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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

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Abstract—Juvenile rainbow trout were exposed to zinc in both moderately hard water (hardness = 120 mg $CaCO_3$/L, pH = 8.0, Zn = 150 μg/L or 450 μg/L) and soft water (hardness = 20 mg $CaCO_3$/L, pH = 7.2, Zn = 50 μg/L or 120 μg/L) for 30 d. Only the 450 μg/L zinc–exposed fish experienced significant mortality (24% in the first 2 d). Zinc exposure caused no effect on growth rate, but growth affected tissue zinc levels. Whole body zinc levels were elevated, but gills and liver showed no consistent increases relative to controls over the 30 d. Therefore, tissue zinc residues were not a good indicator of chronic zinc exposure. After the 30-d exposure, physiological function tests were performed. Zinc was 5.4 times more toxic in soft water (control 96 h LC50s in hard and soft water were 869 μg/L and 162 μg/L, respectively). All zinc-exposed trout had acclimated to the metal, as seen by an increase in the LC50 of 2.2 to 3.9 times over that seen in control fish. Physiological costs related to acclimation appeared to be few. Zinc exposure had no effect on whole body Ca^{2+} or Na^+ levels, on resting or routine metabolic rates, or on fixed velocity sprint performance. However, critical swimming speed (U_{Crit}) was significantly reduced in zinc-exposed fish, an effect that persisted in zinc-free water. Using radioisotopic techniques to distinguish new zinc incorporation, the gills were found to possess two zinc pools: a fast turnover pool ($T_{1/2}$ = 3–4 h) and a slow turnover pool ($T_{1/2}$ = days to months). The fast pool was much larger in soft water than in hard water, but at most it accounted for <3.5% of the zinc content of the gills. The size of the slow pool was unknown, but its loading rate was faster in soft water. Chronic zinc exposure was found to increase the size of the fast pool and to increase the loading rate of the slow pool.

Keywords—Rainbow trout Zinc Acute/chronic toxicity Acclimation Gill metal-binding model

INTRODUCTION

Zinc is an essential micronutrient and a cofactor of over 300 enzymes [1], but it becomes toxic at increased waterborne levels. At extremely high waterborne levels, zinc causes gross morphological alterations at the teleost gill, such as epithelial lifting and lamellar clubbing [2]. The fish usually dies within a few hours as a result of tissue hypoxia secondary to impairment of gas exchange at the gill [3]. At lower waterborne concentrations that more realistically reflect contaminated environments, zinc specifically disrupts calcium uptake across the gills [4–6], leading to hypocalcemia, which may culminate in the death of the fish within a few days, depending on the zinc concentration.

Fish have been shown to acclimate to metals during sublethal waterborne exposure in two ways; first, if a fish survives the metal exposure, then the ionic disturbance may eventually be corrected [7], as is seen with the full recovery of plasma Ca^{2+} during a sublethal exposure to zinc [6]. Second, an increased tolerance (in terms of survival) to the metal may arise upon a threshold exposure. With zinc-exposed trout, this acclimation or increased tolerance was fully acquired within 5 d; the tolerance increased 2.5 times compared with that of unexposed fish, as judged by LC50 tests [8].

Acclimation to metals is thought to occur via a variety of mechanisms, including changes at the gill (i.e., alterations to transport proteins, hypertrophy and hyperplasia of mucous and chloride cells, and a general thickening of lamellar and filamental epithelia) [7,9]. These structural changes could have effects on the maximum O_2 and CO_2 exchange rates, which would limit aerobic swimming performance. In previous studies, critical swimming speed (U_{Crit}) was significantly depressed upon acclimation to aluminum [10] and to copper, the latter depending on the water composition [11].

Zinc toxicity is dependent not only on the zinc concentration but also on the presence of other dissolved ions. Water hardness (as milligrams of $CaCO_3$ per liter) is considered to be the most influential component of water chemistry in modifying toxicity. For example, a decrease in water hardness of approximately 10 times was shown to increase zinc toxicity by 10 times [12]. By analogy, in work done with other metals [13–15], soft water is thought to exhibit less competition from other ions, most importantly Ca^{2+}, with respect to zinc binding sites on the gill surface. There are also fewer ligands in the water to keep zinc from binding to the gill sites.

The objectives of this study were to determine the effects of chronic zinc exposure on juvenile rainbow trout and to determine how they are modified by water hardness. Fish were exposed to 150 μg/L and 450 μg/L Zn, at a water hardness of 120 mg $CaCO_3$/L, and to 50 μg/L and 120 μg/L Zn, at a hardness of 20 mg $CaCO_3$/L. During the exposures, mortality and growth were recorded, and tissue levels of zinc were measured. After the 30-d zinc exposure, the performance of the fish was evaluated with various physiological function tests that included measures of two types of swimming performance, of acclimation as assessed by zinc tolerance (96 h LC50), of metabolic rate, and of the turnover rates of zinc in various tissues.

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The kinetics of zinc turnover in the gills were a particular focus because of recent interest in using gill metal-binding models to predict site-specific toxicity [14,16]. A recent study has demonstrated that because zinc is a micronutrient present at high background levels in gill tissue, zinc binding to gills can only be detected if radioisotopic ^{65}Zn is employed [17]. This being the case, it is essential to understand the kinetics of turnover detected by the radioisotope and to determine whether these kinetics change during chronic sublethal zinc exposure.

MATERIALS AND METHODS

Chronic zinc exposures were performed in two water qualities: moderately hard Hamilton tap water from Lake Ontario and synthetic soft water.

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Rainbow Springs Trout Hatchery (Thamesford, ON, Canada), initially held in aerated 500-L tanks supplied with 3 L/min of dechlorinated Hamilton tap water (''hard 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 [DOM], 3 mg/L; pH 8.0), and allowed to acclimate for 1 week. The fish were then slowly brought to the appropriate water chemistry, if required, and temperature over 7 d (up to 14–18°C, and reduced hardness in the soft water exposure). Soft water (ionic composition: Ca^{2+}, 0.13 mM; Mg^{2+}, 0.04 mM; Na^{+}, 0.13 mM; Cl^{-}, 0.1 mM; hardness, 20 mg $CaCO_3$/L; alkalinity, 15 mg $CaCO_3$/L; DOM, 0.4 mg/L; pH 7.2) was synthesized by mixing one part hard water with six parts ion-reduced water, the latter produced by reverse osmosis (Anderson Water Systems, Dundas, ON, Canada), a procedure that unavoidably raised the temperature to 18°C (relative to 14°C in the hard water exposure). The fish were allowed to acclimate to their new water conditions for at least 3 weeks prior to the initiation of the experiment. Fish were fed twice daily, with a commercial ration totaling 2% body mass/day, during this holding period (fish food composition [partial analysis only]: crude protein [minimum], 52%; crude fat [minimum], 17%; crude fibre [maximum], 2.5%; water, 12%; Ca^{2+}, 1.4%; Na^{+}, 0.4%; zinc (measured), 0.02% [173 μg/g].

One week prior to each experiment, fish ($N = 1,620$) were nonselectively transferred to one of six identical 211-L tanks (270 fish per tank; mean fish weight, 1.68 ± 0.16 g in the hard water exposure and 5.27 ± 0.06 g in the soft water exposure). Water flow into each tank was >0.75 L/min; P_{O_2} was maintained at >90% air saturation through continuous aeration of the tanks. Feces and organic debris were siphoned out of the tanks daily. Photoperiod was set to 10:12 h light:dark to mimic a natural photoperiod.

