Humic Substances Influence Sodium Metabolism in the Freshwater Crustacean *Daphnia magna*

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ABSTRACT

Humic substances are ubiquitous components of natural waters with important roles in alleviating metal toxicity to aquatic organisms. Recent literature reports suggest that humic substances may also exert direct influences on biota. This study investigated the influence of two commercially available humic substances on sodium metabolism in *Daphnia magna*, a hyperregulating freshwater crustacean. Environmentally realistic levels of Suwannee River natural organic matter (SRN) and Aldrich humic acid (AHA) significantly enhanced sodium transport. This effect was described as an uncompetitive stimulation of sodium influx, as characterised by an increased maximal sodium transport rate (J_{max}), accompanied by a decreased uptake affinity (increased K_m). SRN exposure also significantly promoted the unidirectional loss of sodium from the daphnids to the water, an effect not observed in the presence of AHA. A 24-h preexposure to AHA before influx measurement had no effect on AHA-induced stimulation of sodium influx. Conversely, 24-h preexposure to SRN resulted in influx values that returned to control (humic-free) levels. Whole-body sodium levels reduced by SRN exposure were also restored to control levels following 24-h SRN preexposure. The significance and potential mechanisms of these actions are discussed, and the toxicological implications of these findings are assessed.

Introduction

Life in freshwater exerts considerable physiological stress on hyperregulating animals. The maintenance of a relatively constant internal ion concentration in a hypoionic environment relies on mechanisms for reducing diffusive loss of ions and/or ensuring efficient scavenging of ions from the dilute medium (Potts and Parry 1964). Consequently, most osmoregulating freshwater animals devote considerable resources toward the active uptake of ions, such as sodium, across the transport surfaces. Such strategies are of particular importance to small freshwater organisms, whose large surface area to volume ratios result in rapid ion loss across body surfaces, thus necessitating an efficient means for replacing these ions (Bianchini et al. 2002; Grosell et al. 2002).

Freshwater cladocerans are small invertebrates faced with exactly this challenge. Inhabiting waters with diverse salt levels (0.01%–20% seawater; Peters 1987), *Daphnia* exhibit relatively low whole-body ion concentrations and reduced body permeability (Peters 1987; Aladin and Potts 1995) coupled with an active uptake of sodium (Stobbart et al. 1977). Uptake occurs primarily in the ion-transporting regions of the epipodites, gill-like lamellar surfaces located at the base of the legs (Aladin and Potts 1995). Cladoceran sodium transport is concentration-dependent and exhibits saturable Michaelis-Menten kinetic characteristics, with affinity constants ranging from 0.05 to 0.4 meq L^{-1} Na^+ (Stobbart et al. 1977; Potts and Fryer 1979; Bianchini and Wood 2003). The driving mechanism for this uptake appears to be the basolateral Na^+,K^+-ATPase pump, which translocates sodium from transporting cells into the circulation. This generates a gradient for the passage of sodium across the apical uptake membranes via sodium channels or exchangers.

Recent evidence suggests that humic substances, ubiquitous components of natural waters, can influence the permeability of biological membranes. Humic substances are complex, chemically heterogeneous organic materials formed from the breakdown of biotic precursor molecules (McKnight and Aiken 1998). Their heterogeneity stems from differences in precursor entities and the type and magnitude of any physical and/or biological conversion processes endured in transit to or within the waterway (McKnight and Aiken 1998). In algae (*Chlorella pyrenoidosa*, *Selenastrum capricornutum*) and artificial model membranes, humic substances increase membrane permeability to sorbitol or passively diffusing fluorescent probes (Parent et al. 1996; Vigneault et al. 2000). Coupled with the observation that humic substances can adhere to a wide range of biological surfaces (Campbell et al. 1997), it is possible that these important moieties will have ramifications for the ionic maintenance of hyperregulating organisms such as *Daphnia*.

Direct actions of humic substances on ion metabolism would

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have significant implications for environmental toxicology. Copper and silver are metal toxicants with a primary mode of action involving sodium metabolism disruption (see Di Toro et al. 2001; Grosell et al. 2002; Bianchini and Wood 2003). By interfering with various sodium uptake moieties, these metals can cause the depletion of sodium to a level at which mortality results. Such toxic actions are mediated by the free ionic form (M^+) of the metal. Humic substances chelate metals, reducing the abundance of the toxic metal forms, and thus protect against toxicity (see, e.g., Bury et al. 2002). If humic substances influence the permeability of membranes and consequently the passage of ions such as sodium, it is possible that these substances may have modifying actions on the impacts of environmental metal toxicants beyond their protective complexation roles.

This study aims to investigate the actions of two commercially available humic substances (Aldrich humic acid [AHA] and Suwannee River natural organic matter [SRN]) on the sodium metabolism of *Daphnia magna*. Using highly sensitive radiotracer techniques and a kinetic approach, influences of these compounds were investigated over an environmentally realistic range of humic substance concentrations. Significant interactions between humic substances and sodium metabolism will have important implications for the study of freshwater biota in both natural and pollutant-impacted environments.

Material and Methods

Daphnia *Culture*

Adult gravid *Daphnia magna* were obtained from Aquatic Research Organisms (ARO strain; Hampton, NH) and were used to establish a laboratory culture. Daphnids were maintained in a 16L : 8D cycle, at 20°–22°C, in moderately hard reconstituted laboratory water resembling that of Lake Ontario ("synthetic Lake Ontario water": 1 mM $CaCO_3$, 0.15 mM $MgSO_4 \cdot 7H_2O$, 0.6 mM NaCl). To ensure standardised conditions, this synthetic water was made up in a single batch of 1,000 L in a food-grade polyethylene tank, using water purified by reverse osmosis. After bubbling for 24 h with CO_2 to ensure dissolution of $CaCO_3$, this water was further aerated for 48 h with air to rid excess CO_2. Selenium (2.5 μg L^{-1}) and vitamin B_{12} (6 μg L^{-1}) were added to culture water to ensure adequate nutrition (Keating and Dagbusan 1984; Keating 1985). For all experiments juvenile daphnia (6 or 7 d old) were used; average weight was 0.80 mg ± 0.19 (SD).

Exposure Conditions

All flux experiments were performed at a constant calcium concentration (0.5 mM, as $CaCl_2$) and varying sodium constitution (0–2,400 μM) in deionised water (>17.5 megohm-cm; Barnstead Nanopure II), with sodium added from a NaCl stock solution (1 M). Where 24-h exposure periods were included (see below) these were performed in synthetic Lake Ontario water without added selenium and vitamin B_{12}. AHA (Aldrich) was added to solutions at an appropriate concentration (~0–8 mg C L^{-1}) from a nominal stock solution of 2 g L^{-1}. SRN was obtained from the International Humic Substances Society (St. Paul, MN) and was likewise added to test solutions from a 2 g L^{-1} stock to give final concentrations in the range 0–8 mg C L^{-1}.

