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Physiological action of dissolved organic matter in rainbow trout in the presence and absence of copper: Sodium uptake kinetics and unidirectional flux rates in hard and softwater

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

[a] *Laboratory of Ecophysiology and Molecular Evolution, Instituto Nacional de Pesquisas da Amazônia (INPA), Alameda Cosme Ferreira, 1756 – Aleixo, 69083-000, Manaus, AM, Brazil*

[b] *Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, Canada L8S 4K1*

[c] *Department of Biology, Wilfrid Laurier University, Waterloo, ON, Canada N2L 3C5*

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Abstract

We investigated the physiological effects of dissolved organic matter (DOM) on sodium (Na^+) transport in juvenile *Oncorhynchus mykiss* (~2.5 g) in the presence and absence of simultaneous acute exposure to copper (Cu^{2+}; 0, 70, and 300 $\mu g\,l^{-1}$). Trout were acclimated in either hardwater (~1000 μM Ca^{2+}) or softwater (~100 μM Ca^{2+}), and DOM was tested at approximately 8 mg C l^{-1} using a natural (NOM) and a commercial (AHA) source. Ion transport was evaluated based on kinetics estimates (maximum Na^+ uptake rates, J_{max}; substrate affinity, K_m) and unidirectional flux measurements (J_{in}, J_{out}, J_{net}). J_{max} was higher and unidirectional flux rates were greater in softwater-acclimated trout. Fish exposed to DOM alone in hardwater exhibited an increased Na^+ transport capacity indicated by both the kinetics (67% higher J_{max} for AHA) and J_{in} measurements (153% higher for AHA and 125% higher for NOM). In softwater, the effects of DOM alone on kinetic parameters and unidirectional flux rates were negligible. Cu^{2+} affected Na^+ uptake by a mixed-type inhibition (both non-competitive and competitive). In hardwater, only K_m was increased (i.e., affinity decreased), whereas in softwater, K_m was increased and J_{max} was decreased, with more marked effects at the higher Cu^{2+} level. In hardwater, the stimulatory effect of AHA on J_{max} persisted even in the presence of 300 $\mu g\,l^{-1}$ Cu^{2+}, whereas both AHA and NOM prevented the increase in K_m caused by Cu^{2+}; these effects were reflected in J_{in} measurements. In softwater, AHA helped to protect against the increased K_m caused by high Cu^{2+}, but there was no protection against the inhibition of J_{max}. Unidirectional flux measurements indicated that in softwater, Cu^{2+} inhibited J_{in} at 70 $\mu g\,l^{-1}$, whereas at 300 $\mu g\,l^{-1}$ Cu^{2+}, J_{out} was also stimulated. Fish were more affected by Cu^{2+} in softwater, as indicated by the inability to control diffusive losses of Na^+ and a reduced ability to take up Na^+, but in the presence of DOM, losses were

* Corresponding author. Tel.: +55 92 643 3187; fax: +55 92 643 3186.
E-mail address: matsuoaline@aol.com (A.Y.O. Matsuo).

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better controlled at the end of 6 h exposure. We conclude that DOM has direct effects on the gills, as well as protecting fish against acute Cu^{2+} toxicity. This occurs because DOM complexes Cu^{2+}, and because it acts on the transport and permeability properties of the gills. These effects differ depending on both water hardness and the nature of the DOM source.

Keywords: Dissolved organic matter; Copper; Fish; Toxicity; Na^+ transport

1. Introduction

Dissolved organic matter (DOM) includes various organic compounds that have both hydrophilic and hydrophobic moieties, in addition to a number of acidic functional sites such as carboxylic and hydroxyl-phenolic groups (Thurman, 1985). Aquatic DOM either originates from biological processes in the water column in situ, or is a result of the input from soil and sediment through leaching (Thurman, 1985; Hessen and Tranvik, 1998). The DOM fraction of total organic matter is represented by organic compounds that have a molecular weight ranging from 1 to 100 kDa. This 'dissolved' fraction passes through a 0.45 μm porous filter, which provides an operational definition (Thurman, 1985). These compounds are important in aquatic toxicology because they bind metals, altering metal speciation in natural waters (e.g., Cabaniss and Shuman, 1988; Hering and Morel, 1988; Playle et al., 1993b). DOM has a very high ion-exchange capacity that has been suggested to result primarily from ionized carboxylic and hydroxyl-phenolic groups (Perdue, 1998). It has also been shown that DOM can bind to the surface of living cells such as phytoplankton and isolated cells from fish gills at low pH (Campbell et al., 1997). These biotic interactions are apparently related more to the hydrophobic and hydrophilic properties of DOM than to the carboxylic and hydroxyl-phenolic groups. The biological interactions of DOM may potentially lead to changes in the gill microenvironment as well as alter the physiological function of the gills (e.g., ion transport and gas exchange). Only very few studies of DOM acting at the fish gill level have been conducted (Campbell et al., 1997; Richards et al., 1999; Wood et al., 2003), and the potential physiological effects of DOM have been largely overlooked.

Copper occurs in the aquatic environment as a result of the natural geochemistry and through anthropogenic action. Although it is an essential metal for metabolic processes, copper can be acutely toxic to fish in concentrations varying from 10 to 1000 $\mu g\,l^{-1}$ (Spear and Pierce, 1979). The wide range of concentrations over which copper is toxic varies based on the physicochemistry of the water and the chemical speciation of the metal (Pagenkopf, 1983; Santore et al., 2001), as well as the differential tolerance of various fish species (USEPA, 1985). Despite its presence in a number of chemical species and complexes in freshwater, copper toxicity to fish is primarily related to the free-ion form as the divalent cation (Cu^{2+}) rather than the total copper concentration (Morel, 1983; Campbell, 1995).

Fish gills represent a major target site for the toxic action of waterborne metals such as Cu^{2+} (Pagenkopf, 1983; Laurén, 1991) because of the large area in relation to the body (Hughes, 1984) and the critical ionoregulatory functions (Wood, 2001). The mechanism of Cu^{2+} toxicity in freshwater fish involves ionoregulatory disturbances at the gills, specifically disruption of the active uptake mechanisms for Na^+ and Cl^-. At higher Cu^{2+} concentrations, an increase of gill permeability may also occur, which culminates in net ion loss that can lead to death (Laurén and McDonald, 1985, 1986). The active transport sites of the gills play an important role in the Biotic Ligand Model (BLM). The BLM proposes that the toxicity of a given metal results from the action of the freely dissolved metals on these and other sites, in competition for cations which will also bind to those same sites (Di Toro et al., 2001; Paquin et al., 2002). The BLM incorporates the factors affecting the bioavailability of the metal, such as natural organic matter and water hardness, and metal toxicity to aquatic organisms based on the metal's mechanism of action (Paquin et al., 2000; Di Toro et al., 2001).

It is well documented that Cu^{2+} toxicity decreases by an increase in Ca^{2+} concentration in the water (e.g., Spry and Wiener, 1991). Calcium binds to the gill surface and controls the permeability of the membrane and the integrity of the ionoregulatory function (Hunn, 1985). Higher levels of Ca^{2+} (~1000 μM) helped de-

crease the diffusive losses of Na^+ in rainbow trout (*Oncorhynchus mykiss*) exposed to 200 μg l^{-1} Cu^{2+} (Laurén and McDonald, 1985). This is explained by a competition between the hardness metals (mainly Ca^{2+}) and the toxic species for interaction sites at the gills (Pagenkopf, 1983; Playle et al., 1993b). DOM also exerts protective effects on fish, but by a different strategy. DOM forms complexes with Cu^{2+}, which reduces the free form in the water, and therefore the amount of ionic Cu^{2+} available to bind to the gill sites (Stumm and Morgan, 1981; Morel, 1983; Pagenkopf, 1983; Playle et al., 1992; Richards et al., 1999).

A number of studies have examined the influence of water Ca^{2+} and the effects of DOM as modifiers of Cu^{2+} toxicity in freshwater fish (Laurén and Mc Donald, 1985, 1986; Playle et al., 1993a; Hollis et al., 1997; McGeer et al., 2002). However, biological effects of DOM alone in fish are poorly understood and only limited data have dealt with physiological aspects (e.g., Richards et al., 1999; Wood et al., 2003). Apparently, adsorption of DOM on biological membranes is a general process (Vigneault et al., 2000), so the gills of fish may well be primary target sites for the physiological action of these organic compounds. DOM is also likely to be involved in the control of membrane permeability, thereby influencing ion losses at low pH (Kullberg et al., 1993; Wood et al., 2003). We investigated the role of DOM alone and in combination with Cu^{2+} at the fish gills by using measurements of Na^+ uptake kinetics and unidirectional flux rates as sensitive indicators of the initial physiological effects of agents that alter the ionoregulatory functions of the gill (Wood, 1992). Copper effects on Na^+ transport have been particularly well documented in fish (e.g., Laurén and Mc Donald, 1985, 1986, 1987a, b). We evaluated the effects of DOM by using both natural and commercial sources. The same protocols were used for trout acclimated to either hard or softwater to study possible differences in the responses based on Ca^{2+} concentration.

2. Material and methods

2.1. Experimental animals

Rainbow trout (2.5 ± 0.1 g) obtained from Humber Springs Trout Farm (Orangeville, ON) were acclimated to laboratory conditions for at least 14 days in a flow-through system in 500 l polyethylene tanks. The water supply consisted of dechlorinated tap water from the City of Hamilton originating from Lake Ontario ($[Na^+]$ = 600 μM; $[Cl^-]$ = 700 μM; $[K^+]$ = 50 μM; $[Ca^{2+}]$ = 1000 μM; $[Mg^{2+}]$ = 150 μM; dissolved organic carbon (DOC) = 3 mg C l^{-1}; HCO_3^- = 1.5–2.0 mM; background Cu^{2+} in the water = 3 μg l^{-1}; pH 7.7–7.9; temperature 12 ± 1 °C). Water flow rate in the tanks was kept at approximately 500 ml min^{-1}. Fish were fed dry food pellets (Martin Feed Mills, Elmira, ON) at an average of 3% body weight per day. Following initial acclimation, fish were randomly assigned to two groups for acclimation to hard and softwater conditions.

