

## Dissolved protein fluorescence in two Maine estuaries

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### Abstract

Fluorescence of dissolved proteinaceous materials was examined in two estuaries differing primarily in river input. Low-wavelength excitation (220–230 nm) was found to be more useful than the high-wavelength excitation (280 nm) usually reported in the literature. Levels of fluorescence in estuarine samples were of the order to be expected from the probable levels of dissolved amino acids. However, quantitation of protein levels by fluorescence, even in relative terms, is virtually impossible, due to positive interferences among the two amino acid peaks and humic material and negative interference by various types of quenching. Salinity has little or no effect on quantum yield. Proteinaceous fluorescence along estuarine transects was noisy, with some positive correlations with chlorophyll levels. Noisy data are consistent with the short lifetimes of proteins in seawater. Sediments appeared to provide a source of proteinaceous fluorescence. Seaward samples tended to show higher tyrosine peaks while upstream samples were richer in tryptophan emission. © 1999 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

Dissolved organic matter in the oceans has been studied primarily from the standpoint of its elemental components (e.g., DOC) and its molecular constituents, though study of the latter has been inhibited by low concentrations. Spectral techniques for the detection of total organic matter or its various constituents have received some attention. Fluorescence is one such approach, with most work focusing on the principal humic-type fluorophores (e.g., Willey, 1984; Hayase et al., 1987; Laane and Kramer, 1990; de Souza Sierra et al., 1994). However, pro-

teinaceous fluorescence has frequently been detected (e.g., Mopper and Schultz, 1993; Coble, 1996), which may provide a means to ascertain the dynamics of this important component of biological production. Fluorescence provides a potential for rapid and sensitive analysis of dissolved protein, with a much greater ease of use than more rigorous analytical chemical methods.

Fluorescence studies in open ocean environments have shown evidence for elevated dissolved protein in regions with relatively high levels of biological activity (photic zones) (Mopper and Schultz, 1993). This dissolved protein presumably results from exudation and spillage of cell contents during the normal processes of cell growth and grazing. Because estuaries are typically zones of enhanced biological pro-

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duction, they should be excellent areas in which to observe dissolved protein using this approach. In connection with a larger project examining production dynamics of three Maine estuaries (Mayer et al., in preparation), we examined fluorescence of dissolved materials. Our intention has been to identify the important proteinaceous fluorophores and assess the distribution and significance of their fluorescence in the context of other processes occurring in these systems.

## 2. Materials and methods

Water samples were collected in two Maine estuaries—the Kennebec and Damariscotta—in central coastal Maine, USA. These two estuaries are close to one another and similar in morphology. They differ in the amount of river input they receive—with annual averages on the order of  $10^2 \text{ m}^3 \text{ s}^{-1}$  for the Kennebec and  $10^0 \text{ m}^3 \text{ s}^{-1}$  for the Damariscotta. Several cruises were carried out in the summer of 1994. Water samples were obtained using Niskin bottles, and prefiltered through 200–225  $\mu\text{m}$  Nitex mesh to remove zooplankton before further filtration. Samples for fluorescence measurement were then filtered through a 0.45  $\mu\text{m}$  Millipore filter, and stored frozen until analysis.

Fluorescence measurements were made by making emission scans from 200 to 500 nm, at excitation wavelengths every 5 nm from 200 to 400 nm. We used a Hitachi F-4500 spectrofluorometer, with normal operating conditions of 5 nm slit widths (excitation and emission), a PMT voltage of 700 V and a response time of 0.1 s. The spectra were corrected for instrumental bias according to the manufacturer's instructions. Contour plots of emission intensity as a function of excitation and emission wavelength were constructed using the instrumental software and are presented without further data manipulation. Fluorescence intensities are reported as fluorescence units (FU) provided by the instrument.

Mixing experiments were conducted to test for the effect of salinity on proteinaceous fluorescence. Solutions of  $0.2 \text{ mg l}^{-1}$  tryptophan,  $0.4 \text{ mg l}^{-1}$  tyrosine and  $2 \text{ mg l}^{-1}$  bovine serum albumin (BSA), each in  $1 \text{ mM NaHCO}_3$  at pH 8, were added to UV-treated, artificial seawater to make mixtures of

varying salinity. A similar series was conducted using algal protein derived by filtering a culture of *Thalassiosira* spp., sonicating the filter, refiltering, and mixing the resultant solution with seawater.

