

# Influence of waterborne cations on zinc uptake and toxicity in rainbow trout, *Oncorhynchus mykiss*

Derek H. Alsop and Chris M. Wood

**Abstract:** The effects of waterborne cations on  $^{65}\text{Zn}$  uptake, Zn toxicity, and relationships with Ca uptake were examined in juvenile rainbow trout, *Oncorhynchus mykiss*, in soft water. Whole-body Zn uptake (waterborne  $[\text{Zn}] = 100 \mu\text{g}\cdot\text{L}^{-1} = 1.5 \mu\text{M}$ ) was greatly reduced by a variety of cations. This reduction was directly related to the concentration of positive charges, regardless of which ion carried that charge. Thus, 1.0 mM  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{NH}_4^+$ , and *N*-methyl-D-glucamine $^+$  and 0.5 mM  $\text{Mg}^{2+}$  (divalent) reduced Zn uptake to a similar extent (~50%), indicating a relatively nonspecific competition for anionic sites on the gill.  $\text{Ca}^{2+}$  was an exception and was more potent at reducing Zn uptake, likely because only  $\text{Ca}^{2+}$  would also compete for absorption. Although  $\text{Na}^+$  and  $\text{Mg}^{2+}$  were able to markedly reduce Zn uptake, they had no effect on Zn toxicity (measured with 96-h  $\text{LC}_{50}$  tests), a result paralleled by their inability to restore  $\text{Ca}^{2+}$  uptake that was inhibited by Zn. In contrast,  $\text{Ca}^{2+}$  reduced Zn toxicity and restored  $\text{Ca}^{2+}$  uptake. These results partially dissociate Zn uptake from Zn toxicity, implicate disturbed  $\text{Ca}^{2+}$  uptake as the toxic mechanism, and have profound implications for water quality criteria where  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (the two "hardness" cations) are traditionally considered to be equally protective.

**Résumé :** Les effets des cations aqueux sur l'absorption du  $^{65}\text{Zn}$ , la toxicité du Zn et les relations avec l'absorption du calcium ont été étudiés chez des truites arc-en-ciel juvéniles en eau douce. L'absorption par tout l'organisme du Zn présent dans l'eau ( $[\text{Zn}] = 100 \mu\text{g}\cdot\text{L}^{-1} = 1,5 \mu\text{M}$ ) a été fortement réduite par divers cations. Cette réduction était directement liée à la concentration des charges positives, indépendamment de l'ion qui portait ces charges. Par conséquent, 1,0 mM de  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{NH}_4^+$  ou de *N*-méthyl-D-glucamine $^+$  et 0,5 mM  $\text{Mg}^{2+}$  (divalent) réduisaient l'absorption du Zn d'une façon semblable (~50%), ce qui montre une concurrence relativement non spécifique pour les sites anioniques des branchies. Le  $\text{Ca}^{2+}$  faisait cependant exception et provoquait une plus grande réduction de l'absorption du Zn, sans doute parce que le  $\text{Ca}^{2+}$  exerce aussi une concurrence pour l'absorption. Bien que le  $\text{Na}^+$  et le  $\text{Mg}^{2+}$  pouvaient réduire l'absorption du Zn de façon marquée, ils n'avaient aucun effet sur la toxicité du Zn (déterminée par la  $\text{CL}_{50}$  96-h), un effet qui correspond à leur incapacité de rétablir l'absorption du  $\text{Ca}^{2+}$  qui avait été inhibée par le Zn. Au contraire, le  $\text{Ca}^{2+}$  réduisait la toxicité du Zn et rétablissait l'absorption du  $\text{Ca}^{2+}$ . Ces résultats dissocient, du moins en partie, l'absorption et la toxicité du Zn et font supposer une perturbation de l'absorption du  $\text{Ca}^{2+}$  en tant que mécanisme de toxicité. Cela a d'importantes incidences sur l'établissement des critères de qualité de l'eau pour lequel on a toujours supposé que le  $\text{Ca}^{2+}$  et le  $\text{Mg}^{2+}$  (deux cations de la « dureté ») avaient un rôle protecteur semblable.

[Traduit par la Rédaction]

## Introduction

Zinc is an essential micronutrient that can be absorbed by fish via the gill from the aquatic environment as well as from the diet (Spry et al. 1988). The pathway of branchial Zn uptake (as well as other cations) begins with the adsorption of the cation to a negatively charged site on the gill surface (Pagenkopf 1983), followed by absorption into the gill cell (Hogstrand et al. 1996). Different cations can compete for these negative sites

on the gill surface. Handy and Eddy (1991) showed that  $\text{Na}^+$  adsorption (and consequently absorption into the gill) was reduced by competition from other waterborne cations such as  $\text{H}^+$  and  $\text{Ca}^{2+}$  for binding sites.

If waterborne Zn concentrations become high enough, too much adsorption/absorption may ultimately lead to toxic effects. Zn specifically disrupts  $\text{Ca}^{2+}$  uptake at the gill, which can lead to hypocalcemia and even death of the fish (Spry and Wood 1985; Hogstrand et al. 1995). Zn toxicity is not only dependent on Zn concentration but also on the presence of other ions in the water. In water quality regulations (e.g., U.S. Environmental Protection Agency 1980), water hardness (the sum of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  expressed as milligrams of  $\text{CaCO}_3$  per litre) is the only component of water quality that is taken into account as a modifying agent for Zn toxicity. For example, a decrease in water hardness of about 10 times was shown to increase Zn toxicity to juvenile rainbow trout, *Oncorhynchus mykiss*, by 10 times (Bradley and Sprague 1985). Similarly, Alsop et al. (1999) reported that a sixfold

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**Table 1.** Water composition of hard water (Hamilton tap water from nearshore Lake Ontario) and artificial soft water of the Zn and Ca<sup>2+</sup> uptake experiments and LC<sub>50</sub> trials.

	Hard water	Softwater Zn uptake	Softwater Zn LC <sub>50</sub> /Ca <sup>2+</sup> uptake
Ca <sup>2+</sup> (mM)	1.0	0.02	0.06
Mg <sup>2+</sup> (mM)	0.2	0.01	0.02
Na <sup>+</sup> (mM)	0.6	0.04	0.05
K <sup>+</sup> (mM)	0.05	nd	0.01
Cl <sup>-</sup> (mM)	0.7	0.04	0.05
Hardness (as mg CaCO <sub>3</sub> ·L <sup>-1</sup> )	120	5	10
pH	8.0	5.6	5.8

Note: nd, not detectable.

reduction in hardness increased toxicity about six times. The toxic effects of Zn, like most metals, are therefore of particular concern in ion-poor water.