2.2. Acclimation

Hardwater acclimation used the same ion concentrations as the dechlorinated tap water cited above. The fish were ready to be used in the hardwater experimental series after an additional 14 days under these conditions. The Ca^{2+} and Na^+ concentrations during acclimation in the hardwater series were, respectively, 1082 ± 26 and 574 ± 18 μM.

Softwater acclimation involved step-wise exposure to lower ion concentrations. During this process, the flow rate of dechlorinated tap water was gradually reduced in the tank every two days by increasing the flow rate of reconstituted softwater produced using a reverse osmosis system (Anderson Water Systems, Dundas, ON). After the water in the tank had reached the desired concentrations for Na^+ and Ca^{2+} (approximately 68 ± 12 μM $[Na^+]$ and 92 ± 12 μM $[Ca^{2+}]$), fish were kept in these conditions in a flow-through system for 21 days to ensure complete acclimation before the experiments began.

Fish were fed commercial dry food pellets once a day. Ration quantity was kept the same for both hard and softwater-acclimated fish, and feeding was suspended 48 h before beginning the experiments. Both hard and softwater tanks were checked daily for fish mortality and siphoned every other day to avoid residue accumulation in the water. Mortality rates were <1% during acclimation.

2.3. Experimental series

To assess the effects of DOM on Na^+ transport at the gills, all kinetics and unidirectional flux measure-

ments were performed on fish acclimated in both hard and softwater. Experiments were conducted in the presence or absence of nominal 8 mg C l^{-1} of DOM, and under exposure to nominal 0, 70, and 300 μg l^{-1} of Cu^{2+} (as $CuNO_3$, Reference Standard Solution; Fisher Scientific). The copper levels used in this study (70 and 300 μg l^{-1}) were in the range routinely reported in surface waters in the United States (ICA, 2003), and are therefore considered environmentally relevant for physiological studies. Even higher copper concentrations (over 1000 μg l^{-1}) have also been reported in Amazonian surface waters associated with industrial activity (e.g., Sampaio, 2000; Dias, 2001), which still support many fish species.

Two sources of DOM were tested: natural organic matter (NOM) isolated from Luther Marsh (43°57′N, 80°26′W), near Guelph, ON, using a reverse osmosis system (Freshwater Analysis Concentrator, Enviro-Main Filter, Kelowna, BC), and Aldrich humic acid (AHA) purchased from a commercial source (Sigma-Aldrich, St. Louis, MO). Equilibration time for the experimental solutions in the experimental chambers before fish exposure was between 10 and 30 min. Measured concentration of DOM in the experiments averaged 7.5 ± 0.2 mg C l^{-1} for NOM, and 7.9 ± 1.2 mg C l^{-1} for the AHA series.

2.3.1. Na^+ uptake kinetics

For the hardwater experimental series, a NaCl-free solution with average Ca^{2+} and Mg^{2+} concentrations similar to those in the hardwater holding tanks (1000 and 150 μM, respectively) was prepared 2 days before experimentation. The experimental solution was made by adding salts (1.05 mM $CaCO_3$ and 0.15 mM $MgCO_3{\cdot}Mg(OH)_2{\cdot}4H_2O$) to deionized water (∼18 mΩ; Nanopure II, Sybron/Barnstead, Boston, MA). The solution was then bubbled for 12 h with 100% industrial grade CO_2 using a large air-stone to allow the salts to dissolve completely. Finally, the solution was aerated for 6 h to remove excess CO_2 so that pH was returned to circumneutral values (cf. Goss and Wood, 1990). In the softwater kinetics series, experimental solutions were based on the same water used in the softwater holding tanks, with background Na^+ levels of about 70 μM and background Ca^{2+} levels of about 100 μM.

All kinetic studies were run in darkened, aerated polyethylene chambers. Uptake rates (i.e., unidirectional influx rates) were determined based on the amount of ^{22}Na isotope incorporated by the fish during a 2 h-period using terminal analysis. Fish were individually rinsed in distilled water for 30 s before they were transferred to the experimental chambers. To measure Na^+ uptake kinetics, trout were exposed to experimental water in which different Na^+ concentrations were added (as NaCl) to reach approximately nominal concentrations of 50, 100, 200, 400, 800, and 1600 μM, as well as proportionally the same amounts of radiolabelled ^{22}Na (as $^{22}NaCl$, NEN Life Sciences Products, Boston, MA) to yield 0.5, 1, 2, 4, 8, and 16 μCi l^{-1}, respectively. Each fish yielded just one uptake rate measurement at one concentration, with $N = 7$ at each concentration. About 10 ml of water were sampled at the beginning and at the end of the kinetic experiments to measure both the initial and the final specific activity of the sample and the concentration of Na^+, Cu^{2+}, and DOM. Specific activity expresses the counts per minute (cpm) of ^{22}Na per μM of the total Na^+ in the sample. After 2 h of exposure, fish were cold-rinsed in 1 M NaCl to displace any ^{22}Na loosely bound to the surface, killed with an overdose of anesthetic (MS-222, Sigma-Aldrich), blotted dry on a paper towel, and weighed before being transferred to plastic vials for gamma counting.

2.3.2. Unidirectional Na^+ fluxes

Unidirectional Na^+ flux measurements (influx, J_{in}; efflux, J_{out}; net flux, J_{net}) were conducted in the hard or softwater to which the fish had been acclimated, i.e., to the same Na^+ concentrations. Experiments were performed under static conditions in covered, aerated polyethylene chambers, each holding a single fish and filled with 50 ml of the hard or softwater used for acclimation. Water volume in relation to fish mass was kept low to permit greater sensitivity and to detect minor changes in the total Na^+ concentration and radioactivity in the water during short-term exposure (cf. Wood, 1992). Temperature was maintained at 12 ± 1 °C by submersing the chambers in a cooled water bath. The same treatments involving Cu^{2+} and DOM exposure as used in the kinetics assessment were used for the flux measurements. Fish were rinsed in distilled water and transferred to the chambers and allowed to adjust to the container for 1 h before the addition of concentrated Cu^{2+}, DOM, or the combination of both. Equilibration time (DOC-Cu^{2+}) for these experiments were conse-

quently very low (<10 min). Radioisotope was added as 100 nCi l^{-1} of ^{22}Na for the hardwater series, and approximately 20 nCi l^{-1} for the softwater series to keep the specific activity in the water approximately the same in both series. A 10 ml water sample was collected from each chamber after a 5 min mixing period, representing the beginning of the flux period. Subsequent samples were taken after 3 and 6 h. Flux measurements were assessed based on the disappearance of ^{22}Na from the water into the fish, and differences in Na^+ concentrations in the water over time. The influx (J_{in}) was calculated as the incorporation of ^{22}Na by the fish, whereas net flux (J_{net}) was based on the differences in the cold Na^+ concentration in the water at each time interval. The efflux was calculated as the difference between J_{net} and J_{in} (see Section 2.5).

2.4. Analytical techniques

Water samples were acidified with 100 μl of concentrated HNO_3 (Trace Metal Grade, Fisher Scientific) for preservation before analysis. ^{22}Na counts in the water and the fish samples were measured using a gamma counter (Minaxi Auto-Gamma 5000 Series, Canberra-Packard, Meriden, CT). Na^+ concentration was analyzed using flame atomic absorption spectrophotometry (Varian AA- 220 FS, Mulgrave, Australia). Total copper was measured using graphite furnace atomic absorption spectrophotometry (Varian AA-220 GTA), based on Fisher Scientific certified standards, using 10 μl volume injection, nitrogen gas, and operating conditions recommended by the manufacturer. For DOM analysis, water samples were passed through 0.45 μm glass microfiber filters (GD/X Syringe Filter, Whatman). Syringe-filters were rinsed previously with 40 ml of deionized water to flush any organic carbon present in the membrane. DOM was measured as dissolved organic carbon (DOC) in a Total Organic Carbon Analyser (Shimadzu TOC – 5050A, Mandel Scientific, Guelph, ON). Hardwater samples were sparged with nitrogen gas for 10 min before analysis to reduce interference of inorganic carbon on the readings.

2.5. Calculations and statistical analysis

Na^+ uptake was calculated based on the amount of radioactive ^{22}Na incorporated by the fish during exposure based on the equation (Laurén and McDonald, 1987a):

$$J_{in} = \frac{WBA}{SA \cdot W \cdot T}$$

where WBA is the radioactivity in counts per min of the whole body (cpm), SA is the mean specific activity (cpm μM^{-1} Na^+) during the exposure, W the wet weight of the fish (g), and T the experimental time (h).

Maximum uptake rates (J_{max}) and substrate affinity (K_m) were estimated through Michaelis-Menten analysis based on the equation from Wood (1992):

$$J_{in} = \frac{J_{max} \cdot [Na^+]}{K_m + [Na^+]}$$

fitted by a non-linear curve-fitting program (SigmaPlot 2000).

Unidirectional fluxes of Na^+ were calculated based on the following formulas (Wood, 1992):

$$J_{net} = \frac{([ion_1] - [ion_2]) \cdot V}{W \cdot T}$$

$$J_{in} = \frac{([R_1] - [R_2]) \cdot V}{W \cdot T \cdot SA}$$

$$J_{out} = J_{net} - J_{in}$$

where ion_1 and ion_2 are the initial and final Na^+ concentration in the water (μM), V is the water volume in the experimental chamber (l), W the weight of the fish (g), and R_1 and R_2 are the cpm values of the ^{22}Na at the beginning and at the end of the flux period, respectively. SA is the mean specific activity of the isotope in cpm μM^{-1} during the flux time based on the relation:

$$SA = 0.5 \left(\frac{[R_1]}{[ion_1]} + \frac{[R_2]}{[ion_2]} \right)$$

Results are presented as mean ± 1 S.E.M. Data were analyzed in relation to their respective controls by unpaired, two-tailed Student's t-test for the kinetics of Na^+ transport, and by one-way ANOVA for the unidirectional flux measurements. When means were different, Dunnett's multiple-comparison tests were used to check the significance of the difference. Statistical significance was accepted at the level of $P < 0.05$.

