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Effects of water chemistry variables on gill binding and acute toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*): A biotic ligand model (BLM) approach ☆

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ABSTRACT

This study investigated the short-term (3 h) cadmium binding characteristics of the gills, as well as the influence of various water chemistry variables [calcium, magnesium, sodium, pH, alkalinity and dissolved organic carbon (DOC)] on short-term gill accumulation and acute toxicity of cadmium in juvenile freshwater rainbow trout. The cadmium binding pattern revealed two types of cadmium binding sites in the gill: (i) saturable high affinity sites operating at a low range of waterborne cadmium concentration, and (ii) non-saturable low affinity sites operating at a higher range of cadmium concentration. Among the water chemistry variables tested, only calcium and DOC significantly reduced both gill accumulation and toxicity of cadmium. Interestingly, alkalinity (15–90 mg L^{-1} as $CaCO_3$) did not influence the gill cadmium accumulation but a significant increase in toxicity was recorded at a higher alkalinity level (90 mg L^{-1}). Affinity constants (log *K*) for binding of competing cations (Cd^{2+} and Ca^{2+}) to the biotic ligand and for binding of Cd^{2+} to DOC were derived separately from the 3 h gill binding tests and the 96 h toxicity tests. In general, the values agreed well, indicating that both tests targeted the same population of high affinity binding sites, which are likely Ca^{2+} uptake sites on the gills. These parameters were then incorporated into a geochemical speciation model (MINEQL+) to develop a biotic ligand model for predicting acute toxicity of cadmium in trout. The model predictions exhibited a good fit with the measured toxicity data except for high alkalinity and pH.

1. Introduction

Fish gills are the initial target of waterborne cadmium toxicity. Toxicity of cadmium is generally attributed to its free divalent cation (Cd^{2+}) (Pagenkopf, 1983). At the gill surface, Cd^{2+} competes with Ca^{2+} for high affinity calcium binding sites (Playle et al., 1993a,b; Playle, 1998; Niyogi and Wood, 2004a) and after entering the gill chloride cells cadmium blocks calcium uptake by inhibiting the Ca^{2+}-ATPase at the basolateral membrane (Verbost et al., 1987, 1989). The cumulative effect of these two processes can cause severe disruption of calcium homeostasis in fish under acute waterborne cadmium exposure (Verbost et al., 1987; Reid and McDonald, 1988), which ultimately leads to their death.

Certain water chemistry variables [e.g. alkalinity, natural organic matter (NOM)] can influence the concentration of Cd^{2+} ion in the water due to complexation effects, while others [e.g. hardness (Ca^{2+}, Mg^{2+}), Na^+, pH (H^+)] may compete with Cd^{2+}; both may alter the toxicity of cadmium to freshwater fish. Water hardness is often regarded as the major influencing factor on cadmium toxicity. The acute toxicity of cadmium decreases with increasing water hardness (Calamari et al., 1980; Davies et al., 1993; Brinkman and Hansen, 2007). This is believed to be due to the fact that at increased hardness levels, the major hardness cation, Ca^{2+}, out-competes Cd^{2+} for binding sites on the gill and thereby reduces toxicity (Spry and Wiener, 1991). Waterborne calcium has been found to be a strong modifier of cadmium accumulation in fish gills under short-term (2–3 h) acute exposure to cadmium (Playle et al., 1993a; Hollis et al., 1997). However, little is known about the effects of other potential competing ions, such as Mg^{2+} or Na^+, on cadmium accumulation and/or toxicity in fish. Moreover, in many studies quoted above the effects of hardness were difficult to interpret because of the confounding effects of alkalinity, which co-varied with hardness levels. In these studies, differences in cadmium toxicity at various hardness levels may have been caused, at least partially, by a difference in carbonate complexation rather than by competition with hardness cations only.

Similarly, very little is known about the effects of pH on acute toxicity of cadmium in freshwater fish. Playle et al. (1993a) reported that low pH (4.8) reduces cadmium accumulation in gill under short-term acute cadmium exposure, and suggested that it occurs because H^+, like Ca^{2+}, competes with Cd^{2+} on the gill surface. However, it is not yet known whether low pH actually protects fish against acute cadmium toxicity. Similarly, the influence of high pH on acute cadmium accumulation and/

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or toxicity to fish has received little attention. Again, high pH is often associated with increased hardness and/or alkalinity in water, and therefore it is important to understand the effects of pH independent of other surrogate variables. NOM [the dissolved fraction of the NOM is sometimes expressed as DOM (dissolved organic matter), and also in terms of its dissolved organic carbon or DOC (metal binding moiety) content] is also known to reduce the bioavailability and toxicity of cadmium to fish (Giesy et al., 1977; Winner, 1984; Van Ginneken et al., 2001). However, the protective effects of NOM against cadmium are not as strong as those observed against some other metals like copper, since NOM appears to bind cadmium ~10-fold more weakly than copper (Alberts and Giesy, 1983; Playle et al., 1993b).

The development of the "Biotic Ligand Model" (BLM) approach in recent years has provided a flexible framework to assess site-specific bioavailability and toxicity of metals to aquatic life (Di Toro et al., 2001; Niyogi and Wood, 2004b). The BLM is based on the assumption that free metal ions and the corresponding competing cations bind to the fish gill (biotic ligand) with specific affinities (log K) and capacities (B_{max}). The extent of short-term gill binding (e.g. 3 h) is used as a prediction of eventual toxicity (e.g. 96 h mortality). The BLM uses a geochemical equilibrium framework to incorporate the competition of the free metal ions with competing natural cations (e.g. Ca^{2+}, Mg^{2+}, Na^{+}, H^{+}), together with complexation by abiotic ligands (e.g., NOM, carbonates, chlorides, sulfides), to quantify the metal accumulation at the biotic ligand. The level of metal accumulation is then used to predict toxicity (acute and/or chronic) in resident species. Several BLMs have been developed in the last few years to predict acute as well as chronic toxicity of copper and zinc in aquatic animals (fish and daphnia) [see Niyogi and Wood (2004b) for a comprehensive review]. The United States Environmental Protection Agency (USEPA, 2007) has recently approved the use of the BLM approach for directly developing site-specific acute criteria for copper, and indirectly chronic criteria, through application of the BLM in combination with the acute-to-chronic ratio (ACR) approach. To date, there is no published BLM for assessing site-specific toxicity of cadmium, although cadmium is categorized as one of the priority pollutants (Campbell, 2006). An unpublished version of an acute BLM for cadmium in fish is available from the website of Hydroqual Inc. (USA) (www.hydroqual.com), where some affinity constants (log $K_{Cd\text{-}BL}$ and log $K_{H\text{-}BL}$) were taken from the original cadmium gill binding model of Playle et al. (1993a,b) and others (log $K_{Ca\text{-}BL}$, log $K_{Na\text{-}BL}$ and log $K_{Mg\text{-}BL}$) are of unexplained origin (Santore et al., 2002). Overall, the current state of knowledge indicates that a more thorough and comprehensive understanding of the influence of various water chemistry variables on gill accumulation and toxicity of cadmium to fish is required in order to develop a BLM that can be employed to generate ambient water quality criteria for cadmium.

The main objectives of our study were three-fold: (i) to determine the short-term (3 h) cadmium gill binding characteristics; (ii) to characterize the individual effects of various water chemistry variables (Ca^{2+}, Mg^{2+}, Na^{+}, pH, alkalinity and DOC) on the short-term gill cadmium accumulation; and (iii) to determine whether the water chemistry variables that influence gill cadmium accumulation offer comparable protection against the acute toxicity of cadmium as well, as evaluated by 96 h LC_{50} tests. The overall goal was to generate scientific data that will be useful in developing an acute cadmium BLM for freshwater fish. The study was carried out using sensitive freshwater fish, rainbow trout (*Oncorhynchus mykiss*), as a representative species.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (*O. mykiss*) weighing 6–8 g were obtained from Humber Springs Fish Hatchery (Orangeville, Ontario). Fish were held for at least two weeks prior to their use in experiments in 200 L polyethylene tanks supplied with continuous aeration and flowing dechlorinated Hamilton tap water [Lake Ontario water: Ca = 40 mg L^{-1} or 1 mmol L^{-1}, Na = 14 mg L^{-1} or 0.6 mmol L^{-1}, Cl = 25 mg L^{-1} or 0.7 mmol L^{-1}, dissolved organic carbon (DOC) = 3 mg L^{-1} or 0.06 μmol L^{-1}; hardness = 140 mg L^{-1} as $CaCO_3$, alkalinity = 95 mg L^{-1} as $CaCO_3$, pH = 8.0, 12–14 °C]. Fish were fed 1% body weight every other day during the acclimation period, and then fed 1% body weight every day thereafter with Silver Cup Fish Feed for salmon fry [Manufacturer's specifications: 52% Crude protein, 14% Crude fat, 3% Crude fiber, 12% Ash, 1% sodium]. Fish were not fed the day before any exposures.

