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Characterisation of the fluorescence from freshwater, planktonic bacteria

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ABSTRACT

Amino acid-like fluorescence has been used as an indicator of biological activity in wastewater effluent and in natural waters, and can be detected using fluorescence spectroscopy. Little or no work has been able to state conclusively that these so called 'amino acid-like' fluorophores are associated with proteins present as a result of bacterial activity. This work aims to ascertain whether bacteria are one possible cause of these 'amino acid-like' peaks observed in natural waters. In addition, fluorescence derived solely from one bacterial source was determined as a function of the growth time and temperature. 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 growth media. Bacteria were grown at 11, 25 and 37 °C, over a maximum of 10 days. A 3D Excitation-Emission Matrix (EEM) plot was generated from fluorescence analysis of the samples. Both tryptophan and tyrosine-like fluorescence, resembling that observed in natural and waste waters, was observed in these samples, indicating that observed fluorescence signals from aquatic systems in the literature were of biotic origin. Significant differences in fluorescence signals were obtained from planktonic cells grown at different temperatures. At 25 and 37 °C, cells were found to produce predominantly tryptophan-like fluorescence, with some tyrosine-like fluorescence also detected. A further unknown fluorophore was also detected (emission wavelength of approximately 460 nm, with three excitation centres at 225, 260 and 390 nm), likely to be a bacterially produced metabolite. At 11 °C, a more environmentally realistic temperature in temperate environments, quantitative and qualitative differences were observed in fluorescence signals when compared with the higher temperatures, indicating that laboratory observations conducted at higher temperatures may not be easily used to interpret environmental processes.

1. Introduction

The majority of environmental fluorescence research has characterised the fluorescent properties of dissolved organic matter (DOM) in river and estuarine waters (e.g., Reynolds, 2003, Wu et al., 2003, Yamashita and Tanoue, 2003, Baker et al., 2004). In marine systems, Coble (1996) examined variability in fluorescence of natural DOM, attempting to distinguish between humic substances from terrestrial and marine sources. Wastewater and DOM responsible for river pollution has also been a major focus of fluorescence work to date (e.g., Baker et al., 2003, 2004). Tryptophan-like fluorescence was used as an indicator of biological biomass in wastewater effluent (Chen et al., 2003), caused by microbial proteins that are produced during wastewater treatment, in freshwaters (Wu et al., 2003), and in marine/coastal waters (Parlanti et al.,

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2000; Zanardi-Lamardo et al., 2004). Fluorescence spectroscopy, focussed on the intensity of tryptophan-like fluorescence, has therefore demonstrable potential for the detection of waste water pollution, and calibration of tryptophan-like fluorescence intensity against known indicators of pollution (for examples see Baker and Inverarity, 2004) could again permit real-time monitoring of water quality and pollution events.

Much recent work has discussed fluorescence of 'tryptophan-like' and 'tyrosine-like' peaks in natural waters, emitting at around 340–360 and 300–320 nm, respectively, and with excitation at ca 220–280 nm, respectively (Baker et al., 2004; Coble, 1996; Yamashita and Tanoue, 2003; Mayer et al., 1999). Similar peaks, as well as some at 340–460 nm excitation and 430 and 455 nm emission from pure bacterial cultures grown at high temperatures (37 °C) in the lab (Dalterio et al., 1986, 1987) have been observed. In addition, Shelly et al. (1980) used fluorescence for the analysis of single bacterial species within a cultured, mixed-species sample. However, no experimental work has been undertaken to confirm that in freshwater these 'amino acid-like' fluorophores can be derived from a bacterial source. It has only been observed that the peaks seen in natural samples inhabit the same position in optical space as tryptophan and tyrosine standards. Determann et al. (1998) have published the only data from marine systems concerning 'protein-like' fluorescence peaks and pure bacterial (lab) cultures, allowing fluorescence centres to be directly related to the microbial activity (biomass and metabolism) of marine algae and bacteria. All previous work on environmental fluorescence has been performed at elevated temperatures. Research is required for freshwater bacteria at environmentally relevant conditions.

Given a possible bacterial source for some or all of the observed protein-like fluorescence, it is important to consider the behaviour of bacteria in suspension. Bacteria are able to regulate their genetic make-up as a response to growth conditions (Weber and Marahiel, 2003; Jagdale et al., 2005; Kim et al., 2005). For instance, changes in protein expression by the bacteria are used as an indicator of phenotypic differences. Stinzi (2003) found that 336 genes expressed in *Campylobacter jejuni* changed as a response to temperature variation, leading to differences in protein expression, where some proteins were produced as a direct response to the temperature increase (known as 'heat-shock' proteins). Our research attempts to distinguish between protein patterns produced by planktonic bacteria grown in variable temperature conditions, by use of fluorescence spectroscopy. This work also aims to determine whether bacteria are a possible source of the aforementioned 'tyrosine'- and 'tryptophan-like' peaks observed in natural waters. The role of humic substances has also been investigated in this study, both directly as a source of fluorescence, and indirectly as a carbon source for the bacteria.