3. Results

3.1. Hardwater series

Measured concentrations of DOM for control, NOM, and AHA treatments during the experiments were, respectively, 1.8 ± 0.3, 7.5 ± 0.2, and 7.1 ± 0.1 mg C l^{-1}. Cu^{2+} concentrations in the water for the control and exposed fish (nominal 0, 70, and 300 μg l^{-1}) were, respectively, 3.4 ± 0.3, 69.6 ± 3.4, and 298.8 ± 6.3 μg l^{-1}. Na^+ transport in the experiments exhibited saturation kinetics based on the Michaelis-Menten equation. J_{max} and K_m estimates for all series are shown in Table 1.

3.1.1. DOM effects

Of the two sources of DOM tested in rainbow trout, AHA showed significant differences for J_{max}, which increased by 65% relative to control values (Fig. 1a; Table 1). There was a slight decrease in K_m in the presence of both sources of DOM, but the differences were not statistically significant.

Unidirectional flux measurements indicated a significant initial (0–3 h) increase of J_{in}, J_{out}, and J_{net} in fish exposed to both AHA and NOM, but a subsequent adjustment of the fluxes to control values (Fig. 1b).

3.1.2. Cu^{2+} effects

Maximum transport rates (J_{max}) in fish exposed to 70 or 300 μg l^{-1} Cu^{2+} did not show differences in hardwater-acclimated fish (Fig. 2a; Table 1). Affinity for the Na^+ transporters was reduced (i.e., K_m was increased) by more than 130% when fish were exposed to 300 μg l^{-1} Cu^{2+} (Table 1), although such difference was not statistically different.

Unidirectional flux measurements demonstrated that trout exposed to 70 μg l^{-1} Cu^{2+} had an initial stimulation of J_{out} by more than 100%, but they were able to compensate after 3–6 h (Fig. 2b). Net losses (J_{net}) were significantly different in the presence of Cu^{2+}, particularly at 300 μg l^{-1} Cu^{2+}, in which fish did not seem to compensate for the loss. Influx (J_{in}) was reduced by 50%, and it did not increase in the following 3 h period.

3.1.3. Combined effects of DOM and Cu^{2+}

Kinetics data indicated that Na^+ transport in fish exposed to 70 μg l^{-1} Cu^{2+} was stimulated in the presence of AHA, manifested as a significant increase in J_{max} (Fig. 3a). K_m was not affected at this Cu^{2+} concentration, but at 300 μg l^{-1}, the decreased affinity of the Na^+ transporters caused by Cu^{2+} was prevented by both sources of DOM (Table 1). J_{max} was again higher at 300 μg l^{-1} Cu^{2+} in the presence of AHA (Fig. 4a; Table 1). These stimulatory effects on J_{max} were not seen with NOM.

Unidirectional flux measurements (Figs. 3 and 4 Figs. 3b and 4b) indicated that both types of NOM tended to reduce the inhibitory effects of Cu^{2+} on J_{in}. They also demonstrated that Na^+ losses during the initial 3 h period were higher in the presence of AHA but not NOM (Figs. 3 and 4 Figs. 3b and 4b).

3.2. Softwater series

Measured concentrations of DOM for control, NOM, and AHA treatments were, respectively, 1.7 ±

Table 1
Kinetic parameters for Na^+ uptake in rainbow trout acclimated to hardwater (1000 μM Ca^{2+}) and exposed to Cu^{2+} and/or DOM

Treatment	J_{max} (nmol g^{-1} h^{-1})	K_m (μM Na^+)	R^2
Control	605 ± 50	161 ± 44	0.91
NOM	564 ± 68	137 ± 60	0.77
AHA	1011 ± 33*	116 ± 15	0.98
70 μg l^{-1} Cu^{2+}	617 ± 19	130 ± 14	0.98
NOM + 70 μg l^{-1} Cu^{2+}	578 ± 26	103 ± 18	0.95
AHA + 70 μg l^{-1} Cu^{2+}	903 ± 31*	115 ± 15	0.97
300 μg l^{-1} Cu^{2+}	577 ± 70	366 ± 119	0.94
NOM + 300 μg l^{-1} Cu^{2+}	743 ± 94	170 ± 70	0.83
AHA + 300 μg l^{-1} Cu^{2+}	1035 ± 101*	185 ± 67	0.92

(*) indicate significant differences ($P < 0.05$) relative to the first treatment (relative control) in each of the three groups.

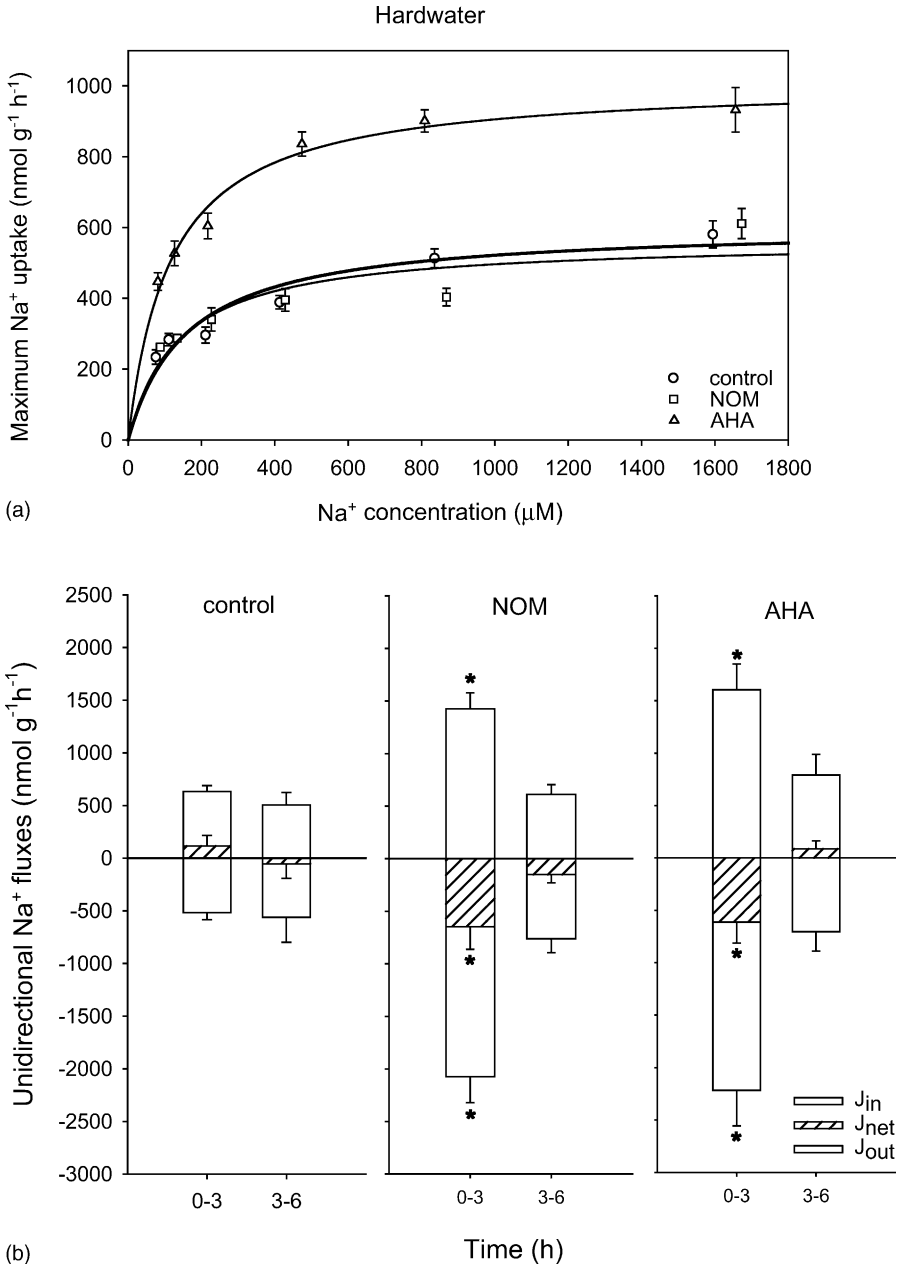

Fig. 1. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to 8 mg C l^{-1} of natural organic matter (NOM) and a commercially available source (AHA) in hardwater (control). Each point in the kinetic curves represents the mean ± 1 S.E.M. ($N = 7$). (*) indicate significant differences relative to the control group ($P < 0.05$). Bars represent mean ± 1 S.E.M. for influx (J_{in}), efflux (J_{out}), and net flux (J_{net}).

