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Freeze/thaw and pH effects on freshwater dissolved organic matter fluorescence and absorbance properties from a number of UK locations

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ABSTRACT

The UV–visible and fluorescence excitation–emission matrix spectrophotometric properties of dissolved organic matter (DOM) were compared for the effects of both pH and freeze/thaw on a wide range of freshwater DOM samples from the United Kingdom. It was observed that the spectrophotometric properties of our freshwater samples were sensitive to pH and that the recorded change varies with fluorescence and absorbance intensity, DOC concentration and the wavelength observed. Large and variable responses to pH were particularly severe at extremes of pH, but within the natural levels typically observed in freshwaters the response to pH was limited. For the same sample set large and variable responses were observed when subjected to freeze/thaw. From our data, knowledge of the original properties cannot be used to determine the amount of change that will occur with freezing and subsequent thawing. It is therefore recommended that in future research, to maintain the natural signal of the DOM, analysis is conducted at natural pH and without freezing to facilitate ease of comparison between studies. Our results also have implications for studies that utilise spectrophotometric techniques to investigate long-term trends in dissolved organic carbon in rivers. Spectrophotometric parameters from upland derived samples show varied responses of samples to pH and there is clear potential to complicate trends in the interpretation of long-term water colour data if pH is changing over time in a system or if samples are treated with different storage protocols with respect to acidification and freezing.

1. Introduction

Aquatic dissolved organic matter (DOM) strongly absorbs energy in the UV–visible (UV-vis) wavelength range, and this has led to the use of UV–vis absorbance spectrophotometry as a method to determine composition and concentration of DOM (Korshin et al., 1997). Typical UV–vis absorbance spectra of DOM, in both isolated and raw states, exhibit featureless trends of decreasing absorbance with increasing wavelength (Kalbitz et al., 1999). This lack of overall resolution has led to the measurement of UV–vis absorbance at single wavelengths or wavelength ratios to determine specific compositional

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variations in DOM (Hautala et al., 2000). For example, a_{465}/a_{665} has been used as a proxy for aromaticity (Chen et al., 2002), a_{254}/a_{410} has been used as a proxy for molecular weight (Andersen et al., 2000; Andersen and Gjessing, 2002) and specific UV absorbance $SUVA_{254}$ ($a_{254}\,m^{-1}$/DOC mg L^{-1}) has been shown to increase with increased aromaticity (Weishaar et al., 2003). The relationship of UV–vis absorbance to DOC concentration in natural waters has been utilised in an attempt to develop a quick and easy analytical technique to determine DOC concentrations. In the water treatment industry, absorbance at 254 nm is measured to monitor DOC concentration (Allpike et al., 2005) and in natural waters ~340 nm is often utilised (Tipping et al., 1988; Hernes and Benner, 2003). Water colour at longer wavelengths is often used by the water treatment industry as a simple proxy for DOM concentration, for example, 400–465 nm (Hongve and Åkesson, 1996; Hautala et al., 2000) in comparison to a standard solution of hexachloroplatinate and cobalt ions in hydrochloric acid (Pt–Co solution) as developed by Hazen (1892).

Three-dimensional fluorescence excitation–emission matrix (EEM) spectra typically cover a range of excitation and emission wavelengths from ~200 nm (short wavelength UV) through to ~500 nm (visible blue–green light), and may contain fluorescence centres which are attributed to both natural DOM groups such as humic and fulvic-like substances, as well as fluorescent protein-like material (Coble, 1996; Baker, 2001; Chen et al., 2003; Stedmon et al., 2003; Baker and Spencer, 2004; Spencer et al., 2007). The exact relationship between fluorescence properties and biogeochemical structure of the organic matter is unknown, but it is recognised that the fluorescence is generated by highly substituted aromatic nuclei, extensive conjugation and high-molecular-weight compounds (Senesi et al., 1989). Nevertheless, careful choice of excitation and emission wavelengths can allow the monitoring of changes in DOM composition (Coble, 1996; Kalbitz et al., 2000; McKnight et al., 2001) and DOC concentrations (Ferrari et al., 1996; Baker, 2002a).

DOM fluorescence and absorbance measurements are sensitive to changes in the environmental conditions of the sample. These conditions were reviewed by Senesi (1990) with respect to fulvic acids and fluorescence and include temperature, pH, metal ions, solvent interactions and other solutes. A typical response in DOM extracted from river water to pH was studied by Patel-Sorrentino et al. (2002) who observed an increase in fluorescence intensity with increasing pH over the range of 1 to 10–11, with a decrease at pH 12. Spectral shifts are also observed in response to changing pH. Mobed et al. (1996) observed a red shift, in fluorescence intensity maxima, with increasing pH at long wavelengths (EXλ~390 nm) and a similar red shift at shorter wavelengths (EXλ~320 nm) in soil derived humic substances. In aquatic derived DOM, shorter wavelength fluorescence peaks have been observed to blue shift with increasing pH (Mobed et al., 1996). Other authors have observed no wavelength change with pH (Tam and Sposito, 1993; Patel-Sorrentino et al., 2002).

