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# An in vitro approach for modelling branchial copper binding in rainbow trout☆

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**Abstract**

The main objective of this study was to characterize the individual effects of water chemistry ($Ca^{2+}$, $Na^{+}$, dissolved organic matter (DOM), pH, alkalinity) on the rapid binding of copper to the gill surface of rainbow trout using an in vitro gill binding assay. In this assay, individual gill arches were exposed for 5 min to $^{64}Cu$ labelled copper solutions ranging from 0.02 to 0.16 μM in water chemistries reflecting the full range of fresh water values for the Great Lakes. The gills displayed saturable Cu binding within this Cu range but gill–Cu binding was completely unaffected over the full range of calcium, sodium and alkalinity concentrations used. Only low pH (pH 4.0) and commercial DOM (Aldrich humic acid at $\geqslant 3$ mgC/l) altered copper binding to rainbow trout gills in vitro. These findings were consistent with the results of geochemical modelling of our water chemistry (using MINEQL+, Version 4.5) which showed that $H^{+}$ and DOM affected the free cupric ion concentration. However, DOM (up to 80 mgC/l) was only able to reduce Cu on the gills by 50%. We hypothesize that in the range of 0.02–0.16 μM Cu there are two high affinity Cu binding sites on the gills, one having a substantially higher affinity for copper than DOM. The absence of a calcium effect on gill copper binding was in accord with in vivo evidence that calcium primarily acts to alter the physiology of the gill binding sites through acclimatory processes, rather than through competitive interactions. It was a surprise that water chemistry parameters influence rapid gill–metal binding in a manner different to their influence on acute toxicity and different from the effects on long-term binding reported in other studies. Currently, the biotic ligand model uses the rapid increase of gill copper (believed to reflect binding to the physiologically active receptor sites) to model gill binding characteristics. The distinction between rapid surface binding and metal uptake obviously plays an important role in determining the toxic effects of copper, especially when regulators need to predict the modifying effects of water chemistry.


*Keywords:* Trout; Copper; Modelling; Gill binding; Toxicity; Water chemistry; Biotic ligand model

## 1. Introduction

Fish gills are the initial target of waterborne copper toxicity (Laurén and McDonald, 1985; McDonald and Wood, 1993; Wood, 2001). Copper binds to physiologically active sites on the gill, which regulate the transport of essential ions across the gill membrane, thus impairing branchial sodium uptake (Laurén and McDonald, 1985; McDonald et al., 1989). The gill surface interaction

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model (GSIM), originally proposed by Pagenkopf (1983), aimed to explain the variability associated with trace metal toxicity by incorporating the differences in metal speciation, alkalinity, hardness and pH with gill–metal interactions. More recently, Playle et al. (1992), Playle et al. (1993a), Playle et al. (1993b) and MacRae et al. (1999a), derived conditional stability constants (log $K$) and binding capacities ($B_{max}$) of the gill for copper ions during 3 and 24 h exposures, respectively. These studies have been deemed toxicologically relevant for modelling the influence of water chemistry, as seen by their inclusion in the biotic ligand model (BLM discussed below; DiToro et al., 2000, 2001; Paquin et al., 2000), even though a condition of the GSIM was that the surface complexation (adsorption) and absorption of metals should be rapid and reversible (Pagenkopf, 1983). At this point, a distinction needs to be made between metal surface binding and that which enters the gill (i.e. metal uptake).

A knowledge of water chemistry, gill–metal binding characteristics (from Playle et al., 1993a,b; MacRae et al., 1999a) and acute toxicity provides the necessary framework in which the most recent GSIM has been developed, termed the BLM (DiToro et al., 2000, 2001; Paquin et al., 2000). In brief, the BLM represents metal interactions with competing cations and complexing ligands found in natural waters and predicts metal accumulation at the gill. The level of accumulation is then used to predict acute toxicity (i.e. lethality). One issue that emerges is whether gill copper accumulations determined in the time frame required for acute mortality are appropriate in understanding the modifying effects of water parameters at the gill surface.

Researchers have documented the influence of water chemistry parameters such as hardness, pH, alkalinity and dissolved organic matter (DOM) on copper toxicity (e.g. Howarth and Sprague, 1978; Erickson et al., 1996; Laurén and McDonald, 1986; Zitko and Carson, 1976; Zitko et al., 1973; Miller and Mackay, 1979; Chakoumakos, 1979). Generally, increases in hardness, pH, alkalinity and DOM provide protection against the toxic effects of copper and also protect against copper accumulation at the gill. Water chemistry influences accumulation by either competition or complexation (Playle et al., 1992). Competition for biotic ligands occurs between Cu and other cations found in natural waters such as $Ca^{2+}$, $Na^{+}$ and $H^{+}$, whereas carbonates ($CO_3^{2-}$), hydroxides ($OH^{-}$) and DOM complex copper thus rendering it less bioavailable. The amount of copper accumulation at the gill is limited by the number of binding sites on the gill and the affinity of the sites for copper (Playle et al., 1993a,b; Reid and McDonald, 1991; MacRae et al., 1999a, also see review by Playle, 1998). These constants were derived on the basis of there being one type of binding site on the gill, however, Taylor et al. (2000) showed that there were at least two types of sites within the toxicological range of copper (i.e. 96 h—LC50 in soft water was 16 μg/l or 0.25 μM and the sites were identified between 1–25 μg/l or 0.016–0.4 μM).

The main objective of this study was to characterize the individual effects of $Ca^{2+}$, $Na^{+}$, pH, alkalinity, and DOM on the initial binding of copper to the gill surface. In order to focus primarily on surface reactions of metal to tissue, a rapid 5-min in vitro gill binding assay was used. The use of isolated gill arches eliminated any changes in water chemistry caused by the organism itself (i.e. through excretion, mucus sloughing, general body surface/skin binding). In addition, gentle aeration allowed mixing of the arch with the exposure water, hence, breaking down the gill boundary layer. Our method adapted from that of Reid and McDonald (1991), involved using radiolabelled copper ($^{64}Cu$) at environmentally realistic and toxicologically relevant concentrations (i.e. μM) in order to determine the 'newly accumulated' gill copper. Copper exposure concentrations were chosen based on our previous finding that high affinity sites saturated at $<0.24$ μM total copper (Taylor et al., 2000). The validity of the in vitro method was evaluated through binding site characterization and included: (1) changes in gill–Cu binding with time, (2) the relationship between gill–Cu binding and copper concentration, (3) the displacement of copper from the gill surface, (4) the resemblance to in vivo gill binding and lastly, (5) the influence of fish size on gill copper binding.

## 2. Materials and methods

### 2.1. Experimental animals

Adult ($147.6 \pm 2.6$ g, $N=90$) and juvenile (2–50 g, $N=30$) rainbow trout were obtained from a commercial supplier (Humber Springs, Orangeville, Ont., Canada) and maintained in dechlorinated Hamilton tap water (14 °C, pH 8, 1.0 mM

$Ca^{2+}$, 0.6 mM $Na^{+}$, 0.7 mM $Cl^{-}$, hardness 120 mg/l as $CaCO_3$, alkalinity 95 mg/l, 3.1 mgC/l DOM) for at least 2 weeks prior to experimentation. Trout were fed commercial trout pellets (Martins Feed Mill, Elmira, Ont., Canada) to satiation three meals per week. The feed contained natural traces of ~3 μgCu/g dry weight. Photoperiod was set to a light/dark cycle similar to the natural photoperiod for Western Lake Ontario.

