The Disruption of *Daphnia magna* Sodium Metabolism by Humic Substances: Mechanism of Action and Effect of Humic Substance Source

Chris N. Glover*
Chris M. Wood
Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

Accepted 5/17/2005; Electronically Published 9/8/2005

ABSTRACT

Humic substances have important functions in aquatic systems. While these roles are primarily indirect, influencing the physicochemical environment, recent evidence suggests these materials may also have direct biological actions. This study investigated the mechanism by which humic substances perturb sodium metabolism in a freshwater invertebrate, the water flea *Daphnia magna*. Aldrich humic acid (AHA) stimulated the maximal rate of whole-body sodium influx (J_{max}) when experimental pH was 6 and water calcium content was 0.5 mM. This effect persisted at pH 8 and 1 mM calcium but not at pH 8 in the absence of calcium. An indirect action of AHA on apical transporter activity was proposed to explain this effect. At pH 4 AHA promoted a linear sodium uptake kinetic relationship, attributed to altered membrane permeability due to enhanced membrane binding of humic substances at low pH. In contrast, a real-world natural organic matter sample had no consistent action on sodium influx, suggesting that impacts on sodium metabolism may be limited to commercially available humic materials. These findings question the applicability of commercially available humic substances for laboratory investigations and have significant implications for the study of environmental metal toxicity.

* Corresponding author. Present address: National Institute for Nutrition and Seafood Research, Postboks 2029 Nordnes, 5817 Bergen, Norway; e-mail: chris.glover@nifes.no.

Physiological and Biochemical Zoology 78(6):1005–1016. 2005. 1522-2152/2005/7806-4153$15.00

Introduction

Humic substances are ubiquitous components of aquatic ecosystems, exercising considerable influence over both the ecology and physiology of the biota therein (Steinberg 2002). Traditionally, these influences are considered to be indirect, resulting from geochemical and physicochemical changes induced by the humic substances rather than the humic materials themselves. For example, humic substances have well-described metal binding properties (e.g., Pagenkopf 1983; Janes and Playle 1995). Metal pollutants primarily exert their toxic effects as free ions ($M^{+/2+}$; Campbell 1995). These entities cross biological surfaces via "accidental transport" through ion channels, by virtue of their physicochemical similarity to the essential ions that normally use these pathways (Bury et al. 2003). Silver ions (Ag^+), for example, cross the apical surface of the branchial epithelia of fish via a sodium channel (Bury and Wood 1999). Once inside the cell, toxic metal ions such as silver may act to inhibit several key ionoregulatory enzymes, thus disrupting ion homeostasis (Morgan et al. 1997, 2004) and ultimately causing death due to ionoregulatory failure (Wood et al. 1996). Humic substance binding reduces free metal ion activity, rendering the metal toxicologically inert and, thus, alleviating toxicity (Janes and Playle 1995; Bury et al. 1999, 2002).

Increasingly, however, direct actions of humic substances on the physiology of aquatic organisms are being elucidated. A number of recent reports detail increased activities of antioxidant defence enzymes (glutathione S-transferase and glutathione peroxidase) in response to humic exposure in a number of invertebrate species, including *Daphnia magna* (Pflugmacher et al. 2001; Meems et al. 2004; Timofeyev et al. 2004). Humic substances have been shown to bind to biological surfaces (Campbell et al. 1997), with resulting increases in membrane permeability (Parent et al. 1996; Vigneault et al. 2000). Humic substances also modify ion movements across fish (Wood et al. 2003; Matsuo et al. 2004) and daphnid uptake surfaces (Glover et al. 2005). In the latter study, two commercially available humic substances (Aldrich humic acid [AHA] and Suwannee River natural organic matter [SRN]) had specific effects on the kinetics of sodium influx. AHA stimulated maximal sodium influx capacity (J_{max}), while SRN stimulated both J_{max} and transport affinity (K_m). Several different mechanisms of action were proposed but were not experimentally tested.

The study described above used two commercially available

humic substances. While these lyophilised powdered substances have many practical benefits, they may not have the same properties as aquatic humic substances that occur in the natural environment (Malcolm and MacCarthy 1986). For example, preparation of the sample may considerably alter humic properties, including acid dissociation constants and metal binding strength (Malcolm and MacCarthy 1986; Gregor and Powell 1987). Humic substances isolated directly from natural waterways by reverse osmosis have not undergone the extensive modifications of the commercially available forms (Aiken and Leenheer 1993) and, thus, may better represent real-world humic materials.

In this study, we sought to extend our earlier observations regarding the effects of humic substances on ion metabolism in *Daphnia magna*. The first objective was to determine whether changes in ion transport properties observed with commercial humic substances were also induced by a real-world humic substance, Luther Marsh natural organic matter (LMN). The second objective was to delineate the potential mechanism by which a commercial humic substance, AHA, promotes changes in daphnid ion metabolism. By varying both water calcium levels and pH, the goal of this study was to distinguish between direct effects caused by humic binding to membrane surfaces at low pH (Vigneault et al. 2000) and indirect effects caused by membrane permeability changes promoted by calcium chelation (Matthews 1986). The activity of Na^+,K^+-ATPase, the enzyme that drives sodium influx in freshwater animals, was also determined in the presence of AHA to examine the possibility of direct humic substance action on ionoregulation.

Material and Methods

Daphnia *Culture*

A laboratory culture of *Daphnia magna* was established from adult gravid animals acquired from Aquatic Research Organisms (ARO strain; Hampton, NH), and maintained in constant light (16L : 8D) and temperature (20°–22°C) conditions. The culture medium was a moderately hard water based on the composition of Lake Ontario (1 mM $CaCO_3$, 0.15 mM $MgSO_4 \cdot 7H_2O$, 0.6 mM NaCl), reconstituted from deionised water generated by reverse osmosis (see Glover et al. 2005). For all experiments 7- or 8-d-old *Daphnia* (~ 1 mg wet weight) were used.

Humic Substances

LMN was collected from Luther Marsh, Ontario, via a portable reverse-osmosis apparatus, resulting in a stock with a concentration of 1,765 mg of carbon per litre (mg C L^{-1}). The collection process has been described in detail elsewhere (Hollis et al. 1996). Once isolated, the LMN sample was passed through a cation exchange resin to remove trace elements that may have been concentrated in association with the humic substances. Aldrich humic acid (AHA; sodium salt) was obtained as a freeze-dried powder from Sigma-Aldrich (St. Louis) and made up to a concentrated stock of ~750 mg C L^{-1}.

Concentrations of humic substances in exposure waters were determined spectrophotometrically (Glover et al. 2005). Briefly, samples of known organic carbon content were used to construct standard curves of absorbance per unit carbon at a wavelength of 300 nm for each humic substance. These standard curves were consequently used to determine the organic carbon content of experimental waters.