2. Methodology

2.1. Sample site

The bacterium used was *Pseudomonas 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, and is in close proximity to a heavily urbanised area in the West Midlands, UK. Extensive studies have been carried out on the Tame, and as a result water quality is well documented (e.g., Beavan et al., 2001; Davenport et al., 2004). The sample site is located at OS grid reference 401575, 295450. The site is downstream of a confluence between the river and two major tributaries, resulting in the site receiving water sourced from residential areas, areas of light and heavy industry, and rural areas.

2.2. Bacterial isolation and growth

Water samples were incubated at 25 °C in phosphate buffered saline solution in an orbital shaker, at 120 rpm. After 24 h, the samples were plated out onto *P. aeruginosa* selective agar and incubated at 37 °C for a further 24–48 h. Catalase tests were performed to confirm the presence of *Pseudomonas*, and the identify of *P. aeruginosa* confirmed by its visual and olfactorary characteristics.

For the purposes of experimentation, *P. aeruginosa* was grown in a Minimal Salts Medium (MSM) containing 0.793 g/l^{-1} $(NH_4)_2SO_4$, 0.123 g $MgSO_4 \cdot 7H_2O$, 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. Hundred milliliter of sterile MSM was inoculated with *P. aeruginosa* and grown overnight in an orbital shaking incubator at 100 rpm, at 11, 25, or 37 °C, at an approximate pH of 7.2. The following morning, 1 ml of overnight culture was transferred into 99 ml of sterile medium. Serial dilutions in phosphate buffered saline solution of 10^{-1}–10^{-6} for first day cultures, 10^{-1}–10^{-9} for second day cultures, and CFU counts, were performed regularly to monitor bacterial growth. The bacteria were then incubated at the relevant chosen temperature (11, 25, or 37 °C), at 110 rpm, for the remainder of the experimental period.

2.3. Fluorescence analysis

Fluorescence measurements were conducted using a Varian Cary Eclipse Fluorescence Spectrophotometer equipped with a Peltier temperature controller. 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 were 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 ~60 s. Fluorescence analyses were performed at 25 °C. The raman peak intensity at excitation of 348 nm and 5 nm slits was repeatedly analysed throughout the experimental period as an instrument standard to permit inter-laboratory comparison and to check machine stability. Mean intensity of the Raman peak intensity was 20.29 arbitary units.

Planktonic *P. aeruginosa* were analysed at $T = 0$ h (addition of 1 ml culture to sterile medium), and then at regular intervals until the end of the experiment (approximately $T = 30$ h at 25 and 37 °C, $T = 12$ days at 11 °C). To avoid inner-filtering effects (Mobed et al., 1996; Ohno, 2002), serial dilutions of sample from 10^{-1} to 10^{-3} were analysed, and the fluorescence intensities of observed fluorophores

measured on the most dilute sample that was above detection limits. Samples were unfiltered; therefore the resulting fluorescence is a combination of bacterial protein biomass and exudates. Media, temperature and pH were maintained at constant values throughout the experimental procedures.

2.4. *Humic Acid addition*

In some samples, International Humic Substances Society Suwanee River Standard Humic Acid (HA) was added at $T = 0$ h, and measured as described in the previous section. The culture medium was as previously described with the