### 2.2. Binding site characterization

A gill binding method, modified from Reid and McDonald (1991), was employed. Adult rainbow trout were sacrificed with a single blow to the head. The three most anterior arches on both sides of the gill basket were removed from each fish (for a total of six arches per fish). The last posterior arches were not used in order to maintain a similar average gill arch weight. Only one arch was placed in each exposure cup, for a total of six cups running at any time. A standard soft water was prepared for all exposures by adding reagent-grade chemicals to deionized water (18 MΩ; US EPA, 1991). Added salts were 0.57 mM $NaHCO_3$, 0.22 mM $CaSO_4$, 0.19 mM $MgSO_4 \cdot 7H_2O$ and 0.03 mM KCl (analytical grade, BDH Inc., Toronto, Ont., Canada). Other parameters of the synthetic soft water included, 40–48 mg/l as $CaCO_3$ hardness, 30 mg/l as $CaCO_3$ alkalinity and a pH of 7.7–7.8. The exposure solutions (50 ml) included radiolabelled $^{64}Cu$ (as copper nitrate, half-life 12.8 h), which was obtained from the McMaster Nuclear Reactor (Hamilton, Ont., Canada). Each exposure cup was fitted with an individual airline in order to provide constant mixing. Arches were exposed for 5 min followed by a 10-s rinse with Cu-free water in order to release all loosely bound copper.

### 2.3. Specific features of Cu binding in vitro

Characterization of the copper binding sites included evaluating several key features which were: (1) Cu accumulation over time, (2) binding vs. [Cu], (3) the fraction of Cu that is surface bound (i.e. reversible), (4) the relationship between in vitro and in vivo binding and (5) the influence of fish size on copper binding. The time dependency experiment involved exposing individual arches ($N=6$ per time) to a nominal concentration of 0.16 μM Cu (specific activity (SA) was 2.42–3.76 mBq/μg) for varying amounts of time ranging from as short as 10 s up to 1 h. To determine the affinity of the gill for copper (log $K$) and the maximum number of binding sites ($B_{max}$), gills were exposed to varying copper concentrations (0–0.5 μM, SA was 0.1–37.66 mBq/μg) using a fixed time (5 min) selected on the basis of the preceding series. The concentrations ⩽0.16 μM total copper (i.e. within the range of saturability) were used to estimate free cupric ion concentrations (0–110 nM; using MINEQL+, Version 4.5, Schecher and McAvoy, 2001, Section 2.5) in order to accurately determine the log $K$. To distinguish the amount of copper that was truly surface bound from the amount that had actually entered the gill cells (i.e. copper uptake), a 'pulse-chase' experiment was conducted. This test involved the standard 5 min exposure to 0.16 μM (i.e. the 'pulse', SA was 3.64–6.06 mBq/μg) but replaced the 10 s rinse in Cu-free water with a rinse containing 100 times unlabelled 'cold' copper (i.e. the 'chase', 16 vs. 0.16 μM nominal Cu concentrations). This concentrated copper rinse was for 30 s, 1, 2.5 or 5 min and was intended to displace any surface bound $^{64}Cu$. Any radioactivity that remained was assumed to be copper inside the cells. The in vitro technique was compared to in vivo by exposing whole adult rainbow trout for 5 min to 0.16 μM Cu (SA was 0.9–1.71 mBq/μg). The exposures were carried out in 3 l of the synthetic soft water contained in black acrylic boxes (1 fish per box). At the end of the 5 min, fish were sacrificed by a single blow to the head and the entire gill basket removed. The same arches were isolated, as mentioned above for the in vitro assay, and counted for radioactivity. Lastly, an experiment was conducted to determine if the amount of copper bound per gram of gill tissue in vitro was dependent upon fish size. An assortment of fish was utilized ranging from 3 to 215 g. Arches were isolated from these fish and exposed to 0.16 μM Cu (SA was 2.33–3.15 mBq/μg) for 5 min.

### 2.4. Effects of water chemistry variables

Water parameters were varied one at a time including: calcium, sodium, alkalinity, pH and DOM (Table 1). These five variables were chosen based on their type of effect on copper binding: cation competition with $Na^{+}$, $Ca^{2+}$ and $H^{+}$ ions and modifiers of copper speciation or complexing ligands including alkalinity, pH and DOM. All

Table 1
Experimental and typical ranges for each water constituent tested using the in vitro gill binding method

| | Test parameter range | Typical range in freshwater | Method of adjustment |
|---|---|---|---|
| $Na^+$ | 0.001–30 mM | 0.043–0.52 mM[a] | NaCl |
| $Ca^{2+}$ | 0.2–1.1 mM | 0.3–1 mM[a] | $CaSO_4$ |
| Carbonate alkalinity | 0.2–2.3 mEq/l | 1–2.6 mEq/l[a] | $KHCO_3$ ($NaHCO_3$ replaced with NaCl) |
| $Cu^{2+}$ | 0–0.5 μM (total) | 3–78 nM (dissolved)[b] | $CuNO_3$ |
| DOM | 2–82 mgC/l | 1–12 mgC/l [c] | Aldrich humic acid |
| pH | 4.0, 7.0, 7.7, 9.0 | 7.8–8.1[a] | HCl or KOH |

A standard synthetic soft water was used in all experiments (Section 2, US EPA, 1991) and the exposure temperature was 20 °C. Each parameter was adjusted individually while all others were held constant.

[a] Beeton et al. (1999).

[b] Batley et al. (1999).

[c] Morel (1983).

experiments were conducted for 5 min at the nominal copper concentration of 0.16 μM (SA ranged from 0.5 to 5.76 mBq/μg). Water samples (5.0 ml) from each exposure cup were collected and acidified with 50 μl concentrated trace metal grade nitric acid (Fisher Scientific, Toronto, Ont., Canada). The effect of added calcium on copper binding was evaluated over the concentration range of 0–1.2 mM $Ca^{2+}$, whereas the range of sodium tested was much larger at 1 μM–30 mM. Alkalinity was altered in the test water by replacing $NaHCO_3$ with NaCl and adding $KHCO_3$, in order to maintain constant sodium levels. The range of alkalinity tested was as low as 0.2 mEq/l increasing to 2.3 mEq/l as $CaCO_3$ equivalents. Water pH was evaluated by testing in acidic, neutral and alkaline conditions (i.e. pH 4.0, 7.0, 7.7 (unadjusted) and 9.0). Lastly, commercial DOM (Aldrich humic acid, Sigma–Aldrich, Oakville, Ont., Canada) was evaluated at environmentally realistic levels up to 20 mgC/l and one extreme level of 80 mgC/l. Solutions for the DOM experiment were allowed to age for 3 h in order to allow interaction time with the copper (as recommended by Ma et al., 1999).