The pH of experimental solutions reported here were unadjusted. Control water pH was 5.6, while the addition of AHA raised the pH to 6.0, 6.2, and 6.6 at AHA concentrations of 1.4, 4.0, and 7.4, respectively. Conversely, SRN addition lowered the pH to 5.1, 4.7, and 4.4 at concentrations of 2.3, 4.0, and 7.4 mg C L^{-1}. Humic-rich waters are often acidic. The pH of the main channels of the Rio Negro, for example, are typically 5.5, with contributing waterways often exhibiting a pH considerably lower than this (Furch 1984), while the Suwannee River has a pH close to 4 (Malcolm et al. 1995). In order to control for possible pH-related effects, follow-up experiments were conducted as detailed in "Concentration Dependence," where pH was adjusted (with 0.1 N KOH or HNO_3) to control values of 5.6 for the highest levels of AHA and SRN examined.

Sodium Influx Kinetics

Concentration Dependence. To determine concentration-dependent effects of humic substances on sodium influx, six exposure chambers (acid-washed 250-mL glass beakers; Pyrex) were set up at each of four concentrations for both AHA and SRN (nominally: control water [no added natural organic matter], 2, 4, or 8 mg C L^{-1}). AHA or SRN was added to 125 mL of exposure water (0.5 mM $CaCl_2$) containing nominal sodium levels of 50, 150, 300, 600, 1,200, or 2,400 μM. Each chamber was also inoculated with ~8 kBq ^{22}Na (as NaCl; Perkin Elmer). Sodium concentrations in exposure waters were measured by flame atomic absorption spectrophotometry (220FS, Varian). Six daphnids were added to each chamber, and unidirectional sodium fluxes were measured over a period of exactly 1 h. Flux measurements were performed in the dark to minimise humic substance photolysis. Daphnids were removed, rinsed (10 s) in a high-sodium displacement solution (~1 M), followed by two 15-s rinses in deionised water, before being blotted dry and weighed on an electronic microscale (UMT2, Mettler-Toledo; 0.001-mg precision). Daphnids were assayed for ^{22}Na activity via γ counting (Canberra-Packard, Minaxi Auto-gamma 5000) and then digested in 50 μL concentrated H_2SO_4 (trace metal grade; Fisher), before being analysed for whole-body sodium content by flame atomic absorption spectrophotometry. Sodium influx (J_{in}) was calculated as follows:

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

where cpm is the γ counts per minute, SA is the specific activity of the exposure water (cpm μeq^{-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. Whole-body sodium concentrations were determined as the sodium level in the whole animal per unit wet weight, again using Stobbart et al.'s (1977) correction factor.

Effect of 24-h Humic Substance Preexposure. In a separate set of experiments, the impact of a 24-h preexposure to humic substances, before determining sodium influx, was assessed. The experiments were set up identically to that described above, with the exception that 24 h before sodium influx measurements, daphnids were exposed to synthetic Lake Ontario water with or without the addition of ~8 mg C L^{-1} of SRN or AHA. Flux measurements were then made on *Daphnia* from each exposure condition (i.e., humic or humic free) in either the absence or presence of ~8 mg C L^{-1} of the respective humic substance. This resulted in four experimental groups: (1) control (humic-free) exposure, control flux, (2) control exposure, humic flux, (3) humic exposure, control flux, and (4) humic exposure, humic flux, for both AHA and SRN treatments. Between transfer from exposure solution to flux solution, daphnids were rinsed successively in three synthetic Lake Ontario water rinses (total rinse time ~1 min). Sodium influx was calculated as described in equation (1).

Sodium Efflux

The impact of AHA and SRN on sodium efflux was assessed in the following manner. Twenty-four hours before efflux measurements, *Daphnia* were placed in one of two different conditions: synthetic Lake Ontario water either with or without natural organic matter (nominally, 8 mg C L^{-1} for AHA and SRN). Both waters contained ~25 kBq of ^{22}Na. This allowed the isotope to fully equilibrate with unlabelled body sodium. Daphnids were then transferred sequentially into a synthetic Lake Ontario water rinse, followed by two rinses of deionised water for ~30 s each before being transferred individually into efflux chambers for a flux period of 15 min. Chambers consisted of 2-mL microcentrifuge tubes filled with 1.5 mL of water of varying sodium composition (50, 150, 300, 600, 1,200, or 2,400 μM), in humic-free or humic (~8 mg C L^{-1}) conditions. Three groups were analysed: (1) control (humic-free) exposure, control (humic-free) flux, (2) control (humic-free) exposure, humic flux, and (3) humic exposure, control (humic-free) flux. Efflux was calculated on the basis of ^{22}Na appearance in the flux water in an identical manner to equation (1):

$$J_{out} = \frac{\text{cpm}}{\text{SA} \times m \times t}, \tag{2}$$

where cpm is the γ counts per minute appearing in the efflux water, SA represents the specific radioactivity in the animal (cpm μeq^{-1}), and m and t are the daphnid mass (g wet weight) and time of exposure (h), respectively. This resulted in an efflux rate expressed as microequivalents per gram wet weight per hour. Sodium concentrations in efflux water were monitored closely and did not differ significantly from the set values, suggesting that trapped radiolabelled carapace water did not contribute to the determined efflux.

Humic Substance Measurement

A standard curve for each humic substance was created covering the experimental concentration range, and samples were analysed for total organic carbon (TOC) using a Shimadzu TOC analyser. Before analysis, each sample was acidified with a single drop of 16 N HNO_3 and sparged with N_2 gas to remove interference by inorganic carbon. Identical duplicate samples were analysed separately for absorbance at 300 nm, and a linear relationship ($f = a + bx$) between absorbance and TOC concentration was established. For AHA, this relationship was described by the equation $f = -0.027 + 0.093x$ ($r^2 = 0.946$, $P < 0.0001$), and for SRN, it was described by $f = -0.001 + 0.026x$ ($r^2 = 0.850$, $P < 0.0001$). Consequently, the A_{300} of water samples from experimental solutions was measured, and TOC concentration was calculated using these formulas. Because of small volumes, exposure water from sodium efflux experiments was not measured but was inferred from measurements of larger volumes of identical composition (i.e., exposure waters used in influx experiments).

Statistical and Kinetic Analysis

Data points have been routinely expressed as means ± SEM (n = number of individual *Daphnia*). Statistical significance was determined by one-way or two-way ANOVA, followed by post hoc LSD analysis (SPSS, ver. 10.05), with the exception of kinetic parameter comparison (discussed below).

Where applicable, the kinetic parameters of sodium influx were determined according to the Michaelis-Menten equation:

$$J_{in} = \frac{J_{max} \times [Na^+]}{K_m + [Na^+]}, \tag{3}$$

where J_{max} is the maximal rate of sodium influx and K_m is the sodium concentration at which sodium influx is half maximal. Kinetic parameters were calculated directly from curves of sodium influx versus sodium concentration using SigmaPlot (ver. 6.0; SPSS). Reported kinetic values represent the sodium influx data from five or six individuals at each of six sodium concentrations.

