

Thermal fluorescence quenching properties of dissolved organic matter

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Abstract

The fluorescence excitation–emission matrices of dissolved organic matter (DOM) are investigated between 10 and 45 °C for river and waste waters and organic matter standards. With increased temperature, fluorescence intensity is quenched. It is demonstrated that for a range of river and wastewater samples, that tryptophan-like fluorescence exhibits a greater range of quenching (between $20 \pm 4\%$ and $35 \pm 5\%$) than fulvic-like fluorescence (19 ± 4 to $26 \pm 3\%$) over this temperature range. Humic substance standards exhibit similar fulvic-like ($23 \pm 4\%$) fluorescence thermal quenching properties to river water samples ($23 \pm 3\%$); however none of the samples exhibit quenching of tryptophan-like fluorescence to the same extent as the tryptophan standards ($\sim 50\%$). Thermal fluorescence quenching is related to the exposure of the fluorophores to the heat source; our findings suggest that the tryptophan-like groups within DOM is more exposed in untreated wastewaters than in treated wastewaters riverine DOM. Thermal fluorescence properties have the potential to be used to source DOM, to provide additional chemical structural information, to temperature correct laser-induced remotely sensed DOM fluorescence, and to characterise DOM through the wastewater treatment process.

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1. Introduction

Dissolved organic matter (DOM) represents an important form of organic matter in fresh waters and is frequently the major source of energy fuelling stream ecosystems (Webster and Meyer, 1997). DOM may be either allochthonous or autochthonous, natural or anthropogenic, and ecosystem limited or limiting. Our understanding of the relative importance of each of these, and their relationship with DOM structure and function, is poor and often limited by the lack of appropriate analytical techniques to characterise DOM.

The development of technologies that would permit an improved understanding of the properties of DOM is essential for the better understanding of freshwater DOM cycling and its role in global carbon cycling; DOM bioavailability; and organic pollution fingerprinting and control (Benner, 2002; Findlay et al., 2002; Ziegler and Brisco, 2004).

The intrinsic fluorescence characteristics of DOM (FDOM) are one such new technology that has demonstrable use in DOM quantification and characterisation. The technique does not require sample pre-treatment and has low (ppb) detection limits, which is important in the determination of low concentrations of often labile DOM that can otherwise not be detected or quantified. With the recent advent of fluorescence

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excitation–emission matrix (EEM) analyses, freshwater DOM intrinsic fluorescence properties are now well understood, particularly at short ultra-violet excitation (200–250 nm) wavelengths. For example, tryptophan-like (with excitation at 220–230 nm and 270–280 nm and emission at ~350 nm) and tyrosine-like (with excitation at 220–230 nm and emission at 300–310 nm) fluorescence have been demonstrated to correlate with waters of poor river water quality (high biochemical oxygen demand (BOD), low dissolved oxygen (DO)) with significant inputs of fresh, bioavailable and often faecal sourced DOM (Baker, 2001, 2002; Baker et al., 2003; Baker and Inverarity, 2004). Longer wavelength fluorescence (often called fulvic-like; with excitation between 300 and 340 nm and emission between 400 and 460 nm) is typically ascribed to aromatic moieties in the high molecular weight (relatively recalcitrant) DOM fraction (Leenheer and Croue, 2003). Paired with conventional techniques such as UV absorbance and total organic carbon (TOC) analyses, fluorescence EEM spectroscopy has become widely used in investigations of DOM sourcing, fingerprinting of river and groundwater pollutants, characterisation of waste waters and understanding DOM transformations in estuarine systems (Baker et al., 2003; Baker and Inverarity, 2004; Baker and Spencer, 2004; Chen et al., 2003; Stedmon et al., 2003).