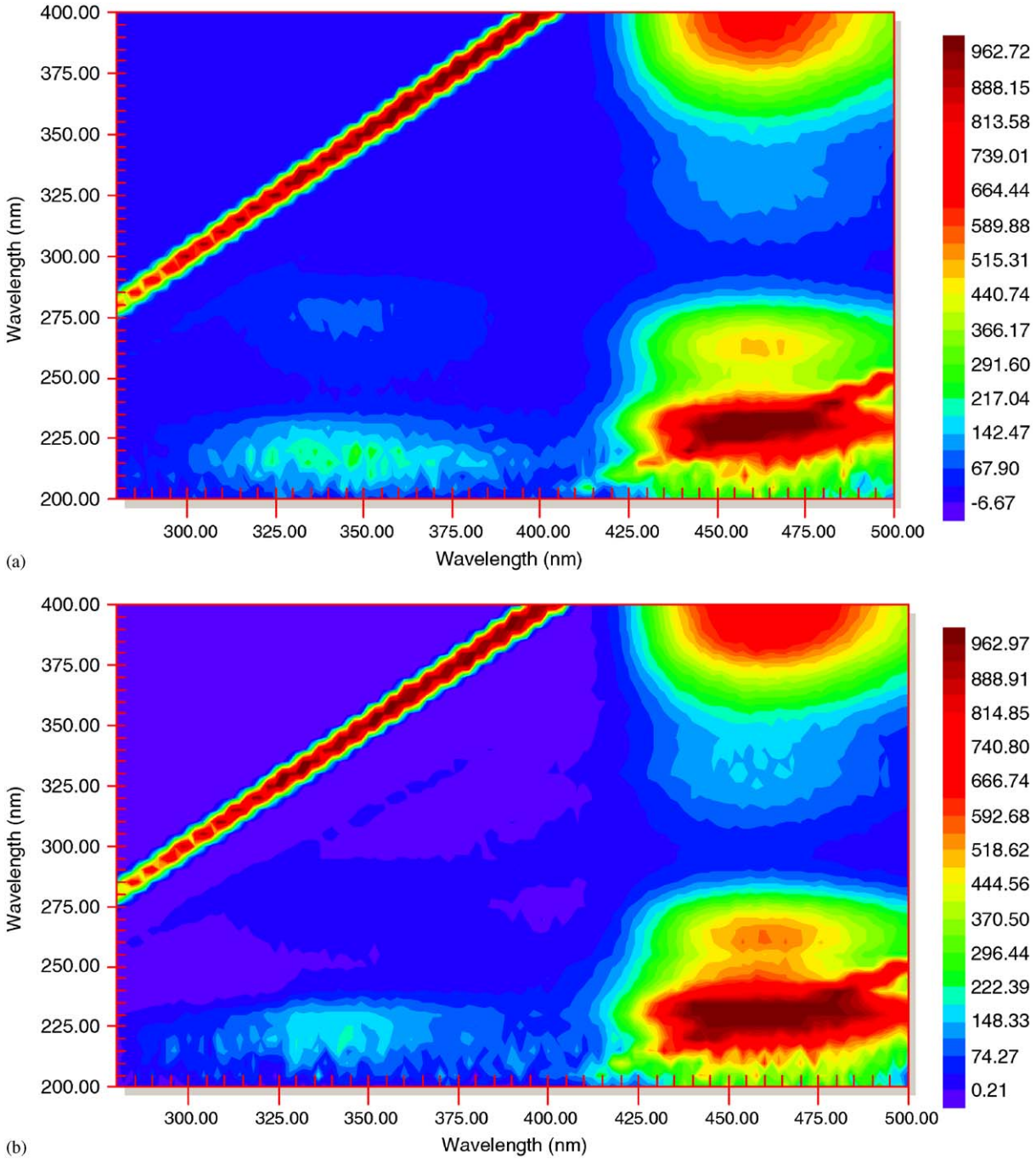
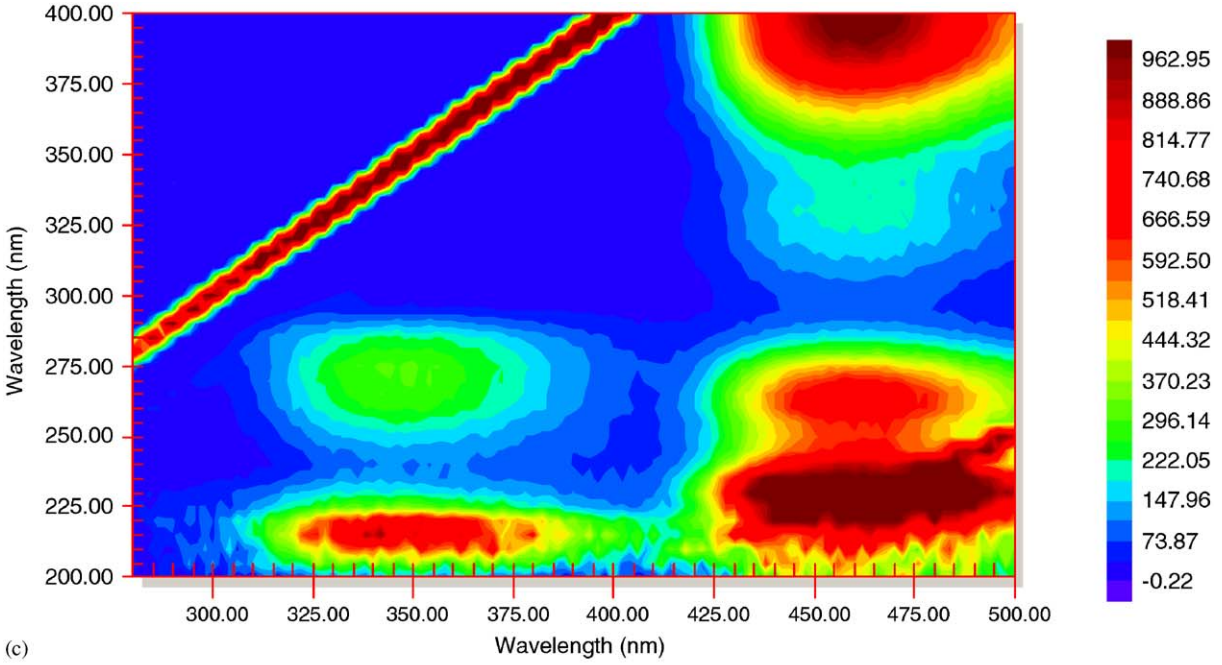


