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Resolving the variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using PARAFAC analysis

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Abstract

Excitation emission matrix fluorescence spectroscopy combined with PARAFAC analysis provides a fast and effective method of characterizing the fluorescent fraction of dissolved organic matter (DOM). Fluorescence measurements can be used as a tracer for quantitative and qualitative changes occurring in the DOM pool as a whole. An earlier study found that the fluorescence signal could be modeled by five fractions. This study presents an analysis on a considerably larger data set (>1,200 samples) resulting from a 1-yr sampling program in Horsens Estuary, Denmark. Eight fluorescent fractions were identified. Four biogenic terrestrial, two anthropogenic, and two protein-like fractions were identified. Analysis of covariation between the components identified source-specific fractions and the presence of common factors controlling the composition of terrestrial DOM exported from different catchments.

Dissolved organic matter (DOM) is present in all natural aquatic environments. Although DOM is one of the largest biologically active carbon reservoirs, a large fraction remains uncharacterized (Benner 2002). This results from the fact that DOM consists of a complex mixture of organic molecules that vary greatly in molecular weight, thus making it extremely difficult to characterize DOM using traditional chemical techniques. The heterogeneity of DOM is a product of its wide variety of sources and its reactivity to physical, chemical, and microbial degradation processes. The major sources of DOM are (1) dissolution of soil organic matter, (2) extracellular release of organic matter by algae, (3) release via grazing and excretion by zooplankton, (4) viral lysis of bacteria and algae cells, (5) degradation and exudation of macrophytes, and (6) release from sediments (Nagata 2000; Aitkenhead-Peterson et al. 2003; Bertilsson and Jones 2003).

In the past it has been difficult to trace changes in the structure of DOM because of the complexity and nonconservative behavior of the composition of DOM in natural environments. Fluorescence measurements can be used to characterize and trace DOM dynamics as a fraction of DOM fluoresces (e.g., McKnight et al. 2003; Stedmon et al. 2003). In early studies DOM fluorescence was used as a proxy for tracing the export of terrestrial organic matter via rivers into the coastal environment and the production of DOM in the open ocean (see Duursma [1974]) and references herein). More recent studies have shown how different components of the DOM pool can be distinguished using spectral measurements (Coble et al. 1990; Mopper and Schultz 1993; de Souza Sierra et al. 1994). Over the last 20 yr, fluorescence excitation emission matrix (EEM) spectroscopy has been successfully applied to identifying terrestrial, marine, and anthropogenic components of DOM (e.g., Coble 1996; Baker 2001). However, it is only recently that all the information contained within the EEM is being used for characterization of the fluorescent organic material. Multivariate data analysis techniques such as principal component analysis and parallel factor (PARAFAC) analysis have been shown to provide a considerable advantage over the traditional methods in interpreting the multidimensional nature of EEM data sets (Persson and Wedborg 2001; Søndergaard et al. 2003; Stedmon et al. 2003). Combining EEM spectroscopy and PARAFAC analysis, Stedmon et al. (2003) have shown how five distinct fluorophore groups, four allochthonous and one autochthonous, can be identified in marine and freshwater DOM. Although it is likely that there are more fluorophore groups present as a result of the complex nature of DOM and the wide range in sites sampled, the number of samples in the data set limited the analysis. This most likely resulted in the merger of two or more different fluorophore groups (with somewhat similar spectral properties) into one group. In this study, we apply the same techniques to a much larger data set (n = 1,276) from the same region, with the aim of characterizing the DOM fluorescence in a more detailed manner. Additionally, the data set now includes seasonal variability, which was lacking in the earlier study.

This approach provides insights into qualitative changes occurring in the DOM fraction in freshwater and estuarine environments, with the assumption that fluorescent DOM is a proxy for the DOM pool as a whole. PARAFAC analysis allows the identification of different, independently variable fluorescence groups and traces changes in their fluorescence intensities (i.e., concentrations) in the environment. These measurements can only be used as relative concentrations, unless the fluorescence of a specific compound is recognized as one of the components, which would then allow for the

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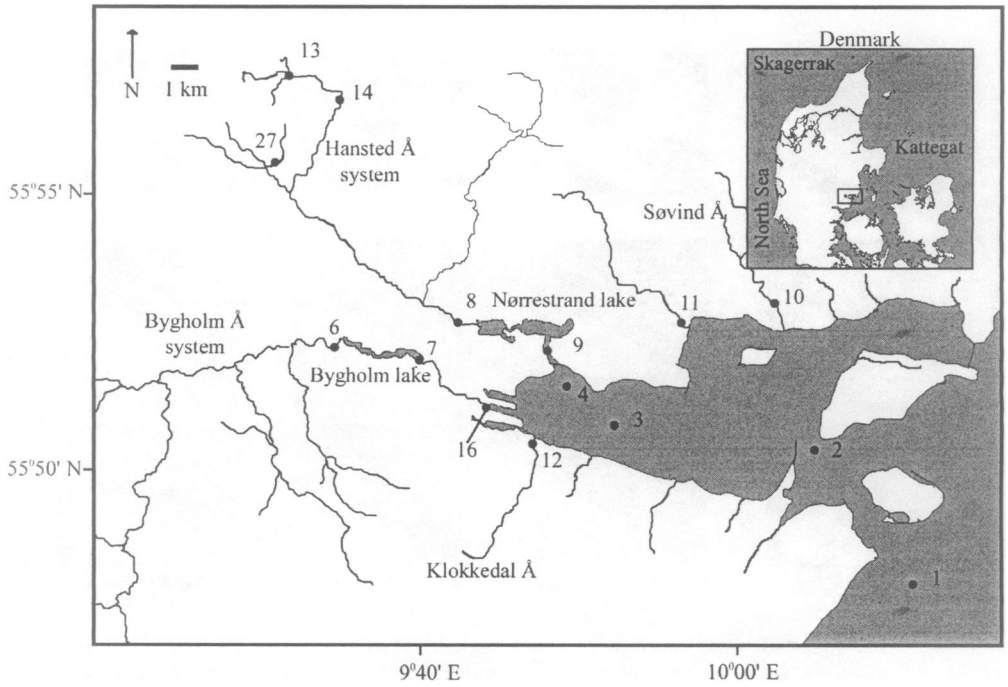

Fig. 1. Location of Horsens Estuary and the sampling stations.

calibration of the measurement to known standards. In this study we analyzed the covariation between the different fractions identified to hypothesize on the sources and fate of different fluorescent groups in a temperate estuary and its catchment. The limited expense and time needed for these measurements make this technique particularly favorable for tracing structural changes in the DOM pool. With increased knowledge of the production, transformation, and removal of different fractions of DOM and the processes responsible for these actions, we can gain a better understanding of the role of DOM in global biogeochemical cycles.

Methods

Sampling—During the period ranging from late August 2001 to early September 2002, samples were collected at 15 stations in Horsens Estuary and within its catchment (Fig. 1). The estuary is on the east coast of the Jutland Peninsula in Denmark. The catchment to the estuary is 517 km^2, and land use within the catchment is dominated by agriculture (75%), with isolated areas of forest (9%), wetlands (4%), and meadows (3%). Approximately 75% of the freshwater input to the estuary is channeled through Bygholm and Hansted stream systems at the western end of the estuary. On both of these systems there are lakes just before the streams flow into the estuary. Both lakes are shallow systems with water residence times of approximately a week. The estuary itself has a mean depth of 2.9 m and a surface area of 77.5 km^2. The marine end member is Kattegat water, with salinity ranging between 12 and 33, and this water originates from the mixing zone between the Baltic Sea and the North Sea. All the freshwater samples (except for that from Sta. 16) were taken from the banks of the streams. Sta. 16 was located at Horsens town wastewater treatment plant, and the samples were taken from the tertiary treated water being pumped into the estuary. The water from the treatment plant represents approximately 4% of the total freshwater input to the estuary.