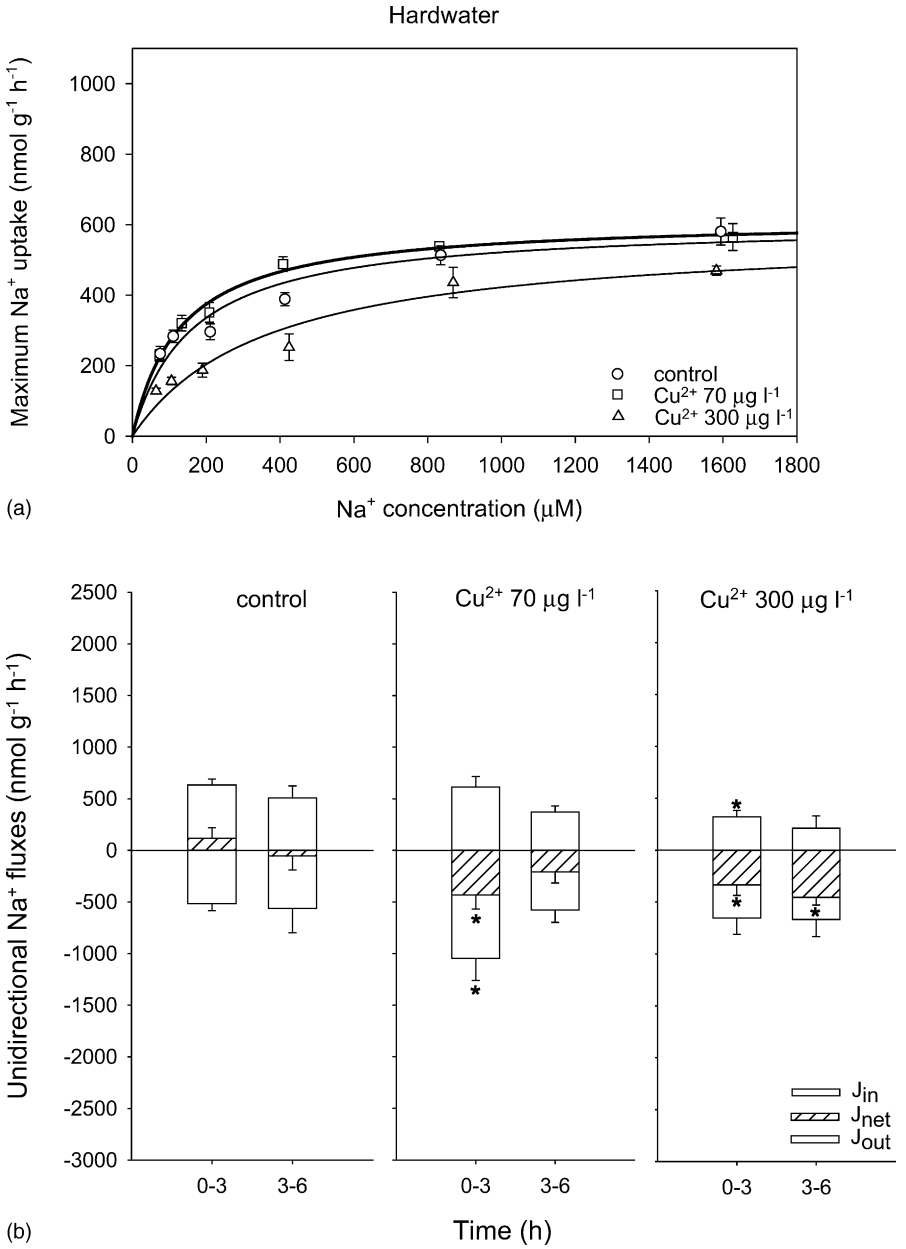

Fig. 2. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to nominal 0, 70, and 300 $\mu g\,l^{-1}$ Cu^{2+} in hardwater (1000 μM Ca^{2+}). Same format as Fig. 1.

0.1, 7.2 ± 0.3, and 7.9 ± 1.2 mg C l^{-1}. Cu^{2+} concentrations in the water for control and exposed fish (nominal 0, 70, and 300 $\mu g\,l^{-1}$) were, respectively, 1.4 ± 0.2, 65.7 ± 4.5, and 273.1 ± 5.9 $\mu g\,l^{-1}$. Na^+ uptake rates were higher on average in softwater compared to hardwater but again exhibited saturation kinetics based on the Michaelis-Menten equation. J_{max} and K_m estimates for all softwater series are shown in Table 2.

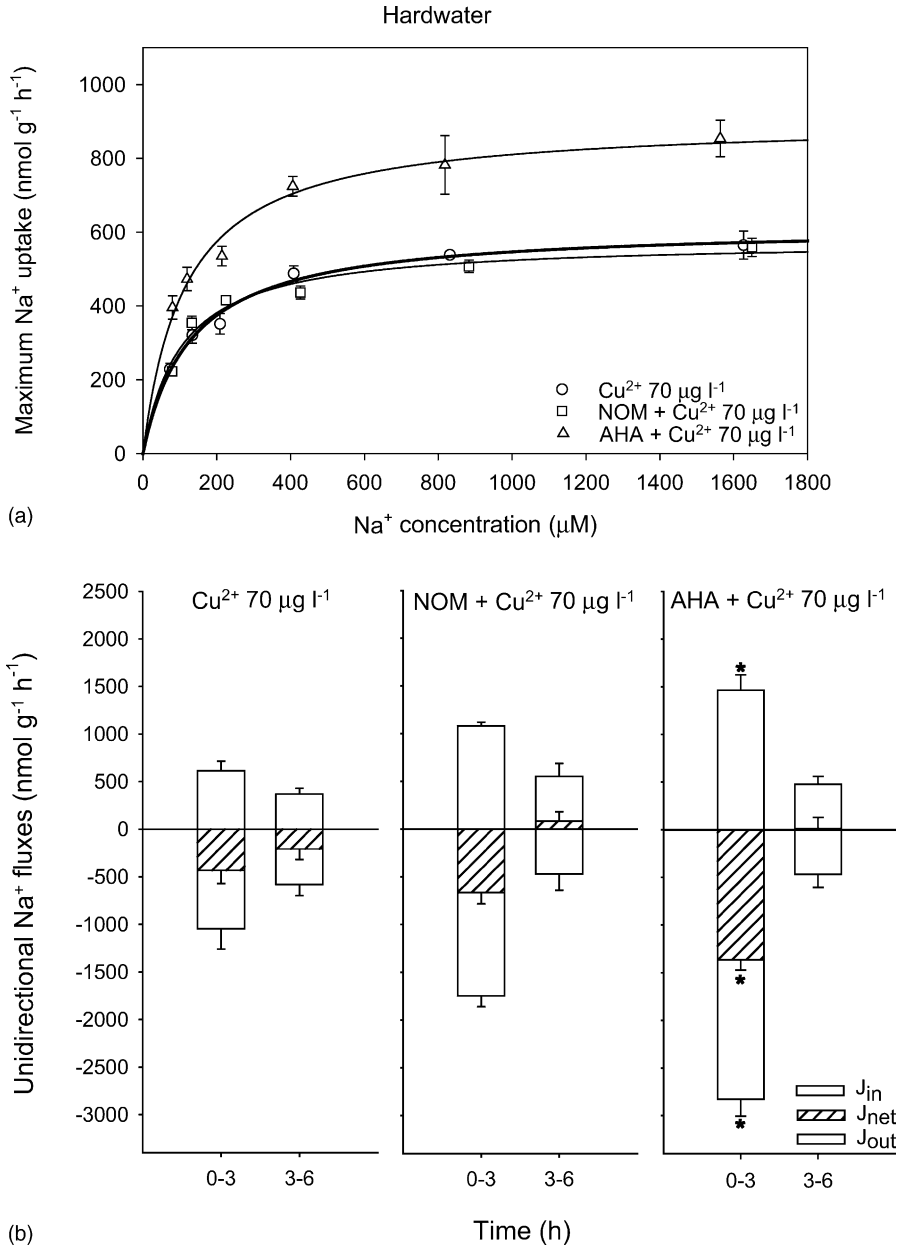

Fig. 3. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to nominal 70 μg l^{-1} Cu^{2+} combined with 8 mg C l^{-1} of natural organic matter (NOM) and a commercially available source (AHA) in hardwater (1000 μM Ca^{2+}). Each point in the kinetic curves represents the mean ± 1 S.E.M. (N = 7). (*) indicate significant differences relative to Cu^{2+} exposure alone (P < 0.05). Bars represent mean ± 1 S.E.M. for influx (J_{in}), efflux (J_{out}), and net flux (J_{net}).

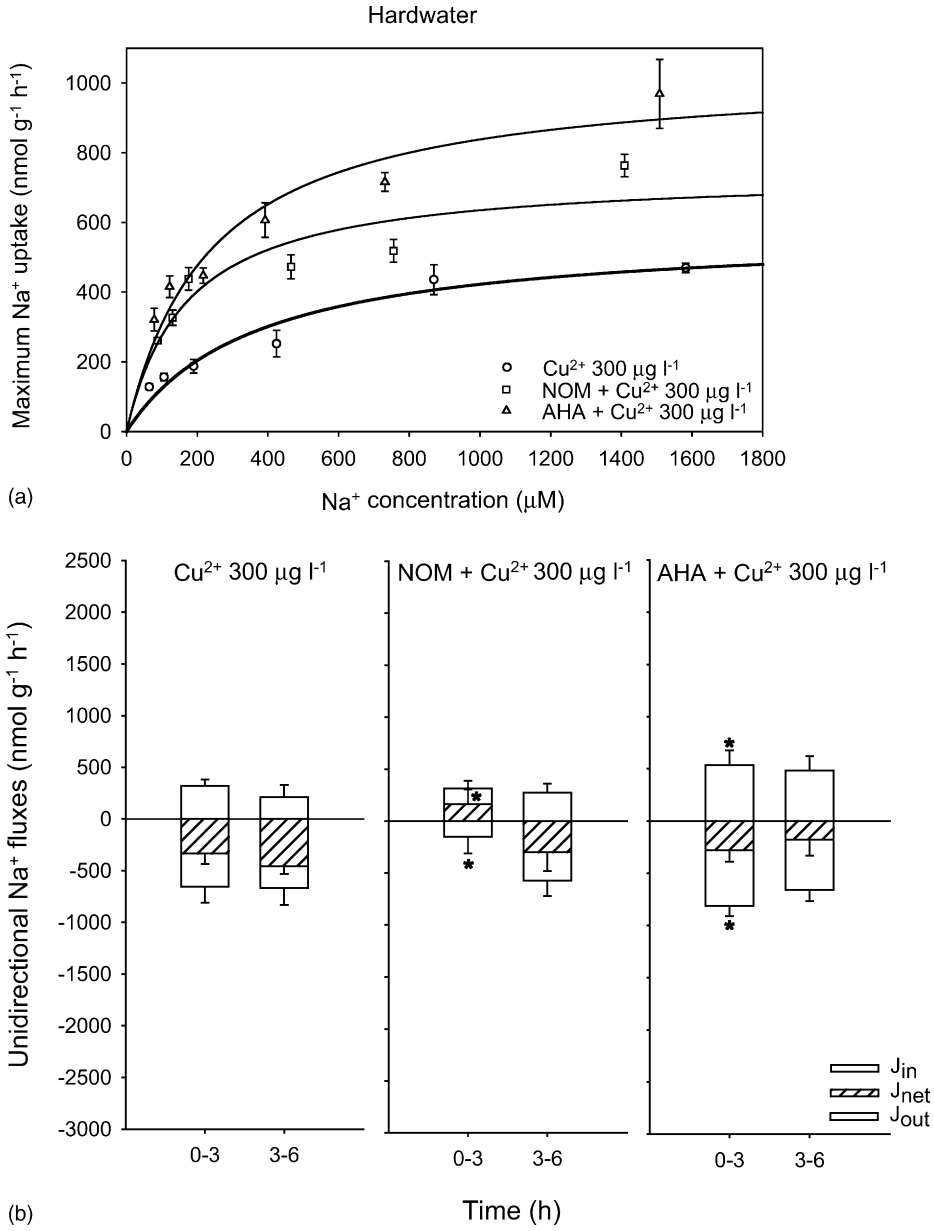

Fig. 4. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to nominal 300 μg l^{-1} Cu^{2+} combined with 8 mg C l^{-1} of natural organic matter (NOM) and a commercially available source (AHA) in hardwater (1000 μM Ca^{2+}). Same format as Fig. 3.

