



## The two faces of DOC

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### ABSTRACT

Dissolved organic carbon (DOC), through its ability to complex metals and thereby reduce their bioavailability, plays a major role in ameliorating metal toxicity in natural waters. Indeed DOC is a key variable in the Biotic Ligand Model (BLM) for predicting metal toxicity on a site-specific basis. However, recent evidence indicates that all DOCs are not alike, but rather heterogeneous in their ability to protect organisms against metal toxicity, at least in fresh water. The degree of protection appears to correlate with optical properties, such that dark, aromatic-rich compounds of allochthonous origin, with greater humic acid content, are more effective in this regard, particularly against Cu, Ag, and Pb toxicity. The specific absorption coefficient of the DOC in the 300–350 nm range ( $SAC_{300-350}$ ) has proven to be a simple and effective index of this protective ability. PARAFAC, a multivariate statistical technique for analysis of excitation-emission fluorescence spectroscopy data, also holds promise for quantifying the humic-like and fulvic-like fluorophores, which tend to be positively and negatively correlated with protective ability, respectively. However, what has been largely missing in the toxicological realm is any appreciation that DOC may also affect the physiology of target organisms, such that part of the protection may occur by a mechanism other than metal complexation. Recently published evidence demonstrates that DOC has effects on  $Na^+$  transport, diffusive permeability, and electrical properties of the gills in fish and crustaceans in a manner which will promote  $Na^+$  homeostasis. These actions could thereby protect against metal toxicity by physiological mechanisms. Future research should investigate potential direct interactions of DOC molecules with the branchial epithelium. Incorporation of optical properties of DOC could be used to improve the predictive capabilities of the BLM.

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### 1. Introduction

Dissolved natural organic matter (NOM) represents ubiquitous components of natural waters formed by the physical breakdown or microbial processing of plant and animal materials (Thurman, 1985). Dissolved NOM is often termed dissolved organic matter (DOM), and is usually quantified as dissolved organic carbon (DOC), which makes up generally about 50% by mass of these heterogeneous molecules. For the sake of simplicity, the term DOC will be used throughout this document.

In the ecological literature, DOC is now recognized as a global regulator of many biotic and abiotic processes (e.g. carbon cycling, nutrient transport, ultraviolet screening) in freshwater ecosystems (Petersen, 1991; Kullberg et al., 1993; Williamson et al., 1999; Steinberg et al., 2006). In the toxicological literature, there is now

general agreement that DOC plays a key role in ameliorating the aquatic toxicity of many metals, by its ability to bind them, thereby reducing bioavailability to target surfaces such as the gills. Indeed, DOC concentration is now incorporated into Biotic Ligand Models (BLM's) used to predict the site-specific toxicity of metals or to derive site-specific ambient water quality criteria (Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004). In practical terms, the DOC component often has a greater protective effect than the traditional "hardness" and "alkalinity" components in the outcome of the model predictions. In the physiological literature, however, the potential importance of DOC has been largely neglected until quite recently. This oversight is remarkable, considering that in many natural waters, DOC concentrations (typical range = 1–15  $mg L^{-1}$  C, representing 2–30  $mg L^{-1}$  NOM), are comparable or greater in abundance relative to most inorganic components (Thurman, 1985), and physiologists have long recognized that other water chemistry characteristics such as pH, hardness (Ca + Mg), and salinity critically affect organism physiology (e.g. Evans et al., 2005).

The objectives of this essay are to delve more deeply into the importance of DOC in both metal toxicology and organism phys-

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iology to show that: (i) DOCs from different natural sources vary greatly in their ability to ameliorate metal toxicity; (ii) certain physicochemical characteristics can be used to predict their differential protective abilities; (iii) DOC has profound effects on the physiology of organisms, which again differ according to the physicochemical characteristics of the molecules; and (iv) at least some of the protective effects of DOC may not be entirely explained by simple complexation of metals, but may also be due to direct beneficial effects on organism physiology. Our hope is that these considerations will stimulate future research interest in this important area.