Stations were initially sampled once a week; however, after the first 2 months, sampling frequency was reduced to twice a month. The data set includes the samples used in Stedmon et al. (2003) for development of the DOM fluorescence PARAFAC modeling technique. Samples were filtered through precombusted GF/F filters and then stored in a refrigerator at 4°C in amber glass 100-ml bottles until analysis in the laboratory. Before analysis, samples were allowed to warm up to room temperature. During the study, samples for dissolved organic carbon (DOC) were also taken and analyzed. The DOC methods are detailed in Stedmon et al. (2003).

Spectral measurements—Absorption spectra were recorded on a Shimadzu UV-2401PC spectrophotometer using 1-, 5-, and 10-cm quartz cuvettes and Milli-Q water as a reference. The technique was identical to that described in Stedmon et al. (2000). Spectra were measured from 240–700 nm at 0.5-nm increments. Absorption coefficients were calculated by dividing the optical density by the path length in meters and multiplying by 2.303.

Fluorescence measurements were made using a Varian Eclipse fluorescence spectrophotometer. An excitation-emission matrix was obtained by combining a series of emission scans made from 240–600 nm while exciting at wavelengths ranging between 240 and 450 nm (every 5 nm). The excitation and emission bandwidths were 5 nm. Because of the high concentration of DOM present in the samples, the EEMs were

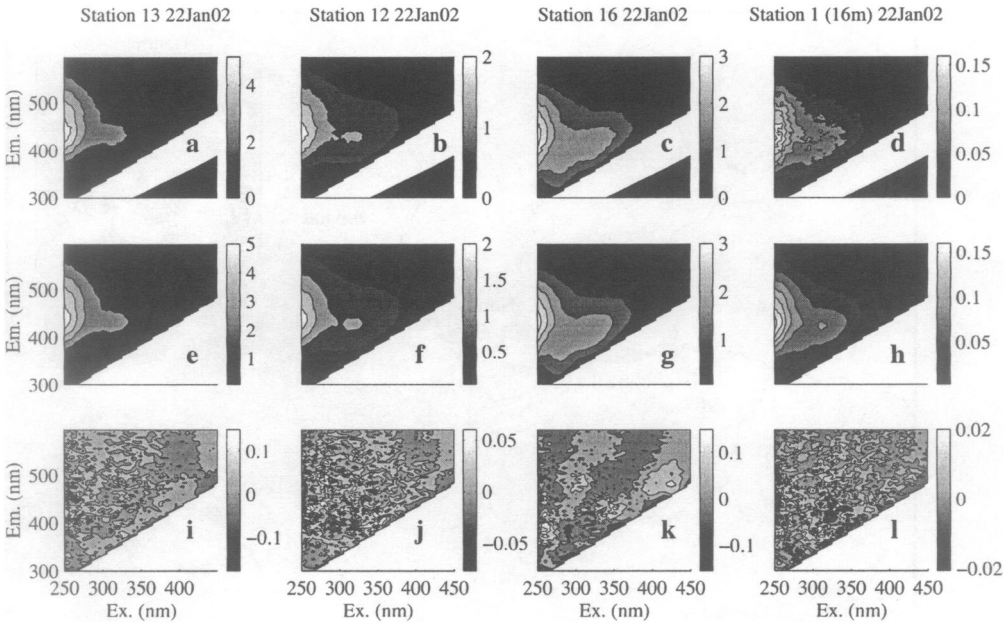

Fig. 2. Examples of (a–d) measured, (e–h) modeled, and (i–l) residual EEMs for four different DOM end members from the catchment. Note the differences in scale between samples and also between the measured and the residual EEMs. Fluorescence is in Raman units.

corrected for inner-filter effects using the measured absorption spectra (Mobed et al. 1996; McKnight et al. 2001). The EEMs were Raman calibrated and corrected for excitation and emission instrument biases using the techniques described in Stedmon et al. (2003). Before further analysis, excitation wavelengths below 250 nm and emission wavelengths below 300 nm were removed from the data set because of a deteriorating signal : noise ratio in this region. An example of the measured EEMs can be seen in Fig. 2a–d.

Modeling procedure—PARAFAC analysis was carried out in MATLAB using the "N-way toolbox for MATLAB" (Andersson and Bro 2000). The reader is referred to Bro (1997) and Stedmon et al. (2003) for a detailed introduction to PARAFAC analysis and its application to DOM fluorescence. All the EEMs measured from Horsens Estuary and catchment were combined into a data array. In addition to the sampling program, the EEMs from microbial and photodegradation experiments conducted using DOM from the catchment were included in the data set. The result of the degradation experiments will not be included in this article, but the data were included in order to facilitate the PARAFAC modeling, as it doubled the number of EEMs in the array and increased the variability of different fluorescence fractions. The data array consisted of 1,276 samples with 151 emission wavelengths and 41 excitation wavelengths. The data array was then split into two random halves of 638 EEMs each, a calibration data array and a validation array. The PARAFAC algorithm was then applied stepwise to both data arrays for 3–15 components. The appropriate number of components (the model rank) was determined by comparing the excitation and emission spectra of the components between the calibration and validation data arrays. Using this technique (split-half validation), we determined that up to eight components could be validated (Fig. 3a–h). When more than eight components were modeled, the results from the two data arrays differed, thereby revealing that the results were not robust. It was also clear from the spectra that the model then was beginning to attempt to model instrument noise.

Residual analysis also revealed that the eight-component model was adequate, explaining the majority of the variation in the EEMs. The fluorescence signal that remained in the residuals was nonsystematic and resembled the instrument noise. Examples of the measured, modeled, and residual (difference between measured and modeled) EEMs from four different environments are shown in Fig. 2. The limited amount of information left in the residual EEMs shows that the eight-component model is replicating the spectral characteristics of the measured EEMs. Figure 4 shows the integrated excitation and emission spectra of the sum of squared errors for five-, six-, seven-, and eight-component models. The presence of peaks in these spectra indicates regions of the EEMs in the data set that are less well described by the model. From examining the integrated excitation spectra it is clear that increasing the number of components from five to seven considerably improved the error (Fig. 4a). The increase from seven to eight components provided only a slight increase in fit, raising the question of whether an eight-component model is necessary for this data set. However, from examining the integrated emission spectra (Fig. 4b) it is clear that the eighth component considerably improves the model's ability to replicate fluorescence below 330 nm.

The shapes of the integrated sum of squares error spectra for the eight-component model reveal that there is some fluorescence in the measured EEMs that is not being explained by the model. The unexplained fluorescence is excited in the UVC and B region and has a broad fluorescence band peaking around 425 nm (Fig. 4a,b). However, normalizing the