Exposures

At the start of each exposure, the six tanks were nonselectively assigned to one of the three zinc exposure concentrations (two tanks/Zn exposure concentration); hard water: control [<1 μg/L Zn], low zinc [150 μg/L Zn], and high zinc [450 μg/L Zn]; soft water: control [<1 μg/L Zn], low zinc ([50 μg/L Zn], and high zinc [120 μg/L Zn]). The levels were chosen on the basis of rangefinder toxicity tests that were performed prior to each exposure. A high zinc exposure level was chosen to produce slight acute mortality, while the low exposure was intended to cause no mortality. To begin the exposure, flow from a Mariotte® bottle of concentrated zinc 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, Aubau, Germany) was begun into a head tank, where the zinc solution mixed with inflowing freshwater by vigorous aeration. Zinc was also added directly to the exposure and the head tanks to rapidly bring each one up to the desired level. Zinc levels in the hard water experiment ranged from 129 to 165 μg/L (mean, 157 μg/L) in the low zinc exposure and from 425 to 465 μg/L (mean, 458 μg/L) in the high exposure. In soft water, the ranges were 45 to 67 μg/L (mean, 53 μg/L) in the low zinc exposure and 109 to 138 μg/L (mean, 118 μg/L) in the high exposure.

After the two exposures were completed, a supplementary third series was conducted in hard water in exactly the same manner used for the first series. This time, however, there was only one control tank and one 250 μg/L zinc exposure tank. The fish were acclimated for 1 month to investigate the effects of zinc acclimation on the oxygen consumption of individual fish in respirometers and aerobic swimming performance (methods described below).

For each exposure, fish were fed three 1%-body mass meals per day, totaling 3% per day. Each meal was calculated as 1% of the bulk weight of each tank, and the meal amount was modified with each bulk weighing. Throughout the exposure, mortalities were recorded and fish were removed daily, weighed, and feeding quantities were adjusted as needed.

Sampling

At 1 d prior to the start of the experiment and at days 2 (hard water) or 5 (soft water), 10, 20, and 30 after exposure initiation, fish ($N = 6$ per treatment) were removed and quickly sacrificed with a blow to the head. The gills and liver were excised and frozen in liquid nitrogen, as was the remaining carcass. Whole fish ($N = 6$) were also removed, sacrificed, and frozen.

Zinc levels in the tissues were determined by digestion in 5 volumes of 1 N HNO_3 (trace metal analysis grade, BDH Chemicals) for 3 h at 80°C. Samples were vortexed and allowed to settle for 24 h; 100 μl of supernatant was diluted to 1 ml with deionized water (NANOpure II Barnstead, Dubuque, IA, USA) and analyzed by atomic absorption spectroscopy (Varian AA-1275, Walnut Creek, CA, USA; an air/acetylene flame was used). Whole-body Ca^{2+} and Na^{+} concentrations were measured from dilutions of the whole-body acid digest in the same manner. Water samples were collected throughout the exposures (20 ml water + 50 μL concentrated HNO_3). Water samples were analyzed by atomic absorption spectroscopy for Zn^{2+}, Ca^{2+}, and Na^{+}.

On the day prior to the start of the experiment and every 6 to 7 days during the exposure, all fish in each tank were bulk weighed using a removable sieve. The specific growth rate (SGR) or percent increase in body mass/day (with 95% confidence limits) was calculated from these bulk-weight measurements by linear regression of the natural logarithm of weight versus time using the statistical package SPSS.

Acclimation tests

After 30 d, the zinc-exposed fish were tested for acclimation to zinc using a 96 h LC50 test. Fifty fish from each treatment were removed and divided into five 18-L tanks (10 fish/tank), with each tank receiving 150 ml/min of control water for 1 h prior to the start of the LC50 trial. After 1 h, the tanks were randomly assigned to one of five zinc concentrations plus a control group (0–4,000 μg/L Zn for hard water groups and 0–1,250 μg/L Zn for the soft water groups). The LC50 test was then begun in the same manner used for the exposures; the concentrated zinc solution flows were started while zinc was added to each tank (apart from the control tanks) to bring them up to the chosen zinc level. Mortalities were recorded over 96 h. Water samples were taken daily and acidified for later zinc analysis. The 96 h LC50s ± 95% CL were calculated by log probit analysis of mortality versus measured waterborne zinc concentration [18].

Oxygen consumption

Routine oxygen consumption. Routine oxygen consumption was measured in-tank after 30 d of exposure in two tanks for each treatment in both exposures. Rates were measured over 1-h periods, starting 2 h after the second feeding of the day and again at 6 h after the final feeding of the day. The surface of the tank was sealed with a tight-fitting, transparent lid made of heavy plastic, and both the aeration and the flow of freshwater to the tanks were stopped. The tank water was then recirculated at 10 L/min by means of a pump (Little Giant, Oklahoma City, OK, USA) that drew water from the bottom and returned it back to the upper region of the tank. P_{O_2} levels were measured over the hour by taking water samples from each tank at 20-min intervals and injecting them into a Cameron E101 oxygen electrode thermostatted to the experimental temperature and connected to a Cameron OM-200 O_2 meter. Water P_{O_2} levels never dropped below 70% of the air saturation values.

The following formula was used to calculate the absolute O_2 consumption rate (M_{O_2}) from changes in P_{O_2} levels.

$$M_{O_2} = \Delta P_{O_2} \cdot \alpha_{O_2} \cdot \text{vol/mass} \cdot \text{time} \quad (1)$$

In Equation 1, ΔP_{O_2} is the change in P_{O_2} values, measured in torr, between the beginning and end of each 1-h test period, vol is the volume of water in each tank expressed in liters (211 L), mass is the total mass, in grams, of fish in the tank, and α_{O_2} is the solubility constant for O_2 in water at the experimental temperature, expressed as micromole per liter per torr [19]. Time is measured in hours.

Resting oxygen consumption. Oxygen consumption was also measured in individual small-volume (3.23-L) Blazka-type respirometers, similar to those described by Beamish et al. [20], using control fish and trout exposed to 250 μg/L Zn for 30 d in the supplementary hard water series (see Exposures). The fish were not fed for 2 d prior to the measurement. The control fish were tested with control hard water, and the zinc-exposed fish were tested with 250 μg/L Zn added to the water. Water velocity was set to 5 cm/s just enough to circulate the water but slow enough that the fish could rest on the bottom of the respirometer without having to swim. Water samples for M_{O_2} measurements were taken at the beginning and at the end of a 1-h time period when the respirometers were closed off. P_{O_2} analyses and M_{O_2} calculations were performed as in the routine oxygen consumption test (Eqn. 1), using individual fish weights and respirometer volumes (3.23 L).

Swimming performance tests

Fixed velocity test. Fixed velocity swimming, a test of sprint performance, was evaluated after 30 d of exposure, using the protocol of McDonald et al. [21]. Fish were not fed on the day of the test. Two sets of 10 fish each were tested from each treatment in both the hard and soft water experiments. Ten fish were removed from one treatment and placed in a flume with control water (no zinc added). The fish were given 5 min to settle at a current velocity of 10 cm/s, and they were then brought up to the test velocity of approximately 7 body lengths/s (57 cm/s in hard water and 63 cm/s in soft water) over a period of 2 min. At the end of the 2-min ''ramp-up,'' the clock was started, and the fish were timed until exhaustion. Fish were deemed exhausted when they became impinged on the rear screen and would not swim after being reintroduced manually into the current. Once exhausted, fish were removed, blotted dry, weighed to within the nearest 0.01 g, and fork length was measured to the nearest millimeter. The lengths of time required to reach a state of fatigue were corrected to a reference body length of 7 cm in the hard water exposure and 10 cm in the soft water exposure. The time to 50% fatigue (±95% CL) was calculated by linear regression of probit fatigue versus log time with SPSS [21].

Critical swimming speed (U_{Crit}). The U_{Crit} tests [22] were performed on the control and 250 μg/L-Zn–acclimated fish from the supplementary hard water series (see Exposures). A modified 100-L Beamish-style swimming tunnel, calibrated prior to use with a Kent Miniflo Type 265 propeller-style flow meter, was employed. The fish were not fed for 2 d prior to the U_{Crit} tests. Control fish ($N = 11$) were tested in the control hard water, while separate batches of the 250 μg/L-Zn–exposed fish were tested in the presence ($N = 13$) and absence ($N = 10$) of 250 μg/L Zn. Fish were allowed an initial 45-min settling time in the swimming tunnel with the current set at 10 cm/s. The U_{Crit} test was then performed by increasing the water velocity by 10 cm/s increments every 45 min until the fish became exhausted. Fish were considered exhausted once they impinged on the rear screen and would not swim after being manually reintroduced into the current. After exhaustion, the fish were blotted dry, weighed, and measured, as in the stamina test.