Comparison of kinetic parameters was performed according to the recommendations of Motulsky (1998). Control (humic-

free) parameters (J_{max} or K_m) were compared to the kinetic parameters of other curves using a paired *t*-test on the parameter values and their SEMs as determined by best-fit Michaelis-Menten analysis. Multiple comparison corrections were not applied (Perneger 1998), although a conservative approach was taken whereby each sodium concentration used in the kinetic analysis was counted as a single point. Thus, for each pairwise comparison, the degrees of freedom used to determine significance ($P < 0.05$) from *t* tables was 10 ($2 \times n - 1$).

Results

The uptake of sodium by *Daphnia magna* was concentration dependent (Fig. 1) and could be described by Michaelis-Menten kinetics. While this relationship persisted in the presence of humic substances, the uptake curves, and consequently the kinetic parameters of uptake, were substantially altered. AHA addition significantly ($P < 0.05$) increased the maximal rate of sodium uptake (J_{max}; Fig. 2*A*). This effect was present even at the lowest AHA concentration tested (1.4 mg C L^{-1}) and peaked at an AHA level of 4.0 mg C L^{-1}, where maximal sodium influx reached 3.69 ± 0.36 μeq g wet weight^{-1} h^{-1}, up from a control (humic-free) level of 2.08 ± 0.15 μeq g wet weight^{-1} h^{-1}. A concomitant trend of increasing K_m (sodium concentration resulting in a half-maximal influx rate) was also observed with AHA addition, representing a tendency for decreasing affinity for sodium uptake. This latter trend was not statistically significant, with *P* values ranging from 0.09 to 0.14. The addition of SRN caused similar, statistically significant increases in both J_{max} and K_m (Fig. 3). These effects were present only at the highest SRN concentration examined (7.4 mg C L^{-1}) but were considerable in magnitude, with J_{max} values increasing from control values of 9.32 ± 1.07 to 20.00 ± 3.43 μeq g wet weight^{-1} h^{-1} and K_m values rising from 685 ± 186 to 3,450 ± 938 μM.

Considerable variability in sodium influx was noted throughout the experiments performed. For example, control values for K_m and J_{max} in Figure 3 for SRN were 7.8- and 4.5-fold higher than the corresponding values in experiments with AHA (Fig. 2). Similar though less dramatic discrepancies were noted between control values in other trials. This variability was of little consequence to the conclusions drawn owing to the simultaneous measurement of influx in both control and experimental animals. Possible explanations for control fluctuations will be discussed below.

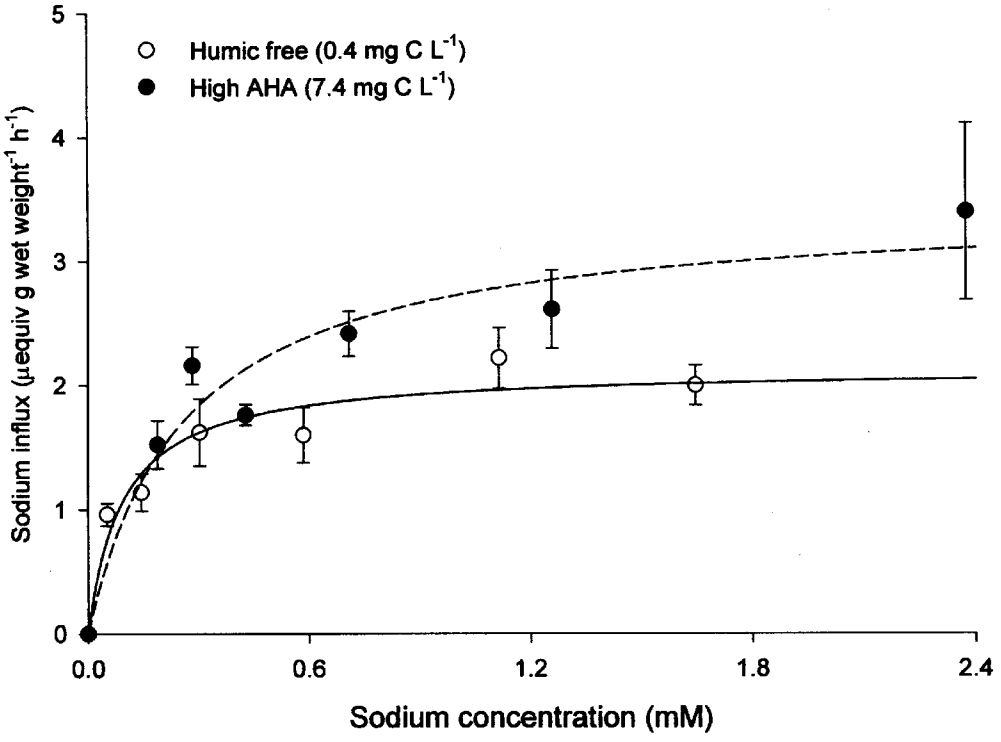

Figure 1: Example curve illustrating the effect of Aldrich humic acid (AHA) on whole-body sodium influx (μeq g wet weight^{-1} h^{-1}) in juvenile *Daphnia magna* as a function of increasing sodium concentration in both control (humic-free) exposure water (*solid line*) and high humic conditions (*dashed line*). Values are the mean ± SEM of five or six individuals at each sodium concentration, with kinetic curve fitted via regression analysis as described in "Material and Methods."