Despite this recent increase in the use of intrinsic EEM fluorescence in studies of DOM, the chemical nature of fluorescent materials in DOM is still poorly understood (Wu et al., 2003a). A key question remains as to the precise location of the observed tryptophan-like fluorescence that seems to provide a diagnostic fingerprint for much anthropogenic DOM. Amino acids in aquatic systems in general are thought to be present as amino acids or peptides absorbed or bound to humic substances (Zang et al., 2000), in living cells as part of the microbial biomass (Simon and Azam, 1989), or as free amino acids (Reynolds, 2003), but despite several decades of research, the quantity and structural location of amino acids within each DOM pool is still poorly understood (Jørgensen and Søndergaard, 1984). This is mostly due to analytical difficulties: HPLC is the standard technique for amino acid analysis and this requires sample pre-treatment; tryptophan is labile during the acid hydrolysis stage and is therefore, not retained for chromatographic analysis unless a separate alkaline hydrolysis is employed (Reynolds, 2003; Wu et al., 2003b), and intrinsic EEM fluorescence alone provides scant structural information. Given that amino acids are carbon and nitrogen nutrients for aquatic microorganisms, and that their availability will depend on their location within the DOM, further techniques that provide source or structural information on the tryptophan-like fluorescence would be invaluable.

Remarkably, to date intrinsic fluorescence EEM properties have been investigated at constant (laboratory) temperature. In contrast, and in particular within optical physics, *thermal quenching* properties are widely used to characterise fluorophores (Curie, 1963; Lakowicz, 1999). Although the fluorescence intensity–temperature relationship is necessary to temperature compensate laser-induced remotely sensed fluorescence (Vodacek and Philpot, 1987), it has not been used to characterise river or waste water DOM. In general, quenching of fluorescence occurs with increasing temperature, as a rise in temperature increases the likelihood that an electron within a molecule will fall back to the ground state by a radiationless process. The extent to which thermal quenching occurs relates to the exposure of the fluorophore to this energy source. This exposure would be expected to vary between free amino acids, tryptophan protected within protein or peptides, tryptophan present in molecules where extensive chemical conjugations provides protection, and that protected due to the formation of colloids. Hence thermal quenching should provide information about the DOM composition (molecular weight, aromaticity, location of amino acid groups within the DOM structure) and hence will also have potential as a new method for characterising DOM. This paper therefore, seeks to demonstrate the thermal fluorescence characteristics of some environmentally relevant DOM, namely river and wastewater samples. Specifically it aims to firstly develop laboratory protocols for fluorescence quenching experiments, before determining whether the addition of a thermal component to EEM analysis improves the characterisation of DOM composition, as well as aiding the fingerprinting of DOM, which could permit the use of thermal fluorescence quenching properties for water quality monitoring, pollution detection and as a tracer in river systems.

2. Experimental section

DOM fluorescence was measured in 4 ml capacity cuvettes using a Varian Cary Eclipse fluorescence spectrophotometer, equipped with a multicell holder with Peltier temperature controller enabling the measurement of EEMs at a variety of precisely controlled (± 0.1 °C) temperatures. Each EEM was generated by scanning excitation wavelengths from 200 to 400 nm at 5 nm steps, and detecting the emitted fluorescence between 280 and 500 nm at 2 nm steps. Scan speed was 9600 nm/min, permitting collection of a complete EEM in ~60 s. For all EEMs, three fluorescence peaks were identified that were always detectable. These were: (1) two centres at excitation wavelengths of 220–230 and 270–280 nm and emission at 350 nm, fluorophores normally attributed to the presence of tryptophan-like

functional groups within the DOM, (2) fluorescence excited between 300 and 340 nm excitation, and emitted between 400 and 460 nm, a poorly characterised fluorophore that is often attributed to fulvic-like substances. For each peak, the excitation and emission wavelengths and the intensity of emitted fluorescence were recorded. To calibrate the fluorescence intensity of FDOM, we also measured the strength of the Raman signal at excitation 348 nm (emitted between 395 and 400 nm) and all results are standardised to a mean Raman peak of 20 intensity units.

For each FDOM sample, we generated an EEM at a range of different temperatures. Different absolute temperature ranges were experimented with as well as differing rates of temperature change, and a protocol of gentle heating 10–45 °C over 30 min, recording EEMs every 5 °C, was decided on. This choice of rate of temperature change and range of temperatures was chosen as an optimal protocol for several reasons. Firstly, a lower temperature limit of 10 °C limits the development of condensation on the outer walls of the cuvette, which could occur at temperatures substantially cooler than laboratory (20–25 °C) temperature and which will interfere with both excitation and emission signals. The upper temperature limit of 45 °C prevents significant sample evaporation that could occur at increasing temperatures; evaporation could lead to the concentration of DOM and therefore, an increase in fluorescence intensity during the course of the experiment. It also limits any changes in EEMs by microbial action: microbial action increases with temperature and would be expected to increase at the higher temperatures used, therefore, a short analysis time is preferable. Finally, it prevents any structural changes in DOM that might occur at both very high (>60 °C) and very low (<1 °C) temperatures and it avoids changes in fluorescence that might be expected to occur at temperatures close to freezing due to changes in matrix viscosity. The rate of ~1 °C/min is at a slow enough rate to prevent the formation of bubbles within the cuvette, that were observed to occur at faster temperature ramping rates and which will alter the apparent fluorescence signal.

