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Effects of filtration and pH perturbation on freshwater organic matter fluorescence

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Abstract

Fluorescence of organic matter from six contrasting freshwaters was analysed after filtration (1.2 μm and 0.2 μm filter sizes) and pH perturbation (±2 pH units from ambient conditions). Two fluorophores were compared in detail: tryptophan-like fluorescence, whose filtration and pH characteristics are relatively poorly understood, and humic-like fluorescence, which is better characterised. Although there was some variability in both fluorophores, the tryptophan-like fluorescence showed the most significant decrease in fluorescence intensity between raw and 1.2 μm filter samples, and a much smaller decrease between 1.2 and 0.2 μm, demonstrating a significant source associated with particulate material as well as a significant <0.2 μm fraction. In contrast, humic-like fluorescence shows little change with filtration, suggesting that the majority of this fluorescence is associated with truly dissolved material. The pH perturbation experiments demonstrate that tryptophan-like fluorescence is less impacted by pH than with filter fraction. For humic-like fluorescence, pH effects are weak and are not as consistent as those reported in the literature for extracted humic substances. pH perturbation of the freshwaters shows a wide range of sample specific pH responses, significantly more variable than that observed in experiments using extracted humic substances and tryptophan standards, demonstrating the natural variability of freshwater dissolved organic matter.

Keywords: Fluorescence; Organic matter; pH; Filtration; Humic substances

1. Introduction

Fluorescence provides important information on the characteristics of organic matter (OM) which can be useful in the elucidation of OM structure and in source apportionment and fingerprinting studies. Most recently this has included recognition that tryptophan-like fluorescence is a measure of biological activity, through either bacterial production or algal growth and its grazing (Cammack et al., 2004; Nguyen et al., 2005; Elliott et al., 2006; Urban-Rich et al., 2006). This fluorescence therefore has a wide range of sources, whose physical speciation covers a potentially wide range of sizes including particulate, colloidal and dissolved matter. All these fluorescent materials may interact in complex ways with other colloidal and particulate forms (Lead and Wilkinson, 2006). In contrast to tryptophan-like fluorescence, humic and fulvic like fluorescence is more widely recognised to be related to both autochthonous (within stream algal and microbial activity) and allochthonous (soil derived organic matter) generation of small colloidal and dissolved organic matter (Senesi et al., 1989; Cammack et al., 2004; Nguyen et al., 2005; Corvasce et al., 2006). The intensity of these fluorophores is more closely correlated to dissolved organic matter concentration (Baker and Spencer, 2004; Cumberland and Baker, in press), suggesting that these make up the greatest mass of the aquatic carbon pool, and fluorescence per gram of carbon and emission wavelength correlate with structural information such as molecular weight and aromacity (Senesi et al., 1991; Kalbitz et al., 1999).

Fluorescence provides useful information on the characteristics and dynamics of OM that can be obtained both

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in situ and non-invasively (Antízar-Ladislao et al., 2006; Saadi et al., 2006). Perturbation experiments utilising fluorescence analysis can therefore provide useful additional information on OM characteristics which are relevant to the natural environment, and can provide a useful and important contrast to experiments undertaken using extracted OM such as humic and fulvic acid (Kalbitz et al., 2000; Sierra et al., 2005). Analysis of different size fractions of OM, while perturbing the pH on each size fraction are simple experiments which will yield a great deal of information about OM. pH is known to affect the intensity of humic and fulvic like fluorescence (Mobed et al., 1996; Patel-Sorrentino et al., 2002), providing information on OM structure, but little is known about the effect of pH on tryptophan-like fluorescence. Filtration provides basic information on the relative size distribution of OM fluorescence, particularly by permitting a separation of nominal particulate, colloidal and dissolved material. The last of these fractions should be free from discrete microbial cells. Here, we present pH and filtration experiments from a range of river water samples representing a wide range of urban to rural waters and stationary to fast flowing. In particular, we focus on the effects of perturbation of tryptophan-like fluorescence, whose source and character within OM is poorly understood, and compare the perturbation response of tryptophan-like fluorescence to that of humic-like fluorescence, which in contrast has been comparatively well characterised.