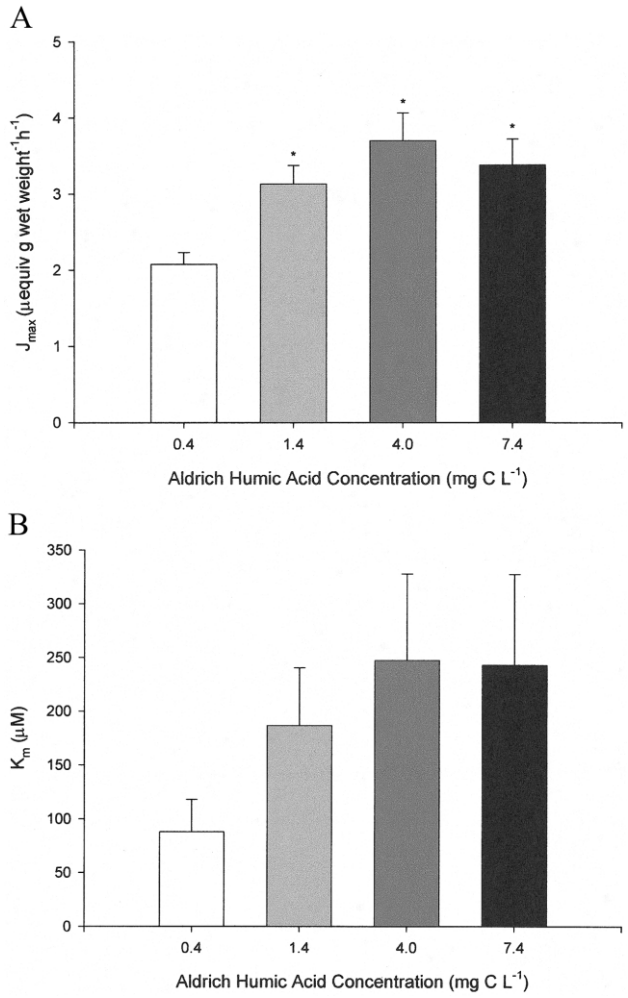

Figure 2: Effect of increasing Aldrich humic acid concentration on Michaelis-Menten parameters of whole-body sodium influx: (*A*) J_{max} (μeq g wet weight^{-1} h^{-1}) and (*B*) K_m (μM). These values and their standard errors were calculated directly from plots of sodium influx with increasing sodium concentration in five or six individuals at six sodium concentrations (nominally: 50, 150, 300, 600, 1,200, and 2,400 μM). Statistically significant differences (*asterisks*) between control (humic-free) and each concentration were assessed at the $P < 0.05$ level using statistical analysis as described in "Material and Methods."

Figures 4 and 5 illustrate the influence of humic substance preexposure on the kinetic parameters of sodium influx. *Daphnia* were preexposed to either control (humic-free) water or water containing 7.4 mg C L^{-1} of AHA or SRN for 24 h, and then sodium flux measurements were made. Again, measurements were made concurrently to account for control variability. There was a significant increase in J_{max} with preexposure to AHA (Fig. 4*A*), even in the absence of AHA during the flux measurements. Affinity constants (Fig. 4*B*) were not distinct, narrowly eluding statistical significance ($P = 0.07$ for control-preexposed, control-fluxed vs. AHA-preexposed, control-fluxed animals). This was very similar to the patterns discerned in Figure 2, suggesting that the modifying actions of AHA persist even after a 24-h preexposure, and these effects do not depend on the presence of AHA in the flux water. In contrast, there were no significant differences in either J_{max} or K_m when *Daphnia* were exposed to SRN (7.4 mg C L^{-1}). This was distinct from the significant actions of high SRN concentrations on uptake capacity and affinity observed in Figure 3.

Differences between AHA and SRN were also noted in sodium efflux experiments. No relationship between sodium ex-

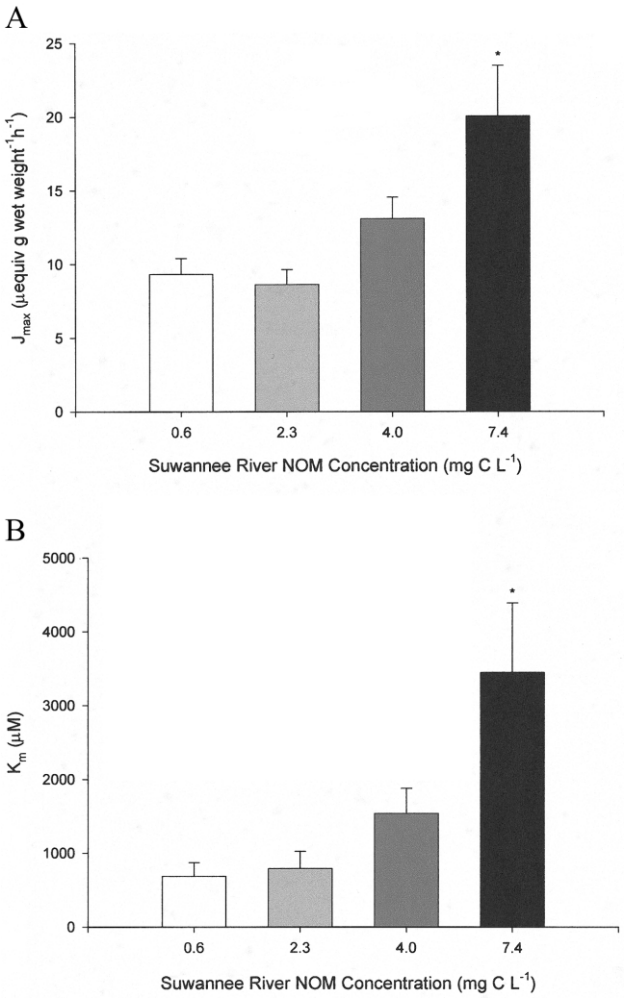

Figure 3: Effect of increasing Suwannee River natural organic matter (NOM) concentration on Michaelis-Menten parameters of whole-body sodium influx: (*A*) J_{max} (μeq g wet weight^{-1} h^{-1}) and (*B*) K_m (μM). These values and their standard errors were calculated directly from plots of sodium influx with increasing sodium concentration in five or six individuals at six sodium concentrations (nominally: 50, 150, 300, 600, 1,200, and 2,400 μM). Statistically significant differences (*asterisks*) between control (humic-free) and each concentration were assessed at the $P < 0.05$ level using statistical analysis as described in "Material and Methods."

