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A Matter of Potential Concern: Natural Organic Matter Alters the Electrical Properties of Fish Gills

FERNANDO GALVEZ,*,†,‡ ANDREW DONINI,‡ RICHARD C. PLAYLE,§,|| D. SCOTT SMITH,⊥ MICHAEL J. O'DONNELL,† AND CHRIS M. WOOD‡,#

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803-1715, Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1, Departments of Biology and Chemistry, Wilfrid Laurier University, Waterloo, Ontario, Canada N2L 3C5, and Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Miami, Florida 33149

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Natural organic matter (NOM) is an important constituent of aquatic environments; however, its influence on aquatic biota remains poorly studied. In the current study, NOM was isolated from nine different sites in southern Ontario, Canada, by the on-site treatment of water by reverse osmosis, followed by cation exchange. NOM from each site was reconstituted to 10 mg of C/L and pH 7.0 and exposed to either adult rainbow trout implanted with indwelling catheters or to in vitro primary cultures of the gill epithelium grown on semipermeable membranes. In both the in vivo and in vitro preparations, NOM was found to hyperpolarize transepithelial potential (TEP), with the magnitude of this change correlating extremely well to the absorptivity of the NOM at 340 nm, which is an index of its aromaticity. Gill hyperpolarization appeared to be independent of Ca^{2+} complexation by the NOM in all but two samples tested. We argue that NOM has direct actions on the ionic transport and/or permeability properties of fish gills. While NOM effects on the bioavailability of contaminants are well-known, NOM actions on such fundamental physiological properties of the gills have previously been overlooked. These may be of comparable or greater magnitude than commonly reported for other water-quality variables (e.g., hardness, pH, salinity) and therefore of critical importance in ecological understanding and risk assessment.

Introduction

Natural organic matter (NOM; commonly known as dissolved organic carbon, DOC) is an important regulator of biogeochemical processes such as global nutrient and carbon cycling, metal redox reactions, and cation complexation (*1*). This functional diversity is largely associated with its high degree of chemical heterogeneity. NOM is often subdivided into two major classifications. Allochthonous NOM consists almost exclusively of terrestrially derived lignin-degradation products, containing high levels of aromatic humic and fulvic substances, enriched in carboxylic and phenolic groups. This high aromatic content imparts significant ultraviolet light absorptivity and makes allochthonous NOM samples darkly colored (*2, 3*). Autochthonous NOM is formed endogenously in water bodies from photosynthetic activity or bacterial degradation of allochthonous NOM (*2*). Chemically, these NOM samples are enriched in carbohydrate and nitrogen functional groups and are characterized by low aromaticity, diminished UV absorptivity, and pale color (*2, 3*).

The ability of NOM to affect aquatic biota indirectly by exerting strong control on the surrounding biogeochemical conditions is well established (reviewed in refs *4* and *5*) due to their ability to complex inorganic cations, including metals (e.g. ref *6*) and a variety of organic compounds (e.g., *7*). In contrast, the potential direct interaction of these amphiphilic compounds with aquatic organisms themselves has been largely overlooked, until recently. However, in the past decade, there has been growing recognition that NOM can directly affect the physiology of organisms (reviewed in refs *5* and *8*), with effects as diverse as activation of glutathione S-transferase (*9*), induction of heat shock proteins (*10*) and CYP1A (*11*), and changes in behavior and the global transcriptome (*12*). Humic substances have been shown to elicit a stress response in aquatic animals, thereby helping fish to survive under extreme environmental conditions and prolonging life in *Caenorhabditis elegans* (*1*).

Of particular interest is recent evidence of NOM interactions with the exchange surfaces of aquatic organisms. As early as 1985, Visser (*13*) and Munster (*14*) described the interactions of humic substances on biological membranes. More recently, Campbell et al. (*15*) provided evidence for the sorption of allochthonous NOM on the surfaces of algae and fish gill cells at acidic pH. Although the exact sorptive mechanism was not deciphered, it was proposed that electronegative functional groups on the NOM formed hydrogen bonds with biological surfaces or, alternatively, that hydrophobic interactions between the NOM and plasma membrane were formed. Vigneault et al. (*16*) found that NOM was able to integrate into phytoplankton and other model membranes, leading to enhanced permeability to fluorescent markers at acidic pH. NOM was reported to influence the flux of Na^+ at the gills of freshwater *Daphnia* (*17, 18*) and fish (*19, 20*) at a near-circumneutral pH, and to protect against the deleterious effects of low pH on Na^+ and Cl^- fluxes in acidic environments (*20, 21*), although no mechanistic basis for these NOM-induced effects was provided.