Fig. 1 – 3D Excitation-Emission Matrix fluorescence intensity plots showing: (a) Tryptophan-like fluorescence at 340 nm emission, and 220/280 nm excitation, at 25 °C, $T = 24$ h (10^{-2} dilution). Peak A is also shown at emission wavelength of 460 nm, and excitation of 250, 300, and 380 nm; (b) tryptophan-like fluorescence at 340 nm emission, and 220/280 nm excitation, at 37 °C. $T = 24$ h (10^{-2} dilution); (c) tryptophan-like fluorescence at 340 nm emission, and 220/280 nm excitation, at 11 °C, $T = 168$ h (10^{-2} dilution); (d) 3D EEM plot at 25 °C, after addition of HA at $T = 0$ h. Taken at $T = 24$ h (10^{-2} dilution).

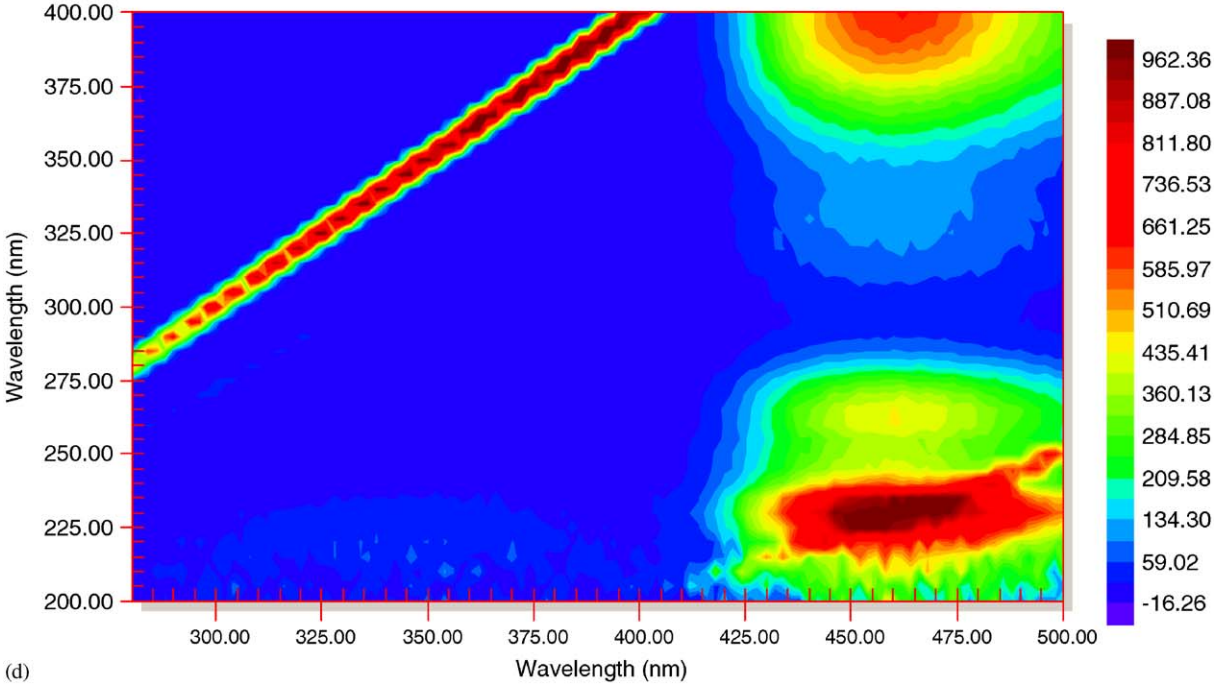
addition of 50 mg/l of HA, creating a medium with a final concentration of 5 mg/l HA.

3. Results

Tryptophan produces two fluorescence centres that excite at 220 and 280 nm (Fig 1a), and emit at 320–370 nm (Lakowicz, 1999). For the purposes of this discussion, unless otherwise stated, tryptophan-like refers to the fluorescence centre at the 220 nm excitation wavelength. The tyrosine-like fluorescence centre excites at approximately 220 nm, and emits at around 240 nm (Fig. 1). Suitable sterile controls, i.e. without bacteria, showed no fluorescence (data not shown). Additionally, we observed an infrequently characterised fluorophore in our experiments: this peak emits at around 460 nm, and has 3 excitation centres, at ca 250, 300, and 380 nm (see Fig. 1). Here we refer to excitation at ca 250 nm, unless otherwise stated, which we call *Peak A*. This peak is a similar region of optical space as those reported in *Bacillus* spores by Giana et al. (2003) and Smith et al. (2004). Lastly, experiments were also performed with the addition of HA. No obvious differences were observed between these solutions and those with no added HA, possibly due to the overlap in optical space



(c)



(d)

Fig. 1 – (*Continued*)

between the HA and Peak A, meaning that if the intensity of Peak A were high, HA may be obscured somewhat.