2. Methods and materials

Six freshwater samples sites were chosen, as detailed in Table 1, which also presents for five of the sites water quality data based on the mean of 36 consecutive monthly measurements performed by the Environment Agency within the period 2000–2004. It can be seen that sites were chosen from very good water quality (e.g. the River Tern, which has been rated between grades A and B for chemical water quality by the Environment Agency for England and Wales, where A is highest water quality grade) to bad quality (e.g. the Tame, rated between grades D and E, where E is the lowest grade). Sites also include free flowing rivers (e.g. the Bournbrook) through to slow moving and heavily regulated waters (Birmingham and Worcester Canal) and a lake (Vale Lake).

Approximately 1.5–2 l of river water sample was taken from each site, and the ambient pH measured and noted. All samples were taken immediately (within 2 h) back to the laboratory and the pH remeasured. Three aliquots of 500 ml of river were taken and the fluorescence of each measured. From the first 500 ml sample, three sub samples of 150 ml each were dispensed. By the addition of small amounts of HNO_3, the pH of one sample was adjusted to approximately 1.5–2 pH units below that of the original sample. Small amounts of NaOH were added to another sample to alter the pH to approximately 1.5–2 pH units above that of the original sample. The third sample was

Table 1
Water quality data and site descriptions

Sample site and location	BOD ($mg\,l^{-1}$)	Ammonia ($mg\,l^{-1}$)	DO (% saturation)	Nitrate ($mg\,l^{-1}$)	Phosphate ($mg\,l^{-1}$)	Ambient pH	Site description
River Tame (UK NGR SP 010980)	4.3 (E)	0.64 (D)	81.1 (D)	37.05 (5)	1.76 (6)	7.5	Urban land use, close to motorway and industrial areas. Channelised river, part of NERC URGENT catchment. High nitrate, phosphate and BOD from sewerage pollution
River Tern (UK NGR SJ 707383)	1.9 (B)	0.11 (A)	92.8 (A)	47.04 (6)	0.42 (5)	7.8	Rural location, agricultural land cover with riparian wetlands Sample site is part of NERC LOCAR catchment. High nitrate from agriculture, good BOD and ammonia
Bournbrook (UK NGR SP 040828)	2.5 (C)	0.13 (B)	106.8 (A)	9.39 (2)	0.18 (4)	7.8	Heavily channelised small river, urban location. Tributary of the Tame. Relatively poor BOD and phosphate from sewerage
River Rea (UK NGR SP 080830)	6.0 (E)	0.41 (C)	105.4 (A)	12.93 (3)	0.19 (4)	8.1	Urban river, channelised. Tributary of the River Tame. High BOD and ammonia from sewerage
Birmingham and Worcester Canal (UK NGR SP 060840)	4.2 (D)	0.92 (A)	95.24 (C)	0.15 (2)	0.10 (3)	7.7	Urban location, heavily regulated flow. Generally good water quality but high BOD due to regulated flow
Vale Lake (UK NGR SP053847)	nd	nd	nd	nd	nd	7.6	Urban location, set in park land and fed by the Chad Brook. Tributary of the Bournbrook. Upstream water quality known to be affected by sewerage failures

BOD refers to biological oxygen demand, DO refers to dissolved oxygen. Grades refer to water quality scales used by the Environment Agency (EA). Chemical and biological data are measured on a scale A–F, where A is very good and F is very bad. Nutrients are measured on a scale 1–6, where 1 is very low and 6 is very high. Location data are United Kingdom National Grid References (UK NGR).

maintained at ambient pH. The pH of the altered samples was allowed to equilibrate for approximately 2 h, and the pH measured again and adjusted if necessary. Fluorescence properties of all three samples were analysed, and then all three samples were filtered at 1.2 μm with Whatman GF/C glass microfibre filters, and the fluorescence and pH of the filtrate measured. The samples were then filtered a second time at 0.2 μm with Millipore polycarbonate filters, the fluorescence of the filtrate measured, and finally the pH recorded.

The second 500 ml aliquot dispensed from the original river water sample was filtered at 1.2 μm before being split into smaller aliquots for pH perturbation and filtration as described above. The third 500 ml aliquot of the original river water sample was filtered at 1.2 μm, and then at 0.2 μm, before being split into smaller aliquots for pH perturbation and filtration as described above. A total of 21 measurements were therefore performed for each site.

