat Sci* 43:1497–1503.
28. Laurén DJ, McDonal DG. 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. *J Comp Physiol B* 155:635–644.
29. Wood CM, Playle RC, Hogstrand C. 1999. Physiology and modelling of mechanisms of silver uptake and toxicity in fish. *Environ Toxicol Chem* 18:71–83.
30. Verbost PM, Van Rooij J, Flik G, Lock RAC, Wendelaar Bonga SE. 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *J Exp Biol* 145:185–197.
31. Comhaire S, Blust R, Ginneken LV, Vanderborght OLJ. 1994. Cobalt uptake across the gills of the common carp, *Cyprinus carpio*, in the water of acclimation and exposure. *Comp Physiol Biochem C* 109:63–76.
32. Hogstrand C, Galvez F, Wood CM. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environ Toxicol Chem* 15:1102–1108.
33. Taylor L, McGeer JC, Wood CM, McDonald DG. 2000. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: An evaluation of chronic endpoints. *Environ Toxicol Chem* (in press).
34. Diamond JM, Winchester EL, Macker DG, Rasnake WJ, Fanelli JK, Gruber D. 1992. Toxicity of cobalt to freshwater indicator species as a function of water hardness. *Aquat Toxicol* 22:163–180.