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Zinc binding to the gills of rainbow trout: the effect of long-term exposure to sublethal zinc

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The effects of sublethal waterborne Zn (2·28 μmol l^{-1}) on Zn binding kinetics to the apical gill surface were studied in juvenile rainbow trout (*Oncorhynchus mykiss*). Two separate radiotracer techniques were employed to ascertain this information. First, *in vitro* binding kinetic experiments were performed at extremely elevated zinc concentrations (up to 20 mmol l^{-1}) to measure relatively low-affinity binding sites at the gill epithelium. There were no differences in Zn binding parameters (K_m and B_{max}) for fish sublethally exposed to Zn for 21 days and their simultaneous controls. Nevertheless, Ca did have an increased inhibitory effect on Zn binding in Zn-exposed fish suggesting that the anionic groups on the gill epithelium of these fish had been altered in some manner. Additionally, *in vivo* Zn binding kinetics were investigated using environmentally relevant waterborne Zn concentrations (low μmol l^{-1} range) to isolate high-affinity Zn binding sites (Ca transporters). No appreciable alterations in the K_m and B_{max} values for Zn binding were seen between the Zn-exposed group and its simultaneous control following 15 days of exposure. Furthermore, no significant differences in CC morphometry were observed between treatments. Despite these lack of treatment effects, there were temporal alterations in K_m, B_{max} and CC fractional surface area in both groups. It is proposed that these fluctuations are controlled by hormonal factors (such as stanniocalcin), believed to play a role in Ca influx.

Key words: zinc; acclimation; binding kinetics; rainbow trout; chloride cells; scanning electron microscopy.

INTRODUCTION

Zinc plays an essential role in the structure and function of over 300 proteins (Vallee & Falchuk, 1993). Due to the importance of Zn as a micronutrient, mechanisms for its uptake and regulation in fish are required. Under most conditions, the gastrointestinal tract is the primary route of Zn uptake, with most being acquired through the diet. The gills become an increasingly important route of uptake during periods of dietary Zn deficiency or at high waterborne Zn concentrations (Spry *et al.*, 1988). In spite of the nutritional demand for Zn, relatively low concentrations of this metal in water will result in various deleterious effects. Acute toxicity caused by very high environmental Zn exposure (i.e. mg l^{-1} range) is manifested primarily as an inflammatory oedema resulting in suffocation due to an increased diffusion distance across the gills (Skidmore & Tovell, 1972; Spry & Wood, 1989). Such gross morphological damage also results in dramatic iono-regulatory disturbances. Adverse

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toxicological responses such as these are relatively non-specific and are typically elicited by most toxicants when present at extremely high concentrations (Mallatt, 1985). However, except for highly polluted sites near industrial inputs, waterborne Zn concentrations rarely reach acute lethal levels. At more environmentally realistic, sublethal concentrations of the metal (μg l^{-1} range), Zn exerts a much more specific response of plasma hypocalcaemia, produced by an impairment of branchial Ca uptake (Spry & Wood, 1985; Hogstrand *et al.*, 1994, 1995).

There is general concensus that Ca passes through the gill epithelium in a transcellular fashion through chloride cells (mitochondrial-rich cells) via a series of transporters (Perry & Flik, 1988; Flik & Verbost, 1993; Verbost *et al.*, 1994; Hogstrand *et al.*, 1996). In this model, a voltage-independent Ca^{2+} transporter allows for passive diffusion of Ca^{2+} across the apical surface of the cell. This inwardly directed diffusion gradient is established by a variety of branchial mechanisms operating to sequester free Ca^{2+} producing low intracellular activities ($\sim 10^{-7}$ mol l^{-1} Ca^{2+}) (Flik & Verbost, 1993). Transport across the basolateral membrane of chloride cells is mediated by a high affinity Ca^{2+}-ATPase or through a low affinity Na^{+}/Ca^{2+} exchanger.

Recent studies on Ca influx across the gill have found flux rates to be inhibited by sublethal Zn exposures (Sayer *et al.*, 1991; Hogstrand *et al.*, 1994, 1995; Hogstrand & Wood, 1996); this effect occurred primarily by competitive inhibition (increase in K_m), with a minor non-competitive component (decrease in J_{max}). In reciprocal studies, elevated waterborne Ca concentrations were shown to inhibit Zn influx competitively (Spry *et al.*, 1988; Spry & Wood, 1989). Consequently, it has been postulated that Ca and Zn share a common uptake mechanism. This model has been supported further by studies of the effects of La, which appears to block apical calcium sites on the chloride cell surface physically without entering the cell (Perry & Flik, 1988). La exposure inhibited the branchial influx of both Ca and Zn, while Na and Cl uptake rates (which are transported via different mechanisms) remained unaffected (Hogstrand *et al.*, 1996). Similarly, $CaCl_2$ injection, which is thought to close apical calcium channels by mobilizing stanniocalcin (Lafeber & Perry, 1988), inhibited both Ca and Zn influxes. Recently, the basolateral high-affinity Ca^{2+}-ATPase was shown to be inhibited competitively by Zn^{2+}, while appearing not to have a direct role in Zn transport (Hogstrand *et al.*, 1995).

During long-term exposure to sublethal waterborne Zn (150 μg l^{-1} = 2·28 μmol l^{-1}) in moderately hard fresh water (Ca = 1 mmol l^{-1}), juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum) exhibit an acclimation response manifested as decreased Zn uptake rates and restored plasma Ca concentrations (following the initial hypocalcaemic effect) (Hogstrand *et al.*, 1994, 1995). This response is accompanied by a chronic increase in both the apparent (measured in presence of Zn) and the true (measured with no Zn present) K_m values for Ca influx, which suggests a persistent change in the intrinsic properties of the Ca/Zn transport system. Since the actual water Ca concentration (1 mmol l^{-1}) is far above the K_m values for Ca uptake (<200 μmol l^{-1}), Ca influx is little affected. Hogstrand *et al.* (1994, 1995) suggested that during acclimation, the affinity of the dual transport system is tuned so as to limit the influx of Zn without impairing Ca uptake significantly. Such a change could occur at several possible

sites in the transport pathway: the present investigation examined the initial binding step to the gill epithelium.

