Excitation–emission–matrix fluorescence spectroscopy applied to humic acid bands in coral reefs

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Abstract

An investigation of the three-dimensional excitation–emission–matrix (EEM) fluorescence spectra of humic acids and other organic matter from corals and other sources is reported, the results of which have implications for the use of corals as paleoenvironmental indicators. Four characteristic excitation/emission peaks were identified in the samples studied, at 310/430, 340/450, 390/490 and 280/(320–350) nm, the last of these attributed to protein fluorescence. The positions of these peaks were essentially invariant between different samples, including coral extracts, solid corals, seawater, and commercially-available humic acids, although their relative intensities showed considerable variation. The use of the EEM technique in the present work has demonstrated that changes in sample concentration can considerably change the intensity distribution of the fluorescence spectra, particularly if strongly absorbant, non-fluorescent species are present. Comparison of the EEM spectra of organic matter extracted from bright and dull coral bands showed the spectroscopic structure of the fluorescence emitted to be essentially identical for both bands; the emissions appear to differ only in their absolute intensities.

Keywords: humic substances; fluorescence spectroscopy; coral; dissolved organic-matter; spectra; excitation

1. Introduction

1.1. Fluorescent banding in massive corals

Fluorescent bands in massive corals were first reported by Isdale (1984) from the study of Porites colonies on the Great Barrier Reef of Australia. The banding, visible under illumination with long-wave UV light, consisted of ‘bright’ (yellowy-green) and ‘dull’ (blue) bands, with each major bright–dull band couplet representing one year’s growth. Furthermore, Isdale found a correlation between the intensity of fluorescence in coral cores taken from large colonies and the record of rainfall and runoff from the adjacent Queensland landmass, with bright bands equating to seasonally high (monsoonal) rainfall.

On the basis of this correlation, Isdale suggested that analysis of fluorescence in cores taken from the skeletons of large, several century old, coral colonies could prove to be a valuable new tool for constructing records of past rainfall and runoff in low-latitude regions. Further investigation of the source of the
fluorescence in the corals led Boto and Isdale (1985), Susic and Boto (1989) and Susic et al. (1991) to suggest that the fluorescence resulted from the incorporation of humic acids (HA) into the coral skeleton, and that in shelf areas, the main source of the HA was the breakdown-products of land plants. Thus, the bright bands in corals equated with the input to coastal waters of large volumes of these terrestrial HA during periods of seasonally high (monsoonal) rainfall and runoff. Although the correlation between rainfall/runoff and fluorescence in coral skeletons clearly holds well for some regions, such as the Queensland shelf (Boto and Isdale, 1985; Susic and Boto, 1989; Susic et al., 1991) and parts of Papua New Guinea (Scoffin et al., 1989), elsewhere the relationship appears to break down. For example, in the region of Phuket, south Thailand, the brightly fluorescent bands are deposited during the dry season (Scoffin et al., 1992), and off the coast of Oman, fluorescent bands have been discovered in corals totally removed from any source of freshwater input (Tudhope et al., in prep.). These anomalies imply that factors other than varying concentrations of terrestrially-derived HA in seawater may influence the nature and extent of fluorescent banding in corals.

In this context, the successful interpretation of the fluorescent bands in corals relies upon an understanding of a number of factors, all of which combine to give the net fluorescence of the system. These factors include:

1. the concentration of fluorescing compounds in the skeleton;
2. the nature and relative abundance of each type of fluorescing compound in the skeleton;
3. the concentration of energy-absorbing compounds in the skeleton;
4. the nature and relative abundance of each type of absorbing compound;
5. the skeletal structure, and in particular, the nature and extent of porosity.

In this paper we address issues relating to the first four of these factors. In particular we investigate some properties of multifluorophore HA systems, which have important general implications for the appearance of fluorescent banding in coral skeletons. A fuller description of the optical properties and banding pattern of these particular Thailand corals can be found in Theodorou (1995).

1.2. Fluorescence of humic acids and marine dissolved organic matter

Humic acids are the major constituent of soil organic matter and of dissolved organic matter (DOM) in the sea. Soil HA is predominantly derived from lignin precursors, which are structural components of land plants only. Since it has been shown that this can precipitate rapidly on mixing with seawater (Sholkovitz et al., 1978), the HA component of marine DOM is believed to be derived instead from melanoidins formed in situ by plankton (Nis- senbaum and Kaplan, 1972). In this paper no distinction is made between humic acid and the smaller fulvic acid molecules, the difference being dependent on the extraction technique (Frimmel and Christman, 1988). However, in recognition that the fluorescent matter in corals, like marine DOM, may also contain protein and other products of biological metabolism, this mixture will be referred to hereafter as coral organic matter (COM), of which HA is the major part.

Fluorescence spectra have been used by soil scientists as an aid to distinguishing different types of HA, but without great success. The normally recorded single line excitation or emission spectra are usually broad and featureless peaks. Slightly more characteristic spectra have been obtained from synchronous scans (constant wavelength interval between excitation and emission) (Senesi et al., 1991; Miano and Senesi, 1992), but these spectra may sometimes be distorted by Raman scattering.