Effect of Humic Substances on Sodium Influx

The effect of LMN on *Daphnia magna* sodium metabolism was examined at three humic substance concentrations (nominally 0, 2, and 6 mg C L^{-1}), in five different water chemistries varying in pH and calcium content (0 mM calcium, pH 4; 0 mM calcium, pH 8; 0.5 mM calcium, pH 6; 1 mM calcium, pH 4; and 1 mM calcium, pH 8). The effect of each water condition on the kinetics of sodium influx was examined by analysing the uptake of radiolabelled sodium at five different concentrations (nominally 50, 150, 300, 750, and 1,500 μM). The radiolabel (^{22}Na; as NaCl, Perkin Elmer) was added to solutions at ~1 kBq mL^{-1}, with the desired sodium concentration achieved by the addition of NaCl from a 1 mM stock. Each test medium was reconstituted from deionised water (>17.5 megohm-cm; Barnstead Nanopure II). The solution pH was initially adjusted at ~16 h, then finally at ~3 h, before daphnid introduction. Six daphnids were added to 100 mL of test water in a 250-mL acid-washed Pyrex glass beaker.

Sodium influx in the presence of AHA was determined at two concentrations (nominally 0 and 8 mg C L^{-1}), in identical water chemistries to those described above for LMN. The experimental protocol used was also identical to that detailed for LMN.

Sodium Influx and Ion Measurement

All water samples were analysed for sodium content by flame atomic absorption spectrometry (220FS, Varian). Influx was determined as described previously (Glover et al. 2005). Briefly, *Daphnia* were exposed to ^{22}Na-labelled solutions for 1 h, then transferred through a series of rinse solutions (1 M NaCl followed by deionised water) that acted to displace surface-bound ^{22}Na and also served to exchange the trapped carapace water with deionised water. These rinses thus eliminated sources of unabsorbed radioactivity. Following weighing (UMT2; Mettler Toledo), daphnids were counted for ^{22}Na γ activity (Canberra-

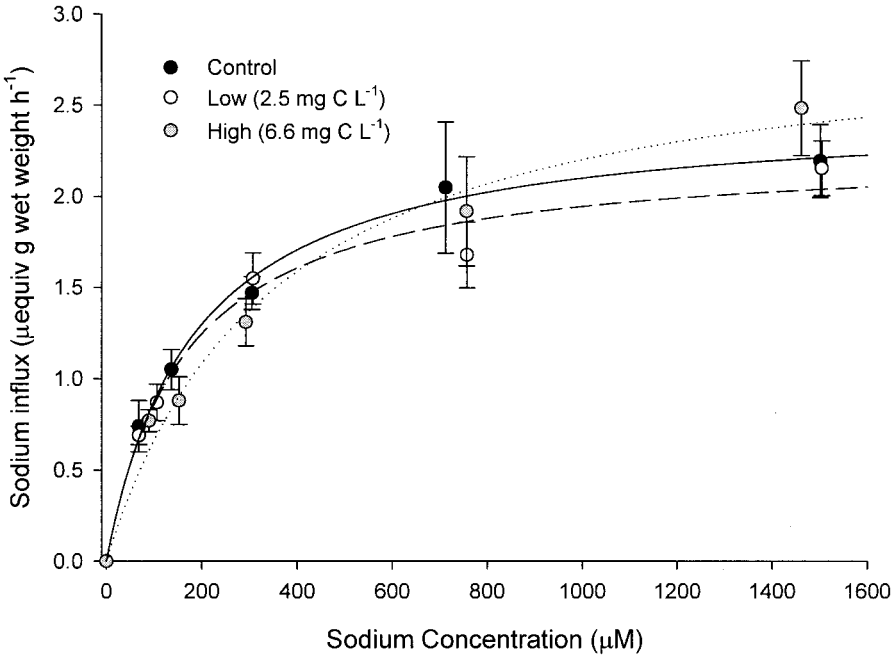


Figure 1. The effect of Luther Marsh natural organic matter (2.5 or 6.6 mg C L^{-1}) on sodium influx (μequiv g wet $weight^{-1}$ h^{-1}) in *Daphnia magna* at pH 6 and a calcium concentration of 0.5 mM. Plotted points represent the means ± SEM of five or six individuals at each sodium concentration.

Packard, Minaxi Auto-gamma 5000). Sodium influx (J_{in}) was calculated according to the following equation:

$$J_{in} = \frac{\text{cpm}}{\text{SA} \times m \times t}, \quad (1)$$

where cpm is the γ counts per minute, SA is the specific activity of the exposure water (cpm $\mu equiv^{-1}$), m represents the daphnid wet mass (in grams; corrected for trapped carapace water by multiplying by 1.25; Stobbart et al. 1977), and t is the time of exposure in hours. This resulted in a J_{in} for sodium expressed as microequivalents per gram wet weight per hour.

Statistical analysis was performed by comparing kinetic parameters of influx curves using *t*-tests (Motulsky 1998), using a conservative approach whereby groups rather than individuals were treated as a single value. This obviated the need to apply multiple comparison corrections that can erroneously inflate the probability of Type II errors (Perneger 1998).

Sodium- and Potassium-Dependent Adenosine Triphosphatase Assay

Following a 1 h exposure to AHA or humic-free conditions in synthetic Lake Ontario water, adult *Daphnia* (~4 mg wet weight) were rinsed in 250 mL of clean synthetic water for ~1 min and blotted dry. Three daphnids were grouped into each replicate, with a total of eight replicates per treatment. Na^+,K^+-ATPase activity was determined according to the method detailed by Bianchini and Wood (2003), a modification of a method earlier described by McCormick (1993). Enzyme activity was determined kinetically at 25°C in a temperature-controlled microplate reader (Molecular Devices, Menlo Park, CA), as the ouabain-sensitive difference in ADP production over 10 min.

Results

The reverse-osmosis isolate of real-world humic material, LMN had little effect on sodium influx in *Daphnia magna* (see Fig. 1). Control (LMN-free) curves conformed to that expected for a Michaelis-Menten kinetic relationship, with a rapid increase in sodium influx with increasing sodium concentration, followed by a saturation of influx as sodium concentration increased further. The addition of LMN had no effect on this relationship (with the single exception noted below).