The U_{Crit} was determined for each fish using the equation given by Brett [22].

$$U_{Crit} = V_f + [(T/t) \times dV] \quad (2)$$

In Equation 2, U_{Crit} is in centimeters per second, V_f is the velocity prior to the velocity at which exhaustion occurred (the last velocity at which the fish swam for the entire 45-min period), dV is the velocity increment (10 cm/s), t is the time the fish swam at each velocity (45 min), and T is the time the fish swam at the final velocity before exhaustion. U_{Crit} was then converted to body lengths/s by dividing by the fork length of the fish.

Zinc turnover tests

After 30 d of exposure, a short-term (14-h) radiolabeled ^{65}Zn exposure was performed with hard water, 150 μg/L Zn–exposed fish. Fish were placed in a 25-L tank containing 150 μg/L Zn in hard water. To the tank was added 25 μCi of radiolabeled ^{65}Zn (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 zinc concentration of the water. At the sampling times of 0.5, 1, 2, 4, 8.5, and 14 h, water samples were taken, and fish were quickly removed ($N = 5$) and sacrificed with a blow to the head. The gills were excised and rinsed vigorously for 10 s in double-distilled water, blotted dry, weighed, and assayed for ^{65}Zn activity in a γ-counter (MINAXI γ Auto-Gamma 5000 Series, Canberra-Packard, Mississauga, ON, Canada). Water samples were similarly assayed for ^{65}Zn activity as well as for total zinc concentration using atomic absorption spectrophotometry in order to allow calculation of the specific activity of the waterborne zinc (Eqn. 3).

A similar experiment with more extensive tissue sampling was conducted over a longer time period with all treatments in both exposures. As in the short-term test, fish were exposed to the same water quality and zinc concentration to which they had been previously exposed. Sampling occurred three times (N = 5–6) between 24 and 75 h. A blood sample was taken from the caudal vein, and the gills were excised and rinsed vigorously in double-distilled water for 10 s. The liver and gallbladder (with bile content intact) were also excised, and the remaining carcass was rinsed in 25 mg/L Zn in deionized water solution for 30 s to displace any surface-bound ^{65}Zn. Each tissue was weighed and assayed for ^{65}Zn along with quadruple water samples; the latter were also analyzed for total zinc concentration using atomic absorption spectrophotometry.

Again, a similar experiment was performed, exposing control and 250 μg/L Zn–acclimated fish to a high level of waterborne zinc (1,125 μg/L), and the gills were sampled in over a 48-h period the same manner described above.

The appearance of ^{65}Zn from the water in the various tissues was calculated by first determining the mean specific activity (SA) of zinc in the water over the time period in question.

$$SA = (cpm/ml)/[Zn] \quad (3)$$

In Equation 3, cpm are the γ-counts per minute, and [Zn] is the concentration of zinc in μg/ml. In practice, zinc total concentrations and specific activities underwent negligible change during these tests. Total zinc appearance in the tissues was then calculated as follows:

$$\text{Total Zn Appearance} = (\text{cpm/tissue weight}) \cdot (1/SA) \quad (4)$$

In Equation 4, total zinc appearance is in μg Zn/g tissue.

For the long-term ^{65}Zn turnover experiments (24–75 h), linear regressions were performed on the relationships between tissue zinc appearance and time. By extrapolating the labeling of this slow turnover pool back to time = 0, the size of the fast turnover pool (in μg Zn/g tissue) could be estimated as y-intercept ±95% CL by SPSS.

Statistics

Data have been expressed as mean ± standard error (N), except for the specific growth rates, 96 h LC50s, fixed velocity performance times, fast turnover pool sizes, and slow turnover pool rates, where means ± 95% CL have been reported. For the latter, values were considered significantly different if the 95% CL did not overlap.

For other data, significant differences were tested with a one-way analysis of variance. If the F value indicated significance, then a Student-Newman-Keuls test for multiple comparisons was applied to test for significant differences among treatments. A Student's *t*-test (two-tailed, unpaired) was used to test for significant differences in the resting oxygen consumption and U_{Crit} experiments. A 5% significance level was employed throughout.

RESULTS

Exposure mortality and growth

Fish exposed to zinc showed little acute mortality apart from the 450 μg/L Zn hard water group (24% mortality in the first 2 d). By the end of the 30-d exposure, the 450 μg/L-Zn group had experienced 25.8% mortality, the 150 μg/L-Zn group had experienced 2.5% mortality, and the control group had experienced 0.7% mortality. In the soft water exposure, there were no mortalities during the first 11 d in any of the groups. By the end of 30 d, however, the 120 μg/L-Zn group had experienced 10.5% mortality, the 50 μg/L-Zn group had experienced no mortality, and the control group had experienced 2.8% mortality.

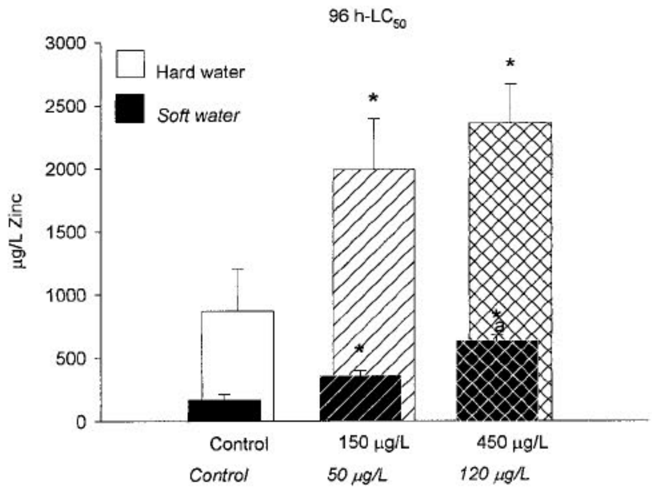

Fig. 1. The 96 h LC50 in hard water (open columns) and soft water (filled columns, italics) measured after 30 d of exposure. The asterisk (*) denotes a significant increase in the LC50 for the zinc-exposed fish groups over the control treatment of the same water chemistry. An (a) indicates a significant increase in the LC50 of the high zinc-exposed trout (hard water = 450 μg/L Zn; soft water = 120 μg/L Zn) over the low zinc-exposed trout (hard water = 150 μg/L Zn; soft water = 50 μg/L Zn) of the same water chemistry. Values expressed as means ± 95% CL.

Even after 25.8% mortality in the 450 μg/L-Zn fish, there appeared to be no effect of zinc exposure on the SGR (SGR = % increase in mass per day). In hard water, the SGRs for control, 150 μg/L-Zn, and 450 μg/L-Zn were 3.47 ± 0.36, 3.59 ± 0.21, and 3.59 ± 0.21, respectively (mean ± 95% CL). This was also the case in the soft water exposure, where the SGRs were 3.33 ± 0.28, 3.25 ± 0.24, and 3.10 ± 0.25 in control, 50 μg/L Zn–, and 120 μg/L Zn–exposed fish, respectively. Growth rate was not significantly different in hard versus soft water ($p < 0.05$).

Zinc toxicity and acclimation tests

Zinc was much more toxic in soft water, where the control 96 h LC50 was 5.4 times less than it was in hard water (869 μg/L vs. 162 μg/L). Both the high and low zinc-exposed groups, in both hard and soft water, exhibited significant acclimation to zinc, as demonstrated by the 96 h LC50 measurements (Fig. 1). In hard water, the increase in the LC50 was 2.3 times (in the 150 μg/L-Zn group) and 2.7 times (in the 450 μg/L-Zn group) over controls. In soft water, the increase was 2.2 times (in the 50 μg/L-Zn group) and 3.9 times (in the 120 μg/L-Zn group) over controls.