We analysed a wide range of environmentally relevant DOM samples. Standards analysed were International Humic Substances Society (IHSS) Suwanee River NOM, Suwanee River FA, Suwanee River HA, Nordic HA and Nordic FA. Details of the spectroscopic properties of these substances can be found elsewhere (Senesi et al., 1989; Mobed et al., 1996). We also investigated the fluorescence quenching of Tryptophan (Aldrich, made to 100 ppb solution). Water samples were sourced from local rivers, waste and storm waters from within storm drain and sewers, and treated and untreated wastewater from within sewage treatment works. River water samples were taken approximately every 3 months from the Bourn Brook, a small urban

river that flows beside the University of Birmingham (52:26:51N, 1:55:49W). Rural river samples were also analysed from the River Tern, Shropshire (52:50 N, 2:32 W) in May 2004. Storm sewer samples were taken between April and October 2004 from a storm water overflow from the University of Birmingham that discharged into the Bourn Brook: this outfall had a thick 'sewage fungus' coating suggesting that it was cross connected. Untreated sewage samples were taken directly from mains sewer between March and October 2004, utilising a pump house facility over the Selly Oak Sewer on the University campus. Finally, in order to investigate any differences in fluorescence thermal properties through the waste water treatment process, influent or settled sewage and final treated effluent were collected from five different activated sludge wastewater treatment works with a maximum treatment rate range of 75–810 Ml/day, between October and December 2004.

DOM samples were collected in 50 ml plastic bottles that had been cleaned in 10% HCl and distilled water. Samples were returned to the laboratory and analysed as rapidly as possible: for sewerage and urban river samples collected on campus this was within 15 min; for rural river and wastewater treatment samples it was within 24 h, with samples transported in a cool box and kept refrigerated prior to analysis. Samples were analysed for pH, electrical conductivity, ammoniacal nitrogen concentration and absorbance at 340 nm using a WTW Multi 350i Meter, a Hanna Ammonia Colorimeter and a WPA Lightwave UV-VIS spectrometer, respectively. All samples were filtered using Whatman GF/C 1.2 µm glass microfibre filter papers to obtain the dissolved fraction. Samples were screened for inner filtering effects: inner-filter occurs at high concentrations due to the absorption and re-emission of emitted fluorescence. Absorbance of all samples was checked using a WPA Lightwave UV-VIS spectrometer, and if greater than 0.1/cm at 340 nm samples were diluted until absorbance fell below this value. This effectively lead to river waters being analysed undiluted, storm water samples analysed at ×2 dilution, final treated effluents at ×2–4 dilution, and mains sewer and wastewater treatment works influent and settled sewage at ×10 dilution. A sub-sample of waters were analysed in duplicate.

3. Results and discussion

Mean and standard deviation results are presented in Table 1 and examples of quenched and unquenched fluorescence EEMs for IHSS and tryptophan standards and a typical urban river sample in Fig. 1. Fig. 2 plots thermal quenching against the ratio of tryptophan-like to fulvic-like fluorescence; the latter ratio has been