This study was stimulated by our casual observation, during the course of an unrelated study on the effects of NOM on silver biovailability, that NOM alone was exerting an apparent influence on the transepithelial potential (TEP) across the gills of freshwater rainbow trout. Therefore, an investigation of the phenomenon was undertaken, incorporating whole-animal and cell-culture-based approaches to study the impact of different sources of NOM on the electrophysiology of the fish gill. On the basis of the fact that NOM may adsorb to (*15*) or integrate into (*16*) or have both effects (*22*) at the surfaces of biological membranes, we predicted that the electrophysiological characteristics of the fish gill may be particularly sensitive to binding of this highly heterogeneous compound. The presence of many charged

* Corresponding author phone: (225) 578-0599; fax: (225) 578-2597; e-mail: galvezf@lsu.edu.
† Louisiana State University.
‡ McMaster University.
§ Department of Biology, Wilfrid Laurier University.
|| R.C.P. passed away after the completion of this research.
⊥ Department of Chemistry, Wilfrid Laurier University.
University of Miami.

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functional groups in many sources of NOM might be expected to influence the electrical potential across the gill epithelium, which may provide an explanation for the NOM-induced effects on Na^+ transport or the alterations in membrane permeability in diverse aquatic biota. To better ascertain the influence of NOM heterogeneity on potential electrophysiological effects, NOM was isolated from nine different sites in southern Ontario. The NOM samples represented a continuum from autochthonous to highly allochthonous in nature, allowing us to assess the influence of NOM aromaticity on the electrical properties of the fish gill. A commercial humic acid (Aldrich) treatment and control Lake Ontario water were also used.

Experimental Section

Isolation of NOM. NOM was collected from Lake Ontario (control water) = C (43°,16′ N, 79°,54′ W), Luther Marsh = LM (43°,37′ N, 80°,26′ W), Sanctuary Pond = SP (41°,59′ N, 82°,33′ W), Trout Lake = Tr (46°,32′ N, 79°,46′ W), Talon Lake = TL (46°,33′ N, 79°,12′ W), Lake Nippissing = LN (46°,15′ N, 80°,47′ W), the Dundas Sewage Treatment Plant = D (43°,16′ N, 79°,54′ W), Four Mile Lake = FM (46°,34′ N, 79°,24′ W), Grand River = GR (43°,27′ N, 80°,22′ W), and Lake Erie = LE (41°,58′ N, 82°,32′ W). Lyophilized humic acid (Sigma-Aldrich Chemical, St. Louis, MO) = AH was used as a positive control. Sample collection involved initial prefiltration using 1 μm glass fiber filters (Geotech Environmental Equipment, Denver, CO), followed by NOM concentration using a portable, stainless steel reverse-osmosis unit with a molecular mass cutoff of 400 Da. These concentrates were treated with a cation exchange resin (Amberlite IR-118H, Sigma) and stored at pH 2.0. Before experimental use, the NOM samples were diluted to 10 mg of C/L with deionized water and neutralized with NaOH. The relative color of the NOM samples was ascertained by application of the specific absorption coefficient (SAC) at 340 nm (pH 7.0) as described previously (*3, 6, 23*). Briefly, the SAC at 340 nm was normalized to the total organic concentration (TOC), measured by subtracting the inorganic carbon concentrations from the total carbon concentration (Shimadzu TOC-5050A, Mandel Scientific, Guelph, ON, Canada). Absorption provided by each NOM sample was measured in polystyrene cuvettes (1 cm path length) at 340 nm using a Milton Roy 301 spectrophotometer. The $SAC_{340\ nm}$ was calculated using the following equation:

$$SAC_{340\ nm} = 2303(\text{absorbance}_{340\ nm})/TOC(2,24) \qquad (1)$$

Experimental Animals. Adult rainbow trout (*Oncorhynchus mykiss*) of both sexes were obtained from the Humber Springs Trout Hatchery (Orangeville, ON, Canada) and held under laboratory conditions for at least two weeks before experimentation. The fish were held in ~400 L polyethylene tanks, supplied with dechlorinated Hamilton tapwater originating from Lake Ontario (control water; water hardness 140 mg/L as $CaCO_3$; ion concentration (mM), 1.0, Ca^{2+}; 0.2, Mg^{2+}; 0.6, Na^+; 0.2, K^+; 0.7, Cl^-; 3 mg/L dissolved organic carbon; temperature of 12 °C) at a rate of ~2 L/min.