Costs and consequences of chronic zinc exposure

There was no effect of zinc exposure on the in-tank routine oxygen consumption rates measured after 30 d of exposure in either hard or soft water (Table 1). There was, however, a 37% higher M_{O_2} in the soft water experiment, which probably re-

Table 1. Routine in-tank oxygen consumption (Mo_2) and resting Mo_2 measured on individual rainbow trout in respirometers after 30 d zinc exposure. Values are expressed as means ± SEM. (Routine $N = 2$ tanks of trout; control resting $N = 19$ trout; 250 μg/L Zn resting $N = 16$ trout)

Zinc exposure level–measurement condition	Hard water (μmol/g/h ± SEM)	Soft water (μmol/g/h ± SEM)
Control-routine	11.8 ± 0.4	16.2 ± 0.5
Low-routine[a]	10.8 ± 0.3	16.3 ± 0.6
High-routine[b]	11.9 ± 0.5	14.7 ± 0.5
Control resting	8.0 ± 0.6	—
250 μg/L-resting	7.1 ± 0.6	—

[a] Hard water = 150 μg/L Zn; soft water = 50 μg/L Zn.
[b] Hard water = 450 μg/L Zn; soft water = 120 μg/L Zn.

flects the effect of the 4°C higher temperature. Resting M_{O_2} values measured on individual fish in respirometers also showed that chronic exposure to 250 μg/L Zn had no effect on metabolic rate in hard water (Table 1). Resting M_{O_2} was not measured in soft water.

Zinc exposure had no effect on the fixed velocity swimming performance in either the hard or soft water experiments (Fig. 2A). However, there was a significant (8.4%) decrease in U_{Crit} when 250 μg/L Zn–exposed fish were tested in 250 μg/L Zn in hard water. This performance remained significantly depressed even when 250 μg/L-Zn fish were tested in control, zinc-free hard water (Fig. 2B).

The zinc exposure in hard water had no effect on whole-body Ca^{2+} and Na^+ levels, which averaged 86.69 ± 2.96 μmol/g and 43.78 ± 0.98 μmol/g, respectively ($N = 30$). In soft water, zinc exposure had no effect on whole-body Ca^{2+}, which was again constant at 83.27 ± 1.43 μmol/g ($N = 30$) over the 30-d exposure. Zinc exposure had a small effect, however, on whole-body Na^+ on day 5, during which the 120 μg/L Zn–exposed fish had a significantly lower level of 30.02 ± 2.34 μmol/g ($N = 6$) compared with the control levels of 37.39 ± 1.39 μmol/g ($N = 6$). This effect did not persist at later times. However, the more striking effect on whole-body Na^+ came with time, where levels decreased progressively in all treatments in soft water from the day −1 level of 41.34 ± 2.97 μmol/g to 29.32 ± 0.53 μmol/g by day 30, a decrease of 29% ($N = 18$).

Zinc was present in relatively high levels in all tissues that were measured in all treatments, including controls (Fig. 3A and B and Table 2). There were only a few significant increases in zinc levels in the gills or livers of the zinc-exposed fish, relative to simultaneous control levels in either the hard or soft water. However, levels in the whole bodies of 450 μg/L Zn–exposed fish (in hard water) were significantly higher than controls at days 20 and 30. In soft water, the 50 μg/L Zn–exposed fish had significantly elevated zinc levels in the liver and whole body by day 30, as did the 120 μg/L Zn–exposed fish, which had elevated zinc level in the gills, liver, and whole body on day 30 and in the whole body on day 20 (Table 2). The zinc levels in the gills of all three treatment groups (i.e., including the controls) in the hard water exposure increased with time by approximately 70% over the 30 d (Fig. 3A). This did not occur in the soft water experiment, in which fish were larger and absolute levels were much higher in the gills at the start of the soft water trial (Fig. 3B). Interestingly, in the soft water exposure, the whole-body zinc levels of the controls continually decreased during the 30 d, becoming significantly lower on days 20 and 30 (Table 2).

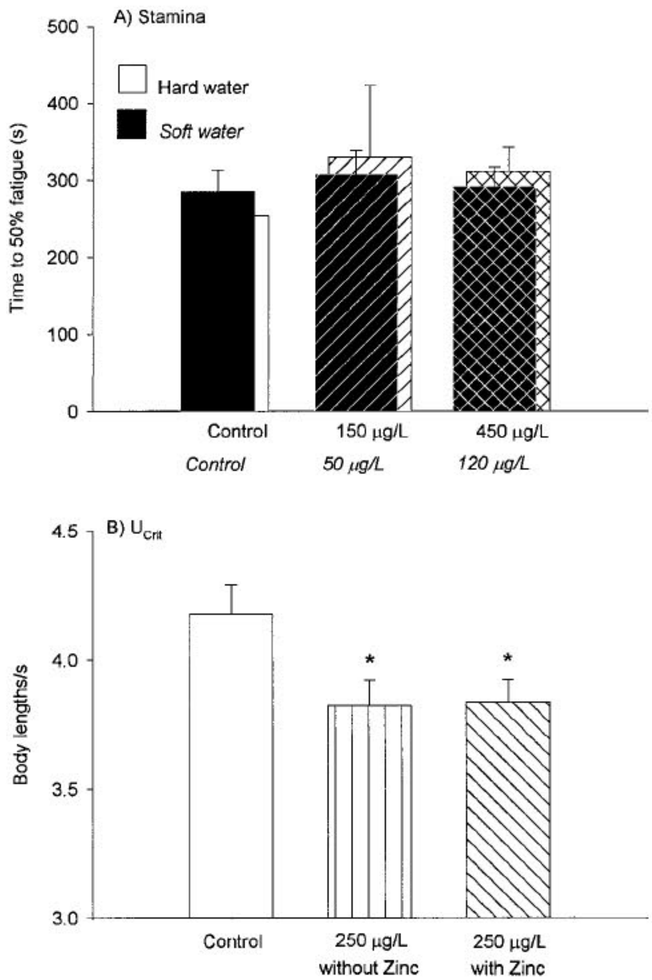

Fig. 2. (**A**) Time to 50% exhaustion during a fixed velocity swimming test. Rainbow trout were tested at approximately 7 body lengths/s in hard water (open columns) and soft water (filled columns, italics). Times were corrected to a body length of 7 cm in hard water and 10 cm in soft water. There were no significant differences between or within treatments. (**B**) Critical swimming speed for control and zinc-acclimated rainbow trout. The zinc-acclimated trout were tested in both water that contained and that was free of the zinc levels to which they had been acclimated. The asterisk (*) denotes a significant difference from the control value, as determined by a Student's *t*-test. $p < 0.05$. Values expressed as means ± SEM ($N = 10$–13).

Zinc turnover in the gills

There appear to be two pools of zinc in the trout gill. The fast turnover pool was characterized by a hyperbolic loading curve (Fig. 4), with a half-time ($T_{½}$) of 3 to 4 h. The slow turnover pool loaded linearly (Fig. 5A and B) for up to 75 h. The rate of appearance of ^{65}Zn in the slow pool (i.e., slope of the line) was higher in chronically zinc-exposed fish in both hard and soft water, and it increased in proportion to exposure concentration (Fig. 5A and B, Fig. 6B). The rates of ^{65}Zn appearance in the slow pool were much greater in soft water than in hard water. For example, the rate of appearance in the soft water group chronically exposed to 120 μg/L Zn was 8.8 times greater than it was in the hard water group chronically exposed to 150 μg/L Zn (Fig. 6B).