Other ions may also compete with Zn binding to the gill, and any ions that impede the initial adsorption of Zn to the gill might be expected to reduce its toxicity (Simkiss and Taylor 1989). For example, Pagenkopf (1983) predicted that H<sup>+</sup> would compete with cationic metals for binding sites on the gill, leading to lower toxicity at sublethal low pH. While data were not available for Zn at the time, the protective effect of sublethal low pH was subsequently shown experimentally by both Bradley and Sprague (1985) and Cusimano et al. (1986). In contrast, Pagenkopf (1983) discounted the protective effect of other monovalents such as Na<sup>+</sup> and K<sup>+</sup> because of their "less extensive" interactions with the gill.

One aim of the present study on freshwater rainbow trout acclimated to soft water was to look at the effect of a range of different cations on radiolabelled Zn (<sup>65</sup>Zn) uptake and the relative potencies of their inhibition. The use of the radioisotope <sup>65</sup>Zn was required to measure Zn accumulation (Galvez et al. 1998; Hogstrand et al. 1998) due to the high levels of Zn already present in fish tissues, which reflect its role as a micronutrient. We predicted that Ca<sup>2+</sup> would have the largest influence on Zn uptake due to the fact that not only would Ca<sup>2+</sup> compete with Zn for binding sites on the gill surface, but it would also compete for uptake. Recent evidence indicates that Ca<sup>2+</sup> and Zn share the same apical transport channel in the gill (reviewed by Hogstrand and Wood 1996). Another goal of this study was to determine whether reductions in Zn uptake by various cations translate into a protective effect against Zn toxicity. Of particular interest was the relative effectiveness of Ca<sup>2+</sup> and Mg<sup>2+</sup>, the two major components of water "hardness," in influencing both Zn uptake and Zn toxicity. Finally, since inhibition of branchial Ca<sup>2+</sup> uptake may be the mechanism of Zn-induced mortality, we examined whether protection against acute Zn toxicity (i.e., increased 96-h LC<sub>50</sub>) by a particular cation was associated with a restoration of Ca<sup>2+</sup> uptake.

## Materials and methods

### Fish care

Juvenile rainbow trout (3–10 g) were obtained from Rainbow Springs Trout Hatchery (Thamesford, Ont.) and initially held for 1 week in aerated 500-L tanks supplied with dechlorinated Hamil-

ton tap water at 3 L·min<sup>-1</sup> (Table 1). The fish were then slowly acclimated to "soft water" over the next 2 weeks by mixing hard water with increasing amounts of ion-reduced water (produced by reverse osmosis; Anderson Water Systems, Dundas, Ont.) until the desired water chemistry was reached (Table 1). The fish were allowed to acclimate to this soft water for at least 1 month before experimentation. Soft water was favored for Zn uptake experiments because >99% of waterborne Zn would be in the free ionic form (Zn<sup>2+</sup>) at the low pH and alkalinity characteristic of this water, as predicted with the aquatic geochemical program MINEQL+ (Schecher and McAvoy 1994). Trout were fed to satiation three times per week with commercial trout feed (fish food composition (partial analysis only): crude protein (minimum), 52%; crude fat (minimum), 17%; crude fibre (maximum), 2.5%; water, 12%; Ca<sup>2+</sup>, 1.4%; Na<sup>+</sup>, 0.4%; Zn (measured), 0.02% (173 µg·g<sup>-1</sup>)). Water temperature was 10 ± 1°C during the experiments.

### Zn uptake

Twenty-litre buckets served as <sup>65</sup>Zn flux units and were lined with polypropylene bags. The buckets were filled with 14 L of soft water (ionic composition given in Table 1) and placed in a wet table surrounded by flowing water for temperature regulation. A stock Zn solution was added to each flux unit (as ZnSO<sub>4</sub>·7H<sub>2</sub>O; Anachemia) to bring each one to 100 µg Zn·L<sup>-1</sup> (1.53 µM). In addition, 10 µCi (0.37 MBq) of <sup>65</sup>Zn (as ZnCl<sub>2</sub>, specific activity = 1.97 mCi·mg<sup>-1</sup>; NEN Life Science Products, Boston, Mass.) was added to each flux unit, an amount that had negligible influence on the total Zn concentration of the water. Each trial was run with a control and with the addition of 0.5, 1.0, and 2.0 mM of a test salt, unless otherwise noted. Once the flux units were prepared and equipped with gentle aeration, 28 fish were removed from their holding tank and divided evenly between the four flux units (control, 0.5, 1.0, and 2.0 mM test salt; N = 7). Flux experiments lasted 7 h, with triplicate 5-mL water samples taken just before the termination of the flux. At the end of the flux period, the fish were removed and rinsed for 1 min in a bath containing a lethal amount of anesthetic (0.5 g MS 222·L<sup>-1</sup>) and 40 mg Zn·L<sup>-1</sup> (as ZnSO<sub>4</sub>·7H<sub>2</sub>O) to displace any surface-bound <sup>65</sup>Zn. This protocol was chosen based on the results of Spry and Wood (1989) who showed that there is a rapid initial adsorption of <sup>65</sup>Zn to sites on the surface of the fish, followed by linear uptake thereafter. The 7-h flux period ensured that most of the uptake was measured over the linear period, and the 40 mg·L<sup>-1</sup> cold Zn rinse served to minimize (by displacement) the initial adsorption component. After rinsing, trout were blotted dry and weighed to the nearest 0.01 g. Fish were assayed for <sup>65</sup>Zn activity in a gamma counter (MINAXI Auto-Gamma 5000 Series, Canberra-Packard). Water samples were similarly assayed for <sup>65</sup>Zn activity as well as for total Zn and the concentration of the cation being tested (Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup>, or Ca<sup>2+</sup>) by atomic absorption spectrophotometry. NH<sub>4</sub><sup>+</sup> concentrations were determined by the method of Verdouw et al. (1978).

The following salts were tested: NaCl, NaNO<sub>3</sub>, KCl, NH<sub>4</sub>Cl, MgCl<sub>2</sub>, and CaCl<sub>2</sub> (BDH Chemicals). There was no indication of ammonia toxicity during the 7-h flux experiment (i.e., there were no mortalities and swimming behavior appeared normal). N-Methyl-D-glucamine chloride (Sigma) was also tested by first preparing a stock solution, which was then titrated to pH 7.0 with concentrated HCl. MgCl<sub>2</sub> and CaCl<sub>2</sub> were tested solely at 0.5 mM against 0.5 mM NaCl and a control. In addition, the effects of pH on <sup>65</sup>Zn uptake were investigated. Two tanks were set up as described above, a control tank (pH 5.6) and a tank at pH 3.7 (addition of ~0.2 mM H<sup>+</sup>). pH levels were achieved by the addition of concentrated HCl to the water, and levels were constantly monitored during the flux and adjusted if needed.