For all conditions tested, transport kinetic parameters were calculated and detailed in Figure 2. Confirming the lack of effect on daphnid sodium metabolism in the presence of LMN, there was no consistent effect of this humic substance on J_{max} and K_m. The only significant action of LMN was in calcium-free water at pH 8, where low LMN (2.5 mg C L^{-1}) significantly increased J_{max} from 1.20 ± 0.09 to 2.19 ± 0.26 μequiv g wet weight h^{-1} ($P = 0.007$). This effect was not concentration dependent as there was no significant effect of the higher LMN

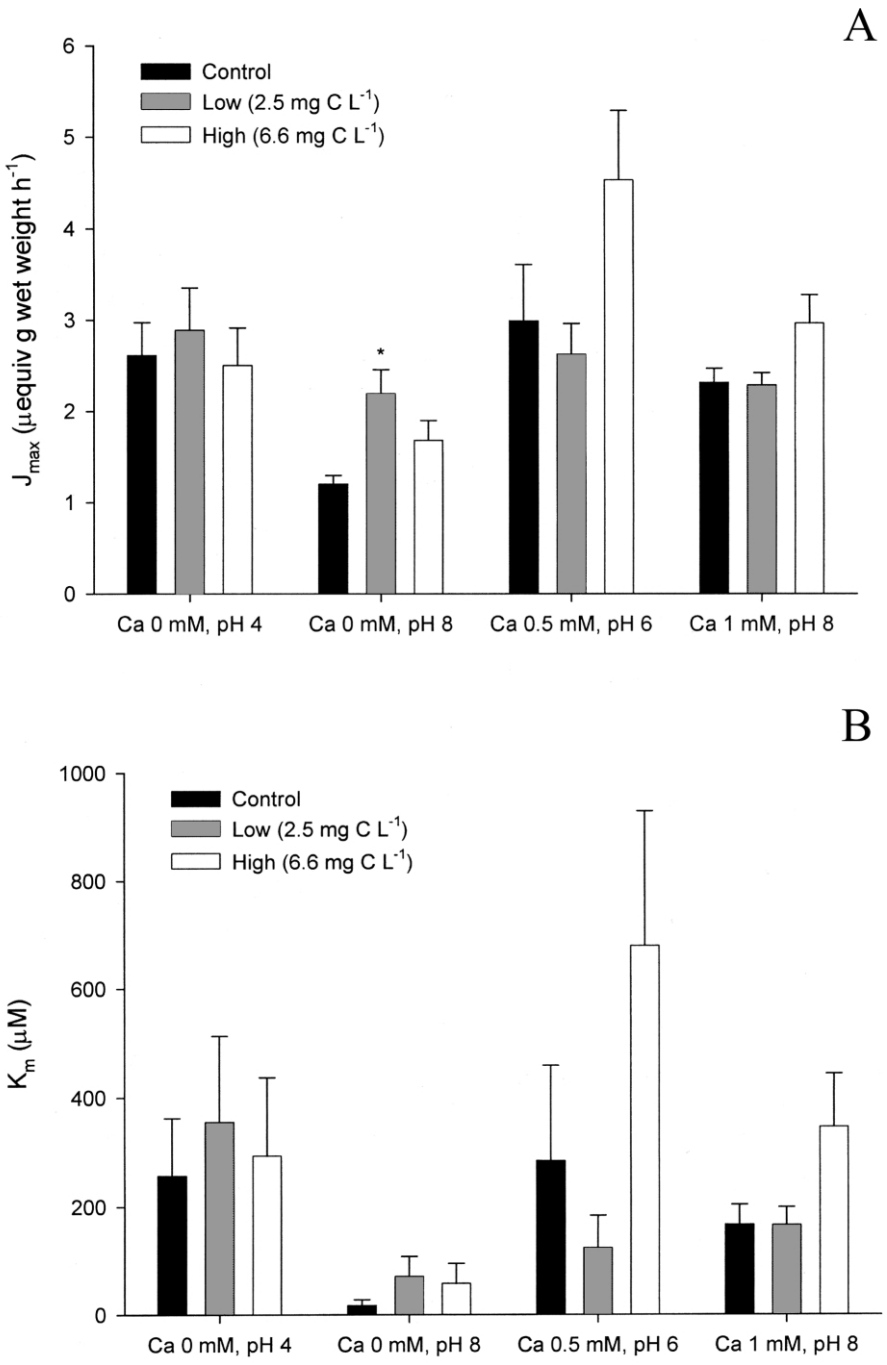


Figure 2. The effect of Luther Marsh natural organic matter (LMN; 2.5 or 6.6 mg C L^{-1}) on the Michaelis-Menten transport kinetic parameters (*A*) J_{max} (μequiv g wet weight h^{-1}) and (*B*) K_m (μM) of sodium influx in *Daphnia magna* in water chemistries of varying pH (4, 6, 8) and calcium concentration (0, 0.5, 1 mM). Significant differences (marked with an asterisk) between control and LMN treatments were tested at the $\alpha = 0.05$ level using the test protocol described in "Material and Methods." Kinetic parameters were calculated directly from plots using SigmaPlot (ver. 8.02; SigmaStat).

level (6.6 mg C L^{-1}) on sodium influx. In no other water chemistry did LMN have an effect on sodium influx. Under conditions of high calcium (1 mM) and low pH (4), sodium influx was an approximate linear function of sodium concentration for control and LMN treatments. The lack of conformation to the Michaelis-Menten transport model resulted in the exclusion of this data from kinetic curve comparison. This effect under high calcium (1 mM) and low pH (4) was consistent with our previous findings regarding the pH and calcium dependence of sodium uptake in *Daphnia magna* (Glover and Wood 2005).

The effect of experimental water pH and calcium level on sodium influx in *Daphnia magna* in the presence of the commercially available humic substance AHA is exhibited in Figure 3. Kinetic parameters calculated directly from Figure 3 are plotted in Figure 4 and show stimulation of sodium transport capacity, without an effect on sodium transport affinity at pH 6, Ca 0.5 mM. Under conditions where pH and calcium were lowered (pH 4, Ca 0 mM), control (humic-free) sodium influx could still be described by Michaelis-Menten kinetics. In the presence of AHA, however, the saturable transport relationship between external sodium concentration and sodium influx was distorted to a linear relationship, for which kinetic parameters could not be calculated. A similar occurrence was observed at pH 4, Ca 1 mM, with a hyperbolic control (humic-free) curve described, in contrast to the linear sodium influx curve in the presence of AHA.

At higher pH (8) J_{max} stimulation was observed only in high-calcium conditions (1 mM). Maximal sodium influx was stimulated 1.8-fold over control, an increase in magnitude similar to that observed at pH 6, Ca 0.5 mM. There were no significant differences in sodium influx parameters between control and AHA treatments at pH 8, 0 mM calcium. AHA had no significant effect on whole-body Na^+,K^+-ATPase activity (Fig. 5).

Discussion

What Is the Mechanism by Which AHA Stimulates Sodium Influx?

The examination of sodium influx in *Daphnia magna* as a function of water calcium, proton, and humic substance concentration permitted a mechanistic analysis of how humic materials influence this important physiological process. In a previous study AHA stimulated maximal sodium influx at a calcium concentration of 0.5 mM and at a slightly acidic pH (~5–6; Glover et al. 2005). An identical action was observed herein. This effect has previously been attributed to an increase in surface binding activity of humic substances (Glover et al. 2005) such as that which may occur at acidic pH's generated by CO_2 at uptake surfaces such as gills (Playle and Wood 1989). An alternative explanation was that humic substance chelation of calcium ions was responsible. The removal of calcium from membranes promotes changes in fluidity and can thus cause altered permeability (Matthews 1986). Both these mechanisms would explain the findings of a mixed stimulatory effect on sodium influx (Glover et al. 2005).