Table 1
Summary of dissolved organic matter fluorescence and chemical properties

| | Tryptophan-like fluorescence (220–230 nm excitation) | | | | | | Tryptophan-like fluorescence (270–280 nm excitation) | | | | | Fulvic-like fluorescence | | | | | Tryptophan intensity/fulvic-like intensity ^c | Absorbance 340 nm (cm ⁻¹) | pH | Electrical conductivity (µS) | Ammonical nitrogen (mg l ⁻¹) |
|----------------------------------|--|--|--------------------------------------|----------------------------|------------------------------------|-----|--|--|--------------------------------------|----------------------------|------------------------------------|--------------------------|--|--|-------------------------------------|----------------------------|---|---------------------------------------|------|------------------------------|--|
| | Excitation wavelength ^a (nm) | Emission wave-length ^a (nm) | Wave-length change ^b (nm) | Intensity ^a (U) | Thermal quenching ^b (%) | | Excitation wave-length ^a (nm) | Emission wave-length ^a (nm) | Wave-length change ^b (nm) | Intensity ^a (U) | Thermal quenching ^b (%) | | Excitation wave-length ^a (nm) | Emission wave-length ^a (nm) | Wavelength change ^b (nm) | Intensity ^a (U) | | | | | |
| Urban rivers (n = 7) | Mean | 232 | 353 | -3 | 223 | -22 | 282 | 353 | -1 | 70 | -24 | 320 | 421 | -1 | 162 | -22 | 0.48 | 0.044 | 7.85 | 621 | 0.34 |
| | Stdev | 1 | 4 | 4 | 151 | 3 | 3 | 3 | 3 | 43 | 5 | 14 | 6 | 2 | 136 | 3 | 0.10 | 0.031 | 0.13 | 408 | 0.36 |
| Rural rivers (n = 6) | Mean | 234 | 352 | -1 | 87 | -23 | 281 | 354 | 0 | 53 | -25 | 333 | 425 | -1 | 138 | -24 | 0.37 | 0.043 | 8.02 | 737 | 0.07 |
| | Stdev | 2 | 3 | 2 | 24 | 4 | 2 | 2 | 4 | 22 | 5 | 4 | 3 | 4 | 40 | 2 | 0.08 | 0.025 | 0.16 | 168 | 0.12 |
| IHSS standards (n = 5) | Mean | 231 | 353 | -3 | 42 | -18 | 283 | 351 | 0 | 15 | Below | 333 | 452 | -9 | 115 | -23 | 0.14 | 0.151 | 6.52 | 21 | 0.00 |
| | Stdev | 3 | 4 | 7 | 15 | 4 | 3 | 2 | 4 | 6 | Detection | 4 | 12 | 19 | 38 | 4 | 0.05 | 0.152 | 0.17 | 9 | 0.00 |
| Storm drain (n = 6) | Mean | 227 | 357 | 0 | 703 | -33 | 280 | 357 | -4 | 265 | -33 | 327 | 421 | 1 | 225 | -20 | 1.04 | 0.203 | 7.53 | 768 | 2.11 |
| | Stdev | 4 | 7 | 8 | 438 | 8 | 2 | 10 | 7 | 155 | 8 | 9 | 2 | 2 | 75 | 2 | 0.41 | 0.135 | 0.07 | 468 | 2.78 |
| Mains sewer (n = 6) | Mean | 223 | 355 | 3 | 4738 | -33 | 280 | 361 | 5 | 1583 | -35 | 332 | 419 | 3 | 778 | -19 | 1.81 | 0.961 | 8.05 | 749 | 19.94 |
| | Stdev | 5 | 10 | 11 | 2795 | 6 | 2 | 6 | 8 | 853 | 2 | 6 | 3 | 4 | 192 | 4 | 0.33 | 0.436 | 0.12 | 97 | 11.61 |
| Waste water treatment works | Mean | 223 | 355 | 8 | 3937 | -35 | 281 | 357 | 2 | 1253 | -37 | 331 | 420 | -3 | 536 | -16 | 2.25 | 0.555 | 7.46 | 1304 | 35.35 |
| Influent/ settled sludge (n = 5) | Stdev | 3 | 11 | 7 | 1240 | 5 | 2 | 7 | 11 | 504 | 4 | 8 | 6 | 7 | 136 | 9 | 0.32 | 0.250 | 0.03 | 101 | 1.06 |
| Waste water treatment works | Mean | 234 | 354 | 0 | 670 | -20 | 283 | 357 | 4 | 230 | -30 | 333 | 420 | -4 | 336 | -26 | 0.70 | 0.055 | 7.35 | 1077 | 0.06 |
| Final Effluent (n = 5) | Stdev | 2 | 4 | 6 | 202 | 4 | 4 | 7 | 7 | 57 | 5 | 4 | 5 | 6 | 73 | 3 | 0.11 | 0.035 | 0.38 | 112 | 0.04 |
| Tryptophan standard (n = 2) | Mean | 227 | 351 | 4 | 104 | -48 | 280 | 357 | 2 | 29 | -48 | n/a | n/a | n/a | n/a | n/a | n/a | 0.000 | 7.10 | 1 | 0.00 |

Duplicate sampls not included.