It has long been recognised that several fluorophores are present in HA, but these were difficult to isolate. Because HA is a multi-chromophoric system, its fluorescence behaviour differs markedly from that of a “normal”, single chromophore molecule. In particular, the wavelengths of the emission maxima are dependent on the excitation wavelength. Consequently, the wide range of excitation wavelengths used in previous studies has resulted in little consistency between results in the literature.

In such a multi-chromophoric system, interpretation of fluorescence spectra is also complicated by photophysical energy transfer and absorption processes. These will be particularly important in the solid corals and extracts with higher concentrations of organic matter than is typical of seawater. How-
ever, intramolecular energy transfer processes between different chromophores which are chemically bound together, possible in such large structures as HA molecules, may occur at all concentrations.

It must be remembered that for every fluorophore in humic acid, there may be many non-fluorescent chromophores. This is apparent from the very low quantum efficiency of HA, typically 1% (Green and Blough, 1994), and from the observation that excitation spectra do not look at all like absorption spectra. Absorption spectra of HA are essentially featureless, showing an exponential decline in intensity from high to low energy, and are thus of little value in characterising HA samples. So the self-quenching (or inner-filter) process, by which fluorescence observed per molecule decreases at high concentrations, will be dominated by these non-fluorescent chromophores. However, as the concentration changes, the relative emission at different wavelengths may also change, as a result of both radiative and non-radiative energy transfer processes and dynamic quenching.

The overall effect of the combination of these processes is a migration of excitation energy from high- to low-energy fluorophores, and thus a shift in the spectral profile towards longer-wavelength emission. This may be highly relevant in explaining the visible fluorescence in coral banding, particularly in cases where a distinct colour difference is apparent between bands. Such energy transfer processes may also be employed deliberately by coral zooanthellae, which produce pigments to convert short-wavelength light (around 350 nm) to the longer wavelengths required for photosynthesis (around 470 nm) (Schlichter et al., 1994).

1.3. The advantages of EEM spectroscopy

As noted above, the use of conventional line spectra for representing the fluorescence of HA may lead to inconsistent results. Recently, marine chemists have been using three-dimensional (3-D) excitation–emission–matrix (EEM) spectra to analyse the composition of DOM, following the work of Coble et al. (1990, 1993), Poryvkina et al. (1992), and Mopper and Schultz (1993). This has shown that marine DOM contains several chromophores, including a protein-like fluorescence, although multiple chromophores have also been distinguished in marine DOM using line spectra (e.g., Sierra et al., 1994). However, the EEM technique is particularly appropriate for the current investigation, because as well as distinguishing different chromophores, it also clearly demonstrates the importance of light absorption, energy transfer and scattering processes, which will all be occurring in the solid coral.

1.4. Experimental methods

Coral samples used in the study were collected from large colonies of *Porites lutea* in Phuket, S. Thailand (8°00'N, 98°20'E). Descriptions of the physical setting, reefs and growth of *Porites* corals in this area are given in Scoffin et al. (1992) and Tudhope and Scoffin (1996). It is worth recalling that in contrast to the Queensland corals, the bright bands here corresponded to the dry season, and there is no major river input nearby to provide a large source of terrestrial HA.

Commercial HA (Aldrich and Fluka), and also tryptophan, riboflavin and isoxanthopterin, were obtained for comparison with the coral extracts.

1.5. Procedure for extracting coral organic matter

Only an outline of the technique is given here, since more detail of the pH changes and reagents used can be found elsewhere (Theodorou, 1995); also Frimmel and Christman (1988).

Bands in slices of solid coral were identified by eye under an ultraviolet lamp. The required bands were cut out and ground to a powder, of which a known mass (1–20 g) was subsequently dissolved by adding HCl solution until all the CO₂ gas (from the CaCO₃ skeleton) had been driven off. The COM was then extracted from this solution (made up to 100 ml) by adsorption onto Amberlite XAD2 resin in a gravity column. Once calcium chloride, and some organic impurities had been washed away, an alkaline solution containing ammonia and methanol (in which no significant fluorescence was found) released the humic acid from the resin. The extract was then concentrated by evaporation to 10 ml, and the pH subsequently adjusted to 7.8, for comparison with seawater.

This method works principally by polarity
changes, and has often been used for marine HA. The chief problems are that pH changes can fragment humic acid molecules, and that the adsorption process may be too selective so some chromophores are under represented, as found by Green and Blough (1994). Ongoing research now suggests that a minimal extraction process, omitting the exchange column, may be preferable. However, the results presented here were derived mainly from samples extracted using the above procedure.

Experiments were carried out to investigate the effects of the extraction process on the EEM spectra of various samples. It was noticed that sometimes substantial quantities of non-fluorescent coloured material were removed in the first wash through the column. The most pronounced effect was observed in the case of Aldrich HA, which will be discussed later. This effect was much less significant for COM samples. However, control experiments showed that the coloured material derived from algae, polyp material and endoliths on the solid corals, which is usually removed by chemical bleaching of the coral surface, could instead be removed effectively by the extraction procedure.