For each sample the initial tryptophan- and humic-like fluorescence of each was measured through the analysis of a fluorescence excitation–emission matrix (EEM). Fluorescence spectra of waters were recorded on a Varian Cary Eclipse spectrofluorometer using a 4 ml, 1 cm path length cuvette equipped with a water-cooled Peltier temperature controller. Following published methods (Baker, 2001), fluorescence EEMs were generated by scanning and recording emission spectra from 300 to 500 nm at 0.5-nm steps with 5 nm increments of excitation wavelength between 250 and 400. The slits for excitation and emission were 5-nm; the temperature of analysis and PMT voltage were set at 20 ± 0.1 °C and 725 V. The spectrophotometer was calibrated by detecting the Raman intensity at 395 nm excitation using a sealed water cell and emission intensity averaged 21.6 ± 0.5 units with no drift during the analytical period.

3. Results

3.1. Fluorescence EEMs of raw samples

For ease of comparison, the fluorescence EEMs of all samples (before experimentation began) are shown in Fig. 1 and summarised in Table 2. The general characteristics of the samples include a tryptophan-like fluorophore observed with two excitation peaks of 225–230 and 280–285 nm, and an emission value of 335–350 nm. Two peaks attributed to humic-like substances were noted at 230–245 and 320–340 nm excitation, and 395–430 nm emission. All of the tryptophan-like emission maxima of the samples are within the same range of 340–350 nm for the lower peak and 335–345 nm for the upper peak. The humic-like peak with excitation at 230–245 nm exhibits a relatively wide range of emission values across the sample sites, ranging from emission at 395 nm in the Canal site, to 430 nm in the river Tern. The Bournbrook, in a heavily urbanised area, yielded the second lowest emission wavelength at 405 nm, and the highest emission wavelength of 430 nm was observed in the Tern samples, which was the most rural of all sites. Coble (1996) and Bolton (2004) reported differences in the emission wavelength fluorescence of humic-like material related to the chemical composition of samples as a result of the source of the humic material. This is in line with knowledge about other aspects of humic substances structure, which vary both spatially and temporally.

Given the relative strength of the humic-like peak at excitation at 230–245 nm compared to that at 320–340 nm, only this peak is considered in the perturbation experiments. Similarly, only the more intense of the two tryptophan-like fluorescence intensities (excitation at 225–230 nm) is analysed.

3.2. Changes in 225–230 nm tryptophan-like fluorescence with pH and filtration

A total of 21 fluorescence EEMs were obtained for each sample, the results for changes in the 225–230 nm tryptophan-like fluorescence are presented in Table 3a–f. These include:

- The raw sample ($n = 1$, as described in the previous section).
- Samples where pH was altered before filtration at 1.2 μm ($n = 3$) and 0.2 μm ($n = 3$) (data rows 1–3 in Table 3, 1.2 μm filtration results in middle column and 0.2 μm results in last column).
- Samples filtered at 1.2 μm ($n = 1$), and subsequently pH perturbed and re- filtered at 1.2 μm ($n = 3$) and 0.2 μm ($n = 3$) (rows 4–6 in Table 3; 1.2 μm filtration results in middle column and 0.2 μm results in last column).
- Samples filtered at 1.2 μm and again at 0.2 μm ($n = 1$), and subsequently pH perturbed and refiltered at 1.2 μm ($n = 3$) and 0.2 μm ($n = 3$), to investigate if the pH change causes aggregation of organic matter (rows 7–9 in Table 3; 1.2 μm filtration results in middle column and 0.2 μm results in last column).
- Results of pH perturbation experiments for the different filter stages can be read from the rows of data in Table 3. Acidified (−2 pH units) sample data is presented in data rows 1, 4 and 7; ambient data in rows 2, 5, and 8; and alkaline (+2 pH units) samples in rows 3, 6 and 9.

Table 3a–f shows that all samples exhibited a decrease in tryptophan-like fluorescence intensity after filtration at 1.2 μm (Bournbrook −5% to −84%; Vale Lake −7% to −84%; River Tern −52% to 79%; Canal −47% to −85%; River Rea −72% to 89%; River Tern −35% to −79%). This consistent decrease intensity contrasts with fluorescence intensity changes observed between filtration at 1.2 and 0.2 μm (Bournbrook −1% to −24%; Vale Lake +9% to −58%; River Tame −1% to −9%; Canal 0% to −34%; River Rea −2% to −10%; River Tern +6% to −31%). This result demonstrates that all samples have substantial tryptophan-like fluorescent material in the >1.2 μm fraction;