The measurement and investigation of DOM in freshwaters by fluorescence and absorbance spectroscopy is increasing in the water sciences. In addition to the spectrophotometric measurements already described, DOM fractionation and concentration methods such as adsorption onto solid phases (Hood et al., 2003; Kaushal and Lewis, 2003), size exclusion chromatography (Allpike et al., 2005), field flow fractionation (Boehme and Wells, 2006) and tangential flow ultrafiltration (Belzile and Guo, 2006) show potential for coupling with spectrophotometric analyses to investigate specific DOM components or size fractions. Environmental effects on DOM fluorescence and absorbance are therefore important to understand to potentially investigate DOM composition through experimental variations of environmental conditions. For example, the wide range of fluorophore responses to pH in the literature reflects the complex nature and heterogeneous composition of DOM and under carefully controlled conditions this may be used to infer DOM composition (e.g. Patel-Sorrentino et al., 2002). Similarly, changes in fluorescence and absorbance properties of DOM due to freezing could also be used to infer DOM properties; however, to the authors' knowledge this has not been previously investigated for freshwater DOM. Here, we compare the effects of both pH and freeze/thaw on a wide range of freshwater DOM samples from the United Kingdom (UK) to investigate the response of spectrophotometric measurements and thus any potential changes in DOM composition. As acidification and freezing are commonly used storage methods for spectrophotometric measurements, it is important to understand any potential impacts these protocols may have on spectrophotometric DOM measurements.

2. Materials and methods

2.1. Study sites and sampling

Samples were collected from a wide number of sites from across the UK in the course of this study (Table 1). Sample sites are dominated by rural, upland, headwater catchments, a large number of which have significant peat cover within their catchments. The Coalburn Experimental Catchment (Northumberland, UK) was used extensively for replicate analyses and to investigate seasonal variability.

Water samples were collected in 'aged' 30 mL polypropylene bottles which had been cleaned in 10% HCl and triple rinsed with distilled water or precombusted glass bottles (450 °C for 4–8 h). Water samples were filtered (Whatman GF/C ashed glass microfibre filter papers) into the bottles and the bottles were rinsed with copious amounts of filtrate before collection of the sample for analysis. All samples were stored at 4 °C in the dark until analysis within less than 24 h or were stored frozen in the dark for the freeze/thaw experiments. To investigate the potential of contamination with respect to spectrophotometric and DOC measurements from the filtration system, sample collection bottles and storage procedures both at each step and for the whole procedure, the sample was substituted with distilled water and no sources of contamination were observed.

2.2. EEM fluorescence spectrophotometric analysis

Fluorescence was measured using a Perkin-Elmer luminescence spectrometer LS-50B. Samples were analysed in a

Table 1 – Sample ID, location, date of collection and source information of all the samples used in the freeze/thaw and pH modification experiments

Sample ID	Location	Date	Source
A1	River Traligill (Assynt) (NC 255219)	08/09/2000	1
A2	River Traligill (Assynt)	08/09/2000	1
A3	River Traligill (Assynt)	08/09/2000	1
A4	River Taw (Devon) (SS 643940)	03/04/2001	2
A5	M_E (Coalburn) (NY 697784)	12/10/2000	1
A6	F_C (Coalburn)	12/10/2000	1
A7	F_E (Coalburn)	12/10/2000	1
A8	River Blyth (NZ 190776)	01/05/2000	3
A9	Glenridding Valley Stream (NX 355157)	02/06/2000	3
A10	Fold Sike (NY 834293)	08/01/2001	3
A11	Chirdon Burn (NY 734847)	11/04/2001	1
A12	Shooter's Clough (SK 005747)	15/08/2000	3
A13	Agill Beck (Lofthouse Moor) (SE 129762)	16/04/2001	1
A14	River Coquet (NT 956035)	17/02/2001	3
A15	CB_{weir} (Coalburn)	30/03/2000	1
A16	CB_{weir} (Coalburn)	30/08/2000	1
A17	CB_{weir} (Coalburn)	16/01/2001	1
A18	CB_{weir} (Coalburn)	24/01/2001	1
A19	CB_{weir} (Coalburn)	11/05/2000	1
A20	P_{weir} (Coalburn)	30/03/2000	1
A21	P_{weir} (Coalburn)	30/08/2000	1
A22	P_{weir} (Coalburn)	11/05/2000	1
A23	PG_{weir} (Coalburn)	30/03/2000	1
A24	PG_{weir} (Coalburn)	15/11/2000	1
A25	PG_{weir} (Coalburn)	20/02/2001	1
A26	PG_{weir} (Coalburn)	11/05/2000	1
A27	Howan Burn (NY 705768)	30/03/2000	1
A28	Howan Burn (NY 705768)	25/05/2000	1
A29	Rookhope Burn (NZ 915425)	09/05/2000	3
A30	Rookhope Burn (NZ 915425)	13/06/2000	3
A31	River Teign (Chagford, Devon) (SX 694879)	18/04/2000	2
A32	River Exe (Exeter) (SS 909936)	20/04/2000	3
A33	Wash Leat (Chagford, Devon) (SX 701876)	23/04/2000	2
A34	Gruntley Beck (NY 826104)	11/05/2000	3
A35	Howgill Sike (NY 826104)	11/05/2000	3

Source 1, waters draining from predominantly peat areas; source 2, rural waters with agricultural land use; source 3, mixed peat and agricultural catchments.

