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Effects of Chronic Waterborne and Dietary Metal Exposures on Gill Metal-Binding: Implications for the Biotic Ligand Model

S. Niyogi* and Chris M. Wood
Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

ABSTRACT

The biotic ligand modeling (BLM) approach has gained recent widespread interest among the scientific and regulatory communities because of its potential for developing ambient water quality criteria (AWQC), which are site-specific, and in performing aquatic risk assessment for metals. Currently, BLMs are used for predicting *acute* toxicity (96 h LC50 for fish) in any defined water chemistry. The conceptual framework of the BLM has a strong physiological basis because it considers that toxicity of metals occurs due to the binding of free metal ions at the physiologically active sites of action (biotic ligand, *e.g.*, fish gill) on the aquatic organism, which can be characterized by conditional binding constants (log K) and densities (B_{max}). At present, these models assume that only water chemistry variables such as competing cations (*e.g.*, Na^+, Ca^{2+}, Mg^{2+}, and H^+), inorganic ligands (*e.g.*, hydroxides, chlorides, carbonates), and organic ligands (dissolved organic matter) can influence the bioavailability of free metal ions and thereby the acute toxicity of metals. Current BLMs do not consider the effects of *chronic* history of the fish in modifying gill-metal binding characteristics and acute toxicity. Here, for Cu, Cd, and Zn, we review a number of recent studies on the rainbow trout that describe significant modifying effects of *chronic* acclimation to waterborne factors (hardness and chronic metal exposure) and dietary composition (metal and essential ion content) on gill metal-binding characteristics (on both log K and B_{max}) and on acute toxicity. We conclude that the properties of gill-metal interaction and toxicological sensitivity appear to be dynamic rather than fixed, with important implications for further development of both *acute* and *chronic* BLMs. Now that the initial framework of the BLM has been established, future research needs a more integrative approach with additional

* Corresponding author: Tel (voice): 905-525-9140, x 23237, Tel(fax): 905-522-6066; niyogis@mcmaster.ca

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emphasis on the dynamic properties of the biotic ligand to make it a successful tool for ecological risk assessment of metals in the natural environment.

Key Words: *chronic* toxicity, copper, cadmium, zinc, gill-binding, Biotic Ligand Model.

INTRODUCTION: A BRIEF OVERVIEW OF THE CONCEPTUAL FRAMEWORK OF THE BIOTIC LIGAND MODEL

The Biotic Ligand Model or BLM is a mechanistic model that relates the bioavailability of a metal in a particular water quality to its toxicity in reference species, thereby potentially providing a cost-effective mechanism to generate site-specific ambient water quality criteria (AWQC) in an infinite variety of different water chemistries (DiToro *et al.* 2001; Paquin *et al.* 2002). While present versions of the BLM predict only *acute* toxicity (*i.e.*, 96h for fish, 48h for daphnids), the ultimate objective is to develop BLMs that successfully predict *chronic* toxicity, thereby providing *chronic* AWQC that are protective throughout the organisms' life cycle. The BLM considers the influence of both biotic and abiotic (organic and inorganic) factors on the bioavailability of metals to aquatic organisms.

The conceptual framework of the BLM (Figure 1) evolved from the gill surface interaction model (GSIM) originally proposed by Pagenkopf (1983) and the free ion activity model (FIAM) of Morel (1983), which provided a rationale for describing metal toxicity in terms of interactions of metal species with cell surfaces (Campbell 1995; Morel 1983; Morel and Hering 1993). This foundation has more recently been extended by others (DiToro *et al.* 2001; Janes and Playle 1995; Hollis *et al.* 1996,1997; MacRae *et al.*, 1999; Marr *et al.* 1999; Meyer *et al.* 1999; McGeer *et al.* 2000; Paquin *et al.* 2002; Playle 1998; Playle *et al.* 1992,1993a,b; Richards and Playle 1998; Santore *et al.* 2001; Wood *et al.* 1999) to yield the BLM as we know it today. The reader is referred to a recent journal issue (*Comparative Biochemistry and Physiology 133C*, 2002) for a compendium of 22 papers addressing various aspects of the BLM.

The general framework of the BLM can be described as a competition for metal complexation between environmental ligands that bind cationic metals (*e.g.*, natural dissolved organic matter [DOM], sulfide, thiosulfate, chloride, carbonate, hydroxide) and the biotic ligand (such as the gill surface in fish), which also binds cationic metals, and is the proximate target for metal toxicity. The modeling approach also takes into account environmental cations such as Na^+, Ca^{2+}, Mg^{2+}, and H^+ that can compete with the cationic metal for binding sites on the biotic ligand and thereby reduce or even prevent the binding of toxic metal ions. All competing reactions are simulated as simultaneous equilibrium reactions and equilibrium metal speciation includes formation of all relevant inorganic and organic metal complexes (Figure 1).

According to the BLM, the toxicity of metals to organisms occurs as the result of free metal ion reacting with the physiologically active binding sites at the site of action or biotic ligand. In practice, it appears that these physiologically active binding sites ("toxic sites") are specific proteins involved in the active uptake of essential ions across the gills in freshwater fish (see below). Standard aquatic geochemistry models [CHESS (Santore and Driscoll 1995); MINEQL+ (Schecher

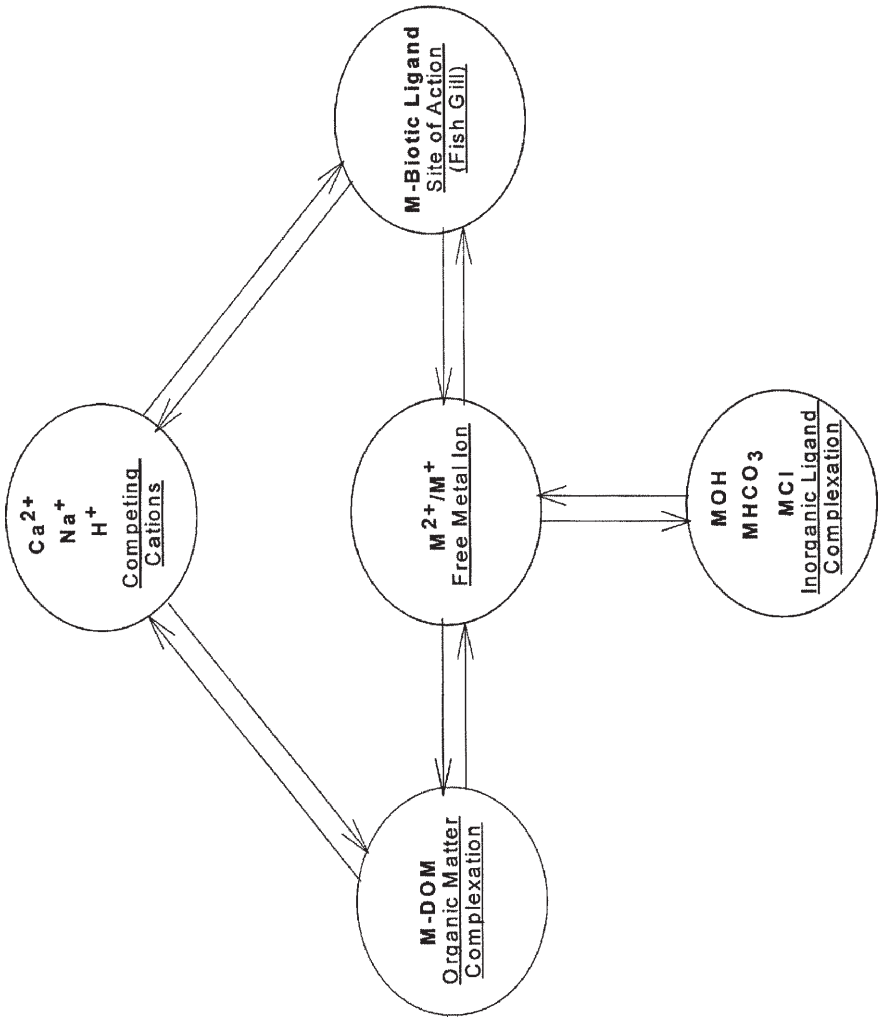

Figure 1. Schematic representation of the biotic ligand model (BLM) framework in freshwater fish (adapted from Di Toro *et al.* 2001).

and McAvoy 2001); MINTEQA2 (Allison *et al.* 1991); WHAM (Tipping 1994)] are used to predict the interactions of the metal ion (at chemical equilibrium) with relevant ligands in the water. The experimentally determined values (log K and B_{max} values, see below) for the biotic ligand (gill) can be integrated into these to evaluate the interaction of the metal ion, and of competing cations, with the toxic sites of interest on the biotic ligand. The relationship between metal binding at the biotic ligand (gill metal burden) and acute toxicity (generally 96 h LC50) can also be experimentally determined. These relationships can then be put together to predict acute toxicity in any particular water chemistry. Toxic responses may start to occur once metal accumulation at the biotic ligand significantly exceeds natural background levels, and mortality occurs when metal binding at the toxic sites crosses a threshold level (critical concentration) that results in the lethal disruption of specific ionoregulatory processes (for most metals). The critical metal concentration at the biotic ligand associated with 50% mortality at 96h is the LA50 ("lethal accumulation").

Characterization of the binding properties [affinity constant or conditional binding constant = K, usually expressed as negative logarithm thereof = log K, and maximum number of binding sites or binding site density (B_{max}, usually expressed as mol or nmol/g wet tissue)] of the gill surface for the free metal ion is a critical part of BLM development, because the amount of metal accumulation at the gill is limited by the number of binding sites and the affinity of those sites for that metal (Playle 1998). Although the goal is to evaluate toxicity at 96 h (the endpoint of importance for environmental regulations), in practice *in vivo* short-term (3 or 24 h) gill binding assays are generally employed (MacRae *et al.* 1999; Marr *et al.* 1999; Meyer *et al.* 1999; Playle *et al.* 1993a,b), because they are more convenient to carry out, and because they yield more consistent data than measurements done over longer periods. This is probably because the gill measurements are made before complications develop from non-specific binding over longer periods, from the disruptions associated with incipient death at higher concentrations, or most importantly from homeostatic adjustments of the properties of the toxic sites.

The fish are exposed to an increasing range of waterborne metal concentrations in a defined simple water chemistry (usually ion-poor soft water) to achieve different degrees of saturation for metal binding to the gill, and then gill metal concentrations are measured at 3 h or 24 h. Radioisotopic techniques may be employed to increase analytical sensitivity, especially when background levels of metal on the gill are high. Affinity constants (log K) and binding site densities (B_{max}) are calculated by Langmuir adsorption or Scatchard analysis (*e.g.,* Janes and Playle 1995; MacRae *et al.* 1999; Meyer *et al.* 1999; Playle 1998; Playle *et al.* 1993a,b). The use of organic ligands of known log K values to set up a competition between the ligands and the gill for the metal in question may help increase precision of the log K estimate for the biotic ligand. Once these binding constants are determined, these values can then be used to calculate gill-metal burden in relation to the dissolved metal concentration in any given water chemistry. Accurate determination of these gill-binding constants is critical to the successful application of the BLM approach in fish, and current versions of the BLM assume fixed values for a particular species. Therefore, any factor influencing these constants would have major implication for the BLM approach.

An alternative approach has also been proposed recently for determining metal-binding constants primarily in the aquatic invertebrate, *Daphnia magna*, an animal that is frequently used for the standard toxicological tests (De Schamphelaere and Janssen 2002; De Schamphelaere *et al.* 2002; Heijerick *et al.* 2002). In this approach, metal-binding constants are derived solely from toxicity data, using the same fundamental concept of the BLM that mortality occurs when metal-binding at the site of action reaches a critical level. This approach may not be preferable because it lacks a mechanistic basis, but it may be more practical because of very small size of the daphnids. This size limitation makes it problematic to readily sample and analyze the gill or other organ that may be the site of toxic action. Nevertheless, once these binding constants are determined, these values are then used to calculate an "imaginary" metal burden at the site of toxic action in daphnids in relation to the dissolved metal concentration in any given water chemistry. Again, any factor influencing these constants would have major implication for the BLM approach.

