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Chapter 12

Fluorescence Excitation Emission Matrix (EEM) Spectroscopy

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The learning objectives of this chapter are the following:

- Understand the theoretical and historical background of fluorescence EEM spectroscopy
- Outline EEM instrumentation and best practice for method development, with consideration of potential shortcomings and interferences
- Present and discuss research literature for fluorescence EEM spectroscopy as applied to the fouling of membrane-based water treatment systems

12.1 INTRODUCTION

Fluorescence is a form of luminescence, whereby light (energy) is absorbed by a substance at a particular wavelength (excitation), and then emitted at a longer wavelength (lower energy, emission). The difference between the absorption and fluorescence maxima is known as the Stokes shift, and the entire process typically takes place over a very short timeframe. This is one of the characteristics which separates it from phosphorescence, which typically takes place over longer timeframes. These processes are best described in general by a Jablonski diagram (Figure 1), named after the Polish physicist Aleksander Jabłonski (1933).

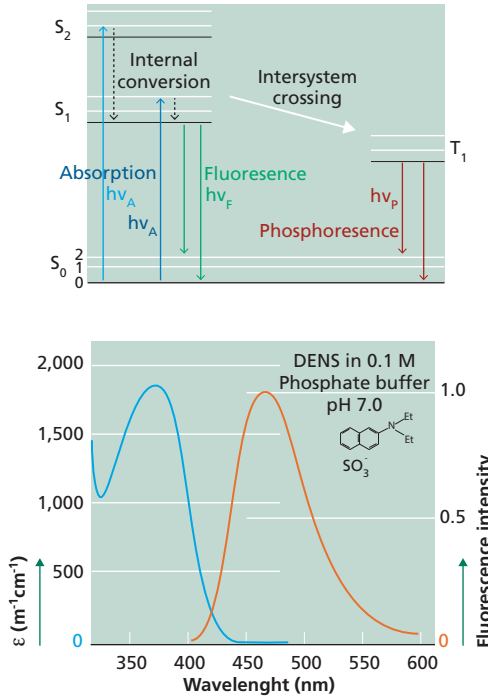

Figure 1 Left Jabłonski diagram; and Right: An example of a compounds absorption (A) and fluorescence emission (F) spectra, where the Stokes shift is the distance between the peaks of the two spectra (Adapted from Lakowicz, 2006b).

Not all substances or compounds are capable of exhibiting fluorescence. However, since it was first referred to in 1852 (Stokes, 1852), fluorescence-based methods have been used to analytically detect and quantify specific compounds with high sensitivity. Fluorescence spectroscopy is often described as being 10 to 1000 times more sensitive than absorbance spectroscopy, due to the nature of the measurement having a 'true' zero. There are many ways which fluorescence can and has been used analytically, from single point to simple 2D emission measurements at a single excitation wavelength, to synchronous fluorescence and excitation-emission matrix (EEM) spectroscopy. Depending on the complexity of the sample, spectra of mixtures such as organic matter, can often be deconstructed into independently varying components using a variety of analytical tools (see section: 11.6 Data Processing). This chapter will focus on fluorescence EEM spectroscopy, and its practical relevance in relation to membrane-based water treatment and fouling in general.

Broadly speaking, the analysis of FDOM (fluorescent dissolved organic matter) with EEM spectroscopy found early use in the natural sciences, the bulk of which was first carried out within oceanography. The methods have also been transferred to freshwater and estuarial research applications, and subsequently have seen applications tied to water treatment within a number of different fields. Fluorescence EEM spectroscopy has now been used to detect and characterise organic content within the aquatic sciences, across a large variety of natural and engineered systems. Oceans (Catalá *et al.*, 2015; Stedmon and Nelson, 2015),

seas, lakes (Kothawala *et al.*, 2014; Osburn *et al.*, 2017), and rivers (Baker and Inverarity, 2004), as well as drinking water (Bridgeman *et al.*, 2011), wastewater (Carstea *et al.*, 2016), water recycling (Hambly *et al.*, 2010; Hambly *et al.*, 2015; Henderson *et al.*, 2009; Murphy *et al.*, 2011), aquaculture (Hambly *et al.*, 2015; Spiliotopoulou *et al.*, 2017; Yamin *et al.*, 2017), and desalination (Drozdova *et al.*, 2017; Shutova *et al.*, 2016) are some of the varied aquatic settings in which fluorescence has been applied, from small scale research studies to large scale industrial uses.