10 mm quartz cell and at a constant room temperature of 20 °C. Sealed water cell blank scans were run every 10–15 samples to test machine stability using the Raman peak of water, at excitation 350 nm and emission 340–420 nm. Raman emission intensity at 390 nm averaged 20.69 ± 2.43 intensity units ($n = 245$). Fluorescence emission intensities were standardised to this peak (Baker, 2002b).

To produce three-dimensional fluorescence EEMs all samples were scanned in the following wavelength regions: excitation 200–500 nm at 5 nm steps and emission 200–600 nm at 0.5 nm steps. Here we report data on three fluorescence peaks. Peak A occurs at an excitation wavelength of 320–350 nm and emission wavelength of 400–450 nm and peak B occurs at an excitation wavelength of 340–390 nm and emission wavelength of 440–500 nm. These two peaks have been described previously and peak A has been related to fulvic-like substances and peak B to humic-like substances (Mobed et al., 1996; Baker, 2001; Newson et al., 2001; Baker, 2002a). Peak C is a fluorophore at 270–285 nm excitation and 340–360 nm emission and is attributed to tryptophan-like fluorescence (Baker, 2001; Baker and Inverarity, 2004). Excitation wavelength (peak $X_{EX\lambda}$) and emission wavelength (peak $X_{EM\lambda}$) were recorded at points of maximum fluorescence intensity (peak X_{Fint}) for peaks A–C in all analyses. Specific fluorescence intensity, peak XS_{Fint}, was determined as a ratio of peak X_{Fint}/DOC mg L^{-1}. In a small number of samples peaks B and C were not identifiable. A humic-like fluorescence peak at 220–240 nm excitation and 430–480 nm emission was observed in all samples, but frequently at an emission wavelength which was red-shifted onto a Rayleigh–Tyndall scatter line and so data for this fluorophore are not reported here.

At high solute concentrations, chromophores and fluorophores interfere with the normal process of excitation and emission resulting in suppression of fluorescence intensity (Bashford and Harris, 1987) which is described as inner-filter effects (IFE). IFE can be reduced by viewing the fluorescence closer to the surface of the cell, reducing the path length, the

use of a triangular analysis cell, dilution, standard additions, measurement at longer wavelengths or application of a correction factor (Senesi, 1990; McKnight et al., 2001; Ohno, 2002; Chen et al., 2003). A number of correction formulae have been derived to combat IFE (Zimmermann et al., 1999; McKnight et al., 2001; Ohno, 2002). The correction equation derived by Ohno (2002) (Eq. (1)) requires no prior knowledge of DOC concentration and therefore is easily applicable:

$$I = I_0(10^{-b(A_{ex}+A_{em})}), \quad (1)$$

where I is the detected fluorescence intensity and I_0 is the fluorescence in the absence of self-absorption. The factor b assumes that both emission and excitation only pass through 0.5× cuvette path length. A_{ex} and A_{em} are the absorbance of the solution at the excitation and emission wavelengths, respectively (Ohno, 2002). Because the majority of samples analysed in this study were coloured, with high absorbance (see Results section), Eq. (1) was applied to the fluorescence intensity data to correct for any IFE. Dilution of samples is also common to overcome IFE; however, this was not used, as dilution was observed to cause changes in pH which has been shown to result in changes in fluorescence intensity and spectral shifts (Mobed et al., 1996; Patel-Sorrentino et al., 2002).

2.3. UV–vis absorbance

UV–vis absorbance was measured using a WPA lightwave UV–vis diode-array spectrophotometer (S2000). Absorption spectra were obtained between 200 and 700 nm and individual absorption coefficients were recorded at a_{254}, a_{272}, a_{340}, a_{365}, a_{410} and a_{465} cm^{-1}. Samples were analysed in a 10 mm quartz cell and were blanked against distilled water. Samples were diluted with distilled water of zero absorbance if the measured absorption exceeded the analytical range (1.999 cm^{-1}). Absorption ratios were calculated as a_{254}/a_{410} and specific UV absorption as $SUVA_{254}$ (a_{254} m^{-1}/DOC mg L^{-1}).