Under conditions where calcium (1 mM) and pH (8) were high, it would be expected that both the calcium chelation effects and the surface binding effects of the humic material would be minimised and sodium influx stimulation would be diminished. Surprisingly, therefore, the J_{max} effect persisted, arguing against both hypotheses. Two important factors need to be considered, however. The experimental waters were unbuffered, meaning that significant acidification of the uptake surface by physiological processes (Playle and Wood 1989) could still have occurred, thus promoting humic binding (Vigneault et al. 2000). Also, the lowered H^+ concentration may have had a secondary effect, whereby decreased competition between H^+ and calcium for chelation by humic substances may have enhanced humate calcium binding. At pH 6 the H^+ concentration is 100-fold higher than at pH 8, while calcium levels were raised only twofold (from 0.5 to 1 mM). The mechanism that allows the J_{max} effect to persist under these water chemistry conditions is unclear.

If the actions observed were the result of calcium chelation and subsequent changes in membrane fluidity, then eliminating calcium from the water should have exacerbated the sodium influx stimulation. At pH 8, where H^+-Ca^{2+} competition effect would have been minimised, J_{max} was unchanged in humic-exposed animals compared to control, thus strongly arguing against a calcium chelation effect. Interestingly, in a recent study that examined the effects of AHA on ion regulation in rainbow trout effects were noted only in high calcium water and not in low calcium water (Matsuo et al. 2004). These results are similar to those noted here and, thus, suggest that mechanisms of AHA action on ion transport may be conserved across phyla and likely do not involve calcium chelation effects on membrane permeability. Conversely, the surface-binding effect promoted by pH cannot be eliminated. The distinct actions of AHA on the pattern of sodium influx at low pH (Fig. 3*B*, 3*D*), coupled with the possibility of uptake surface acidification at higher pH may be a more plausible mode of action.

At low pH the binding of humic substances to biological surfaces is enhanced (Petersen and Persson 1987; Vigneault et al. 2000). This is an action that is attributed to the decreased negative charge of the biological surface that facilitates greater interaction with the polyanionic humic material (see Vigneault et al. 2000). Under conditions of low pH, sodium uptake was greatly reduced in both control and humic-exposed daphnids (note scale differences in Fig. 3). This was likely a consequence of the pH-induced inhibition of sodium uptake that is characteristic of the apical $2Na^+/1H^+$ exchanger (see below). The linearisation of uptake in the presence of AHA at low pH suggests that the change induced by this humic substance completely inhibits the passage of sodium through this transport system. Instead, only a diffusive absorptive pathway remains,

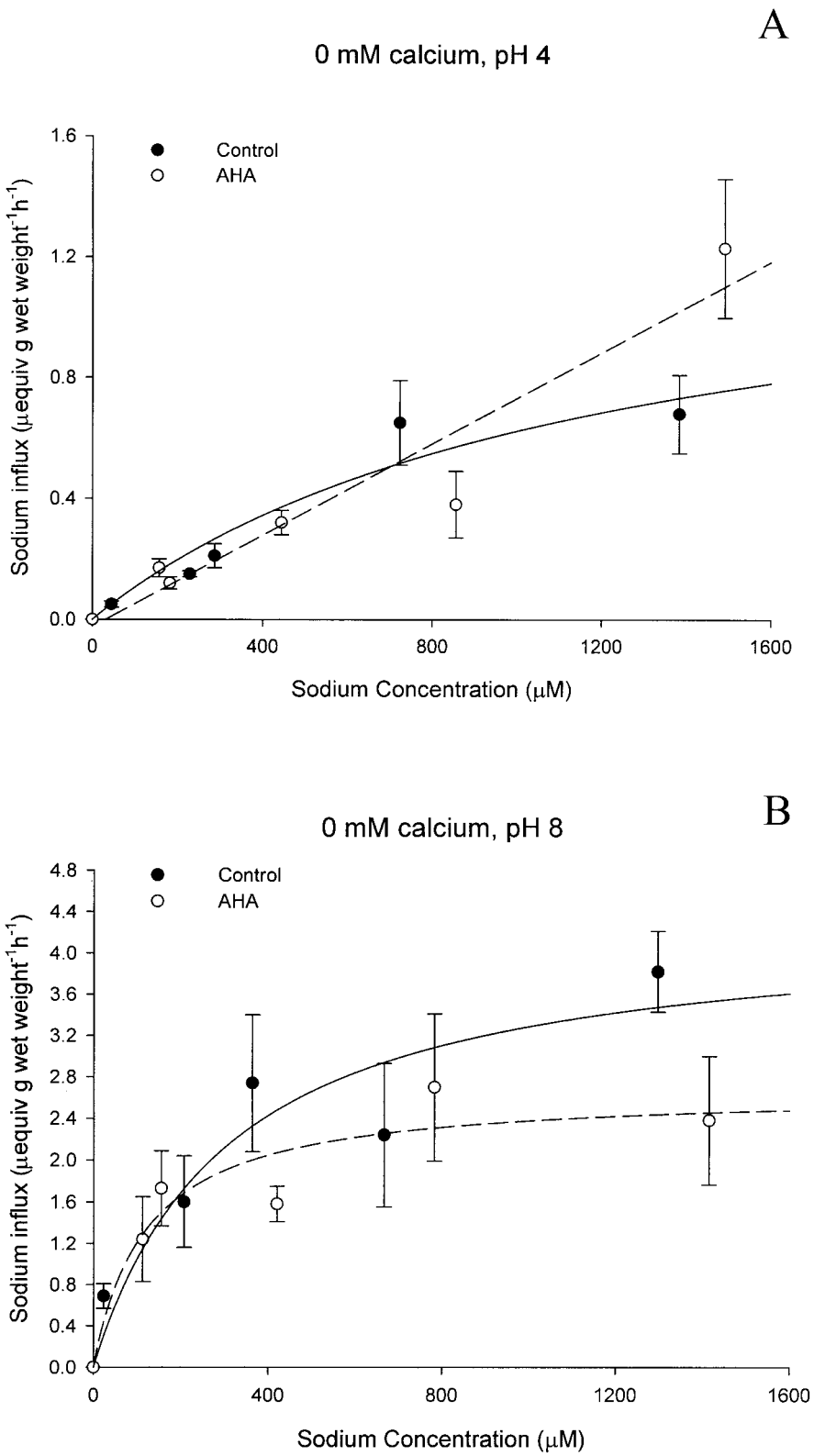
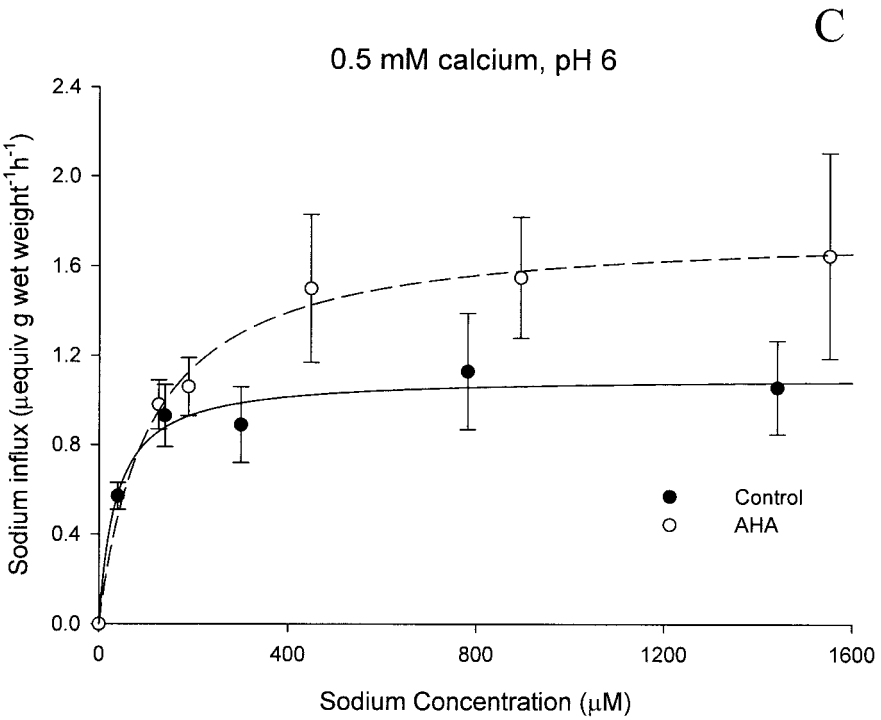


