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PROTECTIVE EFFECTS OF CALCIUM AGAINST CHRONIC WATERBORNE CADMIUM EXPOSURE TO JUVENILE RAINBOW TROUT

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Abstract—Juvenile rainbow trout (*Oncorhynchus mykiss* [Walbaum]) on 1% daily ration were exposed to 0 (control) or 2 μg of cadmium as $Cd(NO_3)_2 \cdot 4H_2O$ per liter added to four different calcium (Ca) concentrations: 260 (background), 470 (low), 770 (medium), or 1200 (high) μM of Ca added as $Ca(NO_3)_2 \cdot 4H_2O$ in synthetic soft water for 30 d. Mortality was highest (~80%) in the background + Cd treatment. Approximately 40% mortality was observed in the low + Cd exposure; mortality was 10% or less for all other treatments. No growth effects were seen for any of the exposures. Kidneys accumulated the greatest concentration of Cd during the 30 d, followed by gills and livers. Accumulation of Cd in gills, kidney, and liver decreased at higher water Ca concentrations. No differences in whole-body or plasma Ca concentrations were found. Swimming performance was impaired in the low + Cd-exposed fish. Influx of Ca^{2+} into whole bodies decreased as water Ca concentrations increased; influx of Ca^{2+} into background + Cd–treated fish was significantly reduced compared to that in control fish. Experiments that measured uptake of new Cd into gills showed that the affinity of gills for Cd ($K_{Cd\text{-}gill}$) and the number of binding sites for Cd decreased as water Ca concentrations increased. Acute accumulation of new Cd into gills and number of gill Cd-binding sites increased with chronic Cd exposure, whereas the affinity of gills for Cd decreased with chronic Cd exposure. Longer-term gill binding (72 h) showed reduced uptake of new Cd at higher water Ca levels and increased uptake with chronic Cd exposure. Complications were found in applying the biotic ligand model to fish that were chronically exposed to Cd because of discrepancies in the maximum number of gill Cd-binding sites among different studies.

Keywords—Cadmium Calcium Gill binding Modeling Rainbow trout

INTRODUCTION

Waterborne cadmium (Cd) can cause severe, acute toxicological and physiological effects to aquatic organisms. However, these effects can be altered by water hardness [1–6]. Carrol et al. [7], Pärt et al. [2], and McDonald et al. [8] have shown that protection against Cd uptake and acute toxicity in freshwater fish is related to the water concentration of calcium (Ca), rather than of magnesium (Mg), illustrating that Ca is the primary cation responsible for the protective action of hard water. This protective action of Ca has been attributed to changes in gill permeability and/or competition between Cd and Ca for gill-binding sites [2,9–13].

The great majority of research has focused on acute Cd toxicity, but a number of studies have shown that Cd may cause sublethal deleterious effects during low-level, chronic exposures [14–20]. Water hardness is protective against chronic as well as acute Cd toxicity in freshwater fish [21], and a hardness correction has been incorporated into ambient water-quality criteria for chronic Cd exposures in Europe [22], the United States [23], and Canada [24]. The primary objective of the present study was to determine the mechanistic basis for the protective effects of Ca against chronic Cd exposure in juvenile rainbow trout. We examined changes in gill Cd burden and the acute Cd-binding properties of the gills accompanying 30-d exposure of trout in synthetic soft water supplemented with various Ca concentrations (260, 470, 770, or 1200 μM) in the presence or absence of 2 μg Cd/L. A particular focus was placing the gill-binding results into a biotic ligand modeling framework, which has recently been advocated for the generation of site-specific water-quality criteria [13,25–28]. Additional goals were to characterize Cd accumulation in other compartments (liver, kidney, and whole body) and to document possible sublethal effects as well as costs of chronic Cd exposure, as expressed in growth on submaximal ration, whole-body and plasma ion content, gill Ca^{2+} influx, and exercise performance.

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MATERIALS AND METHODS

Fish holding conditions

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from Humber Springs Trout Farm in Orangeville, Ontario, Canada, and were held in flowing dechlorinated Hamilton tap water (Lake Ontario water: Ca = 40 mg/L or 1 mM, sodium [Na] = 14 mg/L or 0.60 mM, chloride [Cl] = 25 mg/L or 0.70 mM, dissolved organic matter [DOM] = 3 mg/L or 0.25 mM, hardness = 140 mg/L as $CaCO_3$, alkalinity = 95 mg/L as $CaCO_3$, pH 8.0, 14°C). Trout were held in 600-L aerated polyethylene tanks for two months and then slowly introduced to synthetic soft water during the course of one week. The synthetic soft water (Ca = 10 mg/L or 0.26 mM, Na = 3 mg/L or 0.14 mM, Cl = 4 mg/L or 0.10 mM, DOM = 0.40 mg/L or 0.03 mM, hardness = 20 mg/L as $CaCO_3$, alkalinity = 15 mg/L as $CaCO_3$, pH 7.2, 13°C) was produced by reverse osmosis (Anderson Water Systems, Dundas, ON, Canada) and consisted of one part dechlorinated Hamilton tap water added to six parts ion-reduced water produced by reverse osmosis. Fish were held in soft water for at least three weeks

Table 1. Measured water chemistry parameters and mortality at 30 d for the eight exposure treatments. Means ± 1 SE[a] (n = 16)

Treatment	Nominal concentration	Measured chemistry				Mortality at 30 d
		Ca (μM)	Cd (μg/L)	Na (μM)	Cl (μM)	
Background	260 μM Ca	257 ± 19	0.08 ± 0.06	141 ± 48	111 ± 52	0%
Background +Cd	260 μM Ca +2 μg/L Cd	270 ± 22	3.00 ± 0.02	143 ± 49	112 ± 55	78%
Low	470 μM Ca	445 ± 20	0.02 ± 0.02	140 ± 47	109 ± 50	1%
Low +Cd	470 μM Ca +2 μg/L Cd	502 ± 16	3.20 ± 0.20	140 ± 49	111 ± 51	39%
Medium	770 μM Ca	938 ± 21	0.09 ± 0.09	101 ± 27	66 ± 33	9%
Medium +Cd	770 μM Ca +2 μg/L Cd	602 ± 108	1.60 ± 0.30	104 ± 28	68 ± 34	7%
High	1200 μM Ca	1235 ± 73	0.04 ± 0.04	103 ± 28	67 ± 35	7%
High +Cd	1200 μM Ca +2 μg/L Cd	1218 ± 128	1.60 ± 0.20	96 ± 22	55 ± 31	10%

[a] SE = standard error

before experimentation. Fish were fed 1% body weight per day (as one meal per day) with Martin's Starter Food (Martin Feed Mills, Elmira, ON, Canada; Cd content = 1.06 ± 0.04 [n = 6] μg/g [wet wt]).