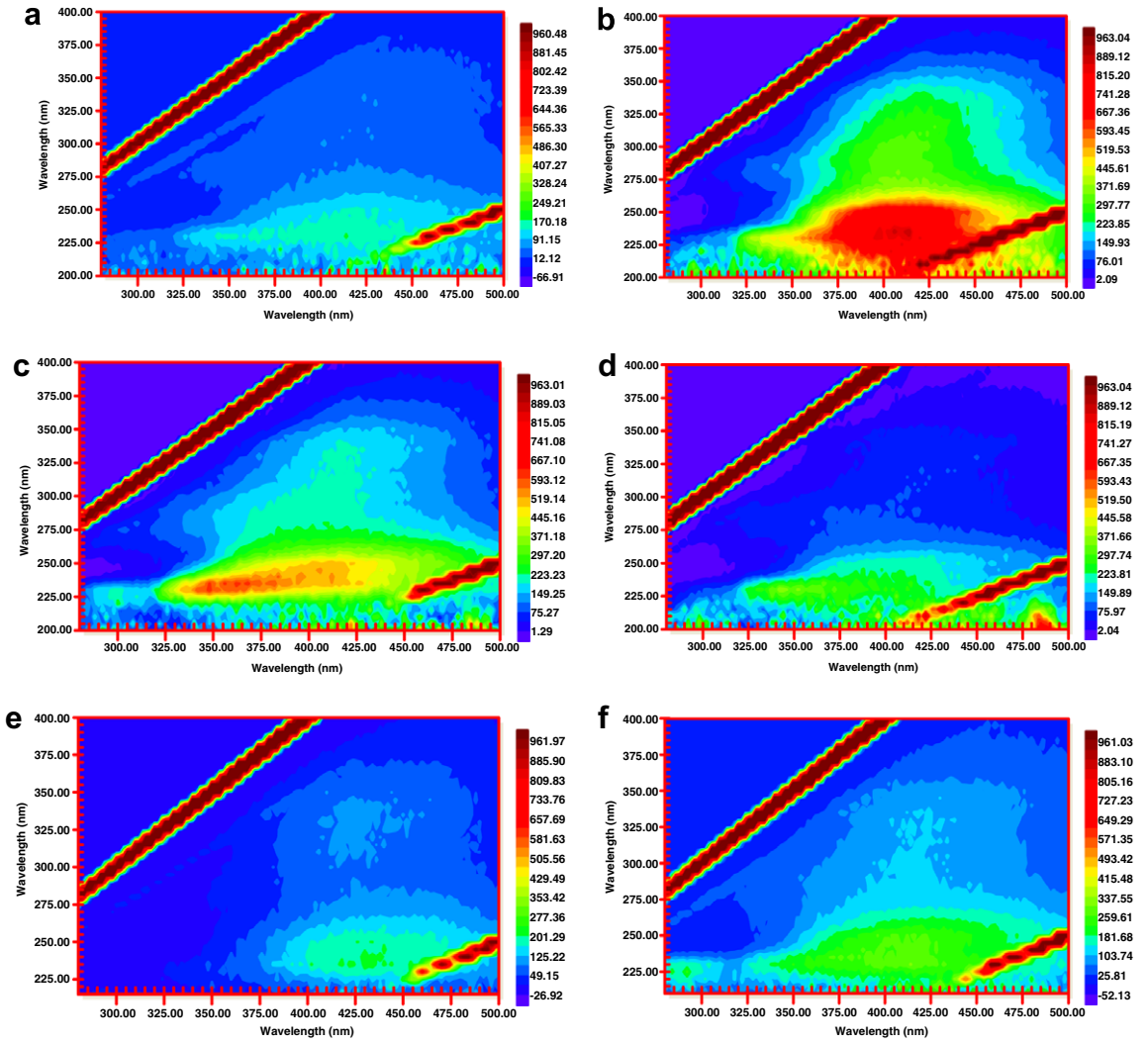

Fig. 1. 3D Excitation–emission matrix plots showing river water sample fluorescence intensity measurements before experimentation: (a) Bournbrook; (b) Vale Lake; (c) R. Tame; (d) Birmingham and Worcester Canal; (e) R. Tern; (f) R. Rea. *X*-axis is the emission wavelength and *y*-axis, the excitation wavelength.