The motivation behind this review is the realization from our BLM-related research of the last few years that the gill-binding constants, at least in the rainbow trout, are in fact not fixed values, but rather appear to vary considerably as a result of the previous history of the fish — its *chronic* history. This variability will clearly impact the accuracy of the *acute* BLM; however, its greater significance may be its impact on BLMs for the prediction of *chronic* toxicity. We argue that this biological reality should be taken into account in future BLM development, so as to provide a more versatile and realistic tool for environmental risk assessment.

A BRIEF OVERVIEW OF GILL PHYSIOLOGY

Since one of the great strengths of the BLM is its mechanistic basis in physiology and chemistry, a brief overview of gill function is provided. The gills in fish serve the functions of both lungs and kidneys in mammals — they are not only the primary organ of gas exchange in fish, but also the primary organ of acid-base balance, nitrogenous waste excretion, and most importantly for the BLM, ionic regulation. The branchial epithelium is finely divided to provide large surface area, and highly permeable to facilitate gas exchange, thereby entraining a severe physiological penalty — the diffusional movements of electrolytes and water along their concentration gradients, and specifically, the metabolic cost of compensating these movements.

In freshwater, the internal environment (blood) of the fish is strongly hypertonic to the outside environment, causing a high rate of diffusive ion loss and osmotic water gain across the gills. To maintain constant ion levels in the blood relative to the ion-poor environment, freshwater fish must take up essential electrolytes (Na^+, Ca^{2+}, Cl^-) from the water by active transport through the gill epithelium, and produce a large volume of very dilute urine. The fine details of active ion transport in freshwater fish remain controversial, and are beyond the scope of this review. However, the generally accepted view is that transbranchial Na^+ uptake occurs via H^+-coupled Na^+ channels (or Na^+/H^+ exchange) on the apical membranes, and via Na^+, K^+-ATPase on the basolateral membranes of the gill ionocytes (Figure 2; see Wood 2001 for additional details). Similarly, Ca^{2+} uptake is thought to occur via apical voltage-insensitive Ca^{2+} channels and via basolateral high affinity Ca^{2+}-ATPase,

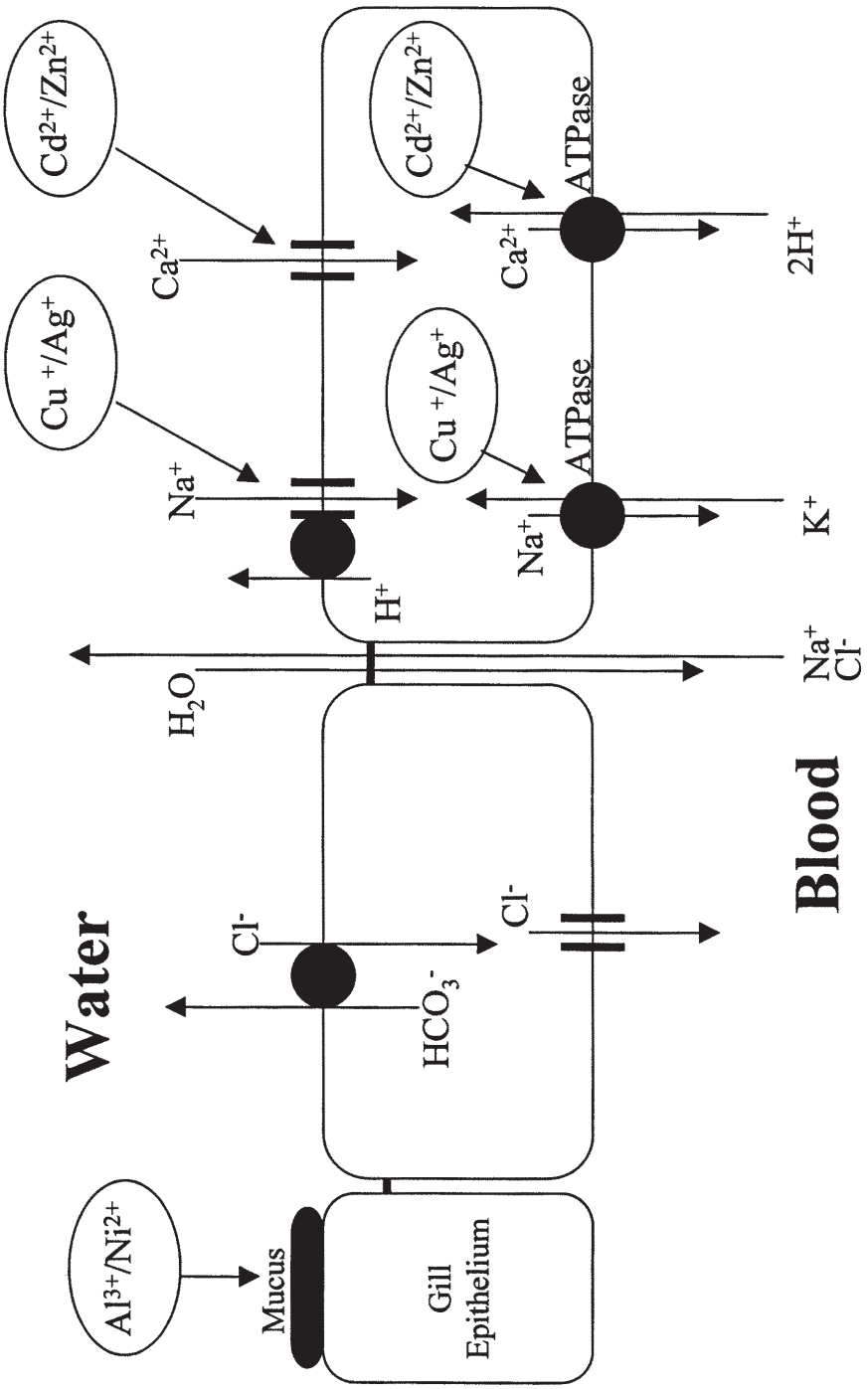

Figure 2. Schematic diagram of transport pathways of essential ions (Na^+, Ca^{2+} and Cl^-) and their interactions with toxic metal ions (Cu^+, Ag^+, Cd^{2+} and Zn^{2+}) in the gill epithelium of freshwater fish.

whereas Cl^- uptake is believed to occur by Cl^-/HCO_3^- exchange at the apical side and via an anion channel on the basolateral side (Figure 2).

These apical and basolateral transport channels, exchangers, and enzymes are proteins, many of which are negatively charged, to which specific positively charged metal ions bind. In practice, they represent the "toxic sites" of the biotic ligand. Cationic metal ions like Cu^+, Ag^+, Cd^{2+}, and Zn^{2+} will compete selectively with Na^+ and/or Ca^{2+} for these specific binding sites at the gills (Figure 2). At acutely toxic concentrations, cationic metals will severely interfere with the ability of the fish to perform normal ion uptake processes across the gill, and therefore passive ion losses will exceed active uptake, leading to eventual ionoregulatory failure and death (McDonald *et al.* 1989; Wood 1992; Wood *et al.* 1996, 1999). The physiological mechanisms of toxicity for most metals can generally be divided into three categories: monovalent metals (*e.g.*, Cu^+, Ag^+) disrupting Na^+ (and Cl^-) uptake, divalent metals (*e.g.*, Cd^{2+}, Zn^{2+}) disrupting Ca^{2+} uptake, and metals such as aluminum (Playle *et al.* 1989) and nickel (Pane *et al.* 2003) that appear to compromise the ability of the gill to take up O_2 and excrete CO_2, probably by increasing the diffusion distance by causing excess mucus secretion or gill cell swelling.

The disturbance of Na^+ and Cl^- homeostasis was originally found to be the direct cause of the acutely toxic effects of acidic pH levels in freshwater fish (McDonald 1983a,b; Milligan and Wood 1982). Later it was shown that elevated levels of certain metals could have similar effects. Disruption of Na^+ and Cl^- homeostasis has been reported as the primary toxic mechanism of Cu and Ag (Laurén and McDonald 1985; Wood *et al.* 1996), probably resulting from metal binding to the apical Na^+ entry mechanism and/or the basolateral Na^+K^+ATPase. Similarly, waterborne Cd and Zn at acutely toxic concentrations are known to disturb Ca^{2+} balance, probably by binding to the apical Ca^{2+} channel and/or basolateral Ca^{2+}–ATPase (Reid and McDonald 1988; Spry and Wood 1985; Verbost *et al.* 1987).

Interestingly, recent studies have demonstrated that chronic sublethal exposure to certain metals (*e.g.*, Cu, Cd, Zn) via either waterborne or dietary routes may produce acclimation in freshwater fish, a phenomenon that results in increased resistance to acute waterborne challenges with these metals (Alsop *et al.* 1999; Hogstrand *et al.* 1995; Hollis *et al.* 1999; Szebedenszky *et al.* 2001; Taylor *et al.* 2000). It has been suggested that gill metal-binding characteristics undergo significant changes as an adaptive response and actually act as a barrier in metal-acclimated fish, thereby minimizing internal metal loading (Hogstrand *et al.* 1998; Hollis *et al.* 1999, 2000a; Kamunde *et al.* 2000a,c). Moreover, acclimation to water chemistry parameters such as water hardness has also been reported to influence the gill metal-binding characteristics and, thereby, affect the acute toxicity of metals (Alsop *et al.* 1999; Hollis *et al.* 1999, 2000a,b; Taylor *et al.* 2000). Dietary loading with normal nutrient ions such as Na^+ and Ca^{2+} may also alter gill metal uptake and/or binding (Kamunde *et al.* 2002c; Pyle *et al.* 2002; Zohouri *et al.* 2001).

EFFECTS OF CHRONIC WATERBORNE AND DIETARY METAL EXPOSURES ON GILL METAL-BINDING

With this background in mind, in this paper we review from recently published works the effects of acclimation to water hardness, chronic metal exposure via water

or diet, and alterations in dietary ionic composition on the gill-binding characteristics of metals. In separate sections, we focus on Cu, Cd, and Zn, the three best-studied metals, and on the potential implications of these effects for the relevant *acute* biotic ligand models. The literature review and discussion is more representative than exhaustive, and mostly limited to the gills of rainbow trout (*Oncorhynchus mykiss*), the model teleost fish on which the bulk of the BLM principles have been developed.

A. Copper (Cu)

Copper is an essential metal in cellular metabolism (Cousins 1985) but is also potentially highly toxic to freshwater fish in the 0.16 to 16 μ*M* concentration range (Spear and Pierce 1979). Copper levels of 0.015 to 0.1 μ*M* are usually reported for uncontaminated natural freshwaters, but concentrations can be in the toxic range for fish in industrialized areas, especially in the vicinity of smelting, refining or metal plating industries, and in marinas and harbor areas due to contamination from Cu-based antifouling agents. Although Cu predominantly exists as a divalent form (Cu^{2+}) in bulk water, it is probably reduced to Cu^{+} via reductases on the gill surface or in the gill microenvironment before it is taken up through the branchial epithelium (Grosell *et al.* 2002; Handy *et al.* 2002). Gills are the primary target organ of waterborne Cu toxicity in freshwater fish, and the accumulation of Cu in the gills is influenced by competition from other cations (Na^{+}, Ca^{2+} and H^{+}), as well as complexation by dissolved organic matter (DOM), carbonates (CO_3^{2-}) and hydroxides (OH^{-}), thus rendering Cu less bioavailable.

Various workers have studied the gill binding characteristics of waterborne Cu in freshwater fish by *in vivo* gill binding assays using "cold" Cu (*e.g.*, MacRae *et al.* 1999; Marr *et al.* 1999; Playle *et al.* 1993a,b). The original gill Cu-binding constants (Playle *et al.* 1993b), which are still used in the *acute* Cu BLM today (DiToro *et al.* 2001; Paquin *et al.* 2002; Santore *et al.* 2001), were derived on the basis of there being only one type of binding site on the gill. However, several different types of Cu-binding sites have now been identified in trout gills by the use of radioactive Cu (^{64}Cu) over a short duration (3 h) of exposure. Taylor *et al.* (2000) and later Kamunde *et al.* (2002a) demonstrated predominantly high-affinity, low-capacity Cu-binding sites at <0.25 μ*M* of waterborne Cu, with low-affinity, high-capacity sites becoming more prominent above this level of Cu (Figure 3). More recently, Grosell and Wood (2002) identified a Na^{+}-sensitive and a Na^{+}-insensitive component of the high-affinity gill Cu-binding sites.