1.6. Procedure for observing solid coral spectra

Preliminary experiments were undertaken to explore the feasibility of determining EEM spectra directly from solid coral samples. A slice of solid coral was attached to a tilting stage, mounted in the sample compartment of the spectrofluorimeter. The coral sample could be positioned such that the excitation beam was incident on only one fluorescent band. The dimensions of the excitation beam focused at the coral surface were about 10 mm by 2 mm. The angle at which the coral sample was presented was adjusted to achieve an acceptable fluorescence intensity. There was much higher intensity of Rayleigh–Tyndall scattered light in the solid coral spectra than in those of the extracts, as would be expected.

1.7. Procedure for generating 3-D fluorescence spectra

Spectra were collected on a Perkin Elmer LS5 spectrofluorimeter. Spectra were recorded using 5-nm bandpass excitation and emission slits and collected at a scan speed of 120 nm/min, a compromise between resolution and the time taken (up to 1 h) to accumulate the data for a complete 3-D plot. The instrument makes an automatic correction for the excitation lamp spectral profile, and any temporal intensity variation, by means of an internal rhodamine dye quantum counter. A correction was later made to the data, for wavelength-dependent response of the photomultiplier tube (1P28 PMT), using manufacturer’s information. This was consistent with our observations of the integrated intensity of the water Raman band. The PMT correction is small within the range 360–460 nm, where most measurements were made, but the response is much weaker at long wavelengths, so the correction at 530 nm (cf. 400 nm) is a factor of two. Solution samples were contained in square section disposable 5-ml cells made of polymethylmethacrylate. This is effectively transparent above 320 nm, although below this absorption begins to increase slightly towards shorter wavelengths (this may slightly affect the excitation spectrum of the peak attributed to tryptophan, but should otherwise be insignificant). The data presented here were collected from individual line spectra and subsequently entered into a computer to produce 3-D plots. Hence the resolution in the plots (grid point spacing) is low, usually 20 nm, or in some cases 10 nm. For this reason, peak heights and positions quoted in the text were mostly taken from the original line spectra. We are confident that these observed peaks cannot be instrumental artefacts, particularly because independently corrected spectra of similar samples analysed on a different instrument (unpublished data) showed exactly the same characteristic peak positions as described in this text.

The maximum fluorescence range covered was from 260 nm (ex) to 580 nm (em), although most spectra presented here show a smaller area. The excitation wavelength was always at least 20 nm shorter than the emission wavelength, to avoid the intense Rayleigh–Tyndall scattering which lies along the \( \lambda_{\text{ex}} = \lambda_{\text{em}} \) diagonal. Grid points in the 3-D EEM plots beyond this \( \Delta \lambda = 20 \) nm line were set to zero. Where scattering is intense and wider than 20 nm, notably in the solid coral samples, this appears as a steep cut-off, which should be ignored.

To allow for a comparison with other work, the fluorescence intensities are reported here relative to
the size of the water Raman scattering peak, as suggested recently by Hoge et al. (1993) and Determann et al. (1994). These authors point out that, although the maximum height of Raman peaks relative to fluorescence peaks varies with the bandpass width and internal optics of the instrument, the area of the Raman peak integrated over wavelength should be independent of these factors and can be used as a standard between instruments. Here the Raman peak will only be used as an external standard, i.e. all intensities are given relative to the integrated water Raman peak in a sample of unconcentrated seawater, within the range 300–400 nm. This integrated area (which was 70% of the full-scale deflection of our instrument with response setting = 1.0) was the average of several Raman peaks measured above the fluorescent baseline on the original line spectra. The resultant fluorescence scale has the dimension nm$^{-1}$, and will be given the symbol RU nm$^{-1}$. We measured that a 10-ppm (mg/kg) solution of untreated Aldrich humic acid (AHA) gives a response of 0.39 RU nm$^{-1}$ at 355/440 nm, which can be compared to the measurement of Determann et al. (1994) of 1.0 RU nm$^{-1}$ for 22.5 ppm of AHA. By referring to a spectrum from a standard quinine sulphate solution, it was possible to estimate that the quantum yield of such a solution of 10 ppm AHA was only 0.85% (absorbance and excitation at 300 nm), compared to 59% for quinine sulphate. This low quantum yield is typical of HA (Hoge et al., 1993; Green and Blough, 1994), and indicates the large proportion of non-fluorescent chromophores within the molecule, as discussed earlier.

Raman scattering is reduced by competition with fluorescence processes, and Determann et al. (1994) use this response as an internal standard to compensate for self-quenching effects. However, this procedure would be inappropriate here, since we are looking at solutions with concentrations of COM and HA which are often much greater than those in typical seawater DOM, in which case the complex quenching processes are unlikely to affect Raman scattering in the same way as they affect fluorescence. An indication of the decline of the Raman scattering signal with increasing COM can be found in Fig. 3. Pure water Raman blanks are sometimes subtracted from EEM spectra of seawater (Mopper and Schultz, 1993) in order to correct for Raman scattering. This practice is clearly only valid for very dilute solutions.

