Effects of copper and cadmium on ion transport and gill metal binding in the Amazonian teleost tambaqui (*Colossoma macropomum*) in extremely soft water

Aline Y.O. Matsuo [a,*], Chris M. Wood [b], Adalberto L. Val [a]

[a] *Laboratory of Ecophysiology and Molecular Evolution, National Institute for Research in the Amazon (INPA), Av. André Araújo, 2936 Aleixo, Manaus, Amazonas 69083-000, Brazil*
[b] *Department of Biology, McMaster University, 1280 Main St. West, Hamilton, Ont., Canada L8S 4K1*

Abstract

Metal toxicity in fish is expected to be most severe in soft waters because of the low availability of cations (particularly Ca^{2+}) to out-compete the metal forms for binding sites on the gills. Natural waters in the Amazon basin are typically soft due to regional geochemistry, but few studies have focused on metal toxicity in fish native to the basin. We assessed the ionoregulatory effects of waterborne copper (Cu) and cadmium (Cd) on tambaqui (*Colossoma macropomum*) in extremely soft water ($10\,\mu mol\,l^{-1}$ Ca^{2+}). Tambaqui had a very high tolerance to Cu (50–$400\,\mu g\,l^{-1}$), as indicated by a complete lack of inhibition of Na^+ uptake and an ability to gradually recover over 6 h from elevated diffusive Na^+ losses caused by Cu. The insensitivity of active Na^+ influx to Cu further supports the notion that Amazonian fish may have a unique Na^+ transport system. Addition of 5–10 mg C l^{-1} of dissolved organic matter (DOM) did not prevent initial (0–3 h) negative Na^+ balance in tambaqui exposed to Cu. Exposure to $40\,mg\,C\,l^{-1}$ DOM prevented Na^+ losses in tambaqui even at $400\,\mu g\,l^{-1}$ Cu, probably because most Cu was complexed to DOM. Tambaqui exposed to waterborne Cd (10–$80\,\mu g\,l^{-1}$) experienced an average of 42% inhibition in whole body Ca^{2+} uptake relative to controls within 3 h of exposure to the metal. Inhibition of Ca^{2+} uptake increased over time and, at 24 h, Ca^{2+} uptake was suppressed by 51% and 91% in fish exposed to 10 and $80\,\mu g\,l^{-1}$ Cd, respectively. Previous acclimation of fish to either elevated $[Ca^{2+}]$ or elevated [DOM] proved to be very effective in protecting against acute short-term metal accumulation at the gills of tambaqui in soft water (in the absence of the protective agent during metal exposure), suggesting a conditioning effect on gill metal binding physiology.
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* Corresponding author. Present address: 3447 Alana Drive, Sherman Oaks, CA 91403, USA. Tel.: +1 818 783 4077; fax: +1 818 981 2747.
E-mail address: matsuoaline@aol.com (A.Y.O. Matsuo).

1. Introduction

Binding of metallic cations to target sites at the gills is the key determinant of the toxicity of met-

als to fish (Di Toro et al., 2001). Toxic responses begin once metal accumulation at these sites reaches a threshold concentration that results in disruption of their physiological function, thus inducing acute toxicity. Geochemical factors, such as competition and complexation, interfere with the amount of free metal available in the water, and can greatly affect the bioavailability and toxicity of metallic cations to fish (Playle et al., 1992, 1993a).

Metal toxicity is expected to be most severe in fish inhabiting soft waters because of the low availability of hardness cations to out-compete the toxic forms for binding sites on the gills (e.g., Pagenkopf, 1983; Playle et al., 1992, 1993a). Complexing agents of organic and inorganic nature determine through the speciation processes which chemical forms of the metal will be present in a given environment. Dissolved organic matter (DOM) is known to exert a protective effect against metal toxicity by forming complexes with metals, thus decreasing the availability of the free metal in the water.

In the Amazon basin, natural waters are mostly soft due to regional geochemistry. Calcium (Ca^{2+}), one of the major hardness cations, is normally present at concentrations between 10 and 180 $\mu mol\, l^{-1}$ (Furch, 1984). Despite the generally low ion content of Amazonian waters, humic substances, the major component of DOM in rivers, are ubiquitous in these environments. DOM concentrations can exceed 35 mg C l^{-1} in some ion-poor blackwater tributaries of the Amazon River (Santos et al., 1984; Walker, 1995). The average content of DOM in freshwaters elsewhere is 0.5–4.0 mg C l^{-1} (Thurman, 1985).

Waterborne metals, such as copper (Cu) and cadmium (Cd), exert initial toxic effects at physiologically active sites on the gill surface, interfering directly with the branchial ion transporting function. Although Cu is essential for metabolic processes, it can be extremely toxic to fish. Cu is known to affect Na^+ homeostasis in freshwater fish by inhibiting branchial Na^+ uptake at moderate concentrations ($\sim$100 $\mu g\, l^{-1}$ in rainbow trout, *Oncorhynchus mykiss*, in hard water; Laurén and McDonald, 1985). The mechanism of Cu toxicity in fish may involve a decrease in the affinity of Na^+ transporters (competitive inhibition) and also a decrease in Na^+ uptake capacity (non-competitive inhibition) (Laurén and McDonald, 1987). At higher concentrations of Cu (>100 $\mu g\, l^{-1}$), disturbance fur-

ther involves stimulation of Na^+ efflux (Laurén and McDonald, 1985). The bioavailability and toxicity of Cu to fish is strongly affected by the concentration of DOM in the water. The role of Ca^{2+} in protecting against Cu toxicity remains controversial (e.g., Laurén and McDonald, 1985, 1986), but most authors accept that Cu toxicity is less severe in hard water.

Cd, unlike Cu, does not constitute an essential element for metabolic processes in living organisms. It can be extremely toxic to fish, even at very low waterborne concentrations, due to its high binding affinity for the gills (Playle et al., 1993a,b). The mechanism of waterborne Cd toxicity in fish involves competition between Cd and Ca^{2+} for Ca^{2+} uptake sites on the gills, probably on the mitochondria-rich "chloride" cells (Verbost et al., 1987, 1989). Cd ultimately disrupts Ca^{2+} homeostasis by inhibition of basolateral Ca^{2+}ATPase in the same cells (Verbost et al., 1987, 1989). DOM does not protect as strongly against Cd toxicity as it does against Cu toxicity, because the binding affinity between Cd and DOM is relatively low (Playle et al., 1993a,b). Ca^{2+} concentration in the water is the major agent protecting against Cd deposition on fish gills (Playle et al., 1993a,b; Hollis et al., 1997).