Changes in amino acid fluorescence for all observed fluorophores over time followed a sigmoidal curve, as is shown in Figs. 2 and 3a–d. The fluorescence data as a function of time coincided well with viable cell counts (Fig. 3e), as expected. As cell numbers increased, fluorescence intensity also increased. Sigmoidal curves are observed for all conditions, consisting of an initial lag phase, rapid exponential growth phase, and the stationary phase.

All fluorescent compounds identified during the experiments were present at much lower concentrations at 11 °C than at higher temperatures (Fig. 3), indicating a lower growth at lower temperatures. For example, at $T = 24$, an average of log 2.9 of tryptophan-like fluorescence intensity was detected in the 11 °C experiment, compared to log 4.4 a.u. at 25 °C, and 4.4 a.u. at 37 °C). At 25 and 37 °C, lower intensities of Peak A are present than the tryptophan- and tyrosine-like fluorescence centres. Peak A exhibits the most intense fluorescence signals by the end of the experiment. Maximum (off-scale) machine intensity readings were obtained after $\sim T = 20$ h for Peak A, whereas tryptophan- and tyrosine-like fluorescence did not reach this maximum level. Although this fluorophore is present initially in very small concentrations, after around 15 h it becomes dominant in the experiment, and remains so until the end of the experiment at 25 and 37 °C.

There is an initial increase in tyrosine intensity between $T = 0$ and 2 h, followed by a steady phase before the

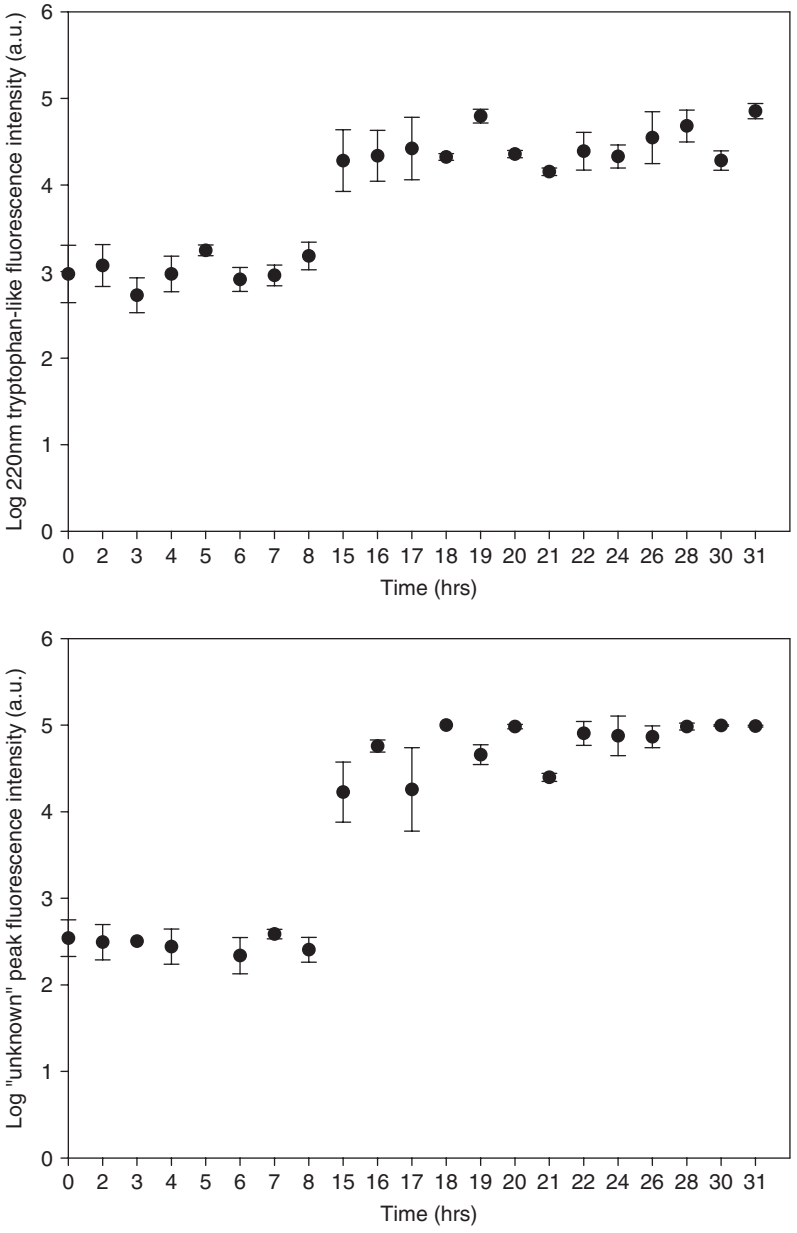


Fig. 2 – Variation of fluorescence intensity over time, error bar graphs showing standard deviation from the mean at 25 °C: (a) Log 220 nm excitation tryptophan-like fluorescence against time; (b) Log Peak A fluorescence intensity against time.