In order to test if acclimation time to low calcium water influenced gill copper binding, one in vivo experiment was also conducted. Juvenile rainbow trout (~10 g, $N=10$) were exposed to 0.27 μM radiolabelled copper (SA was 578 kBq/μg) for 3 h. The gills were excised and counted for radioactivity. These fish had been acclimated for varying amounts of time to soft water ($Ca^{2+}$ 0.02 mM) prior to the 3 h gill binding exposure. Specifically, the first group of hard water acclimated fish ($Ca^{2+}$ 1 mM) were also exposed to copper in hard water (i.e. 0 time in soft water), the second group of hard water acclimated trout were acutely transferred to soft water only for the 3 h exposure to copper, and lastly, the third group of hard water acclimated trout were transferred to soft water for 21 h prior to the 3 h exposure of copper in soft water (i.e. a total of 24 h in soft water).

### 2.5. Analytical techniques

The radioactivity in gill tissues and water samples was measured in a well-type gamma counter with a 7.62 cm NaI crystal (Packard Minaxi Auto-Gamma 5000 Series, Packard Instruments, Meriden, CT). Total copper, calcium and sodium concentrations in water samples were measured using flame or graphite furnace atomic absorption spectrophotometry (Varian SpectrAA-220FS and GTA 110, Walnut Creek, CA) against certified standards (Fisher Scientific). Alkalinity was measured in the bulk solutions by titrating 5.0 ml to pH 4.0 with 0.02 M HCl prepared from a 2.0 N HCl (Sigma–Aldrich) standard and aeration, before and after the exposure time. To prepare solutions containing DOM at desired levels we assumed that 1.0 mg of Aldrich humic acid contained 0.5 mg carbon (i.e. 50%). Actual total carbon and inorganic carbon concentrations were measured on the bulk test solutions using a total organic carbon analyzer (Shimadzu TOC-5050A, Tokyo, Japan). The DOM concentrations were calculated automatically by subtracting inorganic carbon from total carbon, and are reported as dissolved organic carbon (in mgC/l). The pH in individual exposure cups was measured directly

using a PHM-84 meter with a GK2401C combination electrode (Radiometer, Copenhagen, Denmark). Free cupric ion concentrations were calculated using a geochemical modelling program (MINEQL+, Version 4.5, Schecher and McAvoy, 2001) and the above measured aqueous chemistries. All estimations were conducted in a system in equilibrium with the atmosphere with the exception of variable alkalinity, where a closed system simulation was assumed.

### 2.6. Calculations

Newly accumulated gill copper concentrations (nmol/g) in the gill were calculated based on the accretion of radioactivity in the gill:

$$Cu_{gill} = \frac{^{64}Cu_{gill}}{SA}$$

where $^{64}Cu_{gill}$, radioactivity of the gill in counts per min (cpm) per gram of wet gill tissue and SA, specific activity of the water in cpm/nmol.

The binding characteristics of the saturable sites on the gill (binding capacity, $B_{max}$ and affinity, log $K$) were calculated using non-linear regression. The fit of newly accumulated gill copper (nmol/g) against the calculated free cupric ion concentration ($Cu^{2+}$; nM) is given by the equation:

$$\text{Newly accumulated gill Cu} = \frac{B_{max}[Cu^{2+}]}{(K_{gill-Cu} + [Cu^{2+}])}$$

where $B_{max}$ is in nmol/g and $K_{gill-Cu}$ is in nmol/l.

### 2.7. Statistics

All data presented are mean ± 1 standard error ($N$). A Student's $t$-test (two-tailed, unpaired) was used to test for significant differences between two treatments. In cases where treatments were compared to a control group, an analysis of variance was conducted followed by a Dunnett's test to isolate the significant differences. Significance was set at $P < 0.05$. Non-linear regressions were applied using Sigma Plot 2000 allowing for the calculation of saturation kinetic parameters (i.e. maximum number of binding sites on the gill ($B_{max}$), the affinity of the gill for copper (log $K_{gill-Cu}$) and the time to half saturation).

## 3. Results

### 3.1. Site characterization

Initial experiments determined the time course for metal saturation on the isolated gill arches (Fig. 1a). A plateau of newly accumulated gill copper appeared at 2 and 4 min of waterborne copper exposure. Copper binding became increasingly variable at times greater than 8 min. To determine whether the gills were in fact saturating in 5 min, a second more detailed time course evaluated copper binding at times <300 s (Fig. 1b). The fit of non-linear regression to all the data points revealed a time to half saturation ($t_{\frac{1}{2}}$) of 171 s. Unexpectedly, a second plateau may also be present at exposure times less than 50 s, with a $t_{\frac{1}{2}}$ of less than 10 s. We concluded that 5 min was an appropriate exposure time to employ for the remainder of the experiments due to the tech-

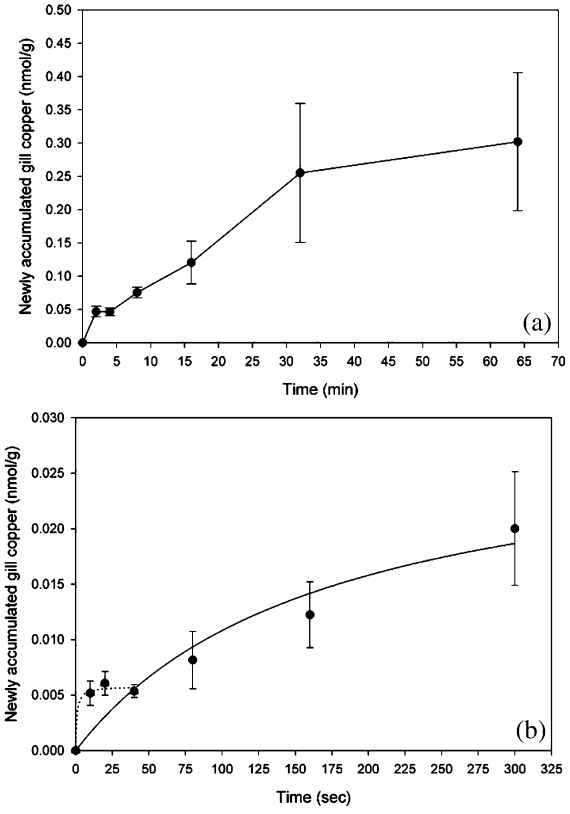


Fig. 1. The amount of newly accumulated gill copper (nmol/g wet gill tissue) at various time intervals. In (a) arches were exposed for 2, 4, 8, 16, 32 and 64 min to 0.20 ± 0.01 μM (24) radiolabelled Cu. In (b), isolated arches were exposed to 0.17 ± 0.01 μM (34) radiolabelled Cu for 10, 20, 40, 80, 160 and 300 s. Each data point represents mean ± 1 S.E. ($N = 6$ arches each from 6 different fish). Non-linear regression was fitted to all the data in panel B ($R^2 = 0.88$, $P < 0.01$) and the time to half saturation ($t_{\frac{1}{2}}$) was 171 s. Newly accumulated gill copper reached a separate plateau at exposure times less than 50 s. A second curve was fit to this data ($R^2 = 0.98$, $P < 0.01$), revealing a $t_{\frac{1}{2}}$ of <10 s.