2.2. Characterization of cadmium binding by the gill

A 3 h acute cadmium gill binding assay was employed in soft water using live fish transferred from the holding tanks. A standard synthetic soft water (USEPA, 1991) was prepared for the exposures using analytical grade chemicals in deionized water (18 MΩ; Nanopure II; Sybron/Barnstead, Boston, MA, USA). The added salts were 0.57 mM $NaHCO_3$ (BDH Inc., Toronto, ON, Canada), 0.22 mM $CaSO_4$, 0.19 mM $MgSO_4{\cdot}7H_2O$ and 0.03 mM KCl (EM Science, Darmstadt, Germany). Other characteristics of the synthetic soft water were 40–48 mg L^{-1} as $CaCO_3$ hardness, 34 mg L^{-1} as $CaCO_3$ alkalinity, and a pH of 7.3–7.5. Cadmium was added to the exposure water as $Cd(NO_3)_2{\cdot}4H_2O$ (Fisher Scientific, Nepean, ON, Canada), and 2 μCi of ^{109}Cd (as $CdCl_2$, specific activity = 3.45 mCi mg^{-1}; Perkin Elmer, Boston, MA, USA) was added to each L of exposure water in order to increase the analytical sensitivity (Hollis et al., 1999). Fish were exposed to a series of increasing cadmium concentrations ranging from 4–80 μg L^{-1}. For each cadmium concentration tested, seven fish were placed in a 5 L clear polyethylene bag containing 3 L of the exposure water. The bags were placed in a wet table to maintain the temperature between 11–14 °C, and each bag was supplied with its own airline. Water samples (2 × 8 mL) were taken at the beginning and end of the 3 h exposure for analysis. Following 3 h of exposure, the fish were euthanized with an overdose of anaesthetic (50 mg L^{-1} buffered MS-222; Syndel Laboratories Ltd., Vancouver, BC, Canada). The gill baskets were removed and rinsed for 30 s in approximately 50 mL of the standard synthetic soft water to remove any loosely bound cadmium, and blotted dry. The gill and water samples were then weighed before analysis.

The 3 h cadmium gill binding assay was also repeated in synthetic soft water with a lower calcium concentration (0.040 mM). The amount of $CaSO_4$ was reduced in the synthetic soft water to achieve the desired calcium concentration, and sulfate levels were maintained using K_2SO_4 (Fisher Scientific, Nepean, ON, Canada). The total waterborne cadmium levels were 4–50 μg L^{-1} for the exposure series at low waterborne calcium concentration.

2.3. Effects of water chemistry variables on 3 h cadmium binding by the gill

Water quality parameters (calcium, magnesium, sodium, pH, alkalinity and DOC) were varied one at a time, in order to establish the effects of each parameter on 3 h cadmium binding by the gill. The other parameters were kept approximately constant, except for pH when alkalinity was varied (due to pH changes induced by the alkalinity changes), and sodium and chloride when DOC was varied [due to the sodium and chloride content of the commercial humic acid (Aldrich humic acid, Sigma-Aldrich, Oakville, ON, Canada) (Glover et al., 2005)]. When necessary, pH was adjusted using 1 N HNO_3 or 1 N KOH to remain within the range pH 7.3–7.5. The 3 h cadmium gill binding assays for each variable were carried out at a cadmium concentration of approximately 18 μg L^{-1} as $Cd(NO_3)_2{\cdot}4H_2O$ (Fisher Scientific, Nepean, ON, Canada) because this was observed to be the approximate saturation concentration for cadmium binding by the gill (see Fig. 1).

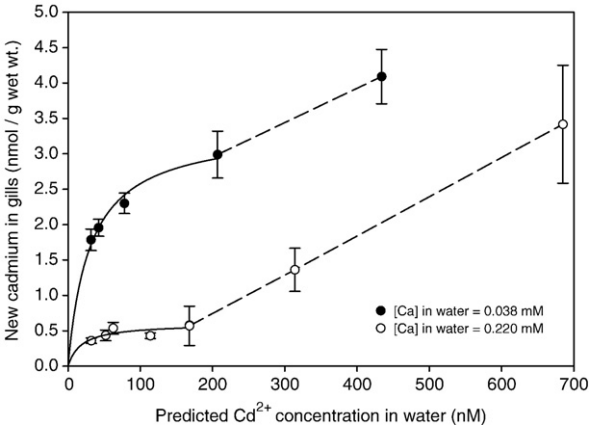

Fig. 1. The effects of different waterborne Cd^{2+} concentrations (~34–725 nmol L^{-1}) on the amount of newly accumulated gill cadmium (nmol g^{-1} wet tissue) in rainbow trout exposed for 3 h at two different water calcium levels: (i) a standard synthetic soft water (control, Ca=0.220 mM±0.007, N=16) and (ii) a synthetic soft water with reduced water calcium (Ca=0.038 mM±0.00055 mM, N=16). Data presented as means±SEM (N=7). The solid lines represent the high affinity, low capacity binding sites, while the dotted lines represent the low affinity, high capacity sites. Cd^{2+} concentrations were estimated using MINEQL+, and Scatchard analyses were employed to calculate the affinity (log $K_{Cd\text{-}Gill}$) and capacity (B_{max}) of saturable high affinity, low capacity cadmium binding sites at the two different water chemistry conditions (see text for details). The analyses yielded log $K_{Cd\text{-}gill}$=7.52, B_{max}=0.62 nmol g^{-1} in the high calcium (control) water, and log $K_{Cd\text{-}gill}$=7.53, B_{max}=3.32 nmol g^{-1} in the low calcium water.

Calcium was altered in the test water by reducing $CaSO_4$ levels to achieve the desired lower concentrations, while maintaining the same sulfate levels with K_2SO_4. Calcium was then added to the water as $Ca(NO_3)_2{\cdot}4H_2O$ (Fisher Scientific, Nepean, ON, Canada) to test the effects on cadmium gill binding over the concentration range 0.04–1.33 mM calcium. Gill cadmium binding was examined over the concentration range 0.20–1.08 mM magnesium by adding magnesium as $Mg(NO_3)_2{\cdot}6H_2O$ (Anachemia, Lachine, QC, Canada). Sodium was altered by first replacing $NaHCO_3$ with $KHCO_3$ (Fisher Scientific, Nepean, ON, Canada), in order to decrease sodium levels while maintaining the same HCO_3^- levels. Sodium was then added to the water as NaCl (Bioshop, Burlington, ON, Canada) to evaluate the effects on gill cadmium binding over the concentration range 0.06–2.20 mM sodium. Water pH was decreased using 1 N HNO_3 stock in deionized water (trace metal grade, Fisher Scientific, Nepean, ON, Canada), and increased using 1 N KOH stock (EM Science, Darmstadt, Germany) to evaluate cadmium gill binding in acidic, neutral and basic conditions (pH 4.6, 5.9, 7.3, 8.1 and 9.4). Alkalinity was changed in the exposure water by replacing $NaHCO_3$ with NaCl, and then adding various amounts of $KHCO_3$ (Fisher Scientific, Nepean, ON, Canada), in order to maintain a constant sodium concentration. The cadmium gill binding were examined at four different alkalinity levels (14, 34, 62 and 86 mg L^{-1} as $CaCO_3$). Finally, the effects of commercial NOM [as Aldrich humic acid (AHA), Sigma-Aldrich, Oakville, ON, Canada] on cadmium gill binding were evaluated over the concentration range 0–40 mgC L^{-1}. The AHA stock solution was prepared in the standard synthetic water recipe to prevent dilution of the exposure water due to the large volumes of stock added. The AHA stock solution was filtered through 0.45 mm nylon filters (Whatman, Clifton, NJ, USA) to ensure that it was completely dissolved. The AHA-treated cadmium exposure waters were matured overnight to allow time for interaction between the AHA and the metal ions (as outlined in Taylor et al., 2002). Additional water samples were taken before and after the AHA -treated cadmium exposures (2×20 mL for each treatment), filtered again as described previously, and stored at 4 °C for later analysis of dissolved organic carbon (DOC) concentrations.