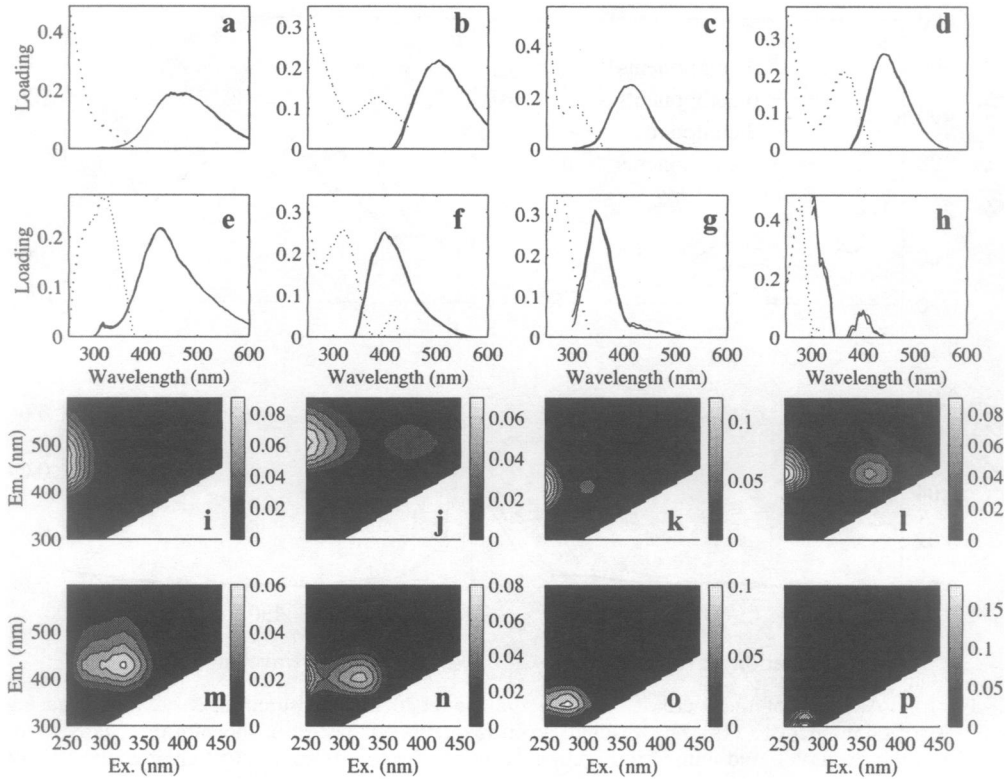

Fig. 3. The validation of the eight-component model and the spectral characteristics of each component. Graphs (a–h) show the excitation (dotted line) and emission (solid line) loadings for each component, obtained from two independent PARAFAC models on random halves of the data array. The fact that they overlap validates the eight-component model. The contour plots (i–p) show the spectral characteristics of each of the components 1–8, respectively.

spectra to the sum of squares of the measured spectra reveals that the residual fluorescence from the eight-component model was minimal with respect to the measured signal at these wavelengths. The residual excitation below 400 nm constitutes less than 0.2% of the measured signal (Fig. 4c). The residual emission, peaking at 425 nm, was also found to be minimal with respect to the measured signal (Fig. 4d). In conclusion, the eight-component model appears to adequately replicate the variability in the DOM fluorescence in these samples. The systematic peaks seen in the residuals are due to fractions with such a low fluorescence that the PARAFAC modeling was unable to resolve them.

Results

EEM component characteristics—Visual analysis of the components identified reveals that they resemble fluorescence characteristics of organic fluorophores, with multiple excitation maxima and single emission maxima (Fig. 3). However, because of the complex nature of DOM, it is unlikely that each component represents a specific fluorophore; rather, it is more likely that each component represents a group of fluorophores with very similar fluorescence characteristics and variability. For example, the presence of shoulders on the emission spectrum of component 8 indicates that this component represents a mixture of fluorophores that the model was not able to separate because of the fact that they appeared to co-vary within the data set (Fig. 3h). Stedmon et al. (2003) identified five components from a data set of only 90 samples from the same area. As the data set used in this study is much larger (leading to more variability), the PARAFAC modeling exercise was able to further decompose the fluorescence signal.

Comparison of the spectral characteristics of the components with those found in the earlier study reveals many similarities (Table 1). Components 1 and 3 from this study had very similar fluorescence characteristics to two UVC humic fractions identified in Stedmon et al. (2003). The combined fluorescence characteristics of components 2 and 4 and the combined fluorescence of 5 and 6 resembled the two UVA humic fractions previously identified (Table 1). Lochmuller and Saavedra (1986) found that a soil fulvic acid extract had an excitation maximum at 390 nm and an emission maximum at 509 nm. These characteristics are very similar to those found for component 2 in this study, so it is likely that this component represents terrestrially derived fulvic acid. Component 7 had similar fluorescence characteristics to the UVB protein-like fluorescence in Stedmon et al. (2003) and resembles the tryptophan-like fluorescence detected in many different aquatic environments (Mopper and Schultz 1993; Coble 1996; Yamashita and Tanoue 2003). Component 8 had similar characteristics to the previously reported tyrosine-like fluorescence peak (Coble et al. 1990; Yamashita and Tanoue 2003).

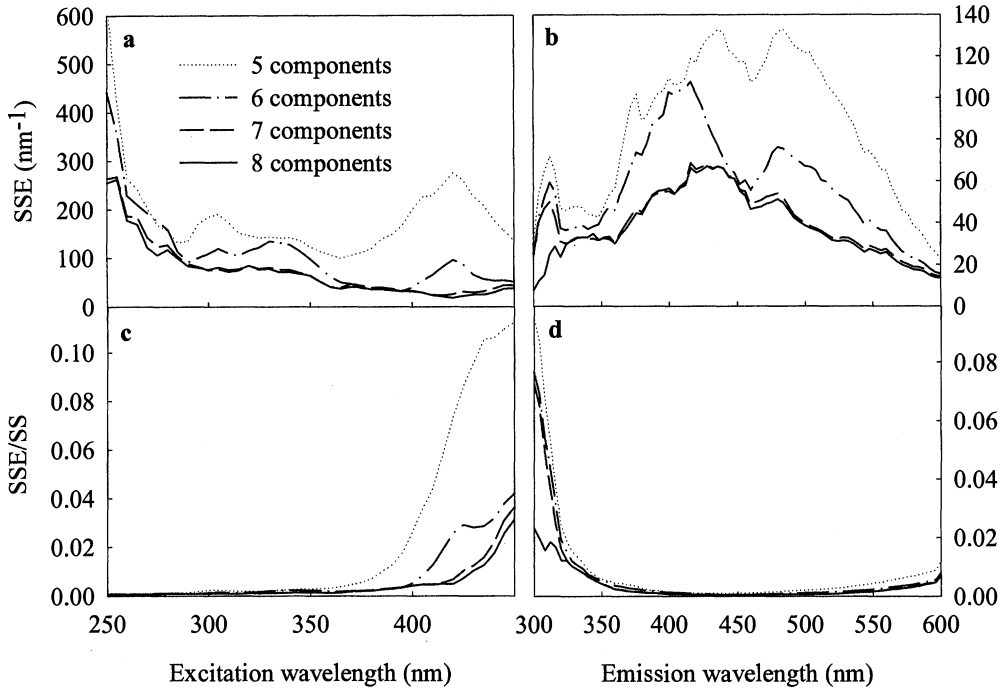

Fig. 4. Analysis of the increase in fit of the model to the measured EEMs with increasing number of components. (a, b) The absolute error as integrated spectra of the sum of squared error (SSE) in the excitation and emission direction, respectively. (c, d) The relative error as the sum of squared error divided by the total sum of squares (SSE/SS). The different lines represent different numbers of components.

Direct comparisons of components 1 through 6 with the results from earlier studies of DOM fluorescence are difficult since the fluorescence peaks identified in the past (e.g., A, C, and M; Coble 1996) have been located visually from the measured EEMs and their spectral shapes and positions have varied slightly depending on the type of DOM and the environment from which it was sampled. However, by comparing the overall shapes of the EEMs measured (Fig. 2a–d) with EEMs from the literature, one can see that the fluorescence of DOM from the Horsens catchments resembles that from other marine, freshwater, and wastewater environments (Coble 1996; Baker 2001; McKnight et al. 2003). There is a characteristic peak fluorescence between 400 and 500 nm, from excitation at 250 nm (often referred to as the A peak). Additionally, there is a broad shoulder around excitation 350 nm and emission 450 nm, which is commonly referred to as the C region. Similarities with the data from the literature indicate that the same eight underlying components identified from this data set could also be present in DOM from a wide range of different environments.

The chemical structure of the fluorophores present is unknown; therefore, the scores from the PARAFAC modeling cannot be calibrated to actual concentrations. From here onward, when the concentration of a component is mentioned, we are referring to F_{max}, the maximum fluorescence (in both excitation and emission) in Raman units. The data can be converted to Quinine Sulfate Units by multiplying by 48.9.