3.2.1. DOM effects

There was no effect of either NOM or AHA on Na^+ uptake kinetics in softwater-acclimated fish (Fig. 5). Affinity for Na^+ transporters did not change in the presence of DOM (Table 2; Fig. 5a) and J_{max} did not differ significantly among the treatments.

Unidirectional flux measurements in fish exposed to either NOM or AHA revealed a similar pattern (Fig. 5b). Diffusive losses (J_{out}) were high throughout

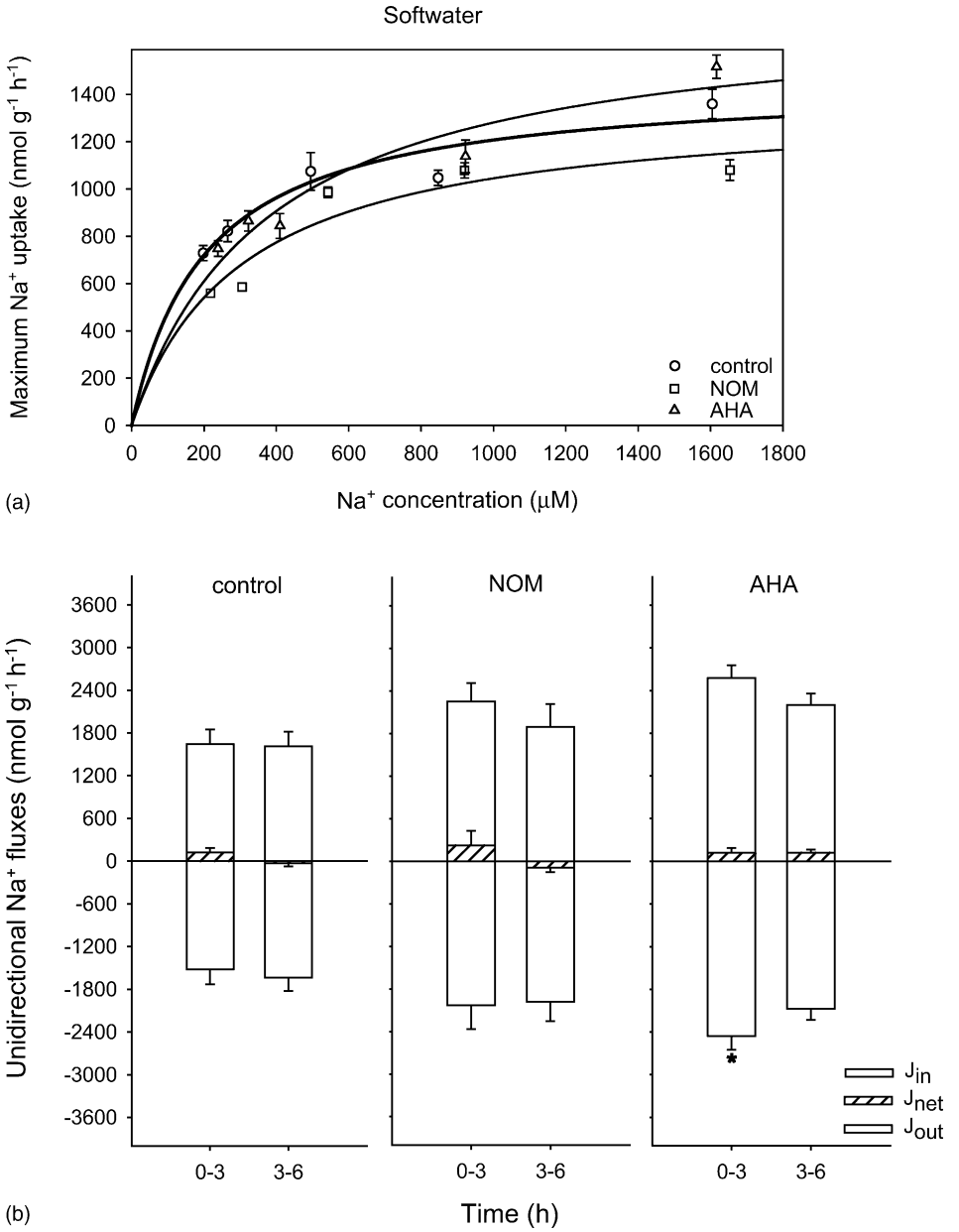

Fig. 5. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to 8 mg C l^{-1} of natural organic matter (NOM) and a commercially available source (AHA) in softwater (control). Same format as Fig. 1.

the flux period, but J_{net} values were very low, indicating a tight homeostatic control in softwater. AHA caused a small but significant increase in J_{out} in the first 3 h only (Fig. 5b).

3.2.2. Cu^{2+} effects

In softwater-acclimated fish, 70 μg l^{-1} of Cu^{2+} decreased J_{max} by 25%, whereas at 300 μg l^{-1}, Cu^{2+} markedly affected both J_{max} and K_m (Fig. 6a; Table 2).

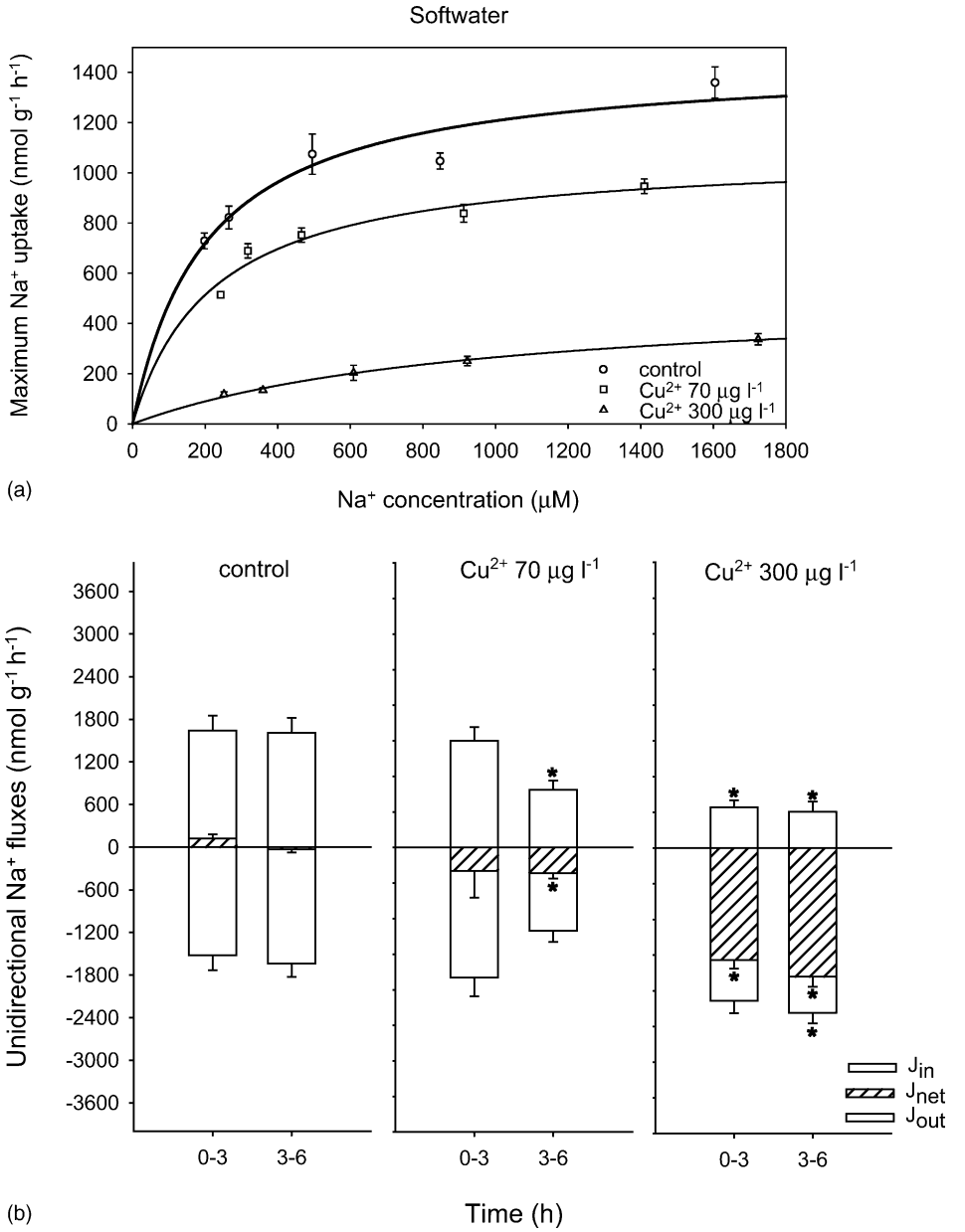

Fig. 6. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to nominal 0, 70, and 300 $\mu g\,l^{-1}$ Cu^{2+} in softwater (100 μM Ca^{2+}). Same format as Fig. 1.

J_{max} was reduced by 64% and K_m increased almost five-fold.

Unidirectional flux measurements further indicated reduction of Na^+ uptake in fish at both Cu^{2+} concentrations tested (Fig. 6b). Net losses in trout exposed to 300 $\mu g\,l^{-1}$ Cu^{2+} indicated a clear and sustained disruption of Na^+ homeostasis ($J_{net} = -1800\,nmol\,g^{-1}\,h^{-1}$) as a result of influx inhibition and efflux stimulation.