Table 2
Wavelengths of excitation and emission maxima of all samples (nm), and fluorescence intensities (a.u.) of each peak

Sample site	Tryptophan-like peak a			Tryptophan-like peak b			Humic substance-like peak		
	Emission	Excitation	Intensity	Emission	Excitation	Intensity	Emission	Excitation	Intensity
Bournbrook	345	230	177	345	280	46	405	235	196
Vale Lake	345	230	491	345	285	152	405	240	897
Tame	350	230	646	345	285	176	410	245	578
Canal	340	230	386	335	285	87	395	230	303
Tern	340	225	69	335	285	31	430	240	253
Rea	340	230	206	335	280	58	420	235	312

Both tryptophan-like peaks, and the humic-like peak are included. Intensity shown is the maximum recorded value for each peak. Tryptophan-like peak 'a' refers to 220 nm excitation and 'b' to 280 nm excitation.

most also have an amount of tryptophan-like fluorescent material in the 1.2–0.2 μm fraction, while some tryptophan-like fluorescence also remained in the <0.2 μm fraction.

pH has a lesser effect on fluorescence intensity than filtration, with a 4 pH unit range leading to a loss in fluorescence at 1.2 μm filtration of <40% and at 0.2 μm filtration of <30%, both values significantly less than the fluorescence decrease at 1.2 μm filtration. For the Bournbrook samples and Vale Lake samples, greater loss of fluorescence occurred in the acid fraction after 1.2 μm filtration (for example, Bournbrook −64% to −82% at acid pH,

Table 3
225–230 nm excitation tryptophan-like fluorescence intensity changes with pH and filtration

		% Decrease from initial fluorescence	pH	pH after adjustment	% Change between raw and 1.2 μm filter	pH value after filter	% Change between 1.2 and 0.2 μm filter	pH value after filter
(a) Bournbrook								
				5.9	−82	5.9	−5	5.9
Raw sample	→	0	7.8	7.8	−64	7.9	−11	7.9
↓				9.9	−47	10.0	−24	9.8
↓								
↓				5.9	−64	6.0	−11	6.4
1.2 μm	→	−5	7.8	7.8	−14	7.9	−12	7.9
↓				9.9	−30	9.8	−25	9.9
↓								
1.2 μm				5.5	−69	5.5	−1	5.5
And 0.2 μm	→	−32	7.5	7.5	−31	7.5	−7	7.4
				9.5	−42	9.0	−5	9.3
(b) River Tame								
				5.6	−76	5.6	−9	5.5
Raw sample	→	0	7.5	7.5	−62	7.5	−9	7.5
↓				9.5	−79	9.5	−3	9.5
↓								
↓				5.5	−70	5.5	−7	5.5
1.2 μm	→	−32	7.5	7.5	−76	7.5	−1	7.5
↓				9.6	−64	9.6	−5	9.6
↓								
1.2 μm				5.5	−70	5.5	−6	5.5
And 0.2 μm	→	−68	7.5	7.5	−62	7.5	−3	7.5
				9.5	−69	9.5	−9	9.5
(c) River Rea								
				6.4	−81	6.4	−7	3.4
Raw sample	→	0	8.1	8.1	−72	8.1	−7	6.4
↓				10.0	−73	10.0	−5	7.1
↓								
↓				4.6	−81	4.6	−7	3.3
1.2 μm	→	−71	7.0	7.0	−76	6.7	−10	3.5
↓				8.6	−85	8.6	−2	6.5
↓								
1.2 μm				4.8	−89	4.8	−5	2.8
And 0.2 μm	→	−79	7.0	7.0	−81	7.0	−5	3.8
				9.0	−86	9.0	−7	6.6
(d) Vale Lake								
				5.7	−57	5.7	−14	5.5
Raw sample	→	0	7.6	7.6	−14	7.5	−38	7.4
↓				9.4	−26	9.4	−58	9.5
↓								
↓				5.6	−84	5.6	−2	5.5
1.2 μm	→	−7	7.6	7.6	−24	7.6	−14	7.6
↓				8.7	−71	8.8	9	8.8
↓								
1.2 μm				5.0	−74	5.0	2	5.0
And 0.2 μm	→	−32	6.9	6.9	−66	7.0	−2	7.0
				8.3	−48	8.3	−21	8.3
(e) Canal								
				5.5	−55	5.5	−34	5.5
Raw sample	→	0	7.7	7.7	−47	7.5	−12	7.5
↓				9.3	−85	9.5	−15	9.3
↓								
↓				6.2	−78	6.2	−16	6.2
1.2 μm	→	−52	8.1	8.1	−56	8.1	−12	8.1
↓				10.0	−65	10.0	−15	10.0
↓								
1.2 μm				5.0	−84	5.0	0	5.0
And 0.2 μm	→	−86	7.0	7.0	−84	7.0	−7	7.0
				9.3	−83	9.3	−7	9.3