## 2. Differential protective ability of different DOCs

Natural DOCs may be broadly classified as ranging from allochthonous (terrigenous) to autochthonous (Thurman, 1985). Autochthonous DOCs, which are produced within lakes and rivers by algae or by microbial and/or photodegradation of allochthonous NOM's, tend to be optically lighter, and composed of smaller molecules with a lower content of aromatic ring structures. Allochthonous DOCs generally originate from land-based sources (thus the term terrigenous) and are mainly derived from the breakdown of lignins (i.e. leaves and wood). These tend to be optically darker, and composed of larger molecules with more aromatic rings (i.e. more phenolic groups). The late Rick Playle and colleagues (1993) were the first to realize the importance of testing DOCs from such different sources in gill metal binding and toxicity experiments and to show that these often exhibited differential protective effects (e.g. Playle, 1998; Richards et al., 2001; Luider et al., 2004; Schwartz et al., 2004). A number of studies have now quantified this variation in protective ability as change in acute LC50 per unit C in DOC, and shown that it can vary considerably amongst different natural DOC sources – for example, 5-fold in protecting fathead minnow (FHM) larvae against Ag (Van Genderen et al., 2003), 11-fold in protecting *Daphnia magna* (DM) against Ag (Glover et al., 2005a,b), 6-fold in protecting FHM (Ryan et al., 2004) and DM (De Schampelaere et al., 2004) against Cu, and 3-fold in protecting marine mussel larvae against Cu (Nadella et al., 2009). Given that the usual tolerance for predictive variability in aquatic toxicology is often taken as 4-fold (i.e. agreement between observed and predicted toxicity within  $\pm$  a factor of 2), it would seem desirable to derive an index of DOC protective quality which would lessen this variability, especially for DOC input into models such as the BLM.

## 3. Physicochemical indices of DOC protective abilities

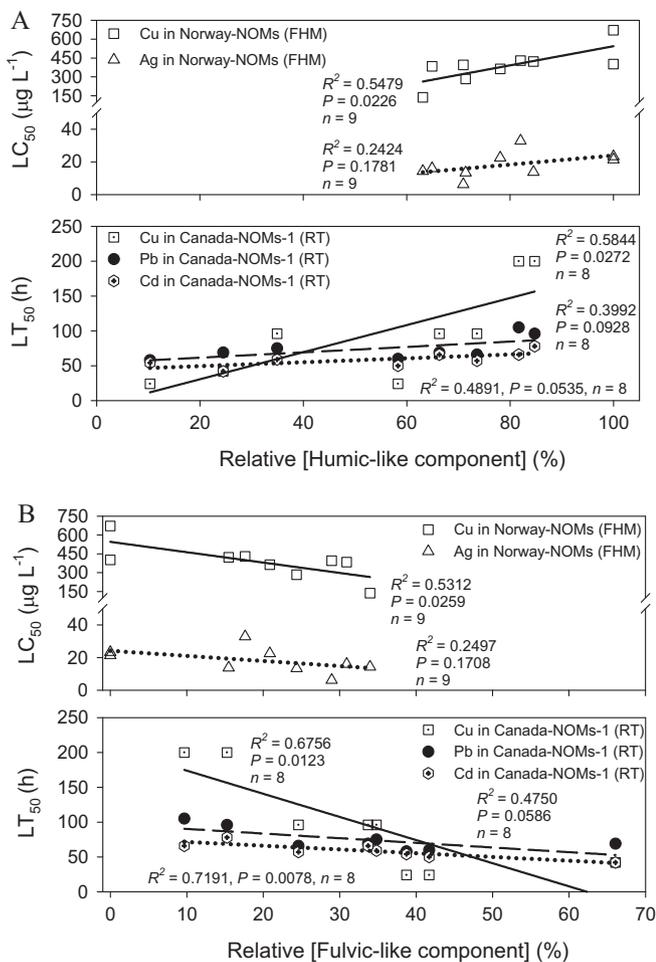
Given the heterogeneous nature of most natural DOCs, it has not been practical or desirable to characterize the properties of individual molecules. Instead, most interest has focused on general indicators that can be applied to natural solutions, and optical properties have received most attention.