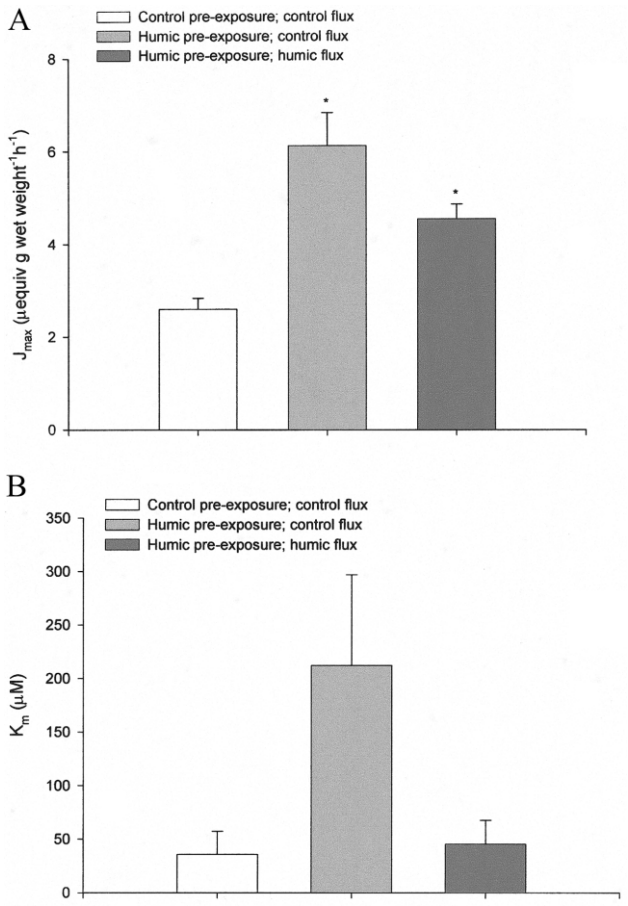

Figure 4: Effect of 24-h preexposure of *Daphnia magna* to Aldrich humic acid on Michaelis-Menten parameters of whole-body sodium influx: (*A*) J_{max} (μeq g wet weight^{-1} h^{-1}) and (*B*) K_m (μM). These values and their standard errors were calculated directly from plots of sodium influx with increasing sodium concentration in five or six individuals at six sodium concentrations (nominally: 50, 150, 300, 600, 1,200, and 2,400 μM). Statistically significant differences (*asterisks*) in kinetic parameters between the control (humic-free) preexposed, control (humic-free) fluxed animals (*white bars*) and the two experimental groups—humic preexposed, control fluxed (*light grey bars*) and humic preexposed, humic fluxed (*dark grey bars*)—were determined at the $P < 0.05$ level by the analysis described in "Material and Methods." Humic preexposure (24 h) and flux (1 h) were in the presence of 7.4 mg C L^{-1} Aldrich humic acid.

posure concentration and sodium efflux rate existed for either AHA or SRN (data not shown), and hence efflux could not be described in terms of Michaelis-Menten kinetics. Efflux rates for all animals under a given experimental regime were therefore pooled. *Daphnia* exposed to control conditions and fluxed in the absence of AHA exhibited no differences in sodium efflux in comparison to AHA-exposed or AHA-fluxed animals (Fig. 6*A*). Conversely, control-preexposed, control-fluxed daphnids had sodium efflux that differed significantly from that of control-preexposed, SRN-fluxed animals (Fig. 6*B*). This increased efflux was not present after 24-h preexposure to SRN. As efflux parameters were determined in a separate set of experiments, and in different animals from those described in influx, it was not possible to obtain relevant net flux parameters.

The influence of AHA on whole-body sodium levels of *D. magna* is shown in Figure 7. For all curves, body sodium levels peaked at exposure concentrations closest to the sodium concentration in which the animals were raised (600 μM). Exposure to concentrations less than or greater than this level decreased total body sodium. The addition of AHA resulted in enhanced

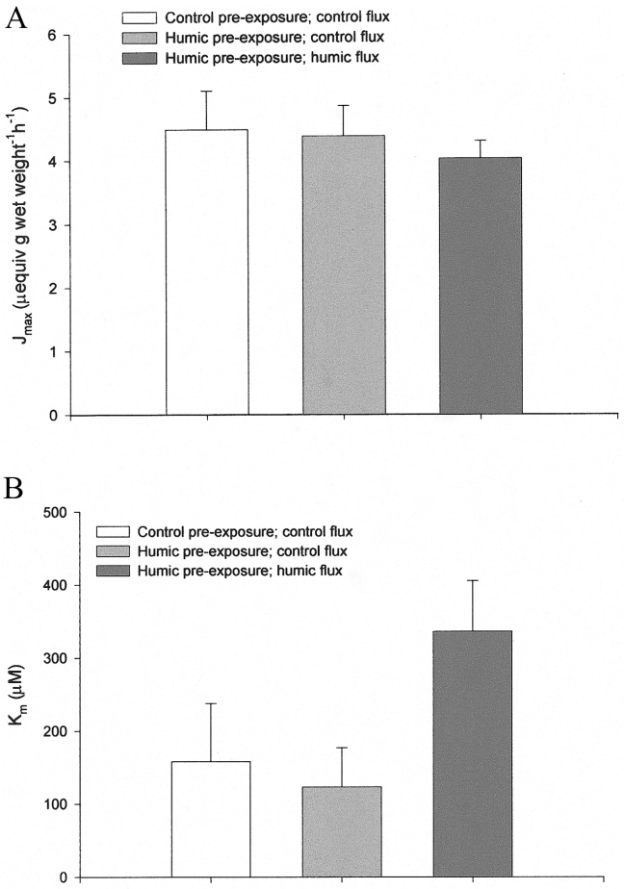

Figure 5: Effect of 24-h preexposure of *Daphnia magna* to Suwannee River natural organic matter on Michaelis-Menten parameters of whole-body sodium influx: (*A*) J_{max} (μeq g wet weight^{-1} h^{-1}) and (*B*) K_m (μM). These values and their standard errors were calculated directly from plots of sodium influx with increasing sodium concentration in five or six individuals at six sodium concentrations (nominally: 50, 150, 300, 600, 1,200, and 2,400 μM). There were no statistically significant differences in kinetic parameters between the control (humic-free) preexposed, control (humic-free) fluxed animals (*white bars*) and the two experimental groups—humic preexposed, control fluxed (*light grey bars*) and humic preexposed, humic fluxed (*dark grey bars*)—as determined at the $P < 0.05$ level by the analysis described in "Material and Methods." Humic preexposure (24 h) and flux (1 h) were in the presence of 7.4 mg C L^{-1} Suwannee River natural organic matter.