2.4. Acute toxicity assays

The 96 h LC_{50} assays were conducted at various waterborne calcium (0.1, 1.0, 2.0 and 3.0 mM), pH (5.8 and 8.8), alkalinity (90 mg L^{-1} as $CaCO_3$) and AHA (5, 10, 15 and 20 mgC L^{-1}) levels in addition to control (standard synthetic soft water). Water chemistry parameters were manipulated in the exposure water as described previously in cadmium gill binding assays. For each treatment condition, fish (N=20 per treatment, equally divided in two replicates) were exposed to six different waterborne cadmium conditions [range: 0 (control) to 20 µg L^{-1}] in a static renewal system that employed 20 L polyethylene tanks under constant aeration. The water in each tank was exchanged every 24 h, and the exposure waters were allowed to equilibrate overnight before adding fish. All tanks were monitored every 12 h during the experiments. Mortality (as overturn) was recorded and water samples

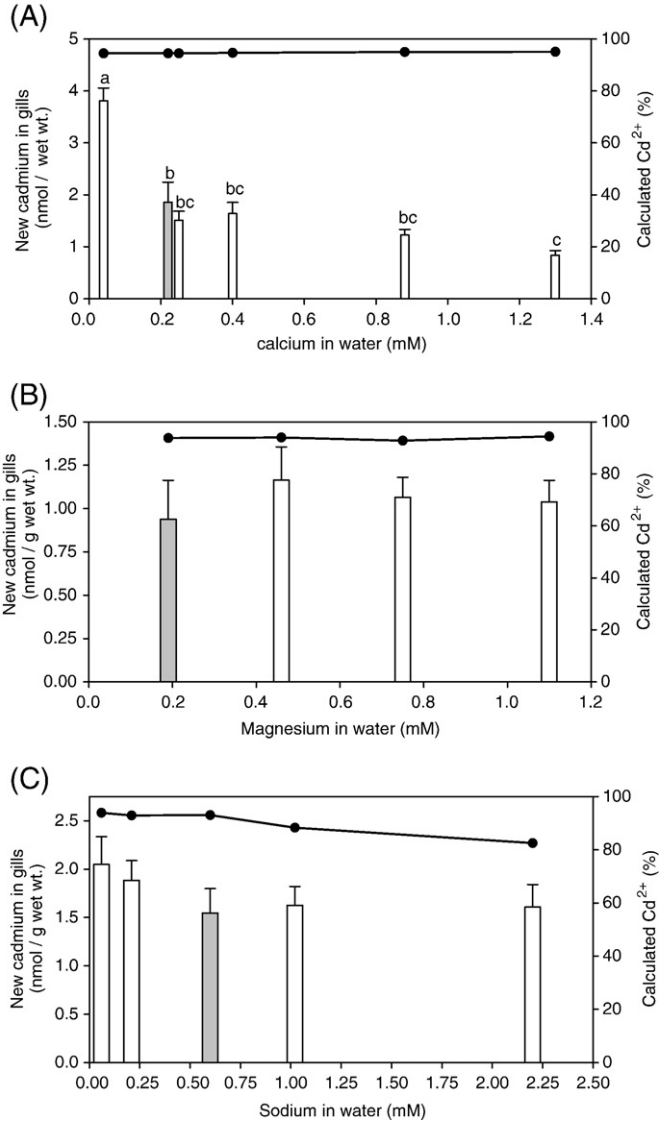

Fig. 2. The effects of varying (A) calcium (0.04, 0.22, 0.25, 0.40, 0.88 and 1.33 mM), (B) magnesium (0.19, 0.46, 0.75 and 1.1 mM), and (C) sodium (0.06, 0.21, 0.60, 1.02 and 2.20 mM), independently of one another on the accumulation of cadmium at the gills of rainbow trout exposed for 3 h to 18.4 µg L^{-1} (164 nM) cadmium in synthetic soft water. Data presented as means±SEM (N=7). Different letters indicate significant differences among various calcium treatments (p<0.05); no significant differences were observed among the treatments for both magnesium and sodium. In all panels, the grey bar represents the control value and the solid line indicates the amount of free cadmium ion (Cd^{2+}) estimated by MINEQL+ as a percentage of total cadmium species present.

(2×20 mL) were collected from each tank for the estimation of total cadmium, calcium and DOC (AHA exposures only) concentrations.

2.5. Analytical techniques

The radioactivity in the gill and water samples was measured on a Minaxi Auto-Gamma 5000 Series Gamma Counter (Canberra Packard, Meridien, CT, USA); tests demonstrated that counting efficiency was constant. Sub-samples of the water samples were diluted (1% HNO_3 and 0.02% $LaCl_3$ in deionized water for calcium and magnesium analysis, 1% HNO_3 in deionized water for sodium and cadmium analysis) for analysis of calcium, magnesium and sodium concentrations in water using flame atomic absorption spectrometry (Varian Spectra AA 220FS, Melbourne, Australia), and for analysis of cadmium concentrations using graphite furnace atomic absorption spectrometry (Varian Spectra AA 220 GTA 110, Melbourne, Australia). The pH of water was measured directly by using a PHM-82 meter with a GK2401C combination electrode (Radiometer, Copenhagen, Denmark). The alkalinity of the water samples was measured using a FF1A Aquaculture Test Kit (Range: 6.4–136 mg L^{-1}; Hach, Winnipeg, MB, Canada). The DOC concentrations in AHA-treated water samples were determined by first measuring the UV absorbance at 300 nm following appropriate dilutions (Ultraspec Plus, LKB, Piscataway, NJ, USA). The total concentration of DOC was then calculated using the standard curve equation:

$$\text{Total[DOC]}\left(\text{mgC L}^{-1}\right) = [(-0.0265 - \text{Absorbance300})/(-0.0934)] \times \text{dilution factor} \quad (1)$$

This standard curve was created for the Aldrich humic acid used by analyzing the total organic carbon (TOC) of samples covering the experimental concentration range. Samples were acidified with a single drop of 16 N HNO_3 and sparged with N_2 gas to remove interference by inorganic carbon. Identical duplicate samples were analyzed separately for absorbance at 300 nm and for TOC (TOC analyzer 5050A, Shimadzu, Tokyo, Japan), and a linear relationship between absorbance and TOC concentration was established ($r^2=0.946$, $p<0.0001$).

Free cadmium ion (Cd^{2+}) and other cation (Ca^{2+}, Mg^{2+} and Na^+) concentrations at various experimental water chemistry conditions were calculated using MINEQL+ (Version 4.5, Schecher and McAvoy, 2001). All estimations were conducted in a system in equilibrium with the atmosphere, using measured water chemistry parameters, and the ionic strengths of the solutions were calculated by the program. The metal-ligand binding constants (log K values) were supplied by the program for each water parameter, except in the case of DOC where the log K values had to be supplied by the user. We used log $K_{\text{Cd-DOC}}=7.4$, log $K_{\text{H-DOC}}=4.0$ and log $K_{\text{Ca-DOC}}=5.0$ (Playle et al., 1993b), and assumed 1 mgC $L^{-1}=2.0\times10^{-8}$ mol of binding sites (Van Ginneken et al., 2001). Note that the log $K_{\text{Cd-DOC}}$ value (7.1, see Results) calculated indirectly by competition with the fish gill in the present experiments was very similar.

2.6. Calculations

The 96 h LC_{50} values (±95% confidence intervals) were determined by log probit analysis using mortality data and average measured waterborne concentrations (SPSS 10.0 for Windows). The accumulation of new cadmium in the gills was calculated based on the accumulation of radioactivity (cpm) in the gill and the specific activity (bc^{-1}) of the water:

$$\text{Cd}_{\text{gill}} = a/(b/c) \quad (2)$$

where a is ^{109}Cd cpm g^{-1} of tissue (wet mass); b is the measured ^{109}Cd counts in the water (cpm L^{-1}); and c is the measured total cadmium concentration in the water (μg L^{-1}). Final values were converted to nmol g^{-1} (wet mass).

