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Characterisation of colloidal and particulate organic carbon in freshwaters by thermal fluorescence quenching

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ABSTRACT

Three-dimensional excitation–emission matrix (EEM) fluorescence with thermal quenching has been applied to raw and size-fractionated freshwaters. To size-fractionate organic matter, sequential filtration through mixed-ester-cellulose membrane filters with nominal pore size of 1.2, 0.1 and 0.025 μm were used. Humic-like fluorophores (peaks A and C) have been found to dominate EEMs of raw and all size fractions of studied waters. Peak A fluorescence intensity has been found to be more thermally sensitive than peak C fluorescence intensity. Humic-like fluorescence intensity was generally size independent, which indicated that it was present mainly in the smallest size fraction (<0.025 μm). This was confirmed by total organic carbon (TOC) measurements. Peak T (tryptophan-like) fluorescence, that is widely associated with biological activity, exhibited a greater thermal sensitivity of fluorescence intensity in the larger size fractions, demonstrating the presence of more than one fluorophore in different size fractions at this location in optical space. Thermal fluorescence quenching provides insights into organic matter variability and associated colloidal characteristics.

1. Introduction

In the aquatic environment, organic matter (OM) is a mixture of compounds with different structures and a wide range of molecular weight (Thurman, 1986; Amy et al., 1987; Wagoner et al., 1997; Jerry and Jean-Philippe, 2003). These compounds might be dissolved in water or aggregate and form different-size colloids and particles (Jones and Bryan, 1998; Mosley and Hunter, 2003; Buffle, 2006; Lead and Wilkinson, 2006). Colloids are an intermediate fraction of natural matter in terms of size, between suspended particles (defined as entities of matter >1 μm) and dissolved molecules (<1 nm) (Buffle and van Leeuwen, 1992). They have a wide range of sizes and are chemically complex, involving organic and inorganic matter, e.g., humic substances, biological cells, polysaccharides, clays, silicon (Buffle, 2006; Lead and Wilkinson, 2006). They are too small to settle out over reasonable timescales and, due to their relatively large surface and ability to bind organic and inorganic substances, play a significant role in transport of nutrients, trace elements, organic pollutants and pathogens in natural waters (Buffle 1990; Lead et al., 1999; Ran et al., 2000; Benaïm and Mounier, 1998).

Many techniques have been applied to quantify organic matter (Doucet et al., 2004; Lead et al., 2005; Caceci and Billon, 1990; Sierra et al., 2005; Lead and Wilkinson, 2006). One of those is 3-D excitation–emission matrix (EEM) fluorescence spectroscopy, which characterises fluorescent properties of naturally occurring organic matter. Three-dimensional (3-D) EEM fluorescence uses scanning emission spectra at specified excitation wavelengths which results in 3-D graphs of the fluorescence intensity with respect to different excitation and emission wavelengths (Coble, 1996; Baker, 2005). This technique has been applied in characterisation and fingerprinting of organic matter in natural waters for 10 years for a wide

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variety of marine, freshwaters and groundwaters (Coble, 1996; Determann et al., 1996; Del Castillo et al., 1999; Mounier et al., 1999; Baker, 2001; Patel-Sorrentino et al., 2002; Chen et al., 2003a, b; Stedmon et al., 2003; Yamashita and Tanoue, 2003; Baker and Spencer, 2004; Sierra et al., 2005; Boehme and Wells, 2006; Lead et al., 2006). In natural waters, two major types of fluorophores have been identified: those attributed to humic-like material and those attributed to protein-like material derived from biological activity (Coble, 1996; Cammack et al., 2004; Elliott et al., 2006a, b). The intensity and location in optical space of these fluorescence peaks can also vary depending on sample character and environmental factors (e.g., pH, temperature) (Sierra et al., 2005). Additionally, it is still not completely understood which types of organic moieties are responsible for humic-like fluorescence. To collect more information on fluorescent organic moieties, fractionation of OM by cross-flow filtration (Mounier et al., 1999), field-flow fractionation (Boehme and Wells, 2006) and split-flow thin-cell fractionation (SPLITT) (Lead et al., 2006) have been applied and fluorescent properties of size-fractionated OM were studied. The impact of pH (Patel-Sorrentino et al. 2002), ultraviolet radiation (Mounier et al., 1999) and temperature (Baker, 2005) on fluorescence EEMs have also been investigated to probe OM character. However, there still remains the question of the how fluorescent OM characteristics vary between size phases, since the available information is rather ambiguous but critical for the understanding of micropollutant fate and behaviour.