To determine if acclimation to the test salts had an effect on their inhibition of Zn uptake, 20 fish were acclimated to soft water plus 1 mM Na<sup>+</sup> (as NaCl) and 20 fish to soft water plus 1 mM Ca<sup>2+</sup>

(as  $\text{CaCl}_2$ ) for 3 weeks. During acclimation, trout were kept in 30-L tanks and 90% of the water was replaced every 2 days. Fish were fed to satiation as usual. Flux tests were then performed in 1 mM  $\text{NaCl}$  and 1 mM  $\text{CaCl}_2$  with acclimated and nonacclimated fish against a softwater control with nonacclimated fish.

Finally, an experiment was performed to examine the distribution of radiolabelled Zn accumulation during the flux tests in the absence (control) and presence of 1.0 mM  $\text{Na}^+$ . The flux test was performed as described above. At the end of 7 h, fish were rinsed and lethally anaesthetized as above, and then, blood was collected in heparinized capillary tubes by caudal severance, the gills were removed and blotted dry, and a piece of skin was removed from the side of the fish underneath the dorsal fin. Any musculature adhering to the skin was scraped off. The height of the blood collected in capillary tubes was measured in millimetres (internal radius of tubes = 1.15 mm) to determine its volume, and the other tissues and remaining carcass were weighed to the nearest 0.001 g. All samples were assayed for  $^{65}\text{Zn}$ . For calculations of partitioning, the blood was assumed to account for 5% of the body mass (Olson 1992), the skin was assumed to weigh  $0.014 \text{ g}\cdot\text{cm}^{-2}$ , and the total skin was assumed to equal  $1.36 \text{ cm}^2\cdot\text{g fish}^{-1}$  based on measurements carried out by M. Grosell (Department of Biology, McMaster University, Hamilton, Ont., personal communication).

Expressed concentrations of Zn and cations are nominal, but were always within 10% of the desired concentrations.

### Toxicity testing

To test whether inhibition of Zn uptake had an effect on acute Zn-induced mortality, 96-h  $\text{LC}_{50}$  tests were conducted. The four test conditions were soft water (Table 1), soft water plus 2.0 mM  $\text{Na}^+$ , soft water plus 1.0 mM  $\text{Mg}^{2+}$ , and soft water plus 1.0 mM  $\text{Ca}^{2+}$ .

Forty trout were removed and divided into five 18-L tanks (eight fish per tank, with each tank receiving 200 mL soft water $\cdot\text{min}^{-1}$ ) and allowed to settle for 1 h. Tanks were then randomly assigned to one of five Zn concentrations including a control group (0–350  $\mu\text{g Zn}\cdot\text{L}^{-1}$ , 0–3000  $\mu\text{g Zn}\cdot\text{L}^{-1}$  for the  $\text{Ca}^{2+}$  trial). To begin the  $\text{LC}_{50}$ , flow of toxicant from a Mariotte bottle was started into a head tank where it mixed with inflowing fresh water by vigorous aeration. The toxicant consisted of  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ , with the appropriate concentration of cationic salt if required, dissolved in deionized water acidified with 0.2 mL concentrated  $\text{HNO}_3\cdot\text{L}^{-1}$  (trace metal analysis grade; BDH Chemicals). At the same time, Zn and the appropriate cation were added to each tank (apart from the control tanks) to immediately bring them up to the desired Zn concentrations. Mortalities were recorded over 96 h. Water samples were taken daily and acidified for later analysis. The 96-h  $\text{LC}_{50} \pm 95\%$  confidence limits (CL) were calculated by log-probit analysis of mortality versus measured waterborne Zn concentration (Finney 1971).

### $\text{Ca}^{2+}$ uptake

The effect of various ions on the ability of Zn to disrupt  $\text{Ca}^{2+}$  uptake was investigated in an attempt to determine their protective mechanism. Six flux units were set up as for the  $^{65}\text{Zn}$  fluxes above. The tanks were designated as (i) control soft water, pH = 5.8, (ii) soft water plus 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$ , pH = 5.8, (iii) soft water plus 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  with pH adjusted to 4.0 with HCl, (iv) soft water plus 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  plus 2 mM  $\text{Na}^+$ , (v) soft water plus 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  plus 1 mM  $\text{Mg}^{2+}$ , and (vi) soft water plus 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  plus 1 mM  $\text{Ca}^{2+}$ , all as chloride salts. Fifteen microcuries of  $^{45}\text{Ca}$  (as  $\text{CaCl}_2$ ; NEN Life Science Products) was added to each flux unit and the fish ( $N = 8$ ) were exposed for 7 h. In a separate experiment, the time course of the effect of low pH on Zn-induced inhibition of  $\text{Ca}^{2+}$  uptake was investigated. Trout were exposed to  $^{45}\text{Ca}$  in soft water, soft water plus 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$ , and soft water plus 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  at pH 4.1, all for 5 h. Sixteen fish were also held in 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  and 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  at pH 4.1 for 8 and 30 h without  $^{45}\text{Ca}$  and then exposed to  $^{45}\text{Ca}$  for 5 h.

At the termination of the  $^{45}\text{Ca}$  fluxes, water samples were taken and fish were removed and rinsed for 1 min in 0.5 g  $\text{MS 222}\cdot\text{L}^{-1}$  and 5 mM  $\text{CaCl}_2$  to displace any surface-bound  $^{45}\text{Ca}$ . Fish were weighed and digested in a vial with five times their weight of tissue solubilizer (TS-2; Research Products International, Mount Prospect, Ill.). Digestion was accelerated by heating to 55°C for 36 h. Triplicate 0.5-mL samples of the digest media and water were assayed for  $^{45}\text{Ca}$  by scintillation counting using an LKB 1217 Rackbeta (Hamilton, Ont.). Differences in counting efficiency between digest media and water were corrected by internal standardization.

### Calculations and statistics

The appearance of  $^{65}\text{Zn}$  from the water into the fish was calculated from the  $^{65}\text{Zn}$  activity of the whole body and the specific activity of Zn in the water. The mean specific activity (SA) of Zn in the water over the flux period was calculated as

$$(1) \quad \text{SA} = (\text{cpm}\cdot\text{mL}^{-1})/[\text{Zn}]$$

where cpm are the gamma counts per minute and [Zn] is the concentration of Zn (micrograms per millilitre). In practice, Zn total concentrations and specific activities underwent negligible change during these tests. Total Zn appearance in the body was then calculated as

$$(2) \quad \text{Total Zn appearance} = (\text{cpm/body weight})(1/\text{SA})$$

where total Zn appearance was in micrograms of Zn per gram of body, which was then divided by 7 h (the duration of the exposure) to yield a final uptake rate in micrograms of Zn per gram of body per hour.  $\text{Ca}^{2+}$  uptake rates using  $^{45}\text{Ca}$  were calculated in an analogous fashion.