2.4. pH and freeze/thaw protocols

The pH of all water samples was measured in the field using a Myron L Company model 6P ultrameter. Modification of pH for method development experiments was performed by the addition of NaOH or HCl and pH measurement using a Jenway bench pH meter, calibrated daily. To assess the impact of sample freezing, 35 water samples (Table 1) were frozen for up to 1 year, before being completely defrosted in the dark at 4 °C for re-analysis.

2.5. Total organic carbon (TOC)

TOC was measured as nonpurgeable organic carbon via the HTCO method incorporating a Shimadzu 5000 TOC analyser and a platinum alumina catalyst. Samples were acidified to pH~2 with HCl and subsequently sparged for 8 min at 100 mL min^{-1} with ultrapure oxygen to remove inorganic carbon. The mean of three to five injections of 100 μL is reported for every sample and precision, described as a coefficient of variance (CV), was <2% for the replicate injections.

2.6. Statistical analysis

Correlation coefficients were calculated using Spearman's rho method (Daniel, 1990) and significant differences were calculated using independent sample t-tests.

3. Results and discussion

3.1. The influence of pH on the spectrophotometric properties of DOM

To establish how natural variation in freshwater pH may influence DOM spectrophotometric properties, a number of pH manipulations were undertaken. Modification of pH was performed on sample numbers A1–A35 (Table 1). The NaOH and HCl used in the pH modification experiments were analysed to ensure there was no intrinsic fluorescence or absorbance derived from them. The observed response to the changes in pH is summarised in Table 2 and Figs. 1a–f, for four representative samples (A4, A11, A13 and A18). These four examples show the range of trends observed in all samples examined and there were no specific trends related to the different sources of DOM.

An overall significant (95% confidence level) red shift in peak $B_{EM\lambda}$ with increasing pH was observed in all samples over varying pH ranges (Table 2; Fig. 1b). This red shift is similar to those seen by Mobed et al. (1996) in a fluorescence intensity peak with similar excitation and emission wavelengths. The contrasting response in peak $A_{EM\lambda}$ and peak $B_{EM\lambda}$

Table 2 – Summary of the response in spectrophotometric properties observed on modification of solution pH (range of pH: 2–10)

Spectrophotometric properties	Response to increase in pH (2–10)
Peak C variables	No response
Peak $A_{EX\lambda}$ and peak $B_{EX\lambda}$	No response
Peak $A_{EM\lambda}$	No consistent response or variation outside the reproducibility of the method
Peak $B_{EM\lambda}$	A significant (95% confidence level) red shift was observed in all samples, over a different pH range and magnitude for each sample
Peak A_{Fint}	Increase, to a maximum at variable pH, decrease at higher pH, mean difference between minimum and maximum 15.75% (s.d. 5.38)
Peak B_{Fint}	Increase, mean difference between minimum and maximum 41.82% (s.d. 7.43)
Peak B_{Fint}/peakA_{Fint}	Increase, some samples exhibited a constant level below pH ~ 8.
a_{340} cm^{-1}	Increase, mean difference between minimum and maximum 17.79% (s.d. 3.45).

