# Thermal quenching of fluorescence of freshwater, planktonic bacteria

S. Elliott, J.R. Lead [*], A. Baker

*School of Geography, Earth and Environmental Sciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*

**Abstract**

This study aims to determine the thermal quenching properties of pure bacterial cultures as a means of aiding the development of fluorescence measurement in natural waters. The bacterium *Pseudomonas aeruginosa* was isolated from the urban River Tame, Birmingham, UK, and planktonic bacteria were grown in sterile, sealed glass jars, in 100 mL of sterile growth media at 37 °C for a maximum of 24 h. Samples were taken at $T = 6$ h and at $T = 24$ h, and thermal fluorescence quenching measured at 5 °C increments between 10 and 45 °C over 30 min. 3D excitation–emission matrix (EEM) plots were generated from the fluorescence analyses over time. It was found that the fluorescence of a microbial culture was significantly thermally quenched, but the results were dependent on the fluorophore type and the stage of the bacterial growth curve. Quenching was sometimes non-linear, presumably due to fluorophore production exceeding thermal quenching during the growth phase of the bacteria. Thermal quenching has the potential to allow us to confirm the importance of microbes in fluorescence signals by the non-linear response to increasing temperature, and to utilise the thermal fluorescence quenching properties of molecules to differentiate between fluorophores present during bacterial growth.
© 2006 Elsevier B.V. All rights reserved.

*Keywords:* Bacterial fluorescence; Thermal quenching; *Pseudomonas aeruginosa*; Planktonic bacteria; Temperature

## 1. Introduction

Fluorescence spectroscopy is now a commonly utilised tool in the characterisation of organic matter (OM) in the environment [1–4]. In addition, amino acid-like fluorescence has often been used as an indicator of biological activity in natural waters [5,6], and in wastewaters [7,8]. Both tryptophan- and tyrosine-like fluorescence centres have been detected in fresh waters, marine waters, and wastewaters [9–11]. In addition, higher fluorescence intensities of these compounds correlated with greater than expected biological activity in coastal and oceanic areas [10]. It is not known what causes this correlation. However, the authors tentatively concluded that tryptophan-like fluorescence from organic matter (OM) is more likely to be a product of bacterial metabolism rather than an energy source for bacteria, and that tryptophan-like fluorescence is an indicator of bacterial-OM interactions in natural waters.

The relationship between amino acid-like fluorescence and bacterial activity has also been examined in a number of studies of cultured bacteria [e.g., 12]. Although fluorescence from

bacterial cultures has been observed, the biochemical causes of fluorescence are not known. The fluorescence occurs in the same optical space as tryptophan and tyrosine [13,2], other reports have implicated lumazines in the fluorescence [14], while it has also been indicated that some fluorescence centres (excluding the tryptophan-like fluorophores) may be artefacts due to the laboratory culturing process [15].

Uniquely in the aquatic sciences, one recent study used fluorescence thermal quenching to improve our understanding of OM, and provide a method of finger printing and source apportionment of OM, as well as to provide some structural understanding of OM. In that work, sample temperature was systematically varied for a range of environmental waters such as wastewaters and river waters. The fluorescence of a molecule may be quenched as temperature is increased because the higher temperature increases the probability that an excited electron within the compound will return to their ground state without fluorescing [16]. Fluorescence of tryptophan standards was quenched by approximately 50%, whereas tryptophan-like fluorescence in untreated sewage was quenched by 30–35%, and by approximately 20% in treated sewage effluent and river water samples. As tryptophan in standard solutions is dissolved, i.e., exposed to the solution phase, it may be that a significant fraction

* Corresponding author. Tel.: +44 121 414 8147; fax: +44 121 414 5528.
  *E-mail address:* j.r.lead@bham.ac.uk (J.R. Lead).

of tryptophan-like fluorescence in waste and natural waters is bound to other materials rather than free in solution.

The study reported here aims to investigate the fluorescence quenching properties of those fluorophores produced by pure bacterial cultures of *Pseudomonas aeruginosa*. In this paper we build on previous work investigating fluorescence in pure bacterial cultures [15] and OM thermal quenching studies [16] in order to: (1) uniquely determine thermal quenching properties of cultured bacterial fluorescence and (2) compare these results with thermal quenching properties of OM as investigated by [16], in order to better understand the source and structure of tryptophan-like fluorescence in OM. The results from this study will provide further evidence as to the utility of the thermal quenching of fluorescence in OM fingerprinting and understanding OM structure.

## 2. Experimental methods

### 2.1. Sample site

The bacterium used was *P. aeruginosa*, chosen because of its ubiquitous nature and environmental relevance, and isolated from the River Tame, Birmingham, UK. The river runs alongside a major motorway, is in close proximity to a heavily urbanised area in the West Midlands, UK, and is downstream of a confluence between the river and two major tributaries. Water quality of the Tame is well documented [e.g., 17,18]. The sample site is located at UK Ordnance Survey national grid reference 401575, 295450.