The goal of the present study was to characterize the binding kinetics of Zn to the gill epithelium, and to determine whether changes in this process could be detected during long-term exposure to Zn under these same conditions. Two recently developed methods were employed. The first, the gill dip method of Reid & McDonald (1991), is an *in vitro* approach which uses high concentrations of radiolabelled metal to measure relatively low-affinity, low-specificity binding sites at the gill epithelium. The second, the *in vivo* method of Playle *et al.* (1993) uses low concentrations of non-radioactive metal to measure relatively high affinity, high-specificity binding sites. To apply the method to Zn, it was necessary to introduce a modification employing radiolabelled metal. To our knowledge, neither method has been used previously to characterize branchial Zn binding, and only the former has been used to assess possible changes during metal acclimation (to Al: Reid *et al.*, 1991). In addition, scanning electron microscopy was employed to examine possible changes in the exposed surface area of branchial chloride cells, the presumptive sites of Ca and Zn transport at the gill epithelium.

MATERIALS AND METHODS

PART I. ZINC BINDING KINETICS BY THE GILL DIP METHOD

Experimental animals and acclimation to zinc

Juvenile rainbow trout (79·2 ± 18·7 g) were obtained from a local fish hatchery (Rainbow Springs Trout Farm, Thamesford, ON, Canada) and acclimated to Hamilton dechlorinated tap water ($[Na^+]$=0·6 mmol l^{-1}; $[Cl^-]$=0·7 mmol l^{-1}; $[Ca^{2+}]$=1·0 mmol l^{-1}; pH=7·9–8·2) for at least 2 weeks prior to experimentation. Experiments were performed from October to the end of March. The 300-l holding tank (fibreglass) was supplied with 0·85–0·95 l min^{-1} of well-aerated water at 14·5 ± 0·6° C throughout this period. Fish were fed to satiation three times per week (Ziegler Brothers Inc., Gardners, PA, U.S.A.). Tanks were siphoned regularly to remove organic waste.

Fish from the main holding tank were divided into two groups and each was placed in a separate 300-l tank (n=150). Water flow to each tank was maintained at approximately 0·925 l min^{-1}. Each group was held under the same conditions as before for at least 1 week prior to the start of the experiment. Following this period, one tank was designated as a control and the other group of fish was subjected to waterborne zinc exposure. To the latter of the two groups, metal stock (2·11 mmol l^{-1} $ZnSO_4 \cdot 7H_2O$; BDH, Toronto, ON, Canada) was infused at a rate of 1 ml min^{-1} using a peristaltic pump achieving a nominal [Zn] of 2·28 μmol l^{-1} (150 μg l^{-1}). Fish were exposed to 2·28 μmol l^{-1} of Zn for 21 days. Flow rates were monitored daily and water samples were taken every second day for analysis of water Zn concentrations by flame atomic absorption spectroscopy (Varian AA-975, Mississauga, ON, Canada).

Binding measurements

Methods followed those of Reid & McDonald (1991) and Reid *et al.* (1991). In brief, each fish was killed by a quick cephalic blow, and the entire gill basket removed. Isolated gills were rinsed in 0·9% NaCl for approximately 2–3 min, followed by a 15-s rinse in nanopure-deionized water, then placed in a 5-mmol l^{-1} solution of ethylenediaminetetraacetic acid (EDTA, disodium salt; Sigma Chemical, St Louis, MO, U.S.A.) for 15 s. The pH of the EDTA solution was adjusted to 7·4 with 0·1 mol l^{-1} KOH. A final 15 s rinse in nanopure-deionized H_2O was performed to remove any EDTA from the gills. These rinses were completed in separate 125-ml plastic beakers, each fitted with

individual air lines, and maintained at 15° C. Following the rinsing protocol, each gill basket was used in one of three separate metal binding experiments outlined below. Gill and water samples were analysed as described in the Analytical Methods section.

Time for half-saturation ($T_{1/2}$). Gill baskets from fish in the main holding tank were exposed to 10·0 mmol l^{-1} ^{65}Zn (as $ZnSO_4 . 7H_2O$, Sigma) with a specific activity of 0·62 kBq μmol^{-1} (New England Nuclear, Boston, MA, U.S.A.). The Zn solution was made up in nanopure-deionized H_2O and the pH was adjusted to 6·7 using 100 mmol l^{-1} KOH. Gills were exposed for 15, 30, 60, 90, 150, 300, 600, 1500 or 1800 s (n=4 gill baskets at each time interval). Based on the $T_{1/2}$ value obtained, the subsequent metal-binding experiments were performed for a period of 300 s.

Zn exposure-acclimation study. Fish from the acclimation study were killed at days 0, 1, 8, and 21. At each time, individual gill baskets (n=4 per concentration) were exposed to one of the following concentrations of ^{65}Zn (as $ZnSO_4 . 7H_2O$; specific activity of 0·62 kBq μmol^{-1}, pH=6·7) for 300 s: 0·5, 1·0, 2·0, 3·0, 6·0, 8·0, 10·0, 15·0 and 20·0 mmol l^{-1}.

Effects of Ca^{2+} on zinc binding kinetics. Gill baskets were exposed for 300 s to solutions containing varying concentrations of Ca [as $Ca(NO_3)_2 . 4H_2O$, BDH, Toronto, ON], while the [Zn] was maintained constant at 4·0 mmol l^{-1} (specific activity of 0·62 kBq ^{65}Zn μmol^{-1}, pH=6·7). The Ca concentrations tested included 0, 0·4, 4·0, 40·0, 400·0 and 1000·0 $\mu mol\ l^{-1}$. Four gills per treatment (control and zinc-exposed fish from day 21 of the exposure) were each exposed to one of the Ca concentrations listed above.