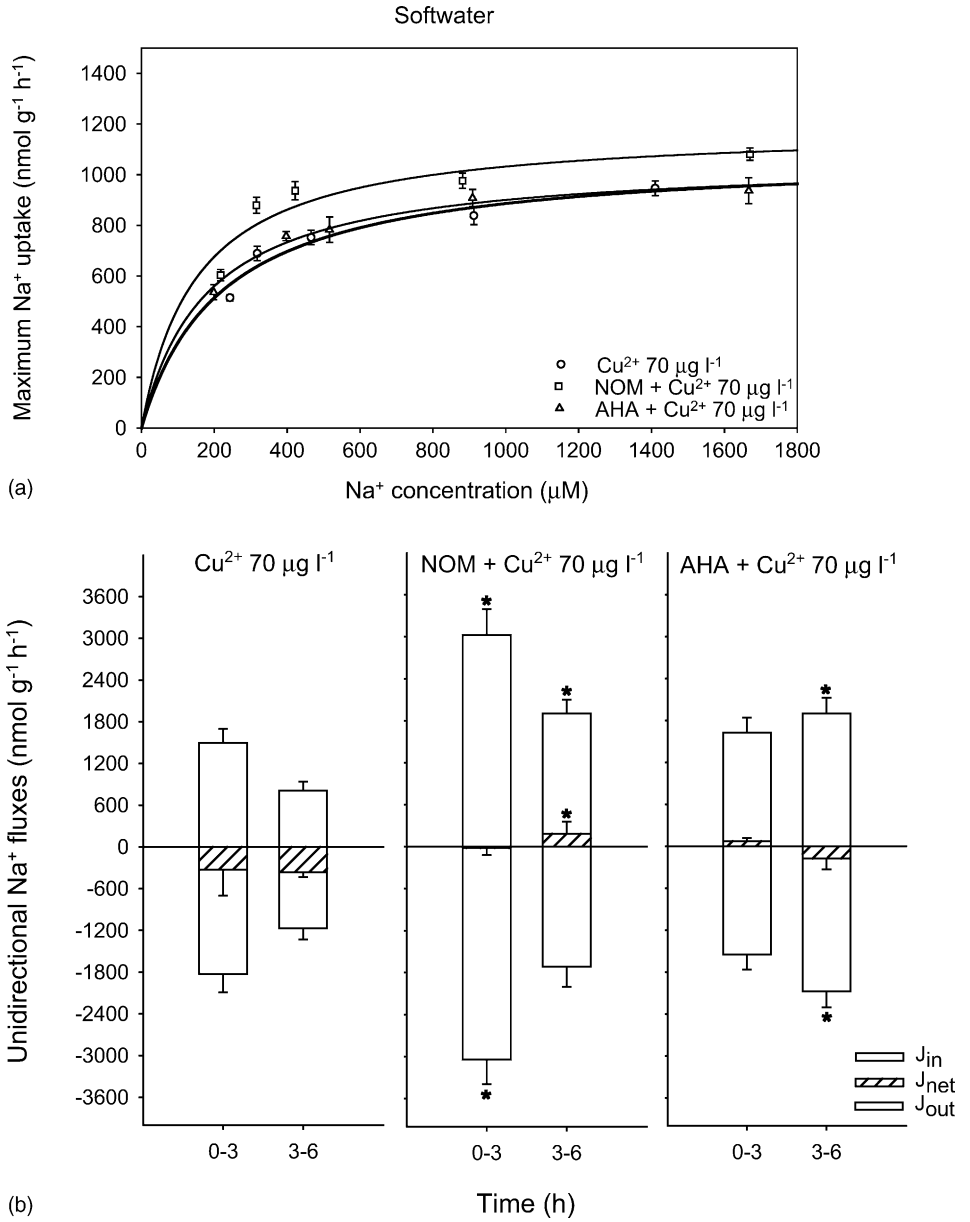

Fig. 7. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to nominal 70 μg l^{-1} Cu^{2+} combined with 8 mg C l^{-1} of natural organic matter (NOM) and a commercially available source (AHA) in softwater (100 μM Ca^{2+}). Same format as Fig. 3.

3.2.3. *Combined effects of DOM and Cu^{2+}*

Kinetic estimates for trout submitted to the combined effects of DOM plus Cu^{2+} were significantly different compared to DOM alone (Figs. 7a and 8a; Table 2). However, compared to fish exposed to Cu^{2+} alone, there were no significant differences in J_{max}, although there was some indication of protection of K_m, which was no longer significantly elevated in the presence of 300 μg l^{-1} Cu^{2+} if either type of DOM was present.

Table 2
Kinetic parameters for Na^+ uptake in rainbow trout acclimated to softwater (100 μM Ca^{2+}) and exposed to Cu^{2+} and/or DOM

Treatment	J_{max} (nmol g^{-1} h^{-1})	K_m (μM Na^+)	R^2
Control	1453 ± 116	204 ± 58	0.91
NOM	1363 ± 162	304 ± 110	0.89
AHA	1767 ± 192	380 ± 111	0.91
70 μg l^{-1} Cu^{2+}	1082 ± 72*	221 ± 51	0.93
NOM + 70 μg l^{-1} Cu^{2+}	1188 ± 102	152 ± 54	0.84
AHA + 70 μg l^{-1} Cu^{2+}	1061 ± 34*	179 ± 23	0.98
300 μg l^{-1} Cu^{2+}	522 ± 30*	967 ± 110 (×)	0.99
NOM + 300 μg l^{-1} Cu^{2+}	478 ± 102*	749 ± 349	0.92
AHA + 300 μg l^{-1} Cu^{2+}	717 ± 97*	592 ± 186	0.94

(*) indicate significant differences in Cu^{2+} exposed fish relative to the respective Cu^{2+}-free control. Significance level: $P < 0.05$.

Unidirectional flux measurements indicated that uptake rate (J_{in}) for trout exposed to NOM + 70 μg l^{-1} Cu^{2+} was double that of the treatment exposed to 70 μg l^{-1} Cu^{2+} alone (Fig. 7b). At 300 μg l^{-1} Cu^{2+} with added NOM, flux data indicated that there was again a protective effect of the natural DOM source (Fig. 8b). Such protection was represented by a reduction in J_{net} as a function of the increased capacity of Na^+ uptake, and not by any particular reduction of the diffusive outflux component membrane permeability. However, diffusive losses were better controlled in the presence of AHA, in addition to a stimulatory effect of AHA on J_{in} (Figs. 7 and 8 Figs. 7b and 8b). Net flux was on average lower when fish were exposed to either source of DOM than it was in the absence of DOM, indicating a beneficial effect of both NOM and AHA in reducing Cu^{2+} toxicity and ionoregulatory impairment in softwater-acclimated fish.

4. Discussion

4.1. Water hardness

Higher Na^+ uptake rates (J_{in} or J_{max}) and higher ion losses (J_{out}) in softwater-acclimated fish over hardwater-acclimated fish reflect the influence of acclimation to different water chemistries on Na^+ transport. In softwater, fish have higher diffusive Na^+ efflux rates, because of the low concentration of Na^+ and perhaps hardness cations, particularly Ca^{2+}. Therefore, softwater-acclimated fish tend to take up ions at a faster rate relative to hardwater-acclimated fish, so as to achieve net balance. Exposure of fish to softwater requires an acclimatory phase to allow physiological adjustments such as the proliferation of gill chloride cells (Laurent and Dunel, 1980; Laurent et al., 1985), which are probably involved in the high uptake rates we found for Na^+ in rainbow trout. Proliferation of this cell type is a common response by fish subjected to ion-poor waters (reviewed by Perry, 1998), although upon acclimation to the media, the cell numbers tend to return to control values (Perry and Wood, 1985). Softwater-acclimated fish in the control situation exhibited a 140% increase in J_{max} and a 27% increase in affinity (i.e., decrease in K_m) relative to hardwater-acclimated fish (Tables 1 and 2). Differences in affinity between hard and softwater treatments were not significant, although one would expect that in softwater, the affinity of the transporters for ions would be higher (e.g., McDonald and Rogano, 1986). This may occur for two reasons. First, fish in the softwater treatment may have been fully acclimated to softwater conditions to the point that K_m returned to a value similar to that found in the hardwater series. Perry and Wood (1985) documented a similar situation in Ca^{2+} uptake studies in which the K_m values in trout seemed to return to control values upon acclimation to softwater. Second, we cannot rule out an effect on eventual decreases in K_m associated with acclimation in the absence of lower Na^+ concentration points in the kinetic determination for the softwater series. The determination of the estimates for the affinity of the ions and transporters at the gill sites is difficult, as reflected by the variability often found in studies of this nature.

Although Mg^{2+}, K^+, and Na^+ represent other important cations, Ca^{2+} is usually the predominant hardness ion, and its physiological importance is well