Within the context of this chapter, fluorescence EEM spectroscopy has shown particular use in the analysis and understanding of membrane-based systems. As an optimised target compound removal is paramount to the performance of any membrane system, any sort of membrane fouling can therefore limit the systems performance. Feed waters often contain a high level of organic compounds, and as such various forms of organic fouling of the membranes can occur. The performance of a membrane system can be evaluated on the FDOM analysis of different aspects of it, and depending on which aspect it is measuring, the appropriate analytical method will require tweaking. Specific details of fluorescence methods are thus contained in the following sections.

Numerous other aspects of the measurement also need to be addressed before fluorescence data is ready for interpretation. While fluorescence measurements are somewhat simpler when compared to e.g., liquid chromatography coupled to mass spectrometry, analysts must consider many questions before measurements can take place. For example, how should samples be taken and how long can they be stored? How exactly should fluorescence be measured, and which analysis strategy is the best for a given study? The following sections 11.2 to 11.7 provide guidance for the practical aspects behind fluorescence measurements within an aquatic context.

12.2 SAMPLING & STORAGE

Samples taken from different stages of membrane filtration consist of particulate and soluble material in water. A fraction of this material is highly bioavailable to microorganisms, while other fractions resist biodegradation for longer periods (Hu and Ren, 2019; Urgun-Demirtas *et al.*, 2008). When it comes to sampling and subsequent storage prior to measurements, the more bioavailable material requires special attention, as microbes naturally target the most available compounds first and can thus alter the sample quickly (Heinz and Zak, 2018). In general, it is advisable to process samples and perform fluorescence measurements as quickly as possible to avoid storage artefacts. However, the constraints of sampling do not always allow for fast sample processing. In such cases, preserving the sample and knowing about possible storage effects is important. Preservation strategies include filtration, storage in cold and dark conditions, freezing, and chemical poisoning. In contrast, autoclaving introduces changes in FDOM (Andersson *et al.*, 2018).

Good practices for characterizing dissolved materials include 1) removing living organisms as quickly as possible through filtration; 2) storing the sample at temperatures below 10 °C in the dark to minimize biological activity; 3) measuring as quickly as possible but preferably at most within 5 days of sampling, and; 3) maintaining the same procedure for sample processing within a study to keep potential biases constant.

Different filter materials and pore sizes are available for filtration. Amongst the available materials, glass fiber filters are the safest option regarding contamination since they can be ashed (> 4 h at > 450 °C, (Coble *et al.*, 2014)). However, glass fibre filters generally only exist with pore sizes of larger than 0.3 μm. The classic GF/F filter with a pore size of 0.7 μm was used to distinguish particulate from dissolved matter. However, at that pore size, studies have reported bacterial passage rates between 10 and 25 % in marine samples (Morán *et al.*, 1999). It should however be noted that passage of microbes through filters with all common diameters can be observed (Obayashi and Suzuki, 2019; Wang *et al.*, 2007, 2008). If initial cell counts are high and assimilable carbon is abundant, microbial regrowth can quickly change sample character despite the usage of 'sterile' filters (< 0.2 μm). These observations emphasize that storages times should be kept short and effects of microbial passage will depend on the original sample. Lastly, the leaching of filter material into the sample should be investigated for the specific batch of filters used in each study. In the context of wastewater, such leaching is likely negligible but can affect primarily the UVA fluorescence emission range due to the leaching of production-related impurities. Filters should be rinsed with ultrapure water followed by sample prior to obtaining a filtrate for analysis. Filtration should occur slowly to avoid the bursting of cells (Rosenstock and Simon, 1993).

Freezing as a means to slow down biological processes can help to facilitate longer sample storage. While some studies recommend freezing as suitable for samples with low carbon concentrations, significant changes in optical indices, sample absorbance, and fluorescence emission characteristics have been observed (Fellman *et al.*, 2008; Heinz and Zak, 2018; Spencer *et al.*, 2007) concluded that the effects cannot be predicted from the composition of the original sample. Thus, the effects cannot be corrected post-measurement.

12.3 BENCHTOP INSTRUMENTATION

Since fluorescence analyses are increasingly popular, users have a range of choices concerning benchtop instrumentation. However, specifications regarding instrument and software can differ significantly between instruments and affect the measurement experience and resulting data quality.