The high-affinity, low-capacity sites are a small proportion of the total sites and are saturable within the environmentally realistic range of waterborne Cu concentration (0.03 to 0.24 μ*M*; Taylor *et al.* 2002) and close to the working range (log K = 7.4) of the acute Cu BLM (Santore *et al.* 2001). It is believed that these high-affinity, low-capacity sites represent the physiologically active binding sites at the gill surface, the sites that are directly involved in ionoregulatory processes and, are therefore relevant from the perspective of the *acute* Cu BLM (Di Toro *et al.* 2001; Santore *et al.* 2001). In contrast, the low-affinity, high-capacity sites are not very specific to Cu and may, in fact, reflect a mixture of different binding sites that are not saturable, do not contribute to Cu uptake at environmentally realistic exposure

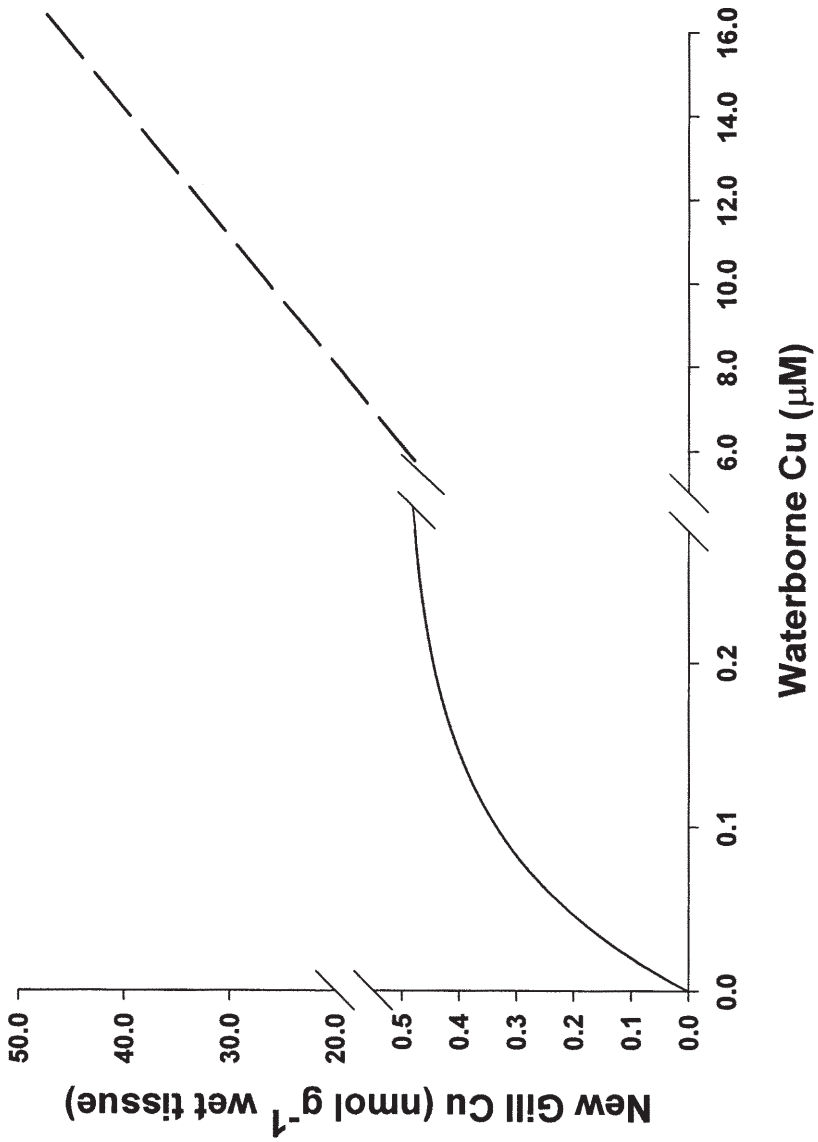

Figure 3. **Short-term *in vivo* gill Cu-binding characteristics in rainbow trout gills showing two types of Cu-binding sites. High-affinity, low-capacity sites at <0.25 *M* of waterborne Cu exhibit saturable binding, and low-affinity, high-capacity sites at >0.25 *M* of waterborne Cu exhibit linear binding (adapted from Taylor *et al.* 2000).** Eight data points (four at <0.25 M and four at >0.25) were used in total for fitting the curve. Each data point represented mean ± SE (N=10).

levels, and are of little relevance to the Cu BLM in fish (Kamunde *et al.* 2002a; Taylor *et al.* 2000).

(i) Effect of Acclimation to Different Water Hardness

Taylor *et al.* (2000) demonstrated the effect of acclimation to different water hardness on gill Cu-binding in rainbow trout acclimated to either hard (hardness 120 mg/L as $CaCO_3$) or soft water (hardness 20 mg/L as $CaCO_3$) for 10 weeks. Gill Cu-binding characteristics were evaluated by exposing the fish in hard and soft water, respectively, for 3 h to waterborne Cu ranging from 0.03 to 0.40 μ*M*. Saturation of high-affinity and low-capacity gill Cu-binding sites was observed at ~0.24 μ*M* of waterborne Cu in both hard and soft water, but there was a more than three-fold increase of binding site density ($B_{max,}$ from 0.6 to 1.9 nmol g^{-1} wet tissue) and a 20-fold decrease in binding affinity (log $K_{gill=Cu}$, from 9.1 to 7.9) in fish acclimated to soft water. The lower B_{max} in hard water acclimated fish was not surprising because Cu-binding to the gills in hard water incorporates the competitive effects of Ca^{2+} and Na^+ thereby reducing the effective bioavailability of free cationic Cu. Interestingly, Taylor *et al.* (2000) also reported a six-fold increase of acute toxicity (*i.e.*, decrease of 96 h LC50) from hard to soft water. The higher affinity of gill Cu-binding in hard water was therefore unexpected. They hypothesized that Ca^{2+}, which is known to be an important factor in regulating membrane permeability and increasing the stability of membrane proteins, might actually regulate both the number and the affinity of binding sites on the gill surface.

Taylor *et al.* (2002) later demonstrated that this probable effect of Ca^{2+} is indeed a chronic rather than an acute effect. Moreover, they did not find any competitive effect of Ca^{2+} in the softwater — hardwater range (0.2 to 1.1 m*M*) on *in vitro* gill Cu-binding determined by exposing isolated gill baskets for 5 min. Laurén and McDonald (1985) also observed no significant short-term effect of hardness (water Ca^{2+} 0.025 to 1 m*M*) on physiological indicators of acute Cu toxicity (Na^+, K^+, and Cl^- loss). Overall, it is reasonable to suggest that acclimation to water hardness, which in fish is largely attributable to Ca^{2+} in water, influences the physiology of gill-Cu interactions. This should not be surprising because water hardness is well known to affect the distribution of ionocytes in the gill (*e.g.*, low hardness, more ionocytes; Laurent *et al.* 1994; Perry and Wood 1985) and passive gill permeability (*e.g.*, low hardness, greater gill permeability; McDonald and Rogano 1986). The present *acute* Cu BLM, which has recently been approved by the U.S. Environmental Protection Agency (USEPA) for regulatory purposes (www.epa.gov/sab/blmresp.pdf), considers only the competitive effects of Ca^{2+} on gill surface ligands and does not incorporate its modulating effects under chronic exposure. In nature, fish would presumably be acclimated to the hardness of the receiving water, so these physiological effects of Ca are important.

(ii) Effect of Acclimation to Chronic Sublethal Waterborne Cu

In nature, fish living in receiving waters would also likely be chronically exposed to sublethal metal levels prior to acute toxic events. Again this is not considered in the present Cu-BLM. The effect of acclimation to chronic waterborne Cu exposure on short-term (3 h) gill Cu-binding in rainbow trout has been reported by both Kamunde *et al.* (2002a) and Taylor *et al.* (2000). Kamunde *et al.* (2002a) demonstrated that

diametrically opposite effects of chronic sublethal Cu exposure could occur, depending on the test concentration for gill Cu-binding. At test waterborne Cu concentrations <0.16 μ*M*, fish acclimated to waterborne Cu for 28 days [0.35 μ*M* in hard water, hardness 120 mg/L as $CaCO_3$] exhibited lower Cu-binding than non-acclimated fish, however, at higher waterborne Cu, the pattern reversed (Figure 4a,b). Taylor *et al.* (2000) observed similar alterations of gill Cu-binding over a concentration range of 0.03 to 16 μ*M* of waterborne Cu in rainbow trout acclimated for 30 days to sublethal waterborne Cu [0.35 and 0.03 μ*M* Cu in hard (hardness 120 mg/L as $CaCO_3$) and soft (hardness 20 mg/L as $CaCO_3$) water, respectively]. Taylor *et al.* (2000) also reported a change of gill Cu-binding properties from saturable to apparently linear within a concentration range of 0.03 to 0.3 μ*M* waterborne Cu in Cu-acclimated fish. These findings indicate that gill Cu-binding properties undergo substantial changes upon acclimation to chronic waterborne Cu, resulting in a decrease in affinity (lower log $K_{gill=Cu}$) of high-affinity, low-capacity binding sites, as well as an increase of binding site density (higher B_{max}) of low-affinity, high-capacity binding sites.

In a related study, McGeer *et al.* (2002) observed significantly higher uptake of Cu in gills over a period of 50 h in rainbow trout acclimated for 30 days to Cu in soft water (0.12 μ*M*, hardness 20 mg/L as $CaCO_3$) relative to nonexposed fish or fish acclimated to waterborne Cu plus elevated Aldrich humic acid (a commercially available DOM) in combination, further substantiating the influence of chronic Cu exposure on gill Cu-binding. Interestingly, humic acid complexed waterborne Cu during chronic Cu exposure, reducing its bioavailability, and thereby resulting in a lack of acclimation effect.

Acclimation to sublethal waterborne Cu in hard water has been reported to produce an increase in lethal tolerance in salmonid fish in several studies (Buckley *et al.* 1982; Dixon and Sprague 1981; Taylor *et al.* 2000), however, no such effect was observed in fish acclimated to Cu in soft water (McGeer *et al.* 2002; Taylor *et al.* 2000). This increase in lethal tolerance in hard water could be interpreted as an adaptive response induced by the decreased affinity in Cu-binding to high-affinity, low-capacity sites at the gills, but why the similar reduction of affinity does not translate into an increase of lethal tolerance in soft water is not clear. McDonald and Wood (1993) 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 repair of the gills (and changes therein) with continued exposure to the metal, leading to acclimation of the fish. Therefore, the answer may be that the chronic Cu exposure concentrations in soft water of all these studies were fairly low and probably did not induce morphological damage to the gills, and thereby did not result in acclimation. Moreover, the adaptive significance of an increased density of low-affinity, high-capacity sites at the gills upon acclimation to chronic waterborne Cu is also not obvious. Notably, however, Kamunde *et al.* (2002a) demonstrated that this increase does not translate into greater internalization or whole body uptake of Cu at the chronic exposure concentration.