Figure 3. The effect of Aldrich humic acid (7.9 mg C L^{-1}) on sodium influx (μequiv g wet $weight^{-1}$ h^{-1}) in *Daphnia magna* under the following water chemistry conditions: (*A*) Ca 0 mM, pH 4; (*B*) Ca 0 mM, pH 8; (*C*) Ca 0.5 mM, pH 6; (*D*) Ca 1 mM, pH 4; and (*E*) Ca 1 mM, pH 8. Plotted points represent means ± SEM of five or six individuals at each sodium concentration.



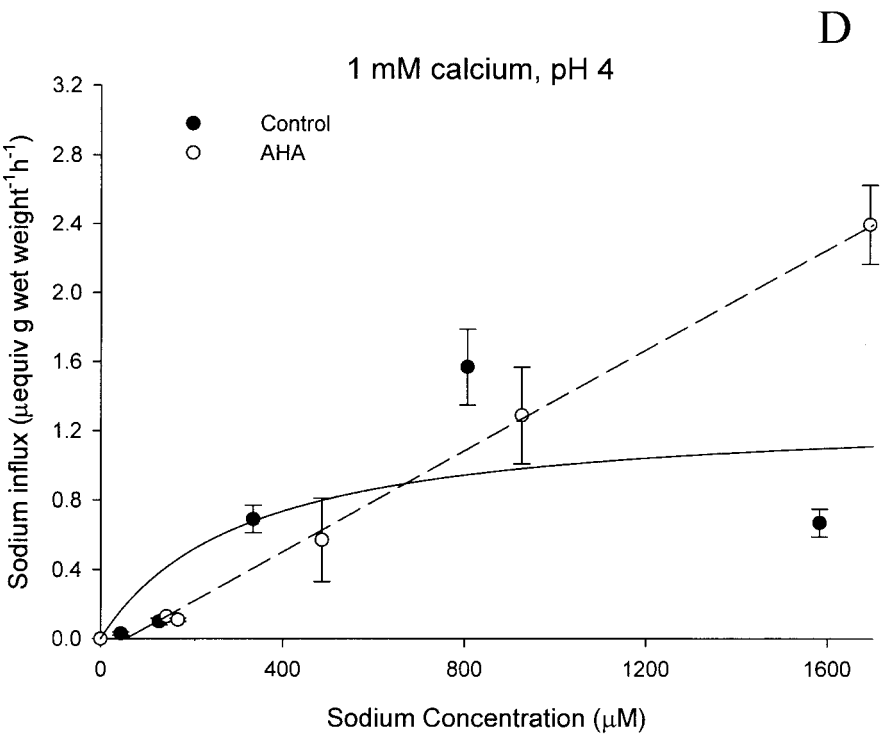


Figure 3 (*Continued*)

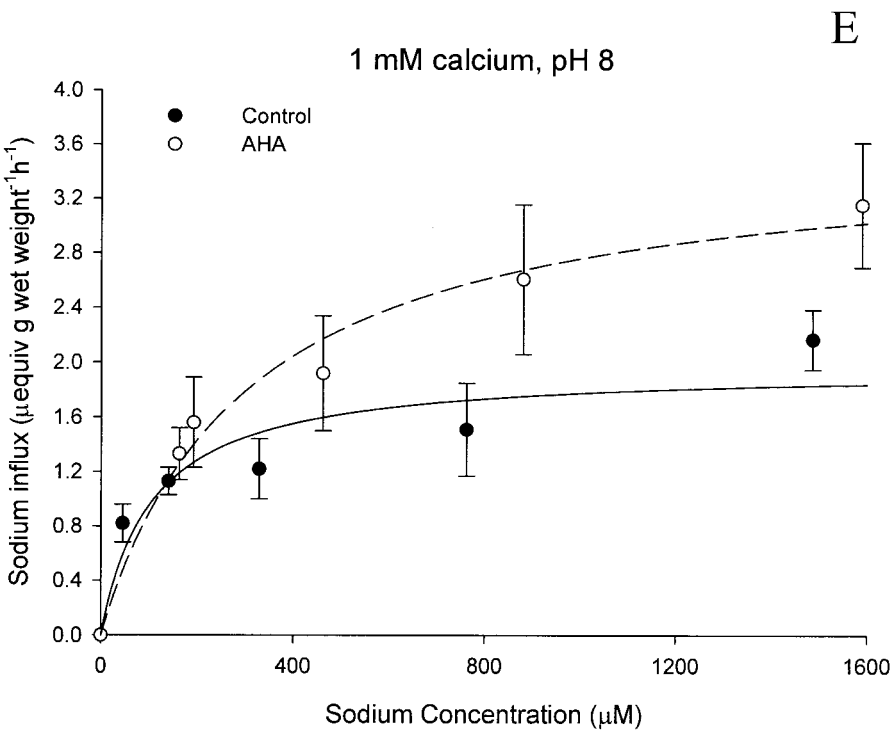


Figure 3 (*Continued*)

which may be a consequence of humic substance increasing nonspecific ion transport via generalised membrane permeability changes.