Metal contamination occurs in some waters in the Amazon basin, and this is mostly attributed to industrial activity. Cu concentrations can exceed 1000 $\mu g\, l^{-1}$ in water bodies associated with effluent disposal in industrial areas in the city of Manaus, Amazonas, Brazil (Sampaio, 2000). Cd concentration can reach 10 $\mu g\, l^{-1}$ in water associated with petroleum extraction along the Amazon River (Oliveira, 2003). Such levels could be toxic to fish given the very low Ca^{2+} content of these waters, but few data are available to address this impact on the diverse fish fauna occurring in the basin.

We assessed the effects of acute waterborne Cu exposure on Na^+ fluxes in a species native to the Amazon basin (tambaqui, *Colossoma macropomum*) in extremely soft water and under various concentrations of added DOM. We also assessed the toxicity of waterborne Cd on whole body Ca^{2+} uptake in tambaqui over 24 h. To assess the role of acclimation on gill metal accumulation, we performed acute short-term exposure to Cu and Cd in tambaqui previously acclimated to either DOM (both natural and commercial forms) or to high Ca^{2+} concentrations.

2. Materials and methods

2.1. Experimental animals

Juvenile tambaqui obtained from a local fish farm (Fazenda Santo Antônio, Rio Preto da Eva, Amazonas, Brazil) were kept in aerated soft water for at least 3 weeks before we began experimentation. The soft water we used was intended to simulate the naturally soft Amazonian waters. Soft water consisted of wellwater with an average composition of: $Ca^{2+} = 11$, $Na^+ = 34$, $Cl^- = 28$, $Mg^{2+} = 0.8$, $K^+ = 15$ (all in $\mu mol\, l^{-1}$); pH 6.3; dissolved organic matter $= 0.9\, mg\, C\, l^{-1}$; background $Cu = 1.7\, \mu g\, l^{-1}$; background $Cd = 0.3\, \mu g\, l^{-1}$; temperature $= 28\,°C$. Fish were fed commercial dry food pellets once a day. Food leftovers and fecal material were removed from the bottom of the tanks every day and there was a minimum of 80% water renewal daily.

2.2. Cu toxicity in tambaqui

2.2.1. Effects on unidirectional Na+ fluxes

We assessed Cu toxicity in tambaqui based on changes in unidirectional Na^+ fluxes over time. Feeding was suspended 48 h before experimentation and fish were individually transferred to polyethylene chambers to adjust to the container's environment overnight.

Tambaqui (0.76 ± 0.02 g; $N = 10$ per concentration tested) were acutely exposed to nominal Cu concentrations of 0 (control), 50, 100, 200, and 400 $\mu g\, l^{-1}$ (added as $CuSO_4 \cdot 5H_2O$). Water in the chambers (50 ml) was renewed 1 h prior to the onset of Cu exposure with the same soft water type used during acclimation. Unidirectional Na^+ fluxes (influx, efflux, and net flux) were measured over 6 h in static conditions following methods described previously (Matsuo et al., 2004) and the general principles outlined by Wood (1992). In brief, radioisotope (50 nCi l^{-1} ^{22}Na, Amersham Pharmacia) and Cu were added to chambers according to the treatments, allowing 5 min for mixture. Unidirectional influx measurements were based on the disappearance of ^{22}Na from the water over time, indicating incorporation of the isotope into the fish. Net flux measurements were based on the change in total Na^+ content of the 50 ml bath, and unidirectional efflux measurements were calculated as the difference. Water samples (5 ml) were taken from each chamber at 0, 1, 3, and

6 h for later analysis of radioactivity and total Na^+ concentration.

2.2.2. Effects on unidirectional Na+ fluxes in the presence of DOM

To determine the effects of Cu exposure in tambaqui in the presence of DOM, we addressed the changes reflected in unidirectional Na^+ fluxes based on the same Cu concentrations tested in the previous section. We used Aldrich humic acid (Sigma–Aldrich) to manipulate DOM concentration in the experimental chambers (5, 10, 20, and 40 $mg\, C\, l^{-1}$). A stock solution of DOM was prepared by dissolving humic acid in deionized water in a darkened beaker and agitating for 12 h using a magnetic stirrer. The stock solution was refrigerated in an amber bottle until use. It was not logistically possible to measure DOM concentrations in the treatments, so we estimated them based on the observation that the Aldrich humic acid is approximately 40% dissolved organic carbon (McGeer et al., 2002).

Unidirectional Na^+ fluxes were performed as described in the previous section. Tambaqui (0.65 ± 0.01 g; $N = 10$ per treatment) were acutely exposed to DOM and Cu and water samples (5 ml) were taken from each chamber at 0, 1, 3, and 6 h for later analysis.

2.3. Cd toxicity in tambaqui

2.3.1. Effects on whole body Ca2+ uptake

To examine Cd toxicity in tambaqui, we measured changes in whole body Ca^{2+} uptake rate (analogous to unidirectional influx rate for Na^+) based on the total radioactivity of ^{45}Ca incorporated into the fish. We did not use the same protocol as that used for the Cu studies because Ca^{2+} uptake occurs much more slowly than Na^+ uptake. ^{45}Ca is also highly affected by adsorption phenomena, which further complicates analysis if using the same protocol used for Na^+ fluxes. Effluxes were not determined because Cd does not strongly affect Ca^{2+} efflux (Verbost et al., 1987; Reid and McDonald, 1988).

Fish were transferred to experimental polyethylene chambers (150 ml) containing the same soft water used for acclimation. A total of 40 fish (0.37 ± 0.02 g) were acutely exposed to each nominal Cd concentration of 0, 10, 20, 40, and 80 $\mu g\, l^{-1}$ (added as $Cd(NO_3)_2 \cdot 4H_2O$).

Radioisotope ($10\,\mu\text{Ci}\,l^{-1}$ ^{45}Ca, Amersham Pharmacia) was added to each chamber and after a 5 min mixing period, water samples (6 ml) were collected for determination of specific activity of the radioisotope within each time interval, and also to measure Cd concentration in the treatments.

Whole body Ca^{2+} uptake was assessed at 3, 6, 12, and 24 h of exposure to Cd. At each sampling interval, 10 fish from each treatment were sampled. Prior to sacrifice, fish were transferred to a concentrated Ca^{2+} solution ($10\,\text{mmol}\,l^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$) for 30 s to displace surface-bound Ca^{2+} ('cold displacement'). Fish were then killed with an overdose of anesthetic ($1\,\text{g}\,l^{-1}$ MS-222, Sigma), blotted dry in paper towel, weighed, and transferred to vials for analysis of incorporated ^{45}Ca.

2.4. Gill metal accumulation in fish acclimated under various conditions

This experimental series examined the effect of prior acclimation to either Ca^{2+} or DOM on gill metal accumulation in tambaqui. Acute short-term (2–3 h) exposure to waterborne metals may be a useful indicator of surface binding at the gills before the metals are internalized or redistributed to other organs (Playle et al., 1992; Hollis et al., 1997).