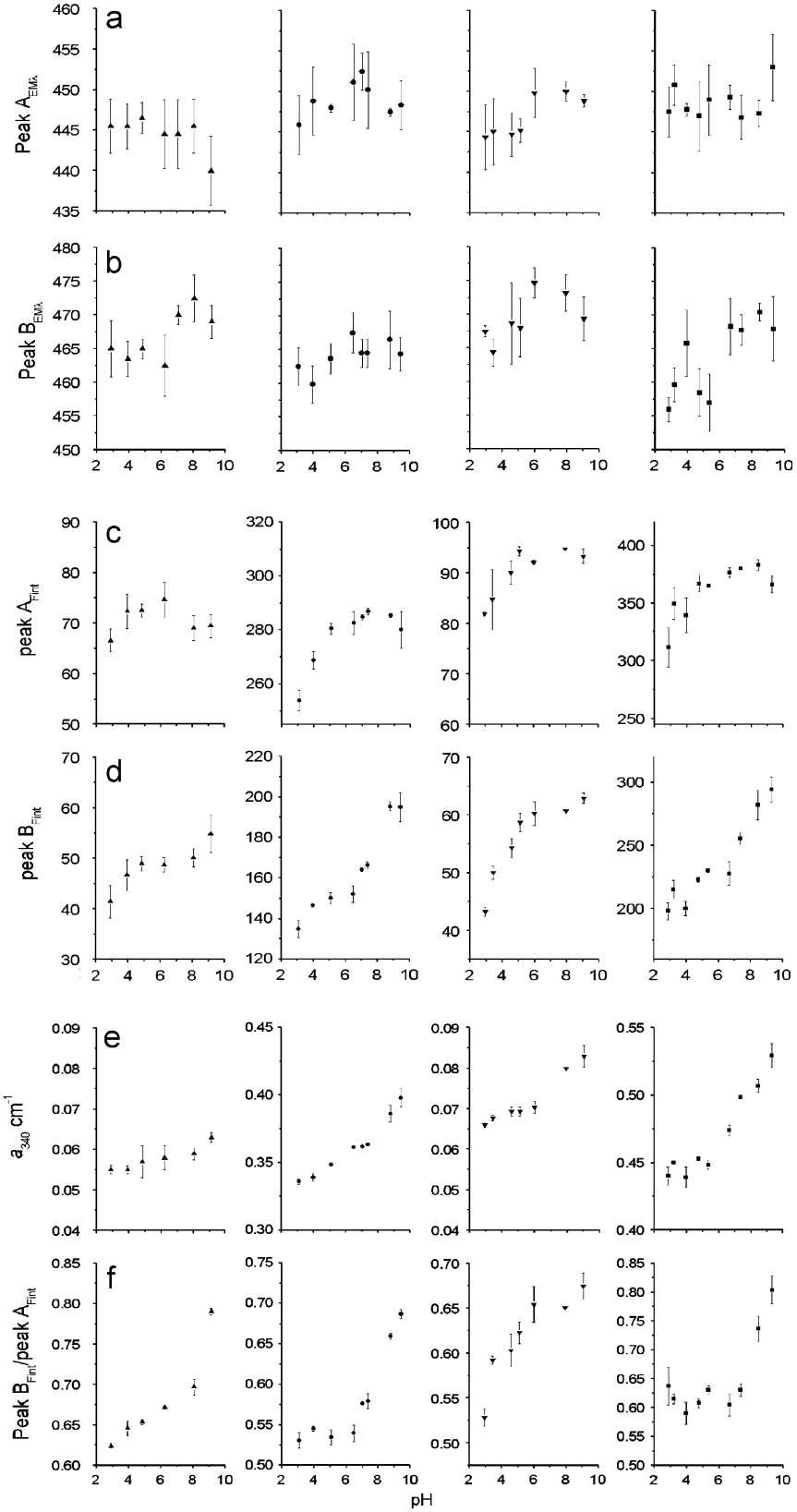

Fig. 1 – Changes in spectrophotometric properties on modification of solution pH: (a) peak $A_{EM\lambda}$, (b) peak $B_{EM\lambda}$, (c) peak A_{Fint}, (d) peak B_{Fint}, (e) a_{340} cm^{-1}, (f) peak B_{Fint}/peak A_{Fint}. (▲) A4; (●) A11; (▼) A13; (■) A18 . DOC concentrations (mg L^{-1}): A4 = 2.52; A11 = 14.91; A13 = 4.68; A18 = 22.25. For sample details see Table 1.

to pH change suggests a different composition between the fluorophores. However, specific functional groups responsible for the different responses are unclear. Fluorescence at shorter wavelengths (peak A) is attributed to the presence of simple structural components with electron donating constituents and long wavelength (peak B) to more conjugated structures with electron withdrawing groups (Senesi et al., 1991). The response known to occur due to changes in pH in electron withdrawing groups is the opposite of that observed for peak $B_{EM\lambda}$. Due to changes in the stabilisation of the excited state of such groups, wavelengths of emission are red-shifted on protonation (Schulman and Scharma, 1999). The opposite, a blue shift, is observed for electron donating constituents. This indicates that firstly it is difficult to predict pH response in compounds of unknown structure (Senesi, 1990).

Fluorescence intensity of DOM is known to increase with increasing pH and then to decline at higher pH levels and this pattern was observed for peak A_{Fint} (Fig. 1c; Table 2). However, the response of peak B_{Fint} to pH changes exhibited an overall increase (Fig. 1d; Table 2). Absorbance has been previously observed to increase with increasing pH (Andersen et al., 2000); Fig. 1e and Table 2 show such a relationship in the response of $a_{340}\,cm^{-1}$ to pH. The amount of change due to pH modification was greater for peak B_{Fint} than for $a_{340}\,cm^{-1}$ (Table 2); however, the increasing trend was similar between $a_{340}\,cm^{-1}$ and peak B_{Fint} (Figs. 1e, d, respectively). The difference in response of fluorescence intensity at different wavelengths is demonstrated in Fig. 1f and Table 2. As with the different response to pH in peak $A_{EM\lambda}$ and peak $B_{EM\lambda}$, the different response in intensity reflects the differing composition of fluorophores responsible for each peak. This study confirms observation made by Patel-Sorrentino et al. (2002) who observed a different response to pH at different wavelengths.

$a_{340}\,cm^{-1}$, peak A_{Fint} and peak B_{Fint} show a greater percentage increase, with increasing pH, if the original sample had higher values of these parameters (Figs. 1e, c and d, respectively). Therefore both fluorescence intensity and absorbance suggest that the response to pH is not only compositionally controlled, but also influenced by the DOC concentration of the original solution. The influence of pH must be considered in the interpretation of spectrophotometric parameters of DOM, especially if samples with a wide range of pH are being examined. Modification of all samples to the same pH is not recommended. As illustrated by the limited number of samples in this study, the DOM from 35 freshwaters exhibits different responses to pH; for example, the increase in peak B_{Fint} with increasing pH ranged from 32.1% to 74.8%. Thus, changing the solution pH may result in varying responses between DOM solutions. Changes in DOM concentration and composition are often related to stream discharge and seasonality which also impact on stream pH. At the typical pH levels observed in freshwater systems, little change was observed on the spectrophotometric parameters measured in this study, thus highlighting that changes in DOM concentration and composition measured by these techniques are typically due to other processes and are not merely a function of pH change.