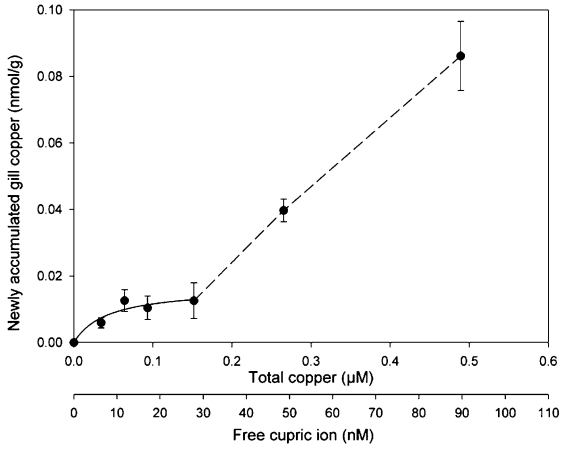


Fig. 2. The effect of various water copper concentrations on the amount of newly accumulated gill copper (nmol/g wet gill tissue) exposed in vitro. Total copper ranged from 0 to 0.5 μM and the free cupric ion concentrations were estimated using MINEQL+. Each data point represents mean ± 1 S.E. ($N=6$ arches each from 6 different fish). At ⩽0.16 μM total copper, the fit of non-linear regression statistics ($R^2=0.91$, $P<0.05$) allowed for the calculation of binding capacity ($B_{max}$) and affinity ($\log K$). $B_{max}$ was 0.0165 nmol/g and $\log K$ was 8.1 for these saturable sites.

nical difficulties associated with <10 s exposures and the fact that saturation was occurring and the response variability was considered minimal.

Isolated arches were exposed to a range of copper concentrations for 5 min in order to characterize the binding sites for copper (Fig. 2). At least two types of sites were identified: (1) saturable high affinity, low capacity sites found below 0.16 μM copper, and (2) lower affinity, higher capacity sites which bound copper in a linear fashion up to 0.5 μM copper. Using the estimated free cupric ion concentration, the affinity or $\log K$ for the high affinity saturable sites was 8.1 and the $B_{max}$ was 0.0165 nmol/g.

The displacement of radiolabelled copper with unlabelled copper allowed for the distinction between surface bound copper and that which had entered the gill cells (Fig. 3). The removal of loosely bound copper was found to increase with longer rinse times. The maximum rinse time of 5 min could only displace up to half of the bound copper. Therefore, the remaining 50% was assumed to have entered the gill cells (i.e. copper uptake).

The in vitro preparation used in this study may be comparable to in vivo gill binding also conducted for 5 min on large fish. Intact whole fish

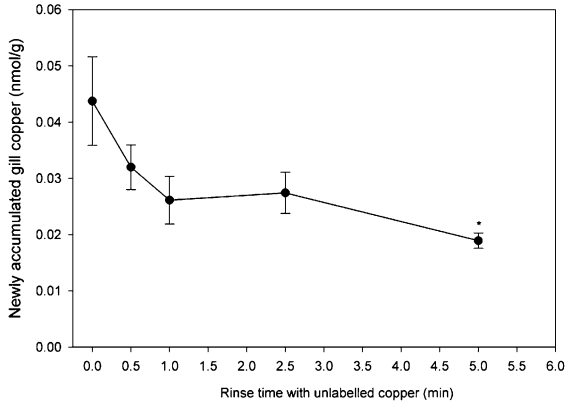


Fig. 3. The displacement of copper from the gill surface (nmol/g wet gill tissue) using varying rinse times. Isolated arches were exposed to 0.15 ± 0.01 μM (30) Cu for 5 min, followed by an unlabelled copper rinse of 15.2 ± 0.1 μM. This rinse was carried out for 0.5, 1, 2.5 and 5 min. Each data point represents mean ± 1 S.E. ($N=6$ arches each from 6 fish). *Indicates significant difference from the control group (i.e. 0 min rinse time) using a one-way ANOVA and Dunnett's test ($P<0.05$).

bound 0.117 ± 0.018 ($N=3$) nmolCu/g of gill tissue, whereas the isolated gill arches bound 0.071 ± 0.012 ($N=6$ from 6 fish) nmolCu/g. Both were exposed to 0.30 μM total copper and the amounts accumulated were not statistically different from each other ($P=0.099$).

The size of the fish greatly influenced in vitro gill copper binding (Fig. 4), an effect which was especially evident in small fish weighing less than

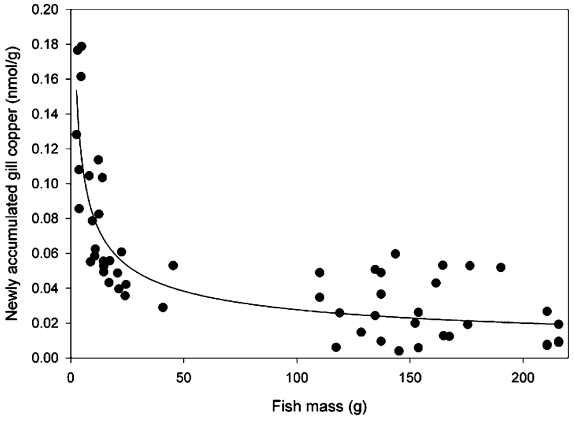


Fig. 4. The effect of fish size on copper binding to isolated arches (nmol/g wet gill tissue). Isolated arches from fish ranging from 2.6 to 216 g were exposed for 5 min to 0.17 ± 0.003 μM (56) Cu. A power relationship best described the data ($y=0.2394x^{-0.4685}$, $R^2=0.72$, $P<0.001$).

50 g. The relationship was best described by a power function ($y=0.2394x^{-0.4685}$, $R^2=0.72$, $P<0.001$). Based on this significant relationship, the size range used in this study (110–220 g) was not likely a factor adding to the variability associated with newly accumulated gill copper binding.

### 3.2. Effects of water chemistry variables

#### 3.2.1. Cation competition

Over the range of calcium tested, there was no significant effect on the amount of newly accumulated copper by isolated gills at 0.17 μM total copper (Fig. 5a). The average gill copper concentration, regardless of $[Ca^{2+}]$, was $0.046\pm0.003$ (33) nmol/g. Despite the absence of a direct calcium effect, there was an impact of varying calcium acclimation times in vivo on the amount of copper bound to the gills (Fig. 5a—inset). Increasing the exposure time to low calcium water from 0 to 24 h increased the amount of gill copper by ~6 times. However, after 12 weeks of soft water acclimation newly accumulated gill copper levels returned to levels in fish acclimated to high calcium water (i.e. 1.85 and 2.75 nmolCu/g of gill tissue, respectively).

Gill copper binding at 0.18 μM copper was also independent of the sodium concentration (Fig. 5b). The average newly accumulated gill copper amount was $0.027\pm0.002$ (65) nmol/g, regardless of sodium concentration. The absence of an effect was evident over the whole range of sodium (1 μM–30 mM).