Tambaqui ($3.8 \pm 0.2\,\text{g}$) were acclimated to different concentrations of either Ca^{2+} or DOM for 10 days in 5 l aquaria. Concentrated Ca^{2+} or DOM was added to soft water to reach the desired concentrations in the acclimation aquaria. Acclimation of tambaqui to high Ca^{2+} concentrations was performed at 100, 200, and $400\,\mu\text{mol}\,l^{-1}$ (as $Ca(NO_3)_2 \cdot 4H_2O$). Acclimation to DOM involved two sources, a natural and a commercial form. The natural source (NOM) used in this study consisted of natural organic matter isolated and concentrated from Luther Marsh (Ontario, Canada). The concentration of dissolved organic carbon (DOC) in the stock of NOM was $1858\,\text{mg}\,\text{C}\,l^{-1}$ (determined using a Total Organic Carbon Analyzer, TOC 5050A, Shimadzu, by R.C. Playle, Wilfrid Laurier University, Waterloo, Ontario, Canada). The commercial source (AHA) consisted of Aldrich humic acid (40% DOC, McGeer et al., 2002). The desired concentrations of DOM in the treatments (20, 40, and $80\,\text{mg}\,\text{C}\,l^{-1}$) were obtained by diluting the concentrated stocks accordingly. Control groups consisted of fish acclimated to the soft wellwater (ion composition given in Section

2.1). The acclimation solution in all aquaria was fully renewed daily.

After 10 days of acclimation, fish were gently rinsed in distilled water and transferred to experimental chambers (500 ml) containing soft wellwater only, where they remained undisturbed for 30 min prior to metal exposure. Note therefore that the acclimation components (elevated [DOM], elevated [Ca^{2+}]) were not present in the test media, so as to evaluate the effects of prior acclimation alone. Tambaqui from each acclimation condition were acutely exposed for 3 h to Cu or Cd at fixed concentrations of 600 and $200\,\mu\text{g}\,l^{-1}$, respectively. Water samples (2 ml) were taken and acidified to measure the actual concentration of the metal in the testing water. Fish were killed and their gill arches removed, rinsed in a solution of $1\,\text{mmol}\,l^{-1}$ EDTA for 30 s, and blotted dry in paper towel. Samples were weighed and transferred to vials for digestion in 1N HNO_3 (48 h, at $70\,^\circ\text{C}$). Cu or Cd concentrations were determined in digested gills.

Gill Cu or Cd concentrations in tambaqui were determined for each acclimation condition to ensure that background concentrations of these metals in the gills were not altered as a result of prior acclimation to the solution prior to exposure to the metals. This was particularly important in fish acclimated to DOM solutions because DOM stocks sometimes retain metals during the concentration process (Hollis et al., 1997). The net accumulated Cu or Cd concentration represents the difference between total metal concentrations after 3 h of metal exposure in each treatment and the measured background concentrations obtained in fish following acclimation but no exposure to the metal.

2.5. Analytical techniques

2.5.1. Cu series

Na^+ concentration in water samples was determined using flame atomic absorption spectrophotometry (AAnalyst 800, Perkin-Elmer). ^{22}Na radioactivity was analyzed in 1 ml aliquots mixed with scintillation fluor (ACS, Amersham Pharmacia) and determined using liquid scintillation counting (LS6500, Beckman & Coulter). Calculations for unidirectional Na^+ influx, efflux, and net flux measurements were based on equations found in Wood (1992).

An experimental variable introduced from using Aldrich humic acid was the elevation of initial Na^+

concentrations in the treatments. Aldrich humic acid is sold in the form of a sodium salt, so it was difficult to disassociate DOM and Na^+. Na^+ concentrations at the start of flux measurements were therefore (mean $\pm$ S.E.M., $N = 5$) 38 ± 1, 48 ± 1, 67 ± 1, and $116 \pm 5 \, \mu mol \, l^{-1}$ for the 5, 10, 20, and $40 \, mg \, C \, l^{-1}$, respectively. Ca^{2+} concentrations also increased due to the input of commercial DOM, but to a much lesser extent.

Total Cu concentration in acidified samples was analyzed using atomic absorption spectrophotometry with graphite furnace atomization (AAnalyst 800-GF, Perkin-Elmer) following operating conditions recommended by the manufacturer. Measured Cu concentrations in $\mu g \, l^{-1}$ (mean $\pm$ S.E.M., $N = 5$) in experimental series of Cu exposure alone, relative to nominal concentrations (in parenthesis), were: 2.8 ± 0.1 (0, control), 43 ± 3 (50), 86 ± 7 (100), 174 ± 6 (200), and 382 ± 11 (400). Measured Cu concentrations in $\mu g \, l^{-1}$ (mean $\pm$ S.E.M., $N = 5$) in experimental series involving exposure to DOM plus Cu, relative to nominal concentrations (in parenthesis), were: 3.3 ± 1.0 (0, control), 58 ± 3 (50), 110 ± 8 (100), 187 ± 4 (200), and 379 ± 11 (400).

2.5.2. Cd series

To determine whole body Ca^{2+} uptake in tambaqui, fish were digested in tissue solubilizer (NCS-II, Amersham Pharmacia) at $60 \, °C$ for 24 h under steady agitation. Tissue digests were then neutralized with glacial CH_3COOH, to which scintillation cocktail for organic samples (OCS, Amersham Pharmacia) was added. The radioactivity incorporated by the fish was analyzed by liquid scintillation counting, corrected for 'quenching' effects, and Ca^{2+} uptake was determined following Perry and Wood (1985).

Specific activity in the water samples was determined based on analysis of Ca^{2+} concentration and ^{45}Ca radioactivity. Ca^{2+} concentrations were determined using flame atomic absorption spectrophotometry. Lanthanum chloride (3% $LaCl_3$) was added to prevent interference of other cations in Ca^{2+} analysis. ^{45}Ca radioactivity was determined as described for ^{22}Na analysis.

Cd concentrations in water samples were determined using graphite furnace atomic absorption spectrophotometry. Measured Cd concentrations in $\mu g \, l^{-1}$ (mean $\pm$ S.E.M., $N = 5$), followed by nominal concen-

trations in parenthesis, were: 0.09 ± 0.02 (0, control), 7.88 ± 0.05 (10), 16.45 ± 0.29 (20), 36.13 ± 1.27 (40), and 72.08 ± 2.94 (80).

2.5.3. Gill metal accumulation

Ca^{2+} concentrations in acclimation treatments were analyzed as described previously. These concentrations, followed by nominal Ca^{2+} in parenthesis, in $\mu mol \, l^{-1}$ were (mean $\pm$ S.E.M., $N = 5$): 103 ± 2 (100), 210 ± 5 (200), and 387 ± 3 (400). Ca^{2+} concentration in acclimation situations involving AHA increased to $70 \, \mu mol \, l^{-1}$ at the highest concentration tested ($80 \, mg \, C \, l^{-1}$ AHA) as a result of the input of Ca^{2+} from the commercial form of DOM.