Based on Cu uptake experiments with marine mussels, Pempkowiak et al. (1999) suggested that protective ability was associated with a higher aromatic content (i.e. more phenolic rings), which in turn is correlated with darker colour. With regard to acute Ag toxicity to FHM larvae, Van Genderen et al. (2003) reported that none of the following were significant predictive indicators of the protective ability of different DOCs: UV absorbance at 254 nm, specific absorbance co-efficient [ $SAC_{254\text{nm}} = \text{Absorbance} \times 2.303/\text{DOC} (\text{mgL}^{-1})$ ], conductivity, or  $\text{H}^+$  or  $\text{Cu}^{2+}$  complexation capacities. However with regard to acute Ag toxicity to DM, Glover et al. (2005a,b) had greater success, finding significant relationships with three optical properties – wavelength of peak fluorescence intensity,  $SAC_{300\text{nm}}$ , and fluorescence index ( $FI_{370\text{nm}} = \text{emission intensity at } 450\text{nm}/\text{emission intensity at } 500\text{nm}$ , both taken at

excitation at 370 nm). There was no relationship with N-content or with chromium reducible sulfur content. Schwartz et al. (2004) had similar success with  $SAC_{340\text{nm}}$  as a predictor of DOC protection against both Cu and Pb toxicity to juvenile rainbow trout (RBT), though the endpoint measured was median survival time (LT50) rather than LC50. Interestingly, there was no such relationship for Cd, which exhibits much weaker binding to DOC. De Schampelaere et al. (2004) also found that UV absorbance at 350 nm, as well as  $SAC_{350\text{nm}}$ , were predictive of protection by natural DOC against acute Cu toxicity to DM. Interestingly, while DOC was still protective in chronic Cu toxicity tests with DM, its source appeared to have relatively little influence (De Schampelaere and Janssen, 2004), suggesting that kinetic limitations on bioavailability during short term tests tend to become less important in long term tests. Perhaps the most detailed study was that of Ryan et al. (2004), who actually fractionated the various DOCs into “humic acid” (insoluble below pH 2.0) and “fulvic acid” components (soluble below pH 2.0) by the classic acid precipitation technique (Thurman, 1985). In this study, both “humic acid” concentration and UV absorbance at 350 nm, as well as  $SAC_{350}$  were strong predictors of the protective abilities of DOC against acute Cu toxicity to FHM larvae. The “humic acid” content and the optical properties were equally strong in this regard, and both more effective than measurements of Cu complexation capacities.

The general picture which emerges is that optically dark DOC of allochthonous origin tends to be more protective, and optically light DOC of autochthonous origin tends to be less protective against metal toxicity, at least for Cu, Ag, and Pb. The optical difference appears to strongly correlate with “humic acid” content, because the larger terrigenous molecules would precipitate in the “humic acid” fraction at low pH, while the smaller molecules would stay in solution in the “fulvic acid” fraction. Interestingly, modern BLM's incorporate a provision for changing the relative “humic” acid versus “fulvic acid” content. For example, in the current Hydroqual BLM (available for download at [www.hydroqual.com/blm](http://www.hydroqual.com/blm)) used by the U.S.E.P.A. (2007), the default input is 10% humic acid, but this value can be manipulated by the user. However Ryan et al. (2004) found that manipulating the % humic acid parameter from 1% to 100% had little influence on the relationship between BLM-predicted and observed LC50 values for acute Cu toxicity to FHM, even though the measured range in the experimental samples was much lower (18–39%). More worryingly, De Schampelaere et al. (2004) reported that there was a positive relationship between the optimized “% active fulvic acid (AFA)” component (i.e. optimal because it gave the best BLM-based prediction of acute Cu toxicity to DM in an earlier Hydroqual BLM) and protection against Cu toxicity. In turn, the optimized “% AFA” was strongly correlated with the UV absorption at 350 nm, which seems exactly counter-intuitive to the preceding discussion. However, *a priori*, the BLM used by these researchers assumed that Cu binding capacity was a positive function of “% AFA”. Therefore, in fact there may not be a conflict but rather the choice of terminology for their modelled parameter may be the source of the confusion. Nevertheless, these two papers raise questions as to exactly how the BLM software handles the “humic acid” versus “fulvic acid” distribution.