The apical uptake of sodium across many invertebrate epithelia is achieved by the actions of an electrogenic $2Na^+/H^+$ exchanger (see Ahearn et al. 2001 for review). This transporter is responsible for the uptake of sodium from the milieu in exchange for the extrusion of intracellular protons. Recent physiological evidence has suggested that such a transporter may be responsible for sodium uptake in *D. magna* (Glover and Wood 2005). This transporter is sensitive to external calcium and proton levels (Ahearn et al. 2001; Glover and Wood 2005). Calcium is an alternative substrate for the transporter and competes with sodium for passage across the apical membrane (Ahearn et al. 2001). High external H^+ concentration (low pH) removes the favourable gradient for proton extrusion generated by intracellular proton production and, consequently, reduces proton efflux and the associated sodium influx. In this study, under conditions that are highly favourable to sodium influx (0 mM Ca^{2+}, pH 8) there was no effect of AHA. In every experiment that was conducted in the presence of inhibitory conditions (either low pH or high calcium), AHA significantly stimulated or linearised sodium influx. The implication of this observation is that AHA may alter the interaction of the transporter with potential inhibitors. This is likely to be an indirect action, caused either by altering the conformation of the transporter in the membrane or by physically precluding transporter access. There is other evidence from the literature suggesting that humic substances can alter transporter function. For example, the hyperpolarisation of internodal cells in the plant *Nitellopsis obtusa* was attributed to an increased permeability to protons, with the author suggesting the possibility of a direct action on the plasma membrane proton pump (Zientara 1983).

The apparent ability of AHA to preclude access to the transporter may be a particularly significant finding for the study of waterborne metal toxicity. Humic substances are believed to ameliorate toxicity primarily by chelating metals and preventing them from accessing sites of toxic action (e.g., Pagenkopf 1983). The capacity of humic substances to preclude access to these sensitive sites may prove to be a further means by which metal ion toxicity may be alleviated.

Sodium Influx Stimulation Is Not due to a Direct Humic Substance Action on Na^+,K^+-ATPase

The basolateral enzyme Na^+,K^+-ATPase powers the apical uptake of sodium ions in freshwater animals (Kirschner 2004). By pumping sodium ions from the intracellular compartment, an electrochemical gradient is generated favouring the influx of sodium from the dilute medium into the absorptive cells of uptake epithelia. In this study humic substances had no effect

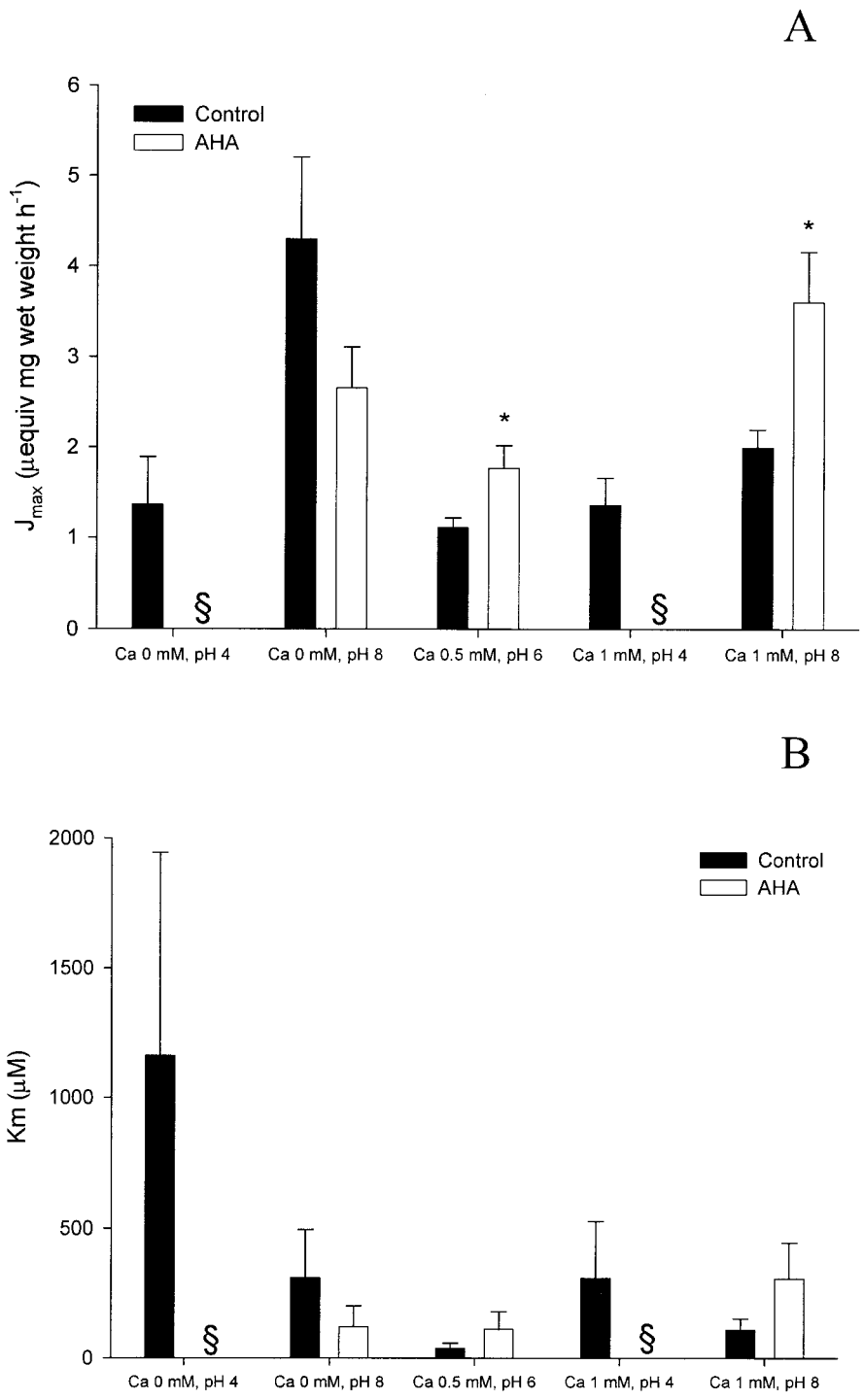


Figure 4. The effect of Aldrich humic acid (AHA; 7.9 mg C L^{-1}) on the Michaelis-Menten transport kinetic parameters (*A*) J_{max} (µequiv g wet weight h^{-1}) and (*B*) K_m (µM) of sodium influx in *Daphnia magna* in water chemistries of varying pH (4, 6, or 8) and calcium concentration (0, 0.5, or 1 mM). Significant differences (marked with an asterisk) between control and AHA treatments were tested at the $\alpha = 0.05$ level using the test protocol described in "Material and Methods." Kinetic parameters were calculated directly from the plots using SigmaPlot (ver. 8.02; SigmaStat). The section symbol (§) indicates that data did not conform to Michaelis-Menten kinetics; consequently, values for these parameters are missing.

