A critical assessment of physicochemical indices used to characterise natural dissolved organic carbon (DOC), their inter-relationships, and the effects of pH

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Environmental context. Dissolved organic carbon (DOC) is ubiquitous in freshwater and concentrations are rising universally while pH is decreasing with climate change. This study demonstrates the interrelationships among DOC characterisation techniques and the pH-sensitive aspects of these techniques that were previously not well understood. As DOC regulates important processes within ecosystems, understanding DOC behaviour at altered pH and identifying techniques to effectively evaluate DOC composition are critical requirements for monitoring aquatic ecosystem health.

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ABSTRACT

Rationale. Dissolved organic carbon (DOC) is both ubiquitous and heterogeneous in freshwater. Freshwaters are browning universally and pH values are decreasing with climate change. DOCs influence water pH, whereas changes in water pH potentially alter the conformation and function of DOCs. The physicochemical properties of DOCs can be characterised by optical and chemical indices, but the inter-relationships among them, and the effects of low pH, are not well understood. **Methodology.** We characterised five naturally sourced DOCs, spanning large differences in origin and composition, at pH 7 and 4, using multiple indices: specific absorbance coefficient at 340 nm, molecular weight index, fluorescence index, octanol–water partition coefficient, molecular charge, proton binding index, size-fractionation, and percentage humic-acid-like, percentage fulvic-acid-like and percentage protein-like components by fluorescence-based parallel factor analysis. **Results.** Many of the indices changed between pH 7 and 4 as reflected in the corresponding absorbance and fluorescence profiles. Generally, apparent aromaticity, apparent molecular weight and molecular charge all decreased with low pH, while lipophilicity increased. Key positive correlations occurred between aromaticity and apparent molecular weight, chemical reactivity and apparent molecular weight, and aromaticity and chemical reactivity, and a negative correlation between lipophilicity and molecular charge. These relationships were pH dependent. **Discussion.** Our results highlight that physicochemical indices used to characterise DOCs from distinct sources should consider pH and be interpreted carefully. The pH-dependent changes in many of the indices are likely alterations in the conformation, reflected in the optical signatures, rather than changes in the composition of DOCs. In contrast, increased lipophilicity and reduced charge at lower pH are due to actual changes in DOC molecules, resulting from proton binding. The ecological functions of DOCs are dependent on source and will likely change with natural acidification events such as increasing atmospheric CO_2.

Keywords: absorbance, acid–base titration, dissolved organic carbon, DOC characterisation, fluorescence signatures, lipophilicity, optical properties, pH-dependent properties.

Introduction

Dissolved organic carbon (DOC) is a universal constituent of freshwater that is increasing worldwide with spatial and temporal variability (Morris *et al.* 2021). Simultaneously, freshwater pH values are generally falling due to climate change (Pagano *et al.* 2014).

DOCs are primarily composed of humic substances (humic and fulvic acids) and various non-humic substances including amino acids, fatty acids, phenols, sterols, natural sugars, hydrocarbons, urea and porphyrins (Boggs *et al.* 1985; Gaffney *et al.* 1996). They are extremely heterogeneous, varying in quality and composition based on origin and spatial, temporal, and seasonal factors. The dissociation of protons from humic substances acidifies water but DOCs also may indirectly alter pH by affecting other natural buffer systems that help to regulate pH (Erlandsson *et al.* 2010). Changes in water pH likewise potentially alter properties of DOCs, inducing changes in their fluorescence behaviour (Pullin and Cabaniss 1995; Patel-Sorrentino *et al.* 2002) and changes in their conformation such as condensation or expansion of the molecules, probably associated with titration of negative charge by protons (Pace *et al.* 2012). As a result, the functional properties of DOC may change with pH (Wenk *et al.* 2021). In freshwater ecosystems, DOCs regulate both biotic and abiotic processes (Petersen 1991; Kullberg *et al.* 1993; Steinberg *et al.* 2006; Al-Reasi *et al.* 2011) and aquatic organisms are very sensitive to changes in DOC quality and concentration (Sucker and Krause 2010; Wood *et al.* 2011; Johannsson *et al.* 2017, 2020; Holland *et al.* 2018). Two well-established functional properties of DOC are their ability to bind cationic metals, thereby reducing toxicity, and their ability to exert generally beneficial physiological effects on osmoregulation in aquatic organisms. Both of these functions appear to vary with DOC type and are sensitive to pH (Wood *et al.* 2011; Morris *et al.* 2021).

The ecological function of DOC is reflected in various optical and chemical indices. For example, there is evidence that DOCs that are more aromatic, more allochthonous, larger in molecular size, with greater humic acid content and with greater reactivity, are more effective at both detoxifying metals and exerting physiological effects (Al-Reasi *et al.* 2011, 2013; Wood *et al.* 2011). These properties have been quantified by a variety of optical and chemical indices, as outlined below, but how they relate to one another and behave at different pH values is not well understood.

Molecular variability of DOCs can be described by optical properties such as absorbance, fluorescence signatures and parallel factor analysis (PARAFAC) of fluorescence data (Al-Reasi *et al.* 2011; Wood *et al.* 2011). Indices have been developed to evaluate: aromaticity (specific absorbance coefficient at 340 nm, SAC_{340} Curtis and Schindler 1997), molecular weight (molecular weight index, MWI; Dahlén *et al.* 1996; Peuravuori and Pihlaja 1997), allochthonous *v.* autochthonous origin (fluorescence index, FI, McKnight *et al.* 2001; DePalma *et al.* 2011), lipophilicity (octanol–water partition coefficient, Gjessing *et al.* 1999; Egeberg and Alberts 2002; Namjesnik-Dejanovic and Cabaniss 2004), size fractionation by ultrafiltration (Wheeler 1976), and relative percentage composition by PARAFAC (Kroonenberg and Heiser 1998; Stedmon and Bro 2008). Non-optical indices of chemical reactivity include the proton binding index

(PBI) (Al-Reasi *et al.* 2013) and molecular charge (an indication of the ionisable fraction of the molecule).

In the present study, we have applied all these techniques to characterise five sources of natural DOCs that were freshly isolated from diverse sites in Ontario, Canada. Two of the sources (Lake Ontario, LO; and Luther Marsh, LM) were selected because some of their physicochemical properties have been characterised in previous collections (Al-Reasi *et al.* 2012, 2013; Duarte *et al.* 2018). These earlier studies established that these sources span extremes with regards to origin (autochthonous *v.* allochthonous) and structure (apparent molecular size, optical and physicochemical properties). The three other DOC collection sites (Preston Flats, PF; Ancient Woods Swamp, AS; and Ancient Woods Pond, AP) represent a gradient of aquatic environments within 'The *rare* Charitable Research Reserve', Ontario, that were predicted to 'fill in the gaps' between the extremes of LM (allochthonous) and LO (autochthonous). With a few exceptions, measurements were made at both pH 7 and 4. Additionally, size fractionation was performed, and the properties were evaluated for each fraction. Previous studies using the MWI have rarely done this. Size fractionation not only assessed the validity of the MWI, it allowed us to determine the importance of each size range considering DOC concentration, molecular weight, relative percentage fluorophore concentration (PARAFAC components) and aromaticity. The indices are related to DOC function; therefore, we were interested in knowing if most of the protective capacity (related to metal toxicity and ionoregulatory function in aquatic organisms) of the DOC was in a specific fraction. Finally, results were analysed to detect important relationships among the various optical and chemical indices and how they were affected by pH.

Our specific hypotheses were: (i) based on previous studies (cited above) on DOC collected from LM and LO, the DOC quality characteristics would differ among sources, with LM DOC being the most aromatic, allochthonous, largest in molecular weight and with the largest relative humic-acid-like fraction, whereas LO would be the least aromatic, autochthonous, smaller in molecular weight, with a lower humic-acid-like fraction; (ii) the physicochemical indices for each source would change with the lowering of pH, due to known H^+ titration of negative charges on DOC, resulting in conformational changes in the molecules; (iii) some of the indices would correlate strongly with one another, in a pH-dependent fashion, whereas others would not. Specifically, based on chemical principles, aromaticity would likely be related to molecular weight, and lipophilicity to molecular charge; and (iv) smaller DOC size fractions would have lower DOC concentration, aromaticity, relative percentage humic-acid-like concentration and molecular weight.