By extrapolating the regression of the slow turnover pool to time = 0, the size of the fast turnover pool was estimated (Fig. 5A and B). The fast turnover pool in the gills increased with exposure concentration and was also much greater in soft

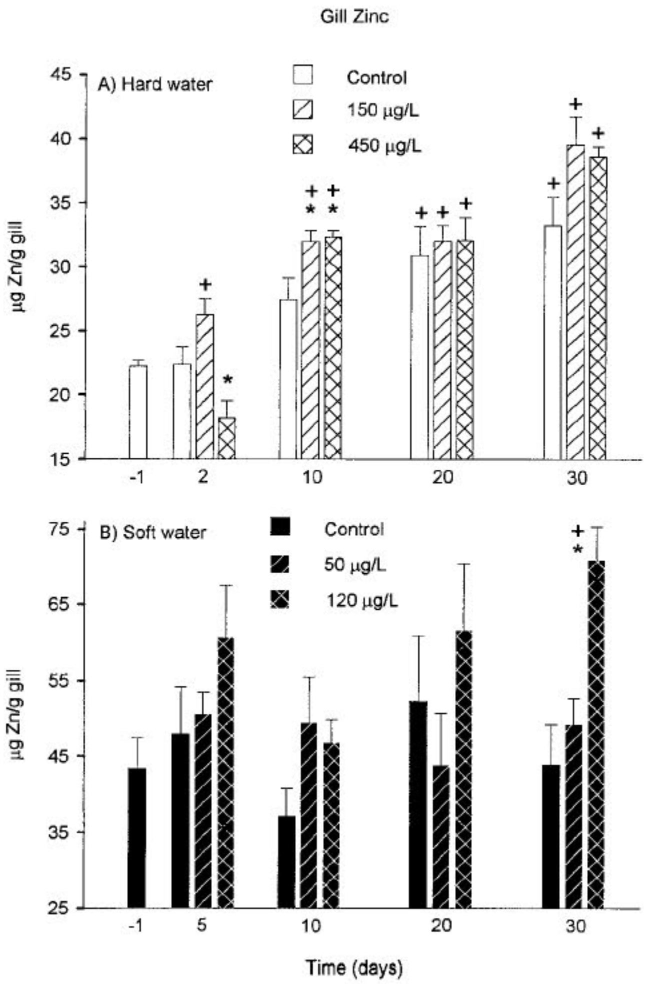

Fig. 3. Total zinc levels in rainbow trout gills at three zinc treatments in (A) hard water and (B) soft water during the 30-d exposures. The asterisk (*) indicates a significant difference (ANOVA followed by a Student-Newman-Keuls multiple comparisons test; $p < 0.05$) from the control zinc level that day, while the plus (+) indicates a significant difference from the control zinc levels measured on day −1.

water than in hard water (Fig. 6A). For example, the fast pool in the gills was 4.3 times greater in soft water at 120 µg/L Zn than it was at 150 µg/L Zn in hard water (Fig. 6A). The size of the fast turnover pool was a very small percentage of the total zinc present in the gill, ranging from 0.14 to 1.18% in hard water and from 0.36 to 3.45% in soft water (compare Table 2). The size of the slow turnover pool could not be estimated from the current data.

Zinc turnover in other tissues

There also appeared to be a two-pool system for zinc operating in the blood (Fig. 7A), with the same patterns as seen in the gill. The two-pool system, however, did not appear to apply to the carcass (Fig. 7B), liver, or whole gallbladder (Table 3), in which the y-intercepts were not significantly different from zero (i.e., no fast turnover zinc pools). The slow pools still followed the same patterns as the gill (an increasing rate of appearance of ^{65}Zn with increasing zinc exposure and a greater rate in soft water than in hard water) (Table 4).

When control and 250 µg/L Zn–acclimated fish were exposed to 1,125 µg/L Zn in hard water (close to lethal levels, cf. Fig. 1), there was a larger fast pool of zinc in the gills in 250 µg/L Zn–acclimated fish compared with control fish

Table 2. Liver and whole-body zinc burdens (µg Zn/g tissue) in rainbow trout over 30 d of zinc exposure in hard and soft water. An asterisk (*) denotes a significant difference from the control measurement on that day. The plus (+) denotes a significant difference from the control value on day −1 (ANOVA followed by a Student-Newman-Keuls multiple comparisons test, $p < 0.05$). Values are expressed as means ± SEM ($N = 6$)

	Hard water					Soft water				
	Day −1	Day 2	Day 10	Day 20	Day 30	Day −1	Day 5	Day 10	Day 20	Day 30
Liver										
Control	21.37 ± 0.59	15.86 ± 2.43	19.56 ± 2.90	20.02 ± 0.94	20.02 ± 0.69	27.59 ± 1.43	26.15 ± 1.92	22.68† ± 0.81	24.24 ± 0.91	23.12† ± 0.39
Low[a]		20.60 ± 0.77	25.27 ± 1.74	24.09* ± 1.15	21.10 ± 0.71		31.03 ± 2.21	27.45 ± 2.32	22.29 ± 0.75	24.67* ± 0.47
High[b]		22.12 ± 2.62	19.89 ± 0.94	25.20*† ± 1.30	21.46 ± 0.32		29.62 ± 4.73	27.98 ± 1.11	25.20 ± 1.16	27.93* ± 0.61
Whole body										
Control	32.57 ± 1.94	28.79 ± 1.05	35.11 ± 3.88	30.44 ± 1.14	28.06 ± 1.18	30.90 ± 2.47	28.29 ± 2.91	25.50 ± 1.44	21.39† ± 0.87	19.17† ± 0.91
Low[a]		30.64 ± 1.36	36.06 ± 1.38	31.44 ± 1.15	30.90 ± 1.37		28.07 ± 1.50	28.16 ± 2.76	24.41† ± 1.84	25.61*† ± 1.00
High[b]		38.55* ± 3.02	36.57 ± 1.30	36.60* ± 0.57	37.28* ± 1.28		26.81 ± 2.23	31.81 ± 3.20	28.28* ± 1.32	33.31* ± 1.39

[a] Hard water = 150 µg/L Zn; soft water = 50 µg/L Zn.
[b] Hard water = 450 µg/L Zn; soft water = 120 µg/L Zn.

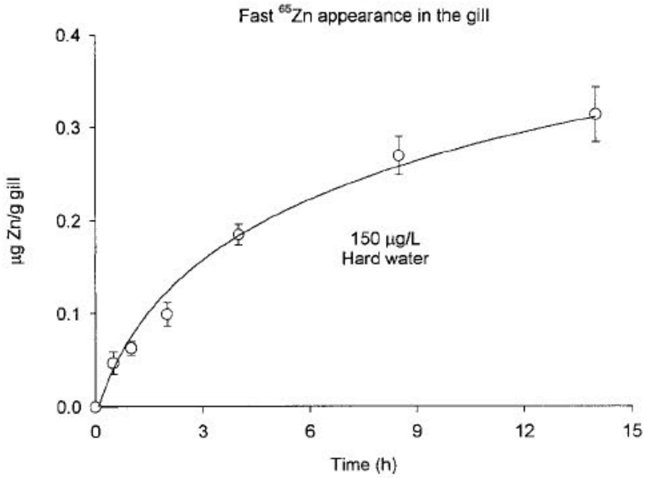

Fig. 4. The appearance of radiolabeled ^{65}Zn in the gills of rainbow trout that had been previously acclimated for 30 d to 150 µg/L Zn in hard water. The trout were exposed for 15 h to a zinc concentration equal to what they had been exposed to for the previous 30 d. ^{65}Zn was also added to the water as a tracer, which had a negligible effect on the total zinc concentration of the water. Values expressed as means ± SEM (N = 5).

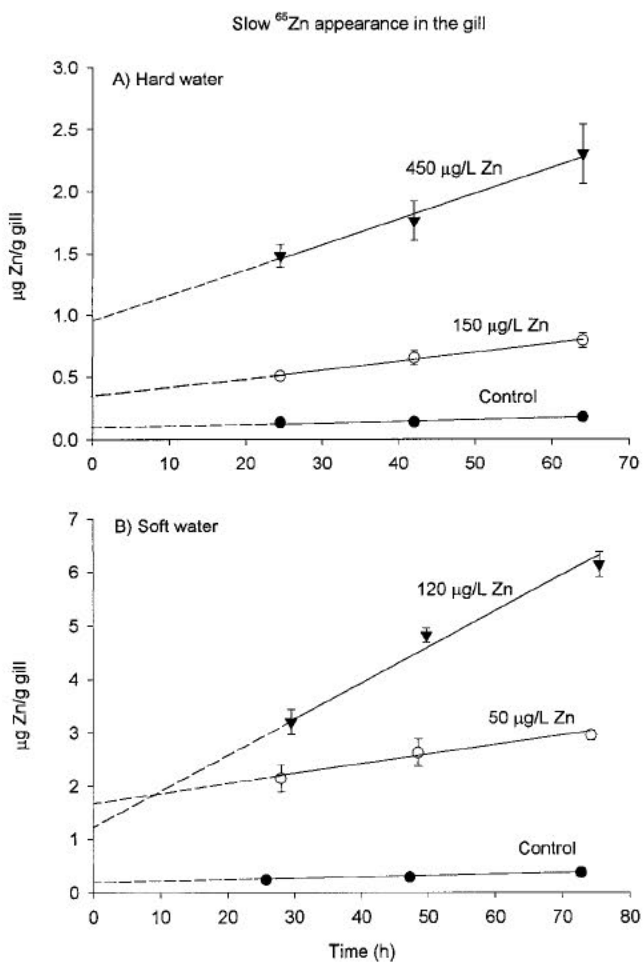

Fig. 5. The appearance of radiolabeled ^{65}Zn in the gills of (A) hard water– and (B) soft water–acclimated rainbow trout. Trout were exposed to a zinc concentration with ^{65}Zn equal to the zinc concentration to which they had been exposed for the previous 30 d. Values expressed as means ± SEM (N = 5–6).