#### 3.2.2. Copper speciation and complexation

The effect of varying alkalinity was tested over the range of 0.2–2.3 mEq/l at 0.18 μM total copper and pH 7.7 (Fig. 6a). There was no significant effect on newly accumulated gill copper binding with all values averaging $0.0369\pm0.004$ (34) nmol/g. According to MINEQL+ estimations in a system open to the atmosphere, the free cupric ion does not vary with changes in alkalinity at pH 7.7 and the $CuCO_3$ species accounted for ~55% of the total copper species. However, in a closed system (i.e. where carbon dioxide cannot be released into the air) the free copper concentration was estimated between 20 and 4% of the total copper species and the copper carbonate species ranged from 47 to 89% at the lowest and highest alkalinities tested, respectively.

Acidic conditions were able to affect newly accumulated gill copper. Gill copper more than doubled at pH 4.0 compared to neutral and pH 9.0 (i.e. 0.0410 vs. 0.0155 and 0.0141 nmol/g, respectively; Fig. 6b). The calculated speciation of copper at 0.20 μM was dramatically different at these three pHs. At pH 4.0, 100% of the copper species was free $Cu^{2+}$, whereas at pH 7.0, 7.7 (unadjusted) and 9.0 it had declined to 70, 19 and 0%, respectively. At the unadjusted pH of 7.7 the only other species of copper (other than $Cu^{2+}$) that was positively charged was $CuOH^+$ (26% of the copper species present). However, at the more alkaline pH of 9.0, $CuOH^+$ was the *only* positively charged species (1.4% $CuOH^+$, 2.5% $Cu(OH)_2$, 53.1% $CuCO_3$, and 42.9% $Cu(CO_3)^{-2}$).

The concentrations of DOM employed in this study were able to complex 0.18 μM copper to varying degrees (from 78 to 100% Cu-humate; Fig. 7). Geochemical modelling of the test water required the use of Playle et al.'s (1993b) Cu–DOC binding constant (log *K*) of 9.1. The resulting change in free copper (from ~20 to 0%) was reflected by a decrease in the amount of copper bound to the gills, however, no dose–response was evident. Approximately half of the newly accumulated gill copper was kept off the gills over the concentration range of DOM used (~0.04 vs. 0.02 nmolCu/g of gill tissue).

## 4. Discussion

The GSIM, conceived by Pagenkopf (1983), provided the foundation on which more recent gill binding models have been formulated. One key aspect of the GSIM, on which the present study was based, states that for surface metal–gill interactions the exchange must be rapid and reversible (Pagenkopf, 1983). Indeed our in vitro model was successful based on these prerequisites because this is the first study to identify the fraction of exchangeable sites on the gill.

### 4.1. Characterization of binding sites in vitro

The in vitro technique employed in this study was adapted from one previously developed by Reid and McDonald (1991). The most important modifications were working at μM (rather than mM) concentrations of radiolabelled copper and eliminating the preparatory rinse of the isolated arches with 5 mM ethlenediaminetetraacetic acid, which was formerly used to remove $Ca^{2+}$ and $Mg^{2+}$ from the gills. We found that our method allowed for consistent determinations of newly