A new development that may shed light on this issue, without the need to resort to the classic acid separation technique, is a more advanced spectral resolution approach entitled parallel factor analysis (PARAFAC). PARAFAC is a multivariate statistical technique for analysis of excitation-emission fluorescence spectroscopy data which allows for greater molecular discrimination (Stedmon et al., 2003; Stedmon and Bro, 2008). Using complete excitation-emission matrices for a particular NOM, PARAFAC resolves the underlying moieties into their peaks, each identified by its corresponding excitation-emission wavelength pair and relative concentrations. “Humic acid-like” and “fulvic acid-like fluorophores”, as well as



**Fig. 1.** The influence of the relative concentrations of the: (A) humic-like fluorophore fraction (%) and (B) the fulvic-like fluorophore fraction (%) of various NOMs, as revealed by PARAFAC analysis, on lethal toxicity measures (LC<sub>50</sub>, LT<sub>50</sub>) for Cu, Ag, Pb, and Cd in larval fathead minnows (FHM) and juvenile rainbow trout (RT). The coefficient of determination ( $R^2$ ) and  $P$ -values are given for each relationship. Data from Al-Reasi et al. (2011).

several proteinaceous components, can be identified and quantified.

Recently, Al-Reasi et al. (2011) have performed PARAFAC on a range of natural DOCs used in many of the freshwater studies cited earlier. This analysis indicates that the humic-like fluorophores confer protection against Cu toxicity to FHM (Ryan et al., 2004) and RBT (Schwartz et al., 2004), resulting in greater LC<sub>50</sub> and LT<sub>50</sub> values (Fig. 1A), whereas fulvic-like fluorophores show the opposite – i.e. greater toxicity at higher fulvic acid-like concentration (Fig. 1B). However, the variation in the protective effects of the humic-like fluorophore (Fig. 1A), and the negative effects of the fulvic-like fluorophore (Fig. 1B) on Ag toxicity to fathead minnow (Van Genderen et al., 2003), were not statistically significant. For Pb and Cd toxicity to RBT (LT<sub>50</sub>'s endpoints; Schwartz et al., 2004), the relationships were in the same direction, but generally not significant statistically, except for the negative relationship between the fulvic-like fluorophore and Cd LT<sub>50</sub> (Fig. 1A and B). However, neither gill accumulation rates of Cu and Cd in blue mussels (Pempkowiak et al., 1999) nor gill burdens of Hg and Cu to RBT (Klinck et al., 2005) were significantly related to the relative concentrations of the two fluorophores (not shown). Clearly, PARAFAC holds promise, but needs further refinement.

Al-Reasi et al. (2011) also critically evaluated all of the various indices of DOC protective ability listed above, as well as additional

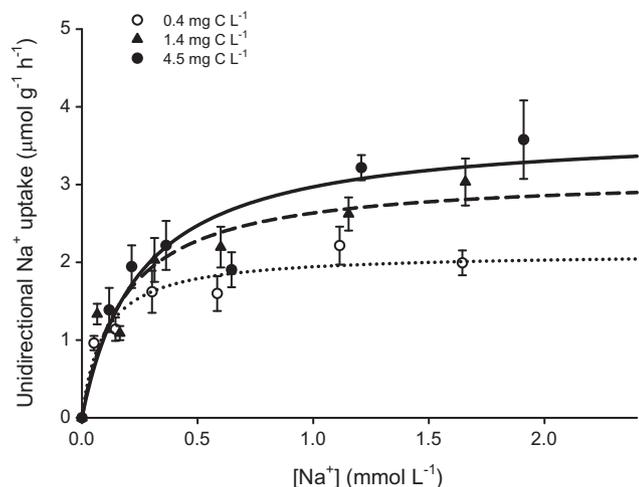
ones based on chemical analyses of the DOCs. From this evaluation, we may conclude that a simple index of colour such as SAC<sub>300–350</sub>nm appears to be just as useful and more practical than more complex excitation–emission analyses such as FI, though PARAFAC may prove eventually valuable in providing a more integrative index of DOC quality. Optical techniques are more diagnostic of protective ability than analytical techniques such as metal-complexation capacity determination by titration, elemental content analysis, protein-to-carbohydrate ratio assay, or lipophilicity assay.