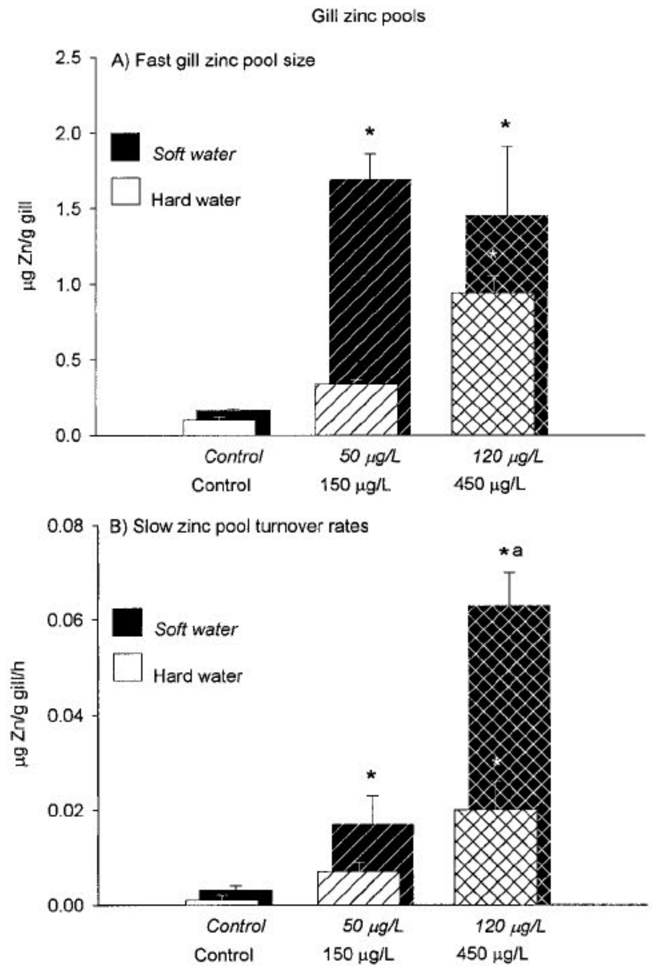

Fig. 6. The calculated sizes of the (A) fast turnover zinc pools and (B) the rates of turnover of the slow zinc pools of the gills after 30 d of exposure in hard water (open columns) and soft water (filled columns, italics). The asterisk (*) denotes a significant difference from the control values from the same exposure. An (a) indicates a significant increase in the zinc pool size or turnover rate of the high zinc-exposed trout (hard water = 450 µg/L Zn; soft water = 120 µg/L Zn) over the low zinc-exposed trout (hard water = 150 µg/L Zn; soft water = 50 µg/L Zn) of the same water chemistry.

(0.991 ± 0.327 µg/g vs. 0.269 ± 0.115 µg/g, respectively). It was also apparent that zinc-acclimated fish took up new zinc more quickly into the slow turnover pool in the gills than did control fish. The rate of new zinc incorporation into the slow pool of the gills of 250 µg/L Zn–acclimated fish was 0.071 ± 0.010 µg/g/h versus 0.039 ± 0.004 µg/g/h in control fish (Fig. 8).

DISCUSSION

Overview

On an acute basis, zinc was 5.4 times more toxic to trout in soft water than in hard water. The zinc turnover rate, as measured by ^{65}Zn, was also higher in the gills and other tissues in soft water than in hard water, which reflects the increased bioavailability of zinc in soft water. Only the 450 µg/L-Zn group in hard water experienced significant mortality, which largely ceased after the first few days of exposure. After 30 d, all zinc-exposed groups had acclimated to zinc, as demonstrated by substantial increases in the LC50. Although the fish must have acclimated through physiological and/or struc-

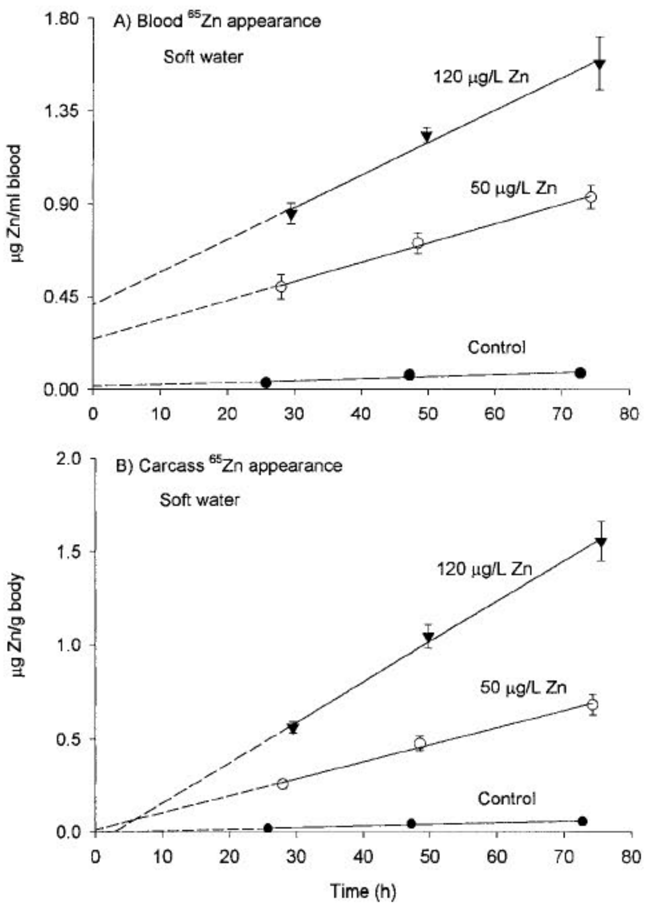

Fig. 7. The appearance of radiolabeled ^{65}Zn in the (A) blood and (B) carcass of soft water–acclimated rainbow trout at the three zinc-exposure concentrations. The carcass was that portion of the rainbow trout remaining after the gills, liver, and gallbladder were excised. The fish were exposed to a radiolabeled ^{65}Zn concentration equal to the zinc concentration to which they had been exposed for the previous 30 d. Values are expressed as means ± SEM ($N = 5$).

tural changes, there appeared to be no marked effect or cost of long-term zinc exposure on growth, whole-body Na^+ or Ca^{2+} concentrations, zinc levels in the tissues, metabolic rate, or fixed velocity swimming performance. There was, however, a significant decrease in the U_{Crit} with zinc acclimation, which persisted in zinc-free water. Acclimation to zinc also involved an increase in the size of the fast turnover pool of zinc in the gills and blood.