In Vivo Experiments. Trout were anesthetized with 0.05 g/L MS-222 and surgically implanted with PE-50 polyethylene tubing (Clay Adams) as described previously (*25*). Cannula were filled with heparinized Cortland saline (sodium heparin, 50 IU/mL) to prevent clotting and sealed until the start of experimentation. Fish were allowed to recover for 2 days in darkened plexiglass containers, which were each supplied with flowing, well-aerated control water. Transepithelial potentials (TEPs) were measured by standard techniques (*26, 27*) using agar-salt bridges (3 M KCl in 2% agar) connected through Ag/AgCl half-cells (World Precision Instruments, WPI) to a high-impedance electrometer (Radiometer pHM 84). One agar bridge was connected directly to the blood-filled cannula entering the animal, whereas another agar bridge was placed in the water adjacent to the operculum. All TEP measurements were expressed relative to the apical side, which was normalized to 0 mV, and corrected for junction potentials.

In Vitro Preparations of the Cultured Fish Gill. Primary cultures of trout gill epithelia (*28*) were prepared using the double-seeded insert technique (*29*), with modifications as outlined by Kelly et al. (*30*). This preparation duplicates the passive properties of the intact gill in vivo, but exhibits only slight active uptake of ions from apical water (*28*). Over the course of two successive days, enzymatically dispersed gill cells were seeded onto semipermeable cell culture inserts (Falcon, pore density 1.6×10^6 pores/cm^2, pore size 0.4 μm), which were held in multiwell cell culture insert plates (Falcon). Both surfaces of the inserts were bathed in Leibovitz's L-15 medium (Gibco) supplemented with 2 mM glutamine, 5% fetal bovine serum (FBS), and antibiotics. The epithelial cultures were monitored daily for changes in transepithelial resistances (TERs) using STX-2 chopstick electrodes connected to a specially modified 200 kΩ EVOM epithelial voltohmmeter (WPI, Sarasota, FL). Once the TER in cultured epithelia reached a plateau (~30 kΩ·cm^2) at approximately 7−9 days, the gill cultures were transferred from symmetrical conditions to asymmetrical conditions by replacing the L-15 medium on the apical side with control water (*30*). The cultures were maintained under these asymmetrical conditions for approximately 1 day to allow the TERs to stabilize. Upon stabilization of these signals, control water was replaced with one of the different sources of NOM (see above) for analyses of the TER and TEP. The TER was measured with the chopstick electrode system described above. The TER of blank inserts (cell culture inserts alone with no gill cells) bathed basolaterally with L-15 medium and apically with the appropriate test water was subtracted from the TER values obtained for cultured gill epithelia. The TER values (kΩ) provided by the voltohmmeter were normalized to the surface area of the cell culture insert by multiplying the blank-corrected TER values by 0.9 cm^2 (the area of each insert). The TEP was measured exactly as described in vivo, with the measurement bridge placed in the basolateral L-15 medium (i.e., "blood side") and the reference bridge placed in the apical test water. All TEP measurements were again expressed relative to the apical side, which was normalized to 0 mV, and corrected for junction potentials.

Exposure of Cannulated Trout and Gill Cell Cultures to NOM. TEPs were measured in vivo via indwelling catheters in the dorsal aortas of adult trout. The volume of the fish chambers were set to 2.5 L, and inflow was stopped, while vigorous aeration was maintained. At the beginning of a test run, the trout was routinely exposed to distilled water, which would cause a large hyperpolarization of the TEP (*26, 31*) (see Figure 2A), to check that the agar bridges were operational. The fish were transferred between NOM-enriched waters rapidly, with minimal air exposure. Since the effects of NOM on the TEP were reversible (see Figure 2B,C), the same fish was routinely exposed to multiple sources of NOM. Typical experiments involved transferring the fish from control water (Lake Ontario water) to one of the NOM samples for approximately 15 min, after which the TEP was almost always stable. The effect of NOM on TEP was not instantaneous but developed over several minutes before reaching an approximate plateau. The TEP was recorded after 15 min posttransfer to the NOM, although the TEP was essentially constant over 3 h of exposure. The animals were then transferred back to the control water and allowed to recover before being transferred to an alternate NOM source. The order of the NOM exposures was randomized to eliminate any bias. The influence of each NOM on the TEP was