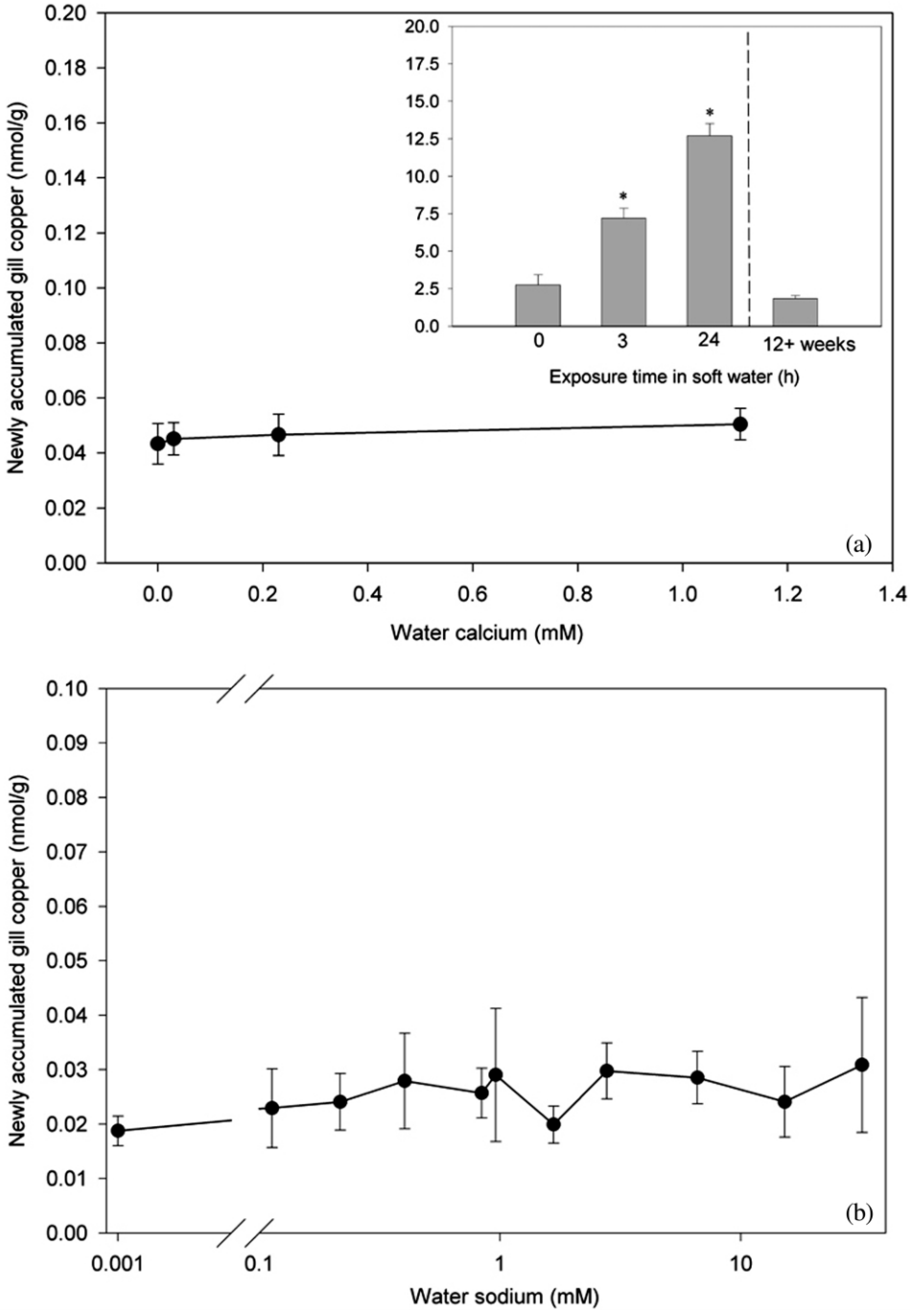


Fig. 5. Cation competition with copper for sites on the gill. In (a), the effect of varying calcium concentrations on newly accumulated gill copper at $0.17 \pm 0.01$ μM (36) Cu. Each data point represents the mean $\pm 1$ S.E. ($N = 8$–9 arches from 6 different fish) in nmol/g of wet gill tissue. In inset (a), the effect of varying acclimation times to low calcium water (0.13 mM; SW) was shown in relation to in vivo gill copper binding. Hard water acclimated (1 mM; HW) juvenile fish were transferred to SW for a time interval which included a 3 h exposure to 0.26 μM $^{64}$Cu (Section 2). The bar to the right of the dotted line represents fish acclimated to SW for 12 weeks followed by a 3 h exposure to radiolabelled Cu in soft water (data from Taylor et al., 2000). Data were mean $\pm 1$ S.E. ($N = 10$). Note: the *y*-axis label also applies to the inset. In (b), the effect of a large range of sodium concentrations was shown in relation to gill copper binding at $0.18 \pm 0.01$ μM (66) Cu. Each data point represents the mean $\pm 1$ S.E. ($N = 6$ from 11 different fish). Note the log scale for the *x*-axis in panel (b).

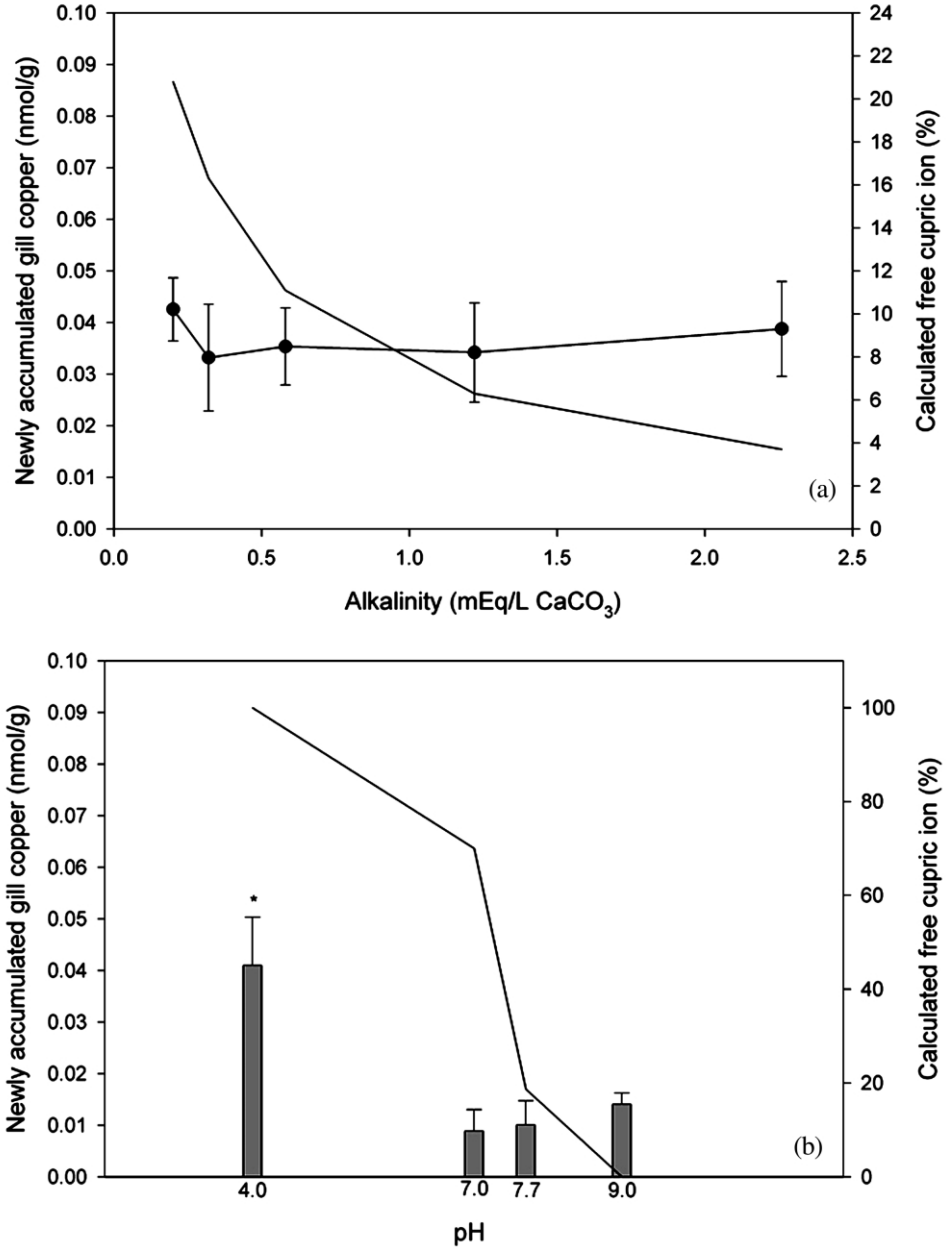


Fig. 6. The effect of varying alkalinity (a) and pH (b), independently of each other, on the binding of copper to isolated gill arches (nmol/g wet gill tissue). The range of alkalinity tested was evaluated at $0.18 \pm 0.01$ μM (30) Cu at pH 7.7. Data in (a) represents the mean $\pm 1$ S.E. ($N=6$ arches from 5 different fish). The effect of pH was evaluated at $0.20 \pm 0.01$ μM (24) Cu. Data in (b) represents the mean $\pm 1$ S.E. ($N=6$ arches from 4 different fish). The solid line in both panels indicates the amount of free cupric ion, as a percentage of the total copper species present, estimated by MINEQL+.

accumulated gill copper (i.e. all 5-min assays conducted at 0.16–0.3 μM Cu ranged from 0.016 to 0.06 ngCu/g of gill tissue). The initial time course study revealed a less variable response of newly accumulated gill copper at exposure times less than 8 min. Beyond this, binding increased greatly and the variability around the mean response also increased, presumably reflecting variability in the viability of the tissue and the increased penetration of the preparation by Cu associated with the deterioration of viability. The second time course which focused on exposures