PART II. ZINC BINDING KINETICS BY THE *IN VIVO* METHOD

Experimental animals and acclimation to zinc

A second batch of 1000 juvenile rainbow trout (initial weight ~5 g) was obtained from the same source as above and held under similar conditions (same water quality) in two 300-l tanks. Over the 4-month holding period the ambient water temperature decreased from 12 to 7° C. Fish were fed a daily diet of commercial trout pellets amounting to a total ration of 1% body weight per day.

In the Zn acclimation study, the water flow rate was maintained at 1000 ml min^{-1} for both the control and Zn-exposed tanks (n=400) and $ZnSO_4 \cdot 7H_2O$ was added by peristaltic pump as before to a nominal Zn concentration of 2·28 $\mu mol\ l^{-1}$. Flow rates and water [Zn] were monitored as above. Fish were exposed to 2·28 $\mu mol\ l^{-1}$ Zn for 15 days.

Binding measurements

Experiments to develop methods were made from October to December at temperatures of 9–12° C using fish of 10–15 g, while the acclimation experiments were performed in January at 7–9° C using fish of 15–25 g. For each exposure, quadruple 5-ml water samples were taken prior to the fish being added, and at the end of the exposure. Gill and water samples were processed as outlined in the Analytical Methods section.

Studies on Zn binding kinetics were performed initially using the non-radioactive techniques developed by Playle *et al.* (1993). Four 6-l transparent polypropylene bags were filled with Hamilton dechlorinated tap water, and varying amounts of $ZnSO_4 \cdot 7H_2O$ were added yielding the following Zn concentrations: 2·4, 4·8, 19·8 and 42·3 $\mu mol\ l^{-1}$. The final volume of water in each bag was 3 l. Each bag was equipped with individual aeration, and partially submersed in water recirculated from holding tanks (to ensure constant temperature for the fish). The exposure period was 3 h.

The lack of resolution obtained using non-radioactive Zn made it necessary to use radioisotope to measure Zn binding accurately. The appropriate time course was determined first.

Time for half-saturation ($T_{1/2}$): Time course for Zn binding saturation was performed using ^{65}Zn, with a nominal specific activity of 0·22 kBq μmol^{-1}. Five bags were filled with 3 l of Hamilton dechlorinated tapwater, with a [Zn] of 5·97 $\mu mol\ l^{-1}$. Six fish were placed in each bag, and exposed for 30, 60, 120, 180 and 240 min (all fish in same bag were exposed for the same duration).

Radio-isotopic modification of basic method. Based on the preceding experiment, a time course of 3 h was deemed appropriate for the kinetic experiments. The same Zn concentration series as in the original non-radioactive trial was used, but this time, ^{65}Zn was added to give a nominal specific activity of 10·1 kBq μmol l^{-1} at each exposure concentration. Fish were exposed to Zn concentrations of 0, 2·0, 4·8, 19·9 and 64·8 μmol l^{-1} (six fish per bag) for 3 h, in dechlorinated Hamilton tapwater. The experiment was then repeated using concentrations of 0·2, 3·1, 5·9, 8·8, 11·5 and 14·6 μmol l^{-1} Zn so as to examine a lower concentration range.

Zn exposure-acclimation study. Experiments on Zn binding during acclimation employed the radio-isotopic modification of the method of Playle *et al.* (1993), described above, with a 3 h exposure period. The nominal specific activity was 0·45 kBq ^{65}Zn μmol l^{-1}. Binding kinetics were determined on the control and Zn-exposed treatment groups on days 0, 1, 5, and 15 using a concentration series of 1·2, 2·3, 4·6, 9·2, and 18·5 μmol l^{-1} Zn (six fish per concentration in each treatment). Day 0 measurements were started 3 h after the acclimation tank was spiked with zinc.

ANALYTICAL METHODS

Part I

Following exposure, gill baskets were given a final 15-s rinse in nanopure deionized H_2O to remove unbound Zn radiometal solution. Gills were weighed and placed in 20-ml scintillation vials for determination of gamma-radiation (Packard Minaxi Auto-gamma). One 5-ml water sample was acidified to 0·5% ultrapure-HNO_3 acid (J. T. Baker, Toronto, ON, Canada) and analysed for total [Zn] by flame atomic absorption spectroscopy. Another 5-ml water sample was assayed for determination of gamma-radiation. The following equation was used to calculate the amount of Zn being bound to the gill:

$$\text{Bound Zn} = R_G/(\text{gill weight}^*A) \quad (1)$$

Where Bound Zn is calculated in μmol kg^{-1}, R_G represents radioactivity of gill (cpm), gill weight in kg and A is the specific activity in cpm per μmol.

Part II

Following each Zn kinetic binding experiment, all fish were rinsed in Zn-free dechlorinated tap water for 1 min, then killed by a cephalic blow. Gill baskets were removed, blotted dry and weighed. For the non-radioactive Zn exposure, gills were digested in five-times volume (by weight) of concentrated nitric acid and heated in an oven for 12 h at 80° C. Samples were then diluted four-times by volume with nanopure deionized H_2O and analysed by flame atomic absorption spectroscopy. The amount of zinc bound could be determined directly from these results. For radioactive Zn exposures, gill and water samples were read on the gamma-counter as described above. Zinc binding was calculated according to equation (1) using the mean A from measurements at the beginning and end of the experiment.

MORPHOLOGICAL STUDY

Possible changes in gill surface morphology were assessed in the acclimation exposure of part II (above).