Characterizing different DOM end members—Land use in the catchment is dominated by agriculture, with minor areas of isolated forests, wetlands, and natural pastures in the upper reaches of the stream systems. The two major types of land use (cultivated and natural) in combination with the tertiary treated wastewater represented the three major freshwater end members. There was imported 'aged' DOM in the bottom waters entering the estuary from the Kattegat and autochthonous DOM produced by phytoplankton in the lakes and estuary. In addition to these five major end members, microbial and photochemical degradation was continually modifying the fluorescence characteristics of DOM.

The differences in the magnitude and type of fluorescence of DOM from the allochthonous end members are apparent through visual analysis of the measured EEMs (Fig. 2a–d). The most noticeable differences in the fluorescence characteristics can be seen by comparing the wastewater DOM (Fig. 2c) with the other more biogenic DOM end members. The wastewater DOM fluorescence is more blue shifted than are the others. The results of the PARAFAC decomposition of the EEMs allow us to examine the quantitative and qualitative differences between end members in more detail, revealing the underlying compositional variability (Fig. 5).

The highest average DOM fluorescence was measured in a forest stream and the lowest in the marine end member. The fluorescence of DOM derived from the forest was dominated by components 1 and 3 (Fig. 5). The EEMs of DOM derived from an agricultural catchment, where they were dominated by the fluorescence of components 2, 3, and 4. The major difference between the agricultural and marine DOM appears to be that component 7 is approximately equal in magnitude to components 2 and 4 (Fig. 5). The treated-

Table 1. Characteristics of the eight components identified in this study compared with those previously identified. Secondary maxima are shown in brackets.

Component Stedmon et al (2003)	This study	Excitation and emission maxima	Origin	Description
1 (UVC humic); terrestrial humic, highest concentration in forest stream + wetlands	1	ex. <250, em. 448	Terrestrial	Humic fluorophore group. Dominating the DOM exported from the natural catchments during the warmer months of the year. Also exported from agricultural catchments. Absent wastewater DOM.
2 (UVC humic), terrestrial humic, highest concentration in forest stream + wetlands	3	ex. <250 (305), em. 412	Terrestrial	Humic fluorophore group. Absent in wastewater DOM.
3 (UVA humic), terrestrial humic, common to a wide range of fresh water environments	2	ex. <250 (385), em. 504	Terretrial/autochthonous	Fulvic acid fluorophore group. Present in all environments.
	4	ex. <250 (360), em. 440	Terrestrial/autochthonous	Fulvic acid fluorophore group. Present in all environments.
4 (UVA humic), terrestrial humic, common to a wide range of freshwater environments	5	ex. 325, em. 428	Terrestrial/anthropogenic	Humic fluorophore group exported from agricultural subcatchments.
	6	ex. <250 (320), em. 400	Anthropogenic	Humic fluorophore, dominating the fluorescence of wastewater DOM. Also correlated to DOM exported from agricultural catchments, possibly due to the spreading of animal waste on fields as fertilizer.
5 (UVB protein-like), autochthonous DOM	7	ex. 280, em. 344	Autochthonous	Tryptophan-like fluorescence. Fluorescence peak almost identical to free tryptophan. Derived from autochthonous processes. Correlated to terrestrial fluorescent material (1–4) in forested catchments.
	8	ex. 275, em. 304	Autochthonous	Tyrosine-like fluorescence. Fluorescence peak almost identical to free tyrosine dissolved in water. Derived from autochthonous processes. Correlated to terrestrial fluorescent material (1–4) in forested catchments. Produced and removed by the same processes as tryptophan fluorescence.

wastewater DOM differed noticeably from the other end members, as it contained on average very little of components 1 and 3, and component 6 was the most prevalent (Fig. 5). Analysis of the time series of measurements of wastewater DOM revealed that for much of the year, components 1 and 3 were absent. However, after periods of high rain, low levels were detected (data not shown). The dominance of these components in the terrestrial end members combined with the synchrony with rainfall events indicates that this is most likely the result of soil-derived organic matter, which is being flushed into the town's sewage system during storm events.

Seasonal changes in DOM end members—There were noticeable seasonal variations in the fluorescence characteristics of DOM from each end member, in addition to the clear differences between their average values (Fig. 6). The fluorescence at Sta. 12 and 13 are shown as an example of the contrasting trends seen at the freshwater stations. Sta. 12 had a catchment that was dominated by agriculture (87% of catchment area), whereas the catchment of Sta. 13 was characterized by forest and natural pastures (45% of area). The other stream sampling sites had catchment characteristics between these two stations and therefore exhibited seasonal trends that were a combination of the two. During the sampling period there were three wet periods; September 2001, mid-January to mid-March 2002, and June–July 2002 (Fig. 6a). The variations in DOM fluorescence in the agricultural stream were tightly coupled to discharge (precipitation, Fig. 6a) because of the drainage characteristics of agricultural soils (Stedmon et al. unpubl. data). Moreover, the seasonal variation closely mirrored that of DOC (Fig. 6a–c). The lowest concentrations were measured during periods with little precipitation. Maximum values for all components were measured after periods of high precipitation in autumn and summer. There were also slightly elevated concentrations for components 1–6 between January and March 2002, correlating to the greater precipitation during this period. Despite the apparent high degree of covariability between the different component concentrations, there were small differences in the composition of the fluorescent DOM pool throughout the year. This was especially evident for the sampling date with the highest DOC concentration on the 12 July 2002, where the fluorescence of component 1 was higher than that of components 2 and 4, in contrast to the rest of the period, during which it was lower (Fig. 6b).

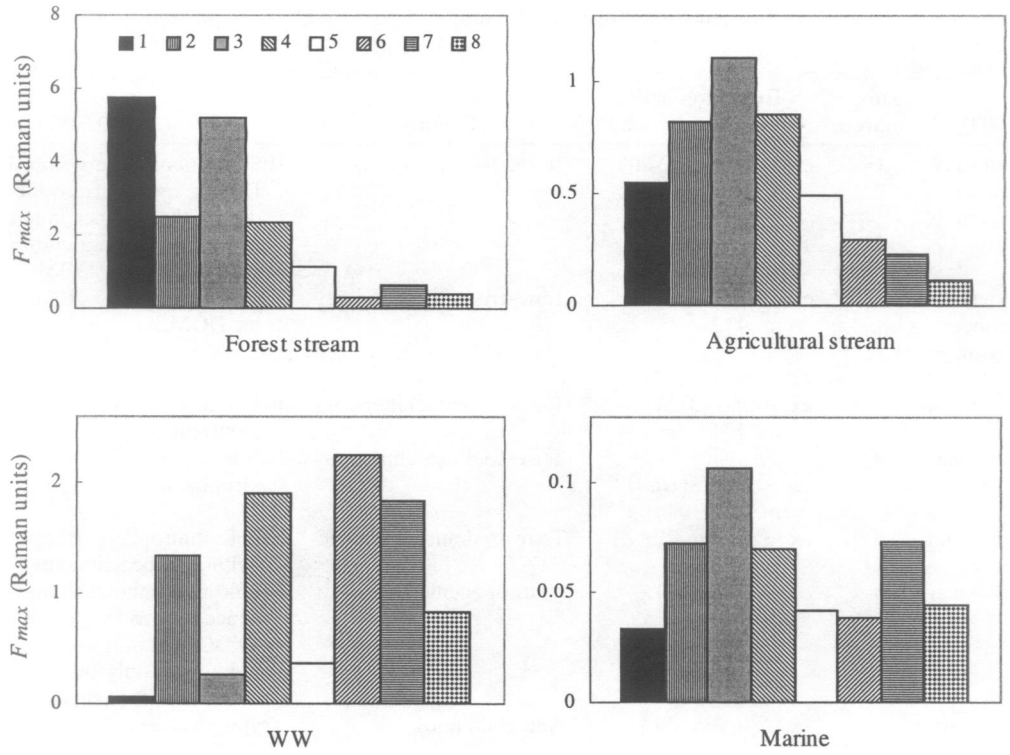

Fig. 5. Composition of the four major fluorescent DOM end members in the catchment with respect to the eight components. Values are annual means. Forest steam (Sta. 13, n = 31, DOC = 1,112 μmol C L^{-1}), agricultural stream (Sta. 12, n = 31, DOC = 440 μmol C L^{-1}), WW (Sta. 16, n = 33, DOC = 759 μmol C L^{-1}), and marine (Sta. 1 at 16.5 m in depth, n = 32, DOC = 186 μmol C L^{-1}). Refer to Fig. 1 for station locations.