### 2.2. Bacterial isolation and growth

Water samples taken were incubated at $25\,^\circ$C in phosphate buffered saline solution (Oxiod: $8.0\,g\,L^{-1}$ NaCl, $0.2\,g\,L^{-1}$ KCl, $1.2\,g\,L^{-1}$ $Na_2HPO_4$, $0.2\,g\,L^{-1}$ $KH_2PO_4$) in an orbital shaker, at 110 rpm. After 24 h, the samples were plated out onto *P. aeruginosa* selective agar (Oxoid, UK) and incubated at $37\,^\circ$C for a further 24–48 h. For the purposes of experimentation, *P. aeruginosa* was grown in a sterile media minimal salts medium (MSM) containing $0.79\,g\,L^{-1}$ $(NH_4)_2SO_4$, $0.12\,g$ $MgSO_4\cdot7H_2O$, $2.67\,g$ $KH_2PO_4$, $11.40\,g$ $Na_2HPO_4$, and $1.80\,g$ glucose. Bacteria were grown in planktonic form in sterile glass flasks. 100 mL of sterile MSM was inoculated with *P. aeruginosa* and grown overnight in an orbital shaking incubator at 110 rpm, at $37\,^\circ$C, at a pH of approximately 7.2. The following morning, 1 mL of overnight culture was transferred into 99 mL of sterile media, and the bacteria were then incubated at $37\,^\circ$C, at 110 rpm, for the remainder of the experimental period. The time of this final dilution is defined as $T = 0$. The samples were exposed to natural light conditions, but not to direct sunlight. Serial dilutions in phosphate buffered saline solution of $10^{-1}$ and $10^{-2}$ for $T = 6$ h cultures (exponential phase), and between $10^{-1}$ and $10^{-4}$ (in a series of steps) for $T = 24$ h cultures (steady state phase) were performed as fluorescence intensity of the initial samples was not within the linear range of the detector. The exponential and steady state phases were previously identified by cell counts as a function of time under identical conditions.

### 2.3. Fluorescence analysis

Fluorescence measurements were conducted using a Varian Cary Eclipse Fluorescence Spectrophotometer equipped with a Peltier temperature controller, allowing precise temperature control. Emission scans were performed from 280 to 500 nm at 2 nm steps, with excitation slit width of 5 nm, and both excitation and emission filters open. Excitation was between 200 and 400 nm at 5 nm steps. The scan rate was 9600 nm/min, with an averaging time of 0.0125 s, resulting in an analysis time of approximately 60 s per sample. The Raman peak intensity at excitation of 348 and 5 nm slits was repeatedly analysed throughout the analysis period as an instrument standard to permit inter-laboratory comparison and to check machine stability. Mean intensity was $21.74 \pm 0.74$ ($n = 128$) arbitrary units.

Samples were gently heated from 10 to $45\,^\circ$C slowly over a period of 30 min, and analysis performed every $5\,^\circ$C. The slow increase in temperature helped to stop bubbles forming in the cuvette. The temperature of $10\,^\circ$C reduced condensation on the outer walls of the cuvette, which can interfere with emission and excitation signals, was also limited. The upper limit of $45\,^\circ$C was chosen to minimise evaporation of the sample (which could lead to sample concentration), and in an attempt to minimise any increase in microbial activity due to temperature increase and to avoid structural changes to the DOM. Structural changes to DOM can occur at temperatures above $60\,^\circ$C and below $1\,^\circ$C [16]. It is also possible that changes in fluorescence may occur at temperatures close to freezing due to changes in matrix viscosity. Thermal quenching fluorescence analysis was conducted at $T = 6$ h (during the exponential phase of bacterial growth), and 24 h (steady state phase), to account for the potentially different fluorescent behaviour at different parts of the growth phase. The maximum fluorescence intensity of each fluorophore was measured.

### 2.4. Filtration

A selection of samples were filtered at $T = 24$ h to determine the proportion of 'free' and bacterially bound amino acid-like fluorescence. Millipore polycarbonate membrane filters were used with a nominal pore size of $0.2\,\mu$m, using a vacuum filtration system, to separate 'free' amino acids from those associated with bacterial cells. Flow rates were minimised to prevent cell rupture and other system perturbations. The fluorescence from the initial sample and the permeate were measured. Subsequently, $T = 24$ h samples were filtered and then thermally quenched to establish whether the thermal properties of the dissolved fraction were different to those of the unfiltered samples.