2. Results and discussion

2.1. General features of EEM spectra

A typical COM EEM 3-D plot (as shown in Fig. 1a) consists of a diagonal ridge formed by a composite of peaks, in which the wavelength of maximum emission increases with excitation wavelength. A straight line section through a plot such as Fig. 1a, i.e. a conventional line spectrum, would cut the diagonal ridge of highest intensity at an arbitrary point, yielding very limited information. This contrasts with the EEM spectrum of a “normal” molecule (a single chromophore), such as riboflavin (Fig. 1c) in which the wavelength of maximum emission is independent of excitation wavelength. Moreover, as will be shown, when the concentration of HA is increased, the peaks at the longer-wavelength end of the diagonal become relatively more intense. This would not have been apparent without observing the whole excitation/emission matrix.

The EEM spectra also enabled Raman scattering from the solvent to be clearly distinguished from HA fluorescence. In an EEM plot, Raman scattering appears as a narrow near-diagonal ridge. This is dominant in dilute samples such as unconcentrated seawater (Fig. 1b). As the concentration of fluorescent material increases, Raman scattering becomes less significant. However, similar scattering was also evident in the case of spectra taken directly from a solid coral (see later) where COM concentrations are high. Here the aragonite skeleton of the coral may behave in a similar way to the solvent molecules in a solution.

2.2. Characteristic peak positions

There was a remarkable consistency of peak positions between the EEM spectra of most of the samples studied, including coral extracts (Figs. 1 and 6), seawater (Fig. 1b), Aldrich and Fluka commercial HA samples (Fig. 2) and solid coral (Fig. 7). In the EEM spectra, however, usually only one or two peaks are prominent, with the others appearing as
Fig. 1. Typical EEM fluorescence spectra of: (a) a coral extract (prepared from 10 g of coral); (b) untreated seawater from near the reef; (c) riboflavin; and (d) tryptophan. One RU nm−1 is equal to the integrated area of the seawater Raman peak on an excitation line spectrum (for further explanation see Section 1.4). Spectra are corrected for variation in lamp intensity and photomultiplier response.

shoulders. In the coral extract spectrum shown in Fig. 1a, for example, at first sight there is only one prominent peak at 340/440 nm. On closer examination, another peak similar to that of tryptophan at 280/350 nm (Fig. 1d) can be made out at the short-wavelength end of the coral extract spectrum. Examination of the original line spectra placed this third peak clearly at 390/485 nm. The position of this latter peak, although usually a shoulder of the 340/440-nm peak, actually maintained the most consistent position between all the HA samples studied, including extracted COM, COM in solid corals, marine DOM, and Aldrich HA both before and after extraction (where it can be seen in Fig. 2). The 390/485-nm peak was also the narrowest peak, and
could be the largest at a certain concentration of Aldrich HA, or in some solid coral spectra (see Fig. 7).

The position of maximum emission of this shoulder remained constant at 485 ± 10 nm while the excitation wavelength was varied. However, the maximum emission of the central peak shifted slightly with a shift in excitation wavelength, suggesting that it may itself be composed of two peaks. Close inspection of the full set of original line spectra (which have better resolution than the EEM) revealed 310/430 and 340/450 nm as possible positions. These are consistent with the results of Poryvkina et al. (1992), using EEM spectra for marine DOM, who find the main marine HA peak at 350/450 nm, and a minor HA peak (possibly derived from freshwater input) at 310/440 nm. Coble et al. (1993) only report one peak in this region at 340/(420–450) nm, but Mopper and Schultz (1993) place it at 310/(420–450) nm. Also, using line spectra, Sierra et al. (1994) recently proposed a two-chromophore model for HA in marine DOM with peaks at 310/440 and 340/500 nm. Finally, the commercial HA from Aldrich and Fluka also showed a peak at 480/540 nm. Such low-energy peaks are known to be more typical of terrestrial (lignin-derived) HA fluorescence.

It is necessary to remember that when such a mixture of peaks is added together as a composite, not all of the component peaks may be visible, and those that are may be shifted by the influence of the neighbouring peaks. For example, in Fig. 6 (extracted COM), a peak is clearly apparent with excitation of 390 nm, but the emission seems to be spread over the range 430–490 nm. This might be due to the combination of the edge of the 340/430-nm peak, and the actual peak at 390/490 nm. Furthermore, due to possible energy transfer processes, an incoming photon matching any observed excitation may result in an emitted photon that corresponds to any observed emission below that excitation. In principle, therefore, for every n fluorophores, we might expect to see n(n + 1)/2 peaks, although some of these would be minor.

In order to estimate the relative contributions of the different peaks to the total intensity distribution, some simulations were undertaken. It was found, for example, that the observed coral extract spectrum shown in Fig. 1a could be adequately simulated by an arithmetical sum of elliptical Gaussian peaks with the following wavelengths and relative intensities: 340/430 nm (100%); 390/490 nm (51%); 280/360 nm (28%); 280/490 nm (26%); and 440/540 nm (19%). The observed intensity distribution could not
be reproduced using a smaller number of peaks than this.