(iii) Effect of Acclimation to Elevated Dietary Cu

In field situations, it is likely that fish under waterborne metal stress may also be exposed to elevated metal levels in the diet. There is no published work to date

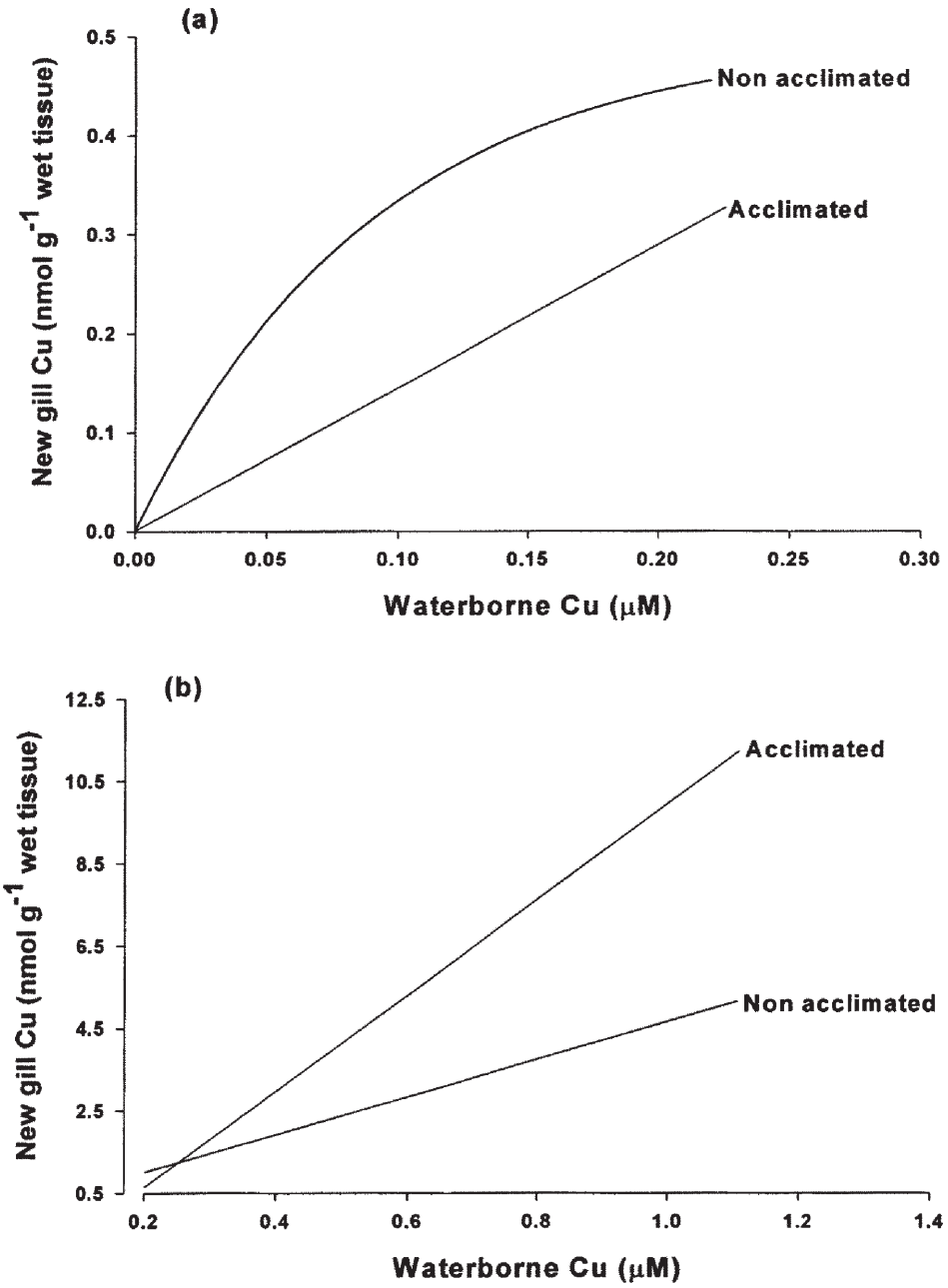

Figure 4. Effect of acclimation to chronic sublethal waterborne Cu exposure on (a) high-affinity, low-capacity binding sites and (b) low-affinity, high-capacity binding sites in short-term *in vivo* gill Cu-binding in rainbow trout (adapted from Kamunde *et al.* 2000a and Taylor *et al.* 2000). Eight data points (four for fitting each line) were used for both Figure 4 (a) and 4 (b), and each data point represented mean ± SE (N=8).

describing the effect of chronic dietary Cu exposure on short-term gill Cu-binding characteristics in fish. However, Kamunde *et al.* (2001) reported a significant reduction of branchial Cu uptake in rainbow trout chronically exposed to elevated dietary Cu (4.68 and 16 μmol/g) for 28 days relative to control diet (0.17 μmol Cu/g) at background levels (0.05 μ*M*) of waterborne Cu in hard water (hardness 120 mg/L as $CaCO_3$). In a more extensive study, Kamunde *et al.* (2002b) found that chronic (7 week) increases in dietary Cu burden [from 0.05μ mol/g (normal) to 4.44 μmol/g] caused a down-regulation of branchial Cu uptake, whereas chronic dietary Cu deficiency (0.013 μmol/g) caused an up-regulation of branchial Cu uptake, suggesting a homeostatic interaction between dietary and waterborne Cu uptake in fish.

These results support the notion that acclimation to elevated dietary Cu probably influences gill Cu-binding at the high-affinity, low-capacity sites, although it is not possible to comment on its effect on BLM gill binding constants from these data. Interestingly, Miller *et al.* (1993) reported increased tolerance to waterborne Cu following dietary Cu exposure (approximately 11 μmol/g) for 42 days, a factor that could be explained by altered gill Cu-binding characteristics. Overall, all these results suggest important implications of chronic dietary Cu exposure for the *acute* Cu BLM in fish.

(iv) Effect of Acclimation to Altered Dietary Na^+ Content

Since the acute toxic mechanism of Cu involves interference with active Na^+ uptake at the gills (Figure 2), and since fish can obtain ions from food as well as from water, two recent studies (Kamunde *et al.* 2003; Pyle *et al.* 2003) have evaluated the influence of alterations in dietary Na^+ content on waterborne Cu uptake and accumulation. Kamunde *et al.* (2003) studied the acclimation effect of chronic elevated dietary Na^+ [1.3 mmol/g vs. 0.26 mmol/g (control)] exposure on short-term (3 h) gill Cu-binding in rainbow trout under background Cu (0.019 μ*M*) as well as chronic sublethal Cu (0.18 μ*M*) exposure in soft water (hardness 20 mg/L as $CaCO_3$, 28 days). They demonstrated that chronic exposure to elevated dietary Na^+ alone decreases the binding of Cu to the gills within a waterborne Cu concentration range of 0.1 to 0.3 μ*M* compared with that in the control fish, indicating an effect on the high-affinity, low-capacity sites at gills (Figure 5). The evaluation of BLM gill Cu-binding constants revealed a significant decrease in binding site density (lower B_{max}) but no change in binding affinity (log $K_{gill=Cu}$) in fish acclimated to dietary Na (Figure 5). These findings suggest strong homeostatic control of Na^+ regulation in freshwater fish, dietary supplementation of Na^+ causing a down-regulation of branchial Na^+ uptake, thereby entraining a reduction of Cu uptake through the branchial Na^+ transport pathway (Figure 2). Furthermore, chronic exposure to a combination of waterborne Cu and elevated dietary Na^+ also decreased gill Cu-binding over the same range of waterborne Cu concentration relative to both Cu-acclimated and nonacclimated fish fed with normal diet (Figure 5). The binding site density (B_{max}) could not be determined in chronic waterborne Cu-acclimated fish, fed with either normal or Na-enriched diet, due to lack of saturation in binding. However, subsequent inspection of the data showed a further decrease of gill Cu-binding affinity (lower log $K_{gill=Cu}$) in fish acclimated to a combination of

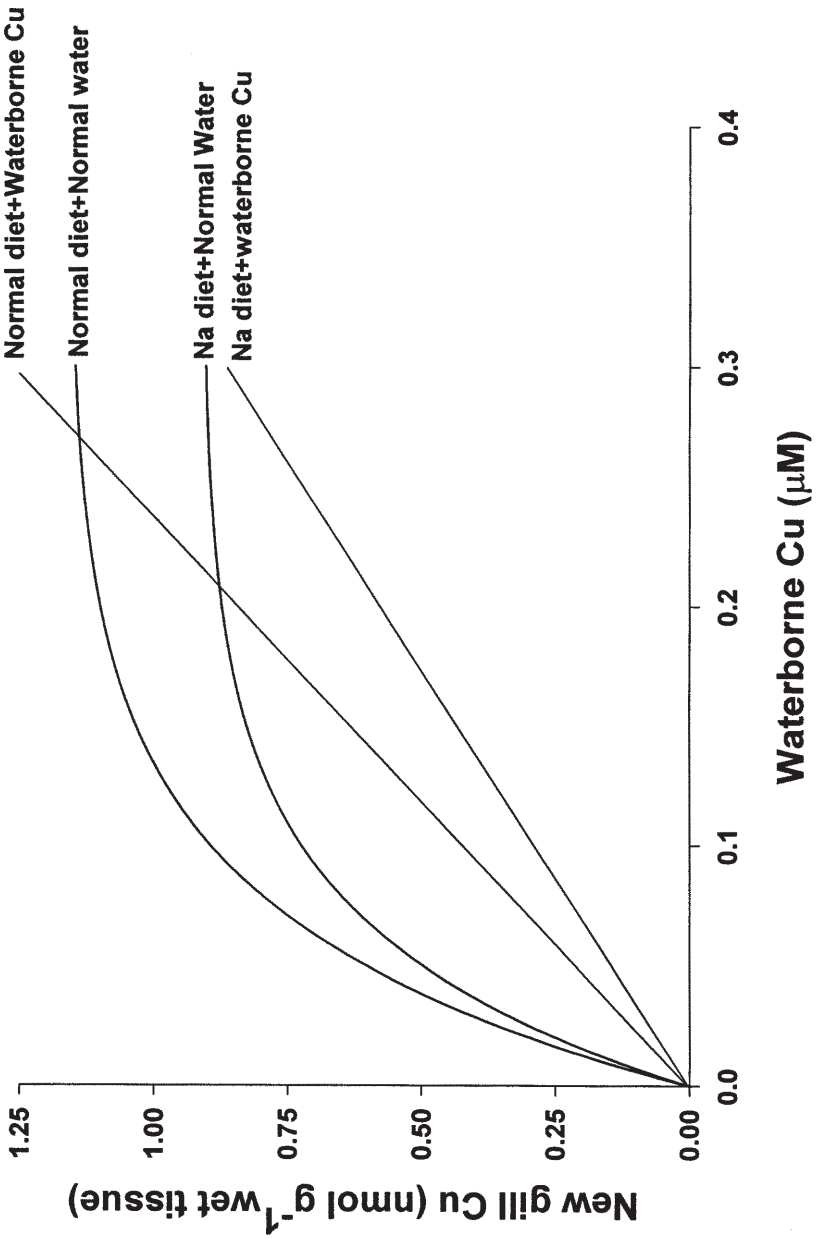

Figure 5. **Effect of acclimation to elevated dietary Na^+ on short-term *in vivo* gill Cu-binding in both chronic waterborne Cu-acclimated and nonacclimated rainbow trout (adapted from Kamunde *et al.* 2000c).** Sixteen data points (four for fitting each line) were used, and each data point represented mean ± SE (N=10).

Na-enriched diet and waterborne Cu relative to fish acclimated to waterborne Cu alone.

Based on the protective effect of waterborne Na^+ on Cu toxicity (Erickson *et al.* 1996), Na^+ binding to the gills is considered in the present *acute* Cu BLM (Santore *et al.* 2001). However, from the above studies it is quite clear that chronic exposure to elevated dietary Na^+ can significantly modify gill Cu-binding characteristics, both under chronic Cu exposure as well as under control conditions. In addition, elevated dietary Na^+ can support internal Na^+ levels in the face of branchial Cu actions, emphasizing the need to consider dietary factors such as Na^+ content in the *acute* Cu BLM because metal binding to the gill has direct bearing on toxicity. However, currently it is not known whether these modifications in gill Cu-binding properties produce any moderating effect on acute waterborne Cu toxicity, an important subject for future research. It is important to note here that acclimation to a Na-enriched diet does reduce waterborne Cu uptake and accumulation in target tissues (gill, liver, and kidney) in rainbow trout under sublethal Cu exposure (Kamunde *et al.* 2003; Pyle *et al.* 2003). Certainly, in field situations, fish under sublethal Cu stress may have the opportunity to increase the Na^+ content of their diets by switching from benthivory to piscivory.