The cadmium binding characteristics [binding site density ($B_{\max}$) and binding affinity (log $K_{\text{Cd-gill}}$)] of saturable sites were calculated by Scatchard analysis. The amount of Cd^{2+} in the water was divided by the amount of cadmium bound to the gills, and then plotted against the amount of Cd^{2+} in water in Sigma Plot 2000 (free/bound *vs.* free), in molar units. The inverse of the slope and log of the inverse of the intercept of the regression line provided the $B_{\max}$ and log $K_{\text{Cd-gill}}$, respectively. The value for log $K_{\text{Cd-gill}}$ was then applied to estimate log $K_{\text{Ca-gill}}$ from the competitive effects of waterborne Ca^{2+} concentrations on the cadmium accumulation at the gill using an approach described by Playle et al. (1993a) with slight modifications. This approach is based on the assumptions that cadmium and calcium compete for the same binding sites and there is a finite number of cadmium/calcium binding sites at the gill. If we consider a situation when 50% of the gill sites are occupied by calcium and the other 50% by cadmium, then $K_{\text{Ca-gill}}$ can be calculated by the following equation:

$$K_{\text{Ca-gill}} = \text{Ki}_{[\text{Ca}]} \cdot K_{\text{Cd-gill}}/[\text{Cd}] \quad (3)$$

where, $\text{Ki}_{[\text{Ca}]}$ represents the Ca^{2+} concentration in exposure water that resulted in 50% inhibition of 3 h gill cadmium accumulation, and [Cd] represents the Cd^{2+} concentration in the water, again in molar units.

We employed the same principle to calculate log $K_{\text{Cd-DOC}}$ using the following equation:

$$K_{\text{Cd-DOC}} = \text{Ki}_{[\text{DOC}]} \cdot K_{\text{Cd-gill}}/[\text{Cd}] \quad (4)$$

where, $\text{Ki}_{[\text{DOC}]}$ represents the DOC concentration in exposure water that resulted in 50% inhibition of 3 h gill cadmium accumulation, and [Cd] represents the free cadmium ion concentration in the exposure water without DOC. Analogous principles were used to estimate log K values for cadmium, calcium, and DOC from toxicity data, as explained in the Results (Eqs. (5) and (6)).

2.7. Statistics

All data have been expressed as means ± SEM (N) except 96 h LC_{50} values, which were expressed as means and 95% confidence intervals. Significant differences in the cadmium accumulation for different treatments were tested with a one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons (SPSS 10.0 for Windows). Mean values were considered different at $p<0.05$. The assumptions of ANOVA, i.e., normality of distribution and homogeneity of variances were examined by Shapiro–Wilk's test and Levene's test, respectively (both at $\alpha=0.05$). All of the data met these assumptions. To test significant differences in cadmium binding characteristics between the two different levels of waterborne calcium concentrations (0.04 and 0.220 mM), the data was converted into Scatchard plots (Sigma Plot 2000), and then the equality of slopes was tested using the t-test for linear regression coefficients (with a 95% confidence interval). For 96 h LC_{50} date, the values were considered significantly different if 95% confidence intervals did not overlap (Litchfield, 1949).

3. Results

3.1. Binding site characteristics of cadmium at the gill

Accumulation of cadmium on the gills of rainbow trout exposed to a range of cadmium concentrations for 3 h in synthetic soft water (Ca = 0.220 mM ± 0.007, $n=16$) was plotted against the calculated Cd^{2+} ion concentrations (Fig. 1). This identified two types of binding sites for cadmium: high affinity, low capacity sites that became saturated at around 20.1 ± 1.1 μg L^{-1} total Cd ($n=4$) (168 nmol L^{-1} Cd^{2+}), and low affinity, high capacity sites that bound cadmium in a linear fashion beyond this point. Scatchard analysis of free/bound versus free cadmium yielded log $K_{\text{Cd-gill}}=7.52$ and $B_{\max}=0.62$ nmol g^{-1} wet wt. for the saturable high affinity sites.

There was a significant increase ($p<0.05$) in cadmium accumulation on the gills in fish exposed to a range of cadmium concentrations

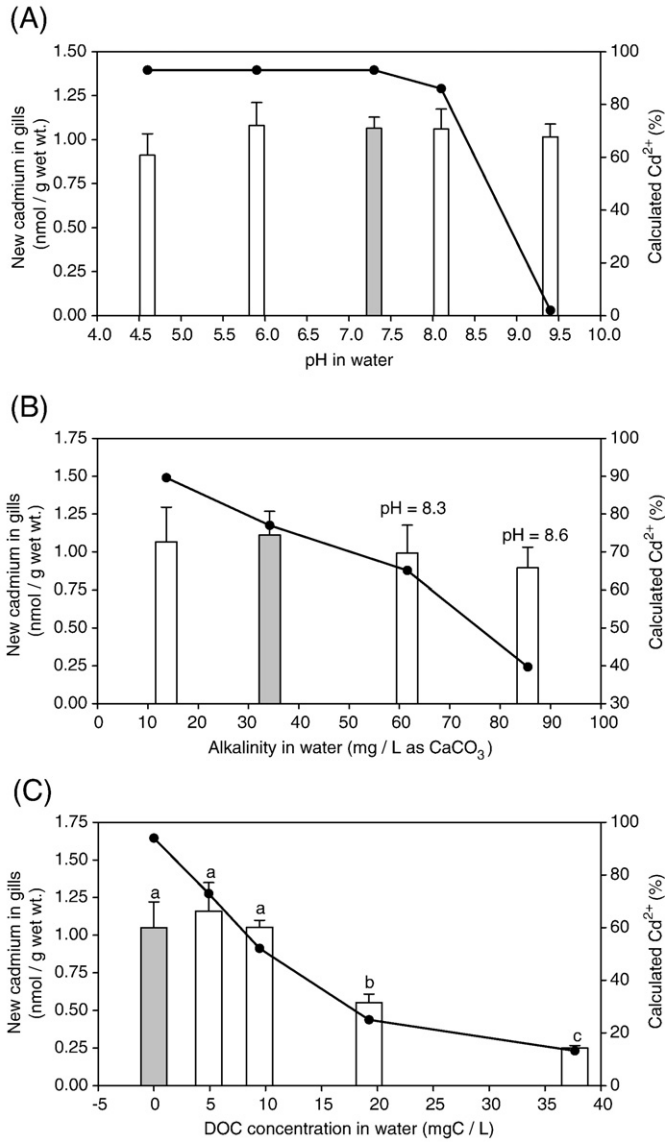

Fig. 3. The effects of varying (A) pH (4.6, 5.9, 7.2, 8.1 and 9.4), (B) alkalinity (13.7, 34.2, 61.6 and 85.5 mg L^{-1} as $CaCO_3$), and (C) DOC (0, 5, 10, 19 and 38 mg C L^{-1}), independently of one another on the accumulation of cadmium at the gills of rainbow trout exposed for 3 h to 18.4 μg L^{-1} (164 nM) cadmium in synthetic soft water. Data presented as means ± SEM (N=7). It is important to note that pH increased from control (pH=7.3–7.5) to 8.3 and 8.6 at 61.6 and 85.5 mg L^{-1} alkalinity as $CaCO_3$, respectively. Different letters indicate significant differences among various DOC treatments (p<0.05); no significant differences were observed among the treatments for both pH and alkalinity. In all panels, the grey bar represents the control value and the solid line indicates the amount of free cadmium ion (Cd^{2+}) estimated by MINEQL+ as a percentage of total cadmium species present.

for 3 h in synthetic soft water with reduced calcium levels (Ca=0.038 ± 0.00055 mM, n=16) compared to controls (Ca=0.220 mM) (Fig. 1). Two types of binding sites were again identified, and affinity remained approximately constant (log $K_{Cd\text{-}gill}$=7.53) while capacity increased almost 5-fold (B_{max}=3.32 nmol g^{-1} wet wt.).