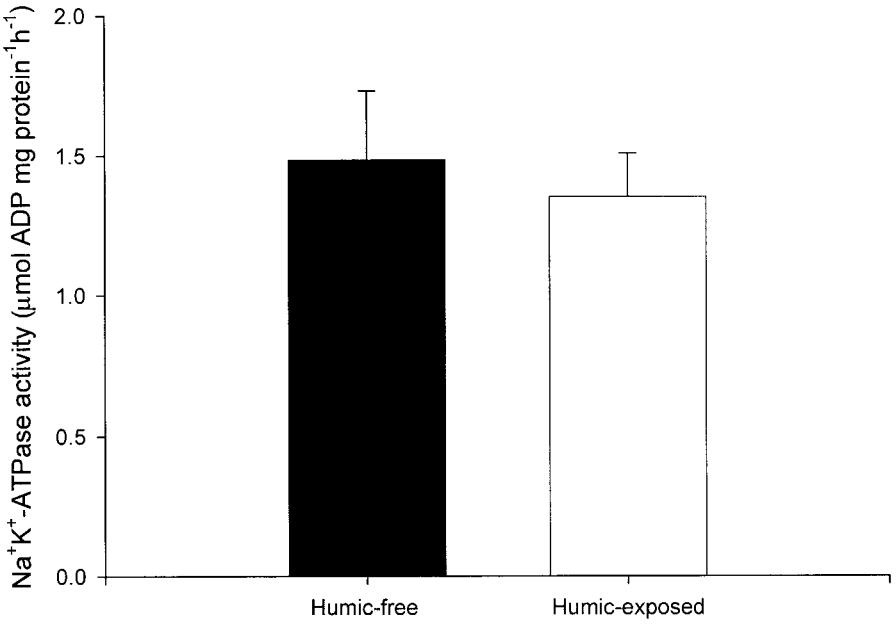


Figure 5. The effect of AHA (8.1 mg C L^{-1}) on whole-body Na^+,K^+-ATPase activity (μmol ADP mg protein^{-1} h^{-1}) in *Daphnia magna*. Plotted data represent the means ± SEM of eight determinations for each condition.

on Na^+,K^+-ATPase activity (Fig. 5). There is some evidence that humic substances can influence the activities of other enzyme systems in invertebrates (e.g., Pflugmacher et al. 2001; Meems et al. 2004; Timofeyev et al. 2004). A direct action such as this would require the absorption of humic substances or a breakdown product. While there is some circumstantial evidence that this may occur (e.g., in plant cell culture; Wang et al. 1999), an indirect effect, such as chelation of a metal cofactor may also explain actions on enzyme systems. The lack of an effect of humic substances on Na^+,K^+-ATPase does not exclude a possible action on other ionoregulatory enzymes. For example, as proposed above, there may be an effect on the major apical transporter ($2Na^+/H^+$ exchanger). Such an effect would not require absorption of the humic substance or a breakdown product but would most likely be an indirect effect acting not on the transporter itself but influencing transport properties by altering membrane fluidity.

Lack of Effect of a Real-World Humic Substance

LMN had no consistent action on sodium influx in *D. magna*. The only statistically significant action (stimulated J_{max} at 0 mM calcium, pH 8) was noted for the lowest concentration of LMN (2.5 mg C L^{-1}) and was not reproduced at the highest level tested (6.6 mg C L^{-1}) suggesting that the effect was likely an anomaly. Similarly, no effect of LMN on the maximal sodium influx rate was noted for rainbow trout exposed to LMN, in a study that did demonstrate stimulation of sodium J_{max} (Matsuo et al. 2004) by the commercially available AHA, in accordance with our results in *Daphnia*.

Humic substances are highly heterogeneous. Precursor substrates are highly variable, and substances can undergo numerous physical, chemical, and biological modifications both before reaching a given water body and within it (McKnight and Aiken 1998). Consequently, every aquatic system is likely to have a unique humic substance composition. Such differences are now being recognised within the field of environmental toxicology, where, for example, copper and silver toxicity amelioration may be influenced by natural organic matter source (e.g., Van Genderen et al. 2003; De Schamphelaere et al. 2004; Ryan et al. 2004). These differences among humic substances are likely to extend to the surfactant-like properties hypothesised in this study. For example, Suwannee River humic acid is more hydrophobic than is the fulvic fraction (Thorn 1995); thus, in an artificial phospholipid membrane vesicle preparation, it was demonstrated to be the better surfactant (Vigneault et al. 2000). The humic nature of AHA, versus the mixed humic/fulvic content of LMN, may thus explain the source-related differences on daphnid sodium metabolism.

It is noteworthy that only commercially available substances have thus far demonstrated effects on sodium influx. Commercial products such as AHA and SRN have numerous practical benefits over reverse-osmosis isolates. These are readily available, more economical to use and obtain, and standardised, thus allowing direct comparison between studies. However, there is considerable evidence that the isolation methods of

commercial substances can substantially alter the properties of these substances (Gregor and Powell 1987) and that this results in materials that differ considerably from natural samples (Malcolm and MacCarthy 1986). As such, results obtained from the use of such substances may not necessarily be applicable to the natural environment. Further testing of naturally obtained humic substances is required to ascertain whether the lack of effect on sodium influx seen with LMN is a standard response to real-world samples. Nevertheless, results showing sodium metabolism disruption in the presence of commercially available humic substances have significant implications for laboratory experiments that use these materials. Studies investigating metal toxicity amelioration by humic materials, for example, could be considerably influenced by altered sodium metabolism. The toxic mode of action of copper and silver ions to freshwater organisms involves inhibition of sodium metabolism (e.g., Laurén and McDonald 1985; Morgan et al. 1997; Bianchini and Wood 2003). Actions of humic substances on sodium metabolism could exacerbate or counter toxic effects in competition or collaboration with the beneficial effects of metal ion chelation by humic matter.

Conclusion

The actions of humic substances on daphnid sodium metabolism are source dependent. AHA stimulates sodium metabolism in *D. magna*, probably via altering the ability of inhibitory substrates to interact with the apical transporter responsible for sodium influx. Such an effect would be consistent with membrane binding of humic substances that alters transporter properties or obstructs inhibitor access to the transport binding sites. These effects have significant implications for environmental toxicology, where sodium metabolism inhibition may be the major mode of toxic action. There is, as yet, no evidence to suggest that the effects of humic substances are mediated by a direct action on the ionoregulatory machinery.

Acknowledgments

This work was supported by a Natural Sciences and Engineering Research Council Collaborative Research and Development Grant Project (CRDPJ 257740-02) with cofunding from Kodak Canada. C.M.W. is supported by the Canada Research Chair program. We are grateful to Dr. Richard Playle at Wilfrid Laurier University for provision of the Luther Marsh natural organic matter. Tammie Morgan and Sonia Sharma are thanked for technical support.

Literature Cited

Ahearn G.A., P.K. Mandal, and A. Mandal. 2001. Biology of the $2Na^+/1H^+$ antiporter in invertebrates. J Exp Zool 289: 232–244.

Aiken G. and J. Leenheer. 1993. Isolation and chemical characterization of dissolved and colloidal organic matter. Chem Ecol 8:135–151.

Bianchini A. and C.M. Wood. 2003. Mechanisms of acute silver toxicity in *Daphnia magna*. Environ Toxicol Chem 22:1361–1367.