It was initially hoped that 3-D EEM fluorescence could provide fingerprint spectra, to identify the source of the HA in the corals by comparison with DOM from local seawater and soils. Because of the similarity of EEM spectra for HA samples from different sources, this proved unsuccessful, although this might be achieved with a more sophisticated technique which incorporates fluorescence lifetime measurement (e.g., McGown et al., 1995).

2.3. The tryptophan peak

It is, however, possible to distinguish the HA peaks from the peak attributed to tryptophan, probably present as part of larger protein molecules. Only three of the amino acids which make up proteins are fluorescent: these are the aromatic amino acids, phenyl alanine, tyrosine and tryptophan. The fluorescence emission spectrum of proteins arises predominantly from only one of these chromophores, tryptophan, since it receives energy transferred from phenyl alanine and tyrosine which have higher excitation energies (Lakowicz, 1983). The characteristic tryptophan excitation/emission peak occurs at 280/350 nm as shown in Fig. 1d. This peak was observed in many of our COM EEM spectra (Fig. 1a), and in our seawater EEM spectra it was higher (but narrower) than the HA peak (Fig. 1b). In the seawater it has been blue shifted to maximum emission at 320 nm, partly due to the spectral interference of the water Raman band, and perhaps also due to the influence of the seawater matrix, as observed by Determann et al. (1994), who also record this peak as being higher than the HA peak in seawater. Tryptophan is very strongly fluorescent, much more so than HA, weight for weight. By comparing the fluorescence of tryptophan solutions to seawater and coral extracts, we estimate that the concentration of tryptophan in the corals was in the range 0.2-2.0 ppm (mg/kg of dry coral) and ~0.01 ppm (mg/kg) in the seawater collected near the reef (within the range estimated by Determann et al., 1994).

Recent work on EEM spectra of DOM in seawater has concluded that the spectrum is a composite of two sources of fluorescence: humic acid, dominant in deeper water, and proteins, dominant in surface water of the open ocean (Coble et al., 1993; Mopper and Schultz, 1993; Determann et al., 1994; Sierra et al., 1994). It has been suggested that the protein fluorescence is directly attributable to planktonic activity in surface waters, where the humic acid fluorescence may be bleached by sunlight. Since such biological activity combined with sunlight are also the conditions around the coral reef, it is not surprising that our coral extract samples also contain a small tryptophan-like peak.

We also observed that the relative intensity of this peak was (compared to the others) non-conservative in the various stages of the extraction process. It also showed a different response compared to the HA peaks, with respect to concentration and pH changes. These differences, which will be expanded on later, are further evidence for this peak being attributable to tryptophan rather than HA.

2.4. Concentration dependence for Aldrich HA, and effect of extraction

Although most of the 3-D EEM plots were composed of the same set of peaks and were thus intrinsically similar, the superficial appearances of the spectra could be strikingly different as a result of variations in the relative intensities of the individual peaks. We postulate that these variations are largely due to inner-filter effects, quenching and energy transfer processes, which are dependent on the concentration of the sample. Since this effect was most pronounced for the Aldrich HA, this will be dealt with first.

The EEM spectra in Fig. 2a and b show the effect of increasing the concentration of a solution of Aldrich HA from 10 to 100 ppm. At first sight there appears to have been a dramatic shift in the position of the main excitation/emission peak from 335/450 to 480/540 nm. On closer inspection, it can be seen that there are actually several shoulders along the diagonal of each spectrum, corresponding to the various characteristic HA excitation/emission peaks identified above. The positions of the peaks themselves have not shifted much, but their relative intensities have changed substantially, such that longer wavelengths are favoured at higher concentration.

Fig. 2c shows the result of putting the 100-ppm Aldrich HA through the extraction procedure, as for
COM. The extract dilution was adjusted such that the concentration would still be 100 ppm if all the HA had been extracted. The extract exhibited a much greater fluorescence intensity than the original solution. Whereas the intensity of the peak at 470/540 nm was not changed significantly by the extraction process, for the 340/450-nm peak there was an eight-fold increase in intensity. The solution washed through the resin in the acid stage of extraction, adjusted to the same pH and dilution, had an absorbance at 300 nm which was about twice that of the extract (it was also visibly more coloured) whereas its fluorescence intensity at the peak wavelength was only one-fifth. This suggests that a large quantity of non-fluorescent material which absorbed strongly in the short excitation wavelength region of HA EEM spectrum had been removed by the extraction. It has been reported that Aldrich HA contains ~8.5% inorganic impurities (Susie et al., 1991) which may be partially responsible for this.