Data have been expressed as means  $\pm$  SE ( $N$ ) except for the 96-h  $\text{LC}_{50}$ s where means  $\pm$  95% CL have been reported. For the  $^{65}\text{Zn}$  and  $^{45}\text{Ca}$  uptake experiments, significant differences from the control uptake rate were tested with a one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons to determine significant differences among treatments.  $\text{LC}_{50}$ s were compared by means of the Bonferroni adjustment to the independent two-tailed Student  $t$  test. The limit of significance was 5, 1, and 0.1% (indicated on the graphs as one, two, and three asterisks, respectively).

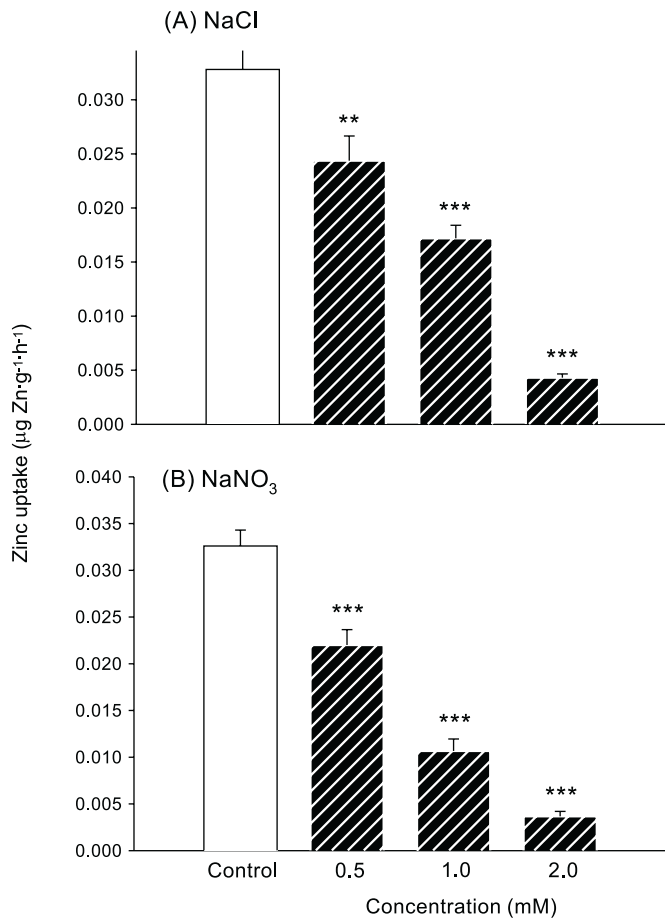
## Results

### Zn uptake

Rainbow trout exposed to 100  $\mu\text{g Zn}\cdot\text{L}^{-1}$  had a control whole-body uptake rate of 0.033  $\mu\text{g Zn}\cdot\text{g body}^{-1}\cdot\text{h}^{-1}$  (Fig. 1A).  $\text{Na}^+$ , as  $\text{NaCl}$ , at 0.5, 1.0, and 2.0 mM reduced the control whole-body Zn uptake by 26, 48, and 87%, respectively (Fig. 1A). As well,  $\text{Na}^+$ , as  $\text{NaNO}_3$ , at 0.5, 1.0, and 2.0 mM also reduced Zn uptake by 33, 67, and 86%, respectively, showing that the effect was independent of the anion (Fig. 1B). The chloride salts of  $\text{K}^+$  reduced Zn uptake by 42, 75, and 93% (Fig. 2A), those of  $\text{NH}_4^+$  by 53, 52, and 80% (Fig. 2B), and those of *N*-methyl-D-glucamine by 37, 52, and 37%, respectively (Fig. 2C), all at 0.5, 1.0, and 2.0 mM concentrations, very similar effects to those of  $\text{Na}^+$ . *N*-Methyl-D-glucamine was tested because it is generally considered to be impermeable across biological membranes.

The addition of 0.5 mM  $\text{Na}^+$  (monovalent), 0.5 mM  $\text{Mg}^{2+}$  (divalent), and 0.5 mM  $\text{Ca}^{2+}$  (divalent), all as chloride salts, decreased Zn uptake by 20, 53, and 85%, respectively, from a control uptake rate of 0.039  $\mu\text{g Zn}\cdot\text{g body}^{-1}\cdot\text{h}^{-1}$  (Fig. 3). The effect of 0.5 mM  $\text{Ca}^{2+}$  was significantly greater than

**Fig. 1.** Effects of (A) NaCl and (B) NaNO<sub>3</sub> on whole-body Zn uptake measured with <sup>65</sup>Zn as a tracer in juvenile rainbow trout. The total Zn concentration of the water was 100 µg·L<sup>-1</sup>. Values are expressed as means ± SE; N = 7. One asterisk denotes a significant decrease in Zn uptake in the control treatment as determined by a one-way ANOVA followed by Tukey's HSD test (*p* < 0.050, two asterisks denote *p* < 0.01, and three asterisks denote *p* < 0.001).



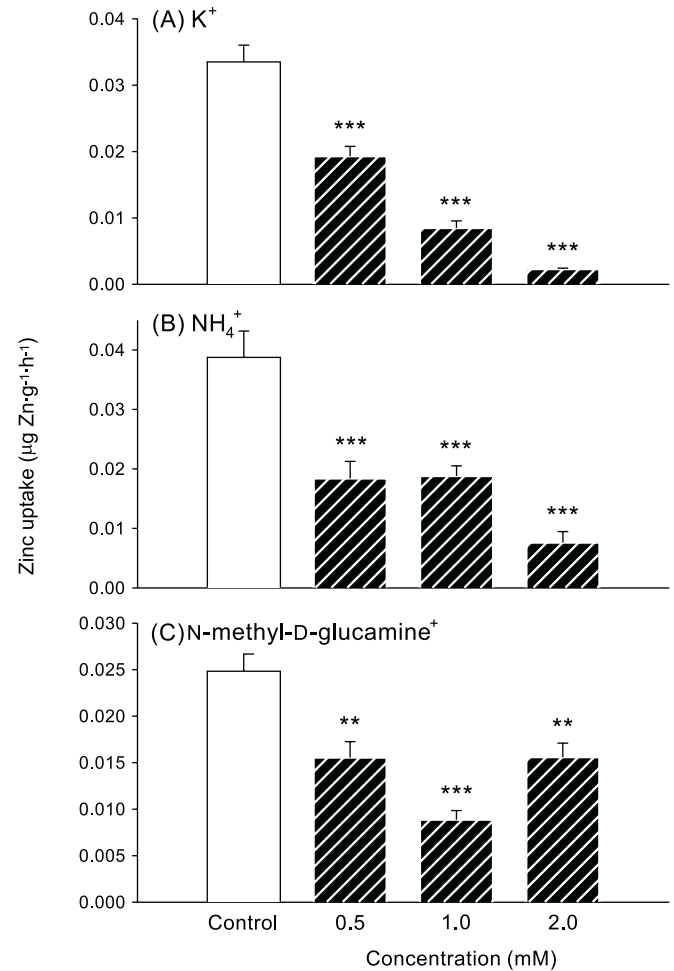
that of 0.5 mM Mg<sup>2+</sup>, which in turn was significantly greater than that of 0.5 mM Na<sup>+</sup>.