3.2. *The influence of freeze/thaw on the spectrophotometric properties of DOM*

The changes in spectrophotometric properties of DOM samples after frozen storage and complete defrosting are summarised in Table 3, Figs. 2a–l and 3. Upon freezing and thawing the intensity and direction of spectral shift (increase and decrease) of the peaks A–C varied significantly between samples (Figs. 2a–i). However, a relative consistency of the intensity and direction of spectral shift of the peaks is observed for the replicate samples from the Coalburn Experimental Catchment (samples A15–A26; Table 1; Figs. 2a–l) which is consistent across season and varying discharge. For most samples and particularly those from the Coalburn Experimental Catchment (samples A15–A26), a greater change in peak C_{Fint} was observed compared to peak A_{Fint} or peak B_{Fint}, as indicated in Figs. 2g, h and i and Table 3. This possibly relates to the stability of the fluorophores that contribute to this fluorescence and may indicate that the protein-like fraction of fluorescent DOM is less stable in response to the freeze/thaw process in comparison to the humic-like and fulvic-like fractions.

It is important to recognise changes in fluorescence intensity ratios if such values are being used as a qualitative measure of DOM. In some cases there was little change from the original signal, however, as expected from the range of

Table 3 – Summary of the response in spectrophotometric properties observed with freezing and thawing

Spectrophotometric properties	Response to freeze/thaw
Excitation and emission wavelengths of peaks A–C	Mean changes were within analytical errors, individual samples exhibited up to ±20 nm shift. The greatest proportion of wavelength change was a blue shift for all wavelengths, except peak $C_{EM\lambda}$. Both direction and magnitude of wavelength change varied.
Peak A_{Fint} , peak B_{Fint} and peak C_{Fint}	80% of the samples exhibited a change in fluorescence intensity greater than the analytical reproducibility, both as increases and decreases Max. change: peak A_{Fint} − 38.24%; peak B_{Fint} − 40.58%; peak C_{Fint} + 52.02%
Peak B_{Fint}/peak A_{Fint}	Range: from −7.89% change to +38.81% change
Peak C_{Fint}/peak A_{Fint}	Range: from −13.01% change to +98.37% change
$a_{340}\,cm^{-1}$	The majority of samples showed a decrease and 77% of the samples exhibited a change outside the analytical reproducibility
Peak AS_{Fint}	Range: from −35.08% change to +30.66% change
$SUVA_{254}$	Range from −34.44% change to +7.03% change