There were also large variations in both the concentration and composition of fluorescent DOM in the forest stream (Sta. 13, Fig. 6d–f). Temperature and DOC concentrations tracked one another throughout the year (Fig. 6d). In general, seasonal variations in the components could be grouped into four patterns. Components 1, 3, 7, and 8 had maximum concentrations during the summer months, and their levels decreased rapidly in an exponential fashion with the onset of winter. Minimum concentrations for these components were measured during February and March 2002. In April 2002 the concentrations increased again, peaking in early June. Their concentrations decreased slightly during the precipitation events in June and July 2002 and then began to increase during the remaining summer months of the study period. Components 2 and 4 had very similar seasonal trends to components 1 and 3; however, the difference between summer and winter concentrations was much less. In addition, after the precipitation events in June and July 2002, the concentrations of components 2 and 4 remained stable.

The seasonal trends for components 5 and 6 in the forest stream differed from both those of the other components and from each other. The concentrations of component 5 decreased almost linearly from the end of September 2001 to a minimum in early March 2002. From late March to May, the concentrations increased sharply to summer levels again. After the precipitation events in June and July 2002, component 5 concentrations decreased noticeably. The maximum concentration of component 6 was measured during September 2001. Its concentration decreased gradually to a minimum in April and May 2002, after which it began to gradually increase throughout the rest of the study period.

Discussion

Number of components identified—The fluorescence characteristics of a fluorophore are determined by its chemical structure. The absorption bands of aromatic compounds are shifted to longer wavelengths as the number of rings increases (Sharma and Schulman 1999). As a result of the heterogeneous nature of DOM, it is difficult to relate the fluorescence spectra found to specific compounds or groups of compounds present. This, however, is not the aim of this study's application of fluorescence spectroscopy. Our aim is to use the fluorescent signal of DOM as a proxy for alterations occurring to the DOM pool as a whole. In an earlier study, only five fluorescent groups could be identified in samples from the same study site (Stedmon et al. 2003). It is clear that the larger number of samples in this data array has considerably increased the variability of DOM fluorescence, as a result of both quantitative and qualitative changes in the pool, and this has in turn increased the resolution of the decomposition of the fluorescence signal. This gives rise to the following question. If the number of samples in the data set was increased further, would the spectral characteristics or the components of the number of validated components change? The nature of this data set indicates that

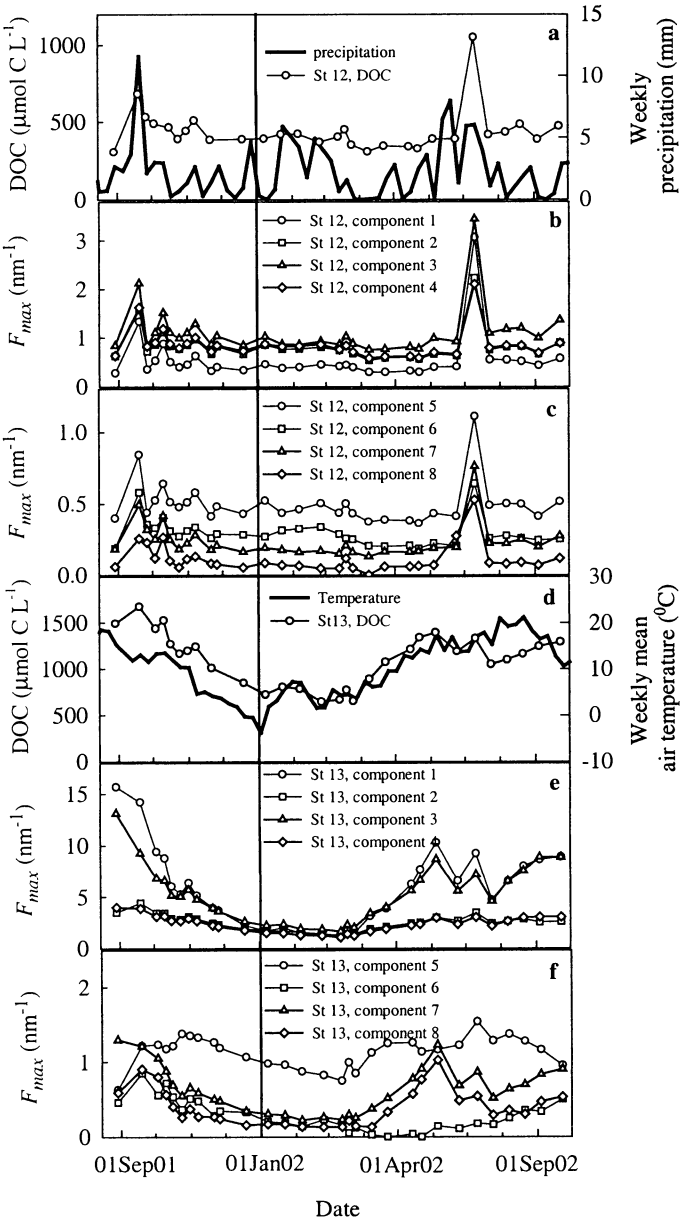

Fig. 6. (a) Weekly precipitation for the catchment during study period and DOC concentration at Sta. 12. (b, c) Variability in fluorescence of components 1–8 in a stream draining an agricultural catchment (Sta. 12). (d) Weekly mean air temperature for the catchment during study period and DOC concentration at Sta. 13. (e, f) Variability in fluorescence of components 1–8 in a stream draining a forested catchment (Sta. 13). Refer to Fig. 1 for station locations.

the majority of the natural variability in DOM fluorescence in this catchment has been captured and modeled. Seasonal and spatial changes were encompassed by the extent of the sampling program. Additionally, degradation experiments on the samples have introduced further variability into the data set. This indicates that it is unlikely that the spectral characteristics of the identified components will change as a result of expanding the data set. Additionally, the absence of large peaks in the residual EEMs (e.g., Fig. 2i–l) shows that the model is explaining the majority of the measured fluorescence. However, it is important to note that this only applies for this particular catchment. A similar study in another catchment and estuary will not necessarily result in the identification of the same eight components. This would be dependent on the DOM end members sampled.

Differing sources of components—From the simple analysis of DOM fluorescence characteristics already undertaken (Figs. 2a–d, 5), we can conclude that components 1 and 3 are of terrestrial origin and that they persist in the estuarine environment. Components 2 and 4 were ubiquitous to all the DOM end members, and component 6 appears to dominate in wastewater DOM. Because of the spectral characteristics of component 7 and 8, we can also conclude that they represent tryptophan- and tyrosine-like structures, respectively. In order to characterize the properties of each component further, it is necessary to examine the spatial and temporal variations in the concentrations of each fluorescent component and to hereby identify similarities in the sources and dynamics of the different DOM fractions.