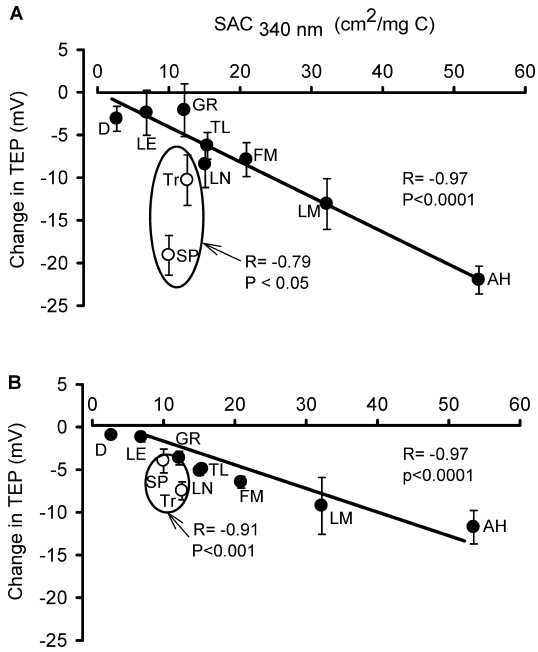

FIGURE 1. Effects of 10 mg of C/L NOM on the TEP of (A) cultured gill epithelia derived from adult rainbow trout ($N = 10$ per NOM) or (B) cannulated adult rainbow trout ($N = 6$ per NOM). This figure describes the relationship between the specific absorption coefficient at 340 nm ($SAC_{340\ nm}$, cm^2/mg of C) and the absolute change in TEP (mV) across epithelia upon transfer from control (Lake Ontario) water and water containing NOM. TEPs are expressed relative to the apical side of membranes and are corrected for junction potentials. The Pearson coefficient of regression (r) and the p values are reported with the inclusion of all data or all the data excluding two NOM sources (Trout Lake and Sanctuary Pond, circled; see the text and Figure 2 for additional details) which were excluded due to their ability to greatly lower the Ca^{2+} activity (see Table 1 and Figure 4). The footnote of Table 1 provides NOM source abbreviations. Data are expressed as the mean ± SEM (M).

calculated as the difference between the mean of the control TEP values before and after a particular NOM exposure and the stable TEP value obtained during an NOM exposure.

In contrast to the in vivo tests, cultured gill epithelia were transferred from control water to only one NOM source to avoid complications of membrane damage during excessive handling. Before exposure of the cultured gill epithelia to NOM, the TER and TEP were measured as described above to obtain pre-0 h values. Subsequently, the apical sides of each preparation were washed three times with 1.5 mL of control water (controls only) or NOM-containing water. The TER and TEP were monitored continuously for up to 3 h post-transfer. As in the in vivo tests, the values changed progressively over several minutes before reaching an apparent plateau; data were recorded at 15 min.

Ca^{2+} Complexation by NOM. Ion activities in the presence of each NOM at 10 mg of C/L were measured using ion-selective microelectrodes (*32*). Microelectrodes demonstrated Nernstian behavior down to 5 μM Ca^{2+}, $CaCl_2$ (slope 32.8 ± 2.3 mV per decade) ($n = 6$). Total Ca concentrations, as well as total Na and K concentrations, were measured by flame atomic absorption spectroscopy, Varian AA-220FS.

Statistics. Data are reported as the means ± standard error of the mean (SEM) (N). Homogeneity of variances was evaluated using the Levene test. A one-way analysis of variance (ANOVA) was used to test for statistical significance. The fiducial level of significance was set at $p < 0.05$. All analyses were conducted using SPSS, version 10.