Exposure system

After three weeks in holding tanks, 85 fish were randomly transferred to each of sixteen 200-L polyethylene exposure tanks, which were flowthrough systems (flow = 500 ml/min) with continuous aeration. Fish were fed a submaximal ration of 1% body weight per day (discussed earlier), compared with a typical ration of 3% body weight per day [20], in the hope of revealing metabolic costs associated with chronic Cd exposure. An acidified Cd stock solution, with Cd added as $Cd(NO_3)_2 \cdot 4H_20$, and a Ca stock solution, with Ca added as $Ca(NO_3)_2 \cdot 4H_20$, were delivered to a mixing head-tank via mariotte bottles [29] to achieve the desired Cd and Ca concentrations in the exposure tanks. Tanks were spiked on the first day of exposure to instantly reach the desired Cd and Ca concentrations. Water chemistry was measured weekly throughout the exposure period. Unless otherwise noted, all chemicals were obtained from Fisher Scientific (Nepean, ON, Canada), and all radioisotopes were obtained from New England Nuclear (Boston, MA, USA).

Fish were exposed to four concentrations of Ca in either the absence or the presence of Cd at a nominal concentration of approximately 2 μg/L. This concentration was chosen based on an initial 96-h LC50 measurement of approximately 2 μg Cd/L in the background Ca concentration of 260 μM. Each of the eight treatment conditions had two replicates, so n = 170 fish per treatment. The eight exposures (nominal concentrations) were (1) background Ca (260 μM) with zero cadmium, (2) background Ca (260 μM) + 2 μg Cd/L, (3) low Ca (470 μM) with no added Cd, (4) low Ca (470 μM) + 2 μg Cd/L, (5) medium Ca (770 μM) with no added Cd, (6) medium Ca (770 μM) + 2 μg Cd/L, (7) high Ca (1200 μM) with no added Cd, and (8) high Ca (1200 μM) + 2 μg Cd/L for 30 d in synthetic soft water. Actual measured water Ca and Cd concentrations are presented in Table 1. Treatments are referred to as background, low, medium, and high Ca, and as background + Cd, low + Cd, medium + Cd, and high + Cd.

Sampling

During the 30-d Cd exposure, 16-ml water samples were taken throughout the exposure period. These samples were acidified with 50 μl of HNO_3 and then analyzed for Na, Ca, Cl, and total Cd content (Table 1). Fish from each treatment tank were bulk weighed every 10 d, and specific growth rates were determined according to the procedure described by Hollis et al. [20].

Six fish from each tank were subsampled at days 0, 2, 10, 20, and 30, and the gills, liver, kidney, and remaining carcass were assayed for Cd content. The remaining carcass was also assayed for whole-body ion content. Fish were sacrificed, and both sets of gills and the liver were excised. Gills were rinsed for 10 s in 100 ml of dechlorinated Hamilton tap water. All tissues plus remaining carcasses were frozen until analysis of Cd and ion content. Six additional fish from each tank were also sampled at days 0, 2, 10, 20, and 30 for plasma Ca concentrations. Fish were sacrificed, and blood samples (40–285 μl) were taken by caudal puncture with 1 cm^3 syringes. Blood samples were centrifuged for 2 min. The plasma was then removed, and the sample was stored at −70°C until the analysis of Ca content.

Testing

Exercise performance. Fish were not fed on the day of swimming tests. Swimming performance was determined using the protocol of McDonald et al. [30], which is a stamina test that uses a fixed velocity (60 cm/s; ~5 body lengths/s) and exhaustion as the endpoint. Sprint times were corrected to a reference body length of 11 cm, and the time to 50% fatigue (± 1 SE) was calculated using 10 fish from each treatment by linear regression analysis in SPSS® (Chicago, IL, USA) of probit fatigue versus log time. Fish were returned to their holding tanks following swim testing.

Ca^{2+} influx. Unidirectional Ca^{2+} uptake into trout was determined by exposing fish (eight fish from each treatment) for 4 h, after the 30-d Cd exposure to radioactive ^{45}Ca, according to the method described by Hogstrand et al. [31].

Acclimation. A 96-h LC50 trial was run after the 30-d exposure to assess possible acclimation of metal-exposed fish. Each test cell consisted of eight fish placed randomly into 15-L polypropylene buckets having aeration and flowthrough (200 ml/min) of dechlorinated Hamilton tap water at the appropriate Ca and Cd level, as added by a mariotte bottle. A total of 48 fish (eight fish for each of the six LC50 test concentrations) were taken from the background and low Ca treatments and were exposed for 96 h to Cd concentrations of 0.07 ± 0.03, 0.28 ± 0.05, 0.95 ± 0.17, 3.28 ± 0.30, 14.58 ± 3.35, or 16.95

± 2.85 μg Cd/L with the appropriate Ca concentration (250 or 470 μM); the number of water samples (*n*) for each Cd concentration was four. Similarly, a total of 48 fish (eight fish per LC50 test concentration) from the medium and high Ca treatments were exposed for 96 h to Cd concentrations of 0.20 ± 0.05, 4.78 ± 1.69, 15.60 ± 0.30, 41.80 ± 4.82, 139.39 ± 27.45, or 247.77 ± 61.76 μg Cd/L with the appropriate Ca concentration (770 or 1200 μM); again, the number of samples for each Cd concentration was four. Dead fish were removed when movement ceased, and times of mortality were recorded. The LC50 values (± 95% confidence limits) were determined by log probit analysis [32].

Acute and longer-term gill Cd binding. In a previous report [20], we showed that use of radiolabeled ^{109}Cd is essential to distinguish new gill Cd uptake relative to the high background cold concentrations already present in the gills of chronically exposed fish. Acute gill metal uptake/turnover of Cd was determined by exposing the fish for 3 h, after the 30-d Cd exposure, to various concentrations of Cd labeled with the radioisotope ^{109}Cd. Fish were not fed on the day of the gill-binding experiments. A total of 25 fish (five per test concentration) from each of seven treatments (the low + Cd treatment was excluded due to the low number of surviving fish available) were placed into 35 clear bags containing 10 L of aerated, soft water (260 μM Ca), which were then placed in a water bath to maintain temperature (13°C). Each treatment group was exposed to the appropriate acclimation Ca concentration (260, 470, 770, or 1200 μM Ca, added as $Ca[NO_3]_2 \cdot 4H_20$) plus 6 ± 1 μg Cd/L, 14 ± 1 μg Cd/L, 27 ± 1 μg Cd/L, 63 ± 2 μg Cd/L, or 115 ± 2 μg Cd/L. The number of samples for each Cd concentration was 14, and Cd was added as $Cd(NO_3)_2 \cdot 4H_20$, with 1 μCi/L of ^{109}Cd added as $CdCl_2$ (specific activity = 1.97 mCi/mg). Water samples (5 ml) were taken at the beginning and end of the 3-h static exposure. Gills were sampled at 3 h. Gills were removed, rinsed, acid digested, and later analyzed for total Cd and ^{109}Cd radioactivity (see the discussion of tissue analyses).