Metal concentrations in water used for testing gill metal accumulation were determined using methods described above (Sections 2.5.1 and 2.5.2). The Cu concentration measured in testing water for the Cu series was $612 \pm 5 \, \mu g \, l^{-1}$ (nominal $600 \, \mu g \, l^{-1}$) and the Cd concentration for the Cd series was $188 \pm 4 \, \mu g \, l^{-1}$ (nominal $200 \, \mu g \, l^{-1}$). Metal concentrations in gill digests were also analyzed using graphite furnace atomic absorption spectrophotometry.

2.6. Statistical analysis

Results are presented as mean $\pm$ S.E.M. for each treatment. Statistical analysis used ANOVA followed by Dunnett's multiple-comparison tests. Significance level was fixed at $\alpha < 0.05$ throughout the study.

3. Results

3.1. Cu toxicity in tambaqui

3.1.1. Effects on unidirectional Na^+ fluxes

Tambaqui did not show inhibition of Na^+ influx over the entire range of Cu concentrations tested, relative to control values at the appropriate time interval (Fig. 1). Indeed, at 100, 200, and $400 \, \mu g \, l^{-1}$ Cu, there were small but significant increases in Na^+ influx rate compared to control values. Cu significantly increased the diffusive Na^+ loss (efflux) in tambaqui, particularly at Cu concentrations between 100 and $400 \, \mu g \, l^{-1}$. Diffusive efflux was not sustained throughout exposure to Cu, as indicated by a gradual reduction in the efflux rate over time. By 6 h, the resulting negative Na^+ net
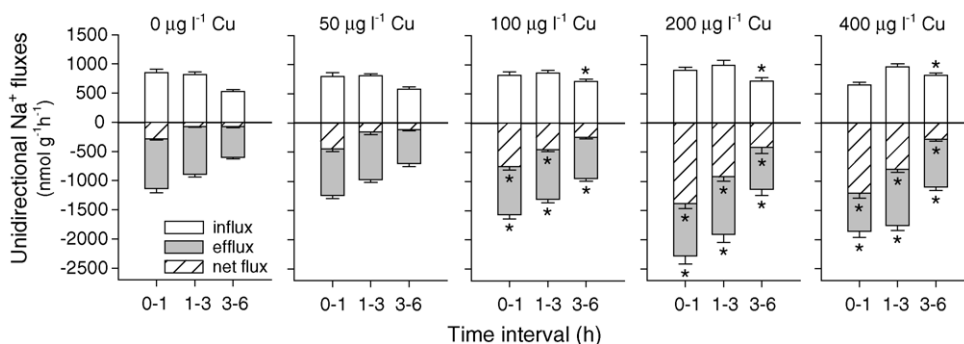
Fig. 1. Unidirectional Na^+ fluxes (mean $\pm$ S.E.M., $N = 10$ per treatment) in tambaqui acclimated to extremely soft water ($10\,\mu mol\,l^{-1}\,Ca^{2+}$, no added DOM) and acutely challenged with Cu. Tambaqui had no inhibition of Na^+ influx upon exposure to 50–$400\,\mu g\,l^{-1}$ Cu, compared to control values at the appropriate time interval. Disturbances were limited to increased diffusive Na^+ loss (efflux), which consequently resulted in significant net Na^+ loss. Tambaqui, however, restored Na^+ homeostasis completely by $6\,h$. Asterisks (*) indicate significant differences relative to control ($0\,\mu g\,l^{-1}$ Cu) at each time interval ($\alpha < 0.05$).

fluxes found in tambaqui exposed to Cu in soft wellwater were minor, although still significant for the 200 and $400\,\mu g\,l^{-1}$ Cu exposures, relative to the control group.

3.1.2. Effects on unidirectional Na^+ fluxes in the presence of DOM

Exposure to DOM concentrations of 5–$10\,mg\,C\,l^{-1}$ did not eliminate the initial (0–$3\,h$) negative Na^+ balance generated by Cu exposure in tambaqui (Fig. 2a and b). At $20\,mg\,C\,l^{-1}$, there was partial attenuation of the effect. Na^+ efflux was controlled only at a DOM concentration of $40\,mg\,C\,l^{-1}$ over the entire range of Cu concentrations tested (Fig. 2d). In the second half of exposure to Cu (3–$6\,h$), however, Na^+ balance was restored to control values in most cases (Fig. 2a–d).

The acute addition of DOM tended to increase Na^+ turnover rates in tambaqui, stimulating both the efflux and influx rates of Na^+, particularly at 20 and $40\,mg\,C\,l^{-1}$. During the initial $3\,h$ exposure to DOM, Na^+ influx in tambaqui increased by 65–80% following exposure to 20–$40\,mg\,C\,l^{-1}$ DOM ($0\,\mu g\,l^{-1}$ Cu), relative to $0\,mg\,C\,l^{-1}$ DOM ($0\,\mu g\,l^{-1}$ Cu) (Figs. 1 and 2). Stimulation of Na^+ efflux was about 30–51% for those same groups, relative to $0\,mg\,C\,l^{-1}$ DOM ($0\,\mu g\,l^{-1}$ Cu).

3.2. Cd toxicity in tambaqui

Tambaqui were very sensitive to waterborne Cd when tested in soft wellwater, as indicated by a significant, dose-dependent inhibition of whole body Ca^{2+} uptake at all Cd concentrations tested (Fig. 3). Within $3\,h$ of exposure, Cd inhibited whole body Ca^{2+} uptake by an average of 42% relative to the control group. The inhibition on whole body Ca^{2+} uptake at $24\,h$ was 51%, 78%, 88%, and 91% at 10, 20, 40, and $80\,\mu g\,l^{-1}$ Cd, respectively. Despite the strong inhibition in Ca^{2+} uptake seen in tambaqui, no mortality occurred during the $24\,h$ exposure.

3.3. Gill metal accumulation in fish acclimated under various conditions

Control fish, acclimated to soft water alone, had an increase of 150% in gill Cu concentration, following $3\,h$ exposure to $600\,\mu g\,l^{-1}$ of Cu (Fig. 4). Tambaqui accumulated significantly less Cu at the gills in acute exposures to the metal in soft water following previous acclimation for 10 days in high concentrations of Ca^{2+} or NOM (Fig. 4). This was true even though these components were removed from the media prior to testing.