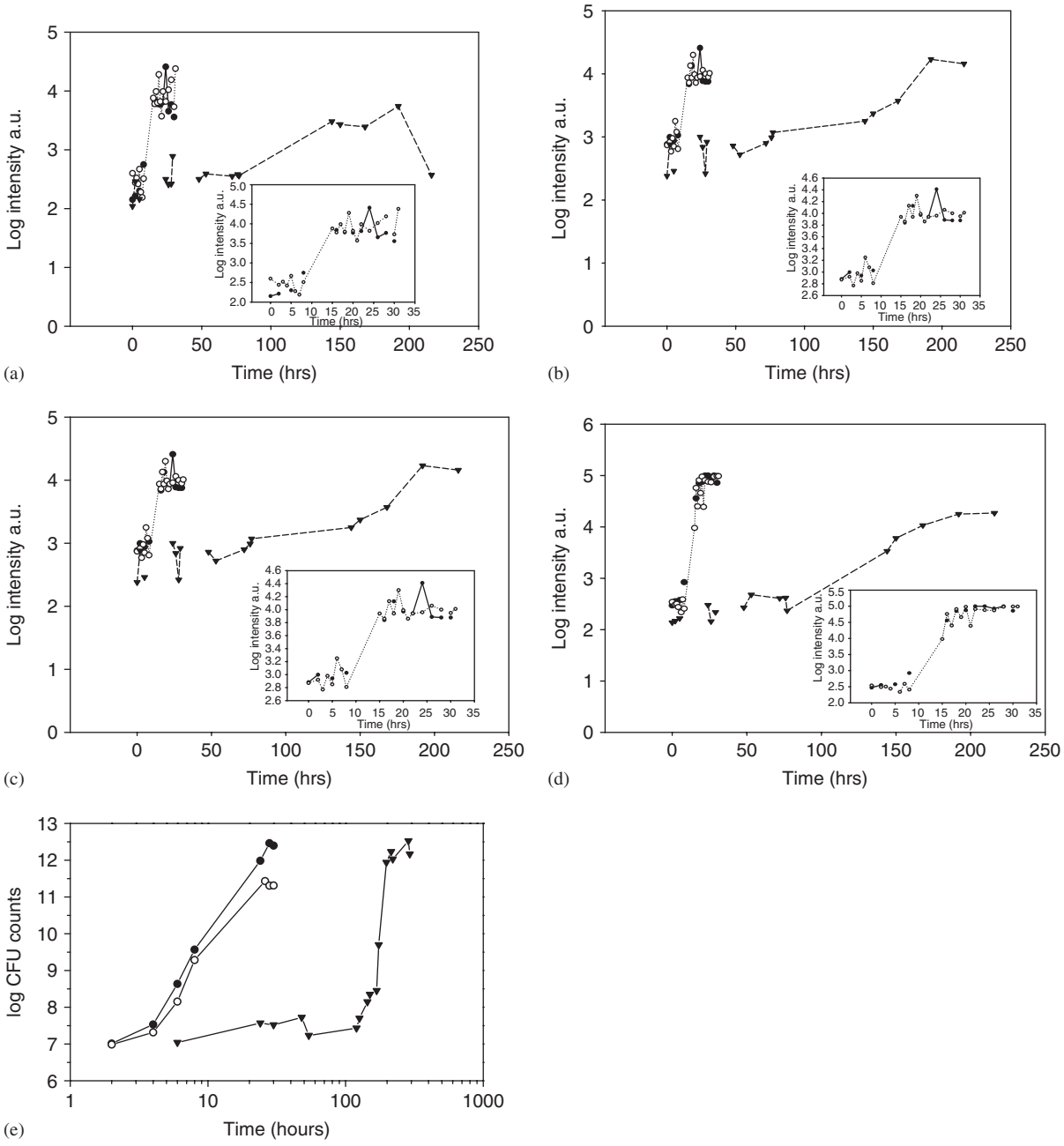


Fig. 3 – Variation of amino acid-like fluorescence intensity over time (average values shown), at all temperatures investigated: (a) Variation of 220 nm excitation tryptophan-like fluorescence intensities over time, at all temperatures investigated. Inset shows magnification of shorter 37 and 25 °C experiments. ● average log 37 °C exp't; ○ average log 25 °C exp'ts; ▼ average log 11 °C exp'ts; (b) Variation of 280 nm excitation tryptophan-like fluorescence intensities over time, at all temperatures investigated. Inset shows magnification of shorter 37 and 25 °C curves. ● average log 37 °C exp't; ○ average log 25 °C exp'ts; ▼ average log 11 °C exp'ts; (c) Variation of tyrosine-like fluorescence intensities over time, at all temperatures investigated. Inset shows magnification of shorter 37 and 25 °C curves. ● average log 37 °C exp't; ○ average log 25 °C exp'ts; ▼ average log 11 °C exp'ts; (d) Variation of Peak A fluorescence intensities over time, at all temperatures investigated. Inset shows magnification of shorter 37 and 25 °C curves. ● average log 37 °C exp't; ○ average log 25 °C exp'ts; ▼ average log 11 °C exp'ts; (e) Variation of cell counts over time, at all temperatures investigated, the average of three experiments. ● average log 37 °C exp't; ○ average log 25 °C exp'ts; ▼ average log 11 °C exp'ts. Note log scale on time axis.

exponential growth occurred. As with tryptophan-like fluorescence, maximum intensities are reached after 30 h at 25 and 37 °C, and the stationary phase is attained in a much shorter period of time than at 11 °C. At the higher temperatures, both tryptophan-like fluorescence curves are very similar in shape.

Figs. 2 and 3 illustrate significant differences between the 11 °C experiments and those performed at 25 °C. No

significant differences between 25 and 37 °C experiments were observed, so only data for the 25 °C experiments are shown. The steady-state phase (where cell death is equal to new production) of the higher temperature curves is reached within 30 h of the start of the experiment. There is no notable increase in tryptophan- and tyrosine-like fluorescence intensity until at least 30 h in the 11 °C experiment. There is little variation in fluorescence intensity of any fluorophore between the start of the experiment and $T = 48$ h (for example, at $T = 0$ 220 nm tryptophan-like intensity is log 2.5 a.u., and at $T = 48$, intensity is log 2.9 a.u. Values for all other fluorophores are of