(*continued on next page*)

Table 3 (*continued*)

		% Decrease from initial fluorescence	pH	pH after adjustment	% Change between raw and 1.2 μm filter	pH value after filter	% Change between 1.2 and 0.2 μm filter	pH value after filter
(f) River Tern								
				5.6	−78	5.6	−10	2.9
Raw sample	→	0	7.8	7.8	−35	7.8	−31	6.9
↓				9.5	−72	9.5	6	6.2
↓								
↓				5.3	−52	5.3	−12	5.7
1.2 μm	→	−43	6.9	6.9	−57	6.9	−17	6.8
↓				8.7	−45	8.7	−12	8.4
↓								
1.2 μm				5.1	−69	5.1	−5	5.1
And 0.2 μm	→	−50	6.9	6.8	−79	6.8	0	6.8
				8.8	−74	8.8		8.8

−30% to −47% at alkaline pH); results from the other sample sites were more variable with respect to fluorescence changes on pH and filtration perturbation.

3.3. Changes in 230–245 nm humic-like fluorescence

Table 4a–f shows the changes in humic-like fluorescence intensity for pH and filtration perturbation; table layout is as per Table 3a–f. Decreases in humic-like fluorescence intensity at all sample sites with filtration were much smaller than those observed in 225–230 nm tryptophan-like fluorescence intensity at the same sites. Fluorescence intensity variations at 1.2 μm filtration were −7 to −35 (Bournbrook); −2 to −31 (Vale Lake); +15 to −16 (River Tame); +13 to −15 (Canal); +3 to −18 (River Rea) and −4 to −38 (River Tern). At the 0.2 μm filtration step, fluorescence intensity varied by a further +5 to −17 (Bournbrook); +6 to −16 (Vale Lake); +21 to −32 (River Tame); +11 to −30 (Canal); +5 to −17 (River Rea) and +14 to −17 (River Tern). These results suggest that although some humic-like fluorescence is associated with a colloidal size fraction, the majority is in the <0.2 μm fraction. There is

Table 4
Changes in 230–245 nm excitation humic acid fluorescence intensity with pH and filtration

		% Decrease from initial fluorescence	pH	pH after adjustment	% Change between raw and 1.2 μm filter	pH value after filter	% Change between 1.2 and 0.2 μm filter	pH value after filter
(a) Bournbrook								
Raw sample	→	0	7.9	5.9	−19	5.9	−17	5.9
↓				7.9 (ambient)	−13	7.9	−2	7.9
↓				9.9	−20	10	−7	9.8
↓								
1.2 μm	→	−2	7.8	5.9	−21	6	−16	6.4
↓				7.8 (ambient)	−11	7.9	5	7.9
↓				9.9	−7	9.8	−8	9.9
1.2 μm								
And 0.2 μm	→	−8	7.5	5.5	−35	5.5	−2	5.5
				7.5 (ambient)	−28	7.5	−7	7.4
				9.5	−26	9	1	9.3
(b) River Tame								
Raw sample	→	0	7.5	5.6	0	5.6	−32	5.5
↓				7.5 (ambient)	−6	7.5	−5	7.5
↓				9.5	4	9.5	−12	9.5
↓								
1.2 μm	→	2	7.5	5.5	15	5.5	−29	5.5
↓				7.5 (ambient)	6	7.5	−18	7.5
↓				9.6	−9	9.6	−6	9.6
1.2 μm								
And 0.2 μm	→	−4	7.5	5.5	−2	5.5	−12	5.5
				7.5 (ambient)	−14	7.5	−7	7.5
				9.5	−16	9.5	21	9.5
(c) River Tern								
Raw sample	→	0	7.8	5.6	−38	5.6	2	2.9
↓				7.8 (ambient)	−4	7.8	−7	6.9
↓				9.5	−34	9.5	14	6.2
↓								