We emphasize that these conclusions about the predictive optical properties of DOC with respect to toxicity amelioration apply only to tests in freshwater at present. In seawater, Nadella et al. (2009) applied PARAFAC analysis of DOC to acute Cu toxicity results with marine mussel larvae, and concluded that DOC-based protection appeared to correlate with higher fulvic acid and lower humic acid content (i.e. opposite the situation in freshwater). However, the conditions of these tests may not have been environmentally relevant, in as much as these were DOCs of freshwater origin tested in full-strength seawater. DePalma et al. (2011a) characterized the optical properties of a wide range of natural NOM samples from estuarine and coastal sources, while DePalma et al. (2011b) evaluated the Cu toxicity of a subset of these same sources to mussel larvae, when the samples were “salted up” to full strength seawater for the tests. Protection correlated well with total DOC concentration, but could not be distinguished by optical properties because positive correlations were equally strong with humic and fulvic fluorophore components, as resolved by PARAFAC. Overall, the situation is presently unclear in seawater, where the high salt environment may alter the properties of the DOC (e.g. cause aggregation into colloids which still are operationally dissolved), as well as the interactions of the metals with organisms.

#### 4. Direct effects of DOC on the physiology of organisms

Numerous observations scattered in the literature indicate that DOC can have direct effects on organismal function (reviewed by Steinberg et al., 2006), though these have generally escaped the attention of environmental physiologists. Our own interest in the direct effects of DOC on fish was catalyzed by a study on the freshwater stingrays which live in the acidic, ion-poor and DOC-rich waters of the Rio Negro in Brazil (Wood et al., 2003). When experimentally exposed to low environmental pH (4.0) in a water whose ionic composition duplicated that of the Rio Negro but virtually lacked DOC (0.6 mg L<sup>-1</sup>), these elasmobranch fish exhibited a marked increase in the passive efflux rates of Na<sup>+</sup> and Cl<sup>-</sup> ions across the gills and an inhibition of their active influx rates. However, when the same acute exposure was carried out in the native DOC-rich water (8.4 mg L<sup>-1</sup>) in which they normally live, there were no increases in Na<sup>+</sup> and Cl<sup>-</sup> efflux rates, and the inhibition of influx rates was attenuated. These effects could be duplicated by experimentally increasing the Ca<sup>2+</sup> concentration of the DOC-free media by 10-fold, suggesting that DOC protects against the deleterious effects of low pH by a similar mechanism, perhaps by stabilizing tight junctions and stimulating the uptake pumps.

Inspired by these findings on the Rio Negro stingrays, we subsequently investigated the influence of DOC on the transport physiology of other systems. Active uptake processes for ions are determined by the kinetic properties (affinity =  $K_m$ , and maximum transport rate =  $J_{max}$ ) of the transporters, the relevant concentration gradients, and the relevant voltage gradients (transepithelial potential = TEP). Passive loss processes are determined by membrane permeability, concentration gradients, and TEP. It is possible that DOC molecules, through their amphiphilic properties, may affect the concentrations of ions in the external micro-environment of the gill transporters, but we are aware of no firm data on this