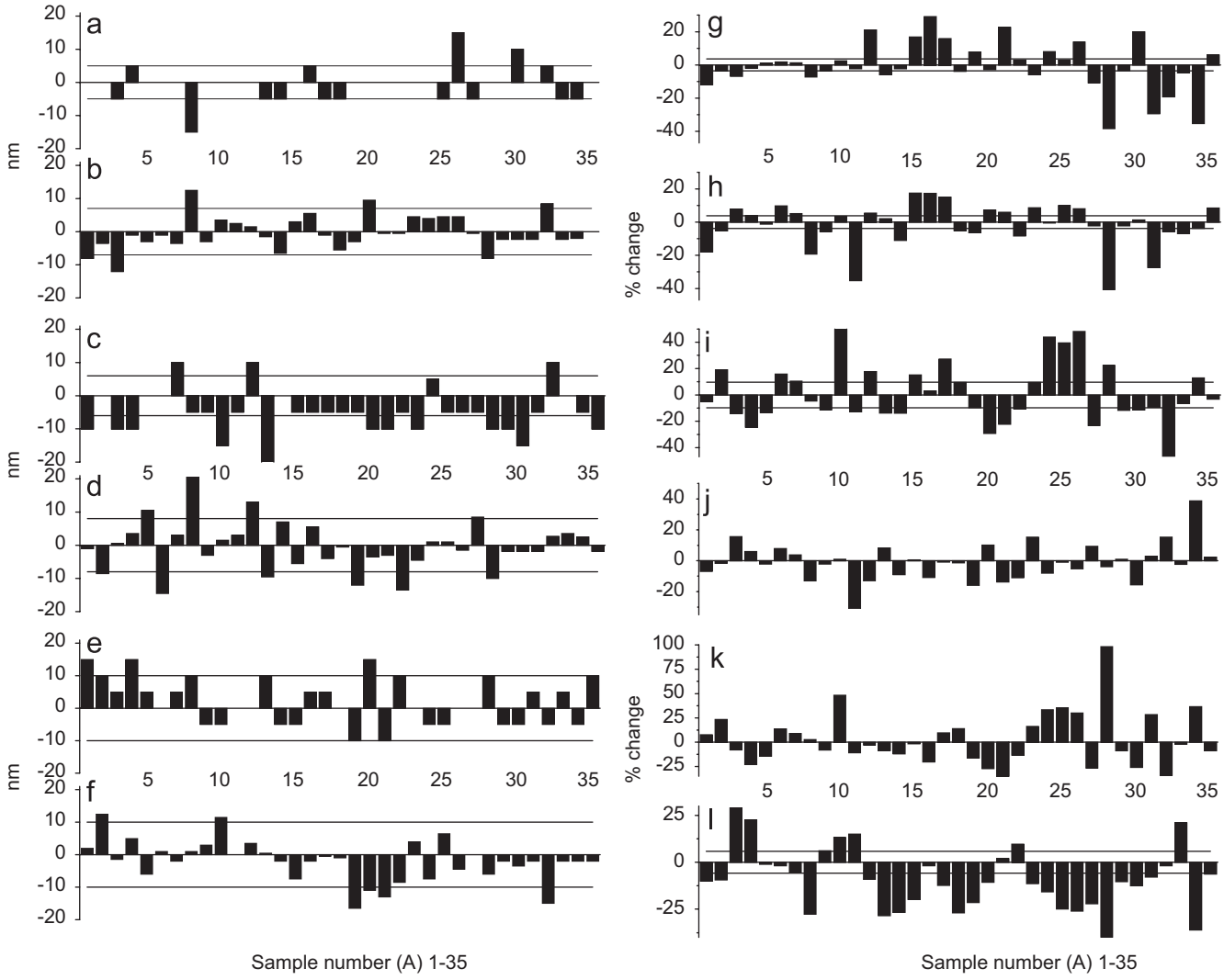

Fig. 2 – Changes in spectrophotometric properties after freeze/thaw: (a) peak $A_{EX\lambda}$, (b) peak $A_{EM\lambda}$, (c) peak $B_{EX\lambda}$, (d) peak $B_{EM\lambda}$, (e) peak $C_{EX\lambda}$, (f) peak $C_{EM\lambda}$, (g) peak A_{Fint}, (h) peak B_{Fint}, (i) peak C_{Fint}, (j) peak B_{Fint}/peak A_{Fint}, (k) peak C_{Fint}/peak A_{Fint}, (l) a_{340} cm^{-1}. ——— Analytical reproducibility. Samples 5, 10, 15, 20, 25, 30 and 35 are numbered between graphs to facilitate finding specific sample numbers of interest. For sample details see Table 1.

responses in fluorescence intensity shown in Figs. 2g–i this was not consistently the case. An extreme example of this is sample A28 which exhibited an increase in peak C_{Fint}/peak A_{Fint} of ~100% (Fig. 2k), effectively doubling the apparent proportion of peak C (protein-like) content. This was due to both a decrease in peak A_{Fint} and an increase in peak C_{Fint}. The changes in fluorescence intensities caused by freezing and thawing could potentially lead to erroneous interpretation of the fluorescence signal. As observed for fluorescence wavelengths, the changes in fluorescence intensities and fluorescence intensity ratios did not correlate with any of the original properties of the samples (95% confidence level). A recent study investigating the effect of freezing and thawing on fluorescence and DOC properties from a number of sediment pore waters observed no change in pore water fluorescence characteristics upon freezing and thawing (Otero et al., 2007) and so in comparison to the results shown here further highlights the variable response of this preservation method depending on DOM characteristics. Interestingly, Otero et al. (2007) observed that pore waters extracted from freeze preserved sediments, which is a common storage method (e.g. Murdoch and Azcue, 1995), resulted in increasing fluorescence intensity and DOC which they attributed to lysis from cells.

Not all samples exhibited the same magnitude of change in absorbance at different wavelengths after freeze/thaw treatment. For example, Table 4 details the change in absorbance in sample A4. In this sample, for example, the ratio of a_{254}/a_{410} which has been linked to molecular weight (Andersen and Gjessing, 2002) changed by +85.60%. This clearly presents problems when using such ratios in examining compositional differences in DOM if the samples were stored frozen and subsequently thawed prior to analysis. This pattern is not typical of those observed and is used as an illustration of the extreme variations in response to freeze/thaw in this data set. Sample A28 showed an ~40% loss in

a_{340} cm^{-1} (Fig. 2l) and this coupled with a loss in peak A_{Fint} and peak B_{Fint} suggests an overall loss of DOC concentration in the sample, as changes in both variables are closely related to concentration. To examine this, a number of defrosted samples (A2 and A15–A28; Table 1) were analysed for DOC concentration. As shown in Fig. 3a, DOC decreases by 4.87% for sample A28. This reduction in concentration cannot explain the greater decrease in absorbance and fluorescence intensity. Similarly, sample A23 exhibited a 7.24% increase in DOC concentration (Fig. 3a), but a corresponding decrease in both a_{340} cm^{-1} (Fig. 2l) and peak A_{Fint} (Fig. 2g).