3.2. Effects of water chemistry variables on cadmium gill binding

3.2.1. Cation competition

Cadmium accumulation by the gill at 18.4 μg L^{-1} total cadmium (in the standard synthetic soft water) decreased with increasing water calcium levels (Fig. 2A). Gill cadmium was significantly higher at 0.040 mM calcium compared to the control (0.22 mM calcium) (p<0.05, 3.81 *vs.* 2.06 nmol g^{-1} wet wt.) and significantly lower at 1.33 mM calcium compared to the control (p<0.05, 0.84 *vs.* 2.06 nmol g^{-1} wet wt.). The calculated Cd^{2+} concentrations did not vary from ~94% of total cadmium concentrations across the calcium range tested. The waterborne Ca^{2+} concentration that yielded 50% inhibition of gill cadmium accumulation ($Ki_{[Ca]}$) was derived by plotting % inhibition of gill cadmium against respective Ca^{2+} concentration in water, which revealed a $Ki_{[Ca]}$ value of 0.25 mM (Fig. 4A). For this derivation, we assumed that all of the gill binding sites were occupied by cadmium at the lowest waterborne Ca^{2+} level tested (~0.04 mM). The derived $Ki_{[Ca]}$ was then applied to Eq. (3) along with the $K_{Cd\text{-}gill}$ value (control) and the Cd^{2+} concentration in the exposure water (calculated by MINEQL+). The Eq. (3) revealed a log $K_{Ca\text{-}gill}$ value of 4.31.

Gill cadmium binding at 18.4 μg L^{-1} total cadmium was independent of both magnesium (Fig. 2B) and sodium concentrations (Fig. 2C) over the ranges tested (0.20–1.08 mM magnesium and 0.06–2.20 mM sodium, respectively). The predicted relative % of Mg^{2+} and Na^{+} concentration in the water did not change across the different treatment levels of total magnesium and sodium, respectively. Calculated Cd^{2+} concentrations did not vary from ~94% of total cadmium concentrations across the magnesium range tested, but did decrease slightly from ~94% to ~83% of total cadmium concentrations at the higher sodium concentrations.

3.2.2. Cadmium speciation and complexation

Varying the pH had no significant effect on cadmium gill binding at 18.4 μg L^{-1} of total cadmium concentration over the range tested

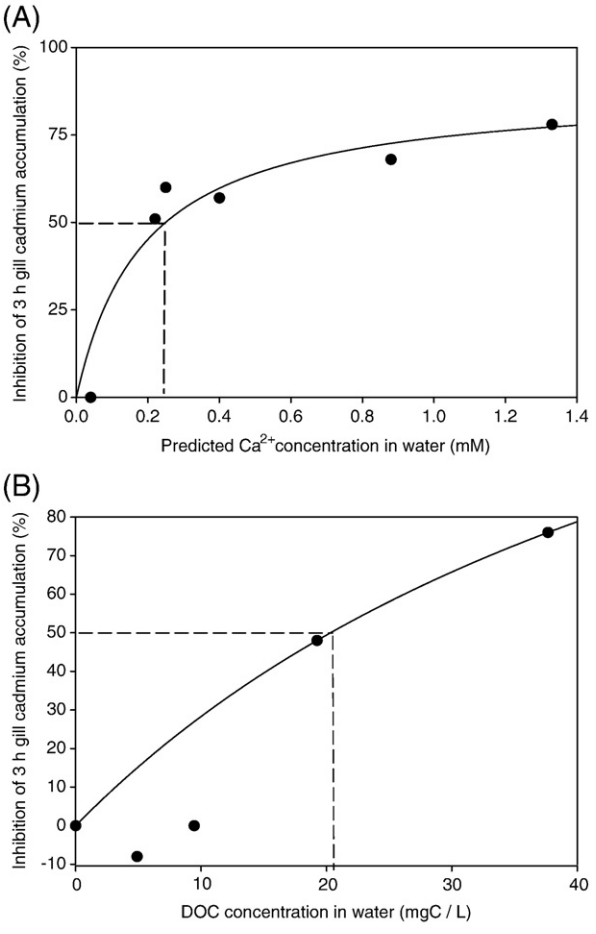

Fig. 4. The percentage inhibition of gill cadmium accumulation in rainbow trout, exposed for 3 h to 18.4 μg L^{-1} (164 nM) cadmium in synthetic soft water, by (A) Ca^{2+} and (B) DOC in water. The dotted line on the *X*-axis in plot (A) revealed the concentration of Ca^{2+} that resulted in 50% inhibition of gill cadmium accumulation ($Ki_{[Ca]}$=0.25 mM), whereas the dotted line on the *X*-axis in plot (B) revealed the concentration of DOC that yielded 50% inhibition of gill cadmium accumulation ($Ki_{[DOC]}$=20 mg C L^{-1}).

(pH 4.6–9.4) (Fig. 3A), although Cd^{2+} concentration (determined by MINEQL+) decreased sharply above pH>8.0. The calculated Cd^{2+} concentration dropped from ~94% of total cadmium concentration at pH 4.6–7.2 to ~89% at pH 8.1, and to only ~2% at pH 9.4.

Over the range of alkalinity tested (13.7–85.5 mg L^{-1} as $CaCO_3$), there was no significant effect on cadmium binding to the gills at 18.4 μg L^{-1} total cadmium concentration (Fig. 3B). However, the amount of Cd^{2+} concentration decreased as alkalinity increased. The Cd^{2+} concentration at 13.7 and 34.2 mg L^{-1} alkalinity was ~90–93% of the total cadmium concentration, whereas at 61.6 and 85.5 mg L^{-1} alkalinity it declined to ~65 and 40%, respectively. The other major positive cadmium species at the latter alkalinities was $CdHCO_3^+$ (~18.5 and 32.5% of total cadmium species, respectively). It is critical to note that the pH increased from ~7.5 at 13.7 and 34.2 mg L^{-1} alkalinity to pH 8.30 at 61.6 mg L^{-1} alkalinity, and to pH 8.60 at 85.5 mg L^{-1} alkalinity, which played a role in changing the predicted cadmium speciation.

The levels of DOC used (5–38 mgC L^{-1}) were able to complex the 18.4 μg L^{-1} of cadmium to varying degrees (from ~25 to 82%). The resulting change in Cd^{2+} concentration corresponded to a significant decrease in cadmium gill accumulation at 19.3 and 37.7 mgC L^{-1} DOC compared to control ($p<0.05$, 1.06 to 0.55 and 0.25 nmol g^{-1} wet mass, respectively; Fig. 3C). The DOC concentration that yielded 50% inhibition of gill cadmium accumulation ($Ki_{[DOC]}$) was derived by plotting % inhibition of gill cadmium against respective DOC concentration in water, which revealed a $Ki_{[DOC]}$ value of 20.0 mgC L^{-1} (corresponds to 4×10^{-7} mol of cadmium binding sites; Van Ginneken et al., 2001) (Fig. 4B). The derived $Ki_{[DOC]}$ was then applied to Eq. (4) along with the $K_{Cd\text{-}gill}$ value (control) and the Cd^{2+} concentration in the exposure water without any DOC (calculated by MINEQL+). The Eq. (3) revealed a log $K_{Cd\text{-}DOC}$ value of 7.10.

3.3. Effects of water chemistry variables on acute toxicity and derivation of affinity constants by using acute toxicity data

All of the water chemistry parameters tested (except pH) significantly influenced the acute toxicity of cadmium in fish (Table 1). Calcium influenced acute toxicity in a concentration-dependent manner. An increase of calcium concentration in water from 0.22 mM (control) to 3.00 mM increased the 96 h LC_{50} by 5.6 fold. In contrast, a decrease in calcium concentration to 0.10 mM increased the acute toxicity by 2 fold. Similarly, an increase of alkalinity from 34 mg L^{-1} (control) to 90 mg L^{-1} (as $CaCO_3$) increased acute toxicity by 2.7 fold. DOC also reduced acute toxicity in a somewhat concentration-dependent manner, an increase in DOC concentration from 0 (control) to 20 mg L^{-1} decreased acute toxicity by 2.5 fold. However, both acidic (5.8) and alkaline pH (8.8) did not produce any significant changes in 96 h LC_{50} values.