So it is probable that inner-filter effects due to the presence of strongly absorbing impurities in the Aldrich HA were a major cause of the observed concentration effect (Fig. 2a and b). We should not dismiss the possibility that a similar effect due to non-fluorescent impurities might be found in some corals, in which case any observed difference in colour between the bands might be simply explained by concentration differences. This may be more relevant for the Queensland corals where the fluorescent material is clearly of terrestrial origin (Susic et al., 1991), than is to the Thai corals investigated here, in which it will be seen that the fluorescence of the COM resembles that of marine DOM more closely than that of Aldrich HA, and the colour difference between bands is much less marked.

2.5. Effect of concentration on coral extract fluorescence

The concentration dependence of the fluorescence of a COM extract was investigated by recording EEM spectra over a wide range of concentrations. The results are summarised in Fig. 3 in which fluorescence intensity is plotted as a function of concentration for various excitation/emission positions in the EEM spectra. It can be seen that there is a non-linear dependence of fluorescence on concentra-

![Image](image.png)

**Fig. 3.** The dependence of fluorescence intensity on concentration for a coral extract. The various lines refer to different excitation/emission wavelengths. The two triangles (open and filled) are either side of the 'tryptophan' peak. The diamonds and squares at the four sides of the central peak. The open circles show the height (multiplied by ten) of the water Raman peak near 400 nm, measured above the fluorescence baseline. The half-height width of the Raman peak was ~7 nm.

Fig. 3 shows the results of Fig. 2 in which the intensity, and particularly, that the intensity curves for different peaks do not run parallel to one another, i.e. the relative intensities are changing. In the case of the peak attributed to protein fluorescence, the intensity eventually begins to decrease as COM concentration and HA fluorescence continues to rise. This behaviour is consistent with the occurrence of energy transfer (radiative and/or non-radiative) from the high excitation energy tryptophan chromophore to lower-energy HA species.

The usual response to the problem of quenching effects is to dilute the sample until these effects are judged to be insignificant (e.g., Willey and Atkinson, 1982). This approach seems inappropriate in the present case since we are trying to explain the visible effects of HA fluorescent banding in real corals, where concentrations are high, as estimated below. Moreover, the relative intensity of the tryptophan
peak, compared to HA, rises with dilution such that it may come to dominate the spectra if the COM extract is diluted to make the concentration similar to that of seawater DOM.

We should also recall that inner-filter effects, quenching and energy transfer processes may have a significant influence on fluorescence properties which are \textit{intrinsic} to the humic acid molecule. Humic acids are macromolecular systems, so that quenching processes and radiative and non-radiative energy transfer may occur as a result of interactions between different chromophores attached to the same macromolecular aggregate, i.e. they may be effectively \textit{intramolecular} processes. Moreover, the large inner-filter effect as discussed above, may also be intrinsic to the macromolecule. Such intramolecular quenching and inner-filter effects would persist at even the lowest sample concentrations.

It was possible to make an order-of-magnitude estimate of the original concentration of HA in the solid corals and seawater samples, by comparison of the fluorescence intensity with that of Aldrich HA whose concentration was known (allowing for the impurity mentioned above). This calculation suggests that HA is \(\sim 100\) ppm in corals (0.1 g/kg of dry coral based on the amount originally dissolved). This is about twice the amount found by Susic et al. (1991), and \(\sim 5\) ppm (5 mg/kg) in seawater taken from close to the coral reef. Although this is higher than a typical value of \(\sim 1\) ppm, biologically active coastal water might be expected to have more HA than average. However, these figures must be treated with caution since unlike HA types are being compared.

\subsection*{2.6. Effect of pH on COM and HA fluorescence}

As a general rule, the pH of all coral extracts was adjusted to 7.8 (\(\pm 0.1\)) before recording fluorescence spectra. However, in the hope of distinguishing the chemical behaviour of the various fluorophores in HA, the EEM spectra of a coral extract and of Aldrich HA extract were recorded over a range of pH. The pH was adjusted using only HCl and/or NH\(_3\) to avoid possible fluorescent interference from buffers. The results are shown in Fig. 4, where the relative intensities of characteristic EEM peaks are plotted as a function of pH for both samples. The

![Figure 4](image-url)

\textbf{Fig. 4.} The dependence of fluorescence intensity on pH for: a typical coral extract and for Aldrich HA extract. Note the different behaviour of the peak attributed to tryptophan (see text).
non-linear increase of intensity of the characteristic HA peaks with increasing pH is similar to that found in previous work on DOM in seawater (Laane, 1982). It can be seen that the intensity of the 280/360-nm peak, attributed to tryptophan, shows a pH dependence which is different from that of the HA peaks. The quantum yield of tryptophan fluorescence is known to be strongly pH-dependent between pH 6 and pH 10, the ionisation region of the α-amine group (Edelhoch et al., 1967). The increase in fluorescence intensity with increasing pH which occurs in this pH range correlates with removal of a proton from the amino group (–NH₃⁺ → –NH₂).