^aMean and standard deviation of all 10 and 45 °C measurements (n = 10–14).

^bThe change in wavelength or intensity between 10 and 45 °C.

^cFor comparison with Baker (2001).

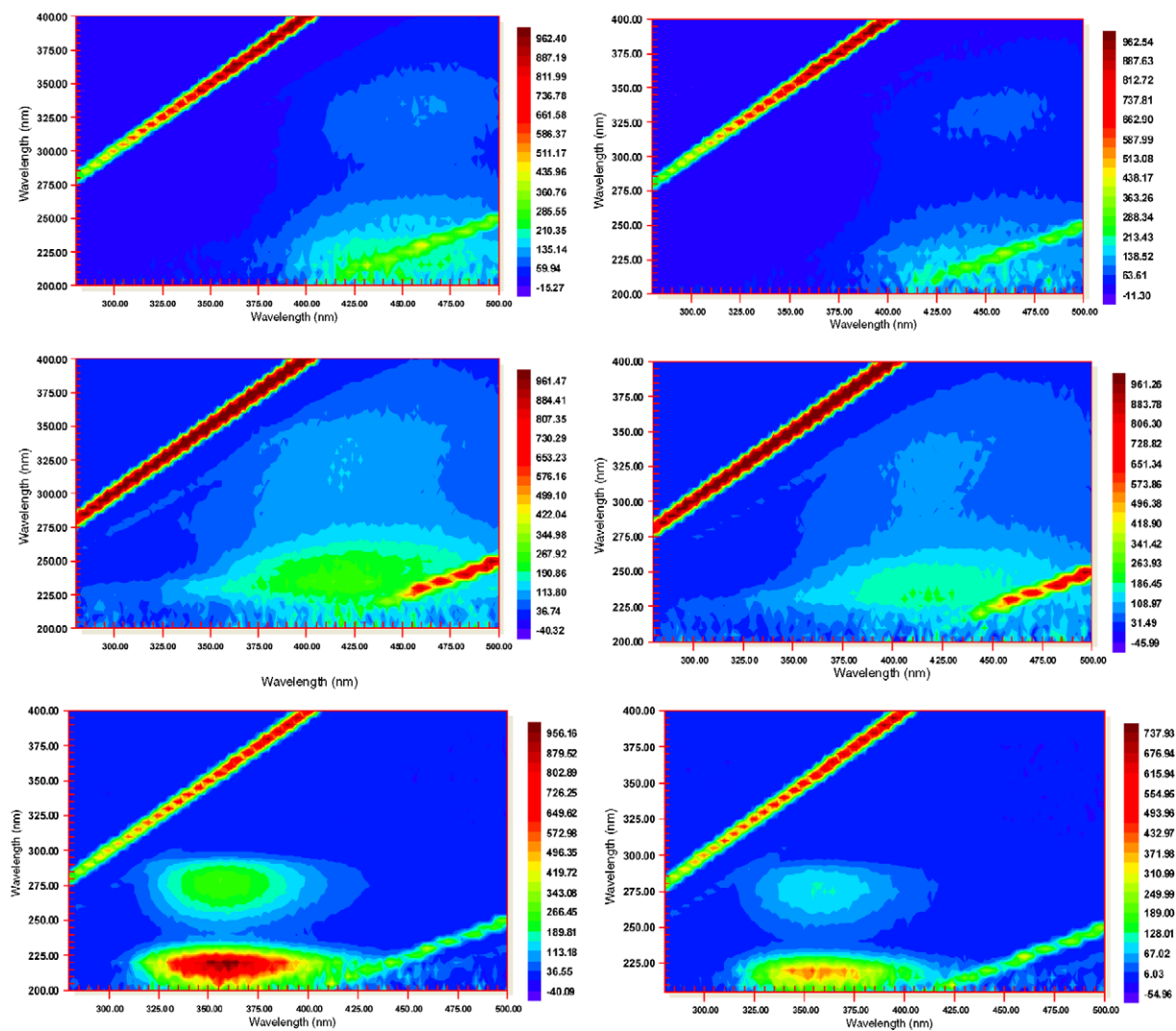


Fig. 1. Fluorescence EEMs at 10 °C (left) and 45 °C (right). Top: Nordic Fulvic Acid. Middle: Urban River (Bourn Brook). Bottom: 100 ppb tryptophan standard.