When pH was decreased by about 2 pH units from 5.6 to 3.7 (a 100-fold increase in H<sup>+</sup> concentration using HCl), Zn uptake was significantly decreased by 74% from 0.007 ± 0.00021 to 0.0018 ± 0.00012 µg Zn·g body<sup>-1</sup>·h<sup>-1</sup> (*p* < 0.001). As pH 3.7 represents the addition of about 0.2 mM H<sup>+</sup>, protons were clearly more potent than the other cations tested in antagonizing Zn uptake.

Acute versus chronic effects of cation exposure were evaluated. Whether trout were acclimated to soft water plus 1.0 mM Na<sup>+</sup> for 3 weeks prior to testing or were acutely exposed to 1.0 mM Na<sup>+</sup> only at the time of the experiment, the presence of 1.0 mM Na<sup>+</sup> in the water had the same effect in decreasing Zn uptake (Fig. 4). This was also the case for Ca<sup>2+</sup>, where acclimation to 1.0 mM Ca<sup>2+</sup> had the same effect on reducing the uptake rate of Zn as did acute exposure to 1.0 mM Ca<sup>2+</sup>, in comparison with control trout (Fig. 4).

In the test to determine the internal distribution of radio-labelled Zn taken up from the water over the 7-h period, the majority (71%) was in the carcass in the control treatment

**Fig. 2.** Effects of (A) KCl, (B) NH<sub>4</sub>Cl, and (C) *N*-methyl-D-glucamine chloride (with a single positive charge) on whole-body Zn uptake. Refer to Fig. 1 caption for more details.



(Fig. 5B). The gills, which accounted for only 2.0% of the body weight, held 17% of the Zn, the blood, which accounted for 5% of the body weight (Olson 1992) held 10% of the Zn, and the skin, which accounted for 0.7% of the body weight, held only 2.5% of the Zn (Fig. 5B). In this experiment, the presence of 1 mM NaCl reduced overall Zn uptake by 30% (Fig. 5A). By far, the largest absolute reduction was in the carcass (29%), with most of the remaining reduction occurring in the gills. There was no significant reduction in the blood or skin components. This result shows that the presence of the competing cation inhibits mainly internal Zn accumulation and is not just acting by displacing Zn from the body surface.

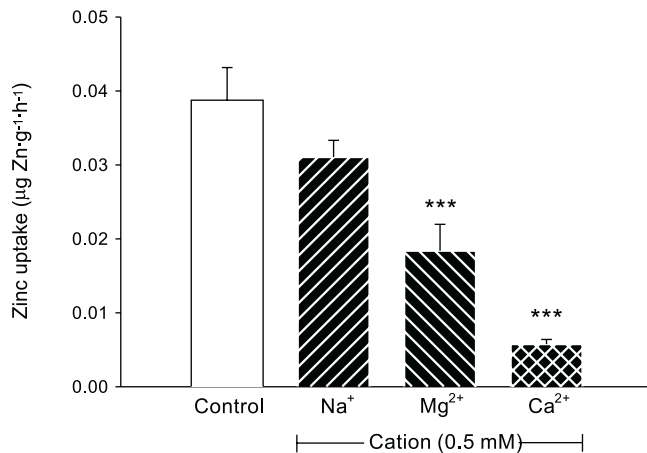
**Toxicity testing**

The addition of 2 mM NaCl or 1 mM MgCl<sub>2</sub> to the water had no effect on acute Zn toxicity, as evaluated by 96-h LC<sub>50</sub> tests (Fig. 6A). In contrast, the addition of 1 mM CaCl<sub>2</sub> greatly reduced Zn toxicity, as indicated by the 18-fold increase in the LC<sub>50</sub> from a control value of 103 µg·L<sup>-1</sup> to 1800 µg·L<sup>-1</sup> (Fig. 6A).

**Ca<sup>2+</sup> uptake**

Exposure to 100 µg Zn·L<sup>-1</sup> in control soft water signifi-

**Fig. 3.** Effects of 0.5 mM NaCl, MgCl<sub>2</sub>, and CaCl<sub>2</sub> on whole-body Zn uptake. Refer to Fig. 1 caption for more details.



cantly reduced Ca<sup>2+</sup> uptake by 87% (Fig. 6B). The addition of 2 mM NaCl, 1 mM MgCl<sub>2</sub>, or 0.2 mM HCl (pH 4.0; data not shown) did not diminish the effect of Zn on Ca<sup>2+</sup> uptake over 7 h. However, the addition of 1 mM CaCl<sub>2</sub> significantly increased Ca<sup>2+</sup> uptake back to 54% of the control rate, a value that was not significantly different from the control (Fig. 6B). In addition, short-term (0–5 h) or long-term (up to 35 h) exposure to Zn with the addition of 0.2 mM HCl (pH 4.1) did not restore Ca<sup>2+</sup> uptake (Table 2).

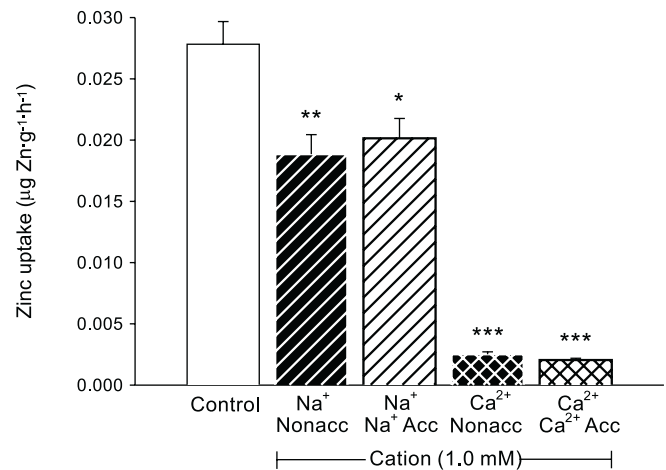
## Discussion

### Zn uptake

Whole-body Zn uptake was reduced in the presence of a variety of positively charged waterborne ions. The magnitude of the reduction was directly related to the concentration of positive charges in the water (e.g., equivalents), regardless of which element or compound carried that charge (except for Ca<sup>2+</sup> and H<sup>+</sup>, as discussed below). Thus, on a molar basis, divalent Mg<sup>2+</sup> was about twice as potent as the monovalent cations. We interpret the slight differences among different monovalent cations on inhibition of Zn uptake as variation of the effect as opposed to real differences in inhibition potency (except for Ca<sup>2+</sup> and H<sup>+</sup>, as discussed below). For example, 0.5 mM Na<sup>+</sup> decreased Zn uptake from control treatments by 26% (Fig. 1A), 33% (Fig. 1B), and 20% (Fig. 3) in three different trials. Free Zn<sup>2+</sup> activity would not have changed significantly with the addition of each salt as a result of ionic strength effects as predicted by the Debye–Hückel equation for the range of concentrations tested in the present study (up to 2 mM). Significant reductions in activity would occur when salts exceed about 10 mM (Stumm and Morgan 1996).