For chlorophyll analysis, estuarine water samples were filtered through 47 mm GF/C filters, frozen and then analyzed using the standard fluorometric technique with an acidification step (Parsons et al., 1984).

## 3. Results and discussion

### 3.1. Fluorescence of amino acids and proteins in seawater

Of the three fluorophores present in proteins, only tryptophan and tyrosine have sufficient fluorescence intensity to be seen as distinct peaks. These fluorophores each have two excitation wavelengths in the UV-tryptophan at 215–220 nm and 275–280 nm and tyrosine at 220–225 nm and 275–280 nm, which can be seen in excitation–emission matrix (EEM) spectra of monomeric amino acids, pure proteins and natural samples (Fig. 1). We found the shorter wavelength excitation peaks to provide clearer evidence for the presence of these two amino acids in estuarine samples, for two reasons. First, the emission intensity ratio of the high-wavelength excitation peak to that of the low-wavelength excitation peak was lower for seawater samples than is the case for pure tyrosine, tryptophan, or a protein such as BSA. Second, the higher wavelength excitation maximum of tyrosine is, at low fluorescence intensities, obscured by the Raman scatter band (Coble et al., 1993). Such interference may be largely eliminated by spectral subtraction of the Raman spectrum of pure water (Coble, 1996), but even this correction is likely to lead to some error in the estimation of peak intensities of protein peaks that have much smaller intensities than the Raman band. Subsequent data in this paper will therefore use emission intensities deriving from the short excitation wavelength maxima for tyrosine and tryptophan.

The use of these low-wavelength peaks creates problems for standardization. Optimally one would use a standard with an excitation/emission wavelength peak near that of the fluorophore being mea-