Fig. 2. (a–d) Unidirectional Na^+ fluxes (mean $\pm$ S.E.M., $N = 10$ per treatment) in tambaqui acclimated to soft water ($10\,\mu mol\,l^{-1}\,Ca^{2+}$) and acutely exposed to a commercial source of DOM (AHA, Aldrich humic acid) and Cu. In most cases, tambaqui had no inhibition of Na^+ influx upon exposure to 50–$400\,\mu g\,l^{-1}$ Cu, and net fluxes indicated recovery of homeostatic Na^+ balance by $6\,h$. Asterisks (*) indicate significant differences relative to control ($0\,\mu g\,l^{-1}$ Cu) at each time interval ($\alpha < 0.05$).
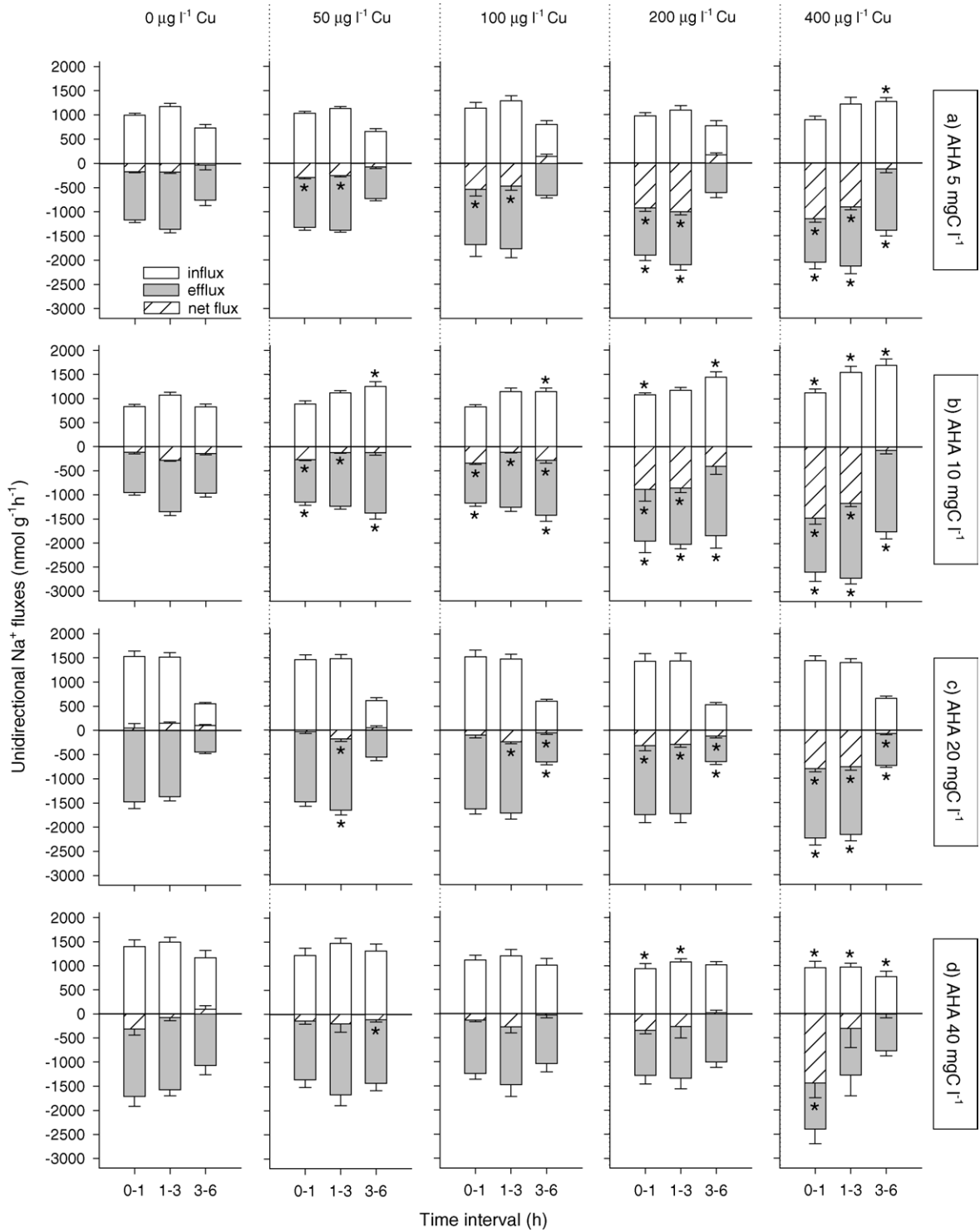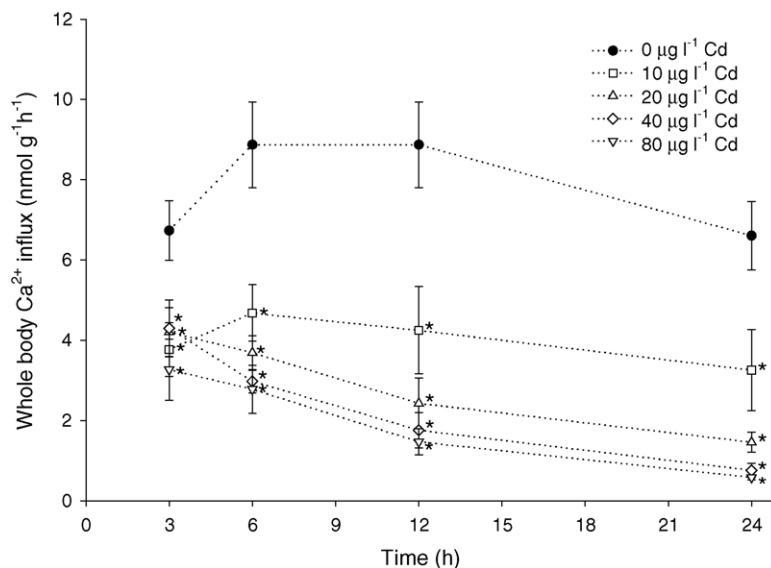
Fig. 3. Whole body Ca^{2+} influx (mean ± S.E.M., $N = 10$ for each sampling time per treatment) in tambaqui acclimated to soft water ($10 \mu mol\, l^{-1}$ Ca^{2+}) and acutely exposed to Cd. Tambaqui had a significant inhibition of Ca^{2+} influx for the entire range of Cd concentrations tested, with no signs of recovery within 24 h. Asterisks (*) indicate significant differences relative to control ($0 \mu g\, l^{-1}$ Cd) at each time interval ($\alpha < 0.05$).
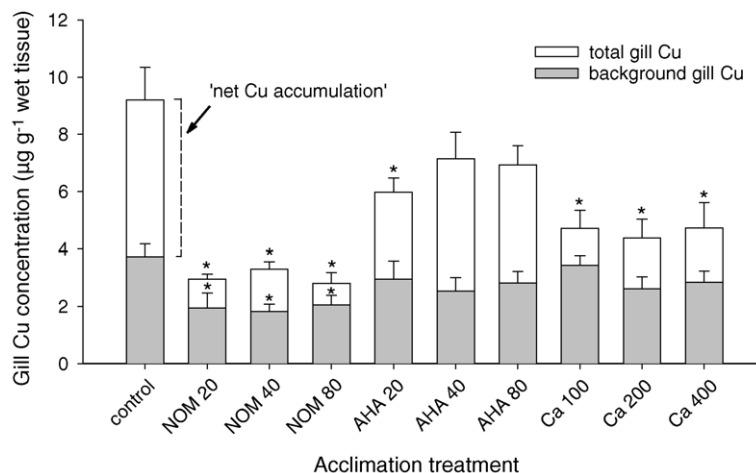