In all the samples studied, neither a change in a_{340} cm^{-1}, peak A_{Fint} or peak B_{Fint} correlated with change in DOC concentration (95% confidence level). Before freezing, peak A_{Fint} and peak B_{Fint} correlated significantly with DOC (Spearman's rho = 0.654, rho = 0.539 respectively, 95% confidence level) and a similar relationship was seen for a_{340} cm^{-1} (Spearman's rho = 0.921, 99% confidence level). Upon thawing these relationships did not exist. These examples suggest a compositional or physical change, such as disaggregation, rather than a concentration related spectrophotometric response to the freeze/thaw processes, but that these processes may also alter DOC concentration. Additionally, as shown in Figs. 3b, and c individual samples show different responses in peak AS_{Fint} and $SUVA_{254}$ values, indicating that after freezing and defrosting DOM has a lower absorptivity (per mg organic carbon L^{-1}) and more fluorescence (per mg organic carbon L^{-1}). As with the other examined properties this was not consistent. For example, sample A23, which showed an increase in DOC concentrations, also shows a decrease in peak AS_{Fint} (Fig. 3b) and $SUVA_{254}$ (Fig. 3c), indicating that the proportion of fluorescent and light absorbing DOM in this sample has decreased. This experiment although only examining a limited number of DOM samples has revealed a variety of combinations of responses to freezing and thawing. This includes varying amounts of both increasing and decreasing fluorescence intensity and absorbance, at different wavelengths, at sample sites which were repeatedly sampled throughout the year.

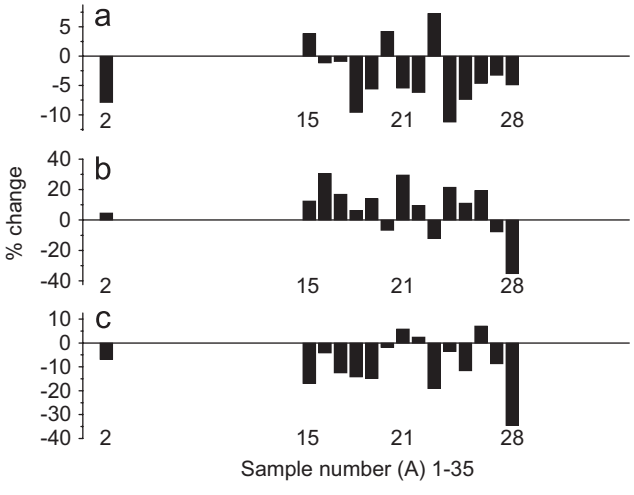

Fig. 3 – Changes after freeze/thaw in (a) DOC (mg L^{-1}), (b) peak AS_{Fint}, (c) $SUVA_{254}$, no bar represents no data. Samples are numbered between graphs to facilitate finding specific sample numbers of interest. For sample details see Table 1.

4. Conclusions

Due to the potential of spectrophotometric techniques for the water sciences community, it is anticipated that fluorescence and absorbance measurements will continue to increase in popularity in a wide range of studies. The ability to produce comparable data without the influence of environmental or storage variations is fundamental to the development of these techniques. From this study the following specific conclusions were drawn:

(1) The spectrophotometric properties of our freshwater samples were sensitive to pH, especially at extremes of pH and to modifications due to freezing and thawing.
(2) Within the natural pH levels typically observed in freshwaters, the response of spectrophotometric measurements was limited. Therefore, to maintain the natural signal of the DOM it is concluded that analyses be undertaken at natural sample pH and if possible the pH of the freshwater sample should be reported or at least the range given to show it is not at an extreme of pH where large variations may be observed.
(3) Rivers may exhibit changes in pH with time e.g. upland areas recovering from acidification and spectrophotometric parameters presented in this paper from upland derived samples show varied responses of samples to pH. There is clear potential to complicate trends in the interpretation of long-term water colour data if pH is changing over time in a system, or if acidified samples from different organic matter sources are compared to one another or even compared to non-acidified samples due to different sampling and storage protocols over time.
(4) Large and variable responses of spectrophotometric measurements were observed within the freeze/thaw experiments and knowledge of the original properties could not be used to determine the amount of change that would occur with freezing and subsequent thawing.

Table 4 – Percentage changes in absorption coefficients at different wavelengths after freeze/thaw in sample A4

Absorption coefficient (cm^{-1})	a_{254}	a_{272}	a_{340}	a_{365}	a_{410}	a_{465}
Change after freeze/thaw (%)	+2.54	+5.43	+22.55	+30.00	−44.75	−77.78

For sample details see Table 1.

Therefore, if possible samples for spectrophotometric analyses should be run as soon as is feasible after collection and preferably without frozen storage.

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