## 3. Results and discussion

Thermal quenching properties of three fluorescence centres were measured. Although a fourth fluorophore, in a similar region of optical space to that of tyrosine, was observed in some samples, it was not present consistently over time. This is possibly due to the rapid production and consumption of tyrosine, and similar processes may be relevant to the other fluorescent
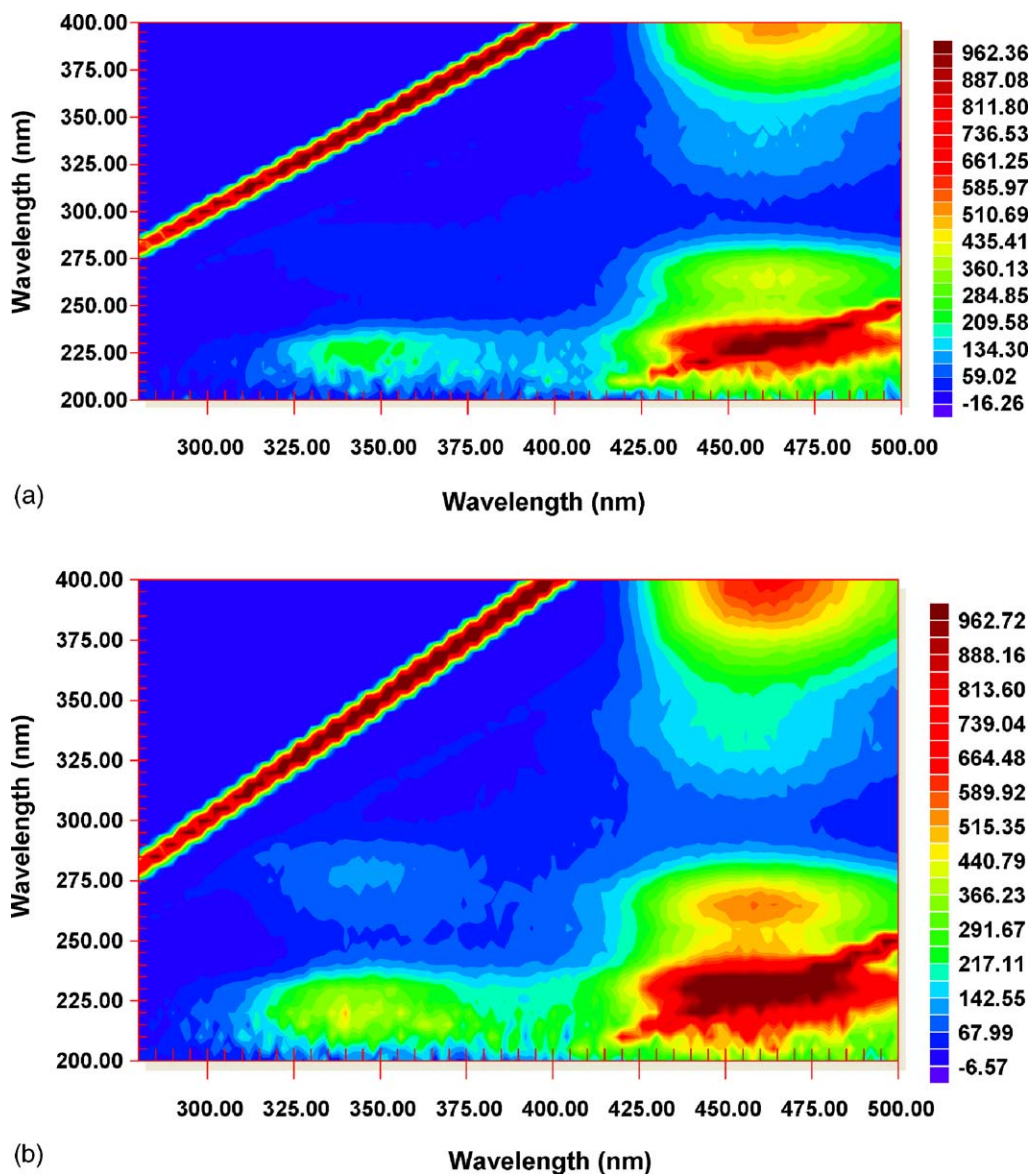
Fig. 1. 3D excitation–emission matrix fluorescence intensity plots showing: (a) fluorescence intensities of measured fluorophores as measured at 10 °C, at $T = 24$ h ($10^{-1}$ dilution). Tryptophan-like fluorescence is seen at 340 nm emission, and 220/280 nm excitation. Peak A is shown at an emission wavelength of 460 nm, and excitation of 250, 300, and 380 nm; (b) fluorescence intensities of measured fluorophores as measured at 45 °C, at $T = 24$ h ($10^{-1}$ dilution). Tryptophan-like fluorescence can be observed at an emission wavelength of 340 nm, but only at 220 nm excitation. Peak A is observed at an emission wavelength of 460 nm, and excitation of 250, 300, and 380 nm.