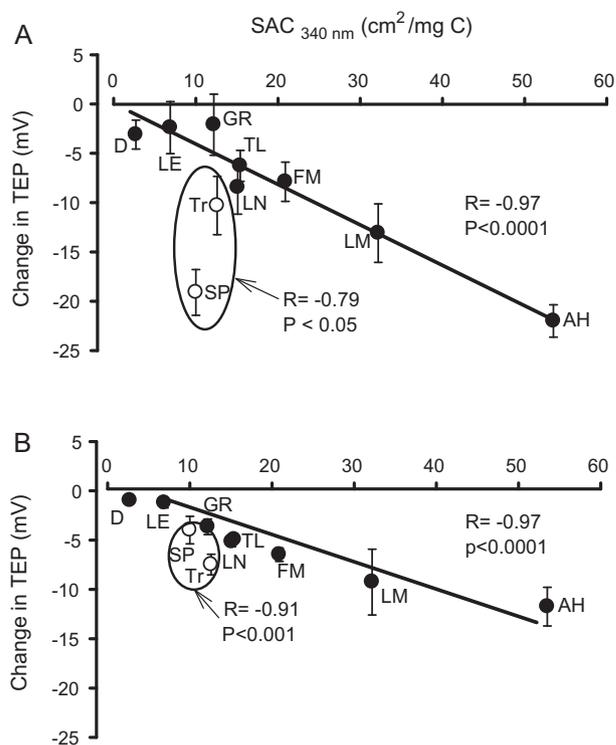


**Fig. 2.** The influence of DOC concentration (Aldrich humic acid) on the concentration-dependent kinetics of  $\text{Na}^+$  uptake in *Daphnia magna*. Note the substantial increases in maximum transport capacity ( $J_{\text{max}}$ ) with greater DOC. Data from Glover et al. (2005c).

issue, so instead our focus has been on the other factors that are amenable to experimental test.

DOC clearly altered the concentration-dependent kinetics of  $\text{Na}^+$  uptake in RBT (Matsuo et al., 2004) and DM (Glover and Wood, 2005; Glover et al., 2005c; Fig. 2). The dominant effect in all these studies was a stimulation of the active uptake of  $\text{Na}^+$  due to an increase in the maximum capacity ( $J_{\text{max}}$ ) of the branchial transport systems, sometimes accompanied by altered affinity ( $K_m$ ). This fits well with the observation that long-term exposure to elevated DOC caused an increase in gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in RBT (McGeer et al., 2002), the enzyme which fundamentally drives gill ion transport (Evans et al., 2005). However this effect on  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was not seen in short-term exposures with DM (Glover and Wood, 2005). To investigate whether DOC altered TEP across the gills, Galvez et al. (2009) employed both intact adult trout fitted with indwelling catheters for TEP measurement, and *in vitro* cultured trout gill epithelial preparations as developed by Fletcher et al. (2000). The advantage of the latter is that in the absence of hormonal support, these cultured epithelia exhibit no active transport of  $\text{Na}^+$  or  $\text{Cl}^-$ , so changes in TEP must reflect diffusion potentials. Interestingly, both *in vivo* and *in vitro*, exposure to NOM's from various natural sources (at a common DOC concentration of  $10 \text{ mg L}^{-1}$ ) resulted in hyperpolarization (i.e. a more negative TEP; Fig. 3A and B). Ionic activity measurements with micro-electrodes demonstrated that gill hyperpolarization was independent of  $\text{Ca}^{2+}$ -complexation by DOC in all but two of the samples tested. Furthermore, the magnitude of this hyperpolarization was strongly correlated with the  $\text{SAC}_{340 \text{ nm}}$ , suggesting that the potency for hyperpolarization is a function of aromaticity of the molecules. Thus the same optical properties that determine protection against metal toxicity appear to have direct effects on branchial membrane physiology.

These results show that DOC clearly affects gill permeability. The TEP in freshwater fish is traditionally interpreted as a diffusion potential predominantly regulated by the relative paracellular permeability to positively- (mainly  $\text{Na}^+$ ) and negatively- (mainly  $\text{Cl}^-$ ) charged ions (Potts, 1984). The more negative TEP associated with higher  $\text{SAC}_{340 \text{ nm}}$ , indicates a greater  $\text{Na}^+$  to  $\text{Cl}^-$  permeability ratio, though not necessarily an increase in  $\text{Na}^+$  permeability. In turn, the hyperpolarized TEP caused by DOC would favour  $\text{Na}^+$  uptake by reducing the opposing electrochemical gradient (an immediate effect), while longer term increases in gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity (McGeer et al., 2002) would reinforce this effect.



**Fig. 3.** The effects of NOM's from different sources, all tested at a DOC concentration of  $10 \text{ mg L}^{-1}$  C on the transepithelial potential (TEP) of: (A) cultured gill epithelia *in vitro* derived from adult rainbow trout, and (B) cannulated adult rainbow trout *in vivo*. These figures describe the relationship between the specific absorption coefficient at 340 nm ( $\text{SAC}_{340 \text{ nm}}$ ; in  $\text{cm}^2 \text{ mg}^{-1} \text{ C}$ ) and the absolute change in TEP (in mV) across epithelia upon transfer from control (L. Ontario) water to water containing experimental NOM's. TEP's are expressed relative to the apical side as 0 mV. The Pearson's co-efficient of regression ( $r$ ) and the  $P$ -values are reported with the inclusion of all data or all the data except two circled NOM sources (Trout Lake = Tr and Sanctuary Pond = SP) which were excluded due to their ability to greatly lower  $\text{Ca}^{2+}$  activity. See original publication for NOM source abbreviations. Means  $\pm$  SEM. Reproduced from Galvez et al. (2009) with permission.