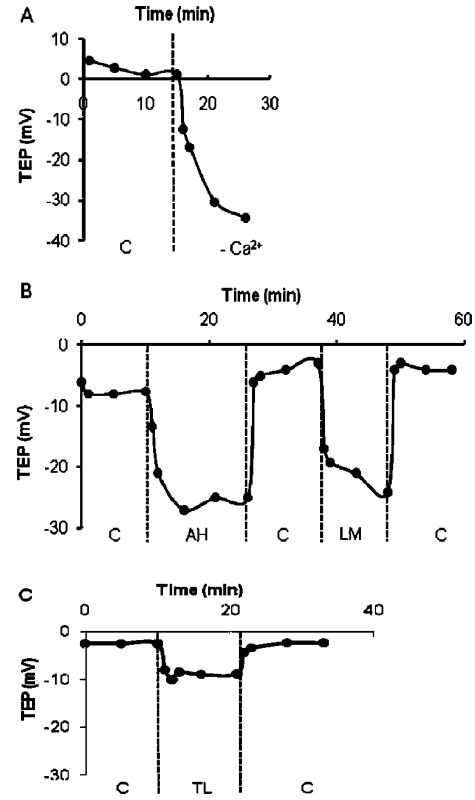

FIGURE 2. Representative profiles of the transepithelial potential (mV) in vivo in cannulated trout exposed to (A) pure distilled water (− Ca^{2+}) as a positive control, (B) control Lake Ontario water (C), Aldrich humic acid (AH), or Luther Marsh water (LM) over time, or (C) control Lake Ontario water (C) or Talon Lake water (TL) over time.

Results and Discussion

In the present study, cultured gill epithelia were kept under asymmetrical conditions with either control (Lake Ontario) or NOM-enriched water on the apical side and a blood serum substitute (L-15 medium) on the basolateral side. Under this arrangement, gill epithelia were monitored for TEP and TER to assess the effects of NOM on the electrophysiological parameters of the gill epithelium. Replacing the control water with NOM-enriched water resulted in a hyperpolarization of cultured gill epithelia (Figure 1A). The magnitude of this hyperpolarization at a standard concentration of 10 mg of C/L was strongly correlated with the $SAC_{340\ nm}$ of the particular NOM, suggesting that the potency for hyperpolarization is a function of the aromaticity of the molecules.

The TEP in vivo measured in cannulated trout showed a similar response, with the magnitude of the hyperpolarization again strongly correlated with the $SAC_{340\ nm}$ (Figure 1B). However, the extent of the hyperpolarization was slightly attenuated to that observed in vitro. The effects in vivo were shown to be completely reversible, since transfer back to control water after NOM exposure led to a typical control TEP of approximately −5 mV (relative to the water side) (Figure 2B,C). These results demonstrated the ability of allochthonous NOM to strongly influence the TEP (Figure 2B), in comparison to autochthonous NOM (Figure 2C).

The TEP in freshwater fish is traditionally interpreted as a diffusion potential predominantly regulated by the relative paracellular permeability to positively (mainly Na^+) and negatively (mainly Cl^-) charged ions (*26, 27, 33*). There are two possible explanations for the somewhat attenuated effect of NOM on the in vivo TEP. First, NOM may simply have had a reduced capacity to influence the paracellular permeability to ions in whole animals relative to cultured epithelia.

TABLE 1. Measured Composition of the Tested NOM Samples[a]

sample	SAC [(cm²/(mg of C)]	total Ca^{2+} concn (μM)	free Ca^{2+} concn (μM)	free:total Ca^{2+} ratio	total Na^{+} concn (μM)	total K^{+} concn (μM)
C	2.2	1000	684	68.4	600	47.7
D	2.7	384	188	49.0	3439	78.2
FM	20.9	243	100	41.2	76	58.8
GR	12.2	172	100	58.1	387	52
LE	6.9	229	227	99.1	98	21.4
LM	32.2	127	40	31.5	2.0	8.5
LN	15.1	389	144	37.0	212	10.9
SP	10	54	1.2	2.2	4.1	11.9
TL	15.4	168	41.7	24.8	29.8	30.4
Tr	12.6	149	14	9.4	25.3	16.7
AH	53.5	194	53	27.3	88	37.1

[a] Color is the spectrophotometric absorbance (SAC) at 340 nm (cm²/mg of C). Total Ca, Na, and K concentrations were measured by flame atomic absorption spectroscopy (μM), the free Ca^{2+} concentration was measured using an ion-selective microelectrode (μM) and the ratio of free to total Ca (%). Key: C, control Lake Ontario; D, Dundas Sewage Treatment Plant; FM, Four Mile Lake; GR, Grand River; LE, Lake Erie; LM, Luther Marsh; LN, Lake Nippissing; SP, Sanctuary Pond; TL, Talon Lake; Tr, Trout Lake; AH, Aldrich humic acid.