Our goal was to critically assess these indices for use in a companion study (C. Morris, S. Zulian, C. J. Brauner, D. S. Smith and C. M. Wood, unpubl. data) probing the physiological effects of these same DOC solutions on freshwater

rainbow trout (*Oncorhynchus mykiss*) at pH 7 and 4. As the indices are indicative of the quality and composition of DOCs, and thus potentially their functions, we predicted that DOCs that have higher aromaticity, larger molecular weight, more humic-acid-like content and a higher proton binding capacity, particularly at pH 4, would be more beneficial to ionoregulation in rainbow trout in our companion study (C. Morris *et al.*, unpubl. data).

Experimental methods

Experimental solutions

Natural DOC sources were collected from five sites in Ontario, Canada. The sites were Luther Marsh (LM) (43°57′42.8N, 80°24′04.0W), Lake Ontario (LO) (44°14′25.3″N 76°27′57.5″W) and three sites within 'The *rare* Charitable Research Reserve': Preston Flats (PF) (43°23′17.5″N 80°22′38.0″W), Ancient Woods Pond (AP) (43°22′30.4″N 80°21′55.5″W) and Ancient Woods Swamp (AS) (43°22′30.8″N 80°22′09.2″W). These are referred to throughout the manuscript by their site designations: LO, LM, PF, AP and AS. There were two types of samples collected, 'grab samples' and DOC concentrates.

Single 'grab samples' of non-processed water were taken at the same time as the collection of the DOC concentrates (described below), for analysis of pH, DOC and cation and metal concentrations (Supplementary Table S1). pH was measured using a sympHony pH electrode (C03243) and meter (SP70P) (VWR International, Radnor, PA, USA). DOC was measured using a Shimadzu total carbon analyser (TOC-L Shimadzu, Mandel Scientific, Guelph, ON, Canada). All samples were well above the instrument detection limit (~0.1 mg C L^{-1}). Metal concentrations were measured by inductively coupled plasma–optical emission spectroscopy (ICP-OES, Optima 8000, PerkinElmer Inc., Woodbridge, ON, Canada). Grab samples were taken from LO, PF, AP and AS; unfortunately, the grab sample from LM was lost.

The DOC concentrates were collected as single stocks in reverse osmosis sessions, each lasting multiple hours from each location. We did not make multiple separate concentrates from each site. To concentrate the DOC, water was pumped from the source through a 1-µm wound string prefilter to remove large debris, and then to a portable reverse osmosis unit fitted with a polyamide thin-film composite membrane (FilmTec BW30-4040, DuPont Water Solutions, Edina, MN, USA) which concentrated organic matter gradually (Sun *et al.* 1995). The concentrates were treated with a cation exchange resin (Amberlite IR-118 (H), Sigma–Aldrich, Saint Louis, MO, USA) to remove cations that may have accumulated during reverse osmosis. For the DOC concentrates, there were two processing procedures, one for optical and chemical characterisation of 'whole' DOC (i.e. not fractionated), and the other for size

fractionation. The concentrates for characterisation of 'whole' DOC were filtered using 47-mm hydrophilic, polyethersulfone polymer membrane filters (0.45-µm pore size) (MS PES Membrane Solutions, Auburn, WA, USA), and stored in sealed bottles at 4°C. Experimental solutions were then prepared for further physicochemical characterisation, including ICP-OES analysis, as well as for use in the fish physiology tests of the companion study (C. Morris *et al.*, unpubl. data). These solutions were made by adding each of the natural DOC concentrates to dechlorinated Vancouver tap water (Na$^+$ = 0.09, Cl$^-$ = 0.10, Ca^{2+} = 0.10, Mg^{2+} = 0.011, K$^+$ = 0.004 mmol L^{-1}, hardness as CaCO$_3$ = 3.3 mg L^{-1}, pH = 7.0, [DOC] = ~1.07 mg C L^{-1}) to nominally equal 10 mg C L^{-1} and the pH was adjusted to nominally pH 7 or pH 4 using HNO$_3$ or NaOH and a sympHony pH electrode and meter (Supplementary Table S2). The pH range among the experimental solutions was typically less than 0.5 pH units. DOC concentrations in the experimental waters (Supplementary Table S2) were measured using a Shimadzu total carbon analyser, as described above.

Size fractionation was performed only at neutral pH, using the experimental solutions. All samples for size fractionation were first filtered using polyether sulfone membrane syringe filters (0.45 µm, 25 mm) (VWR syringe filters 76479-020, VWR International, Radnor, PA, USA) and were prepared at ~10 mg C L^{-1} at neutral pH. Molecular weight centrifugation filters of three sizes (100, 10 and 3 kDa) (MWCO, Vivaspin 20 mL, 100 kDa, GE28-9323-63; 10 kDa, GE28-9323-60; 3 kDa, GE28-9323-58; Sigma–Aldrich) were used in the next step. As these may contain glycerol, which would add carbon, they were preconditioned for 9 days following the soaking protocol outlined by Hourtané *et al.* (2022). The 0.45-µm filtrate was then used to conduct 3-, 10- or 100-kDa ultrafiltration by centrifugation. Aliquots from each individual filtrate (i.e. 45 µm, 3, 10 or 100 kDa) were collected for analysis of DOC concentration, SAC$_{340}$, FI, MWI and fluorescence excitation–-emission matrix (FEEM) as described below.

Optical characterisation of DOCs

An Aqualog optical spectrometer (HORIBA Scientific, Kyoto, KM, Japan) was used to measure the fluorescence and absorbance of each of the experimental solutions. This instrument measures the simultaneous excitation–emission matrices required for PARAFAC of fluorophores (Stedmon and Markager 2005; Stedmon and Bro 2008). All indices were measured at pH 7 and 4 with the exception of those associated with the size fractionation by ultrafiltration.

Specific absorbance coefficient at 340 nm

The SAC$_{340}$, an index of aromatic composition, was estimated by the method of Curtis and Schindler (1997) by examining the absorbance of samples at a wavelength of

340 nm using a 1-cm quartz cuvette (Helma Canada Ltd, Concord, ON, CA) in the Aqualog spectrometer. The instrument was 'blanked' using ultrapure water. Two replicates were performed for each source at pH 7 and 4. Briefly, SAC_{340} (cm^2 mg^{-1} C) was calculated as:

$$SAC_{340} = \frac{(2.303 \times Abs_{340}) \div pathlength}{[DOC] \div 1000} \qquad (1)$$

where DOC is in milligrams of C per litre and pathlength is in centimetres.

Fluorescence index

The FI is an indicator of the DOC source with higher values indicating a more autochthonous origin, and lower values, a more allochthonous origin (McKnight *et al.* 2001). Other FI ratios have been proposed but the original ratio seems to work best for predicting the protective effects of DOC (DePalma *et al.* 2011). FI was calculated by previously described methods (McKnight *et al.* 2001):

$$FI = \frac{Em_{450}}{Em_{500}} \qquad (2)$$

where Em_{450} and Em_{500} refer to emission intensities at 450 and 500 nm, both taken at an excitation wavelength of 370 nm.