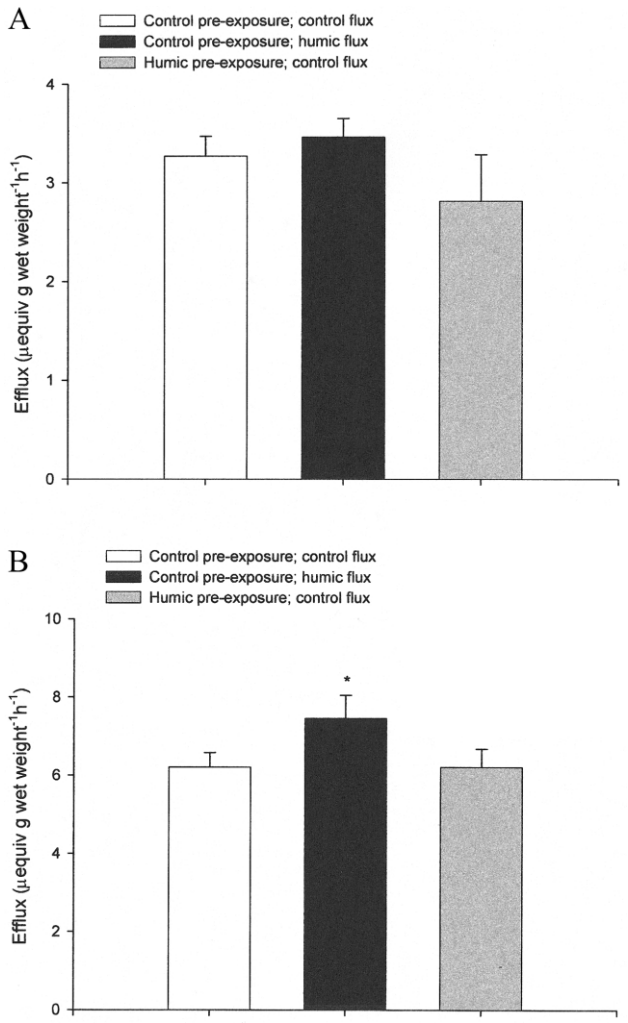

Figure 6: Effect of (*A*) AHA or (*B*) SRN on *Daphnia magna* sodium efflux (μeq g wet weight^{-1} h^{-1}). Efflux in daphnids preexposed to control (humic-free) conditions and fluxed in control water (*white bars*) is compared to efflux in daphnids preexposed to control conditions and fluxed in humic substance (7.4 mg C L^{-1}) (*black bars*) and daphnids preexposed to humic substance (7.4 mg C L^{-1}) and fluxed in control conditions (*grey bars*). Sodium efflux was independent of exposure sodium concentration (data not shown); hence, each plotted value is the pooled mean ± SEM of 30–36 individually fluxed animals exposed to one of six sodium concentrations. Statistically significant differences (*asterisks*; $P < 0.05$) from the control-preexposed, control-fluxed condition were determined by two-way ANOVA, with sodium concentration as an independent factor, followed by post hoc LSD analysis.

sodium body levels in a concentration-dependent manner. This corroborates the findings of the flux experiments, where an increased influx and unchanged efflux would be expected to result in enhanced sodium body status.

SRN, which increased both sodium influx and sodium efflux, had a more inconsistent influence on whole-body sodium, with no unambiguous concentration dependence (data not shown). Figure 8 shows the whole-body sodium levels of *Daphnia* fluxed at the highest SRN concentration (the concentration at which effects on J_{max} and K_m are significant; Fig. 3*A*, 3*B*) in both humic-preexposed and control-preexposed animals. Relative to the control, and in contrast to AHA, SRN tended to decrease whole-body sodium content. After 24 h preexposure to SRN, whole-body sodium levels increased back to control levels.

A follow-up experiment was conducted in which the pH of humic solutions was adjusted to 5.6, the pH of control solutions. This represented a downward pH adjustment with 1 N HNO_3 for AHA solutions and an upward pH adjustment with 1 N KOH for SRN solutions. These results (Table 1) show that similar stimulatory effects of humic substances on sodium influx J_{max} were observed, but there were no longer any significant changes in K_m. However, the SRN levels in the pH experiment were lower than those in the initial experiments (5.3 vs. 7.4 mg C L^{-1}), and this may account for the lack of K_m effect. The decreased whole-body sodium recorded with SRN also suggests that the efflux effect persisted at control pH, while increased whole-body sodium with AHA was in accord with the earlier results.

Discussion

Humic Substances Increase Sodium Influx in an Uncompetitive Manner

AHA and SRN have significant effects on sodium metabolism in *Daphnia magna*. Both act to increase the capacity of sodium transport while lowering the affinity of the influx process. This is indicative of an uncompetitive effect on sodium uptake (Cornish-Bowden 1979) and suggests that these substances do not interact directly with the sodium transport entities but instead have a more generalised action.

There is now considerable evidence that humic substances can adsorb to biological membranes of aquatic organisms (e.g., Gerritsen and Bradley 1987; Parent et al. 1996; Campbell et al. 1997). Furthermore, it has been established that this binding can increase the permeability of the membrane (Parent et al. 1996; Vigneault et al. 2000). An effect of humic substances on membrane properties would explain the findings of an uncompetitive stimulation of sodium influx. Changes in membrane fluidity would make the apical surface more porous, essentially increasing the sodium uptake capacity. Concomitantly, changes in membrane fluidity could alter the membrane phospholipid-transporter interaction (Lagerspetz and Laine 1987), causing a change in protein conformation and resulting in a decreased affinity for sodium. It has been shown that membrane fluidisation leads to an enhanced passage of sodium ions across frog skin (Lagerspetz and Laine 1987), while membrane fluidity has also been observed to modulate the activity of sodium proton exchangers in cultured fibroblasts (Bookstein et al. 1997).

It is important to note that, to date, the binding of humic

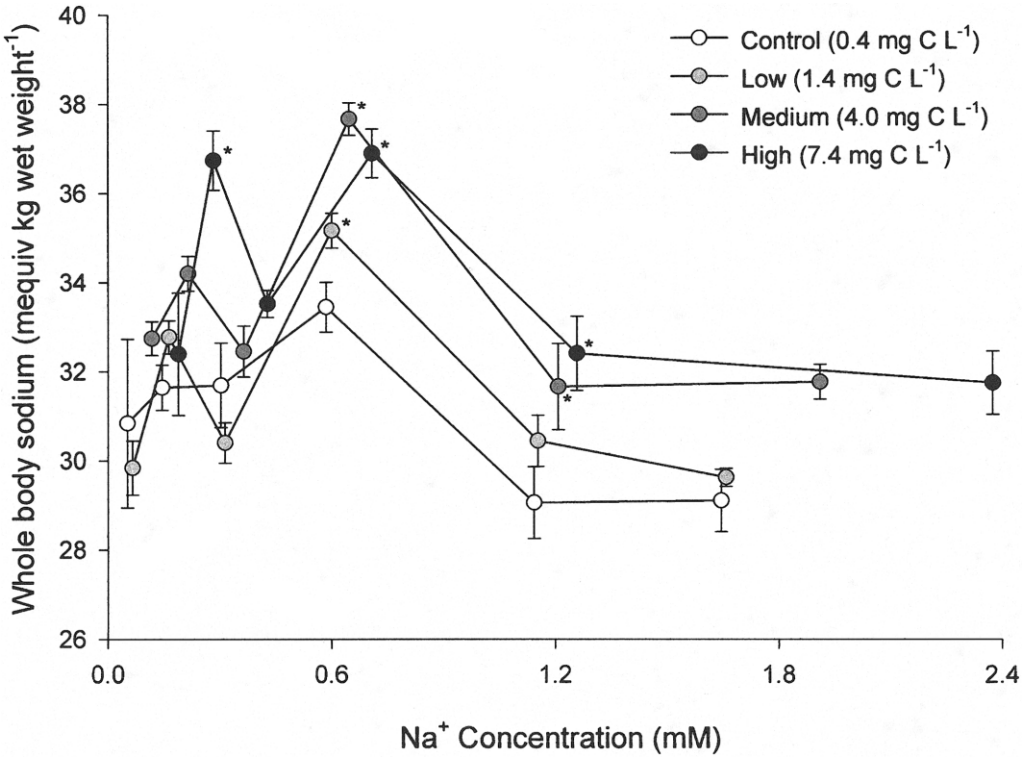

Figure 7: Whole-body sodium content (meq kg wet weight^{-1}) of *Daphnia magna* exposed to increasing concentrations of Aldrich humic acid (humic-free [0.4], 1.4, 4.0, or 7.4 mg C L^{-1}) in exposure waters of increasing sodium concentration (nominally: 50, 150, 300, 600, 1,200, and 2,400 μM). Plotted values represent the mean ± SEM of five or six individuals at each concentration and were taken from the experiment described in Figure 2. Significant differences (*asterisks*; $P < 0.05$) from control whole-body sodium were tested at water sodium values that were relatively consistent for all treatments (300, 600, and 1,200 μM) via one-way ANOVA and post hoc LSD analysis.