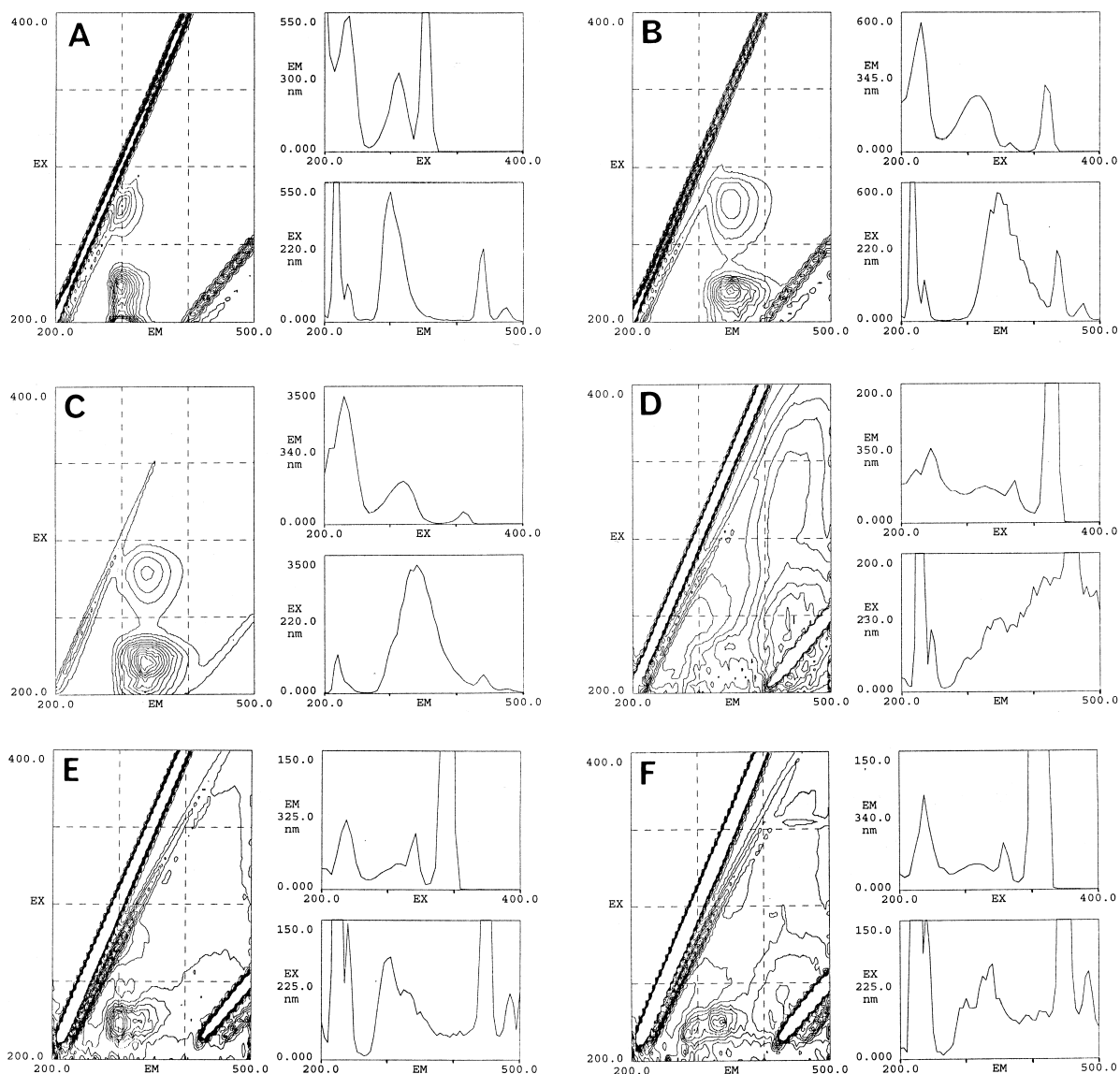


Fig. 1. Excitation/emission (EEM) plots of fluorescence intensity for (A) tyrosine; (B) tryptophan; (C) bovine serum albumen (BSA); (D) August Kennebec, Stn. 9; (E) June Damariscotta Stn. 7, a sample with relatively strong tyrosine fluorescence; (F) June Damariscotta Stn. 9, a sample with relatively strong tryptophan fluorescence. Raman bands in (A)–(D) are barely evident because the proteinaceous fluorescence is so strong. Each plot is accompanied by excitation (top) and emission (bottom) scans at the emission and excitation wavelengths, respectively, shown on the *y*-axis; except for tyrosine (1A) all scans cross the low-wavelength tryptophan peak. Scale on *y*-axis of scans is in FU.

sured. We rejected the use of standards with excitation/emission maxima well above the peaks we measured. Lamp performance degrades relatively rapidly at shorter wavelengths, so that secular changes in lamp intensity in the low UV would not necessarily be seen at the quinine sulfate wavelength.

The usual use of Raman band intensities at the analytical wavelength (e.g., Determann et al., 1994) is hindered because at excitation of 220–230 nm we find significantly lower Raman band intensities for many seawater samples relative to distilled water. These lowered values are related to an inner filter

effect in which both the excitation and emission light is partially absorbed by chromophores in the solution, and this filtering is thereby evident in samples with relatively high concentrations of dissolved organic chromophores. Raman bands at these wavelengths are also complicated by spectral overlap with the Rayleigh scatter band. To provide a means for comparison with our data, we have therefore selected a transect of the Raman band with excitation at 300 nm, a wavelength at which there is minimal overlap between the Raman and Rayleigh bands. The integrated Raman band intensity along an emission transect is 489 (nm FU) on our instrument.

Fluorescence at the shorter excitation wavelengths can be strongly affected by other materials. We have observed three sources of positive interference at these wavelengths. First, fluorescence from terrigenous humic material, usually evident in low-salinity samples from the Kennebec estuary, had broad peaks centering at ca. 240/440 nm and 310/420 nm (excitation/emission), corresponding to 'A' and 'M' peaks of Coble (1996), respectively. These peaks appear to have broad spectral slopes that can contribute some emission intensity at the tryptophan, and perhaps even the tyrosine, wavelengths. Fig. 1d shows a Kennebec sample with significant humic fluorescence, with both tyrosine and tryptophan peaks also evident. The humic interference on tryptophan fluorescence can be gauged from the saddle between the two tryptophan peaks on the excitation scan for this sample. This saddle represents a low-UV extension of the wide humic band to its right on the EEM plot, and is clearly wide enough in the excitation direction to underly both tryptophan peaks. Second, tyrosine and tryptophan each have emission bands that can interfere with one another. For example, tryptophan has a peak width (at half-height) of 50–60 nm, and has some fluorescence at the 305 nm emission wavelength of tyrosine. The wavelength of tryptophan's emission maximum can vary according to the polarity of its microenvironment (e.g., the interior or exterior of a globular protein) so that it is difficult to quantitate the extent of its interference at 305 nm. Third, we find background fluorescence deriving from very low UV excitation wavelengths, causing baselines to rise toward lower wavelength excitation energies with magnitudes often approaching those of the amino acid peaks (cf. the low

wavelength baseline of the excitation scan in Fig. 1d).