Bury N.R., F. Galvez, and C.M. Wood. 1999. Effects of chloride, calcium and dissolved organic carbon on silver toxicity: comparison between rainbow trout and fathead minnows. Environ Toxicol Chem 18:56–62.

Bury N.R., J. Shaw, C. Glover, and C. Hogstrand. 2002. Derivation of a toxicity based model to predict how water chemistry influences silver toxicity to invertebrates. Comp Biochem Physiol C 133:259–270.

Bury N.R., P.A. Walker, and C.N. Glover. 2003. Nutritive metal uptake in teleost fish. J Exp Biol 206:11–23.

Bury N.R. and C.M. Wood. 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. Am J Physiol 277:R1385–R1391.

Campbell P.G.C. 1995. Interactions between trace metals and aquatic organisms: a critique of the free-ion activity model. Pp. 45–102 in A. Tessier and D. Turner, eds. Metal Speciation and Bioavailability in Aquatic Systems. Wiley, New York.

Campbell P.G.C., M.R. Twiss, and K.J. Wilkinson. 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.

De Schamphelaere K.A.C., F.M. Vasconcelos, F.M.G. Tack, H.E. Allen, and C.R. Janssen. 2004. Effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. Environ Toxicol Chem 23:1248–1255.

Glover C.N., E.F. Pane, and C.M. Wood. 2005. Humic substances influence sodium metabolism in the freshwater crustacean *Daphnia magna*. Physiol Biochem Zool 78:405–416.

Glover C.N. and C.M. Wood. 2005. Physiological characterisation of a pH- and calcium-dependent sodium uptake mechanism in the freshwater crustacean, *Daphnia magna*. J Exp Biol 208:951–959.

Gregor J.F. and H.K.J. Powell. 1987. Effects of extraction procedures on fulvic acid properties. Sci Total Environ 62:3–19.

Hollis L., K. Burnison, and R.C. Playle. 1996. Does the age of metal-dissolved organic carbon complexes influence binding of metals to fish gills? Aquat Toxicol 35:253–264.

Janes N. and R.C. Playle. 1995. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 14:1847–1858.

Kirschner L.B. 2004. The mechanism of sodium chloride uptake

in hyperregulating aquatic animals. J Exp Biol 207:1439–1452.

Laurén D.J. and D.G. McDonald. 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson: modulation by water hardness and pH. J Comp Physiol 155B:635–644.

Malcolm R.L. and P. MacCarthy. 1986. Limitations in the use of commercial humic acids in water and soil research. Environ Sci Technol 20:904–911.

Matsuo A.Y.O., R.C. Playle, A.L. Val, and C.M. Wood. 2004. 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. Aquat Toxicol 70:63–81.

Matthews E.K. 1986. Calcium and membrane permeability. Br Med Bull 42:391–397.

McCormick S.D. 1993. Methods for nonlethal gill biopsy and measurement of Na^+,K^+-ATPase activity. Can J Fish Aquat Sci 50:656–658.

McKnight D.M. and G.R. Aiken. 1998. Sources and age of aquatic humus. Pp. 9–39 in D.O. Hessen and L.J. Tranvik, eds. Aquatic Humic Substances. Springer, Berlin.

Meems N., C.E.W. Steinberg, and C. Wiegand. 2004. Direct and interacting toxicological effects on the waterflea (*Daphnia magna*) by natural organic matter, synthetic humic substances and cypermethrin. Sci Total Environ 319:123–136.

Morgan I.J., R.P. Henry, and C.M. Wood. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. Aquat Toxicol 38:145–163.

Morgan T.P., M. Grosell, K.M. Gilmour, R.C. Playle, and C.M. Wood. 2004. Time course analysis of the mechanism by which silver inhibits active Na^+ and Cl^- uptake in gills of rainbow trout. Am J Physiol 287:R234–R242.

Motulsky H. 1998. Comparing dose-response or kinetic curves with GraphPad Prism. HMS Beagle, Issue 34 (http://www.graphpad.com/articles/CompareCurves.pdf).

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.

Parent L., M.R. Twiss, and P.G.C. Campbell. 1996. Influences of natural dissolved organic matter on the interaction of aluminium with the microalga *Chlorella*: a test of the free-ion model of trace metal toxicity. Environ Sci Technol 30: 1713–1720.

Perneger T.V. 1998. What's wrong with Bonferroni adjustments. Br Med J 316:1236–1238.

Petersen R.C., Jr., and U. Persson. 1987. Comparison of the biological effects of humic materials under acidified conditions. Sci Total Environ 62:397–398.

Pflugmacher S., L.F. Tidwell, and C.E.W. Steinberg. 2001. Dissolved humic substances can directly affect freshwater organisms. Acta Hydrochim Hydrobiol 29:34–40.

Playle R.C. and C.M. Wood. 1989. Water chemistry changes in the gill micro-environment of rainbow trout: experimental observations and theory. J Comp Physiol 159B:527–537.

Ryan A.C., E.J. Van Genderen, J.R. Tomasso, and S.J. Klaine. 2004. Influence of natural organic matter source on copper toxicity to larval fathead minnows (*Pimephales promelas*): implications for the biotic ligand model. Environ Toxicol Chem 23:1567–1574.

Steinberg C.E.W. 2002. Ecology of Humic Substances in Freshwaters. Springer, Berlin.

Stobbart R.H., J. Keating, and R. Earl. 1977. A study of sodium uptake by the water flea *Daphnia magna*. Comp Biochem Physiol 58A:299–309.

Thorn K.A. 1995. Nuclear magnetic resonance spectrometry investigations of fulvic and humic acids from the Suwannee River. Pp. 141–182 in R.C. Averett, J.A. Leenheer, D.M. McKnight, and K.A. Thorn. Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures. U.S. Geological Survey Water-Supply Paper 2373, Denver, CO.

Timofeyev M.A., C. Wiegand, B.K. Burnison, Z.M. Shatlina, S. Pflugmacher, and C.E.W. Steinberg. 2004. Impact of natural organic matter (NOM) on freshwater amphipods. Sci Total Environ 319:115–121.

Van Genderen E.J., A.C. Ryan, J.R. Tomasso, and S.J. Klaine. 2003. Influence of dissolved organic matter source on silver toxicity to *Pimephales promelas*. Environ Toxicol Chem 22: 2746–2751.

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

Wang W.H., C.M. Bray, and M.N. Jones. 1999. The fate of ^{14}C-labelled humic substances in rice cells in cultures. J Plant Physiol 154:203–211.

Wood C.M., C. Hogstrand, F. Galvez, and R.S. Munger. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 1. The effects of ionic Ag^+. Aquat Toxicol 35:93–109.

Wood C.M., A.Y.O. Matsuo, R.W. Wilson, R.J. Gonzalez, M.L. Patrick, R.C. Playle, and A.L. Val. 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.

Zientara M. 1983. Effect of sodium humate on membrane potential in internodal cells of *Nitellopsis obtusa*. Acta Soc Bot Pol 52:271–277.