Molecular weight index

The ratio of absorbance measurements at 254 and 365 nm may be used as an indirect indicator of the relative molecular weight (MWI) of DOC (Dahlén *et al.* 1996; Peuravuori and Pihlaja 1997). Note that this is an inverse index, with high values indicating lower molecular weight, and low values indicating a higher molecular weight:

$$Molecular\ weight\ index = \frac{Abs_{254}}{Abs_{365}} \qquad (3)$$

Parallel factor analysis

PARAFAC modelling was conducted in *MATLAB* (ver. 9.12.0.1956245, MathWorks, Natick, MA, USA) using the *PLS Toolbox* (ver. 9.2, Eigenvector Research Incorporated, Manson, WA, USA). PARAFAC is a computational model that uses a multivariate statistical approach to define the components of a sample based on the fluorescence excitation–emission matrix (FEEM) and known predetermined number of fluorophores (Stedmon and Markager 2005; Stedmon and Bro 2008). Two replicates were performed for each source at pH 7 and 4. In the present study, three fluorophore categories were selected: humic-acid-like, fulvic-acid-like and protein-like components.

Octanol–water partition coefficient

The octanol–water partition coefficient (log K_{OW}) is a measure of lipophilicity. The log K_{OW} was measured following the procedure of Gjessing *et al.* (1999). Briefly, DOC solutions (~10 mg C L^{-1}) from each source were made at pH 7 and 4. Octanol (5 mL) was added to a 15-mL aqueous DOC sample which was gently but thoroughly mixed using a three-dimensional nutating mixer (VWR International, Radnor, PA, USA) for 2.5 h. The distribution of the DOC in the water phase and octanol phase was determined by measuring the absorbance in each phase at 254 nm (Gjessing *et al.* 1999). The absorbance baseline zero was set by 'blanking' the machine with ultrapure water or octanol as appropriate. log K_{OW} was calculated as per Gjessing *et al.* (1999):

$$\log K_{OW} = \log\left(\frac{Abs_{254}\ in\ octanol\ phase}{Abs_{254}\ in\ aqueous\ phase}\right) \qquad (4)$$

A higher log K_{OW} values indicate a more lipophilic sample.

Size fractionation

All samples were prepared as described above at ~10 mg C L^{-1} at neutral pH and were filtered through the 0.45-μm syringe filters. The 0.45-μm filtrate was then used to conduct 3, 10 or 100-kDa ultrafiltration by centrifugation. The swinging bucket rotor centrifuge ran at 4300*g* for a duration of 8 min, at 4°C (Allegra X-14 Series Benchtop Centrifuges 120 V, Beckman Coulter, Brea, CA, USA). Aliquots from each individual filtrate were collected for analysis of DOC concentration, SAC_{340}, FI, MWI and FEEM as described above.

Proton binding index and molecular charge

The PBI shows the potential of DOC to exhibit strong tridentate binding of cations and has been used as a general indicator of chemical reactivity. The PBI is based on the protocol of Al-Reasi *et al.* (2013) using acid–base titrations over a wide range of pH values. Titrations were performed on DOC concentrates from each of the five sources (LM, LO, PF, AP and AS). Prior to the transfer to the titration vessel, the ionic strength of each DOC solution was adjusted to 0.01 M by the addition of 5 M $NaNO_3$ (Sigma–Aldrich). The sample was acidified using HCl to pH ~2.0 then titrated by the gradual addition of 1 N NaOH at ~0.1 pH unit intervals until the pH reached 12 using an automated titrator (848 Titrino Plus with 801 magnetic stirrer with support rod, Metrohm Canada, Mississauga, ON, CA) with a pH electrode (Orion 8101BNWP ROSS Half-Cell Electrode, ThermoFisher Scientific, Waltham, MA, USA) and a double junction Ag/AgCl reference electrode (Orion 900200 Sure-Flow Reference Half Cell Electrode, ThermoFisher Scientific). All titrations were performed at room temperature under ultrapure N_2 gas, to create a CO_2-free environment. Three to five replicates were performed for each source. The experimental titration data were fitted to a fully optimised continuous (FOCUS) model using in-house

MATLAB programs as described by Smith and Ferris (2001), to estimate proton binding constants (pK_a) and their site densities (LT, μmol mg C^{-1}). Binding site densities within a specified pK_a range were determined by integration of the area under the curve in the pK_a spectrum. The PBI was calculated as in Al-Reasi *et al.* (2013):

$$PBI = \frac{intermediate}{(acid + basic) \div 2} \quad (5)$$

where, PBI is a function of the measured acidic ($pK_a \leq 5$), intermediate ($5 > pK_a \leq 8.5$) and basic ($pK_a > 8.5$) proton binding site concentrations (determined by integration of the FOCUS pK_a spectrum).

Molecular charge of the DOC molecule, as moles of charge per milligram of carbon, is an indication of the ionisable fraction of the molecule and was determined directly from the experimental data, using interpolation to obtain the appropriate value at exactly pH 7 or pH 4.

Statistical analysis

Error bars shown for indices represent ranges of replicate measurements. The relative percentage fluorophores (PARAFAC components) as individual sources of DOC at pH 7 and 4 were compared using one-way ANOVA with Tukey's *post hoc* test. The overall effect of pH on the relative percentage fluorophores was assessed across all sites using unpaired Student's *t*-test. To do this, we pooled all sites at pH 7 and 4 within their respective relative percentage fluorophore groups (e.g. relative percentage humic-acid-like concentration at pH 7 *v.* relative percentage humic-acid-like concentration at pH 4). Pearson *r* correlation matrices were used to analyse the relationships among the physicochemical characterisation indices (SAC_{340}, FI, MWI, log K_{OW}, absolute charge, PBI, percentage humic-acid-like concentration, percentage fulvic-acid-like concentration, percentage protein-like concentration, percentage <100 kDa, percentage <10 kDa and percentage <3 kDa) at pH 7 or pH 4 separately and at pH 7 and 4 combined. For each index, these analyses were conducted using single observations (or in the case of replicates, the mean values) from each of the five DOC sources. As the various indices were measured on five different DOCs, the degrees of freedom in the Pearson correlation matrices were only three, for pH 7 and 4 separately, so the threshold for statistical significance ($P < 0.05$) for a directional correlation value *r* was ± 0.878; however, values that were ± 0.7 or more were considered strongly correlated (Ratner 2009). For pH 7 and 4 data combined, the degrees of freedom in the Pearson correlation matrices was eight so the threshold for statistical significance ($P < 0.05$) for a directional correlation value *r* was ± 0.632. Simple linear regressions ($P < 0.05$) were used to assess relationships between selected indices at pH 7 and at pH 4, and with the pH 7 and 4 data combined.

All statistical analyses and data plots were done using *GraphPad Prism 10* (ver. 10.2.3, GraphPad Software, San Diego, CA, USA). A significance level of $P < 0.05$ (two-tailed for all but the correlation analysis) was used throughout.

Results

Optical characterisation

At both pH 7 and 4, AP had the highest SAC_{340} value, indicating the highest aromaticity, followed by AS, LM and PF (Table 1). LO had the lowest SAC_{340} value (less than half the value of AP) indicating that it was the least aromatic DOC tested (Table 1). Adjusting the pH from pH 7 to 4 resulted in a lower SAC_{340} value for all but one source, although the changes were relatively small (less than 10%). The exception was LM where the SAC_{340} value increased by 20%.

At pH 7, LO had the highest FI, indicating a relatively more autochthonous origin, and LM had the lowest FI, indicating a more allochthonous origin than the other DOC sources (Table 1). This matched the initial assumption in the sampling design that LM and LO would represent extreme examples of allochthonous and autochthonous sources respectively. The three other DOCs had intermediate

Table 1. The specific absorbance coefficient at 340 nm (SAC_{340}), fluorescence index (FI), molecular weight index (MWI) and octanol–water partition coefficient (log K_{OW}) in Lake Ontario (LO), Preston Flats (PF), Luther Marsh (LM), Ancient Woods Pond (AP) and Ancient Woods Swamp (AS) at ~10 mg C L^{-1} at pH 7 and 4.