B. Cadmium (Cd)

Cadmium is a nonessential element for biological functions in fish and can be highly toxic at relatively low concentrations. Cadmium levels in clean natural freshwaters are usually below 0.009 μM, but in environments impacted by man, concentrations can be several times higher (USEPA 2001). It is bioavailable as a free divalent cation, Cd^{2+}, which causes toxicity to fish (Pagenkopf, 1983). At the gill surface, Cd^{2+} competes with Ca^{2+} for high-affinity Ca^{2+}-binding sites (Playle 1998; Playle *et al.* 1993a,b) and once it enters the ionocytes, it blocks Ca^{2+} transport at the basolateral membrane through the inhibition of Ca^{2+}-ATPase (Verbost *et al.* 1987, 1988, 1989) (Figure 2). The cumulative effects of these two processes cause acute hypocalcaemia in fish leading to their death (Roch and Maly 1979; Verbost *et al.* 1987, 1989).

Acute Cd uptake and toxicity in fish are influenced by various water quality parameters [*e.g.*, hardness, pH, alkalinity, dissolved organic matter (DOM)]. Among these, water hardness appears to be the major factor influencing Cd toxicity (Calamari *et al.* 1980; Hollis *et al.* 1997, 2000b; Pagenkopf 1983; Pärt *et al.* 1985; Richards and Playle 1999). As water hardness increases, acute Cd toxicity decreases (Carrol *et al.* 1979; Davies *et al.* 1993; Pascoe *et al.* 1986). This is because at high water hardness, Ca^{2+}, the major hardness cation for fish, out-competes Cd^{2+} for binding sites on the gills and thereby reduces toxicity (Spry and Wiener 1991). Indeed, water hardness (or waterborne Ca^{2+} to be more specific) is the most important modifier of acute Cd toxicity in fish rather than pH or dissolved organic matter, which plays important role in modifying toxicity of other metals like Cu (Erickson *et al.* 1986; Hollis *et al.* 1997; MacRae *et al.* 1999; Playle *et al.* 1992, 1993a,b). The bioavailability and toxicity of Cd are less affected by DOM than are the bioavailability and toxicity of Cu (Block and Pärt 1986; Giesy *et al.* 1977; Winner 1984) because Cd binds about 10-fold more weakly to DOM than Cu binds to DOM (Alberts and Giesy 1983; Morel and Herring

1993), and binds about 16 times more strongly at fish gills than Cu (Playle *et al.* 1993b).

Cadmium-binding in the gills of freshwater fish occurs through high-affinity, low-capacity binding sites (Hollis *et al.* 1996, 1997, 1999; Playle *et al.* 1993a,b) as well as low-affinity, high-capacity sites (Reid and McDonald 1991). The high-affinity, low-capacity sites exhibit saturatable binding at <0.24 μ*M* of waterborne Cd in short-term (3 h) gill binding assays (Hollis *et al.* 1997, 1999, 2000a; Playle *et al.* 1993b) and probably represent the physiologically active sites (apical Ca^{2+} channels and/or basolateral Ca^{2+}-ATPase molecules) that play an important role in maintaining Ca^{2+} homeostasis in fish. Therefore, these are "toxic sites" that are relevant for the *acute* Cd BLM. In contrast, the low-affinity, high-capacity sites are probably involved in the gradual increase of gill Cd burden over a longer period of exposure, and appear to be much less influenced by water hardness (water Ca^{2+}) (Hollis *et al.* 1996, 1997, 1999), and are therefore not important for the *acute* Cd BLM.

(i) Effect of Acclimation to Different Water Hardness

Hollis *et al.* (1999, 2000a) demonstrated the effect of acclimation to different water hardness levels on *in vivo* 3 h gill Cd-binding in rainbow trout. They reported increased binding of Cd in gills of fish acclimated for 30 days to soft water (hardness 20 mg/L as $CaCO_3$) compared to fish acclimated for 30 days to hard water (hardness 120 mg/L as $CaCO_3$). The affinity of Cd-binding in the gill (log $K_{gill=Cd}$) was reduced twofold (7.6 to 7.3) from hard water to soft water, whereas the binding site density (B_{max}) increased only slightly (1.60 to 1.88 nmol/g wet tissue) (Figure 6). In a separate study, Hollis *et al.* (2000b) evaluated the *in vivo* 3 h gill Cd-binding characteristics in rainbow trout acclimated to four different concentrations of waterborne Ca^{2+} for 30 days. Here again, they observed a consistent trend of a gradual increase of binding site density (higher B_{max}) in the gills with the gradual decrease of the waterborne Ca^{2+} levels (decreasing hardness) of acclimation. However, they also found a gradual increase of Cd-binding affinity (higher log $K_{gill=Cd}$) with gradual decrease of waterborne Ca^{2+} levels (decreasing hardness), which is not in agreement with their earlier findings.

Cadmium shares the same uptake pathway with Ca^{2+} in fish gills and Cd-binding sites in gills are part of the branchial Ca^{2+} transport system (Figure 2). Therefore, the increase of B_{max} of Cd in the gills in the softer water probably results from an up-regulation of the branchial Ca^{2+} transport system as an adaptive mechanism of fish to survive in ion-poor soft water. Recent studies in our laboratory with wild yellow perch (*Perca flavescens*) collected from extremely soft water lakes (hardness 10 to 40 mg/L as $CaCO_3$) of the Sudbury region of Northern Ontario, Canada, also demonstrated a significant increase in density (B_{max}) of both Cd^{2+}- and Ca^{2+}- binding sites relative to those of a yellow perch population reared in hard water in our laboratory (hardness 120 mg/L as $CaCO_3$; Niyogi *et al.* 2003). However, in contrast to rainbow trout, there was no difference in affinity in gill Cd-binding (log $K_{gill=Cd}$) in yellow perch. Interestingly, no difference in affinity in gill Ca^{2+}-binding (log $K_{gill=Ca}$) was recorded either between soft- and hardwater-acclimated yellow perch.

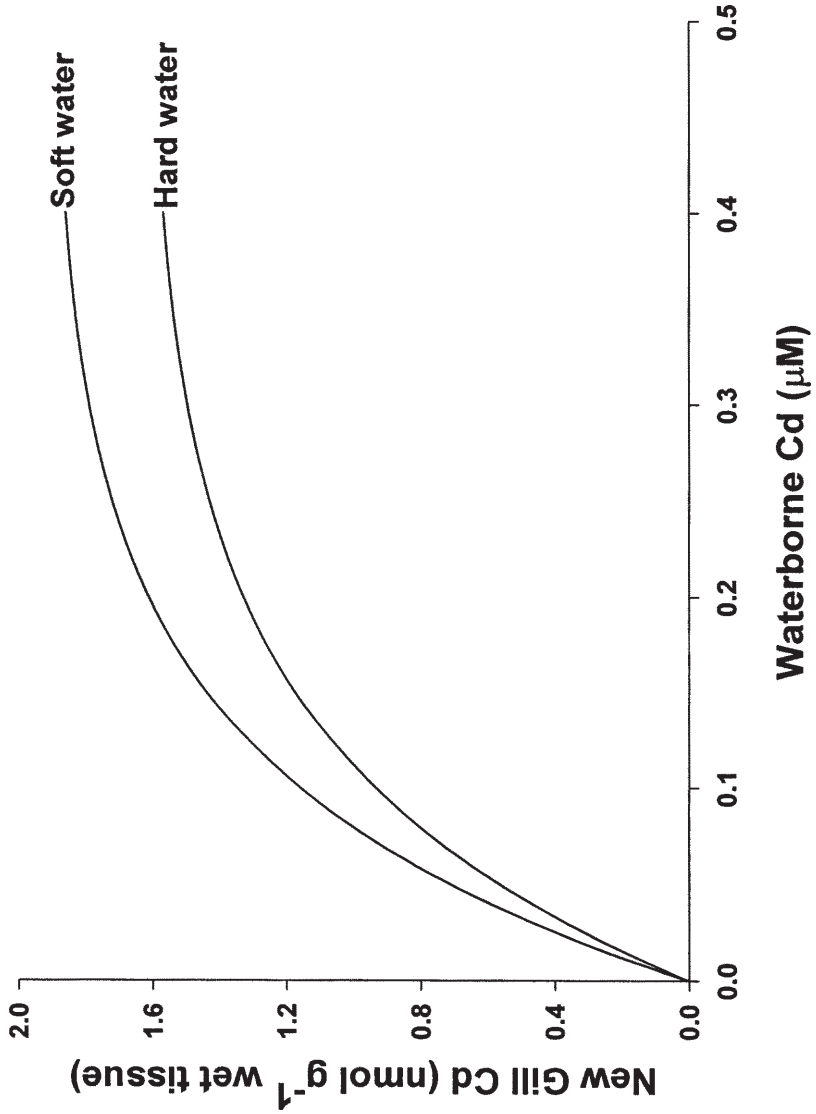

Figure 6. **Effect of acclimation to different water hardness levels on short-term *in vivo* gill Cd-binding in rainbow trout (adapted from Hollis *et al.* 1999 and 2000a).** Eight data points (four for fitting each line) were used, and each data point represented mean ± SE (N=5).

A more than 10-fold decrease of acute Cd tolerance (decrease of 96 h LC50) was reported in trout acclimated to soft water relative to fish acclimated to hard water (Hollis *et al.* 1999, 2000a). This decrease in Cd tolerance could be due to both increased bioavailability of free Cd^{2+} ion in soft water (as a result of less cationic competition and less organic as well as inorganic complexation) and the increase in binding site density (higher B_{max}) in soft water, making fish more vulnerable to increased internalization of Cd.

An *acute* Cd BLM is currently in an active state of development for the USEPA. This provisional BLM considers only the acute effect of differences in water hardness. The gill-binding constants used in this present format have been adopted from the high-affinity gill surface binding model developed for fathead minnow (*Pimephales promelas*) (Playle *et al.* 1993a,b). Although this model is based on studies carried out in synthetic water of extremely low hardness, Hollis *et al.* (1999) reported reasonable success in predicting gill Cd-binding in rainbow trout in hard water using the same gill-binding constants as used in this model. However, there is no information available at present regarding its ability to address the differences of acute waterborne Cd toxicity between hard and soft water.

(ii) Effect of Acclimation to Chronic Waterborne Cd Exposure

The effect of acclimation to chronic sublethal waterborne Cd on short-term (3 h) *in vivo* gill Cd-binding in rainbow trout acclimated for 30 days to Cd concentrations of 0.027 and 0.089 μ*M* in hard water (hardness 120 mg/L as $CaCO_3$) and 0.0006 and 0.001 μ*M* in soft water (hardness 20 mg/L as $CaCO_3$) was examined by Hollis *et al.* (1999, 2000a). The Cd-binding pattern in gills changed from saturable to almost perfectly linear upon acclimation to chronic waterborne Cd (except in fish acclimated to 0.027 μ*M* Cd in hard water) within a concentration range of 0.09 to 0.35 μ*M* of Cd in water (Figure 7). Relative to nonacclimated fish, the binding affinity of Cd (log $K_{gill=Cd}$) in gills decreased considerably in all groups of chronic waterborne Cd-acclimated fish. A 60% increase (from 1.6 to 2.6 nmol/g wet tissue) in binding site density (B_{max}) was reported in fish acclimated to 0.027 μ*M* Cd in hard water; however, no saturation occurred in gill Cd-binding in the remaining chronic waterborne Cd-acclimated groups so binding site density could not be determined in these treatments.

Hollis *et al.* (2000b) extended these findings in a related study, which showed the similar trend of decreasing affinity and increasing site density of gill Cd-binding in rainbow trout acclimated to chronic waterborne Cd (0.018 μ*M*) at variable waterborne Ca^{2+} concentrations. Szebedinszky *et al.* (2001) also reported a decrease of binding affinity (lower log $K_{gill=Cd}$) from 7.05 to 6.54 and an increase of binding site density (3.12 to 4.80 nmol/g wet tissue) following acclimation to 0.018 μ*M* of Cd in hard water (hardness 120 mg/L as $CaCO_3$) (Figure 8). These phenomena were once again reflected in our recent study with wild yellow perch from clean and Cd-contaminated lakes of the Sudbury region. Perch from contaminated lakes exhibited a lack of saturation and a decrease in affinity of gill Cd-binding within a range of approximately 0.09 to 0.45 μ*M* of waterborne Cd relative to perch from clean lakes (Niyogi *et al.* 2003).