2.7. Other small fluorescent molecules

Proteins are not the only fluorescent metabolic products of microorganisms which might be found in seawater. Pteridines and flavines are products of benthic reef organisms, and most of these are strongly fluorescent (Momzikoff, 1969; Chen and Bada, 1992). Although previous research has shown that these are only present at picomolar concentrations in seawater (Dunlop and Susie, 1985), they may contribute up to 13% of total extractable fluorescence (Coble and Gagosian, 1991) and it is possible that in the corals themselves much higher concentrations could be found. Not only do corals concentrate such molecules and the organisms that produce them, they also provide better protection from sunlight which might destroy their fluorescence in open seawater (Kouassi and Zika, 1990). We therefore looked briefly at the 3-D EEM spectra of aqueous solutions of two such molecules: isoxanthopterin and riboflavin, shown in Figs. 5 and 1c, respectively. Also, to investigate the quenching and energy-transfer interaction between these small molecules and COM or tryptophan, mixtures were made and quenching spectra calculated from these as explained below. We are unable to show all of these here, due to space limitation, but Fig. 5 is given as an example of the technique, showing the quenching of isoxanthopterin by COM extract.

The effects of quenching can be revealed by comparing the EEM spectrum of a real mixture of solutions of two different chromophores, [A + B], with the spectrum obtained by arithmetic addition of the EEM spectra of the two individual solutions, [A] and [B]. Subtraction of these spectra gives the EEM spectrum of the quenched fluorescence, as follows:

\[
\text{EEM(quinched fluorescence)} = \text{EEM}[A] + \text{EEM}[B] - \text{EEM}[A + B]
\]

The concentrations of A and B are chosen such that their initial fluorescence intensities are similar, and these concentrations must be the same for each component in the mixture as they were in the pure solutions. It is assumed that A and B do not react chemically.

The EEM spectrum of individual solutions of isoxanthopterin and of a COM extract are shown in
Fig. 5a and b, respectively. The arithmetic sum of these spectra (Fig. 5c) is shown in comparison with the EEM spectrum of the real mixture of the isoxanthopterin and coral extract solutions (Fig. 5d). The difference spectrum, 5c – 5d, shown in Fig. 5e reveals the isoxanthopterin fluorescence which is quenched by the HA in the mixture.

It is not surprising that this occurs, since the main peak of isoxanthopterin, at 340/410 nm, is very close to that of the COM extract, yet the total concentration of chromophores is probably much higher for COM than for isoxanthopterin, considering the low quantum efficiency of HA. So this demonstrates that even if isoxanthopterin were present in the corals, its fluorescence would be masked by that of HA. However, we also observed using EEM spectroscopy, that when the isoxanthopterin solution was put through the extraction procedure it was not efficiently adsorbed onto the resin; most was washed straight through. So for this reason also, it is unlikely that isoxanthopterin could be responsible for much of the coral extract fluorescence.

The fluorescence of riboflavin (Fig. 1c), on the other hand, lies mostly at and beyond the low-energy extreme of the COM extract spectra, and we found that the HA signal could be totally removed by very high concentrations of riboflavin. But when the riboflavin concentration was such that its fluorescence intensity was of the same order of magnitude to that of the other species with which it is mixed, we found that riboflavin neither affected, nor was affected by any of the other substances.

Tryptophan fluorescence was quenched by both isoxanthopterin, and HA and COM extracts (the latter is also apparent in Fig. 3). This is to be expected, since the emission spectrum of tryptophan overlaps well with the excitation spectrum of both isoxanthopterin and HA.

Generally, these results are consistent with those that we would predict considering energy transfer processes, both radiative and non-radiative, as have been outlined earlier. HA in particular is known to quench the fluorescence of smaller molecules (Puchalski et al., 1992), and so although the examples investigated here represent only two of many small fluorescent molecules, it is likely that other such molecules would also interact with HA in a similar way.

2.8. A comparison of bright- and dull-band coral extracts

Typical results obtained in a preliminary comparative study of the EEM spectra of extracts from bright
and dull coral bands are shown in Fig. 6. It can be seen that the structure of the EEM spectra (i.e. peak positions and relative intensities) of the extracts of the bright bands and dull bands of the same coral are essentially identical. Although saturation of the exchange column initially prevented an accurate quantitative comparison between bright- and dull-band COM extracts, nevertheless subsequent experiments have shown that when the exchange column is omitted from the extraction procedure, bright-band extracts show more intense fluorescence than dull-band extracts, yet both bands still exhibit essentially identical EEM spectral structure. This indicates that there is not an intrinsic difference in the nature of the fluorescent materials present in the different bands. These results suggest that fluorescent banding in *Porites lutea* in this location may arise from the presence of different concentrations of fluorescent organic molecules of a similar nature, rather than the presence of organic materials of a different nature, from different sources.