Source	SAC_{340}		FI		MWI		log K_{OW}	
	pH 7	pH 4	pH 7	pH 4	pH 7	pH 4	pH 7	pH 4
LO	16.45 ± 0.28	14.95 ± 0.23	1.30	1.24	8.23	8.25	-1.13	-0.66
PF	21.52 ± 0.16	18.18 ± 0.29	1.24	1.18	7.52	7.04	-1.26	-0.68
LM	26.55 ± 0.32	31.92 ± 0.41	1.13	1.15	5.62	5.90	-1.48	-1.00
AP	36.94 ± 0.02	32.73 ± 0.12	1.24	1.19	5.53	6.11	-1.46	-0.68
AS	27.91 ± 0.03	27.87 ± 0.28	1.28	1.20	5.70	6.71	-1.34	-1.09

SAC_{340} values are mean ± range, N = 2.

FI values. With a decrease in pH, all FI values decreased, except for LM which increased (Table 1); again the changes were modest (~5%).

LO exhibited the lowest apparent molecular weight as indicated by the MWI, an inverse ratio where higher values indicate a lower molecular weight (Table 1). The DOC with the highest apparent molecular weight was AP closely followed by AS and LM which were identical. The decrease of pH moderately increased (less than 20%) the MWI for all sources except for PF (decrease by 6%).

In our PARAFAC analysis, the three resolved fluorophore components were labelled as humic-acid-like, fulvic-acid-like and protein-like moieties (Supplementary Fig. S1), consistent with previous work (Al Reasi *et al.* 2012), where the shortest UV peaks are typically assigned to proteins, the next longest wavelength peaks to fulvic acids and the longest wavelength peaks to humic acids. The FEEM traces revealed a change in the fluorescence profile when the pH was lowered (Supplementary Fig. S1a–j). All sources were primarily composed of fulvic-acid-like components (Fig. 1b), followed by humic-acid-like components (Fig. 1a), with small protein-like components (Fig. 1c). At pH 7, LM had the highest relative percentage humic-acid-like components, AP had the highest relative percentage protein-like components and LO had the highest relative percentage fulvic-acid-like component (Fig. 1). For every source the humic-acid-like component increased with a decrease in pH, however, this was not statistically significant with LM DOC (Fig. 1a). The overall increase at pH 4 considering all DOC sources was statistically significant ($P < 0.0001$). This was mirrored consistently by a significant decrease in the fulvic-acid-like component in all DOC sources (Fig. 1b). Considering all sources together, the decrease was significant ($P < 0.0001$). The protein-like component also decreased at pH 4 in all sources but this was only significant with AP and AS (Fig. 1c). The overall decrease in the protein-like component with a decrease in pH, considering all sources, was not significant ($P = 0.0719$).

The most lipophilic DOC was LO followed by PF (Table 1), as determined by the octanol–water partition
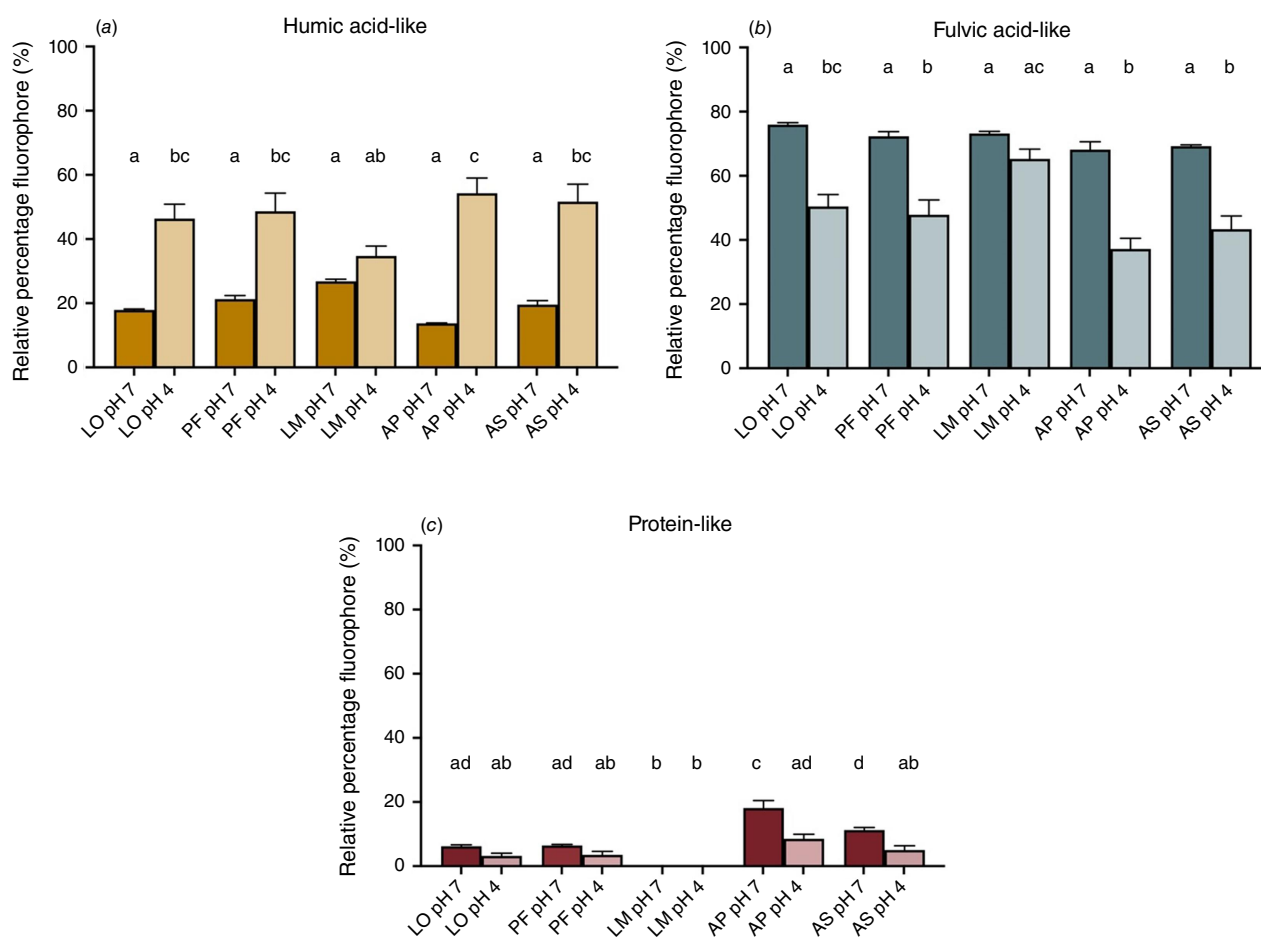

Fig. 1. Relative percentage fluorophores of (*a*) humic-acid-like, (*b*) fulvic-acid-like, and (*c*) protein-like components for Lake Ontario (LO), Preston Flats (PF), Luther Marsh (LM), Ancient Woods Pond (AP) and Ancient Woods Swamp (AS). Values are mean ± range, *N* = 2. Letters that differ indicate statistically significant differences (*P* < 0.05 by one way ANOVA plus Tukey's *post hoc* test).

coefficient (log K_{OW}). AP, AS and LM were less lipophilic and there were marked increases in Log K_{ow} values with the decrease in pH in all sources (Table 1). The total range in log K_{OW} differed by 0.35 from highest to lowest; this corresponds to a factor of ~2 in the underlying linear quantity (K_{OW}).

Proton binding index and molecular charge

At almost 7× greater than the minimum measured value, LM had the highest PBI, a measure of general chemical reactivity, followed by AP, AS and PF, whereas LO had the lowest PBI (Table 2). The molecular charge (mol charge mg^{-1} C) was highest in AS (more than double the lowest value), followed by LM, AP, then LO, which was close to the lowest value, PF, at both pH values. As expected with proton binding sites being titrated, in each source, absolute charge decreased greatly with the lowering of pH (Table 2).

Size fractionation

The DOC concentration of the filtrate generally decreased with decreasing pore size (Table 3). Both LM and PF had the largest percentage change in DOC concentration when looking at the difference between the 0.45-μm and 10-kDa filtrates rather than the 0.45-μm and 3-kDa filtrates (Table 3). However, when considering changes between 0.45 μm and 3 kDa, PF had the lowest percentage change in DOC concentration with filtration. Notably, in all cases, more than 62% of DOC passed through the 3-kDa filter.