Gill sampling

Eight fish per treatment (Zn-exposed and control groups) were killed on days −1, 1 and 15. Day −1 represented the day prior to the onset of the zinc exposure. Gill baskets were removed immediately from each fish and the second gill arch from the right-anterior side was isolated. This section of gill was washed subsequently in the same dechlorinated tap water to which the fish had been exposed, and blood clots were removed carefully with a fine brush. Gills were placed in a wax-filled (Paraffin wax) petri dish containing enough ice-cold 5% glutaraldehyde solution (Marivac, Halifax, NS, Canada) to cover the

preparation. The glutaraldehyde solution was buffered with three parts 0·15 mol l^{-1} Na-cacodylate (Marivac), one part 0·30 mol l^{-1} Na-cacodylate and one part 25% glutaraldehyde (Marivac). This solution was adjusted to a pH of 7·4 with 0·2 mol l^{-1} HCl (Fisher Scientific, Toronto, ON, Canada), yielding an osmolarity of 292 mOsm l^{-1} (Goss *et al.*, 1994). Four paired filaments (still connected at the septum) were isolated randomly from different areas along the gill arch of each fish (Goss *et al.*, 1992). Filaments were placed in glass vials containing 3 ml of ice-cold fixative solution for 1·5 h, during which time they were kept on ice and swirled intermittently. Afterwards, filaments were placed in vials containing 5 ml of 0·15 mol l^{-1} Na-cacodylate buffer (pH 7·4), and stored at 4° C for 2–6 weeks prior to further processing.

SEM preparation and sampling

Paired filaments were prepared for SEM analysis using methodology as outlined by Goss *et al.* (1994). Morphometric gill analyses were performed using a calibrated digitizing tablet (Numonics Graphicmaster) with the aid of Sigmascan software (version 1.10; Jandel Scientific). Only flat sections of the filamental epithelium were analysed, whereas rounded portions of the filamental epithelium and lamellae were not included. Mean CC surface area (CCSA) (μm^2) and surface CC density (amount of cells per mm^2) were determined by tracing individual chloride cells within the designated (flat) region of the epithelium. From these values, the fractional surface area of chloride cells (CC FSA) per unit epithelium could be calculated (μm^2 mm^{-2}) (Goss *et al.*, 1992).

STATISTICAL ANALYSIS

Data are expressed as means ± 1 S.E. (n). Significant differences between the zinc-exposed group and its simultaneous control were evaluated using a Student's t-test (two-tailed, unpaired). Differences were considered to be statistically significant at $P<0·05$. Michaelis–Menten analyses were performed using Lineweaver–Burke linear regressions to yield values for B_{max} and K_m and their estimates of S.E. Note that Lineweaver–Burke transformations yield asymmetrical S.E. values.

RESULTS

PART I

Exposing the gills of juvenile rainbow trout to 0·01 mmol l^{-1} Zn radiometal solution resulted in rapid accumulation of Zn on the gills. Zn binding became saturated at 8000 µmol kg^{-1} after approximately 1800 s (Fig. 1). Using Scatchard plot analysis, $T_{1/2}$ was calculated at 335·2 ± 137·6 s. The effects of a 21-day sublethal Zn exposure (2·28 µmol l^{-1}) on Zn binding kinetics were studied. Zinc binding kinetics (B_{max} and K_m) were not greatly altered by the zinc exposure. The mean B_{max} value was calculated at 3470 ± 280 µmol kg^{-1} and the K_m was estimated at 14 850 ± 3920 µmol l^{-1} for the Zn treatment (Fig. 2). Despite this, the effectiveness of 0·04 mmol l^{-1} Ca to act as a Zn binding antagonist was evident: a 53% decrease in zinc binding in zinc-exposed fish relative to controls was observed after 21 days of exposure (Table I). This inhibitory effect dissipated due to precipitation of the Ca solution at Ca concentrations above 0·4 mmol l^{-1}.

PART II

Accumulation of Zn on the gills of juvenile rainbow trout was measured following 3-h exposures to varying concentrations of waterborne Zn. Despite an 18-fold increase (2·4 to 42·3 µmol l^{-1}) in waterborne [Zn], the amount of Zn bound to the gills during each of the exposures was negligible when compared to

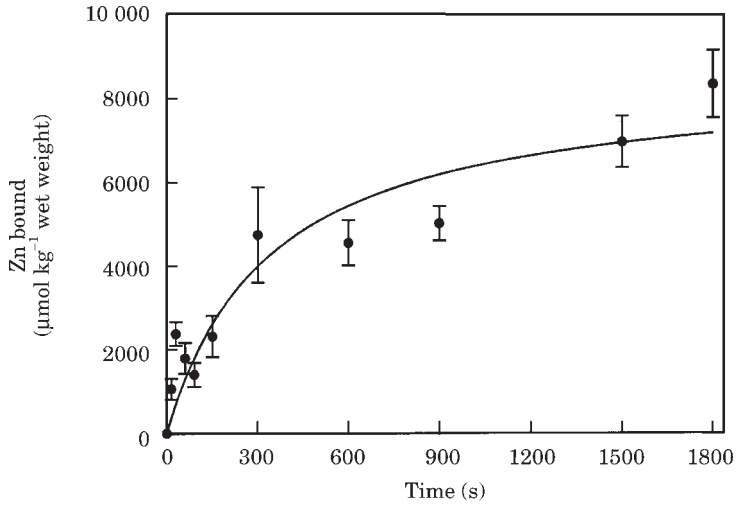

FIG. 1. Time course of zinc binding to the gill epithelium of juvenile rainbow trout, during exposure of gill baskets to a Zn concentration of 10 mmol l^{-1} (specific activity of 0·62 kBq ^{65}Zn μmol Zn). Time required to saturate half the Zn binding sites on the gill was estimated at 335·2 ± 137·6 s using Scatchard plot analysis. Values are means ± 1 S.E. (n=4).