A longer-term gill Cd uptake/turnover experiment was run after the 30-day Cd exposure by exposing the fish for 72 h to the Cd radioisotope ^{109}Cd at a total Cd concentration approximately threefold higher than the nominal chronic exposure concentration. Twenty fish from each treatment (or fewer, depending on availability) were placed into 10 clear bags containing 15 L of aerated, soft water (260 μM Ca), which were then placed in a water bath to maintain temperature (13°C). Each treatment group was exposed to the appropriate Ca concentration (260, 470, 770, or 1200 μM Ca, added as $Ca[NO_3]_2 \cdot 4H_20$) plus 6 ± 1 μg Cd/L (n = 5), with the Cd added as $Cd(NO_3)_2 \cdot 4H_20$, and 1 μCi/L ^{109}Cd added as $CdCl_2$ (specific activity = 1.97 mCi/mg). Water samples (5 ml) were taken daily during the 72-h static exposure. Gills of five fish were sampled at 12, 24, 48, and 72 h. Gills were removed, rinsed, digested in 1 N HNO_3, and later analyzed for total Cd and radioactivity from ^{109}Cd (see the discussion of tissue analyses).

Chemical analyses

Tissue analyses. The concentrations of all measured parameters in tissues were expressed on a per gram wet tissue basis.

Gills, livers, kidneys, and remaining carcass were thawed, weighed, and then digested in one- to 15-fold their weight of 1 N HNO_3 (TraceMetal Grade HNO_3, Fisher Scientific, Nepean, ON, Canada) for 15 h at approximately 60°C. Digests were shaken, left to settle for 10 min, and the supernatant then diluted 20- to 200-fold with deionized water, as appropriate (18 mgohm; Nanopure II, Sybron/Barnstead, Boston, MA, USA). Gill, liver, kidney, and carcass Cd concentrations were measured on a graphite furnace atomic absorption spectrophotometer (Varian AA-1275 with GTA-95 atomizer, Varian Techtron, Springvale, CA, USA) against Fisher certified standards, as outlined by Hollis et al. [33], using 10-μl injection volumes and N_2 gas. Operating conditions were as those described by Varian, with 30 s of drying time at 90°C, 12 s at 120°C, and 4 s at 1800°C, during which Cd was analyzed. The matrices for standards were the same as for unknowns, and samples were always read above the lowest standard on the calibration curve. Reproducibility on duplicate analyses was typically ±10%.

Whole-body Cd was calculated based on the data for individual fish at each sample time using the following equation:

$$WB = [(G \times gwt) + (L \times lwt) + (K \times kwt) + (C \times cwt)]/fwt$$

where *WB* is whole-body Cd accumulation (μg Cd/g wet tissue), *G* is gill Cd accumulation (μg Cd/g wet tissue), *L* is liver Cd accumulation (μg Cd/g wet tissue), *K* is kidney Cd accumulation (μg Cd/g wet tissue), *C* is carcass Cd accumulation (μg Cd/g wet tissue), *gwt* is the weight of the gills (g), *lwt* is the weight of the liver (g), *kwt* is the weight of the kidney (g), *cwt* is the weight of the carcass (g), and *fwt* is the weight of the fish (g). Gills, liver, kidney, and carcass represent 3.0%, 1.5%, 0.5%, and 95.0% of the total whole-body weight, respectively. Levels of Ca and Na in the carcass were measured by atomic absorption spectrophotometry using the dilutions from the acid digests and methods as used for the water and plasma samples (discussed later).

The frozen fish from the Ca influx experiment were transferred to liquid nitrogen and ground to a fine powder with a tissue grinder (Janke and Kunkel GMBH, IKA-Laboratories, Markham, ON, Canada). The powder was weighed out in triplicate samples of 0.5 g in glass scintillation vials. Each tissue sample was digested with 2.0 ml of liquid tissue solubilizer (Soluene-350, Canberra-Packard, Mississauga, ON, Canada) for 48 h at 45°C. The samples were then diluted with 10 ml of scintillation fluor (Hionic Fluor, Canberra-Packard) and counted in a scintillation counter (1217 Rackbeta Liquid Scintillation Counter, LKB-Wallac, Turku, Finland) with quench correction by internal standardization. The inward flux (J_{in}) for Ca^{2+} (in μM/kg/h) was calculated according to the formula described by Hogstrand et al. [31].

Tissue ^{109}Cd concentrations were measured on a Minaxi Auto-Gamma 5000 Series Gamma Counter (Canberra Packard Instrument, Meriden, CT, USA). Tissue ^{109}Cd concentrations were converted to absolute values (new Cd) using the measured specific activity (b/c) of the water:

$$a/(b/c)$$

where a is ^{109}Cd cpm per gram of tissue (wet weight), b is ^{109}Cd counts in the water (cpm/L), and c is the total Cd concentration in the water (μg Cd/L).

Gill Cd dissociation constants and capacity were calculated using Scatchard analysis as outlined by Reid and McDonald [34]. The amount of Cd bound by the gill was divided by the free ionic Cd^{2+} concentration in the water and plotted against the amount of Cd bound by the gill. The K_D and the total B_{max} of the gill were then determined from the slope and x-intercept of the Scatchard plot, respectively. All plots were linear, with

r values ranging from 0.71 to 0.99. Concentrations of Cd^{2+} and other Cd species in the water were calculated using the MINEQL+ aquatic geochemical program [35] and measured water chemistry.

Water and plasma analyses. Water and plasma Na and Ca concentrations were measured using the Varian AA-1275 operated in standard flame absorption mode. Water Cd concentrations were measured using the methods described for tissues. Water and whole-body Cl concentrations were measured on the acid digests using the colorimetric assay described by Zall et al. [36] and read with an MRX microplate reader (Dynatech Laboratories, Chantilly, VA, USA). Water pH was measured using a Radiometer PHM71b meter with GK2401C combination electrode (Radiometer, Copenhagen, Denmark). Dissolved organic matter was measured on a Rosemount Analytical DC-180 automated total organic carbon analyzer (Folio Instruments, Kitchener, ON, Canada).

Duplicate water samples from the Ca flux experiment were acidified and analyzed for calcium by atomic absorption spectroscopy (Varian AA-1275) and for Cd by graphite furnace (Varian AA-1275 with GTA-95 atomizer, Varian Techtron). The remaining duplicate water samples (5 ml each) were diluted with 10 ml of scintillation fluor (Hionic Fluor) and counted for ^{45}Ca.

Statistics

Data are expressed as means ± 1 SE (*n*), except in the case of LC50 values, in which means ± 95% confidence limits are reported. The LC50 values, specific growth rates, and swimming times were compared by means of the Bonferroni adjustment to the independent two-tailed Student's *t* test. For all other data, analysis of variance followed by a Student-Newman-Keuls procedure was used for multiple comparisons of mean values. A fiducial limit of $p < 0.05$ was used throughout.

RESULTS

Effects of exposure

The actual concentrations of Cd and Ca measured in the exposure tanks are reported in Table 1. In the background and low + Cd exposures, Cd concentration averaged 55% greater than nominal, whereas in the medium and high + Cd exposures, it averaged 20% less than nominal. Calcium concentrations were within 7% of nominal in all treatments, except for the medium Ca (22% greater than nominal) and the medium + Cd (22% less than nominal).

Fish mortality during the 30-d exposure was greatest (~80% in total) in the background + Cd treatment, with acute toxicity (~50% mortality) occurring within the first 5 d (Table 1). Approximately 40% mortality was observed in the low + Cd–treated fish during the 30-d exposure, and approximately 10% mortality was observed in all other treatments (Table 1). Despite the submaximal diet, in no instance was the growth of the surviving fish decreased with Ca (mean growth rate of Ca-exposed fish = 0.93 ± 0.20 %/d, $n = 4$) and/or Cd exposure (mean growth rate of Cd-exposed fish = 0.87 ± 0.20 %/d, $n = 4$).