Environmental relevance

The levels of zinc used (150 and 450 µg/L in hard water, 50 and 120 µg/L in soft water) are of environmental and regulatory significance. While normal zinc levels in pristine freshwaters are only a few micrograms per liter or less, concentrations of 50 µg/L are routine in industrialized areas. Maximum zinc concentrations in natural surface waters are reported to range from 130 to 1,170 µg/L in different areas of Canada [23]. There is general acceptance of the principle that acute toxicity is related to hardness [23]. For example, the U.S. EPA [24] employs an equation based on hardness (in mg $CaCO_3$/L) to calculate a numerical limit of total allowable waterborne zinc.

$$[Zn] = e^{(0.83[\ln(\text{hardness})]+1.95)}\ \mu g/L \qquad (5)$$

In the waters used in the present study, the acute limit for zinc

Table 3. Fast zinc turnover pool sizes (µg Zn/ml blood, µg Zn/g tissue) in the blood, liver, and gallbladder of control, low, and high zinc-exposed rainbow trout after 30 d of zinc exposure in hard and soft water. An asterisk (*) denotes a significant difference from the control value. Values are expressed as means ± SEM ($N = 15$)

	Hard water			Soft water		
	Control	150 µg/L	450 µg/L	Control	50 µg/L	120 µg/L
Blood (µg Zn/ml)	0.0012 ± 0.025	0.208 ± 0.116	0.511* ± 0.185	0.014 ± 0.021	0.242* ± 0.028	0.411* ± 0.078
Liver (µg Zn/g)	−0.019 ± 0.013	−0.037 ± 0.065	0.128 ± 0.084	−0.016 ± 0.009	−0.087 ± 0.097	−0.542 ± 0.018
Gallbladder (µg Zn/g)	0.010 ± 0.004	0.041 ± 0.016	0.075 ± 0.039	0.001 ± 0.004	0.013 ± 0.058	0.105 ± 0.108

Table 4. Slow zinc pool turnover rates (μg Zn/ml blood/h, μg Zn/g tissue/h) in the blood, liver, and gallbladder of control, low, and high zinc-exposed rainbow trout after 30 d of zinc exposure in hard and soft water. An asterisk (*) denotes a significant difference from the control value. An (a) indicates a significant increase in the turnover rate of the high zinc-exposed trout (hard water = 450 μg/L Zn; soft water = 120 μg/L Zn) over the low zinc-exposed trout (hard water = 150 μg/L Zn; soft water = 50 μg/L Zn) of the same water chemistry. Values are expressed as means ± SEM (N = 15)

	Hard water			Soft water		
	Control	150 μg/L	450 μg/L	Control	50 μg/L	120 μg/L
Blood (μg Zn/ml/h)	0.002 ± 0.001	0.004 ± 0.003	0.019* ± 0.005	0.0010 ± 0.0002	0.0094* ± 0.0016	0.0157* ± 0.0025
Liver (μg Zn/g/h)	0.002 ± 0.0003	0.007* ± 0.001	0.014* ± 0.002	0.0022 ± 0.0003	0.0218* ± 0.0027	0.0550*[a] ± 0.0057
Gallbladder (μg Zn/g/h)	0.0001 ± 0.0001	0.0004 ± 0.0004	0.002 ± 0.001	0.0003 ± 0.0001	0.004* ± 0.001	0.008* ± 0.002

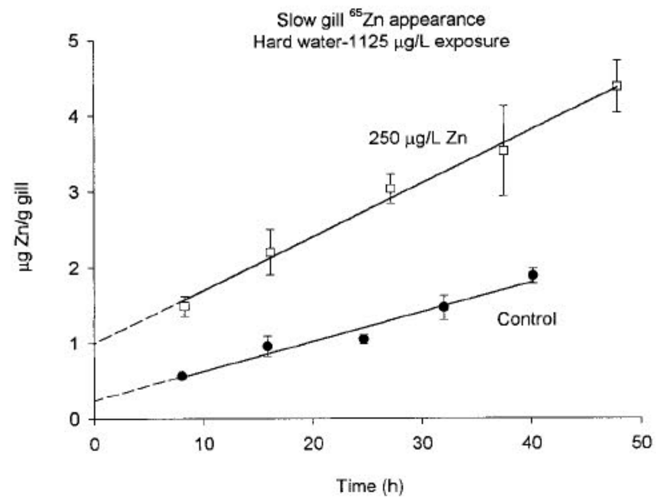

Fig. 8. ^{65}**Zn appearance in the gills of 250 μg/L Zn-acclimated and nonacclimated rainbow trout over time at an exposure concentration of 1,125 μg/L Zn. Values are expressed as means ± SEM (N = 6).**

would be 374 μg/L in hard water (120 mg $CaCO_3$/L) and 85 μg/L in soft water (20 mg $CaCO_3$/L), based on this equation. The hard water value may be too high, based on the 24% mortality of trout in the first few days at 450 μg/L Zn; these fish likely died from hypocalcemia [4,6]. On the other hand, chronic toxicity is generally believed to be unrelated to hardness, and in most jurisdictions, chronic limits around 30 μg/L have been established, independent of hardness [23–25].

Acute toxicity and water chemistry

The acute toxicity of zinc was 5.4 times greater in soft water than in hard water, a result that is in general accord with the literature [12,26,27]. The slightly higher temperature (18 vs. 14°C) in the soft water experiments probably had little effect on the toxicity of zinc. Hodson and Sprague [28] found little difference in zinc toxicity (LC50) with Atlantic salmon acclimated to 11 and 19°C.

The greater toxicity of zinc in soft water is likely explained by the presence of fewer ions, which offer competition to zinc for binding sites on the gill, and of fewer ligands, which could complex with the zinc ion in the water. In hard water, 60% of the zinc was in the free-ion form (Zn^{2+}); the remainder was complexed with DOC and carbonate. In soft water, however, 100% of the zinc was in the free-ion form, as determined with the aquatic geochemical program MINEQL+ [29].

In soft water, the 120 μg/L–exposed fish suffered 10.5% mortality on days 29 and 30. Further investigation into the mechanisms behind these deaths in soft water, particularly of the role (if any) of zinc, would be extremely important in attempting to determine the long-term effects of zinc exposure.

Acclimation

Independent of water hardness or chronic exposure concentration, all zinc-exposed fish acclimated to the metal with a 2.2 to 3.9 times increase in tolerance (96 h LC50); these results are comparable to those of Chapman [30] and of Bradley et al. [8]. For metals such as zinc, which kill fish through their actions on the gills, three mechanisms have been suggested that may provide increased tolerance: (1) alterations to gill barrier properties, such that the rate of metal entry is reduced; (2) increased metal storage and detoxification; and

(3) increased resistance of metal-sensitive processes to metal poisoning [7].

Zinc and calcium competitively inhibit the uptake of each other across the gill, and they share (at least partially) a common uptake pathway [5,31,32]. A series of studies [32,33], in which the actual influx rates of calcium and zinc into the fish were measured (as opposed to the pool turnover rates, which were measured in the present investigation) demonstrated that an interesting combination of mechanisms (1) and (3) certainly applies, at least in the case of rainbow trout chronically exposed to 150 μg/L Zn in hard water. In zinc-acclimated fish, the affinity of the shared branchial transport system was greatly reduced for both calcium and zinc (i.e., concentrations at which 50% of the maximum transport rate is observed [K_ms] were increased), with little change in maximum transport rates. Because of the very different concentrations of calcium and zinc in the water relative to the respective K_m values, calcium uptake rate was little affected, but zinc uptake rate was thereby substantially reduced in acclimated fish. In a related study, Galvez et al. [17] characterized zinc binding to the low-affinity, relatively nonspecific binding sites on the gill surface in comparably treated trout. Calcium more readily displaced zinc from these sites in zinc-acclimated fish.

The results of the present study suggest that mechanism (2) may also contribute. The size of the ''fast zinc pool'' in the gills increased markedly as a result of acclimation (Fig. 6A). Presumably this is either a storage, excretion, or detoxification pool, as discussed in greater detail below. In addition, there is abundant evidence [8,34,35] for the theory that induction of metallothionein and other specific metal-detoxification proteins takes place in the gills and other tissues during chronic sublethal zinc exposure.