peaks discussed. Nevertheless, this does not seem to be dominant (trends are consistent and at much higher overall intensity values for other fluorophores) but may be responsible for some of the uncertainty in the data. Two tryptophan-like centres were observed and measured, the first excites at 220 nm, the second at 280 nm, and both emit at 320–370 nm (see Fig. 1) [19]. For the purposes of this discussion, "tryptophan-like" refers to the fluorescence centre at 220 nm, unless otherwise stated, as this is the more intense fluorophore. The third fluorophore was observed in a similar region of optical space to fluorophores reported by Giana et al. [20] and Smith et al. [21] for *Bacillus* spores, and observed in *P. aeruginosa* [15], although it has been little characterised in the literature. This fluorescence centre emits at 460 nm, and has three excitation centres at 250, 300, and 380 nm (as illus-

trated in Fig. 1). This discussion refers to the centre at 250 nm excitation, unless stated otherwise, and is referred to as Peak A.

### 3.1. Thermal quenching of bacterial samples at $T = 24$ h

At $T = 24$ h, all fluorophores exhibit a general linear decrease in fluorescence intensity with increasing temperature. Both tryptophan-like fluorescence centres exhibited fluorescence intensity quenching (Fig. 2(a)). Each line in Fig. 2(a) (and subsequent figures) represents an individual replicate. The replicates showed the same general trend but did not overlie each other. The variability in data was due to intrinsic natural differences in bacterial growth. Presentation of averaged data reduced the information content and rendered the trends less
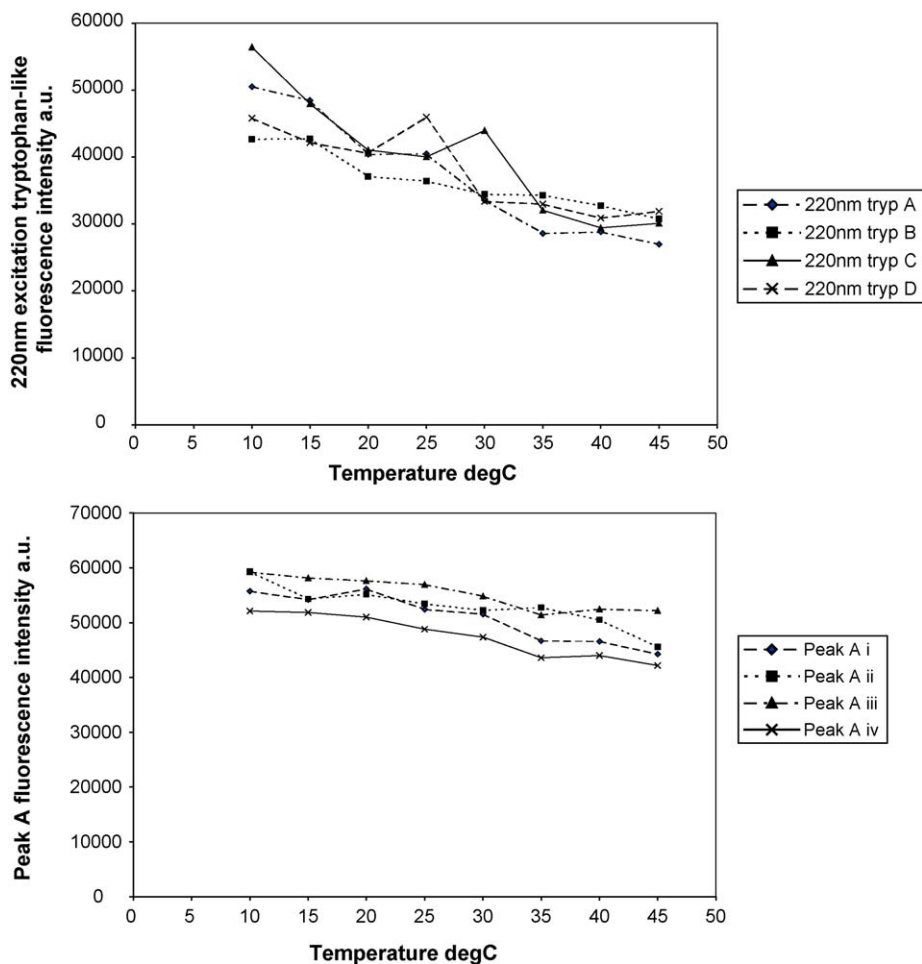
Fig. 2. Quenching of fluorescence intensity with increasing temperature during stationary phase of growth cycle ($T = 24$ h): (a) 220 nm tryptophan-like fluorescence intensity; (b) Peak A fluorescence intensity. Each line shows individual replicates. Differences in absolute numbers are to be expected due to the natural variability of bacteria, but show the same trend.