Significant reductions of Zn uptake were observed at environmentally realistic freshwater concentrations of Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> (see Table 1 for typical freshwater Lake Ontario ionic composition, “hard water”). The Zn exposure concentrations in the uptake studies were also in the range of environmental relevance. While normal Zn levels in clean freshwaters are only a few micrograms per litre or less, con-

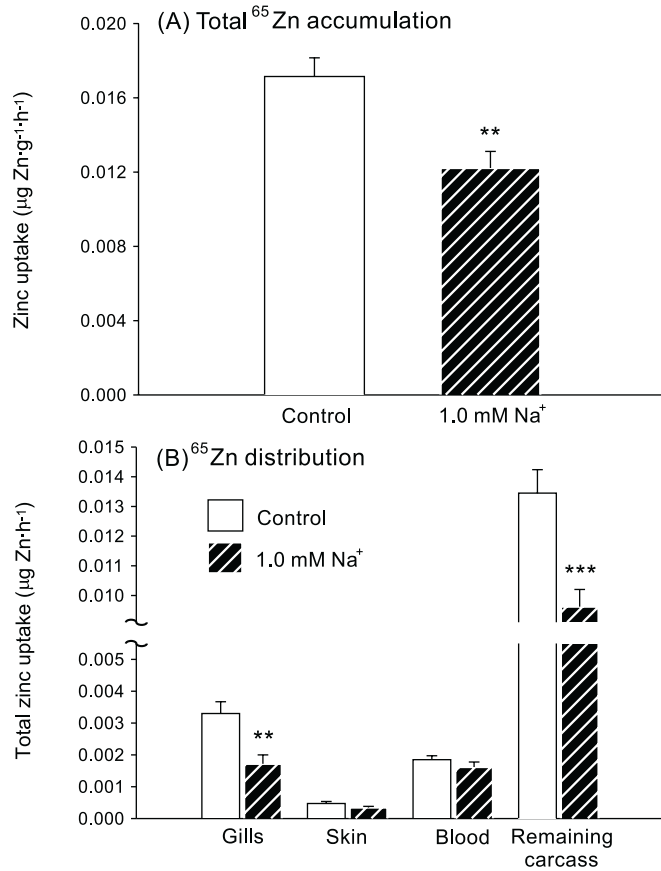
**Fig. 4.** Effects of 1 mM NaCl and 1 mM CaCl<sub>2</sub> on whole-body Zn uptake in 1 mM NaCl and 1 mM CaCl<sub>2</sub> acclimated (3 weeks) and nonacclimated rainbow trout. Refer to Fig. 1 caption for more details.



centrations of 50 µg·L<sup>-1</sup> are found in industrialized areas. Maximum Zn concentrations in natural surface waters are reported to range from 130 to 1170 µg·L<sup>-1</sup> in different areas of Canada (Canadian Council of Ministers of the Environment 1995). The Zn concentration used in the uptake studies was the same as our measured 96-h LC<sub>50</sub> (~100 µg·L<sup>-1</sup>) (Fig. 6A). The Zn uptake rates in the present study were comparable with those reported by Hogstrand et al. (1998) at 100 µg Zn·L<sup>-1</sup> in juvenile rainbow trout in very soft water (Ca<sup>2+</sup> free). They were also comparable with uptake rates at total Zn concentrations near the LC<sub>50</sub> (~900 µg·L<sup>-1</sup>, Alsop et al. 1999) in Hamilton hard water (Hogstrand et al. 1998). Nevertheless, the Zn uptake rate did vary over the course of the present study from a high of 0.039 µg·g<sup>-1</sup>·h<sup>-1</sup> (Figs. 2B and 3) to a low of 0.007 µg·g<sup>-1</sup>·h<sup>-1</sup> (Zn uptake and pH trial) in different control series. The lower rates occurred towards the end of the study when the fish were larger. Notably, Wagner et al. (1985) found that branchial Ca<sup>2+</sup> uptake cycled between periods of low uptake and high uptake in rainbow trout. The difference in Ca<sup>2+</sup> uptake rates varied on the order of fivefold, while the duration of the cycles varied from 7 to 17 days; hormonal changes were thought to be involved, and Zn uptake may follow a similar trend. However, nothing is known about possible corresponding trends in Zn efflux, an important topic for future research.

Ca<sup>2+</sup> had a much greater effect inhibiting Zn uptake than the other divalent cation tested, Mg<sup>2+</sup> (Fig. 3). This is likely because Zn<sup>2+</sup> and Ca<sup>2+</sup> would not only compete on the gill surface for binding sites (adsorption) but would also compete for absorption, since they share the same apical transport channel (Hogstrand et al. 1996). Zn and Ca<sup>2+</sup> may also interact with each other inside the gill cell, where, for example, Zn has been shown to decrease Ca<sup>2+</sup> uptake by both the endoplasmic reticulum and mitochondria (Verboost et al. 1996). Zn is also a potent inhibitor of the basolateral CaATPase transporter in the trout gill (Hogstrand et al. 1996). Increasing waterborne Ca<sup>2+</sup> may also elicit stannocalcin release into the blood, which would cause closure of

**Fig. 5.** (A) Effect of 1 mM NaCl on whole-body Zn uptake and (B) partitioning of <sup>65</sup>Zn into the gills, skin, blood, and remaining carcass of rainbow trout. Uptake was significantly reduced in the gills and remaining carcass exposed to 1 mM Na<sup>+</sup>. The 1 mM Na<sup>+</sup> did not have an effect on the amount of <sup>65</sup>Zn bound to the exterior of the fish. *N* = 8. Refer to Fig. 1 caption for more details.



**Table 2.** Whole-body Ca<sup>2+</sup> uptake (µg Ca·g<sup>-1</sup>·h<sup>-1</sup>) in control soft water and during exposure to 100 µg Zn·L<sup>-1</sup> at pH 5.8 (control pH) or 100 µg Zn·L<sup>-1</sup> at pH 4.1 over 35 h.