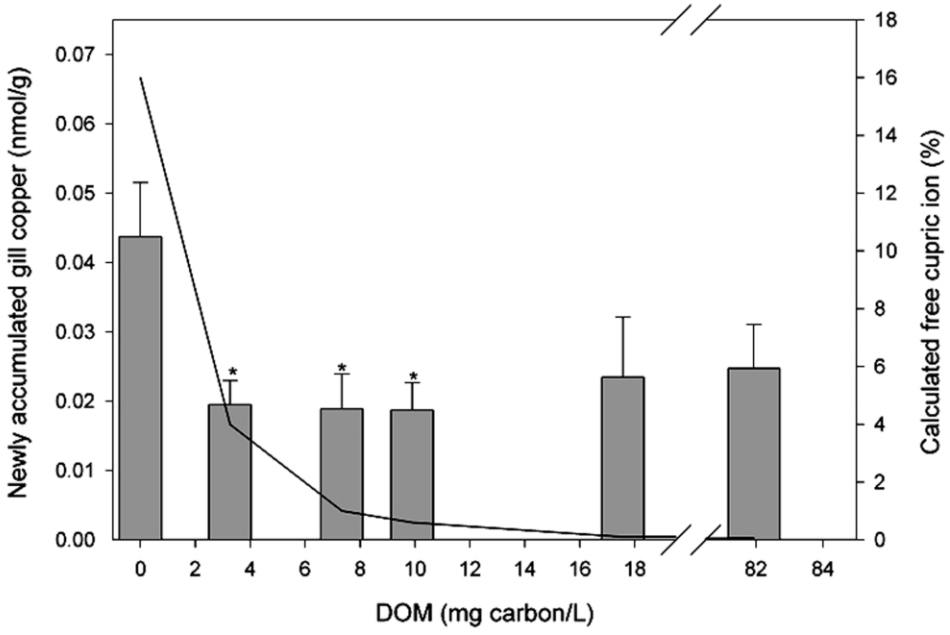


Fig. 7. Newly accumulated gill copper (nmol/g wet gill tissue) at varying concentrations of commercial DOM. The DOM and Cu were allowed to interact for 3 h before the start of the assay. Isolated arches ($N=6$ each from 6 different fish) were exposed for 5 min to $0.18\pm0.01$ μM (36) Cu. The solid line indicates the amount of free cupric ion, as a percentage of the total copper species present, estimated by MINEQL+. *Indicates significant difference from the control group using a one-way ANOVA and Dunnett's test ($P<0.05$).

under 300 s, displayed the gill surface saturating rapidly with copper ($t_{\frac{1}{2}}=171$ s) at a water concentration of 0.16 μM. Beyond this concentration, another set of sites appear to be accessed by copper and are identified as lower affinity, higher capacity sites. This result, of a second set of sites, confirms previous findings conducted in vivo using juvenile rainbow trout exposed to copper radiolabelled with $^{64}$Cu for 3 h, which were also tested within the same range of water copper (Taylor et al., 2000). In the present study using isolated arches, we have verified the same breakthrough phenomenon to lower affinity sites within the range of ~0.2–0.3 μM total copper.

In vivo gill copper binding has been traditionally evaluated in soft water at pH <6.5 for 3 or 24 h (Playle et al., 1992, 1993a,b; MacRae et al., 1999a, also see review by Playle, 1998). Because these studies were conducted without radiolabelled copper, a low pH and long exposure time were employed in order to maximize the amount of free cupric ion (>98% of the copper species present) and to detect differences against the background levels of copper on the gills, respectively. We know that our brief exposure time of 5 min reflects at least 50% of surface binding based on the pulse-chase experiment. Because only 50% of the bound copper could be stripped off of the gill, we can conclude that a portion of the radiolabelled copper had indeed entered the gill tissues in 5 min. The portions of surface-complexed metal and metal uptake across the gill surface in previous traditional gill binding studies is unknown. However, measurable copper accumulation did occur within 20 min in the study by Playle et al. (1992). Indeed, studies on algae (Crist et al., 1990) and yeast (Huang et al., 1990) indicate copper binding to cell surfaces in 5 and 50 s, respectively. Whether surface binding or metal uptake or some combination of the two most accurately predicts metal toxicity is also unknown. Nonetheless, we believe true surface binding, over a shorter time interval, simplifies the task of modelling the influence of competitive and complexing agents in exposure water. We provide evidence for the key assumption originally made by Pagenkopf (1983), which stated that the rates of metal exchange between the gill surface and exposures are rapid when compared to the time required for a bioassay test. The in vitro technique of isolating gill arches also

simplifies some of the confounding factors involved in whole animal gill binding assays.

The gill microenvironment established in a whole fish has been one factor known to influence gill copper binding (Playle et al., 1992). Transfers of carbon dioxide and ammonia can acidify or alkalinize expired water, depending on the water pH. Inspired water would be rendered more acidic at the pH and buffering capacity used in the present study (Playle and Wood, 1989; Lin and Randall, 1990; Playle et al., 1992), a shift which strongly affects the speciation of copper. For example, the amount of the free cupric ion calculated using MINEQL+ changes from 15% at pH 7.7 to 71% at pH 7.0 at 0.16 μM total copper. This magnitude of pH change, (i.e. 0.7 pH units), is reasonable for live trout in our water (Playle and Wood, 1989; Playle et al., 1992). The in vitro protocol of the present study eliminates this effect plus any differences in ventilation frequency and volume between individual fish and any non-specific binding to the skin surface or sloughed off mucus (Reid and McDonald, 1991).

Another confounding factor known to influence copper toxicity is fish size (Howarth and Sprague, 1978). Fish size did indeed have a profound effect on gill copper binding in our study, however, this effect was largely seen in fish less than ~25 g. We conclude that the gills of smaller fish bind more copper per unit gill mass, therefore in using larger fish (110–216 g) we have reduced any associated variability on the amount of copper bound by the gills. The magnitude of our size effect is consistent with Howarth and Sprague's (1978) conclusion that smaller fish were more sensitive to copper toxicity, presumably because they bind more copper at the site of copper's toxic action. According to Howarth and Sprague (1978) a 10-g trout is 2.5 times more resistant to copper than a 0.7-g fish. Our fish size—gill copper binding relationship predicts a 0.7-g fish to have 3.5 times more newly accumulated copper (per unit weight of gill), than a 10-g fish. Kamunde et al. (2001) described a negative exponential relationship between fish mass and gill copper uptake rates in vivo also using radiolabelled copper. By comparison, a theoretical 10 g fish would likely bind ~2 times more Cu in 5 min than the same size fish in 1 h from Kamunde et al. (2001), indicating that significant copper regulation may have occurred during this time frame. In other words, in 1 h a fish could begin to detoxify, store and eliminate copper. MacRae et al. (1999a) used juvenile rainbow trout ranging 15–40 g and Playle et al. (1992), Playle et al. (1993a), Playle et al. (1993b) used 0.5–4 g fathead minnows to model gill copper binding yet neither evaluated the influence of size. A partial explanation for our size effect may be due to differences in surface area per unit volume of gill tissue in actively growing fish (Hughes, 1972).

### 4.2. Effects of water chemistry

In the present in vitro study, we have clearly defined the initial response of gill tissue to copper exposure, as influenced by calcium, sodium, alkalinity, pH and DOM. In vivo, it has been firmly established that all of these water chemistry parameters play a role in mitigating copper binding (Pagenkopf, 1983; Playle et al., 1992, 1993a,b; Taylor et al., 2000; MacRae et al., 1999a; Zitko and Carson, 1976) and copper toxicity (Spear and Pierce, 1979; Taylor et al., 2000; Miller and Mackay, 1979; Chakoumakos, 1979; Laurén and McDonald, 1986; Erickson et al., 1996).