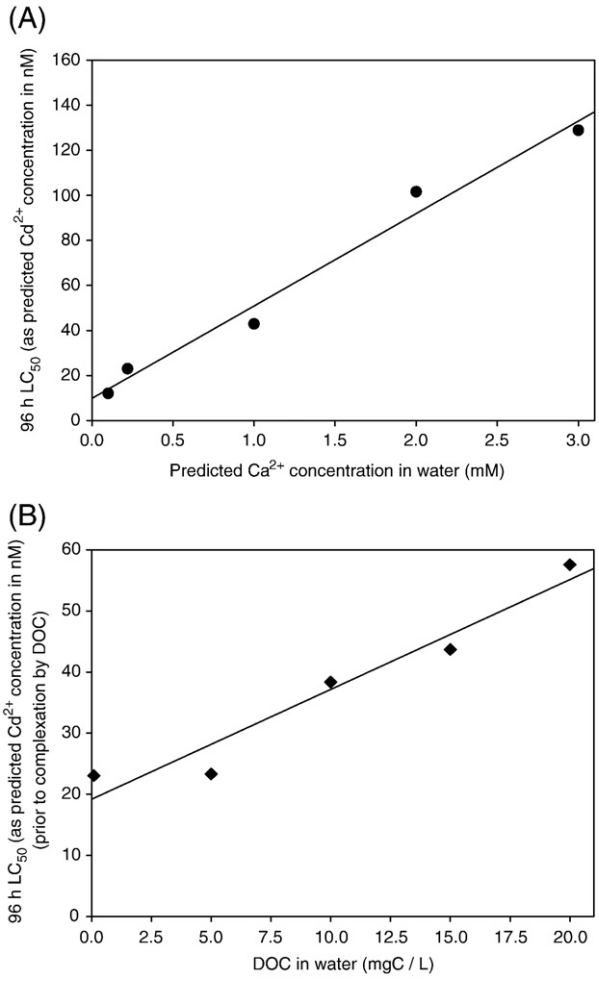

Fig. 5. The correlation (A) between 96 h LC_{50} of cadmium in rainbow trout and Ca^{2+} concentration in exposure water, and (B) between 96 h LC_{50} of cadmium in rainbow trout and DOC concentration in exposure water. Note that although 96 h LC_{50} values are expressed as free ionic cadmium (Cd^{2+}) concentrations in both plots, in plot (B) it represents the Cd^{2+} levels prior to complexation by DOC. This was done to evaluate the competitive interactions between the biotic ligand (gill) and DOC for binding to Cd^{2+} in the exposure water. The linear relationship in plot (A) is represented by the equation: $y=41.05x+9.80$ ($R^2=0.98$), whereas the linear relationship in plot (B) is represented by the equation: $y=1.80x+19.20$ ($R^2=0.94$).

Table 1
Effects of calcium, pH, alkalinity and DOC in water on acute toxicity (96 h LC_{50}) of cadmium in rainbow trout

Treatment parameter		96 h LC_{50} (μg L^{-1})	95% confidence intervals	
			Lower	Upper
Control		2.75	2.06	3.49
Calcium	0.1 mM	1.44*	1.08	1.78
	1.0	5.13*	4.09	6.63
	2.0	12.15*	10.78	15.13
	3.0 mM	15.41*	12.71	18.08
pH	5.8	3.21	1.97	3.91
	8.8	3.08	2.34	4.23
Alkalinity	90 mg L^{-1}	1.02*	0.71	1.82
DOC	5 mgC L^{-1}	2.79	2.14	4.17
	10 mgC L^{-1}	4.59	3.14	5.57
	15 mgC L^{-1}	5.23*	4.03	8.56
	20 mgC L^{-1}	6.88*	4.53	10.21

All exposures were carried out using a standard synthetic soft water (control: calcium 0.22 mM, pH 7.3–7.5, alkalinity 34 mg L^{-1} as $CaCO_3$, and DOC 0 mg L^{-1}), and all different parameters were varied one at a time (except the 90 mg L^{-1} alkalinity exposure where pH increased from 7.5 to 8.6). Significant differences relative to control are indicated by the asterisk.

A linear relationship between acute cadmium toxicity (as Cd^{2+}) and Ca^{2+} concentrations was derived (Cd-LC_{50}=41.05[Ca]+9.80; Fig. 5A) which was then used to derive the affinity constants for cadmium and calcium binding at the biotic ligand that correspond to toxicity (i.e., log $K_{Cd\text{-}tox}$ and log $K_{Ca\text{-}tox}$). The approach is analogous to that used for gill binding, and assumes that 50% mortality at 96 h occurs when 50% of the relevant "toxic sites" at the biotic ligand (gill) are bound with Cd^{2+}. Thus, the Eq. (3) was modified accordingly to fit that assumption:

$$K_{Ca-tox} = [Ca] \cdot K_{Cd-tox}/[Cd - LC_{50}] \quad (5)$$

The *Y*-intercept of the relationship provided by Fig. 5A allowed us to calculate a 96 h LC_{50} of 9.80 nM Cd^{2+} when there is no calcium in water, which transcribed to a log $K_{Cd\text{-}tox}$ value of 8.0 (a true estimate of cadmium binding affinity to the "toxic sites" on the biotic ligand). This value of log $K_{Cd\text{-}tox}$ was then used in Eq. (5) to derive a log $K_{Ca\text{-}tox}$ of 3.9 (average of five different waterborne calcium concentrations tested).

Similarly, a linear relationship between acute cadmium toxicity and DOC concentrations was derived (Cd-LC_{50} = 1.80[DOC] + 19.20; Fig. 5B), which was then used to derive the value of log $K_{Cd\text{-}DOC}$ by the following equation:

$$K_{Cd-DOC} = [DOC] \cdot K_{Cd-tox}/[Cd\text{–}LC_{50}] \quad (6)$$

The Eq. (6) yielded a log $K_{Cd\text{-}DOC}$ value of 7.3 (average of four different DOC concentrations tested).

3.4. BLM development and its predictive capacity

A BLM for predicting acute toxicity (96 h LC_{50}) of cadmium in juvenile rainbow trout was developed by incorporating different parameter estimates for BL-cation and DOC-cation interactions (summarized in Table 2) into MINEQL+. Given the relatively close agreement between log *K* values estimated from gill binding (7.5, 4.3, 7.1 for Cd, Ca, and DOC respectively) and those estimated from toxicity testing (8.0, 3.9, and 7.3 respectively), we were confident that the two techniques were characterizing the same or a similar population of binding sites (see Discussion). As the BLM is intended to predict toxicity, we therefore chose to use the log *K* values for BL-Cd^{2+}, BL-Ca^{2+} and DOC-Cd^{2+} interactions derived from the acute toxicity data in our model. The affinity constants for various cationic interactions with DOC (DOC-Ca^{2+} and DOC-H^{+}) and the total number of binding sites in DOC were adopted from Playle et al. (1993b) and Van Ginneken et al. (2001), respectively. The total number of binding sites at the gill (B_{max}) used in the model was 0.62 nmol g^{-1} wet wt. as recorded from the characterization of high affinity cadmium binding sites under control conditions in Fig. 1. The 96 h LC_{50} under the same control conditions was 22.9 nM as Cd^{2+} (2.75 μg L^{-1} as total cadmium concentration; Table 1). From the equation derived from the Scatchard analysis for characterization of the high affinity sites in Fig. 1, the 3 h LA_{50} (lethal cadmium accumulation by the gill at 3 h that is associated with 50% mortality at 96 h) at 22.9 nM Cd^{2+} was calculated to be 0.27 nmol g^{-1} wet wt. This was in fact the final LA_{50} value used in the model, but upward and downward adjustments of this value were evaluated (while keeping the affinity constants same) to find the best overall fit between measured and predicted 96 h LC_{50} values. The original 3 h LA_{50} value of 0.27 nmol g^{-1} wet wt. was adopted because it provided the optimum overall result.

The predicted 96 h LC_{50} values by the developed BLM were in reasonably good agreement with the measured values (Fig. 6). The predicted values for control, calcium and DOC treatments were more or less within the range of the 95% confidence intervals of the measured values. The predicted LC_{50} value for acidic pH treatment was also in good agreement with the measured data, however the model considerably under-predicted toxicity (i.e. over-predicted 96 h LC_{50}) for the high pH (8.8) and alkalinity treatments (90 mg L^{-1} as $CaCO_3$) relative to control. In latter two treatments, the predicted LC_{50} values were well outside the range of 95% confidence intervals of the measured values.