Commercial instruments on the market today usually feature incandescent or pulsed Xenon lamps. These lamps provide excitation light in the entire ultraviolet-visible spectrum (approx. 220 – 800 nm) and have a relatively continuous emission spectrum. To understand the instrument's limitations, it is important to keep in mind the lamp's output spectrum. For example, incandescent Xe lamps provide little light at wavelengths shorter than 240 nm and resulting emission scans are generally noisier and can be difficult to interpret. Xe flash lamps can provide more light in the ultraviolet range resulting in a wider usable excitation range and a more uniform signal-to-noise relationship across EEMs (Lakowicz, 2006a). However, Xe flash lamps also contain more distinct emission bands, that need to be addressed to obtain spectrally calibrated EEMs. Lastly, incandescent Xe lamps have a lifetime of < 2000 h and thus require more maintenance compared to pulsed light sources.

The optical configuration of spectrofluorometers can differ significantly due to the requirements dictated by the detection system. The classic photomultiplier tube (PMT) is a sensitive photon detector that lacks the ability to distinguish light of different wavelengths but offers superior sensitivity thanks to signal amplification and low noise levels. Spectrofluorometers that utilize PMTs require two monochromators: The first selects a narrow band of light for sample excitation, the second permits a narrow band of fluorescence emission to pass through to the PMT. By scanning through all desired emission wavelengths at all desired excitation wavelengths, an EEM is constructed with typical speeds of 500 nm/min (total time typically between 20-40 min). On the other hand, charge-coupled device detectors (CCDs) allow the simultaneous detection of the entire wavelength range of interest and reduce measurement times considerably.

Due to the necessity to consider non-linearity in fluorescence observations due to the optical density of a sample (discussed below), it is also important to consider the availability of spectrophotometers during the measurement of fluorescence. If potential projects involve field measurements, the use of instruments with a combined absorbance-fluorescence detection system can be advantageous since all required measurements are made within one unit (see Figure 2).

Figure 2 An example of a modern spectrofluorometer instrumentation setup in a research laboratory – in this case an iteration of the Horiba Aqualog with a CCD detector.

As mentioned above, samples generally contain particulate and dissolved material. While both fractions contain fluorescent moieties, different instrument configurations are required to characterize the material. For example, dissolved fluorescent material is quantified after filtration of a water sample through filters in the classic right-angle geometry with a quartz cuvette (Figure 3, left). On the other hand, the measurement of particulate material occurs either directly in the unfiltered, optically thick suspension or by exciting particles directly on a surface. In both cases, the non-transparent nature of the particulate sample necessitates a front-face illumination (Figure 3, right). Front-face measurements of thick suspensions can also occur in cuvettes, but require adapters to either adjust the angle of the incident light relative to the cuvette face or to capture and direct the light at a specific angle toward the cuvette. The remainder of this section will discuss right-angle fluorescence of optically thin solutions since this is by far the most common application of fluorescence.

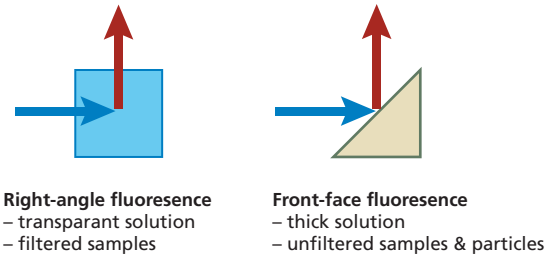

Figure 3 Schematic view (top) of the geometric position of samples and cuvettes.

12.4 QUALITY ASSURANCE

Most of today's spectrofluormeters are spectrally calibrated in full from the factory. However, to generate comparable results, wavelength accuracy and spectral calibration are especially important to monitor over time as the instrument ages. Wavelength accuracy refers to the deviation between true and detected wavelength in nanometres; either the excitation light intercepted by the cuvette or the fluorescence emission captured by the detector. Manufacturers commonly list the specification in the instrument manual and a precision of ± 1 nm is typical. Deviations are monitored by detecting the peak position of Rayleigh scatter at e.g., 467 nm, while the accuracy of the emission detector can be verified by determining the peak position of the 435.8 nm Hg band emitted by common low-pressure mercury vapor lamps (Sansonetti *et al.*, 1996). Monitoring changes in peak positions is especially important after instrument transport.

Spectral calibration refers to the elimination of spectral biases that arise from a biased lamp emission spectrum, and wavelength-dependent monochromator and detector biases. Some of these biases are eliminated with the use of reference detectors, but the remainder of the bias is removed with the use of excitation and emission correction factors that come pre-installed from the factory. It can however be a good idea to verify their appropriateness from time to time. A triangular cuvette of Rhodamine B produces a flat excitation spectrum after the successful elimination of spectral biases (Kopf and Heinze, 1984). The emission calibration is commonly verified with standards available from the National Institute of Standards and Technology (NIST). Different standards cover the ultraviolet-visible emission range and the recorded spectra should fall within the certified values at all times (Gilmore, 2014).