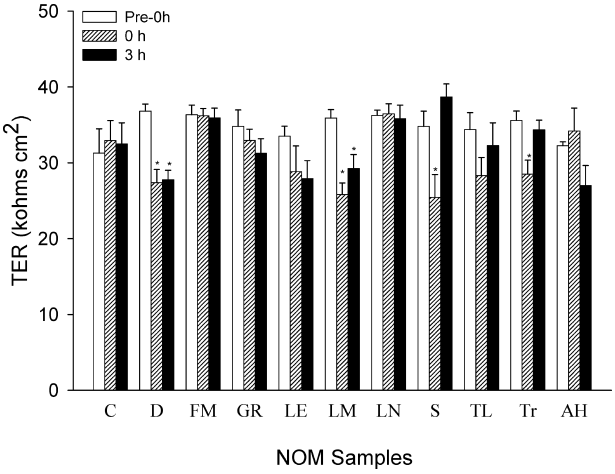

FIGURE 3. TER (kΩ·cm²) in cultured gill epithelia under asymmetrical conditions before exposure to NOM (pre-0 h) or 0−3 h after transfer to 10 mg of C/L NOM. Data are expressed as the mean ± SEM (*N* = 10 per NOM). Asterisks represent $p < 0.05$ from pre-0 h values. See the Figure 1A caption for additional information and the Table 1 footnote for NOM source abbreviations.

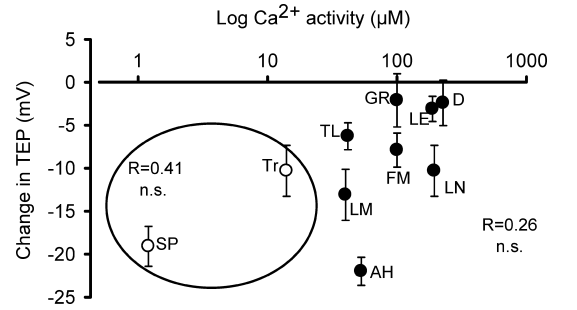

FIGURE 4. Relationship between the free Ca^{2+} activities in each of the sources of water containing 10 mg of C/L NOM and the absolute change in TEP (mV) across epithelia. Pearson's coefficients of regression (*r*) are reported with the inclusion of all data or all the data excluding two NOM sources (Trout Lake and Sanctuary Pond, circled). ns represents no statistical significance. The footnote of Table 1 provides the NOM source abbreviations. Data are expressed as the mean ± SEM (*N*). See the Figure 1 caption for additional information.

However, an alternate explanation is that NOM may have initiated an electrogenic uptake of ions in vivo that tended to counteract the effect on the diffusive permeability (*33*), thereby making the TEP less negative. Current models of Na^{+} uptake in fish suggest that active Na^{+} uptake is driven by an electrogenic H^{+} pump (*34*). Zientara (*35*) provided evidence that humic substances may stimulate the H^{+} pump in the giant alga *Nitellopsis obtusa*. Certainly, there is evidence that allochthonous sources of NOM lead to enhanced Na^{+} influx in *Daphnia* (*17, 18*) and fish (*7, 8*). Also worth noting is that, although the gill epithelial cell cultures mimic the passive properties of the fish gill in vivo (*28*), they exhibit only limited capacity to actively take up ions under asymmetrical conditions (*29, 36*).

In contrast to these marked effects on the TEP, the TER of the gill epithelium, which could only be measured in vitro, was relatively unresponsive to the various NOM samples (Figure 3). The TER is a general indicator of epithelial membrane integrity (*28*), suggesting that it is not affected by these compounds at the concentrations tested. In some cases, there was an initial drop in the TER (at 0 h) upon first exposure, but this is a commonly seen disturbance effect of handling. Only in two instances (Luther Marsh and the Dundas Sewage Treatment Plant) did the fall in TER persist at 3 h. Note that these two samples have different $SAC_{340\ nm}$ characteristics, so the response was not related to aromaticity.