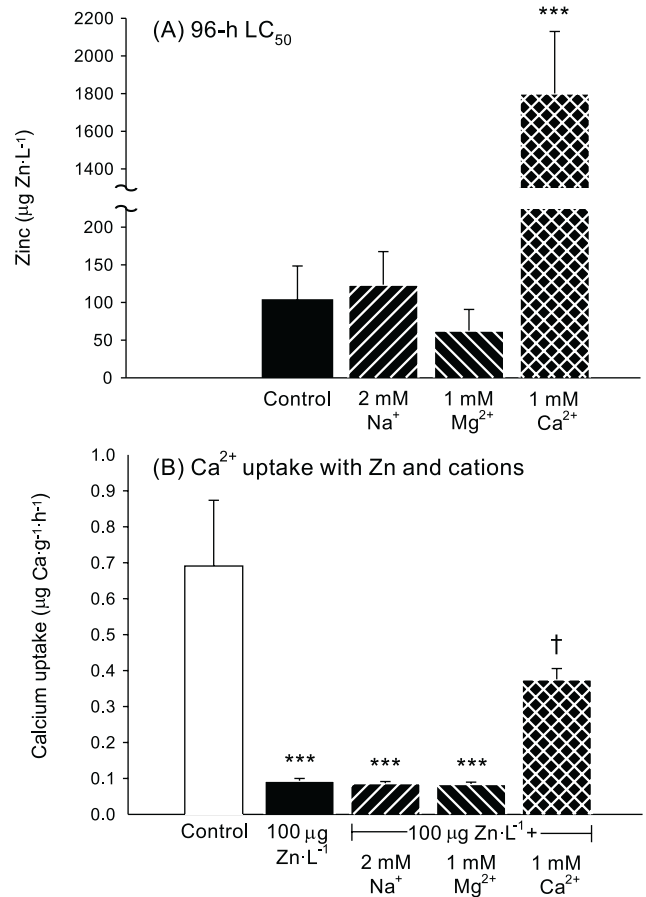
Time (h)	Control	100 µg Zn·L <sup>-1</sup>	100 µg Zn·L <sup>-1</sup> at pH 4.1
-5 to 0	3.05±0.24		
0-5		0.40±0.17***	0.22±0.01***
8-13		0.21±0.01***	0.18±0.01***
30-35		0.20±0.01***	0.17±0.002***

**Note:** For the 8- to 13-h and 30- to 35-h <sup>45</sup>Ca exposures, rainbow trout were initially held in identical Zn and pH waters without <sup>45</sup>Ca until they were used in the <sup>45</sup>Ca flux. Values are expressed as means ± SE; *N* = 8. One asterisk denotes a significant decrease in Ca<sup>2+</sup> uptake from the control treatment as determined by a one-way ANOVA followed by Tukey's HSD test (*p* < 0.05), two asterisks denote *p* < 0.01, and three asterisks denote *p* < 0.001.

the apical Ca<sup>2+</sup>/Zn<sup>2+</sup> channels in the gill and decrease both Zn and Ca<sup>2+</sup> uptake (Wendelaar Bonga and Pang 1991).

NH<sub>4</sub><sup>+</sup> had the same effect in reducing Zn uptake as the other monovalent cations that were tested (Fig. 2B). This indicates that not only can cations from the water affect Zn uptake, but cations that are excreted by the fish at the gills

**Fig. 6.** (A) 96-h LC<sub>50</sub> values in control water alone (no additions except for Zn) and with the addition of 2 mM NaCl, 1 mM MgCl<sub>2</sub>, or 1 mM CaCl<sub>2</sub>. Values are expressed as means ± the average of the upper and lower 95% CL. Refer to Fig. 1 caption for more details. (B) Ca<sup>2+</sup> uptake in control soft water alone (no additions) and with the addition of 100 µg Zn·L<sup>-1</sup> or either 2 mM NaCl, 1 mM MgCl<sub>2</sub>, or 1 mM CaCl<sub>2</sub> in addition to 100 µg Zn·L<sup>-1</sup>. Values are expressed as means ± SE; *N* = 8. One asterisk denotes a significant decrease in Ca<sup>2+</sup> uptake from the control treatment as determined by a one-way ANOVA followed by Tukey's HSD test (*p* < 0.05), two asterisks denote *p* < 0.01, and three asterisks denote *p* < 0.001. A dagger indicates a significant increase in Ca<sup>2+</sup> uptake over the 100 µg Zn·L<sup>-1</sup> treatment (*p* < 0.001). Note that the 1 mM CaCl<sub>2</sub> mean was not significantly different (*p* > 0.05) from the control mean.



may also affect uptake. Zn uptake may therefore be reduced during times of high ammonia excretion, which can occur, for example, after feeding (Kaushik 1980; Beamish and Thomas 1984; Alsop and Wood 1997).

Cationic competition most probably occurs at the apical surface of the gill. *N*-Methyl-D-glucamine<sup>+</sup> is a molecule with a single positive charge that is generally considered to be impermeant to biological membranes (e.g., Song and Chueh 1996), so any effects from the presence of that molecule can only occur in the water or at the gill surface. Therefore, the reduction in Zn absorption (Fig. 2C) was due to decreased Zn adsorption to the gill surface due to competition with the charge carried by *N*-methyl-D-glucamine<sup>+</sup>.

Decreasing the pH to 3.7 (adding 0.2 mM H<sup>+</sup>) reduced Zn uptake to a greater extent than did the other monovalent ions tested. With the drop in pH over this range, there would be no change in Zn speciation as determined by the aquatic geochemical program MINEQL+ (Schecher and McAvoy 1994), which predicts >99% of the Zn to exist in the "free" form (Zn<sup>2+</sup>) in both control and decreased-pH test waters. H<sup>+</sup> is not known to be transported through the Ca<sup>2+</sup>/Zn<sup>2+</sup> channel, and therefore, direct competition should not be occurring there. Handy and Eddy (1991) found an increased potency of H<sup>+</sup> in comparison with Ca<sup>2+</sup> in reducing Na<sup>+</sup> adsorption and absorption in rainbow trout. They attributed this to the increased mobility of H<sup>+</sup> due to its decreased "charge density" in comparison with Ca<sup>2+</sup>. An alternative mechanism may be direct acid damage to the Zn<sup>2+</sup>/Ca<sup>2+</sup> transport system or an indirect effect, such as stimulation of mucus secretion by low pH (McDonald 1983). Mucus production may slough Zn off before it can be taken up into the gill, and once the mucus is sloughed off, it may even bind Zn in the water, keeping it off the gill.