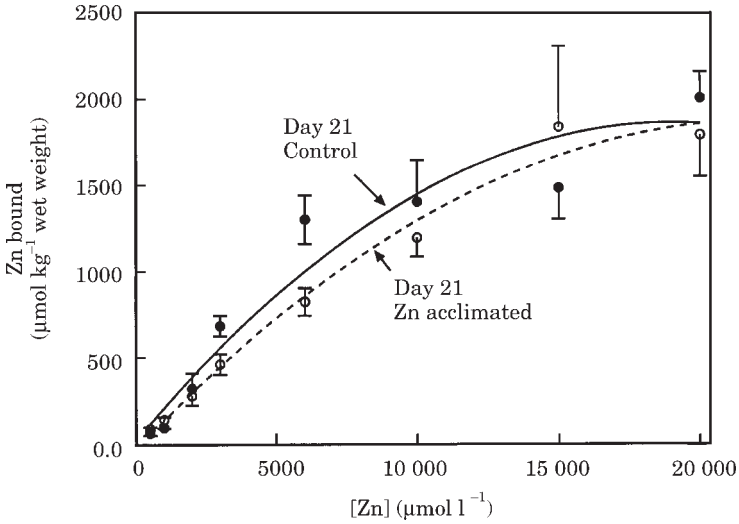

FIG. 2. Zinc binding kinetics of gill baskets from fish exposed to 2·28 μmol l^{-1} Zn for 21 days (open circles) and their simultaneous control no added Zn (solid circles). No appreciable differences in Zn binding kinetics occur in response to adaptation to Zn. The mean B_{max} value was calculated at 3470 ± 280 μmol kg^{-1} and the K_m was estimated at 14 850 ± 3920 μmol l^{-1} for the Zn treatment. Values are means ± 1 S.E. (n=4).

levels of this metal already found in the gill. In the present study, mean Zn concentrations ranged between 565·8–652·6 μmol kg^{-1}; however no correlation between water [Zn] and mean gill Zn content was evident. These values closely approximated the background gill [Zn] (619·0 μmol kg^{-1}) that was determined previously by Hogstrand *et al.* (1995).

TABLE I. Effects of varying [Ca] on branchial zinc binding when gill baskets were exposed to a constant [Zn] of 4·0 mmol l^{-1} for 300 s

[Ca] (mmol l^{-1})	Zinc Binding (μmol kg^{-1})	
	Control (day 21)	Zinc exposed (day 21)
0	660 ± 60	600 ± 60
$4*10^{-4}$	880 ± 220	480 ± 70
$4*10^{-3}$	830 ± 120	530 ± 50
$4*10^{-2}$	570 ± 50	300 ± 30*
0·4	680 ± 40	600 ± 90
1·0	760 ± 50	730 ± 70

Test was performed on control and zinc-exposed fish (2·28 μmol l^{-1} Zn) after 21 days of exposure. Means ± 1 S.E. (n=4).

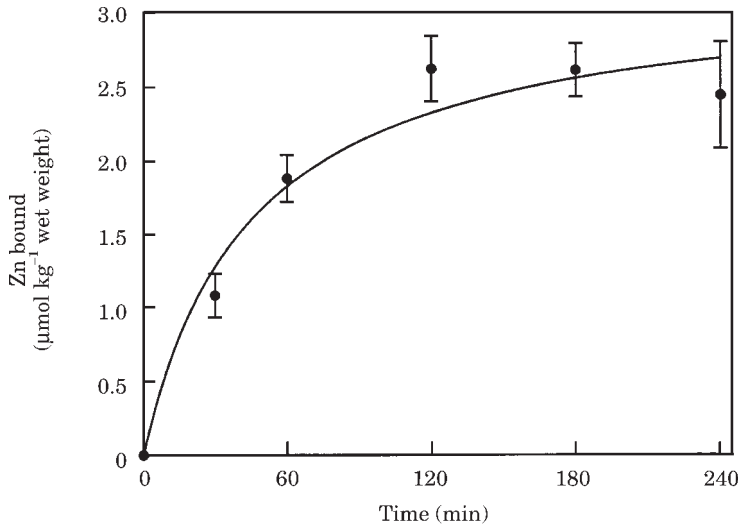

FIG. 3. Time course of zinc binding to gills during exposure of whole fish to 6·08 μmol l^{-1} (specific activity of 0·22 kBq ^{65}Zn μmol $^{-1}$ Zn). Saturation of available Zn binding sites (2·5 μmol kg^{-1}) occurred after approximately 120 min. Values are means ± 1 S.E. (n=6).

When ^{65}Zn was used as a radiotracer, it was possible to differentiate between background gill Zn content and Zn bound during these acute exposures. Gills of juvenile rainbow trout accumulated 2·5 μmol kg^{-1} Zn after approximately 120 min of exposure to 6·08 μmol l^{-1} Zn, henceforth, further accumulation was not observed (Fig. 3).

A linear relationship between water [Zn] and the amount of Zn being bound to the gills was seen at Zn concentrations exceeding 15·0 μmol l^{-1} [Fig. 4(a)]. Linear regression analysis gave the following relationship:

$$\text{Bound Zn} = 0{\cdot}369*[\text{Zn}] + 0{\cdot}234 \qquad (2)$$

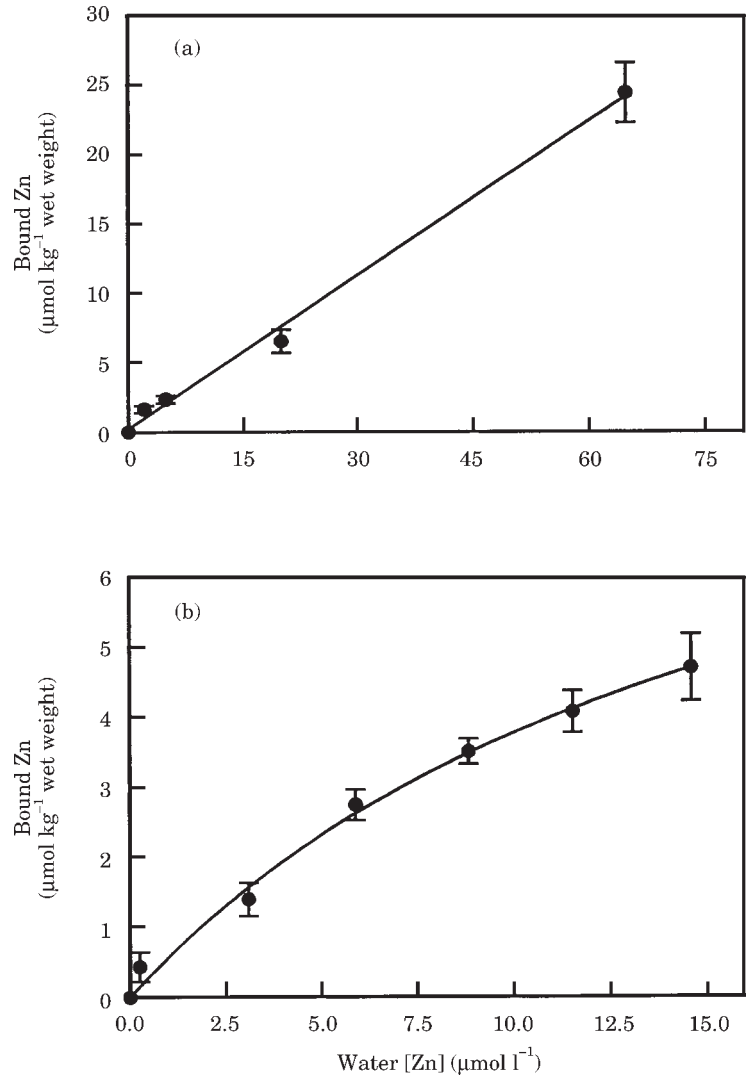

FIG. 4. Zinc binding kinetics for gills of juvenile rainbow trout exposed to various concentrations of Zn for 180 m. (a) Range finder experiment with [Zn]s ranging from 0 to 64·8 μmol l^{-1} (specific activity of 10·1 kBq ^{65}Zn μmol $^{-1}$ Zn). (b) [Zn]s ranging from 0 to 11·54 μmol l^{-1} (specific activity of 0·45 kBq ^{65}Zn μmol $^{-1}$ Zn). All values are means ± 1 S.E. (n=6).

At Zn concentrations below 15·0 μmol l^{-1}, it appeared that gill Zn binding followed a saturable relationship, starting to level off at approximately 5·0 μmol kg^{-1} Zn [Fig. 4(b)]. The equation for the generated curve was:

$$\text{Bound Zn} = -0{\cdot}011^{*}[\text{Zn}]^{2} + 0{\cdot}46\,[\text{Zn}] + 0{\cdot}25 \qquad (3)$$

Zinc binding kinetic constants were calculated for fish exposed to 2·28 μmol l^{-1} Zn over a 15-day period. There were no observed differences in maximal Zn binding (B_{max}) [Fig. 5(a)] and binding affinity (K_m) [Fig. 5(b)] between the

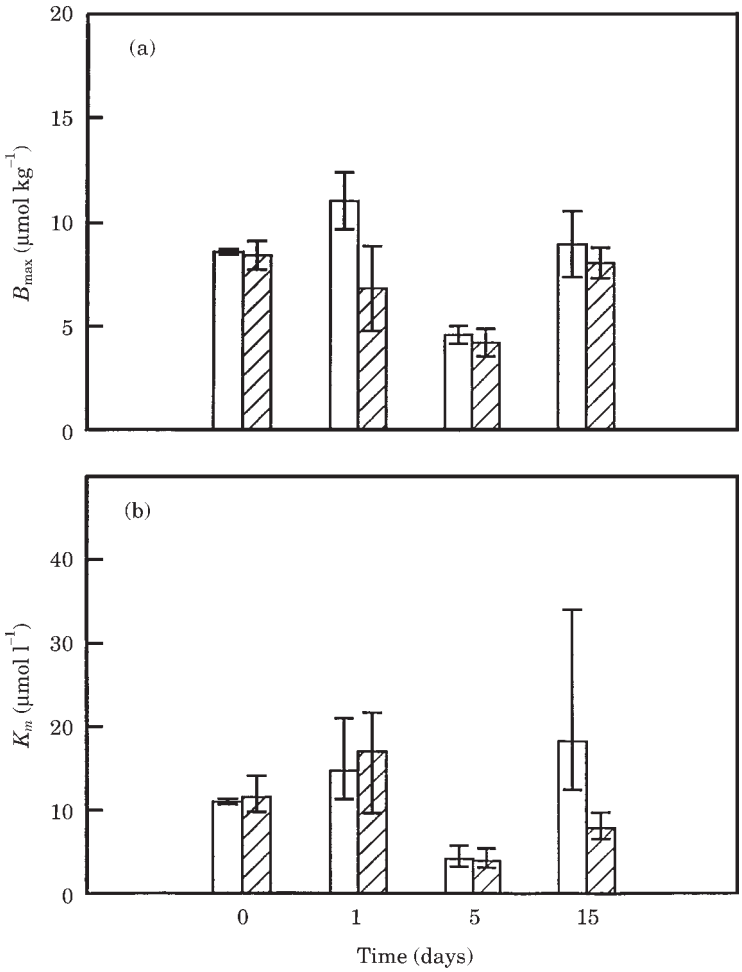

FIG. 5. Zinc binding kinetics for gills of juvenile rainbow trout. Values are means ± 1 S.E. (n=6). Values were obtained by either Lineweaver–Burke or Eadie–Hofstie plots. Asymmetrical S.E. were derived from regression lines fitted to these plots. Cross-hatched bars are values for fish chronically exposed to Zn (2·28 μmol l^{-1}). Open bars show mean values for controls. Analyses for both groups were performed on days 0, 1, 5 and 15 of the exposure. (a) Maximal amount of binding to highly-specific binding sites on the gill epithelium (B_{max}). (b) Dissociation constant (K_m) for the binding of Zn to highly-specific sites.

Zn-exposed group and the simultaneous control over the experimental period. However, there did appear to be a temporal fluctuation in the zinc binding kinetics of both groups, with B_{max} and K_m values being greatest on days 1 and 15 and at their lowest points on day 5.