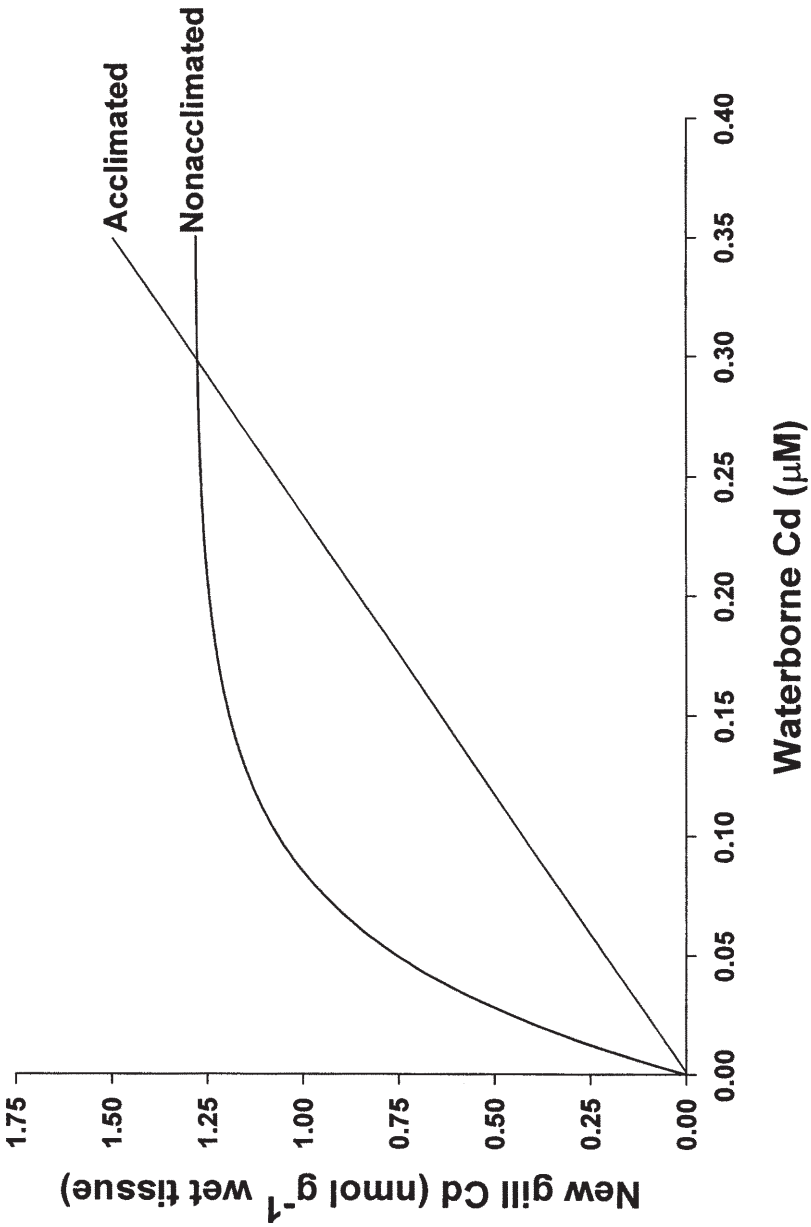

Figure 7. **Effect of acclimation to chronic sublethal waterborne Cd exposure on short-term *in vivo* gill Cd-binding in rainbow trout (adapted from Hollis *et al.* 1999 and 2000a).** Eight data points (four for fitting each line) were used, and each data point represented mean ± SE (N=5).

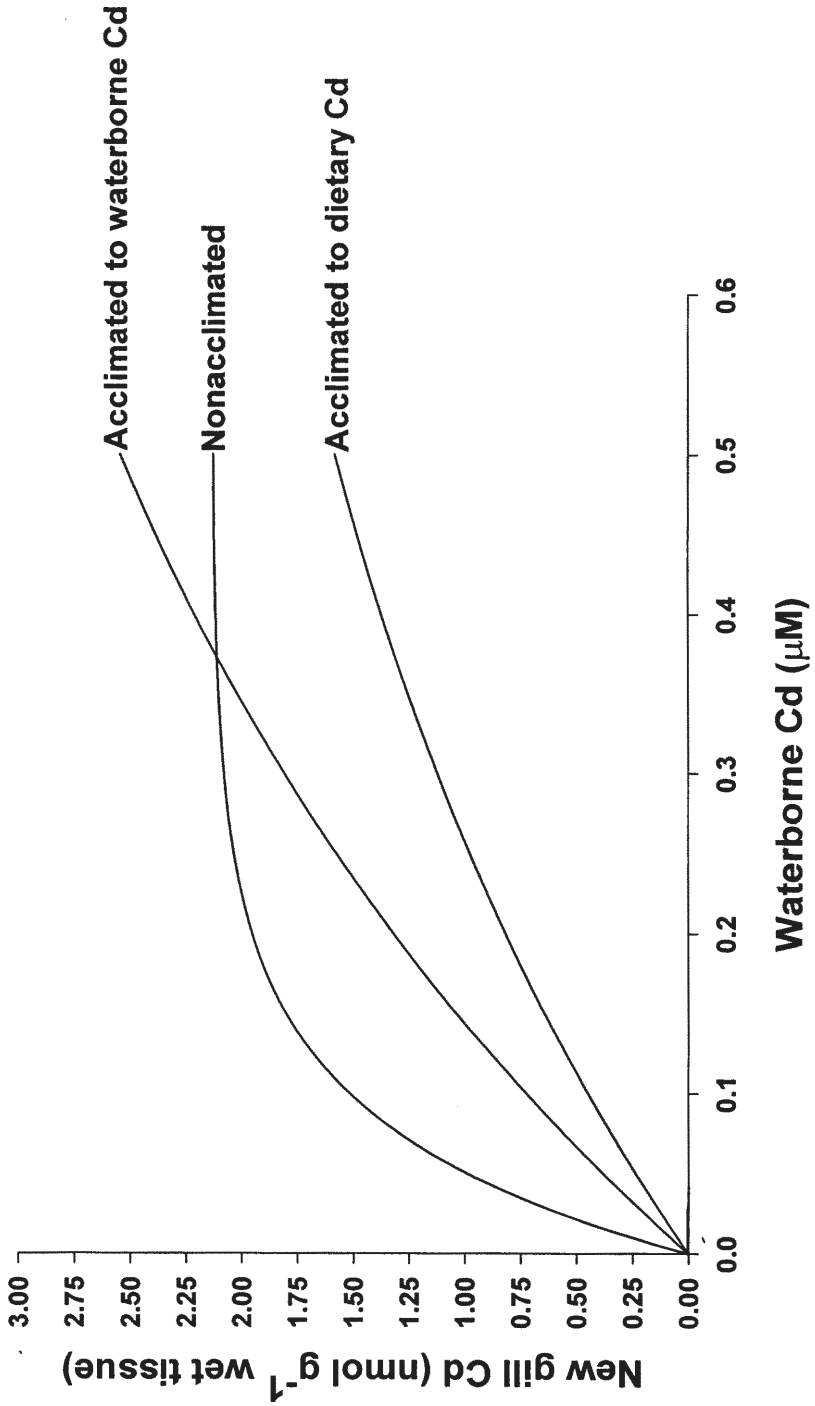

Figure 8. **Effect of acclimation to elevated dietary Cd on short-term *in vivo* gill Cd-binding in rainbow trout (adapted from Szebedinszky *et al.* 2001).** Fifteen data points (five for fitting each line) were used, and each data point represented mean ± SE (N=6).

Subsequent to the changes in short-term gill Cd-binding characteristics, Hollis *et al.* (1999) reported an 11- to 13-fold increase of acute Cd tolerance (increased 96 h LC50) in rainbow trout acclimated to chronic sublethal Cd in hard water. However, no such acclimation effect was observed in fish exposed to chronic waterborne Cd in soft water (Hollis *et al.* 2000a,b). The reduction of binding affinity in gill Cd-binding following acclimation to chronic Cd exposure in hard as well as soft water is probably the protective mechanism for reduced Cd uptake. The increase in binding sites could be a reflection of increased Cd pool sizes in the gill that are related to increased detoxification or temporary storage of Cd, for example in mucus.

However, the question remains as to why toxicological acclimation did not occur in soft water despite the fact that gill Cd-binding characteristics did change in a manner consistent with that in hard water. Interestingly, a similar lack of acclimation was also observed in fish acclimated to Cu in soft water. Therefore, the probable explanation could be, as described in the case of Cu as well, that it is due to the fairly low exposure concentrations of Cd which did not produce any morphological damage to the gills and thereby acclimation (McDonald and Wood 1993). Overall, these findings emphasize the limitations of the present *acute* Cd BLM approach in predicting the gill-Cd binding and acute Cd toxicity under *chronic* waterborne Cd exposure.

(iii) Effect of Acclimation to Elevated Dietary Cd

Szebedinszky *et al.* (2001) described the effect of acclimation to chronically elevated Cd in the diet on short-term (3 h) gill Cd-binding in rainbow trout (Figure 8). Following exposure to high dietary Cd (13.4 μmol/g of food relative to a control level of 0.013 μmol/g) for 30 days in hard water (hardness 120 mg/L as $CaCO_3$), short-term (3 h) gill Cd-binding was evaluated within a concentration range of 0.011 to 0.49 μ*M* of waterborne Cd. It is important to note here that no significant increase in waterborne Cd from the Cd-enriched diet was observed in any exposure tanks over the entire period of acclimation. The gill Cd-binding affinity decreased significantly (from log $K_{gill=Cd}$ 7.05 to 5.92), whereas binding site density increased approximately 75% (from B_{max} 3.12 to 5.50 nmol/g wet tissue) in comparison to the fish fed with normal diet. Szebedinszky *et al.* (2001) suggested that the affinity of the gill for Cd is influenced both by the gill Cd burden promoted by Cd-enriched food as well as by the changes that occur during waterborne acclimation. Szebedinszky *et al.* (2001) also reported a two-fold increase of acute waterborne Cd tolerance (two-fold increase in 96 h LC50) in dietary Cd-acclimated fish compared to fish fed with normal food, strengthening the notion that alteration in gill Cd-binding characteristics due to dietary Cd-acclimation can protect freshwater fish against acute waterborne challenge.

(iv) Effect of acclimation to dietary Ca^{2+} Content

Since elevated dietary Na^+ decreased Na^+ uptake, and therefore Cu uptake through the gills [see section A(iv)] by analogy, elevated dietary Ca^{2+} might be expected to decrease Ca^{2+} uptake, and therefore Cd uptake through the gills. In accord with this idea, a diet only moderately enriched in Ca^{2+} [1320 μmol/g vs. 740 μmol/g (control)] substantially reduced Cd accumulation in target tissues (gill,

liver, and kidney) in rainbow trout chronically exposed for 30 days to sublethal waterborne Cd (0.023 μ*M*) (Zohouri *et al.* 2001). Furthermore, recent work in our laboratory has demonstrated that whole body Cd uptake as well as newly accumulated Cd in gills and liver decreased significantly under short-term (4 h) acute waterborne Cd exposure (0.45 μ*M*) in rainbow trout acclimated for 30 days to moderately elevated dietary Ca^{2+} (750 and 1500 μmol/g) relative to fish fed with normal food (Ca^{2+}: 500 μmol/g) in hard water (hardness 120 mg/L as $CaCO_3$) (Baldisserotto *et al.* 2003). Notably, both Zohouri *et al.* (2001) and Baldisserotto *et al.* (2003) did not report any significant increase of waterborne Ca from the Ca-enriched diet in any exposure tanks during the acclimation period.

These findings indicate that acclimation to elevated dietary Ca^{2+} does influence gill Cd-binding characteristics in rainbow trout under acute waterborne Cd exposure, although we do not know whether it is due to reduced binding affinity (lower log $K_{gill=Cd}$) or binding site density (lower B_{max}) or both. Interestingly, a significant decrease of whole body Ca^{2+} uptake and newly accumulated Ca^{2+} in gills and liver was also observed both in presence and absence of acute waterborne Cd (0.45 μ*M*) in fish acclimated to elevated dietary Ca^{2+} relative to fish fed with normal diet. These findings indicate that dietary supplementation with Ca^{2+} downregulates the branchial Ca^{2+} uptake process, and thereby reduces branchial Cd uptake through the same pathway (Figure 2). Reduction of Cd uptake and new Cd accumulation in target tissues in fish on high Ca^{2+} diets could have potential protective effects against both acute and chronic waterborne Cd toxicity by reducing the internalized Cd load and supporting plasma Ca^{2+} homeostasis. Certainly, in the field, fish under sublethal Cd stress may have the ability to increase the Ca^{2+} content of their diets by foraging more on calcium-rich mollusks and crustaceans, though this idea has never been tested. On the other hand, fish in Cd-contaminated sites might have fewer mollusks and crustaceans to feed on, since they too might encounter Cd-induced problems in Ca^{2+} homeostasis.