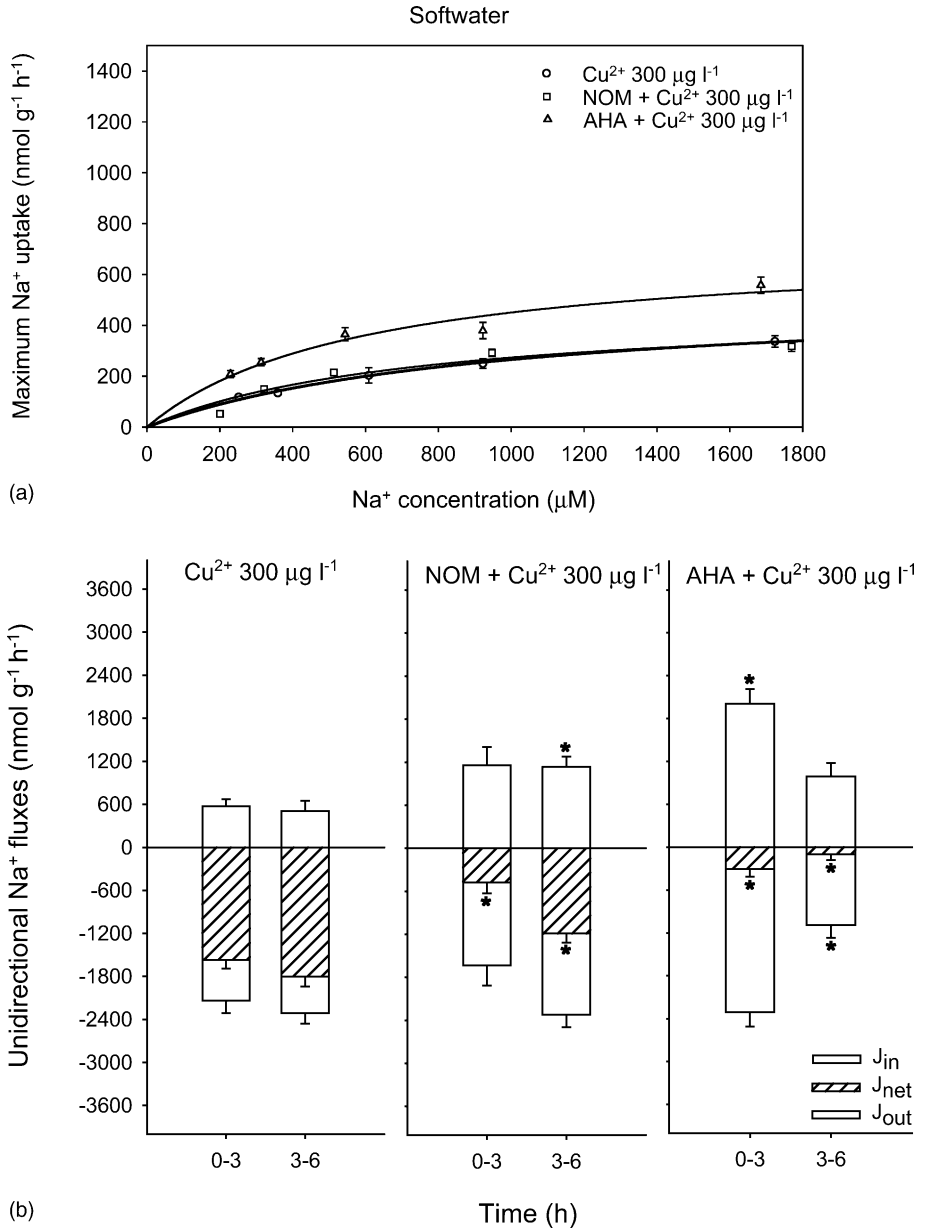

Fig. 8. (a) Kinetic analysis of Na^+ uptake and (b) unidirectional Na^+ flux measurements in rainbow trout exposed to nominal 300 µg l^{-1} Cu^{2+} combined with 8 mg C l^{-1} of natural organic matter (NOM) and a commercially available source (AHA) in softwater (100 µM Ca^{2+}). Same format as Fig. 3.

known. Ca^{2+} affects gill permeability in fish (Cuthbert and Maetz, 1972; Hunn, 1985), and it competes with metals for binding sites on the gills (Pagenkopf, 1983; Playle et al., 1993a, b). Ca^{2+} therefore influences the uptake of certain metals by competitive inhibition, which modulates their toxicity to fish, as was seen here with Cu^{2+}. We should point out that ions, other than Ca^{2+}, varied in the present study and this could have contributed to the observed differences between hardwater- and softwater-acclimated fish.

4.2. DOM effects

Despite a great deal of attention given to DOM with regard to metal complexation, the biological actions of these compounds have been largely overlooked. Campbell et al. (1997), Richards et al. (1999), and Wood et al. (2003) previously studied the biological effects of DOM alone in fish, and here we report data indicating physiological action of DOM at the target sites (the gills) on Na^+ transport in trout. Our kinetic studies indicated that the commercial source of DOM tested, AHA, stimulated J_{max} by 67% (Table 1) in hardwater. Unidirectional flux measurements indicated that Na^+ influx (and also efflux) more than doubled in fish exposed to AHA and NOM in the initial 3 h period (Fig. 1b), then recovered, suggesting that DOM initially acts to increase the Na^+ turnover rates in hardwater until homeostasis is restored to reach steady-state conditions again. Na^+ losses were generally higher relative to the control group, suggesting that a change in membrane permeability occurred when fish were exposed to DOM. Such effects on Na^+ uptake kinetics and loss rates were much less pronounced in softwater-acclimated fish (Fig. 5b), probably because Ca^{2+} was more tightly bound to the gills (Reid, 1995) and therefore the gills would be able to out-complex the NOM for the limited amount if Ca^{2+} in the water.

The mechanism by which DOM stimulates higher uptake rates is not known, but it is likely related to the hydrophobic and hydrophilic moieties of these compounds, which may lead to changes in membrane permeability (Campbell et al., 1997; Vigneault et al., 2000). The association of these amphiphilic sites of DOM with biological membranes may change the fluidity of the lipid bilayer because of their surface-active properties, resulting in alteration of the membrane permeability (reviewed by Vigneault et al., 2000), which in turn could alter the activity of the Na^+ transport sites, or the accessibility of the substrate Na^+ to these sites. Our results suggest that these interactions may be rapid enough to result in changes during short-term exposure.

What further supports our data shown here, are some results obtained for tambaqui (*Colossoma macropomum*) acclimated to NOM at 20, 40, and 80 mg C l^{-1} (A.Y.O. Matsuo and A.L. Val, unpublished data) for 10 days followed later by an acute Cu^{2+} challenge (600 $\mu g\, l^{-1}$ for 3 h). When the fish were acclimated to high NOM, they were able to prevent Cu^{2+} accumulation by the gills (in short-term gill binding assays), even in the absence of NOM in the water, probably because NOM induced changes in the structure/binding capacity of the fish gills.

We also speculate that the diffusive Na^+ efflux seen in the presence of DOM could be attributed either to a displacement of Ca^{2+} by DOM from the paracellular junctions at the gills thus stimulating efflux, or because of the surfactant character of DOM (Thurman, 1985). Surfactants are known to alter membrane permeability by changing their structure (Helenius and Simons, 1975), which probably would help explain the higher diffusive losses seen in our results in the presence of DOM alone.

Long-term effects of DOM have not been assessed in this study, but Richards et al. (1999) did not find physiological differences (respiratory and ionoregulatory effects) in adult rainbow trout exposed to 31 mg C l^{-1} of AHA over a 96 h exposure. Plasma Na^+ and Cl^- remained unchanged, indicating ionic homeostasis in fish upon acclimation to AHA. In contrast, long-term acclimation of rainbow trout to AHA at 3 mg C l^{-1} resulted in a 30% increase in gill Na^+/K^+-ATPase activity relative to the control group after 29 days of the acclimation (McGeer et al., 2002, Fig. 4c). Our results further indicate that the incorporation of DOM in toxicity studies and models should be analyzed carefully because DOM alone results in biologically significant effects on the organisms in a time dependent manner.

4.3. Copper effects alone

Na^+ transport is a sensitive indicator of Cu^{2+} toxicity at the gill sites in fish (Laurén and McDonald, 1985, 1986). Our results corroborate earlier findings on Cu^{2+} toxicity and the disruption of Na^+ ionoregulatory pathways, and they also confirm that such differences can be explained in terms of kinetic and flux measurements (Laurén and McDonald, 1985, 1986, 1987a, b). Cu^{2+} inhibits Na^+ influx by reducing J_{max} and by decreasing the affinity of the transporters for Na^+, particularly at high Cu^{2+} concentration, i.e., by a mixed-type non-competitive and competitive inhibition. These effects are much more prominent in softwater than in hardwater-acclimated fish, reflecting the well known protective actions of Ca^{2+} in this regard

(USEPA, 1985). At 300 μg l^{-1} Cu^{2+}, rainbow trout had a severe impairment of Na^+ balance, which could not be compensated, as indicated by the J_{net} values (Figs. 2 and 6 Figs. 2b and 6b). However, no fish died during the 6 h flux exposure. As reported by Laurén and McDonald (1985), control of Na^+ losses plays an important role in decreasing Cu^{2+} toxicity in trout. Cu^{2+} at acute concentrations inhibits Na^+ uptake, but fish tend to compensate by decreasing Na^+ losses in an attempt to recover homeostatic control.

4.4. Combined effects of DOM and Cu^{2+}

Metal toxicity in aquatic environments can be decreased by the presence of DOM through complexation, thereby decreasing the free metal form for interactions at the organism's target sites (Pagenkopf, 1983; Playle et al., 1993b; Di Toro et al., 2001; Paquin et al., 2000, 2002). A significant increase in J_{max} was observed in hardwater-acclimated fish exposed to AHA combined with Cu^{2+} (Figs. 3 and 4 Figs. 3a and 4a; Table 1) whereas flux measurements further indicated that NOM was also effective in terms of sustaining high Na^+ uptake rates in softwater in the presence of Cu^{2+} (Figs. 7 and 8 Figs. 7b and 8b). Our results indicate that DOM not only affects Cu^{2+} speciation, but also acts at the gill level by producing beneficial changes in the Na^+ balance, the main pathway for Cu^{2+} toxicity. In general, AHA was far more effective than NOM in this regard (e.g., Table 1). At 300 μg l^{-1} Cu^{2+}, however, neither NOM or AHA decreased diffusive Na^+ losses (Figs. 4 and 7 Figs. 4b and 7b), probably because Cu^{2+} complexation capacity was exceeded. Richards et al. (1999) tested the same DOM sources we used in this work, Luther Marsh NOM and AHA, and found that both were effective in decreasing the physiological effects of a Cu^{2+} and Cd^{2+} mixture in rainbow trout.

Differences in the combined effects of DOM and Cu^{2+} in fish acclimated in hardwater versus softwater are probably because Ca^{2+} competes only weakly with Cu^{2+} for the binding sites on DOM (Lu and Allen, 2002), as explained by the lower log K values (log $K_{Cu\text{-}NOM}$ = 9.1 (Playle et al., 1993b; Playle, 1998) versus log $K_{Ca\text{-}NOM}$ = 5.0 (Macdonald et al., 2002)). The inability of DOM to decrease ionoregulatory impairment upon high Cu^{2+} exposure may also be due to the limited number of DOM binding sites (estimated at 100 nmol mgC^{-1}, ~0.7 μM sites; Hollis et al., 1997).

Our results indicate a physiological effect of DOM at fish gills, but it is possible that a longer equilibration time between DOM and Cu^{2+} would result in somewhat different responses. By measuring Cu^{2+} ion activity and toxicity to *Ceriodaphnia dubia*, Ma et al. (1999) determined that the equilibration time needed for complete interactions to occur between DOM and Cu^{2+} exceeded 24 h, suggesting that some studies of the effects of DOM on waterborne Cu^{2+} toxicity may underestimate its maximum protective effects. Lu and Allen (2002) recently found that complexation between Ca^{2+} and DOM apparently reaches equilibrium in a few minutes, much faster than complexation between Cu^{2+} and DOM, in which equilibrium was apparently reached in 10–30 min (even longer at low Cu^{2+} concentrations), indicating that equilibrium is reached even faster than found by Ma et al. (1999). Both studies used AHA as the DOM source.