Cadmium accumulation in all tissues decreased as water Ca concentration increased (Fig. 1). Cadmium concentrations were greatest in kidney (Fig. 1C), followed by gills (Fig. 1A) and liver (Fig. 1B). Gill Cd levels were significantly increased ($p < 0.05$) by 14-, nine-, 12-, and eightfold from the initial (day 0) values (0.30 ± 0.02 μg Cd/g wet tissue, $n = 6$) for Cd + background, low, medium, and high Ca exposures, respectively, after 30 d of exposure (Fig. 1A). Liver Cd concentrations were significantly increased by nine-, seven-, four-, and twofold from the initial values (0.34 ± 0.03 μg Cd/g wet tissue, $n = 6$) for Cd + background, low, medium, and high Ca exposures, respectively (Fig. 1B).

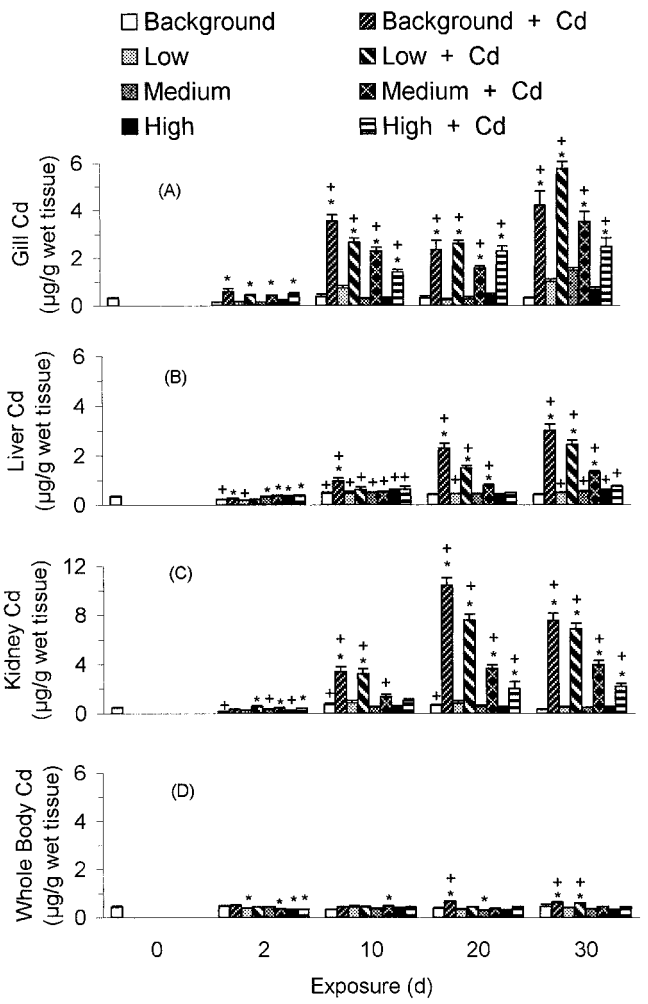

Fig. 1. Accumulation of Cd by gills (**A**), liver (**B**), kidney (**C**), and whole body (**D**) of juvenile rainbow trout exposed to background, low, medium, or high Ca with Cd (patterned bars) or without Cd (shaded bars) for 30 d. Data are presented as means ± 1 SE ($n = 6$). Statistically significant differences ($p < 0.05$) are indicated for Cd + Ca exposures against respective Ca concentrations (260, 470, 770, and 1200 μM) at each sampling day (*) and against background Ca at day 0 (+).

Kidney Cd levels were significantly increased by 16-, 15-, eight-, and fivefold from the initial values (0.47 ± 0.02 μg Cd/g wet tissue, $n = 6$) for Cd + background, low, medium, and high Ca exposures, respectively (Fig. 1C). Remaining carcass Cd levels were not significantly different ($p > 0.05$) from the initial values for any of the treatments, and average carcass Cd concentrations were 0.38 ± 0.03 μg Cd/g wet tissue (data not shown, $n = 33$). Whole-body Cd concentrations were significantly increased ($p < 0.05$) by 1.5- and 1.4-fold from the initial values of 0.43 ± 0.04 μg Cd/g wet tissue ($n = 6$) for background + Cd and low + Cd, respectively (Fig. 1D). The exposures of medium + Cd and high + Cd were not significantly different ($p > 0.05$) from the initial whole-body Cd values (Fig. 1D).

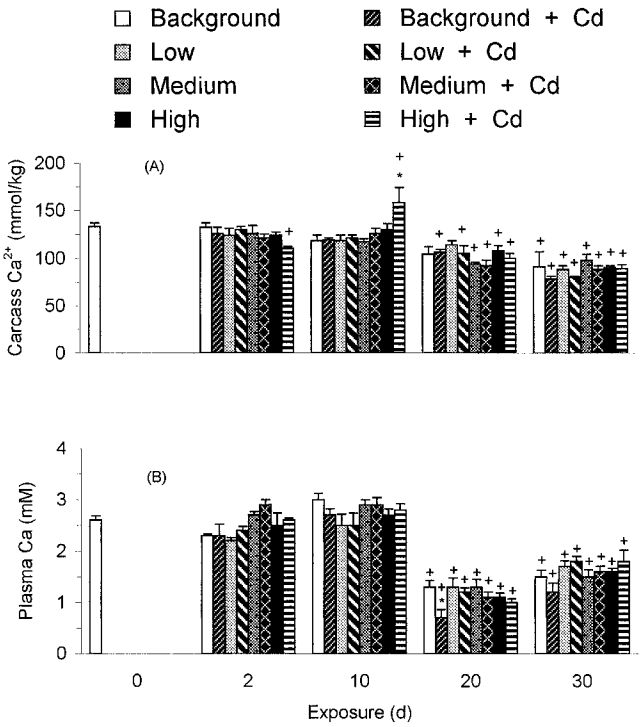

Fig. 2. Carcass (**A**) and plasma Ca (**B**) concentrations of juvenile rainbow trout exposed to background, low, medium, or high Ca with Cd (patterned bars) or without Cd (shaded bars). Error bars represent ± 1 SE (n = 6). Statistically significant differences ($p < 0.05$) are indicated for Cd + Ca exposures against respective Ca concentrations (260, 470, 770, and 1200 μM) at each sampling day (*) and against background Ca at day 0 (+).

Acclimation

These small-scale toxicity tests suggested that toxicological acclimation to Cd only occurred for the background and low + Cd–treated fish. Ninety-six-hour LC50 values were 2.53 ± 1.91, 2.35 ± 1.16, 2.15 ± 2.50, and 1.15 ± 0.58 μg Cd/L for background, low, medium, and high Ca treatments, respectively. By way of comparison, the corresponding 96-h LC50 values for the Cd-exposed fish were greater than 24.33, greater than 17.00, 1.90 ± 1.88, and 6.90 ± 6.84 μg Cd/L, respectively. The fish exposed to Cd + background or low Ca showed a trend of increased tolerance to Cd; however, because less than 50% mortality occurred in the highest concentration tested, 96-h LC50 values could not be calculated for these two groups.