These varied interferences make it necessary to perform scans for positive identification of distinct peaks at the wavelengths of the proteinaceous fluorophores. Simple measurement of emission intensity at the appropriate wavelength may otherwise measure fluorescence deriving from non-proteinaceous fluorophores. Further, although distinct peaks may be found at the amino acid wavelengths, we have found it frequently impossible to correct for background fluorescence satisfactorily in cases where significant interference is present.

Salinity has no apparent effect on quantum yield. A dilution curve of BSA fluorescence versus salinity shows conservative behavior, indicating minimal suppression of fluorescence by seasalt (Fig. 2). Monomer amino acids and the algal protein also showed no quenching by seasalt in similar experiments. Both low and high wavelength excitation peaks behaved similarly in their response to salinity.

Even if emission intensities of amino acid fluorophores could be corrected for positive interferences, they are not likely to be good ordinal measures of protein concentration unless wide variations in intensity are found. There are many opportunities for energy transfers upon light absorption, such as quenching by adjacent amino acids or peptide bonds, with subsequent change in the quantum yield of fluorescence (Wolfbeis, 1985; Permyakov, 1993). Tyrosine has lower fluorescence quantum yield in peptides as compared with its monomer form (Lakowicz, 1983), due to intramolecular quenching.

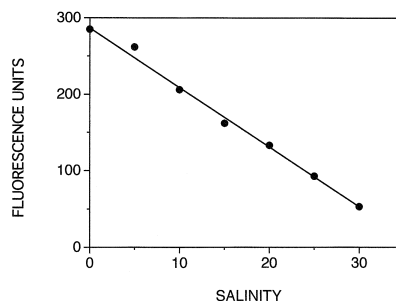


Fig. 2. Low-wavelength tryptophan fluorescence (FU) vs. salinity for BSA. Linear regression line is to help visualization and not to model the data.

Both the quantum yield and wavelength of tryptophan change as a function of polarity in its microenvironment or proximity to quenching groups such as histidine residues. For example, tryptophan buried within hydrophobic domains of proteins can have greater quantum yield than tryptophan exposed at the more polar, protein–solution interface. Exposure to solution can subject these fluorophores to external quenching agents such as transition metals. Using monomeric tryptophan as a standard for estimation of the tryptophan content of proteinaceous materials (e.g., Matthews et al., 1996) is probably not appropriate. In summary, quantitative measurement of protein fluorescence is not yet ready to be interpreted accurately in absolute or perhaps even ordinal terms.

### 3.2. Proteinaceous fluorescence along estuarine transects

Fluorescence of tyrosine and tryptophan in the estuarine samples ranged from undetectable, at which there was background fluorescence but no discernible peak in the spectra (typically with values of about 10 FU), to values as great as 160 FU (Fig. 3). The BSA solution used for mixing experiments had a concentration of  $2 \text{ mg l}^{-1}$ , which yielded a fluorescence intensity of 285 FU at the low-wavelength tryptophan peak. While the discussion above makes clear that no strong inference can be drawn regarding protein concentration, we can use the BSA fluorescence to estimate the order of magnitude concentration of dissolved amino acids in estuarine samples. Applying the calibration from the BSA solution, tryptophan fluorescence intensities in field samples of 50–150 FU imply total amino acid concentrations on the order of  $0.35\text{--}1.05 \text{ mg l}^{-1}$ . This range is not unreasonable, assuming total dissolved organic carbon in the Damariscotta estuary of  $5\text{--}10 \text{ mg l}^{-1}$  (Carlson and Mayer, 1983) with amino acids making up on the order of 1–16% of dissolved organic matter (Ittekkot, 1982; Zhang et al., 1992).