In this study, 3-D EEM fluorescence with thermal quenching has been applied to raw and size-fractionated freshwaters. Thermal quenching is a novel technique for probing the structure of colloidal organic carbon, based on the fact that thermal quenching of fluorescence intensity is proportional to the fluorophore exposure to the energy source (Baker, 2005). However, there is very little information on thermal quenching of freshwater organic matter fluorescence (Baker, 2005; Elliott et al., 2006b), and there is no information on fluorescence quenching in different-size phases of organic matter. We studied thermal fluorescence quenching with size fractionation for six different types of freshwaters, in order to improve our quantification of specific fluorescent moieties, to enable a better understanding fluorescence behaviour of OM and to allow improved fingerprinting and source apportionment through the use of both size fractionation and thermal quenching. To our knowledge, this paper details the first report of thermal fluorescence quenching of size-fractionated colloidal organic matter from natural waters.

2. Methods

2.1. Site details and sample fractionation

All sampling sites were located in West Midlands, UK, chosen to reflect a wide range of fresh water organic matter characteristics, and were sampled between January and March 2006. Sites a–d were located outside Norton-in-Hales, a village situated along the River Tern, a lowland tributary of the River Severn, 16 km from its source (UK National Grid Reference SJ 706 384). The sites have been described in detail by Cumberland and Baker (2007). Sites a and c were located in the River Tern and can be considered to be replicates, site b was a groundwater-fed pond feeding the River Tern adjacent to site c, and site d was a riparian wetland discharge site (that ephemerally feeds the main river). Water quality for the River Tern (representative of sites a and c) as measured by the Environment Agency grades chemical water quality as 'fairly good', with mean Biochemical Oxygen Demand of 1.99 mg L^{-1}, ammonia 0.111 mg L^{-1} and dissolved oxygen 94.1% ($n = 36$, 2003–2005). River Tern nitrate (42.77 mg L^{-1}) and phosphate (0.24 mg L^{-1}) concentrations ($n = 36$, 2003–2005) reflect the impact of agricultural nutrients. Site e was a small urban river, the Bourn Brook, that flows beside the University of Birmingham (UK National Grid Reference SP 041 832). Water quality for the Bourn Brook as measured by the Environment Agency grades chemical water quality as 'fairly good', with mean Biochemical Oxygen Demand of 2.44 mg L^{-1}, ammonia 0.111 mg L^{-1} and dissolved oxygen 93.8% ($n = 36$, 2003–2005). Bourn Brook nitrate (9.45 mg L^{-1}) and phosphate (0.19 mg L^{-1}) concentrations ($n = 36$, 2003–2005), together with chemical water quality, reflect diffuse pollution (cross-connected drains, combined sewer discharges) in this urban river. Site f was Afon Carno, an upland tributary of the River Severn (UK National Grid Reference SO 025 919) at the town of Caersws. Water quality for the Afon Carno as measured by the Environment Agency grades chemical water quality as 'good', with mean Biochemical Oxygen Demand of 1.48 mg L^{-1}, ammonia 0.32 mg L^{-1} and dissolved oxygen 97.6% ($n = 36$, 2003–2005). Afon Carno nitrate (3.73 mg L^{-1}) and phosphate (0.06 mg L^{-1}) concentrations ($n = 36$, 2003–2005), together with chemical water quality, reflect the good quality of this rural river.