Table 2. Proton binding index (PBI) and molecular charge (mol charge mg^{-1} C) at pH 7 and 4 for Lake Ontario (LO), Preston Flats (PF), Luther Marsh (LM), Ancient Woods Pond (AP) and Ancient Woods Swamp (AS).

Source	PBI	Charge at pH 7	Charge at pH 4
LO	0.09 ± 0.007	3.95 ± 0.77	0.95 ± 0.56
PF	0.12 ± 0.02	3.65 ± 0.60	0.59 ± 0.25
LM	0.62 ± 0.17	6.10 ± 1.92	2.17 ± 1.6
AP	0.45 ± 0.10	4.71 ± 0.60	0.95 ± 0.42
AS	0.30 ± 0.04	7.58 ± 0.60	1.68 ± 0.36

Values are means ± range N = 3–5.

Generally, MWI increased with decreasing pore size, indicating a decrease in molecular weight, particularly at 3-kDa filtration. The absolute absorbance at 340 nm and SAC_{340} also decreased indicating that the filtrate was less aromatic (Supplementary Table S3). The FI increased, indicating that the filtrate was fluorescing more like autochthonous DOCs with decreasing pore size. The PARAFAC components also changed with filtration. In each source, when comparing the largest pore size (0.45 μm) and the smallest (3 kDa) there was a decrease in the percentage humic-acid-like component and an increase in the percentage fulvic-acid-like component with the protein-like component being more variable.

Correlations among the physicochemical characteristics of DOC related to pH

At pH 7, there was a significant positive correlation between log K_{OW} and MWI (Fig. 2*a*). There were significant negative correlations between PBI and log K_{OW}, between percentage fulvic-acid-like concentration and SAC_{340}, between percentage protein-like concentration and percentage humic-acid-like concentration, as well as between percentage <10 kDa and percentage humic-acid-like concentration (Fig. 2*a*). Although not significant, there were a number of strong positive correlations (i.e. a Pearson correlation value of +0.7 or more). These occurred between log K_{OW} and FI, between PBI and SAC_{340}, between percentage fulvic-acid-like concentration and MWI, between percentage <100 kDa and percentage protein-like concentration, as well as between percentage <100 kDa and percentage <10 kDa (Fig. 2*a*). In addition, there were many strong negative correlations. These were seen between MWI and SAC_{340}, between log K_{OW} and SAC_{340}, between charge and MWI, between PBI and FI, between PBI and MWI, between percentage humic-acid-like concentration and FI, between percentage protein-like concentration and percentage fulvic-acid-like concentration, between percentage <100 kDa and percentage humic-acid-like concentration, and between percentage <3 kDa and percentage protein-like concentration (Fig. 2*a*).

At pH 4, the correlation relationships differed. PBI and SAC_{340} were significantly positively correlated with each other, as were percentage <10 kDa and FI. There were significant negative correlations between SAC_{340} and MWI,

Table 3. Percentage change in DOC concentration between 0.45 μm and 100 kDa, 0.45 μm and 10 kDa, and 0.45 μm and 3 kDa of Lake Ontario (LO), Preston Flats (PF), Luther Marsh (LM), Ancient Woods Pond (AP) and Ancient Woods Swamp (AS).

Source	0.45 μm to 100 kDa	0.45 μm and 10 kDa	0.45 μm and 3 kDa
LO	−9.25	−11.61	−24.62
PF	−13.64	−24.13	−12.12
LM	−13.89	−31.64	−22.51
AP	−6.38	−15.15	−37.99
AS	−13.13	−19.58	−37.78

between PBI and MW, between percentage protein-like concentration and percentage fulvic-acid-like concentration, and between percentage <3 kDa and percentage humic-acid-like concentration (Fig. 2b). Overall, there were less strong correlations at pH 4 compared to pH 7. The strong positive correlations were MWI with FI, PBI with charge and percentage <10 kDa with percentage <100 kDa. The strong negative correlations were charge with log K_{OW}, PBI with FI and percentage <10 kDa with percentage fulvic-acid-like concentration (Fig. 2b).

When pH 7 and 4 data were grouped together, again the correlation relationships changed. The relationships that remained significantly positively correlated with each other were: SAC$_{340}$ with PBI, percentage fulvic-acid-like concentration with charge, percentage <10 kDa with FI and percentage <10 kDa with percentage <100 kDa (Fig. 2c). The significant negative correlations that remained were: between MWI and SAC$_{340}$, between charge and log K_{OW}, between PBI and MWI, between percentage fulvic-like concentration and log K_{OW}, and between percentage fulvic-acid-
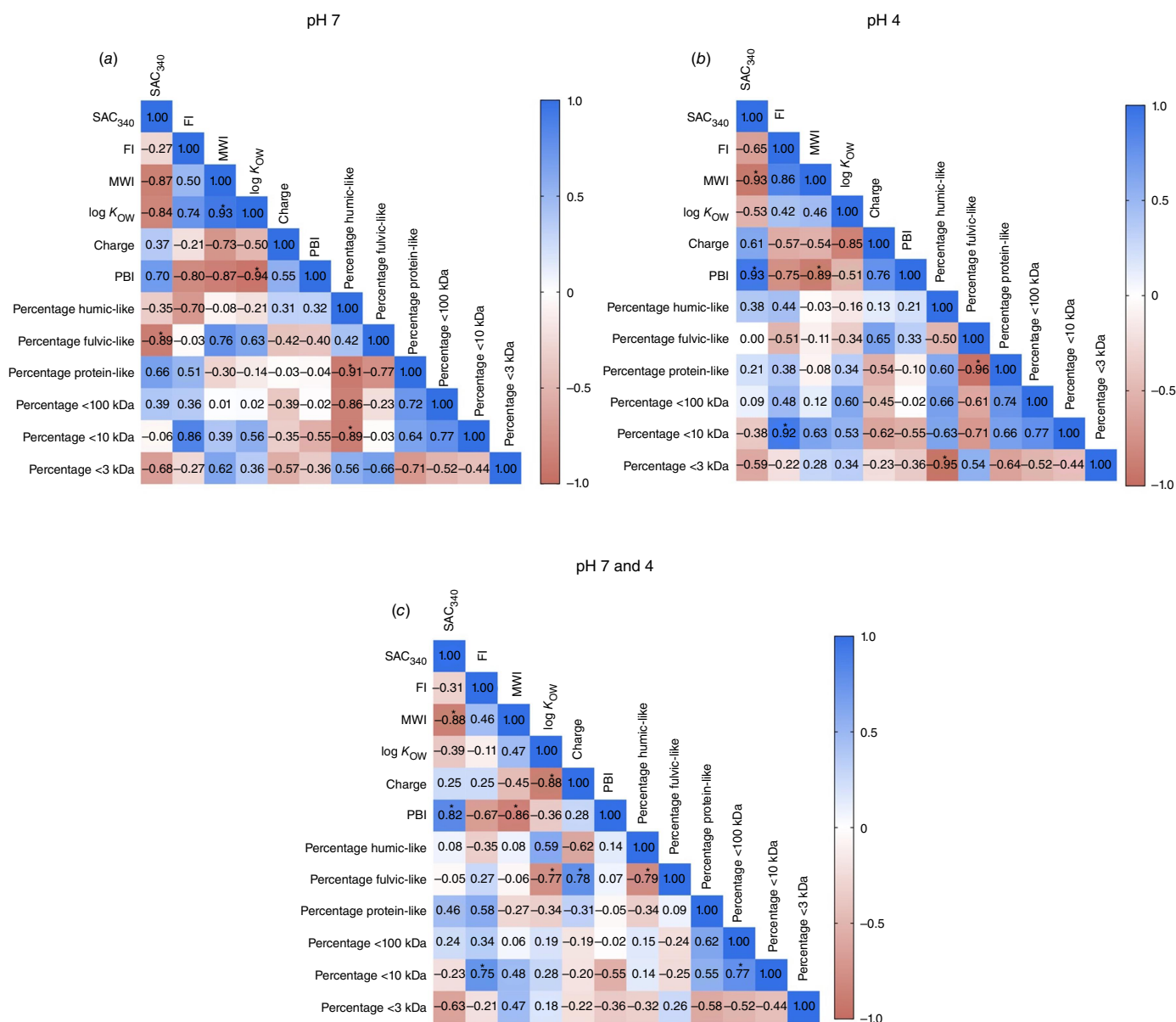