Costs and consequences

Although the fish underwent a physiological change and acclimated to zinc, there was not much of a detectable long-term cost associated with the acclimation process. There was no effect of zinc exposure on growth, in either hard water or soft water, for those fish maintained on a fixed ration of 3% body mass/day. This finding is in accord with other studies in which the effects of zinc on growth were either absent or stimulatory [6,30,36]. There was also a negligible influence of zinc exposure on whole-body Na^+ or Ca^{2+} concentrations. After 30 d of zinc exposure, there was no evidence of an increased maintenance cost-of-living seen in either routine ''in-tank'' M_{O_2} for the whole group of fish or in resting M_{O_2} for individual fish in respirometers in which activity level was controlled. However, given the known time course of acclimation in other sublethal metal studies (generally 5–15 d [7,8,10]), it is quite possible that our metabolic measurements after day 30 would have missed the major initial costs and that remaining costs would no longer be expressed in maintenance metabolism at this time. For example, at day 9 of an exposure to 150 μg/L Zn in hard water, protein synthesis rates in the gills of exposed trout were significantly elevated, but the rates dropped to control levels or below by days 18 and 23 [6,32]. The lack of a persistent effect on maintenance metabolism indicates that zinc did not act as a ''loading stressor'' [37].

However, the depressed U_{Crit} of zinc-acclimated fish (Fig. 2B) indicates that zinc may well have acted as a ''limiting stressor''—one which depresses aerobic capacity without necessarily affecting routine metabolism [37]. This inhibitory effect occurred independent of the presence or absence of zinc in the test water. Thus, the depressed swimming performance was not the result of the presence of zinc but rather of the physiological changes that the fish had undergone as a result of the zinc exposure. With acclimation to aluminum, the ''limiting stressor'' effect has been attributed to a thickening of the respiratory epithelium secondary to mucous cell and chloride cell hyperplasia [9,10]. However, this may not be the case with zinc acclimation, because Galvez et al. [17] found no change in the gill's chloride cell density or surface area after acclimation of trout to 150 μg/L Zn in hard water. Changes in the viscosity of mucus that arise from metal exposure may have been a contributing factor here [38].

Although aerobic swimming performance was depressed, zinc acclimation had no effect on swimming stamina, as measured by the fixed velocity test (Fig. 2A). This type of swim test is thought to involve both aerobic and anaerobic components [21] and may place less overall demand on the cardiorespiratory system than does the U_{Crit} test. Overall, the result suggests that anaerobic capacity was not affected by chronic zinc exposure.

Zinc was present in substantial concentrations in the gills, liver, and whole body in both control and zinc-exposure treatments throughout both the hard and soft water experiments (Table 2 and Fig. 3A and B). This reflects the role of zinc as an essential micronutrient, one that is important as a cofactor for the function of numerous proteins [1]. Relative to these high background levels in control fish, there were no consistent increases in total zinc levels in the gills or liver of zinc-exposed fish in either hard water or soft water. Modest increases occurred in whole-body zinc concentrations. Three previous studies have reported comparable results in rainbow trout chronically exposed to approximately 150 μg/L Zn in Hamilton hard water [6,32,36]. Furthermore, Bradley and Sprague [39] reported modest elevations (40%) in gill zinc concentration and no change in liver concentration in trout exposed for 20 d to over 2,000 μg/L Zn in extremely hard, alkaline water.

Recently, there has been great interest in using tissue metal burdens, especially those in gills, as predictors of acute mortality and as indicators of chronic exposure in freshwater fish (e.g., Bergman and Doward-King [16]). However, the present and previous data (cited above) all clearly indicate that concentrations of this essential metal are subject to remarkable homeostasis in rainbow trout in the face of environmental challenge. Indeed, growth-related changes in zinc tissue content are much more obvious than those resulting from chronic zinc exposure (Fig. 3A). Because of this physiological homeostasis (coupled with high background zinc levels in nonexposed fish), regulatory strategies based on measuring total tissue metal burdens will not work for zinc; clearly, alternate strategies for assessing gill–zinc binding are needed.

Zinc turnover in the gills

The above conclusion was also reached by Galvez et al. [17], who found that it was impossible to determine zinc-binding kinetics to trout gills by measuring total tissue zinc concentration, the approach that has been successfully used with other metals, such as copper, cadmium, and silver [14,40]. Instead, Galvez et al. [17] employed the radiotracer ^{65}Zn with some success in short-term (up to 3-h) binding experiments.

Using the studies of Galvez et al. [17] as a point of departure, in the present investigation we employed much longer exposures to ^{65}Zn in an attempt to characterize the zinc pool(s) in the gills and other tissues as well as their kinetics of turnover. We purposely used low concentration levels that would recruit only high-affinity sites (those with affinities in the micromolar zinc range) rather than high concentration levels that would also recruit the relatively nonspecific low-affinity sites (those with affinities in the millimolar zinc range)[17]. Therefore, rather than looking at concentration dependence within each exposure group, we elected to expose the fish to the radiotracer at the total zinc concentration to which they had been acclimated, and we employed time as the principal variable. With this technique, at least two pools of zinc were found operating in trout gill.

The fast exchanging zinc pool had a time to 50% turnover ($T_{1/2}$) of about 3 to 4 h (Fig. 4). The slower exchanging pool appeared to turn over linearly with time (Fig. 5A and B). The size of the slow pool could not be determined from the present data. However, if we assume that it is the total measured zinc content of the gills, then $T_{1/2}$ was clearly in the range of days to months or more. In fact, $T_{1/2}$ for the slow pool of control fish in hard water was estimated at 3 years. The size of the fast turnover pool could be estimated by extrapolation of the slow pool line back to time zero. For control fish in hard water, this yielded a value of about 0.1 μg Zn/g gill tissue, only 0.14% of the total zinc content of the gill or about 20% of the ''high affinity sites'' determined by Galvez et al. [17]. This difference is explained at least partially by the difference in technique; Galvez et al. [17] attempted to measure the pool when all the high-affinity sites were saturated (i.e., maximum binding capacity), whereas our technique measures the pool size simply at the exposure level.

We interpret the fast pool as a dynamic pool bound to high-affinity sites, one which is in the process of being taken up, excreted, detoxified, or stored. Clearly, the size of the fast turnover pool increased with the concentration of zinc to which the fish were chronically exposed (Fig. 6A). Taken together with the finding that zinc flux rates into the fish (measured in the range of the exposure concentrations) are reduced during chronic exposure [6,32,33; see above], a simple interpretation is that the increased size of the fast pool is related to increased detoxification or temporary storage (e.g., metallothionein). In comparisons at similar exposure concentrations (0 vs. 0 μg/L Zn or 120 vs. 150 μg/L Zn), the fast pool size was clearly much greater in soft water than in hard water (Fig. 6A). A simple interpretation here is that the paucity of calcium in soft water increases the availability of sites for zinc binding [13].

The slow pool presumably represents incorporation into structural components of the gill (e.g., zinc-dependent proteins) in growing fish, though it could also represent long-term detoxification storage in zinc-binding proteins. Without knowledge of the true size of the slow pool or of its $T_{1/2}$, it is difficult to interpret the higher turnover rates in zinc-exposed fish (beyond the fact that they increase with concentration, as expected) (Fig. 6B). However, when compared at similar concentrations in soft water versus hard water (i.e., 0 vs. 0 μg/L Zn or 120 versus 150 μg/L Zn), the labeling of this slow pool was clearly much faster in the soft water fish, a fact that is probably explained by increased access through the fast pool and by reduced calcium levels in the water (Fig. 6B).

This approach may provide a practical tool for modeling zinc binding in the fast pool. For example, we are interested to know whether the binding capacity (i.e., total available site number in the fast pool) or the affinity changes as a result of acclimation. This could be examined by exposing the fish to radiotracer at total zinc levels different from those used during acclimation and then by extrapolating back to time zero at each new concentration in order to determine the pool size. Fig. 8 illustrates one such example, in which the test concentration (1,125 μg/L Zn) was much higher than the acclimation (250 μg/L Zn) or control (0 μg/L Zn) concentrations, so as to estimate the maximum binding capacity of the fast pool. The results indicate that the maximum binding capacity was expanded almost four times as a result of chronic zinc acclimation. This conclusion is in accord with recent findings, using very different techniques, in trout acclimated to both cadmium [41] and copper (L. Taylor, personal communication). Increased maximum binding capacity by high-affinity sites in the gills may be a common feature of acclimation to different metals.

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