C. Zinc (Zn)

Zinc is an essential micronutrient for the fish; however, it becomes toxic at increased waterborne levels due to pathological interactions of Zn with transport functions on the gill surface (Hogstrand *et al.* 1995, 1996; Spry and Wood 1985). The natural and toxic ranges are much higher for Zn than for Cu or Cd. While normal Zn levels in pristine fresh water are only a few n*M* or less, concentrations of 0.75 μ*M* are routine in industrialized areas. Maximum Zn concentrations in contaminated surface waters are reported to range from 2 to 18 μ*M* in different areas of Canada (Canadian Council of Ministers of the Environment 1995). At these levels, Zn severely disrupts Ca^{2+} uptake by the gills in freshwater fish (Figure 2), leading to hypocalcemia, which may end with the death of the fish within a few days, depending on the Zn concentration (Hogstrand *et al.* 1995; Spry and Wood 1985).

In water quality regulations (*e.g.,* USEPA 1980), water hardness is the only water quality component that is currently taken into consideration as a modifying agent for acute Zn toxicity. Ca^{2+}, the major hardness cation, competes with Zn^{2+} for the specific binding sites at the gill surface (Figure 2), thereby reducing Zn uptake and toxicity (Alsop *et al.* 1999; Alsop and Wood 1999; Spry and Wood 1989). Moreover,

an increase in waterborne Ca^{2+} may have the additional benefit of facilitating Ca^{2+} uptake when this saturable process is operating at a less than the maximum rate and probably helps to restore Ca^{2+} homeostasis in fish (Alsop and Wood 1999; Santore *et al.* 2002). Other cations like Na^+, K^+, and Mg^{2+} can also markedly reduce Zn^{2+} uptake, probably through relatively nonspecific competition for anionic sites on the gill; however, they have negligible effect on Zn toxicity (Alsop and Wood 1999). Bradley and Sprague (1985) as well as Cusimano *et al.* (1986) showed a protective effect of sublethal low pH in water against acute Zn toxicity in freshwater teleosts, indicating competition between H^+ and Zn^{2+} for the apical binding sites at the gill.

Zn has been less well studied than Cu and Cd as far as the information needed for derivation of *acute* BLMs is concerned, though a provisional acute BLM for Zn has just been published (Santore *et al.* 2002). In this version, pH has also been incorporated in addition to water hardness (waterborne Ca) as an important modifying factor for acute Zn toxicity. This model predicts lower acute toxicity (higher 96 h LC50) of Zn at pH<6 due to increased competition between H^+ and Zn^{2+} resulting in displacement of Zn from the biotic ligand (gill). It also predicts lower acute toxicity (higher 96 h LC50) of Zn at pH>8, because of increased complexation of Zn^{2+} by hydroxides resulting in its reduced bioavailability.

While there is a great deal of toxicity data available for Zn, there is a paucity of gill binding data. In particular, the characterization of Zn-binding in the gills of fish by using "cold" Zn has not been possible because of the difficulty in distinguishing newly accumulated Zn from the large pool of native Zn already present. However, Galvez *et al.* (1998) and later Alsop and Wood (2000) overcame this problem by using radioactive ^{65}Zn and successfully determined the binding affinity ($\log K_{gill=Zn}$) and binding site numbers (B_{max}) for Zn at the gills in rainbow trout employing a 3 h *in vivo* gill binding assay. Alsop *et al.* (1999) and later Alsop and Wood (2000) reported that the fish gill possesses at least two pools of Zn, a fast and a slow turnover pool. They interpreted the fast pool as a dynamic pool bound to the high-affinity, low-capacity sites (physiologically active sites). These sites show saturation in Zn binding at <8 μ*M* of waterborne Zn concentration under short-term exposure and are probably most important from the perspective of the *acute* Zn BLM. In contrast, the slow pool appears to represent Zn that is bound to the nonspecific, low-affinity, high-capacity sites that exhibit linear binding over longer period of time, and actually are of little significance to the *acute* Zn BLM.

(i) Effect of Acclimation to Different Water Hardness

At present there is no published report addressing specifically the effects of acclimation to different water hardnesses on the BLM gill Zn-binding characteristics in fish. Alsop *et al.* (1999) studied the turnover of the slow pool of Zn between 24 to 75 h in the gills of rainbow trout acclimated for 30 days to both hard (hardness 120 mg/L as $CaCO_3$) and soft water (hardness 20 mg/L as $CaCO_3$) at 2.3 μ*M* of waterborne Zn. By extrapolating the regression of the slow turnover pool to 0 h, they estimated the fast turnover pool of Zn in both hard and softwater-acclimated fish. The size of the fast pool, which represents the Zn-binding sites that are relevant to the *acute* Zn BLM, was significantly larger in the softwater-acclimated fish relative to the hardwater-acclimated fish. This increase in the fast pool indicates an increase in

the number of high-affinity, low-capacity Zn binding sites (higher B_{max}) upon acclimation to soft water; however, it is not possible to say anything about alterations in binding affinity (log $K_{gill=Zn}$) from these data. Barron and Albeke (2000) reported significantly lower branchial uptake of Zn in rainbow trout acclimated for 14 to 22 days to high waterborne Ca^{2+} (3.28 mM) relative to the fish acclimated to low waterborne Ca^{2+} (0.16 m*M*) over a period of 24 h exposure in 1.56 μ*M* of waterborne Zn. This observation further helps to substantiate the acclimation effect of water hardness (waterborne Ca^{2+}) on Zn-binding characteristics in the gills of fish.

Spry and Wood (1989) studied the effect of waterborne Ca^{2+} on Zn uptake kinetics in rainbow trout acclimated to hard water (Ca: 1 m*M*). They observed that acutely raising the concentration of waterborne Ca^{2+} (2.35 and 4.85 m*M*) over a concentration range of 0.8 to 23 μ*M* waterborne Zn produced little change in maximum rate of Zn uptake (J_{max}) but caused large decreases in affinity (increased K_m, concentration at which 50% of the maximum transport rate is observed), thereby suggesting Ca^{2+} inhibits branchial Zn uptake directly by competitive interaction at the gills of fish. Both Alsop *et al.* (1999) and Bradley and Sprague (1985) reported that the Zn was much more acutely toxic in softwater-acclimated trout relative to hard water-acclimated trout. The greater toxicity of Zn in soft water is likely explainable by the presence of fewer Ca^{2+} ions, which offer competition to Zn^{2+} for the binding sites on the gill, and of fewer ligands (carbonates, hydroxides, sulfates), which could complex Zn^{2+} in the water. The pH is not expected to have any impact because the pH values in both soft (7.2) and hard (8.0) water were within the range of 6 to 8. Nevertheless, it is unclear whether changes in Zn-binding characteristics induced by acclimation to different levels of water hardness have any role in modifying acute Zn toxicity.

(ii) Effect of Acclimation to Chronic Waterborne Zn

Fish chronically exposed to sublethal waterborne Zn are often able to acclimate physiologically (Hogstrand *et al.* 1994, 1995), and this acclimation can involve regulation of Zn-binding characteristics in the gills. Alsop and Wood (2000) evaluated the effect of acclimation for 30 days to waterborne Zn (3.9 μ*M*) in hard water (hardness 120 mg/L as $CaCO_3$) on 3 h *in vivo* gill Zn-binding in rainbow trout. A remarkable 9-fold increase of binding site density (from B_{max} 7.03 to 64.7 nmol/g wet tissue) and two-fold decrease (from log $K_{gill=Zn}$ 5.6 to 5.3) in binding affinity was observed following acclimation to waterborne Zn (Figure 9). Alsop *et al.* (1999) earlier reported a significant increase of the size of fast pool of Zn in the rainbow trout gill following acclimation to waterborne Zn in both hard and soft water, which also indicates an increase in binding site density (higher B_{max}). Interestingly, they also reported 2- to 3-fold increases in acute Zn tolerance (*i.e.*, increases in 96 h LC50) following acclimation to sublethal waterborne Zn in either hard or soft water (120 and 20 mg/L as $CaCO_3$, respectively). These increases in Zn tolerance could very well be induced by the alterations in gill binding characteristics for Zn.

Zn^{2+} and Ca^{2+} share a common branchial uptake pathway and competitively inhibit the influx of each other across the gill (Figure 2; Hogstrand *et al.* 1996; Spry and Wood 1989). Hogstrand *et al.* (1994, 1998) observed a decrease in the branchial affinity (increased K_m, concentration at which 50% of the maximum transport rate

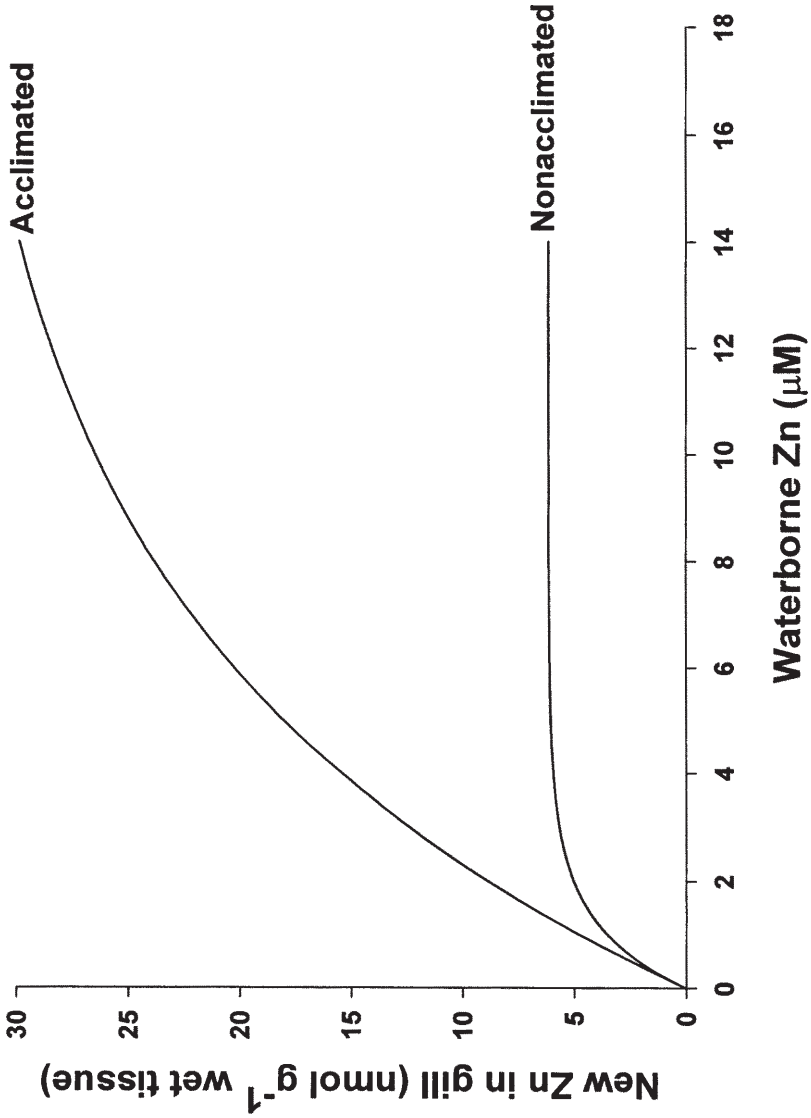

Figure 9. **Effect of acclimation to chronic sublethal waterborne Zn exposure on short-term *in vivo* gill Zn-binding in rainbow trout (adapted from Alsop and Wood 2000).** Ten data points (five for fitting each line) were used, and each data point represented mean ± SE (N=7).

is observed) for the transport systems of both Zn^{2+} and Ca^{2+} with sublethal waterborne Zn acclimation in rainbow trout. Because of the very different concentrations of Ca^{2+} (1000 μ*M*) and Zn (2.3 μ*M*) in the water relative to the respective K_m values, Ca^{2+} uptake rate was little affected, but Zn uptake rate was substantially reduced in Zn-acclimated fish. Based on these Zn transport data, there was very likely a decrease in the affinity of Zn-binding (lower log $K_{gill=Zn}$) in the gills of the waterborne Zn-acclimated fish. The increase in binding site density (higher B_{max}) due to acclimation presumably indicates greater storage or detoxification capacity in waterborne Zn-acclimated fish. Regardless, it is clear that acclimation to chronic waterborne Zn alters the short-term gill binding characteristics and thereby, influences the acute Zn sensitivity. Once again, these findings have important implications for an *acute* Zn BLM in fish.