Physiological effects and costs of chronic exposure

No consistent treatment or time-related effects were seen in carcass and plasma Ca, which averaged 111 ± 5 mmol/kg (n = 198) and 2.0 ± 0.1 mM (n = 198), respectively (Fig. 2). Carcass Na^+ and Cl^- concentrations averaged 37 ± 5 mmol/kg (n = 198) and 40 ± 3 mmol/kg (n = 198), respectively, also with no consistent treatment or time effects.

Swimming stamina was not significantly altered by exposure to different water Ca concentrations in the absence of Cd. However, in the presence of chronic Cd exposure (30 d), a tendency for swimming impairment at low water Ca concentrations was seen (Fig. 3). Swimming performance, as determined by time to 50% fatigue, was significantly decreased ($p < 0.05$) with chronic exposure to low + Cd (Fig. 3).

Unidirectional influx of Ca, as assessed by ^{45}Ca appearance

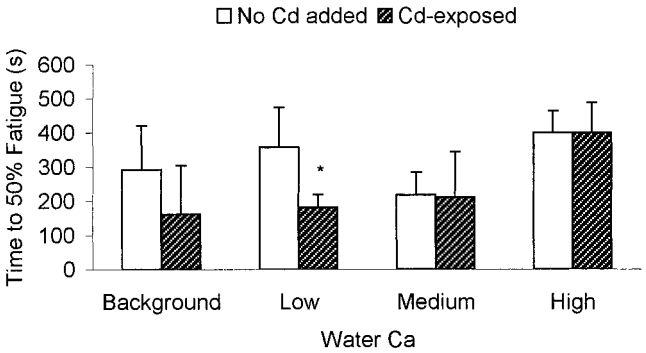

Fig. 3. Swimming performance (stamina) of juvenile rainbow trout after 30 days exposure to background, low, medium, or high Ca with Cd (striped bars) or without Cd (clear bars). Swimming times were corrected to a reference length of 11 cm (average length of fish tested [30]). Data are presented as means ± 1 SE (n = 10). Statistical comparisons were made for Cd + Ca exposures against respective Ca concentrations (260, 470, 770, and 1200 μM). * = $p < 0.05$.

in whole bodies, decreased significantly ($p < 0.05$) compared with background Ca at medium and high water Ca concentrations in the absence of Cd (Fig. 4). Unidirectional Ca influx was decreased by 50% with chronic exposure to Cd at background water Ca compared with controls, but it was not affected at higher water Ca levels.

Cadmium uptake/turnover in gills of acclimated trout

In all groups of the acute (3-h) Cd uptake/turnover test, uptake increased with increasing acute exposure concentrations of radiolabeled Cd, with uptake tending toward saturation at higher concentrations (Fig. 5). Fish in the background Ca treatment reached approximate saturation when exposed to 115 μg Cd/L for 3 h, with approximately 1.15 μg/g of new waterborne Cd bound to the gills (Fig. 5A). With increased water Ca concentrations, uptake of new Cd by gills decreased. Trout that had been exposed to low, medium, and high water Ca concentrations for 30 d had 37%, 50%, and 64% decreases in new gill Cd uptake, respectively, compared with controls at the highest Cd concentration tested (115 μg/L; $p < 0.05$; Fig. 5A). Similar trends with increasing water Ca levels (470, 770, and 1200 μM) were seen in fish that had been exposed to Cd for 30 d (Fig. 5B).

At each Ca concentration, new Cd accumulation in the gills

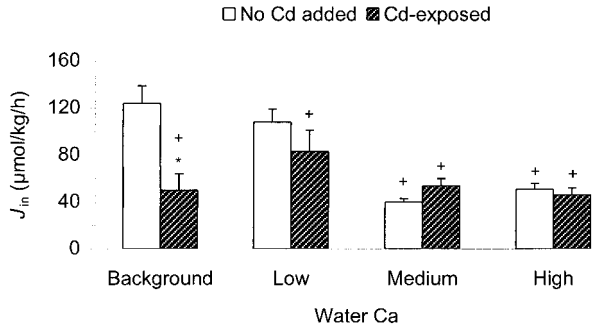

Fig. 4. Unidirectional influx of Ca, as measured with ^{45}Ca, into juvenile rainbow trout after 30-d exposure to background, low, medium, or high Ca with Cd (striped bars) or without Cd (controls; clear bars). Error bars represent ± 1 SE (n = 8). Statistically significant differences ($p < 0.05$) are indicated for Cd + Ca exposures against respective Ca concentrations (260, 470, 770, and 1200 μM; *) and against background Ca (+).

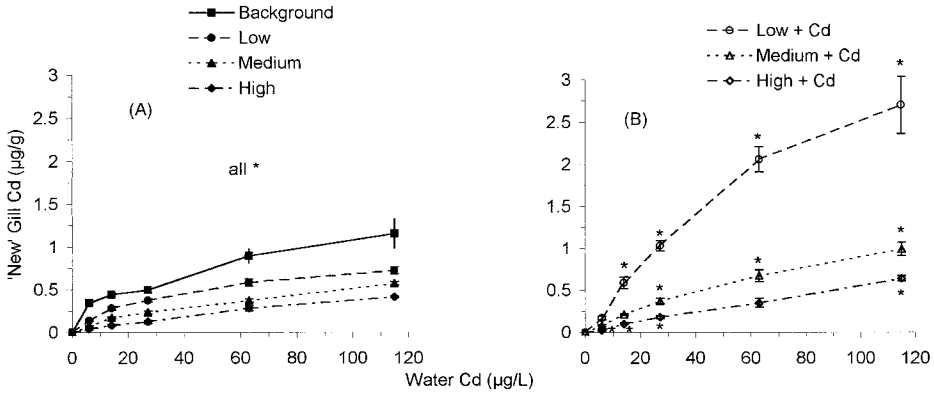

Fig. 5. Accumulation of new Cd by gills of Ca-exposed (**A**) and Cd + Ca (background [squares], low [circles], medium [triangles], and high [diamonds]–exposed trout (**B**) acutely exposed (3 h) to ^{109}Cd with total Cd concentrations of 6, 14, 27, 63, and 115 μg/L. Data are presented as means ± 1 SE ($n = 5$). Statistical comparisons were made against background Ca exposure at each sampling concentration for **A** and against respective Ca concentration from **A** at each sampling concentration for **B.** * = $p < 0.05$.

increased significantly ($p < 0.05$) with chronic Cd exposure (Fig. 5B). At the highest test concentration (115 μg Cd/L), uptake of new Cd by gills increased 3.8-, 1.7-, and 1.6-fold for the low + Cd, medium + Cd, and high + Cd groups, respectively (Fig. 5B), compared with their respective control groups at the same Ca levels (470, 770, or 1200 μM) without Cd (Fig. 5A).