Analysis of covariability of components—The concentrations and ratios of each component in a given sample vary depending on time of the year and locality, as can be seen in Fig. 6. To further analyze the variability in the fluorescence, each component was plotted against each other. The samples were indexed by season and, in addition, were classified according to catchment/environment. Five different groups were defined: >50% natural pastures and forests, >50% agricultural pastures, >75% agricultural pastures, outflow from the two lakes, estuarine, and wastewater. For some component pairs there was no discernible covariation, indicating that these components did not have a common factor controlling their concentrations within the data set. None of the components were linearly related across the whole data set, as the PARAFAC algorithm does not permit it. If two components co-varied linearly for all samples within the data array, the model is unable to distinguish between them and therefore combines them as one component. The spectral properties of the components would then be the combined spectra of the two.

Nonlinear relationships were found between several of the components in certain environments and in some cases across all environments. An example of the analysis is shown in Fig. 7a, where the fluorescences of component 1 and 2 are plotted against each other. A logarithmic relationship is apparent for all samples except those from wastewater. Wastewater DOM often did not contain any component 1 fluorescence (Fig. 5) and therefore did not follow the trend for the other samples. After log-transformation, a linear relationship was found. However, values from the lake samples taken in spring and summer and from the estuarine samples seemed to fall below the line (Fig. 7b), and the best relationship was obtained if they were excluded (Fig. 7c). A similar analysis was carried out for all components, and the results are summarized in Table 2.

This type of analysis allows us to investigate the similarities and differences in the distribution, in time and space, of each component. If the concentrations of two components are controlled by a common exponential factor, for example,

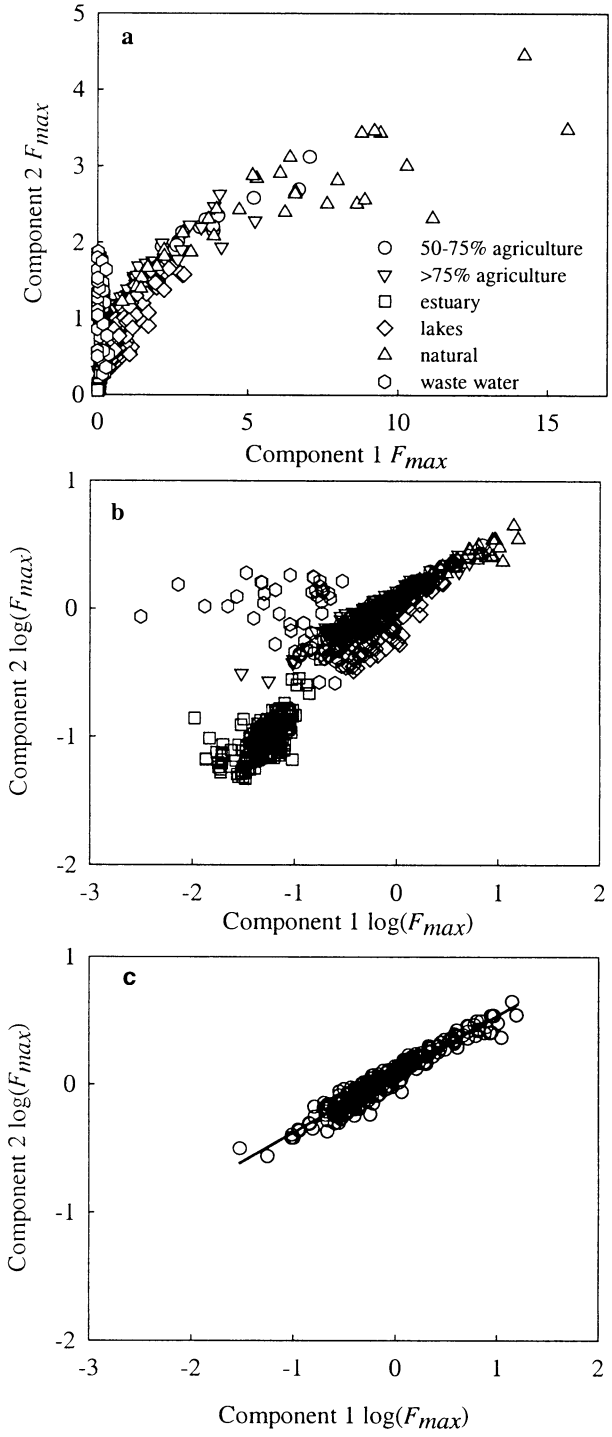

Fig. 7. (a) Covariation of the fluorescence of components 1 and 2 indexed by catchment characteristics and environment. (b) Same as a, but for the logarithms of each component. (c) Logarithmic relationship between the fluorescence of components 1 and 2 for freshwater samples, excluding the samples taken in the outflow of the two lakes during spring and summer. Refer to Table 2 for the regression equation.

temperature, as could be the case for Sta. 13 (Fig. 6d–f), their concentrations could be modeled by these equations:

$$c_x = c_x^0 e^{k_x T} \quad (1)$$

$$c_y = c_y^0 e^{k_y T} \quad (2)$$

where c is the fluorescence of a given component, T is the controlling exponential factor, and k is the net rate of change depending on the factor T. As T is common to both equations, these equations can then be solved for T (Eq. 3), to result in a linear relationship between the logarithms of the fluorescence of each component represented by Eq. 5.

$$T = \ln\left(\frac{c_x}{c_x^0}\right)\frac{1}{k_x} = \ln\left(\frac{c_y}{c_y^0}\right)\frac{1}{k_y} \quad (3)$$

$$c_y = \frac{c_y^0}{c_x^0} c_x^{k_y/k_x} \quad (4)$$

$$\log c_y = \log\left(\frac{c_y^0}{c_x^0}\right) + \left(\frac{k_y}{k_x}\right)\log c_x \quad (5)$$

The slope of the regression represents the ratio of the net production rates of the two components with respect to T. From here onward, for simplicity; k will be referred to as a net production rate. However, the reader is reminded that these values could also be negative and therefore represent a net removal rate. The intercept is the logarithm of the ratio of the fluorescence of each component at a reference condition ($T = 0$). Equation 5 implies that a linear relationship will be observed between the logarithms of two components if they are both controlled by the same factor in an exponential fashion (Eqs. 1, 2). The analysis provides a tool for identifying components that are being controlled by the same factors and for identifying deviations from an overall pattern. For example, at Sta. 13 it appears that temperature is influencing not only the quantity of fluorescent DOM but also the composition of the fluorescent DOM (Fig. 6d–f). However, the rate constants differ for each component. Table 3 presents examples of the four possible outcomes of the analysis, which aid in the interpretation of the results.

Covariation of humic fluorescence—The results in Fig. 7c show that for the freshwater samples, taken at a variety of stations within the catchment, components 1 and 2 co-vary, with component 1 having a greater net production rate (k_1) than component 2 (Table 2). The fact that the data from the estuary and the samples taken during spring and summer in the water flowing out of the lakes differ from the rest of the freshwater samples indicates that in these environments, additional and/or different processes are influencing the relative concentrations of these two components. A possible explanation for this pattern is photodegradation. In the streams (and in the lakes during the winter), the exposure to light is low, and the concentrations of components 1 and 2 are therefore controlled by their production and leaching from the soils in the catchment alone. As the DOM is exported from the streams to the lakes and the estuary during summer, the considerably higher surface irradiance and the longer residence time (especially during summer low-flow conditions)

Table 2. Results from linear regression on log-transformed fluorescence between pairs of components found in this study (Eq. 6). c_y^0/c_x^0 is the intercept of the regression and corresponds to the ratio of fluorescence at a reference condition (Eqs. 1 and 2, $T = 0$). k_y/k_x is the slope of the regression representing the ratio of the net production rates of two components. * Denotes data included in the regressions; (f,w), fall and winter samples only; (s,s), summer and spring samples only. WW, wastewater; Est, estuary; lakes, outflow from Bygholm and Hansted Lakes; >75% Agri, stream samples in which >75% of catchment was agricultural; >50% Agri, stream samples in which >50% of catchment was agricultural; >50% Nat, stream samples in which >50% of the catchment was forest.