Samples were collected in 3 L plastic bottles, previously cleaned in 10% HNO_3 and ultra-pure water ($R = 18.2\,\Omega\,cm^{-1}$), and rinsed thoroughly three times in the collected water before sampling. The washings were discarded. In the field, the waters were analysed for pH, electrical conductivity, dissolved oxygen and temperature (Table 1). After collection, samples were transported to the laboratory and filtered immediately using Millipore mixed-cellulose-ester filters with nominal pore sizes of 1.2, 0.1 and 0.025 μm. Slow flow rates and small filter volumes were used to minimise sample alteration. Measurement of flow rates of ultra-pure water before and after sample filtration did not show a change in flow rate, indicating minimal clogging of the membrane. Raw water and all filtrates were analysed in terms of fluorescence and total organic and inorganic carbon. Blanks were performed using ultra-pure water.

2.2. Chemical and fluorescence analysis

Total organic and inorganic carbon was measured with a Shimadzu TOC analyser. TOC/TIC was obtained from the bulk sample, although tubing width within the TOC analyser effectively defines the TOC/TIC fraction to be <2 mm, as well as from all size fractions. In all instances, total carbon was determined by high temperature combustion incorporating a platinised alumina catalyst. Inorganic carbon was determined by phosphoric acid digestion. Organic carbon was determined

Table 1 – Physical and chemical characteristics of the freshwater samples

			OC ($mg L^{-1}$)	IC ($mg L^{-1}$)
Site A				
Conductivity (μS)	381	A raw	11.2	35.6
Temperature (°C)	5.7	A<1.2	11.3	35.5
Dissolved oxygen (%)	116	A<0.1	11.2	34.9
pH	7.89	A<0.025	8.3	37.7
Site B				
Conductivity (μS)	300	B raw 8.8	36.5	
Temperature (°C)	4.7	B<1.2	8.7	36.0
Dissolved oxygen (%)	113	B<0.1	8.1	34.9
pH	7.96	B<0.025	8.0	35.6
Site C				
Conductivity (μS)	385	A raw	9.5	37.8
Temperature (°C)	5.6	A<1.2	10.0	36.5
Dissolved oxygen (%)	114	A<0.1	10.2	35.7
pH	7.89	A<0.025	11.7	35.0
Site D				
Conductivity (μS)	350	A raw	6.9	41.2
Temperature (°C)	9.5	A<1.2	7.7	41.1
Diss. oxygen (%)	Not measured	A<0.1	6.1	40.3
pH	7.10	A<0.025	6.8	40.2
Site E				
Conductivity (μS)	400	A raw	7.0	26.4
Temperature (°C)	6.5	A<1.2	7.9	26.0
Dissolved oxygen (%)	102	A<0.1	5.0	27.4
pH	7.78	A<0.025	8.6	25.8
Site F				
Conductivity (μS)	81	A raw	9.7	0
Temperature (°C)	5.0	A<1.2	9.3	0.6
Dissolved oxygen (%)	117	A<0.1	7.2	2.1
pH	7.13	A<0.025	6.7	2.6

by the difference. The mean of three to five injections of 100 μl was used to determine total and inorganic carbon for every sample and precision, described as a coefficient of variance (CV), was <2% for the replicate injections. Sample replicates, when analysed, agree within $\pm 1 mg L^{-1}$. The instruments were calibrated using total and inorganic carbon standards. Glass vials used for analysis were washed in 4% Decon 90© and 10% HCl and pre-rinsed with ultra-pure water and sample. Detailed analysis of the accuracy of TOC analyses can be found in Aiken et al. (2002).