5. Conclusion

We conclude that DOM has a physiological role on the active sites at fish gills, particularly by increasing the Na^+ transport capacity, an effect that is pronounced in hardwater but not in softwater. AHA appears more potent than NOM in this regard. In the presence of Cu^{2+}, DOM helps sustain higher Na^+ uptake rates relative to Cu^{2+} exposure alone, and it counteracts the effects of Cu^{2+} on the affinity of the Na^+ transporters in both media. Both NOM and AHA sustained equally high uptake rates, but AHA helped reduce the negative effects of Cu^{2+} better than did NOM, possibly due to a greater Cu^{2+} binding capacity. These results support the inclusion of DOM in the Biotic Ligand Model, but indicate that not all DOM sources are alike, and further indicate that different DOM sources may exert different effects on basic gill physiology, even in the absence of Cu^{2+}.

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References

Cabaniss, S.E., Shuman, M.S., 1988. Copper binding by dissolved organic matter II: variation in type and source of organic matter. Geochim. Cosmochim. Acta 52, 195–201.

Campbell, P.G.C., 1995. Interactions between trace metals and aquatic organisms: a critique of the free-ion activity model. Wiley, New York.

Campbell, P.G.C., Twiss, M.R., Wilkinson, K.J., 1997. Accumulation of natural organic matter on the surfaces of living cells: implications for the interaction of toxic solutes with aquatic biota. Can. J. Fish. Aquat. Sci. 54, 2543–2554.

Cuthbert, A.W., Maetz, J., 1972. The effects of calcium and magnesium on sodium fluxes through the gills of *Carassius auratus* L. J. Physiol. 221, 633–643.

Dias, C.M., 2001. Estudo físico-químico da água de três igarapés da região do Distrito Industrial de Manaus, AM. Unpublished Master's Thesis. Federal University of Amazonas, Manaus, Brazil, 114 pp.

Di Toro, D., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic Ligand Model of the acute toxicity of metals 1. Technical basis. Environ. Toxicol. Chem. 220, 2383–2396.

Goss, G.G., Wood, C.M., 1990. Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. I. Responses to environmental hyperoxia. J. Exp. Biol. 152, 521–547.

Helenius, A., Simons, K., 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta 415, 29–79.

Hering, J.G., Morel, F.M.M., 1988. Kinetics of trace metal complexation: role of alkali earth metals. Environ. Sci. Technol. 19, 187–192.

Hessen, D.O., Tranvik, L.J., 1998. Aquatic humic matter: from molecular structure to ecosystem stability. In: Hessen, D.O., Tranvik, D.O. (Eds.), Aquatic Humic Substances, Ecological Studies, 133. Springer-Verlag, Berlin, Heidelberg, pp. 333–342.

Hollis, L., Muench, L., Playle, R.C., 1997. Influence of dissolved organic matter on copper binding, and calcium on cadmium binding, by gills of rainbow trout. J. Fish Biol. 50, 703–720.

Hughes, G.M., 1984. General anatomy of the gills. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, XA. Academic Press, New York, pp. 1–72.

Hunn, J.B., 1985. Role of calcium on gill function in freshwater fishes. Comp. Biochem. Physiol. 82A, 543–547.

ICA (International Copper Association), 2003. Diffuse Sources of Environmental Copper in the United States. ISBN 0-943642-12-15, International Copper Association Ltd., New York.

Kullberg, A., Bishop, K.H., Hargeby, A., Janssen, M., Petersen, R.C., 1993. The ecological significance of dissolved organic carbon in acidified waters. Ambio 22, 331–337.

Laurén, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. J. Comp. Physiol. 155, 635–644.

Laurén, D.J., McDonald, D.G., 1986. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. Can. J. Fish. Aquat. Sci. 43, 1488–1496.

Laurén, D.J., McDonald, D.G., 1987a. Acclimation to copper by rainbow trout, Salmo gairdneri: physiology. Can. J. Fish. Aquat. Sci. 44, 99–104.

Laurén, D.J., McDonald, D.G., 1987b. Acclimation to copper by rainbow trout, Salmo gairdneri: biochemistry. Can. J. Fish. Aquat. Sci. 44, 105–111.

Laurén, D.J., 1991. The fish gill: a sensitive target for waterborne pollutants. In: Mayes, M.A., Barron, M.G. (Eds.), Aquatic Toxicology and Risk Assessment, vol. 14, STP 1124, American Society for Testing and Materials, Philadelphia, pp. 223–244.

Laurent, P., Dunel, S., 1980. Morphology of gill epithelia in fish. Am. J. Physiol. 7, R147–R159.

Laurent, P., Hobe, H., Dunel-Erb, S., 1985. The role of environmental calcium relative to sodium-chloride in determining gill morphology of fresh water salmonid fish. Cell Tissue Res. 240, 675–692.

Lu, Y., Allen, H.E., 2002. Characterization of copper complexation with natural dissolved organic matter (DOM) – link to acidic moieties of DOM and competition by Ca and Mg. Water Res. 36, 5083–5101.

Ma, H., Kim, S.D., Cha, D.K., Allen, H.E., 1999. Effect of kinetics of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 18, 828–837.

Macdonald, A., Silk, L., Schwartz, M., Playle, R.C., 2002. A lead-gill binding model to predict acute lead toxicity to rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. 133C, 227–242.

McDonald, D.C., Rogano, M.S., 1986. Ion regulation by the rainbow trout, *Salmo gairdneri*, in ion poor waters. Physiol. Zool. 59, 318–331.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2002. The role of dissolved organic carbon in moderating the bioavailability and toxicity of Cu to rainbow trout during chronic waterborne exposure. Comp. Biochem. Physiol. 133C, 147–160.

Morel, F.M., 1983. Principles of Aquatic Chemistry. Wiley, Toronto, ON, Canada.

Pagenkopf, G.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environ. Sci. Technol. 17, 342–347.

Paquin, P.R., Santore, R.C., Wu, K.B., Kavvada, C.D., Di Toro, D.M., 2000. The biotic ligand model: a model of the acute toxicity of metals to aquatic life. Environ. Sci. Policy 3, S175–S172.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002.

The Biotic Ligand Model: a historical overview. Comp. Biochem. Toxicol. 133C, 3–35.

Perdue, E.M., 1998. Chemical composition, structure, and metal binding properties. In: Hessen, D.O., Tranvik, L.J. (Eds.), Aquatic Humic Substances, Ecological Studies, 133. Springer-Verlag, Berlin, pp. 41–61.

Perry, S.F., 1998. Relationships between branchial chloride cells and gas transfer in freshwater fish. Comp. Biochem. Physiol. 119A, 9–16.

Perry, S.F., Wood, C.M., 1985. Kinetics of branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. J. Exp. Biol. 116, 411–433.

Playle, R.C., 1998. Modelling metal interactions at fish gills. Sci. Total Environ. 219, 147–163.

Playle, R.C., Gensemer, R.W., Dixon, G., 1992. Copper accumulation on gills of fathead minnows: influence of water hardness, complexation and pH of the gill micro-environment. Environ. Toxicol. Chem. 11, 381–391.

Playle, R.C., Dixon, D., Burnison, K., 1993a. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. Can. J. Fish. Aquat. Sci. 50, 2667–2677.

Playle, R.C., Dixon, D., Burnison, K., 1993b. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modeling of metal accumulation. Can. J. Fish. Aquat. Sci. 50, 2678–2687.

Reid, S.D., 1995. Adaptation to and effects of acid water on the fish gill, In: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes, 5. Elsevier, Amsterdam, pp. 213–227.

Richards, J.G., Burnison, K., Playle, R.C., 1999. Natural and commercial dissolved organic matter protects against the physiological effects of a combined cadmium and copper exposure on rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 56, 407–418.

Sampaio, A.Q., 2000. Caracterização física e química dos sedimentos na área do Distrito Industrial de Manaus (AM). Unpublished Master's Thesis, Federal University of Amazonas, Manaus, Brazil, 81 pp.

Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E., Meyer, J.S., 2001. Biotic Ligand Model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and Daphnia. Environ. Toxicol. Chem. 20, 2397–2402.

Spear, P.A., Pierce, R.C., 1979. Copper in the aquatic environment: chemistry, distribution, and toxicology. National Research Council of Canada, Environmental Secretariat Publication No. 16454, Publications, NRCC, Ottawa, Ontario, 227 pp.

Spry, D.J., Wiener, J.G., 1991. Metal bioavailability and toxicity to fish in low-alkalinity lakes: a critical review. Environ. Pollut. 71, 243–304.

Stumm, W., Morgan, J.J., 1981. Aquatic Chemistry: An Introduction Emphasizing Chemical Equilibria in Natural Waters. Wiley, New York.

Aquatic Humic Substances. Organic Chemistry of Natural Waters. Martins Nijhoff/Dr. W. Junk Publishers, Dordrecht.

USEPA, 1985. Ambient water quality criteria for copper. USEPA Office of Water, Criteria & Standards Division, Washington, DC, EPA 440/5-84-031.

Vigneault, B., Percot, A., Lafleur, M., Campbell, P.G.C., 2000. Permeability changes in model and phytoplankton membranes in the presence of aquatic humic substances. Environ. Sci. Technol. 34, 3907–3913.

Wood, C.M., 1992. Flux measurements as indices of H^+ and metal effects on freshwater fish. Aquat. Toxicol. 22, 239–264.

Wood, C.M., 1995. Toxic responses of the gill, In: Schlenk, D., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, 1. Taylor and Francis, London, pp. 1–89.

Wood, C.M., Matsuo, A.Y.O., Wilson, R.W., Gonzalez, R.J., Patrick, M.L., Playle, R.C., Val, A.L., 2003. Protection by natural blackwater against disturbances in ion fluxes caused by low pH exposure in freshwater stingrays endemic to the Rio Negro. Physiol. Biochem. Zool. 76, 12–27.