Fig. 4. Gill Cu accumulation (mean ± S.E.M., $N = 6$) in tambaqui. Fish were previously acclimated for 10 days to either natural or commercial sources of DOM (NOM and AHA, respectively) at 20, 40, or $80 \, mg\, C\, l^{-1}$ or high concentrations of Ca^{2+} (100, 200, or $400 \mu mol\, l^{-1}$) and subsequently exposed to Cu ($600 \mu g\, l^{-1}$) in soft water ($10 \mu mol\, l^{-1}$ Ca^{2+}) for 3 h. Prior acclimation decreased Cu accumulation at the gills, even in the absence of the organic ligands (NOM) or the competing cations (Ca^{2+}) in the water. The net Cu accumulated at the gills represents the difference between the total Cu concentration following acute exposure to the metal and the background Cu concentration in each acclimation condition. Asterisks (*) indicate significant differences of the Cu accumulated at the gills relative to control for total or differences relative to background gill Cu ($\alpha < 0.05$).
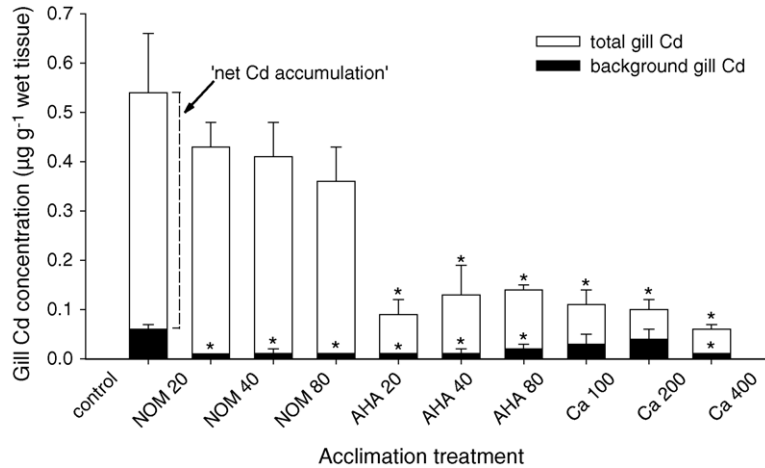
Fig. 5. Gill Cd accumulation (mean ± S.E.M., $N = 6$) in tambaqui. Fish were previously acclimated for 10 days to either natural or commercial sources of DOM (NOM and AHA, respectively) at 20, 40, or 80 mg C l^{-1} or high concentrations of Ca^{2+} (100, 200, or 400 μmol l^{-1}) and subsequently exposed to Cd (200 μg l^{-1}) in soft water (10 μmol l^{-1} Ca^{2+}) for 3 h. Prior acclimation decreased Cd accumulation by the gills in most cases, even in the absence of the organic ligands (AHA) or the competing cations (Ca^{2+}) in the water. The net Cd accumulated at the gills represents the difference between the total Cd concentration following acute exposure to the metal and the background Cd concentration in each acclimation situation. Asterisks (*) indicate significant differences of the Cd accumulated at the gills relative to control or differences relative to background gill Cd ($\alpha < 0.05$).

Background Cu concentrations in the gills were also significantly lower upon acclimation to NOM (Fig. 4).

Prior acclimation to NOM at 20 mg C l^{-1} prevented gill Cu accumulation by 86% relative to the control group. Increased concentrations of NOM, however, did not result in further decreases of gill Cu accumulation relative to 20 mg C l^{-1} NOM.

Prior acclimation to commercial DOM (AHA) did not as effectively protect against Cu accumulation. At 20 mg C l^{-1} AHA, gill Cu accumulation was only 45% lower relative to control group.

Prior acclimation to a Ca^{2+} concentration 10× higher (100 μmol l^{-1} Ca^{2+}) than the control water resulted in 77% less Cu accumulated at the gills during acute exposure. Increasing the acclimation Ca^{2+} concentration from 100 to 200–400 μmol l^{-1} did not result in a further decrease of Cu binding to tambaqui gills.

For the Cd series (Fig. 5), control fish, acclimated to soft water alone (10 μmol l^{-1} Ca^{2+}), had an eight-fold increase in gill Cd concentration following 3 h exposure to 200 μg l^{-1} of Cd.

Prior acclimation to NOM did not effectively prevent Cd from accumulating in the gills of tambaqui, but it did reduce background gill Cd concentrations. AHA from 20 to 80 mg C l^{-1} decreased acute Cd accumula-

tion in the gills by an average of 77% relative to the control group.

Prior acclimation to high Ca^{2+} concentrations (10–40× greater than the control) had a marked protective effect against acute Cd accumulation in the gills of tambaqui. Prior acclimation to 100 μmol l^{-1} Ca^{2+} prevented Cd accumulation in the gills by 83%, whereas 200 and 400 μmol l^{-1} Ca^{2+} resulted in a similar blockade of 87% and 90% less Cd accumulated at these sites, respectively.

4. Discussion

4.1. Effects of Cu on Na$^+$ fluxes

Inhibition of Na$^+$ influx in freshwater fish is a typical response to waterborne Cu (Laurén and McDonald, 1985; Pelgrom et al., 1995). Such inhibition is believed to occur as a result of interactions between Cu and the sulfhydryl groups present in the basolateral Na$^+$K$^+$ATPase, consequently blocking the apical entry of Na$^+$ (Playle et al., 1992; Wood, 2001). Tambaqui had a very high tolerance to Cu toxicity in soft water. There was no inhibition of Na$^+$ influx, even at Cu con-

centrations as high as 400 μg l^{-1} (Fig. 1). The effects of Cu on Na$^+$ fluxes in tambaqui were limited to stimulation of Na$^+$ losses, which tended to decrease over 6 h (Fig. 1). This effect is believed to result from a Cu-induced opening (and subsequent gradual closure) of the paracellular diffusion pathway (Laurén and McDonald, 1985; Wood, 2001). Long-term control of Na$^+$ losses plays an important role in minimizing the disruption of Na$^+$ homeostasis induced by Cu (Laurén and McDonald, 1985, 1987).