easy to observe, and so replicates are presented individually. The quenching of tryptophan-like fluorescence can be observed qualitatively by the comparison of Fig. 1(a) and (b); the former illustrates fluorescence intensity when measured at 10 °C, and the latter the intensity at a 45 °C measurement temperature. The mean decrease in intensity for 220 nm excitation tryptophan-like fluorescence was 41% (±9), and 45% (±2) for 280 nm excitation tryptophan-like intensity. These results are much higher than the thermal quenching of fluorescence data given in Baker [16] for untreated sewage (approximately 30–35%) and rural rivers (approximately 23–25%), and slightly lower than the tryptophan standard figure (approximately 50%) quoted by the same author (results are compared in Table 1). These comparisons would indicate that whilst some of the tryptophan in our experiments was bound to bacterial cells, some of it is again likely to be in solution, as quenching is related to exposure to the solution phase as discussed in Section 1.

Peak A exhibited linear fluorescence thermal quenching at $T = 24$ h (as illustrated in Fig. 2(b)), the mean decrease in fluorescence intensity being 19%. This is much lower than the 41% and 45% decreases quoted for 220 and 280 nm excitation (respectively) tryptophan-like fluorophores above. It may be the

case that Peak A is much less exposed than the tryptophan-like fluorophores, as the amount of quenching is related to exposure of each fluorophore to the light source [16]. This confirms initial ideas [15] that Peak A and the tryptophan-like

Table 1

Comparison of *P. aeruginosa* thermal fluorescence quenching and thermal quenching properties of river and wastewater samples (river and wastewater data from Baker (in press))

|  | Tryptophan-like fluorescence (220 nm excitation), thermal quenching (%), mean |
|---|---|
| $T = 6$ h[a] | $-17 \pm 4$ |
| $T = 24$ h[a] | $-38 \pm 3$ |
| Urban rivers[b] | $-22 \pm 3$ |
| Rural rivers[b] | $-23 \pm 4$ |
| Storm drain[b] | $-33 \pm 8$ |
| Wastewater treatment works (influent)[b] | $-20 \pm 5$ |
| Wastewater treatment works (final effluent)[b] | $-20 \pm 4$ |
| Tryptophan standard[b] | $-48$ |

[a] Refers to data from this study.
[b] Refers to data from [16].

fluorescence centres are very different molecules. It appears that either Peak A is a compound of bacterial origin produced during the exponential phase of growth ($T = 6$ h; see next section), but not during stationary phase ($T = 24$ h) [16], or that the fluorophore is being produced and consumed very quickly. It also seems that Peak A is a relatively large molecule – or is associated with large molecules – compared to tryptophan, explaining its lower energy of excitation (thus higher wavelength) and lower thermal quenching values. However, Peak A is not seen in EEMs from natural river water and wastewater samples, suggesting that it is an artefact of growth under laboratory conditions.

### 3.2. Thermal quenching of bacterial samples at $T = 6$ h

Both tryptophan-like fluorescence centres exhibited an overall decrease in fluorescence intensity with increasing temperature after growth of 6 h in batch culture (Fig. 3). However, the decrease is non-linear, as after 30–45 °C, fluorescence intensity begins to increase, contrary to results reported in Baker [16]

(see Fig. 3(a)) and here in Section 3.1. For example, 220 nm excitation tryptophan-like fluorescence was measured at 30 °C as ca. log 3.6 a.u., and again at 45 °C approximately 3.7 a.u. (see Table 1). It is likely that this is a result of the quenching effect being outweighed by growth of bacterial cells, as the higher temperatures (30–45 °C) would increase bacterial growth (at $T = 6$ h the growth curve is in the exponential phase). Cell growth, and therefore increased bacterial fluorescence, may become dominant over the thermal quenching effect, leading to the observed increase in fluorescence intensity later in the experiment.