Fluorescence was measured using a Varian Cary Eclipse spectrophotometer, equipped with a Peltier temperature controller. Emission scans were performed from 280 to 500 nm at 2 nm steps, with excitation wavelengths from 200 to 400 nm at 5 nm intervals. Slit widths were 5 nm and photomultiplier tube voltage set to 725 V. Spectrophotometer output was monitored by regular measurement of the Raman intensity of ultra-pure water in a sealed cuvette at 348 nm excitation and 5 nm slit widths. Over the analytical period, mean intensity was 23.0 ± 1.1 units. Fluorescence quenching was measured at 5 °C intervals over a temperature range of 10–45 °C. The temperature range and the sample-heating rate have been studied previously (Baker, 2005; Elliott et al., 2006b) and have been shown to avoid problems with condensation and evaporation.

3. Results and discussion

3.1. *Organic carbon concentrations*

Organic carbon concentration of raw samples were in the range of 6.9–11.2 $mg L^{-1}$, and was the highest for sites a and c and the lowest for site d (Table 1). The applied mixed-cellulose-ester membrane filters have not shown any influence on organic carbon in filtrates of pure water (Karlsson et al., 1999); therefore possible particle retention on the filters is not anticipated. However, organic carbon concentration was generally consistent for all fractions of every sampling site, predominantly within the precision of TOC analyser, confirming that the majority of the organic carbon was in the smallest size fraction and that potential loses by adsorption were minimal. In all the blank samples using ultra-pure water, in both filtrates and bulk ultra-pure water, organic carbon was below the detection limit (and fluorescence spectra did not show any significant peaks).

3.2. *Fluorescence EEMs*

For fresh water samples, three major fluorescence peaks have been found. Two are associated with humic substances (peak C: excitation at 300–340 nm and emission at 400–460 nm, and peak A: excitation at 220–250 nm and emission at 400–460 nm) and the third, a tryptophan-like fluorophore (peak T: excitation at 220–230 nm and emission at approximately 350 nm) (Coble, 1996; Mounier et al., 1999), that is commonly associated with microbial action (Cammack et al., 2004; Elliott et al., 2006a). As representative EEM spectra at 10 and 45 °C for all size fractions, EEMs for site e are shown in Fig. 1. At all sample sites, peaks A and C were present in all EEMs. Peak T fluorophores were found only for sites b and e, at site b the probably source is from wildfowl whereas at site e the probable source is cross-connected storm drains, so peak T was only further analysed for these two sites. The dominance of fluorescence peaks A and C indicate that humic substances dominate the fluorescence EEMs of our investigated waters, which has been previously observed for other fresh waters (Baker, 2001). For all samples, peaks A and C did not show significant differences between fractions, again agreeing with