Fig. 2. Pearson *r* correlation matrices demonstrating the correlations between optical characterisation indices, specific absorbance coefficient$_{340}$ (SAC$_{340}$), fluorescence index (FI), molecular weight index (MWI), log K_{OW}, molecular charge, proton binding index (PBI), percentage humic-acid-like, percentage fulvic-acid-like, percentage protein-like, percentage <100 Da, percentage <10 kDa, percentage <3 kDa at (a) pH 7 and (b) pH 4 separately and (c) pH 7 and 4 combined. Asterisks (*) denote significance at *P* < 0.05. The threshold for significance for panels A and B is a directional correlation value *r* = ±0.878 and for C is *r* = ±0.632 based on the degrees of freedom. Values with *r* = ±0.7 or more are considered highly correlated. See Experimental methods for additional details.

like concentration and percentage humic-acid-like concentration (Fig. 2c). There were no other important correlations as the directional correlation value r for significance was ± 0.632 for the combined pH values.

Based on the Pearson r correlation matrices described above, important relationships were further assessed using simple linear regressions at pH 7 and at pH 4, and with pH 7 and 4 data combined (Fig. 3). The regression of SAC_{340} against PBI was significant at pH 4 and when pH 7 and 4 data were combined, but not at pH 7 (Fig. 3a). When SAC_{340} was regressed against MWI, again the relationship was significant at pH 4 and when pH 7 and 4 data were combined, but not at pH 7 alone (Fig. 3b). The regression of log K_{OW} against PBI was significant at pH 7 only (Fig. 3c). The regression of PBI against MWI was significant when pH 7 and 4 data were combined and at pH 4, but not at pH 7 (Fig. 3d). Finally, when log K_{OW} was regressed against charge, the relationship was significant only when pH 7 and 4 data were combined (Fig. 3e).

Discussion

Overview

With respect to our original hypotheses, there were (i) substantial physicochemical differences among our five DOC sources. Indeed, at pH 7, LM DOC was among the most aromatic, and was the most allochthonous, with the largest apparent molecular weight and the largest relative humic-acid-like component, whereas LO was the least aromatic, most autochthonous, with the smallest apparent molecular weight and lowest relative humic-acid-like component. As expected, the indices for the other three sources lay intermediate between these extremes.

We (ii) confirmed that most of the physicochemical indices used to characterise the DOCs changed as pH was lowered from 7 to 4, and these changes were reasonably consistent in a qualitative manner. Thus, SAC_{340} and FI values decreased slightly at lower pH and MWI increased, although in each case there was one exception (Table 1). We attribute these shifts to pH-dependent changes (proton binding at lower pH) in the molecules that alter their optical properties, rather than real changes in origin and molecular weight respectively. For example, there is evidence that DOC molecules tend to condense at low pH and expand at higher pH (Pace *et al.* 2012). This would fit with the higher MWI (i.e. lower apparent size) at pH 4 *v*. 7. It is unlikely that the pH changes alter the actual aromaticity as there are no associated oxidising agents, only protonation–deprotonation reactions based on pH changes. Pace *et al.* (2012) speculated that condensation of the molecules at low pH would limit their exposure to light, thereby lowering their specific absorption, which would explain the small decreases in SAC_{340}. By contrast, effects on fluorescence may be different.

At the lower pH, by PARAFAC, samples consistently exhibited apparent increases in the percentage humic-acid-like component, and decreases in the percentage fulvic acid-like and percentage protein-like components, although the latter was not significant (Fig. 1). Patel-Sorrentino *et al.* (2002) reported that pH affected the fluorescence of both humic-acid-like and fulvic-acid-like components in a qualitatively similar manner, but their ratio would be altered to favour the humic-acid-like components at low pH. This would also explain the shift to lower FI values (again an index based on fluorescence) at low pH, suggesting that the DOC manifests as though it is more allochthonous in origin. Again, conformational changes, rather than real compositional changes, were the likely explanation. Fluorescence spectra for protonated *v*. deprotonated fluorophores are different. Therefore, although the conformational and optical changes with pH are real, they do not reflect actual changes in the characteristics that the indices are generally thought to represent. Caution in interpretation is needed. On the other hand, the substantial and consistent decreases in charge (Table 2) and increases in log K_{OW} (Table 1) at lower pH reflect real changes in the molecules, as proton binding to anionic sites on the DOCs reduces their negative charge, thereby making the molecules more lipophilic.

We also confirmed (iii) that several of the indices used to characterise DOC correlated strongly with one another, with particularly positive correlations between aromaticity and apparent molecular weight, between chemical reactivity and apparent molecular weight, between aromaticity and chemical reactivity, and a negative correlation between lipophilicity and molecular charge. These and other relationships were pH-dependent and are explored below.

We found that (iv) smaller DOC fractions did have lower DOC concentration, aromaticity, relative percentage humic-acid-like concentration and molecular weight, supporting our hypothesis. However, apart from the SAC_{340} values, the changes we saw in DOC concentration, relative percentage humic-acid-like concentration and molecular weight were more modest than we would have predicted. Our results indicate that the aromatic groups are within larger fractions of the DOC. However, although the overall DOC concentration, molecular weight and relative percentage humic-acid-like concentrations did decrease with decreasing pore size, they were somewhat retained even in the smallest fraction that we tested.

The physicochemical characteristics of DOCs: ecological and physiological implications