Pagenkopf (1983) hypothesized that H<sup>+</sup> would compete for the negative sites on the gill surface, forming Lewis acid-base complexes, unlike Na<sup>+</sup> and K<sup>+</sup>. Playle et al. (1993) showed that H<sup>+</sup> had a high affinity for Cd-binding sites on the gills, with log *K* stability constants for Cd, H<sup>+</sup>, and Ca<sup>2+</sup> of 8.6, 6.7, and 5.0, respectively. Like Zn, Cd disrupts Ca<sup>2+</sup> homeostasis (Verboost et al. 1989). Galvez et al. (1998) estimated the log *K* value for Zn<sup>2+</sup> and trout gills to be about 5.5. Richards and Playle (1998) found a similar pattern of potency in their study of Co binding to rainbow trout gills, and Co is also known to interfere with Ca<sup>2+</sup> uptake (Comhaire et al. 1994). Richards and Playle (1998) reported that H<sup>+</sup> was the most potent cation reducing Co accumulation in the gills, followed by Ca<sup>2+</sup> and then Na<sup>+</sup>.

### Toxicity testing

From the present study, it appears that any cation may possess the capability of reducing the uptake of Zn at trout gills. Simkiss and Taylor (1989) stated that anything that obstructs the initial adsorption of a metal will reduce its toxicity. The present study showed that this is not always the case. Although Na<sup>+</sup> and Mg<sup>2+</sup> were able to reduce Zn uptake, presumably through decreased adsorption to the gill (Pagenkopf 1983) (Figs. 1 and 3), they did not ameliorate Zn toxicity (Fig. 6A). In contrast, Ca<sup>2+</sup> strongly reduced both Zn uptake and Zn toxicity (Figs. 3 and 6A).

One explanation may be that Na<sup>+</sup> and Mg<sup>2+</sup> may indeed reduce Zn uptake by decreasing adsorption to Zn uptake sites, but these may not be the sites related to Zn toxicity. Ca<sup>2+</sup> may reduce adsorption to both nontoxic Zn uptake sites and to a separate population of toxic Zn binding sites. Another explanation may be that cations such as Na<sup>+</sup> are reducing nontoxic Zn uptake, while Ca<sup>2+</sup> reduces both toxic and nontoxic Zn uptake (as opposed to binding). A third possibility relates to effects on Ca<sup>2+</sup> uptake. Zn specifically induces hypocalcemia by inhibiting branchial Ca<sup>2+</sup> uptake (Spry and Wood 1985; Hogstrand et al. 1995), and increased waterborne Ca<sup>2+</sup> may simply reduce the symptoms of Zn toxicity by preventing this effect. Indeed, we found that 2 mM Na<sup>+</sup> and 1 mM Mg<sup>2+</sup> did not restore Zn-induced Ca<sup>2+</sup> uptake inhibition (Fig. 6B), nor did they reduce Zn-induced

mortality (Fig. 6A). However, 1 mM Ca<sup>2+</sup> was able to restore branchial Ca<sup>2+</sup> uptake that had been inhibited by 100 µg Zn·L<sup>-1</sup> (Fig. 6B), a Zn concentration that was equivalent to the 96-h LC<sub>50</sub> in control soft water (Fig. 6A). In addition, 1 mM Ca<sup>2+</sup> simultaneously increased the LC<sub>50</sub> to 1800 µg Zn·L<sup>-1</sup>, a Zn concentration that was probably high enough to inhibit branchial Ca<sup>2+</sup> uptake again. These results partially dissociate Zn uptake from acute Zn toxicity and reinforce the view that Zn-induced inhibition of Ca<sup>2+</sup> uptake is the direct cause of toxicity.

Both Bradley and Sprague (1985) and Cusimano et al. (1986) observed a protective effect of decreased pH (increased H<sup>+</sup>) at nontoxic levels (pH ≥ 4.7) against waterborne Zn toxicity. Cusimano et al. (1986) speculated that H<sup>+</sup> interference with metal uptake at the gill was the primary mechanism that decreased metal toxicity (interestingly, they found that H<sup>+</sup> also protected against Cu and Cd toxicity). In the present study, however, a higher [H<sup>+</sup>] (pH 4.0, close to the toxic range) did not restore Ca<sup>2+</sup> uptake over 7 h of exposure. Hobe et al. (1984) found H<sup>+</sup> (pH 4.0–4.2) alone initially reduced Ca<sup>2+</sup> uptake in rainbow trout by two thirds from 0 to 12 h, but Ca<sup>2+</sup> uptake was fully restored by 18–24 h. We suspected that if pH was protective, it may require a longer period of time. However, we found no effect of H<sup>+</sup> (pH 4.1) on restoring Zn-induced Ca<sup>2+</sup> uptake inhibition again over short-term (0–5 h) or long-term (up to 35 h) exposures (Table 2). If H<sup>+</sup> is truly protective against Zn toxicity, it may protect via a different mechanism than does Ca<sup>2+</sup>.

With respect to Zn, there is also a general acceptance of the principle that acute toxicity is related to the levels of waterborne Ca<sup>2+</sup> and Mg<sup>2+</sup>, which is termed "hardness" (Canadian Council of Ministers of the Environment 1995). For example, the U.S. Environmental Protection Agency (1980) employs an equation based on hardness (milligrams of CaCO<sub>3</sub> per litre) to calculate a numerical limit of total allowable waterborne Zn:

$$(3) \quad [\text{Zn}] = e^{(0.83[\ln(\text{hardness})]+1.95)} \mu\text{g}\cdot\text{L}^{-1}.$$

The present study has shown that while Ca<sup>2+</sup> protects against acute Zn toxicity, Mg<sup>2+</sup> does not. Therefore, eq. 3 would overestimate safe Zn levels in waters where a significant percentage of the total hardness was contributed by Mg<sup>2+</sup> (e.g., coastal freshwaters), and aquatic life would not be adequately protected. The higher the Mg<sup>2+</sup> levels of the water, the more eq. 3 would overestimate the total allowable Zn levels. For example, eq. 3 would calculate that in the control soft water used in the LC<sub>50</sub> trials (Table 1), 40 µg Zn·L<sup>-1</sup> would be the total allowable limit, and presumably protective against acute Zn toxicity to aquatic life. The 96-h LC<sub>50</sub> was determined to be 103 µg Zn·L<sup>-1</sup> in soft water (Fig. 6A), and in this case, eq. 3 may be protective. With the addition of 1.0 mM Ca<sup>2+</sup> to soft water, eq. 3 would calculate a total allowable limit to be 343 µg Zn·L<sup>-1</sup>, while the LC<sub>50</sub> was 1800 µg Zn·L<sup>-1</sup>, evidently protective. However, with the addition of 1.0 mM Mg<sup>2+</sup>, eq. 3 would similarly calculate a total allowable limit to be 343 µg Zn·L<sup>-1</sup>. This is 5.5-fold greater than the measured LC<sub>50</sub> of 63 µg Zn·L<sup>-1</sup> (Fig. 6A). Clearly, this may pose harmful consequences to aquatic life.

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