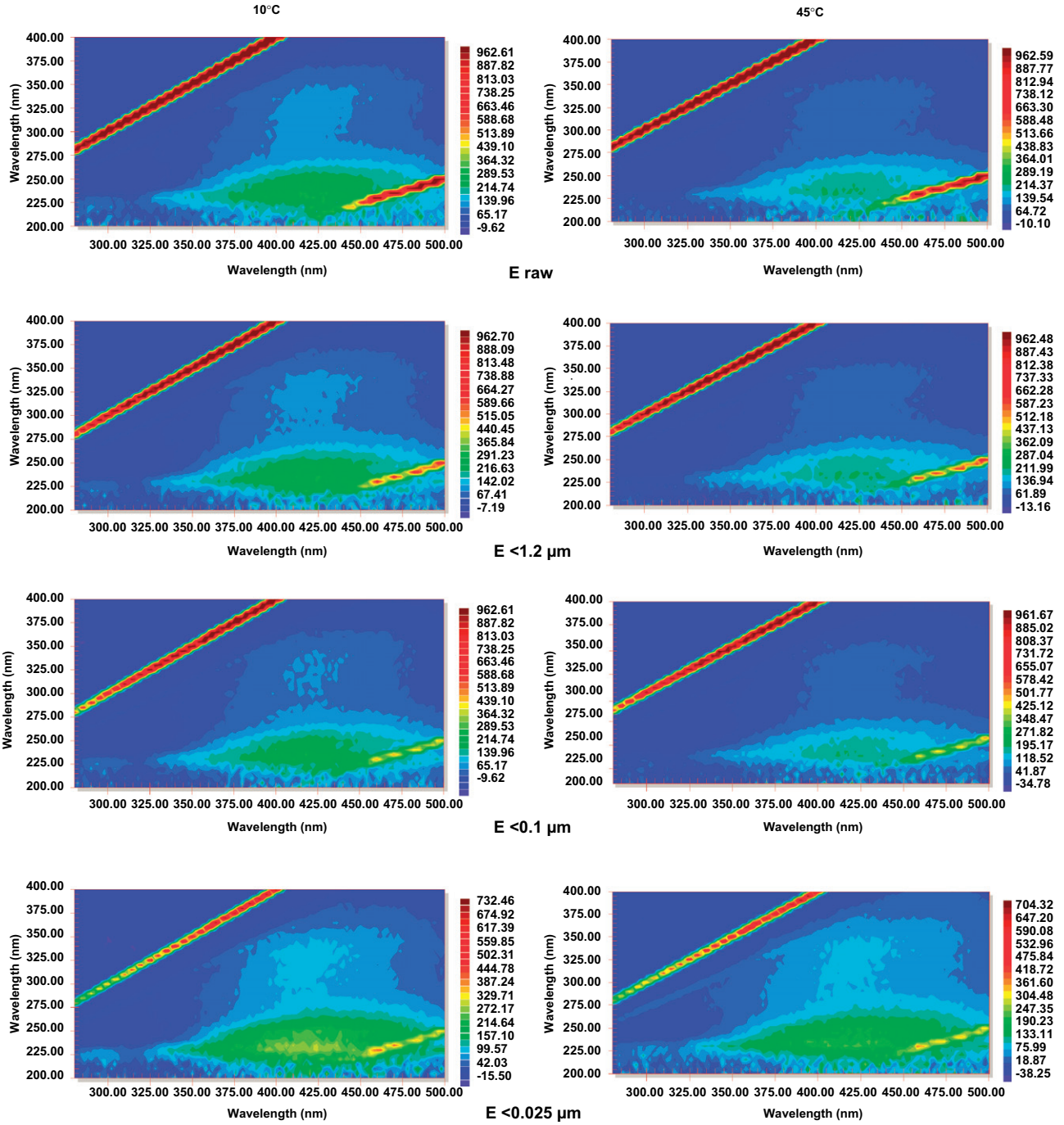

Fig. 1 – Fluorescence EEMs of site e at 10 and 45 °C.

organic carbon results, confirming observations that these fluorophores are predominantly in the smallest size fraction (Lead et al., 2006; Baker et al., 2007). For both sites b and e, the highest peak T fluorescence intensity was obtained for the raw samples, indicating that fluorophores associated with the particulate fraction contributed significantly to peak T fluorescence in these waters. This agrees with Lead et al. (2006) and Baker et al. (2007) who found peak T fluorescence mainly in the particulate fraction in fresh waters. For all raw samples, additional scatter artifacts that could potentially occur from interactions between excitation beam and the particulate fraction were not observed.