Table 2
Input data that were incorporated into MINEQL+ to develop a biotic ligand model (BLM) for predicting the acute toxicity of cadmium in juvenile rainbow trout

Parameter	Value
log $K_{Cd\text{-}BL}$	8.0
log $K_{Ca\text{-}BL}$	3.9
log $K_{DOC\text{-}Cd}$	7.3
log $K_{DOC\text{-}Ca}$	5.0[a]
log $K_{DOC\text{-}H}$	4.0[a]
Total number of binding sites on the gill (BL) (B_{max})	0.6 nmol g^{-1} wet wt.
Lethal cadmium accumulation on the gill associated with 50% mortality (LA_{50})	0.2 nmol g^{-1} wet wt
Total number of binding sites in AHA	2×10^{-8} mol per mgC L^{-1} DOC[b]

[a] Adopted from Playle et al. (1993b).
[b] Adopted from Van Ginneken et al. (2001).

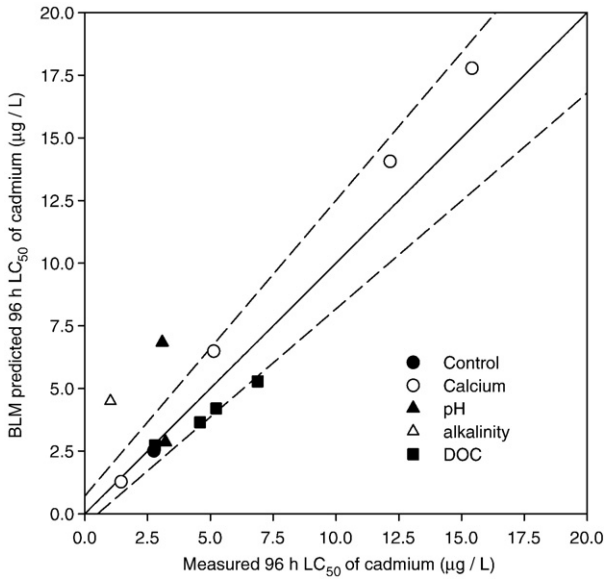

Fig. 6. Comparison of measured and predicted acute toxicity of cadmium (96 h LC_{50}) to juvenile rainbow trout. The solid line shows the line of perfect agreement among the measured values and the broken lines show the range of 95% confidence intervals (upper and lower). Different water chemistry treatments in acute toxicity assays are indicated by different symbols in the plot. The composition of the control water was 0.22 mM calcium, 0.19 mM magnesium, 0.57 mM sodium, 0.03 mM potassium and chloride, 40–48 mg L^{-1} water hardness as $CaCO_3$, 34 mg L^{-1} alkalinity as $CaCO_3$, and a pH of 7.3–7.5. The experimental treatments include variations in calcium (0.1, 1.0, 2.0 and 3.0 mM), pH (5.8 and 8.8), alkalinity (90 mg L^{-1} as CaCO3) and DOC (5, 10, 15 and 20 mgC L^{-1}).

4. Discussion

4.1. Characterization of cadmium binding sites at the gill

The short-term in vivo cadmium gill binding assay employed in our study revealed two distinct types of binding sites: (i) high affinity, low capacity sites which are saturable at ≤200 nM of waterborne cadmium, and (ii) low affinity, high capacity sites that exhibit linear increase in binding beyond 200 nM of cadmium. Previous studies have also demonstrated that cadmium binding in the gills of freshwater fish occurs through high affinity, low capacity sites at a relatively low cadmium concentration in water (Hollis et al., 1997, 1999, 2000; Playle et al., 1993b; Niyogi et al., 2004), and through low affinity, high capacity sites at higher cadmium level (Reid and McDonald, 1991). A similar pattern of gill binding in freshwater fish was also reported for copper, where the high affinity, low capacity sites were predominant at <250 nM of waterborne copper and low affinity, high capacity sites became more prominent above that level of copper (Taylor et al., 2000; Kamunde et al., 2002). Hollis et al. (1997, 1999, 2000) and Playle et al. (1993b) reported saturation of high affinity, low capacity sites at <240 nM of cadmium in water, which is quite similar to our observation. These sites which show saturation at low, environmentally realistic waterborne cadmium concentration range likely represent the physiologically active sites (apical Ca^{2+} channels and/or basolateral Ca^{2+}-ATPase molecules) in the gill that play an important role in maintaining calcium homeostasis in fish (Niyogi and Wood, 2004a,b). Therefore, we assumed that these represented the "sites of toxic action" that are relevant for developing a BLM for cadmium in fish. In contrast, the low affinity, high capacity sites probably bind to cadmium non-specifically, contributing to the gradual increase of gill cadmium burden over a longer period of exposure. They appear to be much less influenced by waterborne calcium (Hollis et al., 1997,

1999), and therefore not important from the perspective of the BLM approach.

According to the current principles of the BLM approach, the water chemistry variables such as hardness cations can influence gill-metal accumulation through competition, but they do not influence gill-metal binding characteristics (e.g., log K or B_{max}). We examined the gill cadmium binding pattern at two different waterborne calcium concentrations [0.22 mM (control) and 0.04 mM], and observed a significant increase in binding at low calcium in water. The analysis of binding characteristics revealed that the binding site density (B_{max}) increased by approximately 5-fold at low waterborne calcium concentration, although the affinity of cadmium binding by the gill (log $K_{Cd\text{-}gill}$) remained the same. This change in capacity cannot be explained by a simple competitive effect. However, this phenomenon is very similar to the 5-fold up-regulation of Ca^{2+} transport sites on the gills reported by Perry and Wood (1985) when rainbow trout were exposed to a similar low calcium water for 1 day. In our study, fish acclimated to moderately hard water (1 mM calcium) were transferred directly to synthetic soft waters [0.22 (control) and 0.04 mM calcium], which might have led to increased cadmium binding under both experimental conditions as well. Since Ca^{2+} is known to compete with Cd^{2+} for high affinity cadmium binding sites at the gill (Playle et al., 1993a,b; Playle, 1998; Niyogi et al., 2004) and *vice versa* (Niyogi and Wood, 2004a), this provides additional evidence that the same population of sites is targeted by both metals. Furthermore, this finding indicates that the cadmium binding characteristics of the gill can change rapidly depending on the chemistry of the ambient water contrary to the current BLM principles.

The log $K_{Cd\text{-}gill}$ value (7.5) of high affinity sites of our study is comparable with those previously reported in rainbow trout exposed to both soft water [7.3 at 0.12 mM calcium in water (Hollis et al., 2000)] and hard water [7. 6 (Hollis et al., 2000) and 7.3 (Niyogi et al., 2004), both at 1.0 mM calcium in water]. However, the log $K_{Cd\text{-}gill}$ value of our study is much less than the value (8.6) reported by Playle et al. (1993b) in fathead minnow, which corresponds to a more than 12 fold greater affinity of fathead minnow gills for cadmium compared to rainbow trout. It is to be noted that the synthetic soft water used in our study had much greater concentrations of natural cations (e.g., more than four fold higher calcium) relative to the water used by Playle et al. (1993b). This might have led to considerably greater competitive inhibition of gill cadmium binding, thereby reducing the apparent affinity of the gill for cadmium binding. In this regard, the log $K_{Cd\text{-}tox}$ estimate (8.0) derived by extrapolation to zero [Ca^{2+}] in our toxicity studies (Fig. 5A, Table 2) was much closer to the value of log $K_{Cd\text{-}gill}$ (8.6) in fathead minnow reported by Playle et al. (1993b).

The B_{max} value (0.62 nmol g^{-1} wet wt.) at control water (0.22 mM calcium) in our study was about 4 fold lower than the value of 2.27 nmol g^{-1} wet wt. reported in fathead minnow by Playle et al. (1993b). However, as noted above, we observed a higher B_{max} value (3.32 nmol g^{-1} wet wt.) than theirs when the calcium level in water was low (0.04 mM). Other B_{max} values reported for rainbow trout in previous studies are in this same general range — 1.78 nmol g^{-1} wet wt. in both soft and hard water (0.12 and 1.0 mM calcium in water, respectively) by Hollis et al. (2000), and 0.85 nmol g^{-1} wet wt. in hard water (1.0 mM calcium in water) by Niyogi et al. (2004). The variations likely reflect species, size, batch, and especially feeding differences in fish (Baldisserrotto et al., 2004; Wood et al., 2006), as well as differences in water calcium levels. As long as log $K_{Cd\text{-}gill}$ (or log $K_{Cd\text{-}tox}$) does not change, these variations can be dealt with practically by appropriate adjustment of the LA_{50} in BLM development. Nevertheless, these issues should be given greater attention in further refinement of the BLM approach.