Cadmium speciation analyses from MINEQL+ [35] indicated that 60 to 94% of the total Cd existed as the free ionic species Cd^{2+} for exposures ranging from 6 to 115 μg/L, respectively. Calcium concentrations of the test waters (background, low, medium, and high Ca) did not affect Cd^{2+} speciation to a large extent. A less than 1% decrease in free ionic Cd^{2+} concentration was found as water Ca levels increased from background to high (from 54.9% to 54.7% of total Cd as Cd^{2+} for background and high Ca, respectively, with the remainder bound to DOM). Scatchard analysis of Cd uptake/turnover for the 3-h exposure to radioactive ^{109}Cd (Fig. 5A) was done using ionic Ca^{2+}. This analysis indicated a trend for decreasing conditional stability constants (log $K_{Cd\text{-}gill}$; decreasing affinity) and for decreasing B_{max} values (number of gill Cd-binding sites) for Ca-exposed fish as the chronic Ca exposure level increased (Table 2). Furthermore, at each calcium concentration, conditional stability constants decreased with chronic exposure to cadmium. The number of gill Cd-binding sites (B_{max} values) tended to increase with chronic Cd exposure, particularly for the low + Cd–treated fish (Table 2).

The data set for the longer-term gill-binding exposure (72 h) is incomplete due to mortality during the 72-h exposure (Fig. 6) as well as mortality during the 30-d chronic exposure to cadmium (Table 1), which limited the number of fish that could be tested. The longer-term gill-binding exposure to 6 μg Cd/L for 72 h again revealed pronounced influences of both the water Ca concentration and the effects of chronic Cd exposure. Gill uptake of new Cd decreased as water Ca increased, but gill accumulation of new Cd increased with chronic exposure to 2 μg Cd/L (Fig. 6). The total Cd concentrations in gills remained constant during the 72-h exposure (data not shown).

Table 2. Log $K_{Cd\text{-}gill}$ (conditional stability constants) and B_{max} (number of gill Cd binding sites) of juvenile rainbow trout, calculated against ionic Cd^{2+}, after 30 d of exposure to background, low, medium, or high Ca or without Cd

	No Cd added		2 μg/L Cd added	
Ca concentration	Log $K_{Cd\text{-}gill}$	B_{max} (μmol/g)	Log $K_{Cd\text{-}gill}$	B_{max} (μmol/g)
Background	7.0	0.010	—	—
Low	6.9	0.007	6.2	0.042
Medium	6.6	0.006	6.3	0.012
High	6.3	0.005	5.8	0.015

DISCUSSION

Environmental relevance

The U.S. Environmental Protection Agency (U.S. EPA) freshwater-quality criteria for aquatic life regarding water hardness ranging from 20 mg/L as $CaCO_3$ (close to our low Ca exposure) to 120 mg/L as $CaCO_3$ (close to our high Ca exposure) recommend an acute limit of 0.6 to 4.8 μg Cd/L, respectively, for acute Cd exposures [23]. Limits set by the U.S. EPA for chronic Cd exposures are 0.3 and 1.3 μg Cd/L for water hardness, ranging from 20 to 120 mg/L as $CaCO_3$, respectively [23]. Note, however, that U.S. EPA criteria may not be implemented at low hardness, because the hardness relationship is thought to be incompletely characterized at less than 50 mg/L. We used a nominal Cd concentration of 2.0 μg/L for our experiment, which is also relevant to Canadian water-quality guidelines of 0.2 and 1.3 μg Cd/L for protection of freshwater life exposed chronically to cadmium in soft and hard water, respectively [24].

Costs of Cd exposure

Despite the restricted ration, no significant effects on growth were observed during the 30-d exposure to Cd plus increasing water Ca concentrations. Therefore, growth was not a sensitive indicator of ongoing sublethal Cd exposure. Other reports on chronic Cd exposure, many run at higher ration levels, support these findings of no adverse effects on growth [6,20,37,38].

Whole-body and plasma Ca concentrations were not consistently changed with chronic Cd exposure and increasing water Ca concentrations (Fig. 2). Hollis et al. [20] also found no significant differences in whole-body ions with chronic Cd exposures in hard water (140 mg/L as $CaCO_3$). Giles [15] showed that plasma calcium and magnesium concentrations were not significantly changed at exposures of 3.6 μg Cd/L at a water hardness of 82 mg/L as $CaCO_3$, but that they were

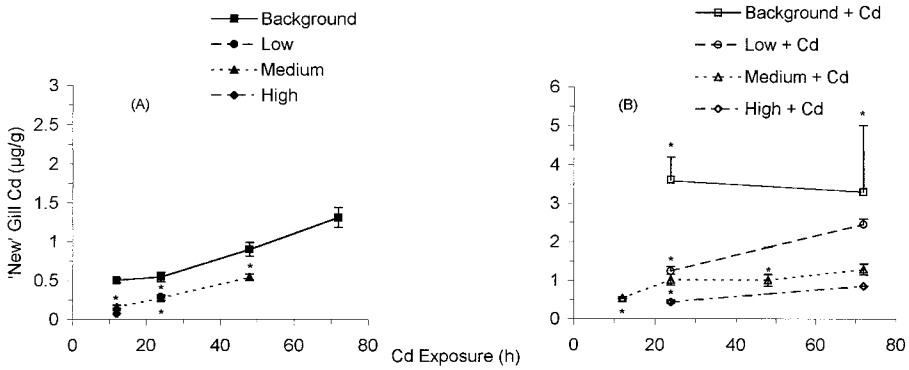

Fig. 6. Accumulation of new Cd by gills of rainbow trout exposed for 72 h to ^{109}Cd, with total Cd concentration of 6 μg/L, after 30-d exposures to background (squares), low (circles), medium (triangles), or high (diamonds) Ca without Cd (**A**) or with Cd (**B**). Data are presented as means ± 1 SE (n = 1–5). Statistical comparisons were made against background Ca exposure at each sampling concentration for **A** and against the respective Ca concentration from **A** at each sampling concentration for **B**. Note the scale difference in **B**. * = $p < 0.05$.

decreased at 6.4 μg Cd/L exposures. Reid and McDonald [39] demonstrated no adverse effects from 6.5 μg Cd/L on plasma Ca and Na levels during a 24-h exposure in both hard (Ca = 1 mM) and soft (Ca = 0.04 mM) water; however, whole-body influx of Ca^{2+} from the water was significantly reduced.

In contrast, swimming performance responded to Cd exposure at the lower Ca concentrations. Sprint performance was significantly reduced for the low + Cd-exposed fish (Fig. 3). Scherer et al. [40] reported impaired swimming performance, as represented by foraging ability of adult lake trout exposed to 0.5 μg Cd/L at a water hardness of 90 mg/L as $CaCO_3$; however, escape of the prey (rainbow trout fingerlings) from the lake trout was not impaired by the Cd exposure.