As discussed, the non-linear decrease in fluorescence is in contrast to the experiments performed at $T = 24$ h, where the increase in fluorescence after 30–40 °C (at $T = 6$ h) is not observed. After 24 h, the cells were no longer exponentially multiplying even at the higher experimental temperatures, because the sample was taken from a solution where the bacteria were already at the steady state phase of growth. At $T = 6$ h, the mean overall thermal fluorescence quenching effect on 220 nm excitation tryptophan-like fluorophore is 15%, and 12% for
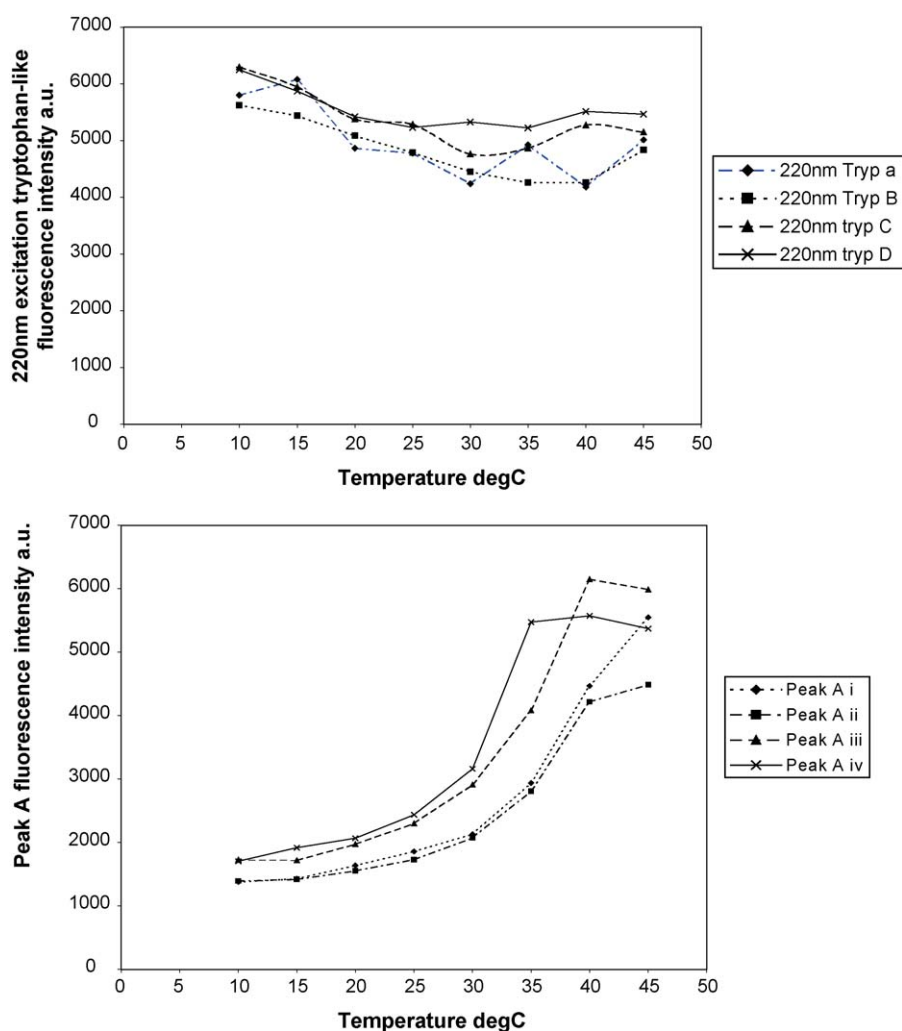


Fig. 3. Quenching of fluorescence intensity with increasing temperature during exponential growth phase ($T = 6$ h): (a) 220 nm tryptophan-like fluorescence intensity; (b) Peak A fluorescence intensity (each line indicates a replicate of the experiment). Each line shows individual replicates. Differences in absolute numbers are to be expected due to the natural variability of bacteria, but show the same trend.

280 nm excitation tryptophan-like fluorophore. If we calculate the decrease in fluorescence intensity between 10 and 35 °C (rather than 45 °C to account for uncertainty due to cell growth), thermal quenching is 20% and 25% for the 220 and 280 nm tryptophan-like fluorescence, respectively. If these unexpected data values between 35 and 45 °C are removed, a linear regression line can be fitted to the remainder of the results, and the 220 nm tryptophan-like fluorophore would exhibit a mean hypothetical thermal quenching of fluorescence of 56% ($r^2 = 0.88$) if the remainder of the results are extrapolated. This can also be expressed as a 1.9% decrease in fluorescence intensity for every 1 °C increase in temperature. This value is close to the tryptophan standard value reported by Baker [16], as well as our results in Section 3.1. When the same procedure is applied to the 280 nm tryptophan-like fluorophore, the decrease in fluorescence becomes $18 \pm 5\%$ (uncorrected value, i.e., over entire temperature range = 12% decrease), which is a relatively small value compared to 220 nm excitation tryptophan, possibly because the intensity of the 280 nm peak is generally lower from the start of the experiment. While this does take the value closer to that reported by Baker [16], it is still a small decrease in comparison to the 220 nm excitation tryptophan-like fluorophore. The difference in quenching between the two tryptophan fluorophores suggests that there are two sources of tryptophan-like fluorophores: one free (exudates) and one bound (cells), and that one has relatively more sensitivity to 220 nm excitation than the other.