x	y	n	r^2	c_y^0/c_x^0	k_y/k_x	WW	Est	Lakes	>75% Agri	>50% Agri	>50% Nat
1	2	293	0.94	1.19	0.46			* (f,w)	*	*	*
1	3	321	0.98	1.72	0.64			*	*	*	*
		263	0.76	1.03	0.65		*				
1	4	293	0.91	1.23	0.42			* (f,w)	*	*	*
1	5	235	0.96	0.72	0.45			* (w)	*	*	
1	7	34	0.96	0.20	0.70						*
1	8	34	0.84	0.10	0.76						*
2	3	293	0.91	1.35	1.31			* (f,w)	*	*	*
		292	0.94	1.55	1.00		*	* (s,s)			
2	4	321	0.97	1.04	0.93			*	*	*	*
		264	0.97	1.14	1.08		*				
		34	0.98	1.39	1.06	*					
2	5	551	0.99	0.60	1.09		*	*	*	*	
2	6	34	0.92	1.58	1.15	*					
2	7	91	0.79	0.20	1.24					*	*
3	4	293	0.89	0.87	0.64			* (f,w)	*	*	*
3	5	287	0.92	0.49	0.69			*	*	*	
		264	0.80	0.39	1.03		*				
3	7	34	0.95	0.14	0.91						*
3	8	34	0.81	0.07	0.98						*
4	5	551	0.98	0.58	0.97		*	*	*	*	
4	6	34	0.93	1.11	1.09	*					
		494	0.94	0.34	0.84		*	*	*		
4	7	91	0.84	0.20	1.28					*	*
7	8	619	0.81	0.44	0.94	*	*	*	*	*	*

increase the exposure to microbial and photochemical degradation processes. This in turn causes the relation between components 1 and 2 to disintegrate. Since the observed values from the lakes during summer fall below the line (Fig. 7b,c), we can hypothesize that in this environment, component 2 is more susceptible to photodegradation than component 1. Earlier studies of the effects of photodegradation on the fluorescence characteristics of DOM have shown that fluorescence in the regions of component 1 and 2 is susceptible to photodegradation (fig. 2 in Boehme and Coble 2000). Support for this hypothesis can also be found by comparing the excitation (absorption) spectra of these two components (Fig. 3a,b) with that expected for the underwater light field, where lower wavelength light is rapidly attenuated. Component 2 has a strong absorption band in the UVA region, whereas component 1 is mainly restricted to UVB and UVC. The rapid attenuation of UVB and C light in the water column would therefore restrict the photodegradation of component 1 to a thin layer at the surface. In contrast, the volume of water exposed to UVA light is considerably larger, resulting in the accelerated removal of component 2.

Logarithmic relationships were also attainable between components 1 and 3. As with the previous example, the wastewater samples do not correlate because of the fact that they are lacking component 1. Between these two components there are two different relationships: a freshwater and an estuarine (Table 2). In the freshwater relationship, samples taken in the outflow of the lakes were included, since no systematic deviations were identified. The slope of the regression on the estuarine samples was the same as that for the freshwater samples; however, their intercepts differed (Table 2). This indicates that the rates of the removal and production processes controlling the concentrations of these two components are the same in both the freshwater and estuarine environments (Table 3, example 3). However, the fact that the intercepts are different implies that the two environments have a different reference ratio of the two components (c_3^0/c_1^0). This can be explained by the fact that in the estuary, "aged" DOM from the Kattegat is mixing with the local supply of "fresh" terrestrial DOM, whereas in the streams, there is only the local terrestrial source of these two components. Although the "aged" DOM has a strong terrestrially derived component, because of the large riverine inputs into the Baltic Sea, it is unlikely that it still has the same fluorescence characteristics (c_3^0/c_1^0) as the local terrestrial DOM.

In the freshwater samples, the fluorescence of components 1, 2, 3, and 4 were related to a common factor, as can be seen from the regressions between them presented in Table 2. Component 5 was also controlled by the same factor; however, only in DOM from agricultural catchments. By comparing the slopes of the relationships found one can con-

Table 3. Example of the different scenarios that explain the outcome of the log–log component fluorescence analysis (Eqs. 1–5, Fig. 7, and Table 2).

No.	Example	Log–log regression
1	Both components vary in concentration independently and are not controlled by a common factor.	No relationship
2	Both components have a common source and their production and removal rates are controlled by a common environmental factor (e.g., two dissolved soil organic matter fractions). Levels found in the estuary are purely due to local inputs.	One linear relationship for freshwater and estuarine samples (common slope $[k_y/k_x]$ and intercept $[c_y^0/c_x^0]$)
3	Same as example 2; however, both components are not only due to local inputs but are also present in the estuarine mixing end member (e.g., older DOM from coastal seas).	Two linear relationships with the same slope (k_y/k_x) but different intercepts (c_y^0/c_x^0)
4	Both components controlled by the same factor; however, different factor depending on environment.	Two linear relationships with different slopes (k_y/k_x) and intercepts (c_y^0/c_x^0)

clude that $k_1 > k_3 > k_2 > k_5 > k_4$. Component 1 was the most variable fluorescent DOM fraction in the data set, with net production rates that were 2.4 times greater than those associated with component 4. From these results, it is clear that concentrations of components 1 to 4 in the freshwater environment were controlled by the same environmental factor.

The relationship between components 2 and 5 was the same for samples from the freshwater streams draining agricultural catchments, the outflow from the lakes, and the estuarine samples, whereas samples from the wastewater and catchments dominated by forests behaved differently. Similar trends were also seen between components 4 and 5 and between components 4 and 6. It can therefore be concluded that saline mixing in the estuary and increased exposure to degradation processes in the lakes and estuary did not additionally influence the net production rates of these components. The fact that the freshwater and estuarine samples fall on the same regression line and therefore have a common intercept implies that the presence of component 5 and 6 in the estuarine DOM is purely due to local inputs (Table 3, example 2).

Samples from the estuary and samples taken from the lake outflow during summer had a different logarithmic relationship between components 2 and 3 and that found for the other freshwater samples (Table 2). The slope for this regression was 1, meaning that for these samples, the net production rates of the two components were the same (i.e., covaried linearly). For the regressions between components 2 and 4 and 3 and 5, the estuarine samples had both different slopes and intercepts to the freshwater samples. This result indicates that the net production rates of these components differed in the estuarine environments compared to the freshwater environment. The difference in intercepts indicates that there is mixing of the local freshwater supply of these components with the marine end member (Table 3, example 4).

As wastewater DOM contained very little or none of components 1, 3, and 5 (Fig. 5), there were no relationships found that included these components. There were, however, wastewater DOM–specific relationships found between components 2, 4, and 6. The presence of components 2 and 4 in the absence of the other terrestrial components (1, 3, and 5) indicates that there is an additional source of component 2 and 4 fluorescence in wastewater. This result agrees with the findings of recent work (McKnight et al. 2001, 2003) that have shown that fluorescence in the region of components 2 and 4 can be derived from both terrestrial and microbial (autochthonous) sources. This will be discussed in more detail later.

Protein-like fluorescence—In the DOM exported from the more natural catchments, the fluorescence of components 7 and 8 co-varied with components 1-4, indicating that in these environments, the concentrations of components 7 and 8 are linked to the concentrations of components 1–4. The relationship between components 7 and 8 was the only one that was the same across the different DOM types, regardless of season (Table 2). These two components have earlier been identified as tryptophan and tyrosine, respectively, and they are associated with the autochthonous production of DOM (Mopper and Schultz 1993; Determann et al. 1996; Coble et al. 1998). The results here illustrate that common processes control their concentrations. The fact that the fluorescence of these two components co-varies with the terrestrially derived components in the streams draining natural catchments (e.g., forest streams) indicates that they are associated with the degradation of terrestrial organic matter in these environments.