2.9. EEM spectra of solid corals

The EEM spectra obtained from solid corals were found to be similar, but not identical, to those obtained for extract solutions. However, it was not possible to compare quantitatively the fluorescence from two different samples or two bands in the same sample. This is because the overall intensity of the measured fluorescence was extremely sensitive to the angle of the exposed face of the coral relative to

![EEM spectrum of solid coral](image)

*Fig. 7. EEM spectrum of solid coral. Note, besides the fluorescence peaks, the diagonal scattering line where emission \( \lambda \) is \( \sim 100 \) nm greater than excitation \( \lambda \), and the absorption dip at excitation \( \lambda = 300 \) nm. A contour interval is given in RU nm\(^{-1}\) for comparison with the solution spectra, but the optical pathway is different here: the light is scattered off a dry solid coral surface, with no cuvette.*
the excitation beam and the emission slit, so that moving the coral resulted in loss of comparability. A variation in rugosity of the cut coral surface may be partially responsible (Bak and Laane, 1987). Our preliminary results show that the solid corals, like the extracts, have EEM spectra with similar structure for both bands. The qualitative and quantitative comparison of bright and dull bands is currently being further investigated. Eventually, for this purpose we hope to connect an automated EEM fluorimeter system to a fiber-optic sensor [as used originally by Boto and Isdale (1985), and recently by Milne and Swart (1994)].

A typical spectrum is shown in Fig. 7. It exhibits the same characteristic HA peaks as were observed in the extract spectra, although with different relative intensities. At $\lambda_{ex} = 300$ nm a strong absorption (a dip extending across all fluorescence wavelengths) with a half-height width of $\sim 20$ nm can also be seen, presumably due to absorption of the exciting light by the aragonite matrix. This absorption is sufficiently close to the 340-nm HA excitation peak to reduce its intensity, also having the effect of shifting the maximum to 350 nm, as observed. On the other side of the absorption dip, ‘‘fluorescence’’ increases steadily towards high energy, which is a general feature of solid spectroscopy and of little interest analytically.

The fluorescence peaks should also be distinguished from another interesting feature of Fig. 7, which is a ridge running diagonally, similar in width ($\sim 10$ nm at base) to that from water Raman scattering. However, the solid coral was dry and the offset of this ridge ($\Delta \lambda$) from the ex = em line is greater than observed for water Raman scattering. We suggest that this feature is due to Raman-like scattering by the aragonite (CaCO$_3$) matrix of the coral skeleton.

Over a number of solid coral spectra, the HA excitation/emission peak at 390/490 nm was, on average, $\sim 30\%$ more intense than the other prominent peak at 350/440 nm. The latter peak is slightly shifted in excitation wavelength and reduced in relative intensity compared with coral extracts: in extract spectra it occurs at 340/440 nm and is generally the most intense HA peak. There are two factors which may account for these differences. Firstly, the absorption already noted reduces the exciting light available at 340 nm. Secondly, concentration and geometric effects in the solid corals may enhance energy transfer processes, leading to a shift in intensity to longer wavelengths.

3. Conclusions

The consistency of the characteristic peak positions between EEM spectra of HA samples of coral extracts, seawater, solid corals, and commercial HA, has been found to be much greater than might be expected from the literature on line spectra. This demonstrates the advantage of the EEM technique in revealing the complete photophysical system of the multi-chromophore macromolecular humic acid, in the presence also of proteins and possibly other small molecules, and alongside scattering processes which can be clearly distinguished by this method. By using such pictures, we could also observe the effect of concentration changes on the relative intensities of the various peaks, and thereby discern possible pathways for energy transfer and quenching processes. We suggest these processes should be given more attention when considering apparent differences in colour between fluorescent bands in corals.

Four characteristic HA EEM spectral peaks were identified in all of the natural samples studied, occurring at the following excitation/emission wavelengths: 280/350, 310/430, 340/450 and 390/490 nm. The first of these corresponds to the emission from tryptophan in proteins, and the central peaks are already well-documented in the literature as typical of HA in marine DOM. The coral and seawater samples did not show the longer-wavelength peak which we found in Aldrich and Fluka HA at 460/540 nm. This, combined with the presence of the protein-fluorescence peak, suggests that the fluorescence in the Thai corals is more typical of marine DOM than of terrestrial lignin-derived HA. The protein fluorescence led us to consider the possibility that other small biomolecules may also contribute to fluorescence in corals. Experiments with a few such molecules suggested that, even if present, their fluorescence would be strongly quenched by HA.

Since we also found no apparent spectral difference between the bright- and dull-band extracts, we conclude that there is not an intrinsic difference in
the nature of the fluorescent material in different bands of these corals. An alternative explanation is therefore required to explain any observed colour difference between the bands, which is anyway much less marked here than in the Queensland corals. Since the EEM spectra have demonstrated that changes in concentration of HA can dramatically alter the intensity distribution of the fluorescence spectra (and hence the colour that meets the eye), this could be one possible explanation of yellow-blue (bright–dull) visible banding. However, we recognize that other physical factors such as porosity of the coral skeleton may also be important.

A preliminary investigation of solid coral samples also showed EEM spectra which had similar structure for both bright and dull bands. The relative intensities of the peaks in the solid coral spectra were different from those in the extract spectra, perhaps indicating the occurrence of inner-filter and/or quenching effects in the solid which are not reproduced in the extract. The EEM technique is a particularly useful tool for following such energy-transfer processes, and should contribute further to understanding the factors which give rise to the fluorescence banding phenomenon in the solid corals.

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References