shown to be a useful indicator of anthropogenic DOM inputs. Table 1 and Fig. 2 demonstrate that IHSS standards, river waters, untreated sewage and treated sewage samples cluster in groups with distinctive mean fluorescence intensities and wavelengths of the tryptophan-like and fulvic-like fluorescence centres. In particular, untreated wastewaters had the highest intensities of tryptophan-like fluorescence, whereas the IHSS standards had significantly lower tryptophan-like fluorescence than all other samples and significantly higher fulvic-like emission wavelengths. Samples from the cross-connected storm drain had the widest range of fluorescence properties, reflecting the mixture of samples representing high flow, rainwater dominated DOM and low flow, sewage contaminated DOM. The range of

intensities and wavelengths observed here is in agreement with previously published results for river, storm and waste waters (Baker, 2001; Baker et al., 2003; Baker and Inverarity, 2004).

Table 1 shows that by increasing temperature from 10 to 45 °C, fluorescence intensity is quenched for all three fluorescence centres for both river and wastewater DOM as well as IHSS and tryptophan standards. In order to quantify the accuracy that could be obtained for fluorescence quenching, both sample replicates and instrument replicates were analysed. For six river and wastewater samples, sub-samples were taken and analysed in duplicate. For these, the quenching of the tryptophan-like fluorescence excited at 220–230 nm excitation was reproduced between sample duplicates

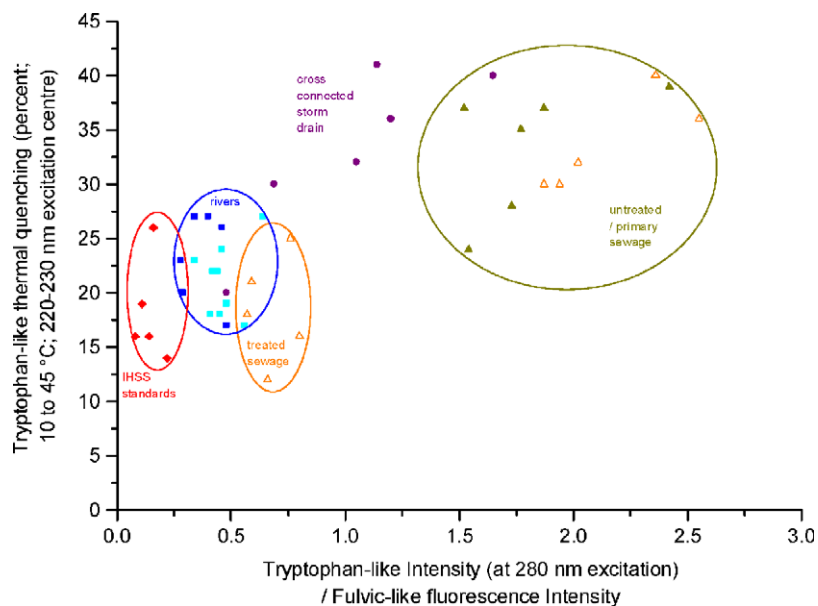


Fig. 2. Percentage tryptophan-like fluorescence quenching plotted against the relative proportions of tryptophan-like to fulvic-like fluorescence.

at $\pm 3\%$, the quenching of the tryptophan-like fluorescence excited at 270–280 nm $\pm 4\%$, and the fulvic-like peak at $\pm 5\%$. Instrument reproducibility was determined by analysing individual samples in triplicate; instrument variability of fluorescence intensity was determined to be $\pm 3\%$, giving an instrumental thermal quenching reproducibility of 4.2%. The observed variability of fluorescence quenching within the groups of river, storm, sewer and waste waters is therefore, indistinguishable from both instrument precision and within sample variability of DOM fluorescence, and significantly less than the observed differences in fluorescence quenching of between $\sim 18\%$ and $\sim 50\%$ (Table 1).