The primary mechanism of acute Cd toxicity is fatal hypocalcemia resulting from irreversible blockage of Ca^{2+} uptake across the gills, apparently by noncompetitive inhibition of an essential transport enzyme, high-affinity Ca^{2+} ATPase [41,42]. Uptake of Ca^{2+} did not recover within a 12-h period once the Cd (a high level of 730 μg Cd/L) was removed, suggesting that Cd causes permanent damage to the Ca^{2+} ATPase [39]. In the present study, unidirectional influx of Ca^{2+} into trout was significantly reduced with chronic Cd exposure at the background Ca concentration (Fig. 4). Unidirectional influx of Ca^{2+} into trout was also significantly reduced at the medium and high water Ca concentrations, with or without Cd (Fig. 4), whereas whole-body and plasma Ca^{2+} concentrations were not significantly different at the various water Ca concentrations (Fig. 2). Reid and McDonald [39] reported similar results with significant inhibition of Ca^{2+} influx in rainbow trout exposed for 24 h to 6.5 μg Cd/L. In addition, the degree of inhibition of Ca^{2+} influx due to Cd exposure relative to control fish was greater in soft water–acclimated (Ca^{2+} = 0.04 mM) than hard water–acclimated (Ca^{2+} = 1 mM) trout [39].

Because both water Ca and Cd effects on unidirectional Ca^{2+} influx occurred in the present study without effects on whole-body Ca^{2+} concentrations or growth, compensating changes in Ca^{2+} efflux are likely to have occurred. Verbost et al. [42] demonstrated that transepithelial Ca^{2+} efflux in the gills of rainbow trout is less sensitive than the Ca^{2+} influx to Cd^{2+}. Efflux of Ca^{2+} did not occur in the gills of trout exposed to 0.1 μM Cd (11 μg Cd/L); however, Ca^{2+} efflux was significantly stimulated at a Cd concentration of 1 μM (112 μg Cd/L). In contrast, Reid and McDonald [39] found no significant effects on Ca^{2+} efflux in whole bodies of rainbow trout during a 24-h exposure to 6.5 μg Cd/L in both hard water (Ca = 1 mM) and soft water (Ca = 0.04 mM).

Acclimation

Toxicological acclimation to cadmium may have occurred at the background and low chronic Ca exposures with Cd. However, acclimation did not occur at the higher Ca concentrations, because subsequent challenge to Cd after the 30-d exposure did not yield significantly higher 96-h LC50 values compared with those of controls. The apparent acclimation at the low Ca levels may have resulted from selection for fitter fish, because total mortality was approximately 80% and approximately 40% for the background + Cd and low + Cd exposures, respectively, after the 30-d exposure to cadmium. However, mortality was minimal (<10%) for the higher Ca–treated fish (Table 1). The chronic Cd exposure concentration of 2 μg/L (nominal) was, therefore, less than the threshold for acclimation at the higher Ca levels. Significant accumulation of Cd was observed in the gills of these fish (Fig. 1); however, the exposure concentration of approximately 2 μg Cd/L may not have been great enough to induce morphological damage to the gills and, thereby, acclimation. McDonald and Wood [43] proposed a damage-repair hypothesis that describes an initial shock phase involved with metal exposure that results in morphological damage to the gills, followed by changes and repair of the gills with continued exposure to the metal, leading to acclimation of the fish. Lack of this damage repair may explain the observed acclimation of trout at the lower Ca exposures but not at the higher chronic Ca exposures to Cd.

Internal Cd distribution

Although the diet had appreciable Cd (1.06 μg/g), we are confident that the great majority of Cd accumulation observed in the present study came from the water, because the control fish on the same diet exhibited negligible Cd buildup in the tissues during the 30-d period. Indeed, the most sensitive indicator of chronic waterborne Cd exposure appears to be tissue accumulation of the metal. Kidneys accumulated the greatest concentration of Cd during the 30-d exposure, followed by gills and liver (Fig. 1). Several other studies have demonstrated equal or greater concentrations of Cd (relative to gills or liver) in kidneys of chronically exposed trout. Benoit et al. [14] and Kumada et al. [38] showed that kidney Cd concentrations remained high once the fish were returned to Cd-free water,

indicating the importance of the kidney as a storage organ for Cd.

The protective effects of Ca against Cd uptake into the organs was observed for the gills, liver, and kidney, with decreased Cd accumulation at higher water Ca concentrations (Fig. 1). However, we cannot rule out the possibility that some of this apparent protective effect resulted from measured Cd levels in the medium and high treatments being less than nominal, whereas in the background and low treatments, such levels were substantially greater than nominal. Nevertheless, Wicklund and Runn [4] similarly showed the protective effects of Ca against Cd uptake with slower uptake of Cd into the gills with increasing water Ca levels (0.2–5 mM), causing lower Cd accumulation in the liver and kidney. Verbost et al. [41,42] demonstrated that Cd^{2+} and Ca^{2+} compete for the same apical channel in the initial uptake step into the gills, whereas the basolateral transports appear to differ. Cadmium noncompetitively inhibits the high-affinity Ca^{2+} ATPase, which moves Ca^{2+} across the basolateral membrane into the bloodstream. Pärt et al. [2] reported a strong, inverse relationship between water Ca^{2+} concentrations as great as 3 mM and Cd uptake into perfused rainbow trout gills; however, Cd retention in gill tissue was not sensitive to water Ca and, therefore, was different from Cd transfer.

Implications for biotic ligand modeling

The biotic ligand model involves use of the fish gill, which is the primary site of toxic action, as a generalized biotic ligand for complexing metals [13,28]. Conditional equilibrium stability constants for the affinity of this biotic ligand for a particular metal, along with relevant water chemistry, are entered into aquatic geochemistry programs (e.g., MINEQL+) to predict metal binding to the gills [44,45]. The predicted accumulation by the gills correlates with the toxicity of the metal to the fish [46].

In our previous study, we demonstrated that the biotic ligand model could be successfully applied to fish in hard water [20]. In the present study, however, control fish (260 μM Ca) had much higher new gill Cd concentrations (higher B_{max} values; Table 2) compared with our earlier study in hard water [20]. This discrepancy in the number of gill Cd-binding sites between studies may result from size or batch differences in fish or from differences in feeding regime, which was a 1% daily ration in the present study versus 3% in the previous one. In the current study, fish were fed a submaximal ration of 1% body weight per day in the hope of revealing metabolic costs associated with chronic Cd exposure. Whatever the explanation for the differences in B_{max} values between studies, this creates problems when trying to apply the biotic ligand model to different sets of experiments. In our previous study [20], the maximum number of binding sites was similar to the fathead minnow value of 0.2 nmol/fish or 2 nmol/g of gill from Playle et al. [44,45]. The present study yielded gill Cd-binding site numbers closer to that reported by Hollis et al. [47] for rainbow trout (1 nmol/fish or 10 nmol/g of gill). Hence, the biotic ligand model would only be successful at predicting new Cd accumulation in the gills of the Ca-exposed fish from the present study (Fig. 5A) if the number of Cd-binding sites on the gill was increased.