Peak A did not exhibit any quenching of fluorescence with increasing temperature and fluorescence at $T = 6$. In fact, intensity increased as a function of temperature in a sigmoidal manner (Fig. 3(b)). The increase in fluorescence intensity is small between 10 and 30 °C (mean intensity at 10 °C was ca. log 3.2 a.u., and ca. log 3.4 a.u. at 30 °C), increases rapidly between 30 and 40 °C (mean intensity was approximately 5000 a.u. at 40 °C), followed by a smaller comparative increase to 45 °C (mean intensity ca. log 3.7 a.u.). The mean increase in fluorescence intensity over the experiment was 71%. It is possible that the amount of this molecule being released into solution due to cell multiplication is a more dominant factor than the quenching effect. Thus, the increase in fluorescence due to these factors will outweigh the potential decrease through quenching. In previous research, there has been some question as to the identity of Peak A [15] but these results suggest that Peak A and the tryptophan-like molecule are two different compounds. Dalterio et al. [14] investigated bacterial fluorescence at higher wavelengths (430 nm excitation, emission at 514 nm) and put forward some suggestions as to the identity of this fluorophore. As the fluorophore identified by Dalterio et al. [14] occupies a similar region of optical space as our Peak A, it is possible that the compounds may be similar. Dalterio et al. [14] suggest that compounds which fluoresce at higher wavelengths may be flavins, pyridine coenzymes, and heterocyclic pteridines, of which pterins are highly fluorescent and lumazines are moderately fluorescent. In comparison to tryptophan-like fluorescence, Peak A is highly fluorescent, so it is possible to speculate that Peak A may be one of the afore-mentioned pterins.

## 3.3. Thermal quenching of filtered samples (T = 24 h)

Filtration, without subsequent thermal quenching (Table 2), has suggested that approximately 50% of the 220 nm tryptophan-like is dissolved, i.e., will pass through a filter of nominal pore size 0.2 μm. For instance, the mean intensity of 220 nm tryptophan-like fluorescence in the filtration experiments at $T = 24$ h was approximately log 4.3 a.u., and the intensity of 220 nm excitation tryptophan-like fluorescence in the filtrate was log 3.9 a.u.. These results suggest that approximately half of the tryptophan-like fluorescence is 'free' tryptophan in solution and half is bound to bacterial cells. These results are not in total agreement with the work of Reynolds [3], which proposed that most tryptophan found in river and wastewaters is dissolved, and also helps to explain the results of Baker [16], where clean river waters exhibited tryptophan quenching of approximately 10–25%, whereas wastewaters showed quenching of approximately 30–40%. This suggests that wastewaters may be a mix of microbially derived 'free' tryptophan (e.g., exudates) together with microbially bound tryptophan. The proportion of the fluorescence intensity that is quenched is similar to that of free tryptophan reported in [16], thus suggesting that the bacterially bound tryptophan may have similar thermal properties to the free fluorophore.

After filtration, all fluorophores were detected in the permeate, but at lower fluorescence intensity. The mean decrease in fluorescence intensity of the 220 nm intensity tryptophan-like fluorophore with temperature in the filtrate was $35 \pm 10\%$ (Table 3). The quenching of fluorescence intensity was not completely linear (data were not corrected for this) although the overall trend is negative. The decrease in the unfiltered fraction is $41 \pm 9\%$ which was not significantly different to the $35 \pm 10\%$ quenching observed in the filtered samples. The loss of fluorescence after heating the filtrate was similar to those reported by Baker [16] for untreated sewage (30–35%), and also higher than the 20% quenching reported for treated sewage effluent. The thermal quenching of the filtered samples is a priori more comparable with the work of Baker [16] because the samples were filtered prior to quenching in that work also. However, no significant quantitative difference in thermal quenching was observed between filtered and unfiltered samples. Presumably, the less than expected quenching observed in the filtrate, compared with the free tryptophan standard, is due to the protective effect of larger molecules such as exudates present in the perme-

Table 2
Fluorescence of filtered and unfiltered samples of *P. aeruginosa* grown at $T = 24$ h

| Unfiltered/ filtered | log 220 nm tryptophan-like fluorescence intensity (a.u.) | 280 nm tryptophan-like fluorescence intensity (a.u.) | Peak A fluorescence intensity (a.u.) |
|---|---|---|---|
| Unfiltered | 4.3 | 3.8 | 6.7 |
| Filtered | 3.9 | 3.6 | 5.4 |
| Difference (%) | 51 | 70 | 56 |

Tryptophan-like fluorescence intensity is shown at $10^{-2}$ dilution, Peak A fluorescence intensity at $10^{-3}$ dilution.