the same magnitude). All fluorophores increase in intensity after $T = 48$, although tyrosine-like fluorescence increases at a slower rate (at $T = 150$, 220 nm tryptophan-like intensity is log 3.9 a.u., whereas it is log 3.5 a.u. for tyrosine-like intensity). None of the fluorophores are as intense by the end of the experiment as the same measured in the higher temperature experiments—a logical conclusion would be that there are less of the fluorophores present at 11 °C than at 25 or 37 °C.

4. Discussion

We have demonstrated that at least some of the tyrosine- and tryptophan-like fluorescence observed in river systems can have a direct bacterial origin (Fig. 1). Further research is now needed, both using cultures of other common individual freshwater bacteria to compare the intrinsic fluorescence properties of different species, as well as a comparison of cultured and freshwater samples. In addition, studies (Smith et al., 2004) have noted fluorescence signals in a similar position to that of our fluorophore "Peak A" from *Bacillus* endospores. However, the same authors did not observe this fluorescence with *P. aeruginosa* alone, as we have done, although the reason for this disagreement is not clear. All possible contaminant sources were ruled out as a result of investigation by the appropriate use of blanks and controls without bacteria present, and the fluorophore was found to be bacterial in origin. As the Peak A is present in increasing concentrations during the exponential cell growth phase, it would indicate that it is a bacterially produced metabolite. It has been suggested by Smith et al. (2004) that these substances may be pteridine compounds, important in bacterial metabolism and reported as exhibiting some fluorescence. The fluorophore is not well characterised in the literature, although it inhabits optical space in a similar position to HS and therefore identification of these two separate peaks (HS and unknown metabolite) in mixtures such as natural waters may not be easy. Caution should therefore be exercised when interpreting fluorescence peaks unambiguously in terms of chemical structures, without supporting evidence. To our knowledge, nothing similar to Peak A has been noted in previous environmental fluorescence work concerning river and waste waters. As such, a further possible explanation for the detection of this peak may be as a result of bacterial stress due to storage under lab conditions, leading to production of different proteins.