SAC_{340} values have been reported between 1 and 73 cm^2 mg^{-1} with most natural waters being below 40 cm^2 mg^{-1} (Al-Reasi *et al.* 2012; Johannsson *et al.* 2017; Sadauskas-Henrique *et al.* 2021). We observed similar trends in SAC_{340} values in the two sources that have been previously characterised at pH 7, LO and LM (Al-Reasi *et al.* 2012), with LM being more aromatic
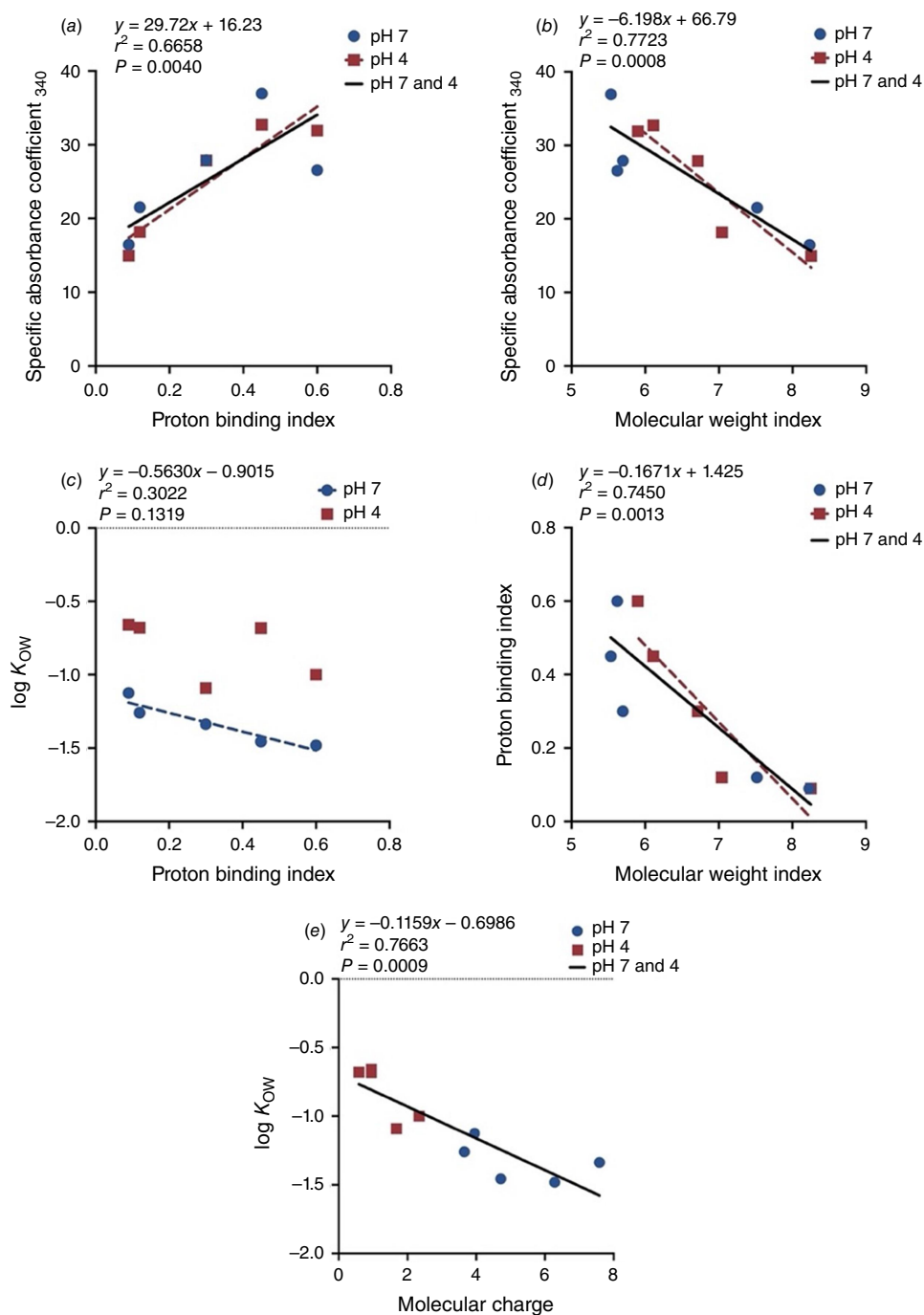
Fig. 3. Simple linear regressions of relationships between indices at pH 7 (blue circles and blue dashed lines) and at pH 4 (brown squares and brown dashed lines) separately, as well as with the pH 7 and 4 data combined (solid black line). Significance ($P < 0.05$) is indicated by the presence of a line in the corresponding colour. The equations of the regression equations for pH 7 and 4 data combined are shown on each panel, together with the r^2 and P values. Additionally, for the pH-specific regressions, the following were significant ($P < 0.05$): (*a*) specific absorbance coefficient$_{340}$ regressed against proton binding index (pH 4, $y = 34.87x + 14.25$, $r^2 = 0.8696$, $P = 0.0208$). (*b*) specific absorbance coefficient$_{340}$ regressed against molecular weight index (pH 4, $y = -8.107x + 80.29$, $r^2 = 0.8638$, $P = 0.0223$). (*c*) log K_{OW} regressed against proton binding index (pH 7, $y = -0.6371x - 1.133$, $r^2 = 0.8902$, $P = 0.0160$). (*d*) proton binding index regressed against molecular weight index (pH 4, $y = -0.2086x + 1.731$, $r^2 = 0.7999$, $P = 0.0405$). (*e*) log K_{OW} regressed against molecular charge ($y = -0.1159x - 0.6986$, $r^2 = 0.7663$, $P = 0.0009$).

compared to LO (Table 1). However, our absolute values differed. At pH 7, LM and LO were 26.55 and 16.45 respectively (Table 1) *v.* 39.03 and 4.85 reported by Al-Reasi *et al.* (2012). The SAC_{340} reported by Sadauskas-Henrique *et al.* (2021) for LM DOC collected in 2010 was 39.70. These differences were perhaps due to spatial, temporal and seasonal variation in collections. In an earlier study, SAC_{340} has been positively correlated with relative percentage humic-like concentration (PARAFAC component) and negatively correlated with the relative fulvic acid-like concentration (Al-Reasi *et al.* 2012). Our data only partially agreed: at pH 7 only, there was a significant negative correlation between SAC_{340} and percentage fulvic-acid-like concentration (Fig. 2a). It is notable, however, that with size fractionation, for each DOC, there were consistent large increases in the percentage fulvic-acid-like component, large decreases in the percentage humic-acid-like component and substantial decreases in SAC_{340} in the smallest filtrate, <3 kDa (Supplementary Table S3).

SAC_{340} appears to be an important index in predicting DOC effects on aquatic systems and organisms, and it exhibited a very strong negative correlation with MWI under all pH conditions in the present study (Fig. 2, 3b). Thus, larger DOC molecules appear to be more aromatic, which agrees with the substantially lower SAC_{340} values in the <3-kDa fraction (Supplementary Table S3), as noted above. The chemical composition of DOC greatly affects its biodegradation in aquatic ecosystems (Mann *et al.* 2012; Wickland *et al.* 2012). DOCs with higher aromaticity and higher molecular weight are generally less biodegradable, and therefore are responsible for lower CO_2 production, which is environmentally favourable in the current context of climate change (Palviainen *et al.* 2022). The aromaticity of DOC molecules is also often used as an indicator of their ability to protect against metal toxicity and to influence ionoregulation in aquatic organisms (Al-Reasi *et al.* 2011, 2012, 2013; Duarte *et al.* 2016; Sadauskas-Henrique *et al.* 2019). We noted a decrease in SAC_{340} with the decrease in pH in all DOC sources with the exception of one, however, it remains unknown if this optical change is mirrored in the physiological effects. Interestingly, DOCs seem to be more effective in providing beneficial physiological effects at low pH (Wood *et al.* 2011; Morris *et al.* 2021), yet are less effective in binding metals at low pH (Tipping 1994; Christensen and Christensen 2000). This suggests that different sites on the large aromatic molecules are involved in the two different protective processes.

FI is a well recognised indicator of DOC origin (McKnight *et al.* 2001). LM DOC was previously reported to be of allochthonous origin with relatively smaller FI values compared to LO (Al-Reasi *et al.* 2012; Sadauskas-Henrique *et al.* 2021) and our results agree (Table 1). We observed that LO DOC had the smallest FI with high relative fulvic-acid-like concentrations and low relative humic-acid-like concentrations at both pH values (Table 1, Fig. 1). FI was previously reported to be negatively correlated with the humic acid-like

component and positively correlated with the fulvic-acid-like component (Al-Reasi *et al.* 2012). However, only in our combined pH 7 and 4 data is this trend present, and the correlations are neither significant nor strong (Fig. 2c). Notably, FI values were highest in the <3-kDa filtrates (Supplementary Table S3), indicating that the smaller molecules in each DOC sample were more autochthonous in nature.

As noted earlier, the change in FI values with the change in pH (Table 1) potentially reflected a change in the conformation of DOC towards a fluorescence profile that is normally associated with that origin (i.e. either of allochthonous or autochthonous origin) and the fulvic-acid-like component is more sensitive to a change in pH relative to the humic-acid-like component (Patel-Sorrentino *et al.* 2002). The change in fluorescence with pH is likely due to the molecular orbital of excitable electrons being altered by ionisation (Laane 1980; Patel-Sorrentino *et al.* 2002). Macromolecular configuration could also offer an explanation. More rigid structures give better fluorescent yields; humic substances have a more linear structure at high pH and coil as pH decreases which may mask some of the fluorescence (Ghosh and Schnitzer 1980). Generally, allochthonous DOCs have been described as highly protective against metal toxicity and physiological disturbances (Al-Reasi *et al.* 2011; Wood *et al.* 2011; Al-Reasi *et al.* 2013). However, Al-Reasi *et al.* (2011) suggested that FI was a less successful predictor compared to SAC_{340} because FI was unable to explain the variability in protection offered by DOC against copper and silver toxicity in fish. Based on our results we agree that FI is not as strong a predictor of DOC quality because of the mechanistic explanation offered above, and the implication that a change in FI is not necessarily a direct change in origin of the DOC.