Comparison to the Fluorescence Index—In the past, ratios of fluorescence at different wavelengths have been used to indicate changes in the composition of the DOM pool (e.g., Coble 1996; McKnight et al. 2001). The results presented here also show that there are considerable changes in DOM fluorescence depending on its original source and the processes acting on it. McKnight et al. (2001) found that the ratio of fluorescence at 450 nm to that at 500 nm, from excitation at 370 nm, was correlated to the source of fulvic acid fluorescence. This ratio is called the fluorescence index and microbially derived fulvic acids have a higher fluorescence index than terrestrially derived fulvics. Looking at the fluorescence characteristics of the components identified, the results from McKnight et al. (2001) indicate that both component 2 and component 4 are fulvic acids. Their relative proportion is dependent on the proportion of terrestrial and microbially generated DOM. Three different logarithmic relationships were found between these two components in the

data set (Table 2), one each for freshwater, wastewater, and estuarine samples. Although not directly comparable, c_4^0/c_2^0 from the regression is proportional to the fluorescence index, and the results appear to agree with the stated sources of the fulvic acids. The lowest values were found in the freshwater samples, in which terrestrial fulvic acid would be expected to dominate the DOM pool (Table 2). In the wastewater, the ratio is highest, and with the limited input of terrestrial DOM (e.g., components 1 and 3), the fulvic acids are most likely microbially derived. The value for the estuarine DOM lies in between. This is to be expected, as both of the other DOM sources discharge into it, and one can also presume that there is autochthonous production occurring within the estuary.

Fluorescent DOM is only a subfraction of the DOM and can therefore only describe part of the dynamics occurring in the pool. Currently we cannot assess how much of the total DOM pool is represented by fluorescence. However, fluorescence spectroscopy combined with PARAFAC analysis has proven to be a useful tool for tracing the dynamics of DOM in natural ecosystems (this study; Søndergaard et al. 2003; Stedmon et al. 2003). Because of the fact that the technique is relatively inexpensive, it allows the analysis of both spatially and temporally intensive sampling programs, thus providing a more detailed picture of the quantitative and qualitative changes occurring in DOM than is possible with many chemical techniques. Although the exact chemical compounds responsible for the fluorescence of these groups are still unknown, there is much knowledge to be gained about their ecological relevance through tracing their dynamics in the field and in laboratory experiments. The incorporation of fluorescence PARAFAC analysis into studies on DOM dynamics has the potential to identify groups of samples (i.e., DOM end members) that may be worthy of more detailed chemical characterization.

References

AITKENHEAD-PETERSON, J. A., MCDOWELL, W. H., AND J. C. NEFF. 2003. Sources, production and regulation of allochthonous dissolved organic matter inputs to surface waters, p. 26–70. *In* S. E. G. Findlay and R. L. Sinsabaugh [eds.], Aquatic ecosystems: Interactivity of dissolved organic matter. Aquatic Ecology Series, Academic Press.

ANDERSSON, C. A., AND R. BRO. 2000. The N-way Toolbox for MATLAB. Chemometrics Intelligent Lab. Syst. **52:** 1–4.

BAKER, A. 2001. Fluorescence excitation-emission matrix characterisation of some sewage impacted rivers. Environ. Sci. Technol. **35:** 948–953.

BENNER, R. 2002. Chemical composition and reactivity, p. 59–90. *In* D. A. Hansell and C. A. Carlson [eds.], Biogeochemistry of marine dissolved organic matter. Academic Press.

BERTILSSON, S., AND J. B. JONES. 2003. Supply of dissolved organic matter to aquatic ecosystems: Autochthonous sources, p. 26–70. *In* S. E. G. Findlay and R. L. Sinsabaugh [eds.], Aquatic ecosystems: Interactivity of dissolved organic matter. Aquatic Ecology Series, Academic Press.

BOEHME, J. R., AND P. G. COBLE. 2000. Characterization of colored dissolved organic matter using high-energy laser fragmentation. Environ. Sci. Technol. **34:** 3283–3290.

Bro, R. 1997. PARAFAC. Tutorial and applications. Chemometrics Intelligent Lab. Syst. **38:** 149–171.

COBLE, P. G. 1996. Characterisation of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. Mar. Chem. **51:** 325–346.

———, C. E. DEL CASTILLO, AND B. AVRIL. 1998. Distribution and optical properties of CDOM in the Arabian Sea during the 1995 Southwest Monsoon. Deep-Sea Res. II **45:** 2195–2223.

———, S. A. GREEN, N. V. BLOUGH, AND R. B. GAGOSIAN. 1990. Characterisation of dissolved organic matter in the Black Sea by fluorescence spectroscopy. Nature **348:** 432–435.

DE SOUZA SIERRA, M. M., O. X. F. DONARD, M. LAME, C. BEELINE, AND M. EWALD. 1994. Fluorescence spectroscopy of coastal and marine waters. Mar. Chem. **47:** 127–144.

Determann, S., Reuter, R., and R. Willkomm. 1996. Fluorescent matter in the eastern Atlantic Ocean. Part 2: Vertical distribution and relation to water masses. Deep-Sea Res. I **43:** 345–360.

DUURSMA, E. K. 1974. The fluorescence of dissolved organic matter in the sea, p 237–256. *In* N. G. Jerlov and E. Steemann Nielsen [eds.], Optical aspects of oceanography. Academic Press.

LOCHMULLER, C. H., AND S. S. SAAVEDRA. 1986. Conformational changes in a soil fulvic acid measured by time dependent fluorescence depolarization. Analyt. Chem. **38:** 1978–1981.

MCKNIGHT, D. M., E. W. BOYER, P. K. WESTERHOFF, P. T. DORAN, T. KULBE, AND D. T. ANDERSEN. 2001. Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity. Limnol. Oceanogr. **46:** 38–48.

———, E. HOOD, AND L. KLAPPER. 2003. Trace organic moietes of dissolved organic material in natural waters, p 71–96. *In* S. E. G. Findlay and R. L. Sinsabaugh [eds.], Aquatic ecosystems: Interactivity of dissolved organic matter. Aquatic Ecology Series, Academic Press.

MOBED, J. J., S. L. HEMMINGSEN, J. L. AUTRY, AND L. B. MCGOWN. 1996. Fluorescence characterisation of IHSS humic substances: Total luminescence spectra with absorbance correction. Environ. Sci. Technol. **30:** 3061–3065.

MOPPER, K., AND C. A. SCHULTZ. 1993. Fluorescence as a possible tool for studying the nature of water column distribution of DOC components. Mar. Chem. **41:** 229–238.

NAGATA, T. 2000. Production mechanisms of dissolved organic matter, p. 121–152. *In* D. L Kirchman [ed.], Microbial ecology of the oceans. Wiley Series in Ecological and Applied Microbiology. Wiley-Liss.

PERSSON, T., AND M. WEDBORG. 2001. Multivariate evaluation of the fluorescence of aquatic organic matter. Analyt. Chim. Acta **434:** 179–192.

SHARMA, A., AND S. G. SCHULMAN. 1999. Introduction to fluorescence spectroscopy. Wiley.

SØNDERGAARD, M., C. A. STEDMON, AND N. H. BORCH. 2003. Fate of terrigenous dissolved organic matter (DOM) in estuaries: Aggregation and bioavailability. Ophelia **57:** 161–176.

STEDMON, C. A., S. MARKAGER, AND R. BRO. 2003. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. Mar. Chem. **82:** 239–254.

———, ———, AND H. KAAS. 2000. Optical properties and signatures of Chromophoric Dissolved Organic Matter (CDOM) in Danish coastal waters. Estuar. Coastal Shelf Sci. **51:** 267–278.

YAMASHITA, Y., AND E. TANOUE. 2003. Chemical characterisation of protein like fluorophores in DOM in relation to aromatic amino acids. Mar. Chem. **82:** 255–271.

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