Table 4 (*continued*)

		% Decrease from initial fluorescence	pH	pH after adjustment	% Change between raw and 1.2 μm filter	pH value after filter	% Change between 1.2 and 0.2 μm filter	pH value after filter
1.2 μm	→	−11	6.9	5.3	−15	5.3	−17	5.7
↓				6.9 (ambient)	−11	6.9	−2	6.8
↓				8.7	−6	8.7	−4	8.4
1.2 μm								
And 0.2 μm	→	−14	6.9	5.1	−13	5.1	−1	5.1
				6.8 (ambient)	−11	6.8	−2	6.8
				8.8	−13	8.8	1	8.8
(d) Vale Lake								
Raw sample	→	0	7.6	5.7	−2	5.7	−9	5.5
→				7.6 (ambient)	−12	7.5	−11	7.4
↓				9.4	−25	9.4	−8	9.5
↓								
1.2 μm	→	−22	7.6	5.6	−5	5.6	−7	5.5
↓				7.6 (ambient)	−23	7.6	−5	7.6
↓				8.7	−31	8.8	−8	8.8
1.2 μm								
And 0.2 μm	→	−30	6.9	5	−9	5	−9	5
				6.9 (ambient)	−12	7	−16	7
				8.3	−32	8.3	6	8.3
(e) Canal								
Raw sample	→	0	7.7	5.5	−15	5.5	−3	5.5
↓				7.7 (ambient)	−10	7.5	−7	7.5
↓				9.3	−5	9.5	−30	9.3
↓								
1.2 μm	→	−9	8.1	6.2	−1	6.2	−2	6.2
↓				8.1 (ambient)	−11	8.1	−1	8.1
↓				10	−11	10	−11	10
1.2 μm								
And 0.2 μm	→	−13	7	5	13	5	−23	5
				7 (ambient)	−3	7	11	7
				9.3	−13	9.3	−7	9.3
(f) River Rea								
Raw sample	→	0	8.1	6.4	−9	6.4	0	3.4
↓				8.1 (ambient)	−10	8.1	−5	6.4
↓				10	−11	10	−3	7.1
↓								
1.2 μm	→	−12	7	4.6	−18	4.6	−2	3.3
↓				7 (ambient)	−12	6.7	5	3.5
↓				8.6	−13	8.6	−5	6.5
1.2 μm								
And 0.2 μm	→	−9	7	4.8	3	4.8	−17	2.8
				7 (ambient)	−10	7	−11	3.8
				9	−16	9	−4	6.6

a strong possibility that tryptophan-like fluorescent material is associated with larger biological particles, whereas smaller humic-like material (1 nm to several hundred nm in size, Lead et al., 2000; Lead and Wilkinson, 2006) is less likely to be removed by the filtration processes used during our experiments.

It was expected that the largest decreases in humic-like fluorescence intensity would occur at acidic pH due to aggregation which has previously been observed in the literature (Mobed et al., 1996; Avena et al., 1999; Patel-Sorrentino et al., 2002; Chen et al., 2003). However, none of the samples behaved in the predicted manner. For example, the highest decreases in fluorescence intensity in the samples from the Vale Lake site generally occurred at alkaline pH (−25% to −32%) and mostly in the 1.2–0.2 μm fraction. In the River Tame the highest decreases in pH did occur at acid pH (−12% to −32%) but only in the 0.2 μm filter fraction. For the other samples, very little variation occurred in fluorescence intensity with pH.

4. Discussion

The differences in tryptophan-like and humic-like fluorescence with filtration observed in our experiments fits our understanding that extracted humic substances are in the size range of 1 nm to several hundred nm (Lead et al., 2000; Lead and Wilkinson, 2006). Thus the reason for smaller decreases in humic-like fluorescence intensity compared to tryptophan-like fluorescence intensity is that generally humic substances pass more easily through the

0.2 μm pore size filter paper. As a small decrease in humic-like fluorescence is also sometimes observed in the filter stages, some humic substances are in these size ranges, most likely not as free humic material but sorbed to the surface of other material (Lead and Wilkinson, 2006). Measurement of the humic-like fluorescence properties of different size fractions should allow us to selectively probe the surface sorbed humic material. Tryptophan-like fluorescent molecules are more likely to be associated with larger biological molecules of a size greater than 0.2 μm (tryptophan residues are often embedded within a protein (Lakowicz, 1999), which may be associated with microbial cells), hence they are unable to pass through the filter, therefore leading to lower tryptophan-like fluorescence intensities in the permeate. However, we observe a bigger decrease in tryptophan-like fluorescence between the raw and <1.2 μm samples, rather than between the <1.2 and <0.2 μm fractions, suggesting that in our samples more tryptophan-like fluorescence derives from particulate and the largest colloidal (cellular) material.