Our log K_{OW} values ranged from -1.48 to -1.13 for pH 7 and -1.09 to -0.66 for pH 4 (Table 1) which were similar to ranges for other DOC sources at pH 7, -1.92 to -0.97 (Al-Reasi *et al.* 2013) and -1.05 to -0.48 (Gjessing *et al.* 1999). The lipophilic nature of DOC is dependent on pH (Gjessing *et al.* 1999; Egeberg and Alberts 2002; Namjesnik-Dejanovic and Cabaniss 2004). It is likely that this change in log K_{OW} reflects actual changes within the DOC molecules with proton binding to anionic sites making the molecules more lipophilic; the charges on weak acid sites are neutralised thus decreasing the molecule polarity and less polar molecules will partition more readily into the non-polar octanol phase. This conclusion was reinforced by the significant negative regression relationships between log K_{OW} and charge for the combined pH 7 and 4 data (Fig. 3e), and between log K_{OW} and PBI at pH 7 (Fig. 3c). More ionised DOC molecules (higher molecular charge) will more readily partition into the polar water phase ('like dissolves like') and result in a lower log K_{OW}.

At pH 7 the DOCs (AP and LM) that were the most aromatic, more allochthonous, and had generally higher

relative humic-acid-like fluorophore concentrations, were also the least lipophilic, but at pH 4 the log K_{OW} values of these DOCs lay intermediately within the range (Table 1). At both pH values, LO and PF were the most lipophilic and were the least aromatic, the most autochthonous and were among the lowest relative humic-acid-like fluorophore concentrations (Table 1, Fig. 1). Al-Reasi *et al.* (2013) likewise described LO to be more lipophilic when compared to LM. At pH 7, log K_{OW} and SAC_{340} were strongly negatively correlated in our Pearson correlation matrix, such that more aromatic DOCs were less lipophilic (Fig. 2a), however, these indices were only moderately correlated at pH 4 (Fig. 2b). Al-Reasi *et al.* (2011) concluded that there was no relationship between lipophilicity and the ability to bind metals; the present results suggest that at best, there is a weak negative relationship. At face value, greater lipophilicity might favour interaction with the cell membranes of the gills, and this might explain why the physiological effects of DOC, in contrast to the metal-binding actions of DOC, are more pronounced at low pH where DOC lipophilicity is greater. Further biological studies should assess how DOCs with varying lipophilicity affect physiological measurements at neutral and acidic pH.

PBI, an indicator of the chemical reactivity of DOC, was developed for predicting the metal binding capacity of DOC by Al-Reasi *et al.* (2013) who proposed that it may also be useful to predict physiological influences of DOC. The PBI range for our DOC sources (Table 2) was from 0.09 for LO (lower than the previously reported 0.20) to 0.60 for LM (higher than the previously reported 0.44) (Al-Reasi *et al.* 2013). DOCs with high PBI have been described to also have high SAC_{340} values (Al-Reasi *et al.* 2013). Our data show a similar trend: the two DOCs with the highest PBI also had the highest SAC_{340} values (AP and LM), likewise LO had the lowest PBI and SAC_{340} value (Table 1). PBI and SAC_{340} were strongly correlated at pH 7 and significantly correlated at pH 4, as well as in the combined data set (Fig. 2). Note though that PBI is a pH-independent value because it is calculated from all pH values measured during acid–base titration. log K_{OW} and PBI were significantly negatively correlated at pH 7 only. As molecules become more lipophilic (i.e. higher log K_{OW}) the PBI (i.e. chemical reactivity) decreases (Fig. 2a and 3c).

The MWI is considered an inverse index of the mean molecular weight of DOC molecules (Dahlén *et al.* 1996; Peuravuori and Pihlaja 1997). Our size fractionation data generally showed that as DOCs were filtered through decreasing pore size, MWI increased (i.e. molecular weight decreased) and FI increased indicating more autochthonous DOC (Supplementary Table S3). The MWI for LM was slightly higher than previously reported and LO was slightly lower (Table 1) (Al-Reasi *et al.* 2012; Sadauskas-Henrique *et al.* 2021). Relatively higher values of MWI have been noted for autochthonous DOC which are composed of a large proportion of low molecular weight

macromolecules (Al-Reasi *et al.* 2012). A higher MWI indicates that LO has the lowest molecular weight followed by PF, with LM, AP and AS being very similar at both pH values (Table 1). This trend is generally supported by the percentage change in DOC concentration with size fractionation by ultra-filtration (Supplementary Table S3). At pH 4, FI and MWI were significantly correlated (Fig. 2b). Sadauskas-Henrique *et al.* (2021) reported that SAC_{340} and MWI were negatively correlated. We noted a strong negative correlation that was significant at pH 4 and when the pH values were combined (Table 1, Fig. 3b). As MWI is an inverse index, this indicates that more aromatic DOCs also have larger molecular weights. The specific wavelength of 254 nm may represent the maximum absorbance for all aromatic structures present in humic samples whereas 280 nm is where electron transition occurs for some aromatic groups including phenolic arenes, polyenes and polycyclic aromatic hydrocarbons with two or more rings (Peuravuori and Pihlaja 1997). It has been suggested that the relative amount of aromatic moieties in the humic fraction increases with increasing molecular weight (Peuravuori and Pihlaja 1997).

Conclusions

Mechanistic understanding of DOC effects is crucial for future monitoring of aquatic environments and interpreting how different systems with different DOCs interact with the organisms that reside there. Overall, our results highlight that the physicochemical indices used to characterise DOCs are pH dependent, and as such should be interpreted carefully as it is likely that changes in the conformation rather than changes in the composition are altering the optical signatures. It should be noted that in previous works on optical indices they were all determined at a fixed pH (usually 7) because the pH dependence of absorbance and fluorescence spectra is well known. So in the application of those indices to characterise source waters the comparisons are valid when compared at the same fixed pH. On the other hand, higher log K_{OW} (indicative of greater lipophilicity) and reduced charge at lower pH are undoubtedly real, resulting from proton binding. Our major findings that apparent aromaticity, apparent molecular weight and molecular charge all generally decreased with low pH while lipophilicity increased shows the importance of pH-specific characterisation of DOC. Additionally, these results indicate that the ecological function of DOC likely changes with natural acidification events such as increases in atmospheric CO_2 or anthropogenic influences such as acid mine drainage and acid rain. Further research is needed to determine if these changes are reflected in the physiological responses of aquatic organisms to DOC. Indeed, the present study has provided the background for a companion investigation on the rainbow trout (C. Morris *et al.*, unpubl.)

evaluating the effects of these same DOC solutions on the transepithelial potential (TEP) across the gills, a sensitive indicator of biological effect (Galvez *et al.* 2008). In particular, we have established that the five chosen DOC sources differ widely in physicochemical indices and have critically evaluated the relationships among them. An important caveat is that our goal in this study was not to perform a comprehensive characterisation of spatial and temporal variation within sites, but rather to collect discrete DOC stocks that had a range in characteristics in order to evaluate the relationships among indices, the effects of pH on these indices and the relationships of these indices on fish physiology at two pH values in the companion study (C. Morris *et al.*, unpubl. data). Some indices are strongly related to one another in a positive or negative fashion, whereas others are not, but these relationships vary with pH (Fig. 2 and 3). DOC quality has major impacts spanning trophic levels, DOC concentrations are rising in freshwaters and pH values are falling with climate change (reviewed by Sucker and Krause 2010; Pagano *et al.* 2014). Indeed, pH should be considered when determining the ecological role of DOCs within aquatic ecosystems.

Supplementary material

Supplementary material is available online.

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