Occasionally the higher wavelength tryptophan peak (280/340 nm) could be seen, but tryptophan fluorescence was generally 2–4 times stronger at its lower excitation wavelength peak (230/340 nm). Furthermore, the ratio of high wavelength to low wavelength peak intensity in estuarine samples was

usually lower than for monomers or pure proteins (e.g., compare the excitation scans in Fig. 1f vs. b). This loss of the high wavelength peak suggests either selective bleaching or quenching. The higher wavelength peak may be similar to that recently observed in filtered samples in biologically enriched waters from the Arabian Sea (Coble et al., in press), and labelled peak 'N', though their emission maximum is at a higher value of 360–370 nm. The Raman peak obscured the higher wavelength tyrosine peak (at 280/305 nm) in our samples.

Higher values of protein fluorescence were typically found within the estuaries relative to the waters entering from the land or seaward ends (Fig. 3). Within the estuaries, there was only occasional correlation with chlorophyll, and this correlation was negative as often as positive. For example, significant ( $p < 0.05$ ) negative correlations were found between tryptophan fluorescence and chlorophyll in the Kennebec June and Damariscotta August transects, but positive correlations were found between both protein fluorophores and chlorophyll in the Damariscotta June transect. Relatively high values of proteinaceous fluorescence were sometimes found in areas that also contained high chlorophyll, though overall correlations between these two variables were not usually statistically significant. An example was high values of tyrosine fluorescence associated with elevated chlorophyll values (due to a bloom of *Skeletonema costatum* diatoms; Wong and Townsend, submitted) in the high salinity region in the Kennebec transect of August. These co-occurrences suggest either exudation or sloppy feeding as a source of dissolved proteinaceous material. This association is similar to previous observations of protein fluorescence associated with upper water column biological processes in open ocean vertical profiles (Mopper and Schultz, 1993), association with a plankton bloom (de Souza Sierra et al., 1994) and exudation by laboratory *Skeletonema costatum* cultures (Traganza, 1969).

Humic fluorescence showed excellent inverse correlations ( $p < 0.001$ ) with salinity in all transects (Fig. 3), as has been found previously (e.g., Willey, 1984). Humic fluorescence was stronger in the Kennebec than in the Damariscotta estuary, due to the higher river inflow and consequently lower salinities found in the former system. The humic fluorescence