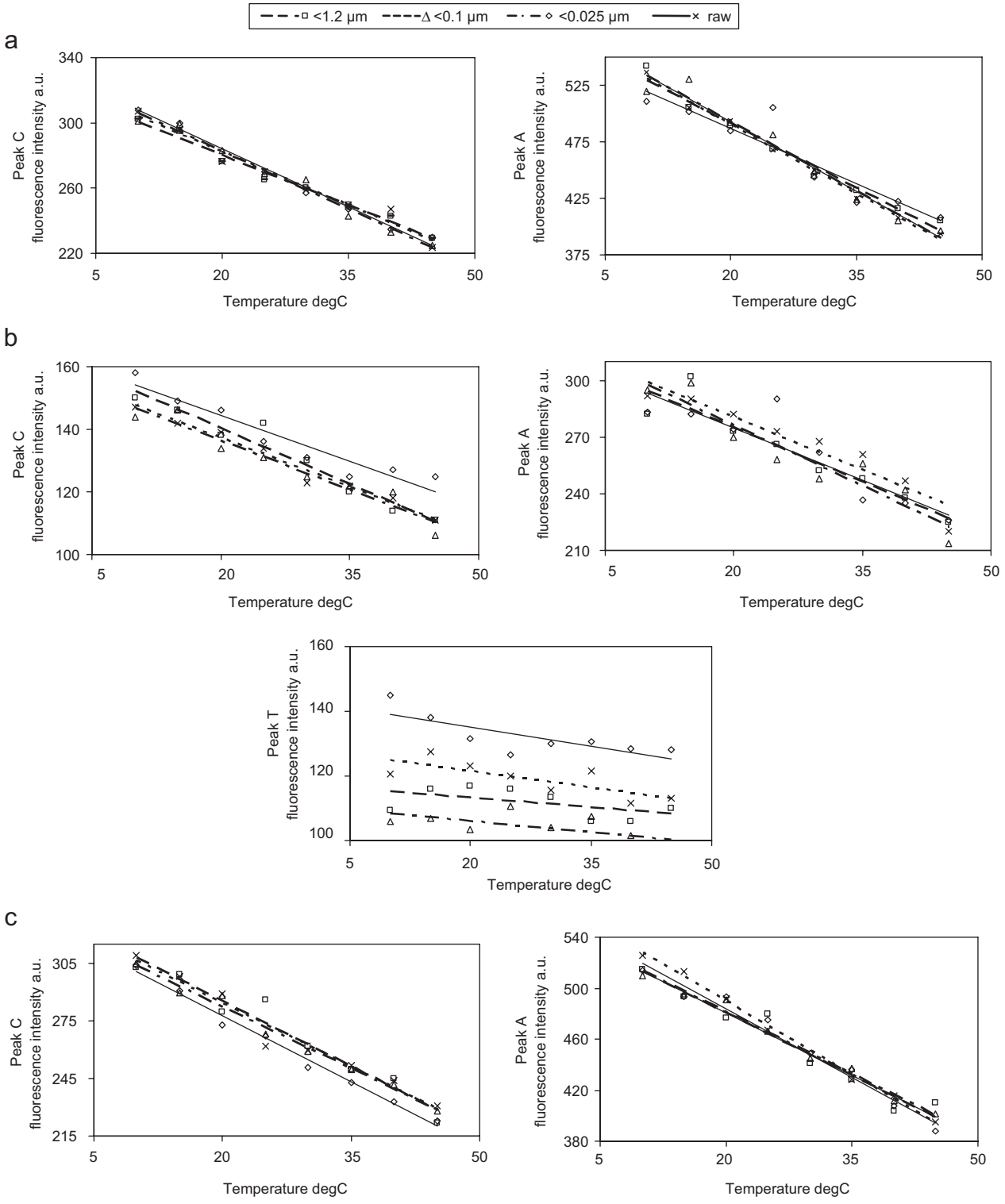

Fig. 2 – Change of fluorescence intensity with increasing temperature.