To obtain valid results, the measured sample needs to meet certain criteria to ensure that the instrument is capable of delivering the best results possible. For example, fluorescence counts should not exceed the linear range of the detector. Fortunately, the instrument control software usually warns users when the linearity threshold is exceeded. In such cases, settings can be adjusted (e.g., integration time) or samples diluted.

In cases with high concentrations of chromophores, the sample transmission can be too low to deliver quantitative fluorescence results. Kothawala *et al.*, (2013) defined an absorbance of 0.05 cm^{-1} (89 % transmittance) as the cutoff below which such effects can be safely

neglected (Kothawala *et al.*, 2013). On the other hand, an absorbance of 1.5 cm^{-1} (3 % transmittance) was found to be the upper limit after which no quantitative fluorescence results can be obtained even if correction methods are applied (see below). It is thus important that samples meet the second criterion *during* the fluorescence measurements, as no post-measurements for linearity will be possible.

Prior to every study, choosing appropriate measurement settings is important to ensure appropriate fluorescence counts in the relevant ranges of the EEM are accumulated. Regarding range and resolution, the types of observed fluorophores and their properties will govern which parts of the EEM should be captured. For example, if protein-like material, phenolic compounds, and / or oils are present a high resolution in the excitation range below 300 nm is especially important. To distinguish these highly similar fluorophores and quantify their fluorescence, it can also be important to capture emission spectra with a resolution below 3 nm if bandwidth characteristics of monochromators permit this. For instruments with incandescent Xe lamps, it generally makes little sense to capture emission below 240 nm even if sample fluorophores exhibit strong absorbance bands since signal-to-noise ratios deteriorate in the UV region. Capturing the emission up until 800 nm is necessary should the sample contain algae or fluorescent pigments. Moreover, to enable the correction of inner filter effects, it is important to measure the samples absorbance spectrum covering all excitation and emission wavelengths. Otherwise, such corrections can become difficult to implement.

12.5 INTERFERENCES

As an extrinsic property of fluorophores in solution, fluorescence fingerprints are vulnerable to changes due to interferences. Such changes can impact fluorescence yields (per mol of substance) and spectra and are caused by physicochemical properties of the sample. When comparisons between samples are made, it is thus important to consider whether physicochemical properties remain stable or are subject to change. In the following, we will list *some examples* (not all) of the physicochemical parameters known to influence DOM fluorescence.

The sample's temperature can affect the fluorescence intensity observed for a given sample. When solvent temperatures increase, observed fluorescence generally decreases. However, there is no evidence to suggest that spectral characteristics change due to temperature (McKay *et al.*, 2018). This effect is of great importance for *in situ* measurements with sensors since temperature can vary systematically over longer periods of time. However, a compensation is trivial if the sample's temperature is known (Watras *et al.*, 2011). For benchtop instruments, temperature effects are usually not an issue since jacketed cuvette holders and climate-controlled laboratories eliminate the chance for systematic biases.

Changes in pH can lead to spectrally-dependent changes in a sample's fluorescence. Numerous studies have investigated the effect of pH on organic matter fluorescence, and the different results reported in each study hints at sample-dependent, complex effects (e.g. Esteves *et al.*, 1999; Groeneveld *et al.*, 2022; Mobed *et al.*, 1996; Murphy *et al.*, 2018; Spencer *et al.*, 2007). These complex changes make it effectively impossible to compensate

the pH-induced interference. It is thus best to avoid sample-to-sample differences in pH to facilitate comparisons between samples.

Beyond temperature and pH, ionic strength, the presence of metal ions, and particle attenuation have also been reported to affect fluorescence measurements. As with all physicochemical properties, it is advisable to obtain reference measurements for samples coming from the system that is subject of a study. This will help to ascertain if issues with certain parameters are to be expected and if so, whether great variation (e.g., pH or ionic strength) might introduce artefacts that complicate interpretation of the fluorescence readings in a given dataset.

12.6 DATA PROCESSING

Fluorescence measurements require several steps of processing before further analyses can take place (Figure 4). While some software offers comprehensive features that contain the most critical steps, we believe it is most advisable to export data from proprietary formats and subsequently use open software environments to process and analyze fluorescence data. This gives the user more control over processing steps, freedom of choice regarding analysis strategies, and maximizes the impact of the conducted research by extracting as much information as possible. Amongst the most common languages for statistical computing, Matlab and R have community-driven software packages (Matlab: drEEM, EEMlab; R: eemR, StaRdom, albatross) that facilitate all steps in Figure 4 and enable a range of multivariate analyses (Micó *et al.*, 2019; Murphy *et al.*, 2013; Pucher *et al.*, 2019). While theoretically possible, an analysis of EEMs in spreadsheet software is not recommended since workflows are not easily automatable.