Table 3
Fluorescence of filtered, quenched $T = 24$ h samples

|  | log 220 nm tryptophan-like fluorescence | log Peak A fluorescence |
|---|---|---|
| Unfiltered | $4.3 \pm 0.09$ | $4.73 \pm 0.08$ |
| Temperature (°C) |  |  |
| 10 | $4.34 \pm 0.6$ | $4.58 \pm 0.1$ |
| 15 | $4.03 \pm 0.1$ | $4.59 \pm 0.1$ |
| 20 | $3.97 \pm 0.1$ | $4.58 \pm 0.1$ |
| 25 | $3.94 \pm 0.1$ | $4.58 \pm 0.1$ |
| 30 | $3.97 \pm 0.1$ | $4.56 \pm 0.1$ |
| 35 | $3.90 \pm 0.2$ | $4.54 \pm 0.1$ |
| 40 | $3.90 \pm 0.2$ | $4.51 \pm 0.1$ |
| 45 | $3.90 \pm 0.2$ | $4.49 \pm 0.1$ |
| Thermal quenching (%) | $35 \pm 10$ | $18 \pm 2.5$ |

'Unfiltered sample' refers to the fluorescence on the sample before filtration. Samples were filtered and subsequently thermally quenched. Shows both 220 nm excitation tryptophan-like fluorescence intensity, and that of Peak A.

ate. A similar decreasing trend in fluorescence intensity to that of the 220 nm intensity tryptophan-like compound is observed for 280 nm intensity tryptophan-like fluorophore. The mean decrease in fluorescence intensity is $30 \pm 5\%$, again close to the values reported by Baker [16], and is somewhat lower, but not significantly lower, than the thermal quenching value for the unfiltered sample of $41 \pm 9\%$. Importantly, the 30–35% thermal quenching observed in the filtrate is lower than 50% thermal quenching in tryptophan standard indicating that at least a proportion of the fluorescent moieties in the filtrate are bound to other chemical species and therefore partially protected from quenching as stated above.

Peak A also exhibits a more linear decrease in fluorescence intensity than the two tryptophan-like fluorophores, and is quenched by approximately $18 \pm 2.5\%$ after filtration. This is similar to the values for Peak A at $T = 24$ h ($19 \pm 5\%$) obtained from the unfiltered samples, suggesting that Peak A is largely associated with the larger material, protecting it from thermal quenching.

## 4. Conclusions

In this work we have shown that the technique of thermal quenching produces information which improves quantification of fluorescence signatures from bacterial sources which in turn improves our ability to fingerprint fluorescent compounds, identify their sources and provide structural information about the fluorophores. The results have significant implications for fluorescence analysis in the fields of microbiology and environmental sciences.

## References

[1] D.M. Reynolds, S.R. Ahmad, Water Res. 31 (8) (1997) 2012.
[2] S. Determann, J.M. Lobbes, R. Reuter, J. Rullkötter, Mar. Chem. 67 (1998) 137.
[3] D.M. Reynolds, Water Res. 37 (2003) 3055.
[4] A. Baker, R. Inverarity, Hydrological Processes 18 (2004) 2927.
[5] E. Parlanti, K. Wörz, L. Geoffroy, M. Lamotte, Org. Geochem. 31 (2000) 1765.
[6] E. Zanardi-Lamardo, C.A. Moore, R.G. Zika, Mar. Chem. 89 (2004) 37.
[7] W. Chen, P. Westerhoff, J.A. Leenhee, K. Booksh, Environ. Sci. Technol. 37 (2003) 5701.
[8] F.C. Wu, E. Tanoue, C.Q. Liu, Biogeochemistry 65 (2003) 245.
[9] P.G. Coble, Mar. Chem. 51 (1996) 325.
[10] Y. Yamashita, E. Tanoue, Mar. Chem. 82 (2003) 255.
[11] A. Baker, D. Ward, S.H. Lieten, R. Periera, E.C. Simpson, M. Slater, Water Res. 38 (12) (2004) 2934.
[12] W.K.L. Cammack, J. Kalff, Y.T. Prairie, E.M. Smith, Limnol. Oceanogr. 49 (6) (2004) 2034.
[13] R.A. Dalterio, W.H. Nelson, D. Britt, J.F. Sperry, D. Psaras, J.F. Tanguay, S.L. Suib, Appl. Spectrosc. 40 (1) (1986) 86.
[14] R.A. Dalterio, W.H. Nelson, D. Britt, J.F. Sperry, J.F. Tanguay, S.L. Suib, Appl. Spectrosc. 41 (2) (1987) 234.
[15] S. Elliott, J.R. Lead, A. Baker, Water Res., submitted for publication.
[16] A. Baker, Water Res. 39 (2005) 4405.
[17] L. Beavan, J. Sadler, C. Pinder, Hydrobiology 445 (1–3) (2001) 97.
[18] A.J. Davenport, A.M. Gurnell, P.D. Armitage, Riv. Res. Appl. 20 (2004) 687.
[19] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 2nd ed., Kluwer Academic/Plenum Publishers, New York, 1999, pp. 15–18.
[20] H.E. Giana, S. Landulfo, A.Z. Renato, T.T.P. Marcos, J. Fluor. 13 (6) (2003) 489.
[21] C.B. Smith, J.E. Anderson, S.R. Webb, Spectrochim. Acta, Part A 60 (2004) 2517.