pH perturbation experiments generally showed that the highest loss of tryptophan-like fluorescence occurs in the acidic solutions, but with significant variability between samples. This is probably due to the many potential sources of tryptophan-like fluorescence. For example, if the tryptophan-like fluorescence is associated with colloidal material (as demonstrated by the loss of fluorescence with filtration), then pH-related conformational changes can occur within the colloids associated with the fluorescent moieties (such as aggregation). Additionally, there may be direct changes in the behavior of the fluorophore resulting in, for example, increased exposure to the solution phase. Folding and unfolding reactions of proteins (probably associated with other particles, colloids or dissolved OM in this case) with pH changes may be considered as a further possible explanation for the observed differences in final tryptophan-like fluorescence intensities (Lakowicz, 1999). Finally, the acid–base behavior of an amino acid in solution may also be important in controlling fluorescence characteristics, as the carboxylic and amine functional groups present within the structure can allow the molecule to act either as an acid or as base depending upon the pH of the solution.

It was expected that the highest losses of humic-like fluorescence intensity would likely occur in the most acidic samples due to aggregation losses, although this was not observed in our experiments. Chen et al. (2003) suggested that changes in the protonation states of functional groups such as amines and hydroxyls can increase fluorescence intensities. Based on the work of Mobed et al. (1996), it would be expected that as pH increases, the fluorescence intensity of humic-like substances also increases (also observed by Patel-Sorrentino et al., 2002), and that changes in the acidic functional groups caused by increasing pH will lead to changes in fluorescence of the molecule. It is important to consider that the current work is based on heterogeneous natural samples, unlike the majority of the work quoted here (with the exception of Patel-Sorrentino et al., 2002), which is based on extracted humic substances. Our understanding of the structure of humic substances is still poor, with competing evidence for them as permeable spheres, micelles, polymers and fractal aggregates (see review in Lead and Wilkinson, 2006). Our variable pH responses may reflect the possibility that a range of humic structures is present in unperturbed samples.

5. Conclusions

In the majority of samples it has been shown that tryptophan-like fluorescence showed the most significant decrease in fluorescence intensity between raw and 1.2 μm filter samples, with smaller decreases between 1.2 and 0.2 μm, demonstrating a significant source associated with particulate material as well as a significant <0.2 μm fraction. The wide size distribution of tryptophan-like fluorescence agrees with its many potential sources; further research is needed to investigate whether a constant tryptophan-like fluorescence intensity – organic carbon concentration relationship occurs for all size fractions. In contrast, humic-like fluorescence shows little change with filtration, suggesting that the majority of this fluorescence is associated with truly dissolved material. pH perturbation experiments demonstrate that tryptophan-like fluorescence is less impacted by pH than by filter fraction. For the humic-like fluorescence, pH effects do not reflect those reported in the literature. pH perturbation shows a wide range of sample specific pH responses, significantly more variable than that observed in experiments using extracted humic substances and tryptophan standards, demonstrating the natural variability of freshwater dissolved organic matter.

Much of the published literature concerns humic acid and fulvic acid isolates and/or laboratory standard solutions such as those from the International Humic Substances Society, whereas our samples were complete and unperturbed natural river water samples. Compounds would have been present in our samples which are not present in humic substances standards and as such may have led, in part, to the differences in pH and filtration behavior exhibited, as the natural river samples are far more complex. Our work also includes OM from sources including small urban catchments, which are not commonly investigated, thus to some extent we would expect the results to be somewhat different to published literature. Most published work involving freshwaters has been concerned with larger rivers where OM will be more mixed, relatively more processed, and potentially less labile; whereas in smaller catchments such as those studied in the current work OM might be expected to be more variable, less processed and possibly very different in urban rivers compared to clean rivers. What is clear is that work involving extracted OM standards can only go some way towards explaining the behavior of OM in the natural environment because of the inherent complexity and heterogeneity of both the aquatic and terrestrial environments.

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