The high insensitivity of branchial Na$^+$ uptake in Amazonian species has also been documented in a number of other studies focused on acid tolerance. Amazonian fish, particularly those living in naturally acidic waters of the Rio Negro (pH 3.0–6.5, see review by Matsuo and Val, 2003), are extremely tolerant to low pH. Tambaqui withstand pH levels around 3.5 with only modest disturbance of Na$^+$ balance (Wood et al., 1998; Wilson et al., 1999). Because the mechanism of both Cu and H$^+$ toxicity in fish appears to be inhibition of Na$^+$ transport (McDonald, 1983; Laurén and McDonald, 1985; Wilson and Taylor, 1993), it is not surprising that Na$^+$ influx in tambaqui was insensitive to Cu. The lack of inhibition of Na$^+$ influx in tambaqui exposed to high Cu concentrations (400 μg l^{-1}) supports the hypothesis that Na$^+$ transport in some Amazonian fish occurs through a unique mechanism that differs from those currently proposed for other freshwater species (Gonzalez and Preest, 1999). Recently, Preest et al. (2005) reported that Na$^+$ uptake in neon tetras (*Paracheirodon innesi*), another teleost native to the acidic, dilute waters of the Amazon, was completely insensitive to a variety of pharmacological drugs known to effectively block pathways of branchial Na$^+$ uptake. Inhibition of Na$^+$ uptake (by 95%) in neon tetras was reported only upon exposure to low levels of silver (6.5 μg l^{-1} Ag), a metal that is believed to have a similar mechanism of toxicity as Cu and H$^+$ (Wood, 2001).

Inhibition of Na$^+$ influx resulting from Cu exposure in tambaqui may never occur at all, or if it does, the exposure to the metal would have to be much higher than the concentrations we tested. Oliveira (2003) found a 96 h-LC$_{50}$ value of Cu in tambaqui of 735 μg l^{-1} using the same soft water we used in our experiments. This value is almost double the concentration of the highest Cu level we tested for the effects on Na$^+$ fluxes.

Our data suggest that Cu toxicity in tambaqui occurs through large Na$^+$ losses due to increased gill membrane permeability at increased Cu concentrations (Fig. 1). The elevated branchial Na$^+$ efflux in fish exposed to high Cu concentrations may be explained by an out-competition of Cu in relation to Ca^{2+} at branchial sites that control gill permeability (Pagenkopf, 1983; Playle et al., 1993a). Increased Cu concentrations promote the displacement of Ca^{2+} at these sites, thus resulting in increased permeability and ion 'leakage' (Laurén and McDonald, 1985). Even still, tambaqui were better than trout at resisting and controlling the diffusive Na$^+$ loss (Matsuo et al., 2004). This may reflect adaptation of the tambaqui to extremely soft waters, possibly involving a high affinity of the gills for Ca^{2+} (Wood et al., 1998).

4.2. Effects of Cu and DOM on Na$^+$ fluxes

DOM decreases the toxicity of Cu to fish by decreasing the number of free Cu ions through complexation, thus decreasing the availability of the metal to interact at the gills (Playle et al., 1993a; Hollis et al., 1997). DOM was protective at 40 mg C l^{-1} over the entire range of Cu concentrations tested (Fig. 2d). The absence of a significant diffusive Na$^+$ efflux in fish exposed to Cu at such a high concentration of DOM suggests that most Cu was indeed complexed to DOM. The small inhibition of Na$^+$ influx relative to controls at 200–400 μg l^{-1} of Cu occurred only in the presence of high [DOM] and was probably a feedback response of fish to adjust Na$^+$ homeostasis instead of an inhibitory effect induced by Cu (Fig. 2d).

Lower concentrations of DOM (5–10 mg C l^{-1}) did not completely protect tambaqui against initial (0–3 h) Na$^+$ loss induced by Cu (Fig. 2a and b). This was probably because the Cu complexation capacity of DOM had been exceeded (Matsuo et al., 2004) and there was still enough free Cu in the water to induce diffusive Na$^+$ efflux in the fish. Regardless, tambaqui restored Na$^+$ homeostasis by 6 h in virtually all cases.

When tambaqui did not appear to control Na$^+$ losses at higher Cu concentrations and low DOM in the initial 3 h, they stimulated Na$^+$ influx to help recover from the losses and restore net fluxes to control values by 6 h (Fig. 2a and b). Rainbow trout had a similar response when exposed to 300 μg l^{-1} Cu and

7 mg C l^{-1} commercial DOM (Matsuo et al., 2004). Although Matsuo et al. (2004) attributed the stimulatory effect of Na$^+$ influx in rainbow trout to the presence of DOM in the water, the same may not necessarily apply for tambaqui, which were able to stimulate Na$^+$ influx even in the absence of DOM in the water (Fig. 1).

Acute exposure to DOM alone induced higher Na$^+$ turnover rates in tambaqui, as indicated by a stimulation of both the efflux and influx of Na$^+$, particularly at 20 and 40 mg C l^{-1} (Figs. 1 and 2, at 0 µg l^{-1} Cu). This effect could be attributed either to increased gill membrane permeability as a result of the surfactant nature of DOM (Vigneault et al., 2000), or to the removal of Ca^{2+} by DOM from the paracellular tight junctions upon complexation, as previously discussed by Matsuo et al. (2004). The stimulation of Na$^+$ influx upon acute exposure to DOM appears to be a physiological response of the fish to counterbalance these losses and restore homeostatic control.

4.3. Effects of Cd on whole body Ca²⁺ uptake

The large inhibition of Ca^{2+} uptake found in tambaqui exposed to waterborne Cd suggests that the species is highly susceptible to the toxicity of this metal in soft water (Fig. 3). Even still, the susceptibility seen in tambaqui is less severe than that of the rainbow trout, in which the 96 h-LC$_{50}$ value of waterborne Cd is only 2 µg l^{-1} when tested in soft water of 130 µmol l^{-1} Ca^{2+} (Hollis et al., 2000). Although we do not have data on the 96 h-LC$_{50}$ for Cd in tambaqui, we can safely say that the relative tolerance of tambaqui to Cd is greater than in trout, because we recorded no mortality during exposure to Cd, even at a Ca^{2+} concentration in the water of only 10 µmol l^{-1}.

The mechanism of Cd toxicity in tambaqui is presumably the same as that reported for other freshwater fish, in which Cd enters through the apical Ca^{2+} channels and inhibits the basolateral Ca^{2+}ATPase of the mitochondria-rich "chloride" cells (Verbost et al., 1987, 1989). In our study, Cd significantly inhibited whole body Ca^{2+} uptake in tambaqui within 3 h of exposure to waterborne Cd concentrations between 10 and 80 µg l^{-1}. Moreover, such inhibition increased gradually over time, which is in agreement with the time-dependent relationship described by Verbost et al. (1989).