What mechanism then explains the NOM-induced hyperpolarization in fish epithelia? Glover et al. (*17, 18*) suggested that NOM could lead to indirect biological effects by complexing biologically active ions such as free Ca^{2+} from the water. Ca^{2+} is an important constituent of epithelial tight junctions, and reductions in this ion to very low levels are known to cause a general increase in diffusive ion losses, as well as to selectively enhance the permeability of the gills to Na^{+} relative to Cl^{-}. The resulting hyperpolarization of the gills (more negative TEP) has been documented in a variety of species in vivo (*26, 27, 33*), including the rainbow trout (*37*), as well as in cultured trout gill epithelia in vitro (*38*). To test whether allochthonous NOM samples were exerting their effects on fish epithelia by complexing Ca^{2+}, ion-selective microelectrodes were used to measure free Ca^{2+} activities at 10 mg/L NOM (Table 1, Figure 4). Although all of the NOM samples tended to complex Ca^{2+} to a certain degree, only the samples from Trout Lake and Sanctuary Pond had free Ca^{2+} activities (14.0 and 1.2 μM) approaching that of distilled water. Eddy (*39*) found that total waterborne Ca^{2+} levels below 0.5 mM lead to a hyperpolarization of the TEP in vivo in goldfish, with the most pronounced effect occurring below 0.1 mM. In the current study, if NOM from Trout Lake and Sanctuary Pond was excluded from the analyses described in Figure

1A,B, the relationships between TEP hyperpolarization and the $SAC_{340\,nm}$ were greatly improved (Figure 1A, from $R = 0.79$ to $R = 0.97$; Figure 1B, from $R = 0.91$ to $R = 0.97$). Ca^{2+} complexation alone could not explain the TEP response (Figure 4). Whether the data from Trout Lake and Sanctuary Pond are included in the analyses or not, there was no significant relationship between waterborne Ca^{2+} activities and TEP hyperpolarization (Figure 4).

The results therefore suggest that NOM, rather than acting to reduce Ca^{2+} activity, in the water (an indirect effect), in some way acts directly on the gills in a fashion analogous to that of low Ca^{2+}. The time course of the effect is not instantaneous as would occur if NOM had an artifactual effect on the reference electrode, but rather asymptotic over several minutes (Figure 2B,C), similar to the well-documented effect of varying environmental Ca^{2+} on the TEP across the gills (e.g., Figure 2A). This could be an action on the paracellular/tight junction pathway to increase the relative permeability ratio of Na^+ versus Cl^-, with aromatic, allochthonous molecules being most effective in this regard. Direct evidence that NOM can accumulate on the surfaces of fish gill cells, as well as algae and microbial cells, has been provided (*13*−*15*), as well as evidence that NOM can integrate into algal and model lipid membranes, thereby changing their permeability (*16*). In both studies, the effects were pH-dependent, with greater effectiveness in acidic environments. This suggests that, in future investigations, the interactive effects of NOM and pH on gill function should be studied at a range of acidic pH values. In this regard, it is interesting that NOM reduced ion losses at fish gills at moderately low pH (4.0), similar to the action of Ca^{2+} (*39*), but this did not occur at circumneutral pH (*20*).

The TEP is a key component determining the electrochemical gradient through which the active and passive transport of all ions must occur. Source-dependent effects of NOM on Na^+ flux rates in freshwater *Daphnia* (*18*) and fish (*19*) have recently been reported; changes in the TEP may provide the explanation. As with the TEP effect, the amelioration of metal toxicity in aquatic animals by NOM also seems to be governed by aromaticity (*6, 18*), so the functional groups and mechanisms involved in the two processes may be similar, providing a guidepost for future research.

To conclude, while NOM effects on the bioavailability of contaminants are well-known, NOM effects on organismal physiology have not been widely recognized by environmental scientists. In the present study, we have demonstrated that NOM may alter the fundamental physiological properties of fish gills. While the influence of water-quality characteristics such as salinity, calcium (water hardness), and pH on gill physiology, including ion fluxes and TEP, are universally acknowledged (*33, 40, 41*), these physiological effects of NOM have been curiously overlooked until now. The present study indicates that the effects may be large. Such effects may be of key ecological importance in the real world because of the primary role of the gill epithelium in diverse life-support functions such as gas exchange, acid−base regulation, nitrogenous waste excretion, immunity, and ion transport (*40, 41*). The present data add to the growing body of evidence that the nature of ambient NOM, in terms of both concentration and chemical characteristics, can have a major impact on the physiology of aquatic organisms (see the Introduction). The impact of NOM may be of comparable or greater magnitude than commonly reported for other water-quality variables (e.g., hardness, pH, salinity) and therefore of critical importance in ecological understanding and risk assessment. Certainly these matters are of potential concern.

Acknowledgments

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