4.2. Effects of cationic competition and cadmium complexation on gill binding and acute toxicity of cadmium

We observed a strong correlation between the effects of calcium on short-term cadmium gill binding and acute toxicity in rainbow trout, suggesting that the same population of gill sites was involved in both phenomena. A decrease in the waterborne calcium concentration significantly increased gill cadmium accumulation and toxicity, whereas the increase of calcium level in the water resulted in the decrease of both gill cadmium accumulation and toxicity. This observation is in very good agreement with the BLM assumption that the short-term metal accumulation at the biotic ligand can be the indicator of metal toxicity in fish. Both Hollis et al. (1997) and Playle et al. (1993a) recorded a concentration-dependent effect of waterborne calcium on short-term gill cadmium accumulation, although our study is the first to specifically investigate how that effect corresponds to 96 h acute toxicity. Interestingly, we did not observe any competitive effects of either Mg^{2+} or Na^{+} on short-term gill cadmium accumulation, although it is to be noted that we examined their effects at 6–7 fold higher concentration of cadmium relative to its acutely toxic level. Both Mg^{2+} and Na^{+} may have some protective effects against gill cadmium accumulation at low exposure level and acute toxicity of cadmium, a possibility which needs to be evaluated in future studies. The effects of water hardness on the acute toxicity of cadmium in fish are quite well documented (Calamari et al., 1980; Davies et al., 1993; Brinkman and Hansen, 2007). Based on our observation, it seems that this influence of hardness occurs primarily due to the calcium, not magnesium, in water.

Since the fractional concentration of Cd^{2+} ion in the exposure water did not change across the range of calcium concentration examined, the effects of calcium on cadmium binding can be attributed to the competition between Cd^{2+} and Ca^{2+} for the same binding sites at the gill. The log $K_{Ca\text{-}gill}$ and log $K_{Ca\text{-}tox}$ values derived in our study by competition against Cd^{2+} were 4.3 and 3.9, respectively, not far from the value of 3.7 determined directly for rainbow trout by Niyogi et al. (2004) using radioactive ^{45}Ca gill binding assays. All these values were somewhat lower than that reported in fathead minnow (5.0) by Playle et al. (1993b), but as noted earlier, these workers also reported a higher log $K_{Cd\text{-}gill}$ (8.6 versus 7.5–8.0), so the difference is to be expected. Playle et al. (1993b) also reported a significant reduction in gill cadmium accumulation at acidic pH (4.8) and suggested that it is due to the competition between H^{+} and Cd^{2+} at the gill surface. The result of our study is in contradiction to their observation since we did not notice any decrease in gill cadmium accumulation even at pH 4.5 relative to control pH 7.5. It is possible though that the relatively higher cadmium level in our assay water (18.4 μg L^{-1}) relative to the concentration (6.0 μg L^{-1}) used by Playle et al. (1993b) might have contributed to the lack of competitive effects of H^{+} on gill cadmium binding. The use of this higher cadmium exposure concentration could also explain the lack of protective effect against gill cadmium accumulation by alkalinity and low DOC level in water.

As observed with pH, we also did not observe any significant decrease in gill cadmium accumulation at alkaline pH (9.4) although predicted Cd^{2+} level in the water dropped sharply. Similar to our observation at the gill, we did not record any effects of pH (either acidic or alkaline) on the acute toxicity of cadmium in fish. Speciation calculation by MINEQL+ revealed that $CdCO_3$ and $CdOH^{+}$ were the two dominant cadmium species in our alkaline pH exposures. It is likely that either one or both of these cadmium species can bind to the gill, but that binding does not translate to toxicity.

The effects of alkalinity in our study are also quite intriguing. We did not find any significant decrease in gill cadmium accumulation across a broad range of alkalinity (10–90 mg L^{-1} as $CaCO_3$) although predicted Cd^{2+} concentration dropped gradually with increasing alkalinity. However, a significant increase in toxicity was recorded at 90 mg L^{-1} alkalinity level relative to control alkalinity (34 mg L^{-1}). Speciation calculations by MINEQL+ revealed $CdHCO_3^{+}$ to be the other major cadmium species besides Cd^{2+} at higher alkalinity levels. These findings imply that $CdHCO_3^{+}$ may be bioavailable as well toxic, although there is no mechanistic basis of such a notion at the present time.

DOC significantly decreased both gill cadmium accumulation and acute toxicity in fish at higher concentrations (≥10 mgC L^{-1}). These effects correlated quite well with the concomitant drop in Cd^{2+} concentrations in the exposure water. The ameliorative effects of natural humic acids on acute cadmium toxicity in fish have been demonstrated by several previous workers (Giesy et al., 1977; Winner, 1984). Van Ginneken et al. (2001) also reported that cadmium uptake in gills and whole body of carp (*Cyprinus carpio*) exposed to AHA followed the measured variations in Cd^{2+} ion activity in the exposure water, as observed in our study as well. Interestingly, the log $K_{Cd\text{-}DOC}$ values derived from gill binding as well as acute toxicity data (7.1 and 7.3, respectively) were quite comparable to that (7.4) derived in fathead minnow by Playle et al. (1993b). It should be appreciated that all these values are calculated indirectly, using the fish-ligand as a competitor, so are operationally defined.

4.3. BLM development and validation

The BLM was developed by adopting the affinity constants for BL-Cd^{2+}, BL-Ca^{2+} and DOC-Cd^{2+} binding derived from the acute toxicity data instead of those derived from gill binding results, although the two sets of corresponding values were not markedly different. This is based on the notion that parameter estimates derived from acute toxicity may be more appropriate since the predictive endpoint of the model is acute toxicity. The BLM developed in this study has some important differences with the cadmium gill binding model proposed by Playle et al. (1993b). The affinity constants for cadmium and calcium (log $K_{Cd\text{-}tox}$ and log $K_{Ca\text{-}tox}$) adopted in our model correspond to about 4 fold and 13 fold weaker binding of cadmium and calcium to the biotic ligand, respectively. Moreover, the present model has no affinity constants for H^+ since we did not record any competitive effects of H^+ either on cadmium gill binding or toxicity. By integrating our measured toxicity data (Table 1) with our measured gill binding data (Fig. 1), we derived a 3 h LA_{50} value of 0.27 nmol g^{-1} wet wt. This corresponds to ~45% of the total high affinity binding sites (0.62 nmol g^{-1} wet wt) on the biotic ligand occupied with cadmium, and is very close to the 3 h LA_{50} value reported by Niyogi et al. (2004) for rainbow trout — 0.35 nmol g^{-1} wet wt, or 40% of the high affinity binding site B_{max} recorded in their study. The model performed quite well in capturing the effects of calcium and DOC on acute cadmium toxicity; all of the predicted 96 h LC_{50} values were within the natural variability of the experimental results. However, the model was not able to reflect the influence of alkalinity and high pH on the acute toxicity of cadmium. This is because the model is based on the assumption that only Cd^{2+} ions are bioavailable and toxic to the fish gill, which may not be entirely true as our experimental results suggest. In this regard, De Schamphelaere et al. (2002) were able to account for the increase in the acute toxicity of copper at alkaline pH in *Daphnia magna* by incorporating affinity constants for binding of $CuOH^+$ and $CuCO_3$ to the biotic ligand in addition to Cu^{2+} in a BLM framework. Further studies are needed to determine whether such an approach can be applied for cadmium to capture the effects of pH and alkalinity in trout.

5. Conclusion

Overall, our study suggests that calcium and DOC in water are the most protective factors against acute cadmium toxicity in freshwater fish. Since the typical ranges of calcium and DOC concentrations in freshwater vary between 0.3–1 mM (Beeton et al., 1999) and 1–12 mgC L^{-1} (Morel, 1983) respectively, both will be important (c.f. Fig. 5) although calcium is likely to be more effective than DOC in most natural waters. The developed BLM, based on the estimates of interactions of different cations with the biotic ligand and DOC, was able to account for the effects of calcium and AHA, but not of high pH and alkalinity, on acute cadmium toxicity in rainbow trout. Future studies should focus on refinement of the model to address the pH and alkalinity effects as well as validation of the model by examining its ability to predict toxicity of cadmium in fish across a wide range of natural waters.

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