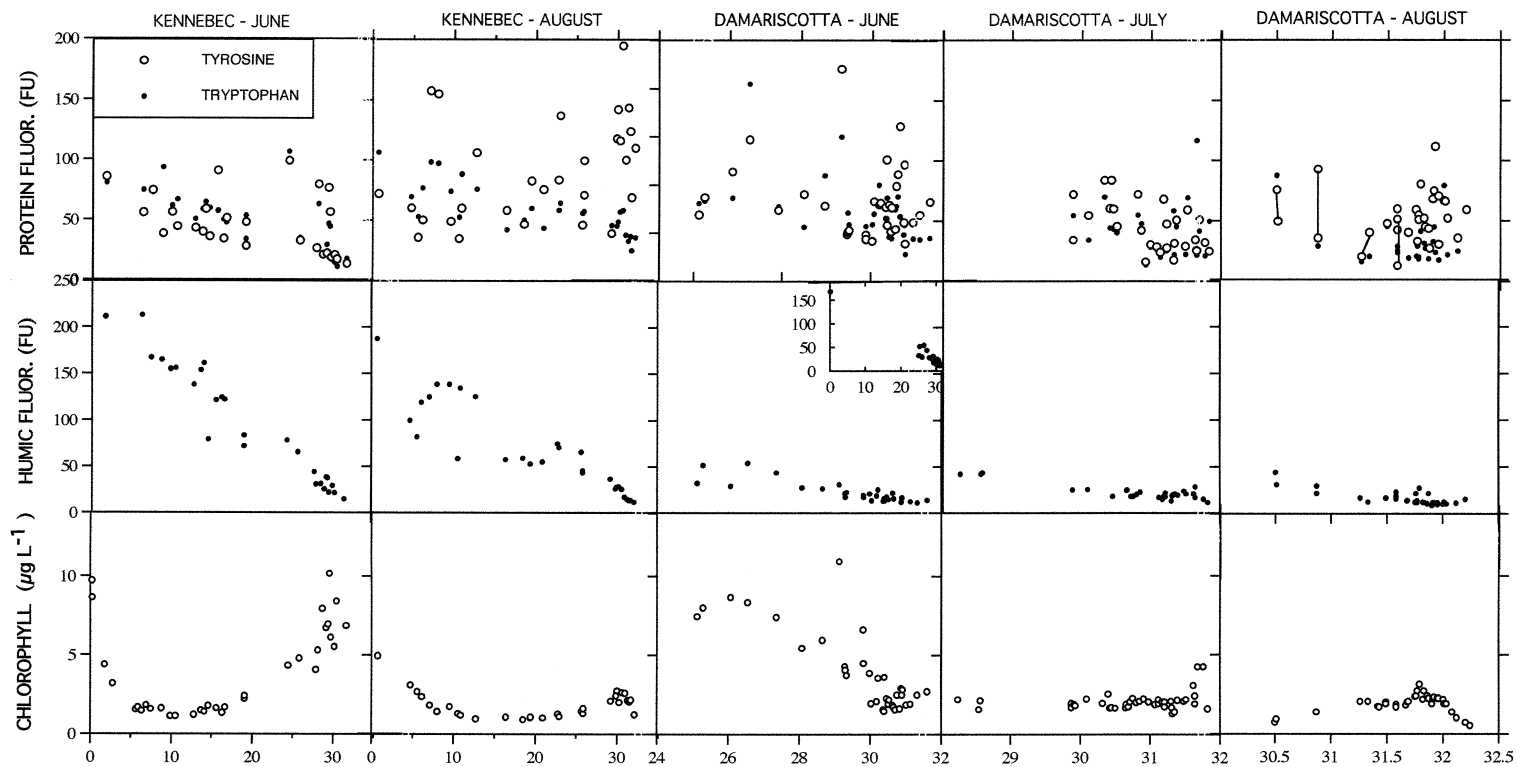


Fig. 3. Fluorescence of dissolved tyrosine, tryptophan, and humic materials, and particulate chlorophyll vs. salinity in the Damariscotta and Kennebec estuaries in summer, 1994. Inset in the June Damariscotta plot of humic fluorescence shows the entire salinity range, to show that the high salinity data extrapolate to the freshwater endmember value. Vertical lines in August Damariscotta plot connect air–water (bottom of line) to sediment–water interface (top of line) values at four upstream stations; water depths at these stations ranged 4–11 m.

in the Damariscotta estuary, albeit smaller, was also due to freshwater input (see inset in the Damariscotta June transect).

Humic fluorescence influenced protein fluorescence. In the June Kennebec transect, especially, a significant fraction of the tryptophan fluorescence appeared to be due to positive interference from the broad humic band centered at approximately 240/440 nm. Tryptophan fluorescence hence had a similar inverse relationship with salinity and its emission intensities showed significant positive correlation ( $p < 0.05$ ) with humic fluorescence in all but the Damariscotta July transect. More distinct tyrosine peaks were evident and were less well correlated with humic fluorescence, suggesting independent control on this fluorophore. However, tyrosine fluorescence intensities were strongly influenced by interference from fluorophores with very low excitation wavelengths. Variability in the shape of the broad humic band obviates using a constant peak shape to subtract out humic fluorescence. This interference emphasizes the danger in using fluorescence intensities at protein wavelengths indiscriminately, even when distinct peaks can be discerned.

There was some evidence that the sediments could act as strong sources for protein fluorophores. One striking example was an anchor station at which the bottom water sampled was contaminated with both anomalously high suspended particulate material and fluorescence of both tyrosine and tryptophan (the June Damariscotta sample with  $S = 29.1$ ). This input is consistent with the elevated levels of protein fluorescence found in sedimentary pore waters by Coble (1996). Further evidence for a sedimentary source is seen in the relatively shallow upstream stations of the Damariscotta in August. In this warmest month of the year, this area shows evidence for strong heterotrophic activity in the form of nutrient regeneration (unpublished data). The four shallow, upstream stations show considerably higher tyrosine fluorescence in the samples collected near the sediment–water interface than the corresponding samples from the air–water interface (Fig. 3). This enrichment was not significantly influenced by humic material. Three of the four stations showed the same trend for humic fluorescence, though tryptophan showed no such trend. Similar evidence for a sedimentary source was not evident in other profiles, suggesting that detec-

tion of protein fluorescence from the sediments requires a high source signal.