Considering the amount of fluorescence thermal quenching of each of the three fluorescence centres for the river and waste water samples, the tryptophan-like fluorescence (excitation at 220–230 nm and emission at 350 nm) exhibits the greatest variation, between $\sim 20\%$ for final effluent from wastewater treatment works and river water samples, and $\sim 30\text{--}35\%$ for untreated sewage. Fulvic-like (excitation at 300–350 nm and emission at 400–460 nm) fluorescence exhibits the least variation in quenching between river and wastewater samples ($\sim 20\text{--}25\%$ for all samples). Tryptophan-like fluorescence (excitation at 270–280 nm and emission at 350 nm) exhibits an intermediate range of quenching, due to the significant contribution to the fluorescence intensity at this location from the tail of the fulvic-like fluorescence centre (this can be seen in Fig. 1 for the urban river sample). Given that the extent to which

thermal quenching occurs relates to the exposure of the fluorophore to heat source, these results suggest that the tryptophan-like fluorophores are relatively more exposed in the untreated waste water samples compared to river and treated final effluent. In contrast to fluorescence intensity, changing sample temperature does not change fluorescence excitation or emission wavelengths for either tryptophan-like or fulvic-like fluorescence centres. Changes in emission wavelength of the fulvic-like fluorescence centre have in particular been attributed to changes in molecular weight or aromaticity: the lack of change in fluorescence excitation and emission wavelengths could be interpreted as indicating that little compositional changes in DOM occurred within the thermal experiments.

The fluorescence thermal quenching properties of river and wastewater samples can be compared to the properties of standard DOM solutions. We investigated the thermal quenching properties of both tryptophan standard and IHSS humic and fulvic substances. Results for these samples are also shown in Table 1 and Figs. 1 and 2. IHSS standards exhibit similar fluorescence quenching properties to river water samples ($18 \pm 4\%$ for tryptophan-like fluorescence and $23 \pm 4\%$ for fulvic-like fluorescence), although they do have a lower absolute intensity of tryptophan-like fluorescence relative to fulvic-like fluorescence than river waters. Tryptophan standard, in contrast, has significantly greater fluorescence quenching of $\sim 50\%$ over the same temperature range. The tryptophan standard comprises free amino acids that are apparently easier to thermally

quench than tryptophan-like fluorophores within river and wastewater DOM. The difference in thermal quenching between tryptophan standard and river and waste waters suggests that in the latter, free tryptophan is not the dominant component of DOM tryptophan-like fluorescence (as opposed to the suggestion of Reynolds (2003)), although there may be a greater proportion of this form of tryptophan in untreated wastewaters.

4. Conclusions

Fluorescence thermal quenching experiments have demonstrated that tryptophan-like fluorophores within river and wastewater DOM are not as exposed in the DOM as in tryptophan standard solutions, suggesting a smaller amount of free tryptophan is present. This finding is of importance in studies investigating the uptake of energy from DOM within stream ecosystems, where free tryptophan is easier to utilise than tryptophan-like fluorescence bound within higher molecular weight DOM. Thermal quenching also demonstrates that IHSS standards reflect riverine DOM in terms of their fluorescence thermal properties, although they have relatively low amounts of tryptophan-like fluorescence compared to riverine DOM. Thirdly, significant differences between untreated and primary settled sewage, and treated final effluent and river water samples, are observed. This suggests that thermal quenching properties could have an application in fingerprinting DOM pollutants from different sources, or in process control within wastewater treatment. Fig. 2 demonstrates that the ratio of tryptophan-like to fulvic-like fluorescence intensity has a greater range, and therefore, discriminatory power, than that observed by thermal quenching. However, in situations where the tryptophan-like to fulvic-like fluorescence intensity fails to discriminate DOM, thermal quenching properties may be of benefit. Fifthly, we demonstrated that techniques utilising laser-induced remote sensing of fluorescence will require different temperature compensation equations, varying with DOM character. Finally, thermal fluorescence quenching provides another technique for researchers who are interested in relatively non-invasive analysis of DOM to obtain information on DOM composition.

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