Overall, no significant differences on gill CC morphology were seen between the zinc-exposed group and its simultaneous control at each sample period (Fig. 6). Nonetheless, there appeared to be a temporal fluctuation of CC fractional area for both treatments [Fig. 6(c)]. Chloride cell FSA values for Zn-exposed fish decreased from 87 751 to 49 340 μm^2 mm^{-2} between day −1 to day 1 of the exposure, however by day 15, the CC FSA had returned to approximately its pre-exposure value (84 241 μm^2 mm^{-2}). Similar temporal

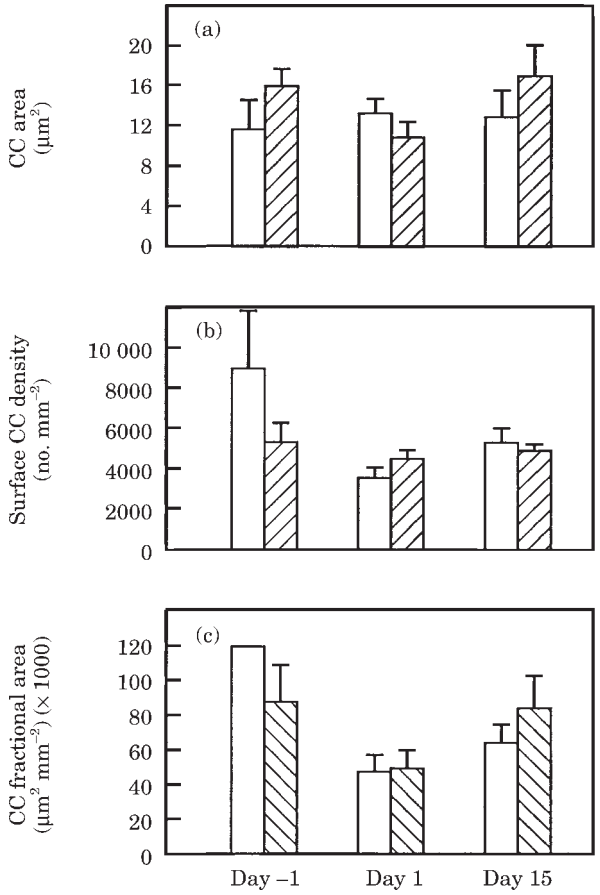

FIG. 6. The effects of chronic sublethal Zn exposure on gill filamental chloride cell (CC) morphometry including: (a) mean surface area of individual CCs, (b) CC density on gill filament, and (c) CC fractional surface area. All values are means ± 1 S.E. (n=6). Cross-hatched bars are values for fish chronically exposed to Zn (2·28 μmol l^{-1}). Open bars represent mean values for controls.

fluctuations in CC FSA were seen for control fish. Once again CC FSA values were highest on days −1 and 15. In representative SEM photographs, CCs showed characteristic microvilli projections which were distinct from the microridged formations found on pavement cells. Qualitative examination of SEM images revealed no differences between control and zinc-exposed fish from day −1 to 8 of the experiment. Following 15 days of zinc exposure, gills appeared to have an increased number of chloride cells characterized by smooth, dark surfaces, although chloride cells with microvilli projections at the surface were still common and typically the larger of the two types.

DISCUSSION

The gill micro-environment contains an extensive number of molecules possessing a combination of phosphate, carboxylate and sulphate functional groups (Seimiya & Ohki, 1973). Typically, pK values for these constituents

range between 2·6 and 4·0 (Dawson *et al.*, 1986); therefore at environmentally relevant pH levels these groups are fully ionized. The resultant formations of negative charges on the gill surface act as metal receptors, attracting positively charged metal ions such as Zn^{2+}. Classical pharmacological approaches can be used subsequently to quantify metal ligand-gill binding parameters such as maximal binding (B_{max}) and affinity (K_m). In the present study, alterations to Zn binding kinetics at the gill epithelium were investigated in response to sublethal Zn exposure. Procedures adopted from Reid *et al.* (1991), and Reid & McDonald (1991), were employed to discern these binding characteristics. Isolated gill baskets exposed to 10 mmol l^{-1} Zn became saturated at approximately 8000 μmol kg^{-1}, with a $T_{1/2}$ for zinc binding of 335 s (Fig. 1). The $T_{1/2}$ for maximal zinc binding was similar to values obtained for Ca, La, Cd and Cu from Reid & McDonald (1991). Zinc binding kinetic experiments were performed with brief 300-s exposures, identical to those used by Reid & McDonald (1991) for determining the binding kinetics of other metals. Since both studies were performed with similar water qualities and experimental protocols, direct comparison of results between investigations could be made.

The gill epithelium of rainbow trout has been shown to accumulate approximately seven times more Cu than La, while maximal binding estimates for Ca and Cd were intermediate between these (Reid & McDonald, 1991). The ability of Cu to bind avidly to the gill has been attributed to its high affinity for a large number of electronegative sites (Nieboer & Richardson, 1980). Potentially, Cu binding could occur at either oxygen, nitrogen or sulphur sites on the gill. In comparison, binding of class A metals such as La and Ca is limited to oxygen centres, while Cd (class B metal) binds preferentially to nitrogen or sulphur-containing molecules. In the present study, high levels of zinc accumulated on the gill epithelium. Approximately eight times more Zn was capable of binding to the gill compared to Cu. Since both metals possess borderline tendencies (have class A and B characteristics), differences seen in maximum binding capacity between them must be due to some other factor. One possible explanation for the large binding capacity of Zn on the gill epithelium may be its ability to form complex coordination geometries (hexagonal); in comparison, Cu is able to associate with other sites up to a total coordination number of only four (square-planar geometry) (Williams, 1984; Reid & McDonald, 1991). It is this flexible metal-ligand binding that makes Zn an efficient cofactor for a large number of diverse proteins (Williams, 1984).