For example, if the conditional stability constant for Cd binding to the gill (log $K_{Cd\text{-}gill}$ = 8.6) from Playle et al. [44,45] and the number of Cd-binding sites (B_{max}) on the gill (1 nmol/fish or 10 nmol/g of gill) from a different source, such as Hollis et al. [47], were used in the biotic ligand model, we could successfully predict new Cd accumulation in the gills of the Ca-exposed fish. Interestingly, whereas the log $K_{Cd\text{-}gill}$ value was derived for fathead minnow, the high B_{max} value of 10 nmol/g, which is similar to the present data, was derived from work on rainbow trout [47]. In that study, the fish were fed once per day on a limited ration (L. Hollis, personal communication), as in the present study, pointing to feeding as the cause of the variation in B_{max}. In our previous study [20], fish were fed a threefold higher ration (3% per day as three daily meals). Possibly, fish on restricted ration may upregulate Ca^{2+} transport from the water to augment limited dietary calcium, thereby increasing the B_{max} for Cd^{2+} binding on the gills. However, other important factors, such as age and size of the fish, cannot be ruled out as possible explanations for the observed changes in the number of gill-binding sites between studies.

The biotic ligand model is able to predict Cd accumulation in control fish. However, for several reasons, the model cannot be used to predict gill Cd loading and toxicity in trout that are chronically exposed to Cd. Both the log $K_{Cd\text{-}gill}$ and B_{max} values for new Cd were altered as a result of chronic Cd exposure, with the affinity decreasing and the site number increasing (Table 2). Furthermore, total cold Cd burden was increased during chronic exposure by approximately fourfold (4.2 and 2.5 μg Cd/g for background and high Ca, respectively; Fig. 1) saturation of the B_{max} values (1.1 and 0.6 μg Cd/g for background and high Ca, respectively; Table 2) for acute toxicity. The acute toxicity threshold itself appeared to change as well, but not in proportion to changes in gill Cd-binding kinetics. In addition, it was necessary to use a radiotracer technique, rather than the cold technique described by Playle et al. [44,45], to detect the small increases in new gill accumulation against the high background Cd pools accumulated in the gills during the chronic 30-d exposure (Fig. 1).

Scatchard analysis was applied to the kinetic binding curves for Ca and for Ca + Cd-exposed fish (Fig. 5), which resulted in conditional stability constants of log $K_{Cd\text{-}gill}$ = 5.8 to 7.0 (Table 2), much lower than that of Playle et al. [44,45] for fathead minnows (log $K_{Cd\text{-}gill}$ = 8.6) but reasonably similar to that of controls from our previous study for hard water–exposed fish (log $K_{Cd\text{-}gill}$ = 7.6; [20]). At least in part, the difference in our log $K_{Cd\text{-}gill}$ values compared with that of Playle et al. [44,45] results from the difference in methods for calculating conditional equilibrium stability constants. We calculated these values from Cd loading in gills of the fish, whereas Playle et al. [44,45] used competitive ligands to reduced Cd accumulation of gills, a method that is less sensitive to the competitive effects of Ca^{2+} and H^+. In the present study, as the water Ca^{2+} concentrations increased, the affinity of Cd for the gill decreased (Table 2). Using the log $K_{Ca\text{-}gill}$ value of 5.0 from Playle et al. [44,45], the log $K_{Cd\text{-}gill}$ value should theoretically move 0.6 log units (i.e., log $K_{Cd\text{-}gill}$ of 7.4 decreases to 6.8) as the water Ca increases from 260 to 1200 μM. This reduction is identical to the shift seen in the present study (7.0 to 6.3; Table 2), although the absolute values are slightly lower.

Trout that had been chronically exposed to 2 μg Cd/L accumulated more new Cd in their gills compared with their respective Ca controls when exposed acutely to higher total Cd concentrations, ranging from 6 to 115 μg/L (Fig. 5B). This influence of chronic Cd exposure (increased uptake of new Cd by gills during high Cd exposure of trout previously chronically exposed to low levels of Cd) was also evident in the longer-term (72-h) gill-binding exposure to radioactive ^{109}Cd

(Fig. 6). These results are similar to those of our hard-water study [20], in which fish chronically exposed to 3 and 10 μg Cd/L had higher gill concentrations of new Cd with an acute exposure to 100 μg Cd/L but lower gill concentrations if the acute exposure was only 10 μg Cd/L.

The affinity of the gills for Cd was reduced by chronic exposure to 2 μg Cd/L, whereas the number of binding sites increased (Table 2). These changes in gill-binding characteristics are in good agreement with our previous results [20], in which the affinity of the gill for Cd (log $K_{Cd\text{-}gill}$) decreased and the number of gill Cd-binding sites (B_{max}) increased with chronic sublethal Cd exposures in hard water. Alsop et al. [48] have similarly shown that the affinity of the gill for zinc (Zn) was consistently reduced by chronic acclimation to sublethal Zn, and that B_{max} was greater for Zn-exposed fish compared with controls. Gill Zn pool size was also much larger in soft water (20 mg/L as $CaCO_3$) than in hard water (120 mg/L as $CaCO_3$) [48]. Thus, two metals, one essential (Zn) and one nonessential (Cd), appear to be handled in similar ways by the gill during chronic exposure. During acute exposures, these two metals interfere with branchial Ca^{2+} uptake in freshwater fish, causing hypocalcemia [41,42,49]. Therefore, it seems likely that the binding sites for both metals are part of the branchial transport system for Ca (e.g., Ca channels or Ca-transporters), and that changes occurring in log K and B_{max} values as a result of chronic Cd or Zn exposure represent changes in the nature or expression of the proteins that, in some way, are involved in Ca uptake. Increases in B_{max} values likely reflect increases in the amount of protein available for transport, but the cause (or causes) of decreases in log K values is unknown. The molecular basis for these changes will be an exciting area for future research.

CONCLUSIONS

In conclusion, discrepancies are found in the maximum number of gill Cd-binding sites found in this study compared with those in earlier gill-binding studies [20,44,45], but our results are in agreement with those of Hollis et al. [47]. Therefore, difficulties exist in using the biotic ligand model as a tool for predicting toxic effects of Cd to fish at various water Ca concentrations. In addition, the acute toxicological threshold appears to change in an unpredictable manner as a result of acclimation in some instances but not in others. Furthermore, the adaptive changes that occur in the gills of fish that are chronically exposed to Cd cannot be easily incorporated into the model. Cold Cd concentrations in the gills increased during the 30-d exposure to Cd, and these changes were accompanied by decreases in the affinity of the gill for Cd as well as by increases in the number of gill Cd-binding sites. These adaptive changes are currently not accounted for by the biotic ligand model. Until the discrepancies in B_{max} values between studies and changes in toxic threshold and in gill-binding characteristics with chronic Cd exposure can be resolved for the biotic ligand model, current chronic ambient water-quality criteria for Cd at different Ca concentrations are reasonable limits for protecting aquatic life. These regulations do not take water Mg^{2+} levels, DOM concentrations, and so on into consideration, but they are based on water hardness, which is the most influential factor on Cd uptake and toxicity. Recommended Canadian water quality guidelines for cadmium (0.2–1.3 μg Cd/L for a hardness range of 20–120 mg/L as $CaCO_3$) [24] are currently set at Cd concentrations well below the chronic level (2 μg Cd/L), which caused substantial fish mortality at the various water Ca concentrations in the present study. However, further research is needed to determine whether these limits for the protection of freshwater life exposed chronically to Cd are appropriately protective or overprotective, particularly on a site-specific basis.

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