substances to biological surfaces has been observed only in vitro and is promoted by acidic conditions (Parent et al. 1996; Campbell et al. 1997; Vigneault et al. 2000). While the SRN effects on J_{max} were observed at acidic pH's (4.4), the similar action of AHA at circumneutral pH (6.6), and of both humic materials in pH-corrected experiments (5.6), suggests these effects are unlikely to be a consequence of the acidic water environment. The action of SRN on K_m, however, dissipates with increased pH (Table 1), suggesting that this effect may be pH dependent, although this may have been a consequence of the reduced SRN levels in the pH-corrected experiments. This does not preclude the possibility that pH may be responsible for the observed actions because the potential for acidification of the uptake surface microenvironment, as occurs at the piscine gill (Playle and Wood 1989), exists.

While pH facilitation of humic substance binding to gill surfaces cannot be excluded at this stage, an additional putative mechanism of action is proposed. Calcium sequestration by humic substances has been hypothesised to influence the passage of ions across the branchial epithelium of fish (Gonzalez et al. 1998; Wood et al. 2003). Calcium is proposed to have important roles in the permeability of both the tight junctions and the cell membrane itself (see Matthews 1986). In particular, it is proposed that decreases in bioavailable calcium, such as those likely to occur via humic substance chelation, would stimulate an increase in cell membrane permeability (Matthews 1986). This would promote an effect similar to that seen in this study. Further investigations into the mechanism of humic substance action on ion metabolism are required to delineate whether these entities are modulating sodium uptake by direct (i.e., membrane-binding) or indirect (i.e., cation chelation) actions.

SRN, but Not AHA, Promotes Sodium Efflux and Engenders an Acclimation Response

While both humic substances examined had influences on sodium influx, only SRN induced changes in efflux. Ion efflux in daphnids is stimulated by acidic conditions (Potts and Fryer 1979; Havas et al. 1984). This does not, however, explain the significant efflux effect observed in the presence of SRN, as it persisted at control pH (significantly decreased whole-body sodium; Table 1). Humic substances are characterised by their heterogeneity, an attribute that is likely to extend to their com-

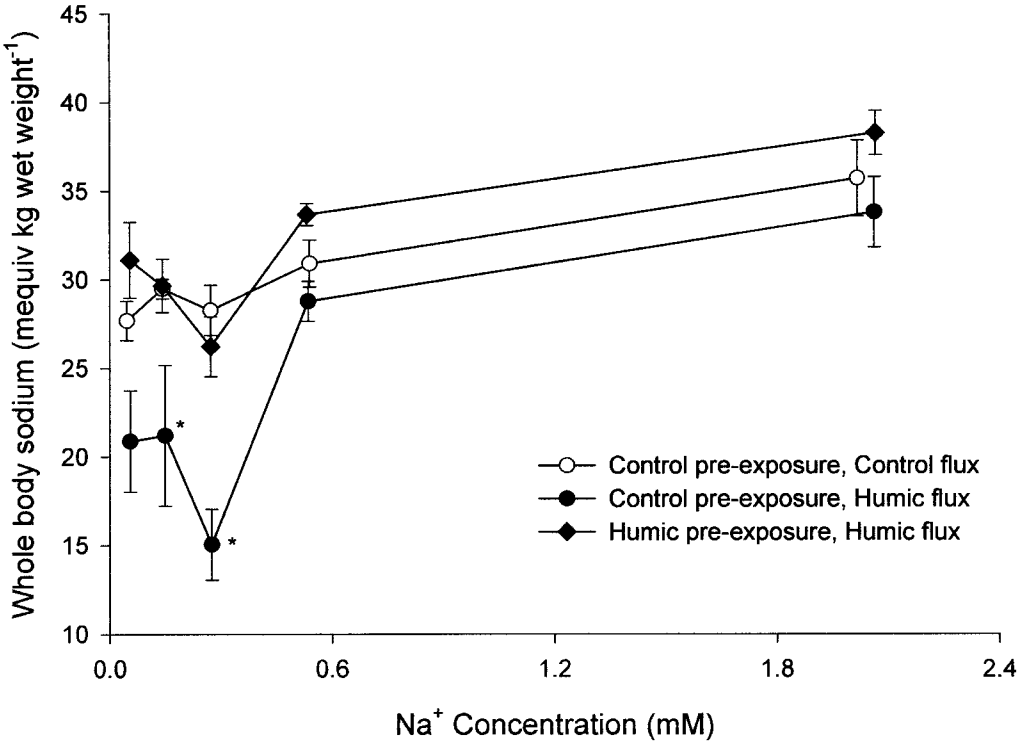

Figure 8: Whole-body sodium content (meq kg wet weight^{-1}) of *Daphnia magna* from three different exposure regimes: control preexposure, control flux (*open circles*); control preexposure, fluxed in 7.4 mg C L^{-1} Suwannee River natural organic matter (SRN; *solid circles*); or 24-h preexposure to SRN (7.4 mg C L^{-1}) fluxed in 7.4 mg C L^{-1} SRN (*solid diamonds*). Each plotted point represents the mean ± SEM of five or six individuals at each sodium concentration (nominally: 50, 150, 300, 600, and 2,400 μM) and were taken from the experiment described in Figure 5. Statistically significant differences (*asterisks*) between control preexposed, control fluxed animals and other groups were tested at the $P < 0.05$ level by one-way ANOVA followed by post hoc LSD analysis.

plexation affinities for divalent cations. In fact, recent research from our laboratory has shown that although humic substances are considered to be equal in terms of their protective effects for the purposes of predictive toxicity modelling (see, e.g., Di Toro et al. 2001), they actually have highly variable protective effects on silver toxicity to *Daphnia*, probably due to different complexation affinities for the metal toxicant (C. N. Glover, R. C. Playle, and C. M. Wood, unpublished manuscript). It is therefore postulated that the differences between SRN and AHA (stronger effects of SRN on influx [see Fig. 3*A*, 3*B*; cf. Fig. 2*A*, 2*B*] and an influence on efflux [Fig. 6]) may be a consequence of an enhanced ability of SRN to sequester calcium and thus more greatly influence epithelial permeability. This hypothesis is the focus of ongoing research.