Tambaqui did not show signs of recovery of whole body Ca^{2+} uptake within 24 h of waterborne Cd exposure (Fig. 3). The degree of Ca^{2+} uptake inhibition seen in tambaqui is likely to result in hypocalcemia similar to that reported for rainbow trout (Verbost et al., 1989).

In extremely soft water environments, such as those found in the Amazon basin (Furch, 1984; Walker, 1995), waterborne Cd contamination represents a major threat to fish diversity (Oliveira, 2003), because of the limited amount of Ca^{2+} in the water to outcompete Cd for binding sites on the gills (Playle et al., 1992, 1993a,b). Moreover, the environmental effects of soft water on gill morphology may also contribute to increased susceptibility of tambaqui to Cd toxicity. Proliferation of chloride cells is one of the most common responses of fish to soft water (see review by Perry, 1997). Zia and McDonald (1994) found that acclimation of rainbow trout to soft water (50 µmol l^{-1} Ca^{2+}) significantly increased branchial Cd uptake by 2–3-fold relative to hard water (1000 µmol l^{-1} Ca^{2+}) acclimated fish. This increase was directly correlated with a 2.6-fold increase in the chloride cell surface area in soft water-acclimated fish compared to hard water-acclimated fish. If the proliferation of chloride cells also occurs in tambaqui acclimated to soft waters, it would be a major contributor to the susceptibility of the species to Cd (Fig. 3). Data on gill morphology for tambaqui, however, are not currently available.

4.4. Effects of prior acclimation to DOM or Ca²⁺ on gill metal accumulation

Acclimation to high Ca^{2+} or DOM concentrations in the water generally decreased the accumulation of Cu or Cd at the gills in tambaqui, even when these components were not present in the testing water during exposure to the metals (Figs. 4 and 5). The amount of metal that accumulates on the gills during short-term acute exposure is believed to reflect the free metal concentration in the water after integration of effects resulting from both complexation and competition with other ligands (Playle et al., 1993a,b). If metal accumulation on the gills was dependent on these phenomena alone, the removal of the complexing agents (DOM) or the competing cations (Ca^{2+}) from the acute testing solution would eliminate these geochemical effects, thereby removing their protective action against metal accumulation at fish gills. Our results indicated, how-

ever, that upon acclimation to high Ca^{2+} or high DOM and subsequent 3 h acute exposure to a metal in very soft water, tambaqui still retained substantial protective effects of the prior acclimation against metal deposition on the gills (Fig. 4). Cu and Cd concentrations in tambaqui gills (background and accumulated metal) were much greater than values reported for trout (e.g., Hollis et al., 1999; Taylor et al., 2000) and fathead minnows (*Pimephales promelas*; Playle et al., 1992, 1993a,b) also suggesting that tambaqui has a higher number of sites to which Cu or Cd can bind.

Tambaqui previously acclimated to high concentrations of Ca^{2+} were able to prevent both Cu and Cd accumulation by, on average, 77% and 86%, respectively (Figs. 4 and 5). Our results contrast with the findings for rainbow trout acclimated to hard water and subsequently tested in soft water. Trout acclimated to hard water and acutely transferred to soft water for a 3 h Cu exposure accumulated 3× more Cu than fish acclimated in soft water for 12 weeks and acutely transferred to soft water for a 3 h Cu exposure (Taylor et al., 2002). Calamari et al. (1980) found a similar effect for Cd toxicity when trout acclimated to hard water and subsequently tested in soft water had a 48 h-LC$_{50}$ about 7× higher than that of trout acclimated and tested in the same soft water. The high susceptibility of trout to ionoregulatory distress in soft water (e.g., Laurén and McDonald, 1985; McDonald and Rogano, 1986) compared to the high adaptability of tambaqui to ion-poor environments (e.g., Wood et al., 1998; Wilson et al., 1999) helps explain the differences found between the two species. Regardless, full acclimation to water Ca^{2+} concentration prior to metal toxicity tests appears to be fundamental for toxicity tests because of an apparent conditioning of the gills that interferes with metal accumulation. Taylor et al. (2000) suggested that acclimation of fish to water Ca^{2+} regulates both the number of binding sites and its affinity for metals at the gills.

Tambaqui accumulated significantly less Cu following previous acclimation in NOM and significantly less Cd following acclimation to AHA (Figs. 4 and 5), suggesting that acclimation to DOM concentrations in the water is likely to result in changes in gill binding properties much in the same way as acclimation to water Ca^{2+} concentrations. The source of DOM may be important in modulating this branchial conditioning for metal binding, and it may also differ in response to the

metal type (Cu or Cd). Prior acclimation of tambaqui to NOM was more effective in preventing Cu accumulation at the gills relative to AHA. Moreover, prior acclimation to NOM at 20, 40, and 80 mg C l^{-1} prevented gill Cu accumulation by 86%, 73%, and 81%, respectively. This indicates that increasing concentrations of NOM over 20 mg C l^{-1} does not improve the protective effect or does not change gill conditioning to prevent any further Cu accumulation. By contrast, prior acclimation to AHA was more efficient in preventing gill Cd accumulation in tambaqui than NOM, although again, increased concentrations of AHA (>20 mg C l^{-1}) did not further improve the ability of the gills to accumulate less metal. Pre-acclimation to AHA at 20, 40, and 80 mg C l^{-1} prevented gill Cd accumulation by 82%, 75%, and 75%, respectively.

Our studies on the short-term accumulation of metals in the gills of tambaqui were limited by the fact that we did not perform an additional series involving metal exposure in previously acclimated fish with either the competing cation (Ca^{2+}) or the complexing agent (DOM) present in the testing solution. Despite the geochemically protective role of Ca^{2+} and DOM on metal toxicity in fish, it is becoming increasingly clear that, in addition to Ca^{2+}, DOM also has biological roles that influence metal accumulation at the gills. Therefore, surface binding of metals on physiological active sites in fish is not only a function of metal complexation and competitive interactions between cations, but it is also a function of the biological component, the gills. Moreover, acclimation to DOM significantly reduced background gill metal concentrations.

5. Conclusions

Tambaqui have a very high tolerance to high concentrations of Cu, which is demonstrated by the insensitivity of active Na^+ influx to waterborne Cu and by the ability to recover from diffusive Na^+ losses. Moreover, a complete lack of inhibition of Na^+ influx in the species upon acute exposure to Cu also supports the idea of a unique Na^+ transporting system. Waterborne Cd toxicity can be a major threat to tambaqui living in soft waters because of the low availability of Ca^{2+} to protect against Cd toxicity. Prior acclimation of tambaqui to either high Ca^{2+} or DOM concentrations in the water generally resulted in protection against gill

metal accumulation, which is likely attributed to the biological effects exerted by these components on the gills themselves.

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