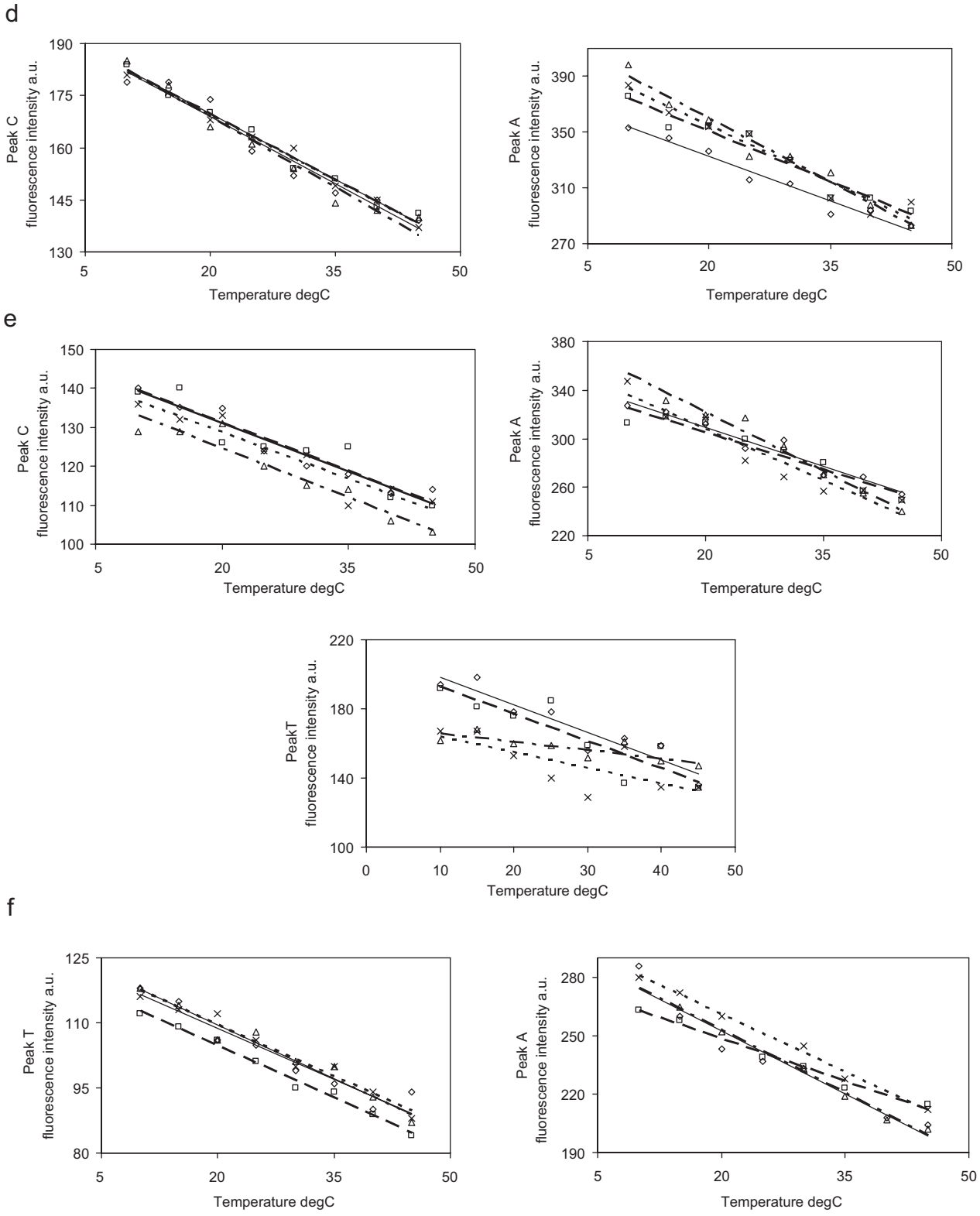

Fig. 2 – (*Continued*)

3.3. *Fluorescence thermal quenching*

Peaks A, C and T (where observed) exhibited a linear decrease in fluorescence intensity with temperature, as presented in Fig. 2. The gradient of this decrease in fluorescence intensity per °C is summarised in Table 2. It has been previously suggested that the peaks A and C are generated by a different fluorophores within humic substances (Mounier et al., 1999);

Table 2 – Gradient of fluorescence quenching (arbitrary units per °C) of raw and filtered freshwater samples