The scatter in the proteinaceous fluorescence, as compared with virtually all of the other variables measured (including humic material fluorescence) implies considerable analytical variance or environmental variability. Similar scatter for protein vs. humic fluorescence was observed in a smaller sampling of Columbia River estuary samples (Prahl and Coble, 1994). The large differences in protein fluorescence between the bottom and upper water column samples in the well-mixed Damariscotta estuary in August implies that this fluorescence persists for times less than the vertical mixing time of the water column. As the sampled water depths in this region of the estuary ranged from 4 to 11 m, and are essentially isohaline with depth, this persistence is likely no more than on the order of hours to days. Likewise, the presence of a distinct mid-estuarine minimum in protein fluorescence in this estuary implies persistence for less than the horizontal mixing times within the estuary, on the order of days. These estimates are maxima, and lifetimes may be much shorter.

This lack of persistence is consistent with studies of dissolved proteins in estuaries using radiotracer methods (Keil and Kirchman, 1993), typically showing turnover of protein spikes in several hours. Other studies, in systems with much larger time scales of mixing, have shown spottiness of protein fluorescence also consistent with short persistence (e.g., Determann et al., 1994, 1996). This ephemeral nature of protein fluorescence suggests that it is dominated by the relatively labile fraction of dissolved combined amino acids (DCAA) and not the more long-lived fractions that also appear to be present (Keil and Kirchman, 1993).

The short persistence of dissolved protein fluorescence, combined with the ease of measurement and great sensitivity, implies that it may be of greater use in high-frequency temporal sampling of dissolved protein dynamics than of controls manifested in spatial surveys. Again, however, care must be taken not to interpret intensities of fluorescence as indicators of protein concentration, at least until more development is made of this method.

Most samples showed fluorescence from both tyrosine and tryptophan, but there were salinity-depen-

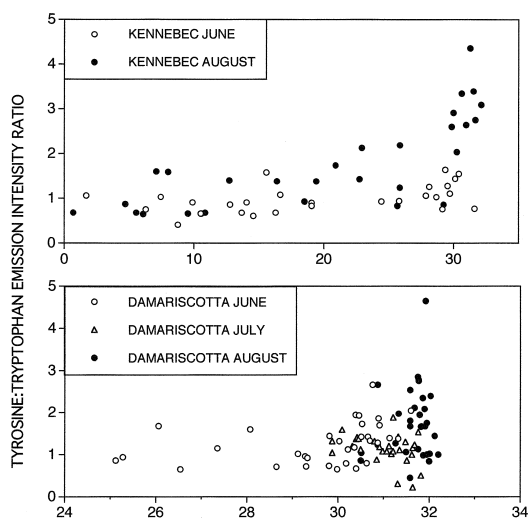


Fig. 4. Ratio of tyrosine to tryptophan fluorescence intensities versus salinity in the Damariscotta and Kennebec estuaries in summer, 1994.

dent trends in their intensity ratios (Fig. 4). The seaward samples in most months had higher tyrosine than tryptophan fluorescence (with the exception of the July Damariscotta data). The sudden increase at the extreme seaward end in the August Kennebec data set is similar to sudden spectral changes found in humic fluorescence at the seawater end of the Gironde estuary (de Souza Sierra et al., 1997). These authors explained their trend as resulting from mixture of a riverine endmember with a high concentration of fluorophores with a seawater endmember having a low concentration, so that the characteristics of the seawater endmember become evident only at very high salinities at which the oceanic component becomes dominant (cf. their Fig. 4). This explanation seems reasonable here as well.

Intact proteins containing both tyrosine and tryptophan residues are generally dominated by tryptophan fluorescence, due to both its higher quantum yield and energy transfers from tyrosine absorption to tryptophan emission (Permyakov, 1993). Tyrosine fluoresces well only in monomer form or in peptides in which no tryptophan is present, and its quantum yield is enhanced in denatured relative to folded proteins (Longworth, 1971). Hence, the presence of significant tyrosine fluorescence may be more indicative of presence of tyrosine monomers or pep-

tides containing tyrosine but not tryptophan, the latter being either free or combined in humic-like condensates. Samples with a high tryptophan and low tyrosine fluorescence, which were occasionally found, may indicate the dominance of intact dissolved proteins of normal composition — perhaps fresher material recently derived. The common increase in tyrosine:tryptophan intensity ratio toward the ocean endmember, on the other hand, may signal more degraded, dissolved peptide material coming from offshore. These peak ratios may be fertile ground for future exploration of protein decay.

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