SRN, but not AHA, induces an acclimation effect. After a 24-h preexposure to SRN, the effects of SRN on sodium metabolism seen under acute conditions (Figs. 2*B*, 3*B*) have been lost. Kinetic parameters were returned to levels similar to those in control animals. Conversely, for AHA, the J_{max} stimulation effect is still present after a 24-h preexposure. This suggests that these two humic substances have different modes of action. SRN effects appear to engender a physiological compensation, whereas AHA effects persist independently of AHA in flux waters.

This apparent physiological compensation may be related to the decline in whole-body sodium observed with SRN exposure (Fig. 8). When whole-body sodium levels fall to a certain threshold, a response to minimise the loss is induced and acclimation occurs, represented by a return of whole-body sodium levels to control values (Fig. 8). In the presence of AHA, whole-body sodium levels do not fall, and consequently the trigger to acclimate is not forthcoming.

Sodium Metabolism Was Highly Variable in Control (Humic-Free) Conditions

The values obtained for J_{max} and K_m in this study fall reasonably close to previously reported values for cladocerans. Maximal uptake rates are not easily comparable between studies because of different methods of calculation; however, the affinity constants in humic-free water (0.09–0.7 μeq L^{-1}) are similar to those ascertained previously (0.05–0.4 μeq L^{-1}; Stobbart et al.

Table 1: Effect of pH adjustment to 5.6 on kinetic parameters of sodium uptake and whole-body sodium levels in *Daphnia magna* exposed to humic substances

	AHA		SRN	
	Control (Humic Free)	Humic	Control (Humic Free)	Humic
J_{max} (μeq g wet weight^{-1} h^{-1})	2.59 ± .37	4.11 ± .65[a]	1.42 ± .13	2.65 ± .21[a]
K_m (μM)	135 ± 83	153 ± 79	21 ± 13	31 ± 18
Whole-body sodium (meq kg wet weight^{-1})	33.2 ± .7	34.6 ± .8[b]	32.9 ± .8	31.3 ± .7[b]

Note. Values represent the mean ± SEM calculated from five or six individuals at each of six sodium concentrations as described in "Material and Methods." Whole-body sodium concentrations reported here are pooled means for *Daphnia* at all tested sodium levels. Total organic carbon levels were as follows: in humic-free conditions, <0.6 mg C L^{-1}; for AHA and SRN exposures, 5.0 ± 0.0 and 5.3 ± 0.4 mg C L^{-1}, respectively.

[a] Significant differences ($P < 0.05$) between exposed and corresponding control (humic-free) conditions were determined by statistical analyses described in "Material and Methods."

[b] Significantly different ($P < 0.05$) whole-body sodium level compared to at least one corresponding control sodium concentration as determined by pairwise comparison of values by *t*-test.

1977; Potts and Fryer 1979). It is interesting to note that the variation within our study encompasses a wider range of values than those described between different cladoceran species, inhabiting considerably different waters, with affinity determined using different techniques. Potts and Fryer (1979) examined the kinetics of sodium influx in three different cladoceran populations. Two of these populations were of the same species (*Daphnia magna*), but in relatively recent times, one had become isolated in a comparatively salt-enriched environment. Noting the apparently superfluous high uptake affinity of this population, it was suggested that adaptation of sodium transport was a process that occurred over centuries. Furthermore, differences in K_m were only 3.1-fold between two populations inhabiting waters of sodium content of 0.146 and 12.4 mM. In this study, experiments were run on consecutive days on animals raised from the same mixed population of neonates, kept under identical conditions, yet control (humic-free) affinity constants varied by up to 7.8-fold. While this finding does not discount that adaptation may take many years, the huge variation in control values from day to day suggests that sodium uptake is far more labile. This variability also strongly enforces the concept of running control experiments parallel with exposure regimes to ensure that any conclusions drawn are not the result of fluctuations caused by inherent physiological rhythms.

The cause of such flexibility in uptake parameters is unknown but may be related to the moulting cycle. *Daphnia* moult frequently. Each instar lasts 2–4 d and terminates with a moult (Peltier and Weber 1993). Zare and Greenaway (1998) noted increased sodium uptake in postmoult freshwater crayfish, coupled with enhanced activity of V-ATPase and Na^+,K^+-ATPase, enzymes important for creating the gradient for sodium influx. The twofold increase in enzyme activities noted in crayfish was much smaller than the changes in influx noted herein. However, given the smaller size of *Daphnia* (~1 mg vs. 16 g) and the concomitant increased body surface to volume ratio, the relatively greater sodium turnover in *Daphnia* (Bianchini et al. 2002; Grosell et al. 2002) would be accompanied by a parallel larger increase in postmoult sodium demand.

Environmental Significance

The significant effects on kinetic parameters described in our study were discerned at environmentally realistic levels of humic substances. Dissolved organic matter concentrations in natural waters range from 1 to 15 mg C L^{-1} (Thurman 1985). Significant actions for AHA were discerned as low as 4.0 mg C L^{-1}, suggesting that even at moderate concentrations, humic substances may have important influences on the physiology of aquatic organisms. It is important to note, however, that commercially available humic substances, such as those used in this study, may differ from humic substances isolated directly from natural waters (Malcolm and MacCarthy 1986). In a recent report, a commercial organic carbon source (AHA) failed to re-create the protective effects of natural Rio Negro blackwaters on sodium and chloride fluxes in freshwater stingrays exposed to low pH (Wood et al. 2003). In fact, the commercial form exacerbated the increases in efflux. Given such an observation, the conclusions drawn from this investigation should be treated with caution until sodium metabolism is investigated in the presence of "real-world" humic substances.

The ability of humic substances to bind, and therefore detoxify, environmental metal pollutants is well described (see, e.g., Di Toro et al. 2001). For metals such as copper and silver, this chelating action reduces the levels of the toxic free ionic form of the metal. This consequently limits the capacity of these elements to interact at toxic loci. Copper and silver both exert their actions by interfering with sodium metabolism (Bianchini et al. 2002; Grosell et al. 2002). Both these elements appear to traverse the apical membrane of uptake surfaces via

sodium channels (Bury and Wood 1999; Grosell and Wood 2002) and subsequently inhibit the basolateral Na^+,K^+-ATPase (Laurén and McDonald 1987; Bianchini and Wood 2003), the enzyme responsible for creating the electrochemical gradient driving sodium through these channels. It is likely that both copper and silver also interfere with carbonic anhydrase (Grosell et al. 2002), the enzyme providing acid equivalents assisting the passage of sodium across the apical surface. The enhanced uptake of sodium in the presence of humic substances (by J_{max} stimulation) would assist in the maintenance of sodium levels, while the reduced affinity of the sodium uptake process may restrict the passage of silver and copper across the apical membrane. By such mechanisms, humic substances could help protect against metal toxicity independent of their ability to bind metals. Conversely, the reduced whole-body sodium in response to SRN exposure may make daphnids more vulnerable to metal toxicants. Our investigation suggests that the presence of humic substances may have considerable implications in the toxic response of aquatic organisms to ionoregulatory toxicants.

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