	Peak C	Peak A	Peak T
A raw	−2.37	−3.27	
A<1.2	−2.03	−3.81	
A<0.1	−2.35	−4.12	
A<0.025	−2.18	−4.07	
B raw	−0.97	−1.84	−0.79
B<1.2	−1.19	−1.92	−0.39
B<0.1	−1.04	−2.15	−0.45
B<0.025	−1.03	−1.87	−0.67
C raw	−2.29	−3.58	
C<1.2	−2.26	−3.25	
C<0.1	−2.15	−3.25	
C<0.025	−2.21	−3.83	
D raw	−1.28	−2.11	
D<1.2	−1.26	−2.39	
D<0.1	−1.35	−3.05	
D<0.025	−1.25	−2.68	
E raw	−0.83	−2.13	−1.59
E<1.2	−0.83	−2.05	−1.58
E<0.1	−0.84	−3.21	−0.47
E<0.025	−0.80	−2.81	−0.90
F raw	−0.78	−2.16	
F<1.2	−0.80	−1.45	
F<0.1	−0.83	−2.16	
F<0.025	−0.79	−1.98	

therefore, it might be expected to have a different quenching per °C. Table 2 shows that the rate of fluorescence decrease per °C temperature increase is higher for peak A for all sample sites and all size fractions. Peak C fluorophore intensity showed the smallest variability in the amount of thermal quenching between size fractions for almost all of the sample sites (Table 2), suggesting that this fluorophore might be associated with a fraction that is less easily environmentally perturbed than peak A. Differences in rates of thermal quenching between filter fractions for peaks A and C were the least for sites a–d, and highest for sites e and f, which is likely to represent differences in organic matter composition. The lowland Tern sites a–d are all known to be groundwater dominated and the sites are, therefore, likely to have a similar organic matter composition. In contrast, site e is an urban river and site f an upland river with a peat and upland grass dominated catchment. At the urban site e in particular, the peak A <0.1 and <0.025 μm fractions show significantly greater thermal quenching than the larger size fractions, suggesting the peak A fluorophore is associated with more than one size fraction at this urban site, probably due to differences in colloid size and character, and in contrast to fluorophore peak C.

Where present, peak T fluorescence demonstrated the largest decrease in fluorescence intensity with filter fraction (Fig. 2). For site e, a significant difference in thermal quenching gradient at the 0.1 μm size fraction is observed, with a steeper fluorescence quenching gradient (greater environmental sensitivity) in the larger size fraction (Table 2). This change in peak T thermal quenching properties at site e occurs in the same size fraction as the change observed in peak A fluorescence at this site. The 0.1 μm cut-off will separate microbial from dissolved material, and we therefore hypothesise that the >0.1 (<0.1) μm fraction is typified by organic matter with peak A fluorescence less (more) sensitive to thermal quenching and peak T fluorescence more (less) sensitive to thermal quenching, demonstrating differences in colloidal interactions between humic substances and microbial matter.

4. Conclusions

We have applied 3-D excitation–emission fluorescence to characterise colloidal and particulate organic matter in freshwaters to produce the first data on thermal fluorescence quenching of size-fractionated natural water samples. We confirmed the distinction between peaks A and C fluorescence peaks in surface waters EEMs. We found that peak A has a steeper gradient (decrease in fluorescence intensity per °C), and therefore temperature sensitivity, than peak C; although this typically was independent of size fraction, suggesting both fluorophores were associated with a <0.025 μm fraction. That peak A gradient of fluorescence quenching was steeper than peak C was true for all sites, either rural or urban, upland or lowland. However, at one site (e), our urban site, some size dependency was observed, with differences in the gradient of thermal quenching occurred at a 0.1 μm cut-off, with more thermally sensitive peak A fluorescence in the smaller size fraction. This suggests the importance of a microbial fraction that interacted with at least part (peak A) of the humic fraction in the >0.1 μm fraction. This site was notable for high intensities of peak T fluorescence, suggesting that in this urban river at least, the microbial fraction may significantly affect humic substance behaviour through the formation of colloids (for example via exopolymeric substances), with associated environmental implications for trace element and pollutant transport.

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