(iii) Effect of Acclimation to elevated Dietary Zn

Grosell *et al.* (2003) recently studied the effects of elevated dietary Zn on branchial Zn transport in rainbow trout. There were only small, insignificant increases in the maximum rate of branchial Zn uptake (J_{max}) and decreases in transport affinity (increased K_m) following acclimation for 42 days to elevated dietary Zn concentrations [34 μmol/g] relative to control levels in the food (3 μmol/g)], both alone and in combination with waterborne Zn (4 μ*M*) in hard water (hardness 120 mg/L as $CaCO_3$). Moreover, no significant increase in Zn accumulation was observed in any tissues either in the dietary or combined dietary plus waterborne Zn-acclimated treatments over the entire period of exposure, indicating strong homeostatic regulation of Zn in fish. Spry *et al.* (1988) studied waterborne Zn uptake in rainbow trout acclimated to 0.016, 1.4, and 9.2 μmol/g of dietary Zn simultaneous with 0.11 (ambient), 0.6, 2.3, and 8.3 μ*M* of waterborne Zn. They also suggested that waterborne Zn uptake is probably independent of intestinal Zn uptake in fish since at any dietary Zn concentration, increasing the waterborne Zn concentration resulted in increased whole body Zn.

At present there is no direct evidence of an acclimation effect of dietary Zn, either alone or in combination with waterborne Zn, on Zn-binding characteristics in the gills. Although these studies suggest very little or no effect, further research is needed to address this issue specifically. Grosell *et al.* (2003) observed a 3.5-fold increase in acute waterborne Zn tolerance (increase in 96 h LC50) in rainbow trout acclimated to elevated dietary Zn, alone or in combination with elevated waterborne Zn. Since the primary objective of the *acute* BLM is to predict toxicity, chronic exposure to elevated dietary Zn through contamination of the food chain therefore could have important implications for the *acute* Zn BLM.

(iv) Effect of Acclimation to Altered Dietary Ca^{2+} Content

Waterborne Zn causes toxicity in freshwater fish essentially by reducing branchial Ca^{2+} uptake, an action that leads to a disruption of internal Ca^{2+} homeostasis (Figure 2). Feeding on a diet enriched with Ca^{2+} (*e.g.*, mollusks and crustaceans) therefore, could help fish to compensate the reduction of branchial Ca^{2+} uptake, and by further reducing Ca^{2+} uptake at the gills, could help to reduce Zn uptake, just as for Cd (see Section B.iv). Notably, Hogstrand *et al.* (1996) reported that injections of

Ca^{2+} salts to raise blood Ca^{2+} levels resulted in a decrease in both Ca^{2+} and Zn^{2+} uptake at the gills of trout. So far very little information is available on the effects of dietary Ca^{2+} on gill Zn-binding and toxicity. However, a recent study in our laboratory (Niyogi S and Wood CM, unpublished results) showed that unidirectional waterborne Zn uptake decreased significantly in rainbow trout acclimated for 28 days simultaneously to elevated dietary Ca^{2+} (1 mmol/g) and waterborne Zn (2.3 μ*M*) in hard water (hardness 120 mg/L as $CaCO_3$) relative to fish acclimated to the same concentration of waterborne Zn and fed with normal food (Ca^{2+} content = 0.5 mmol/g). This study also revealed that the former group accumulated significantly less new Zn in the gill compared to the latter group over a period of 24 h of exposure to 2.3 μ*M* of Zn labeled with ^{65}Zn. No significant change in water Ca level due to the Ca-enriched food was recorded in any exposure tanks during the acclimation period. These results strongly indicate a parallel situation with Cd uptake — dietary Ca^{2+} influences Zn-binding characteristics in fish under chronic sublethal waterborne Zn exposure. Further studies are needed to evaluate specifically how dietary Ca^{2+} affects the gill Zn-binding constants (log $K_{gill=Zn}$ and B_{max}).

Interestingly, rainbow trout acclimated to elevated dietary Ca^{2+} and waterborne Zn exhibited the greatest tolerance to acute waterborne Zn challenge (in terms of median lethal time or LT50) followed by fish acclimated to waterborne Zn alone and control conditions (normal food and water; Niyogi S and Wood CM, unpublished results). This increase in tolerance was likely mediated by the changes in gill Zn-binding characteristics, which emphasizes the need to consider the acclimation effect of dietary quality (Ca^{2+} content) in the *acute* Zn BLM for fish.

SUMMARY

The effects of *chronic* waterborne and dietary exposures on the gill-binding characteristics and acute toxicity of Cu, Cd, and Zn in rainbow trout are summarized in Table 1. Clearly, acclimation to altered water hardness, sublethal metal exposure both via water and diet, and dietary quality [essential ion (Ca^{2+} and Na^{+}) content] can significantly modify gill-binding characteristics and thereby, also influence acute toxicity. As a general trend, acclimation to low levels of water hardness (low Ca^{2+}, soft water) decreases the affinity (lower log K) of metal binding and increases the binding site density (higher B_{max}) in the gill of fish. Acclimation to sublethal waterborne metals also reduces the affinity (lower log K) of gill metal-binding for that particular metal and increases the binding site density (higher B_{max}). Notably, saturation generally does not occur for gill metal-binding in fish chronically acclimated to sublethal waterborne metal levels in soft water in short-term gill binding assays, and therefore binding site density cannot be determined under these conditions. At least for Cd, chronic preexposure to elevated dietary Cd seems to induce changes in gill metal-binding characteristics similar to those observed following chronic waterborne exposure. However, not enough information is available at present regarding the effects of dietary Cu and Zn acclimation.

Finally, acclimation to elevated dietary ion content (Na^{+}) reduces the binding site density (lower B_{max}) for Cu in the gills but does not affect the binding affinity (no change in log $K_{gill=Cu}$) in Cu-unexposed fish. In contrast, it decreases the gill Cu-binding affinity (lower log $K_{gill=Cu}$) in Cu-exposed fish, however, the lack of satura-

Table 1. The effect of different chronic acclimation conditions on gill metal-binding constants and acute toxicity in rainbow trout. Increases and decreases in gill metal-binding affinity, site density and 96 h LC50 are indicated by (↑) and (↓) respectively. 'ND' represents absence of data.

Metal	Exposure condition	Gill-binding affinity (Log K)	Binding site density (B_{max})	96 h LC50	Reference
Cu	Low water hardness	↓	↑	↓	Taylor *et al.* 2000
	Waterborne Cu	↓	↑	↑	Taylor *et al.* 2000
	Dietary Cu	ND	ND	↑	Miller *et al.* 1993
	Dietary Na	↓[a]	↓[b]	ND	Kamunde *et al.* 2002b
Cd	Low water hardness	↑↓[c]	↑	↓	Hollis *et al.* 1999, 2000a, b
	Waterborne Cd	↓	↑	↑	Hollis *et al.* 1999, 2000a, b
	Dietary Cd	↓	↑	↑	Szebedinszky *et al.* 2001
	Dietary Ca	ND	ND	ND	
Zn	Low water hardness	ND	↑	↓	Alsop *et al.* 1999
	Waterborne Zn	↓	↑	↑	Alsop and Wood 2000; Alsop *et al.* 1999
	Dietary Zn	ND	ND	↑	Grosell *et al.* 2002
	Dietary Ca	ND	ND	↑[d]	Niyogi and Wood (unpublished data)

[a] Rainbow trout acclimated to sublethal waterborne Cu. [b]Rainbow trout not acclimated to sublethal waterborne Cu .
[c]Both increases as well as decreases have been reported. [d]Data represent median lethal time or LT50

tion in gill Cu-binding did not allow the determination of binding site density in this case. Although acclimation to elevated dietary Ca^{2+} reduces acute waterborne Cd and Zn uptake, it is not yet known whether this is due to a decrease in binding site density or binding affinity. Moreover, acclimation to low water hardness (soft water) induces an increase in acute toxicity (lower 96 h LC50), whereas acclimation to sublethal levels of waterborne and dietary metals results in a decrease in acute toxicity (higher 96 h LC50 value). In addition, elevated dietary Ca^{2+} content also leads to a decrease in acute Zn toxicity (higher LT50 value), although it remains to be seen whether elevated Na^+ and Ca^{2+} in the diet produce the same effect on acute Cu and Cd toxicity respectively.

IMPLICATIONS FOR BIOTIC LIGAND MODELS (BLMs) AND CONCLUDING REMARKS

The scientific foundations of the present acute BLMs are sound. The strength of the models lie in the fact that they are built upon a mechanistic paradigm with a strong chemical and physiological basis, *i.e.*, predicting binding at the site of action (gill of fish) and the mechanism of acute toxicity (blockade of Na^+ and Ca^{2+} uptake)

for metals (Cu, Cd, Zn). Present versions of the *acute* BLMs assume that only water chemistry variables such as competing cations (*e.g.*, Na^+, Ca^{2+}, Mg^{2+}, and H^+), inorganic ligands (*e.g.*, hydroxides, chlorides, sulfides, carbonates) and organic ligands (dissolved organic matter) can influence the bioavailability of free metal ions and thereby the acute toxicity of metals. The conditional binding constants of the metal-biotic ligand and of the various cation-biotic ligand complexes are considered to be unchangeable with the changes in environmental conditions. Thus the log K (binding affinity) and B_{max} (binding site density) values used for a certain metal in the present BLMs are fixed for any particular aquatic organism, irrespective of its background or *chronic* history.

However, the biotic ligand (*e.g.*, fish gill) is a part of a living organism, which is capable of regulating its physiological and metabolic functions in response to environmental perturbations, thereby changing the properties of the biotic ligand. The changes in the gill metal-binding characteristics (log K, B_{max}) due to *chronic* sublethal exposures to metals, both via water and diet, and different water hardness and dietary ion levels, are a reflection of this phenomenon. Notably, among the studies reviewed here, wide variability was observed in binding site densities (B_{max}) for Cu and Cd among different sets of trout maintained under control conditions (normal food and water). However, the binding affinities (log K) were found to be fairly consistent in most cases under the same conditions. These discrepancies in B_{max} values between the studies could be due to the size, age, or batch differences in fish and also differences in the feeding regime, and certainly comprise an issue that has to be given greater attention in future. Overall, these observations strongly indicate that the dynamic properties of the biotic ligand (fish gill), rather than just water quality parameters alone, need to be considered for the future development and refinement of the BLM, especially since *chronically* induced changes in gill-metal binding characteristics can directly affect the *acute* toxicity of metals.

The ultimate objective of the BLM approach is to serve as a predictive tool for not only *acute* AWQC, but also for *chronic* AWQC providing lifetime protection. In the natural environment, water hardness, metal levels in water, dietary metal contents, and dietary ions may vary greatly from time to time, and from place to place. Therefore, incorporation of the *chronic* acclimation-based changes in gill metal-binding into BLMs, is critically important for their successful application in the real environment, particularly from the perspective of their potential use for predicting *chronic* toxicity. Future research should examine whether this can best be achieved by incorporating some long-term acclimation-based characteristics into the present versions of BLMs, or through the development of time-variable exposure models. Moreover, other factors, as yet unidentified, may also be important in dynamically modifying the properties of the biotic ligand. Now that the initial framework of the BLM has been laid down, future research needs a more integrative approach with emphasis on the dynamic properties of the organism itself in order to make it a more meaningful tool for ecological risk assessment of metals in aquatic ecosystems.

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