The lower fluorescence intensities at 11 °C, compared with the higher temperatures, are likely explained by the decreased number of viable cells present and decreased metabolic rate, as the temperature is far below the optimum for *P. aeruginosa* growth. At 11 °C, cell numbers reach approximately 10^{12} per ml during the final stages of growth. In addition, steady state is achieved over longer periods of time. Weber and Marahiel (2003) reviewed the concept of cold shock response in bacteria, a mechanism of bacterial adaptation to a sudden decrease in temperature, which is applicable to the 11 °C growth curves as the initial culturing in our experiments was performed at higher temperatures. Cells can reprogram gene expression as a response to changes in growth conditions in order to minimise adverse effects on cell function. The review identifies an "acclimatisation phase" which occurs after the initial drop in temperature, and leads to a reduction in growth rate whilst protein synthesis is adjusted. It is likely that the apparent slower production of tryptophan- and tyrosine-like fluorescence observed at 11 °C is as a result of this cold shock response, because of initial growth at 37 °C. A further fluorescence centre was identified that emits at 395 nm, and excites at 225 and 325 nm, and is only present at 11 °C. This centre has not been seen in natural river waters, although there are possible explanations as to its appearance in the experiment. It could be that tryptophan-like fluorescence intensity maxima can shift to higher wavelengths if it unfolds for any reason, and is exposed to the aqueous solution, as documented by Lacowicz (1999). It is possible that, if we are observing tryptophan fluorescence, the sudden drop in temperature could cause a change in the exposure of tryptophan. This could be related to the cold shock response, as noted above. The signal from this centre can be detected throughout the experiment, following a similar pattern to the usual tryptophan-like fluorescence peak. One further possible explanation for this is that the compound is produced by the bacteria, and released into solution. If production of the compound is as a result of bacterial cold-shock response, it is possible that it aids *P. aeruginosa* survival at the low temperature.

Weber and Marahiel (2003) also state that whilst growth of cells will continue at the lower temperature, it will be at a reduced rate. Taking this into account, it can be assumed that fewer cells (with a lower metabolic rate) are present at the lower temperature, therefore fewer proteins produced (assuming that proteins produced are relative to the number of cells present in growth media). This would help to explain the overall lower levels of tryptophan- and tyrosine-like fluorescence intensity present.

The differences observed between lower and higher temperatures are marked and perhaps not unexpected. However, the results indicate that microbial work performed at higher temperatures may not be a reliable model for processes operative in lower temperature environmental systems. Our results suggest that it is necessary to undertake environmental microbial research at more environmentally realistic temperatures, to enable results to be applied to these systems. It can also be shown by this research that different fluorophores become dominant depending on temperature. At higher temperatures, tryptophan- and tyrosine-like fluorescence intensities are similar. This is interesting to note, as tyrosine-like fluorescence is rarely seen in UK rivers, but is

present in untreated sewage and in slurries (Baker, 2002). It is possible that our lab cultures are growing in a way that is more similar to bacteria found in sewage samples, rather than behaving as river water samples. This leads to the possible conclusion that the experimental methods may need further work to ensure they reproduce the desired environmental conditions.

For all samples measured, there appears to be a fairly constant ratio of 3:1–4:1 of tryptophan-like fluorescence intensity at 220 nm excitation compared to excitation at 280 nm. Values of this ratio calculated from published data on river systems (Baker and Inverarity, 2004; Baker and Spencer, 2004), are also in the same range of between 1:1 and 4:1. This would indicate that the tryptophan-like fluorescence observed in our cultures might be similar in nature and origin to the fluorescence observed in a natural environment. However, it is interesting that Peak A is not observed in natural waters. It is possible that this peak may be present as a result of stress induced by keeping the bacteria under unnatural lab conditions, or as result of a lack of some components of natural waters. When it is present at very high levels, Peak A obscures the HA sign (and possible vice versa). In addition, it was noted that HA has little effect on other fluorescent signals under these conditions. For instance, it does not act as a significant energy or carbon source in the presence of glucose in the growth media.

Expressed graphically (data not shown), it is noted that both the tryptophan-like peak and Peak A exhibit a similar range of intensity values at each time point, in both the experiments where HA was present, and in those where it was omitted. There are marked differences between the intensities of all fluorophores illustrated in Fig. 3. It can be observed that there is a much higher concentration of Peak A compared to tryptophan-like fluorescence, which is present only in small quantities (refer to Fig. 2). This is consistent in both 25 °C experiments, with and without HA present, and would indicate that HA appears to have little or no effect on the concentrations of fluorophores produced.

5. Conclusion

We have demonstrated that at least some of the tyrosine- and tryptophan-like fluorescence observed in river systems can have a direct bacterial origin. Fluorescence Peak A, however, was observed in our cultures but has not been reported in freshwaters, and may be as a result of bacterial stress due to storage under lab conditions, leading to production of different proteins. We observe a temperature dependence of fluorescence properties, with significant differences at 11 °C to those at 25 and 37 °C, explained as a possible cold-shock response by the *P. aeruginosa* that had been cultured at a higher temperature. The results indicate that microbial work performed at higher temperatures may not be a reliable model for processes operating at lower temperature environmental systems. In contrast, the addition of humic substances appears to have little or no effect on the concentrations of fluorophores produced in our cultures.

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