In searching for a mechanistic explanation for these physiological effects of DOC, our attention was drawn to an earlier seminal study by Campbell et al. (1997). These workers demonstrated that DOC molecules can actually bind directly to the surface membranes of living cells (algae and isolated fish gill cells), and that the effect increases as the water pH is lowered, suggesting either a hydrogen-bonding sorption mechanism, or the formation of hydrophobic bonds. This same group later demonstrated that DOC could alter the permeability of algal cell membranes in a pH-dependent fashion (Vigneault et al., 2000). We speculate that the binding of these amphiphilic DOC molecules may stabilize, and/or alter the net charge on tight junctions in paracellular channels of the gills, thereby altering diffusion potentials by altering  $\text{Na}^+$  to  $\text{Cl}^-$  permeability ratios. They may also change the fluidity of the lipoprotein bilayers in the transcellular pathway, which in turn could alter the activity or accessibility of the  $\text{Na}^+$  transport sites in the short term, and the number of transport sites in the longer term. These ideas await future testing.

### 5. Some protective effects of DOC against metal toxicity may be due to direct effects on organism physiology

The observations summarized in the previous section raise the prospect that at least some of the protective effects of DOC against metal uptake and toxicity may be due to DOC effects on the organism, rather than to complexation of metals by DOC. This is reinforced by the finding that the same optical property (SAC)

that protects against metal toxicity also correlates with at least one physiological action (Fig. 3). There is a need to test whether this is true of other direct physiological actions of DOC.

It is now generally accepted the primary action of metals such as Cu and Ag in causing acute toxicity in freshwater organisms is inhibition of active Na<sup>+</sup> uptake, while higher levels of these and other metals also destabilize the paracellular pathway, increasing ion leakage rates (Wood, 2001; Niyogi and Wood, 2004). If DOC, especially allochthonous DOC rich in aromatic groups, stimulates active Na<sup>+</sup> uptake (Matsuo et al., 2004; Glover et al., 2005a; Glover and Wood, 2005), augments Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (McGeer et al., 2002), hyperpolarizes the TEP (Galvez et al., 2009), and reduces paracellular permeability (Wood et al., 2003), then these direct beneficial effects would oppose the toxic effects of metals. Experimentally, these two faces of DOC are difficult to separate. Nevertheless, Matsuo et al. (2004) concluded that the complex protective effects of DOC against Cu-induced disturbance of Na<sup>+</sup> uptake and efflux in RBT reflected both metal-binding to DOC, and direct DOC actions on Na<sup>+</sup> uptake kinetics. It is also apparent that long term exposure to DOC prior to metal exposure may alter the nature of the target surface, thereby reducing subsequent Ag body burden in DM exposed to elevated waterborne Ag (Glover and Wood, 2004), and gill Cu and Cd burdens in tambaqui (a tropical fish) exposed to elevated waterborne concentrations of these metals (Matsuo et al., 2005). These effects occurred even when DOC was absent during the metal exposures, emphasizing that the nature of the target surface was fundamentally changed by DOC pre-exposure.

At the very least, these observations suggest that the protective effects of DOC should be re-assessed in light of the two face hypothesis. They also indicate that for experimental tests designed to parameterize the BLM or other predictive models, the test animals should be acclimated to the appropriate DOC concentrations prior to test. A DOC quality factor should be incorporated into models, and an SAC-based adjustment would seem the best choice at present. To conclude, we paraphrase the prescient words with which Campbell et al. (1997) concluded their ground-breaking paper: "We recognize that our views of DOC–organism interaction are somewhat heretical. It is hoped, however, that they will stimulate further discussion and debate on the relative importance of surface actions (i.e. direct effects on organism) versus solution phenomena (i.e. metal complexation) in this important area".

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