#### 4.2.1. Alkalinity and pH

It is generally accepted that calcium (reported as water hardness) competes with copper for binding sites on the gills and that the accompanying bicarbonate/carbonate concentrations (usually present as $CaCO_3$ alkalinity) complex copper, thereby reducing the amount of free or available cupric ion. By contrast, we found no effect of calcium or alkalinity on newly accumulated gill copper binding over the range found in the Great Lakes. Playle et al. (1992) also found no effect of calcium on copper binding at pH 6.3 but found gill copper accumulation was eliminated at pH 4.8 by $Ca^{2+} \geqslant 2100$ μEq/l. It was believed that the increase in hydrogen ion concentration at the lower pH may have added to the competition by calcium. In contrast, we found a decrease in pH (at constant $[Ca^{2+}]$) caused an increase in gill copper binding. Apparently, the increase in free cupric ion concentration at pH 4 outweighed the competitive effect of $H^+$ ions for these high affinity sites. At pH 9.0 and 7.0, there was no difference in newly accumulated gill copper, thus confirming the availability of copper hydroxide species to the gill (Chakoumakos, 1979). The lack of an alkalinity effect on the rapid binding of copper to the gill is somewhat supported by the previous finding which

showed that alkalinity did not alter copper *uptake* over 24 h (Laurén and McDonald, 1986). This is despite the fact that the free cupric ion concentration in our study (as estimated by MINEQL+ in a closed system), varied ~20% over the range of alkalinities tested. We assumed that a system closed to the atmosphere was appropriate for modelling changes in alkalinity because of the short exposure time. Realistically, it would likely take more than 5 min for the release of carbon dioxide from the water, whereas the geochemical model assumes equilibrium conditions. Also in support of this assumption, the alkalinity and pH were measured before and after the exposure time and both remained unchanged despite the gentle aeration required for mixing. The copper complex present in the largest amount was $CuCO_3$. However, it has been reported that copper carbonate complexes are not available for binding/uptake and hence, are considered non-toxic copper species (Chakoumakos, 1979).

*4.2.2. Calcium*

Although our data did not exhibit a competitive effect of calcium at the gill, our in vivo experiment did show that calcium may influence the gill through acclimatory processes. This result is in agreement with hypotheses previously stated by Taylor et al. (2000), where the role of calcium in regulating membrane permeability and stabilizing membrane proteins may also include regulating the number and affinity of binding sites on the gill surface. Fish which were fully acclimated to hard water or soft water bound similar amounts of newly accumulated copper, likely as a result of their ionoregulatory homeostatic stability. In striking contrast, hard water acclimated trout acutely transferred to soft water for 3 or 24 h bound increasingly larger amounts of copper, a result likely due to their ionoregulatory homeostatic upset. The effect of ion poor water on the morphology of the gills has been reported (see review by Laurent and Perry, 1991) and therefore it is reasonable to suggest calcium may affect the physiology of gill–metal interactions. Gundersen and Curtis (using an isolated arch technique; 1995), reported the effect of calcium on gill permeability and identified different calcium binding sites responsible for the membrane permeability function.

*4.2.3. Sodium*

We also found no effect of sodium on the initial response of the gill to copper. This effect was absent over the large range of sodium concentrations tested (1 μM–30 mM). The rationale for the extended test range (i.e. outside the range of North American freshwaters) was to encompass the sodium sensitive ($<200$ μM $Na^+$) and sodium insensitive copper *uptake* pathways over 2 h, proposed by Grosell and Wood (2002). In addition, the mechanism of acute copper toxicity is to impair sodium balance, and trout have been shown to compensate by altering sodium and copper uptake from the diet (Pyle et al., submitted for publication). We agree with Pyle et al. (submitted for publication), that sodium plays an important role in modifying copper uptake and toxicity, nonetheless our results indicate that it does not affect the rapid high affinity binding of copper to the gills.

*4.2.4. Humic acid*

Commercial DOM reduced copper binding to isolated arches but over the range of DOM tested (3–82 mgC/l) a dose–response was not exhibited. Previous studies have shown that the presence of DOM at $\geqslant 5$ mg/l reduced copper to background levels on the gills (Playle et al., 1993a,b; Hollis et al., 1997; Richards et al., 2001). One reason we were not able to completely keep Cu off of the gills may be due to the increased level of detection or sensitivity of our assay (i.e. radiolabelled Cu and 5 min exposures). In addition, there has been a range of reported values for the affinity of DOM for copper, presumably because many types of sites exist on organic acids. Morel (1983) found that Cu–DOM binding constants vary with log *K*s ranging from 6 to 11 in waters with pH $>6$. Playle et al. (1993a), Playle et al. (1993b) specifically estimated stability constants for the high affinity copper binding sites on DOM to be $\log K_{\text{Cu-DOM}}$ of 9.1 and on the gill to be $\log K_{\text{Cu-gill}}$ of 7.4. Accordingly, copper would likely bind to DOM rather than the gill surface. MacRae et al. (1999b) reported two types of binding sites for Cu on Aldrich humic acid; low and high affinity sites with log *K*s of 6.15 and 8.14, respectively. The higher affinity sites characterized by Playle et al. (1993a), Playle et al. (1993b) are most relevant at the low copper concentrations employed in our study. For modelling free copper concentrations in the present study, we employed Playle et al. (1993b) $\log K_{\text{Cu-DOM}}$ of 9.1. Therefore, in com-

parison to our calculated affinity constant for copper to the gill, $\log K_{Cu-gill}$ of 8.1, Cu would certainly be bound by DOM rather than the gill. In contrast, at any DOM concentration used in our experiment, copper accumulation was reduced by only 50%. We must conclude then the possible existence of another set of high affinity sites on the gills for Cu. These sites would have a stronger affinity for copper than does DOM. The only other possible explanation would be that the Cu–DOM complex itself was able to bind to the gill surface. Wilkinson et al. (1993) suggested this possibility with aluminum as a ternary complex, where the metal plays a bridging role (i.e. $(H_2O)_x$–(DOM)–Al–ligand–gill). Moreover, fulvic acids (a major component of DOM) have been shown to bind to isolated Atlantic salmon gill cells (Campbell et al., 1997). However, these studies also report the necessity for acidic conditions in order for this to occur and at our pH of 7.7 the binding of a Cu–DOM complex at the gill surface would be unlikely.

### *4.3. Conclusions*

The in vitro technique used in this study was successful in modelling the initial or rapid response of copper binding to the gill surface and in understanding the influence of water chemistry, one variable at a time. The use of isolated arches simplified the gill microenvironment and ultimately research will be necessary to combine acute toxicity, water quality and gill binding in whole fish. It was a surprise that water chemistry parameters influence gill–metal binding in a manner different to their influence on acute toxicity and different from the effects on long-term binding reported in other studies. This was evident by the absence of a protective effect from the competing ions calcium, sodium and hydrogen, no effect at all of alkalinity or high pH and the fact that commercial DOM did not prevent copper from binding to the gills. Currently, the BLM uses the rapid increase of gill copper (believed to reflect binding to the physiologically active receptor sites) to model gill binding characteristics (DiToro et al., 2000). The distinction between rapid surface binding and metal uptake obviously plays an important role in determining the toxic effects of copper, especially when regulators need to predict the modifying effects of water chemistry.

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