EFFECTS OF ZINC ACCLIMATION ON ZINC BINDING KINETICS

Generally, adaptive responses to sublethal exposure of metals entail a complete recovery of the resulting iono-regulatory disturbance. For instance, sublethal Cu exposure was shown to increase the K_m and cause a massive reduction in J_{max} for Na influx within 24 h (Laurén & McDonald, 1987). However, after continued sublethal exposure (after 28 days), Na influx would begin to normalize with an almost complete restoration of J_{max} and a partial recovery of K_m. In comparison, sublethal Zn exposure created a persistently low affinity for the proposed common Ca/Zn transporter(s), which did not recover with time (Hogstrand *et al.*, 1994; Hogstrand & Wood, 1996). Despite this, Ca influx was affected only marginally, whereas Zn influx was reduced in half by

lowering the affinity for part of the uptake carrier mechanism. Attempts were made to correlate changes in Zn uptake kinetics (measured during previous experiments) with possible alterations in Zn binding at the gill epithelium during sublethal Zn exposure.

Zn binding kinetics (B_{max} and K_m) were not altered in response to sublethal Zn exposure (following 21 days) (Fig. 2), suggesting that the overall ability of the gill epithelium to bind Zn externally did not change. Nevertheless, calcium was shown to have an increased inhibitory effect on Zn binding in zinc-exposed fish compared with controls (Table I). This is consistent with results showing a greater competitive interaction of Ca on Al binding following Al-acclimation (Reid *et al.*, 1991). These data suggest that, although the number of anionic groups at the gill is not changing, a shift in the types of Zn binding sites may be occurring. The increased inhibitory action of Ca might represent a switch from class B to class A metal-seeking binding sites (such an alteration would allow Ca to bind more effectively to the gill). Meanwhile, the overall binding capacity for Zn to the gill epithelium could remain unaltered.

The high concentrations of Zn used during these *in vitro* assays made it impossible to note changes which may have occurred at high-affinity binding sites in response to Zn-acclimation: it is these sites, most likely Ca transporters, which are believed to be involved in the process of Zn uptake. Presumably, these Ca transporters should make up only a small proportion of the anionic groups on the gill epithelium. Accordingly, even if alterations in the kinetic binding parameters of these transporters were to occur, non-specific Zn binding would interfere with their assessment. Nevertheless, this procedure did allow us to discern the ability of the gill surface to sequester waterborne metals. It has been proposed that binding of metals to non-specific sites may reduce toxicity by decreasing the bioavailability of free metal ions for specific carrier-mediated transporters (Pagenkopf, 1983). Consequently, determination of the number and affinity of these non-specific sites may be required to understand fully the extent to which elevated waterborne metal concentrations are able to elicit deleterious effects.

The methodology used during the *in vivo* Zn binding experiments was implemented in an attempt to characterize the binding kinetics of Zn to highly-specific sites at the gill. The question which arises here is, how can we be sure that the Zn concentrations used were low enough to observe alterations to these high-affinity sites (Ca transporters)? Figure 4(b) illustrates Zn binding kinetics performed between Zn concentrations of 0 to 14·61 μmol l^{-1}; concentrations which closely approximate the [Zn] required to evoke Zn adaptation. It is apparent that Zn binding to the gill starts to become saturated as waterborne Zn concentration increases. However, as Zn concentration was increased above 15 μmol l^{-1}, Zn binding kinetics became linear in nature [Fig. 4(a)], possibly suggesting a spill-over of Zn onto non-specific sites once high-affinity binding sites had become saturated. No discernible effects on Zn binding kinetics between the Zn-exposed group and its simultaneous control were observed (Fig. 5). Based on previous studies (Hogstrand *et al.*, 1994), this [Zn] should have been adequate to elicit adaptive-type responses on Zn and Ca influxes. This may suggest that changes in the affinity for Zn uptake mechanisms are not associated with modifications to high-affinity Zn binding kinetics at the gill.

Further work is needed to determine whether changes in Zn uptake kinetics are mediated via non-Ca apical Zn pathways or by altered storage capacity of the gill cells for Zn.

Many studies have tried to correlate alterations in gill function (iono-regulation, acid/base regulation, nitrogenous waste excretion and gas transport) with changes in the morphology of the gill epithelium, such as acid regulation (Audet & Wood, 1993; Goss *et al.*, 1994; Wilkie & Wood, 1994) and Ca uptake (Perry & Wood, 1985). Morphometric analysis of the gill epithelium was performed to observe whether changes to CC availability could be correlated with other physiological changes also seen during Zn adaptation. Morphometric analyses showed no significant differences in CC FSA, CC density and mean CCSA between control and Zn-exposed fish during the experimental period (Fig. 6). Despite the lack of effects seen on Zn binding kinetics and CC morphology, temporal fluctuations in CC FSA were seen for both treatments. According to Wagner *et al.* (1985, 1993), Ca^{2+} influx in juvenile rainbow trout varied on a natural cycle of *c.* 11 days, presumably in an attempt to optimize growth. Both the control and Zn-exposed groups showed their greatest CC FSA at days -1, and 15, while being at their lowest value on day 1. B_{max} and K_m values were also determined to follow the same pattern. There is some evidence that this response is regulated by stanniocalcin, a hormone that has been shown to decrease Ca influx. Verbost *et al.* (1993) have suggested that stanniocalcin reduces Ca influx by controlling transporters on the apical surface of the gill epithelium.

According to McDonald & Wood (1993), metal acclimation resulting from sublethal exposures involves a two-fold process which includes toxicant-induced gill damage, followed by tissue repair. They argue that cellular damage is a necessary component of toxicant acclimation. The lack of morphological damage to the gills during the early phases of Zn exposure puts in question whether an acclimation response to Zn is in fact being elicited (at least during exposure to 2·28 μmol l^{-1}). As mentioned by Hogstrand *et al.* (1994), deliberately keeping the affinity of the carrier mechanism low caused a decrease in Zn influx. Since the study was conducted in relatively hard water (1·0 mmol l^{-1} Ca) which was well above the K_m for Ca influx, the decrease in transporter affinity produced only a moderate decrease in Ca influx over time. Whether Ca influx would be altered if the experiment was conducted in soft water (near the K_m for Ca influx), is uncertain. Future investigations should attempt to answer this question in order to understand fully the adaptive response being observed.

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