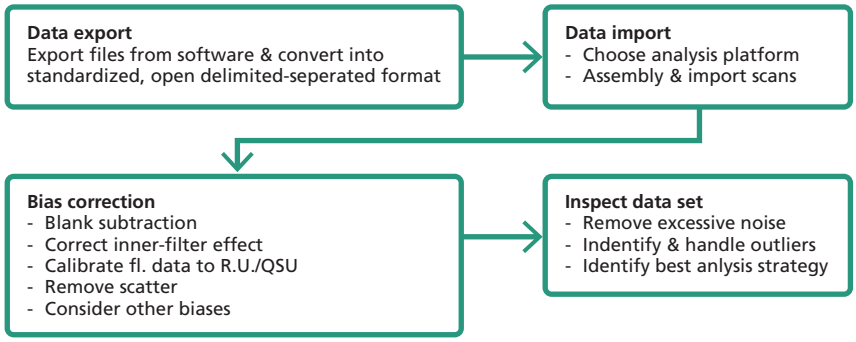

Figure 4 Steps involved in the processing of fluorescence measurements.

After the successful import into the programming environment of choice, the next processing steps concerns the correction or removal of different measurement biases. For example, a blank should be subtracted from each sample fluorescence landscape to remove the impact of background signals and reduce the abundance of scatter. Such blanks should be measured daily and always be made from the sample solvent (e.g. water, buffered water,

organic solvent). Blanks can also function as a standard for the calibration of fluorescence signals into Raman Units (Lawaetz and Stedmon, 2009). Next, inner-filter effects (IFEs) are compensated since they disturb the linearity of observed fluorescence due to the partial or complete absorbance of emitted fluorescence by chromophores (Parker and Rees, 1960). Such effects are easily corrected by applying correction factors derived from the sample's absorbance scan – provided the maximum absorbance be below approx. 1.5 cm^{-1} (Kothawala *et al.*, 2013).

The removal of Rayleigh and Raman scatter can be an important step if the subsequent analysis strategy (see below) includes the decomposition of fluorescence EEMs into statistical components according to Beer Lambert's law (Bahram *et al.*, 2006). The open-source software packages mentioned above include functions for scatter removal and thus simplify this task considerably.

12.7 DATA ANALYSIS

Once data is measured and fully processed, the data analysis can occur. Analysis strategies (overview in Table 1) can range from simply comparing fluorescence intensities (known as 'peak picking') and fluorescence indices to multivariate analysis such as parallel factor analysis (PARAFAC).

Table 1 Overview of most common strategies to analyse fluorescence EEMs.

Analysis strategy	Description	References
Peak picking	Extraction of fluorescence intensities from EEMs at defined wavelengths.	(Coble 2007)
Fluorescence regional integration (FRI)	Integration of fluorescence in wavelength regions with predefined interpretation.	(Chen *et al.*, 2003)
Fluorescence indices	Qualitative descriptors of FDOM with insights into humification, aromaticity, freshness and microbial processing.	(Huguet *et al.*, 2009; Maie *et al.*, 2006; Ohno 2002; Parlanti *et al.*, 2000)
Parallel factor analysis (PARAFAC)	Multivariate decomposition of EEMs into components.	(Murphy *et al.*, 2013)

The comparison of fluorescence intensities usually occurs at predefined wavelengths that typically have letters assigned to them (see Figure 5, table 2). For example, peak T, extracted at excitation / emission 275 / 340 is typically ascribed to tryptophan- or protein-like material. Peaks A and C on the other hand are often described as humic-like material. It should be noted that the interpretation of fluorescence peaks should only be informed by comparison with studies of the same sample material and take into account potential issues (e.g. pharmaceuticals fluorescing like amino acids).

Table 2 Position of predefined peaks and fluorescence indices as listed or described in Coble (2007), Maie *et al.* (2006), Huguet *et al*. (2009), Ohno (2002).

Peak / index	$\lambda E_x / \lambda E_m$	Tentative interpretation in natural environments
A	260/400-460	Humic-like, terrestrial
B	275/305	Autochthonous
T	275/340	Autochthonous
M	290-310/370-410	Anthropogenic contaminants
C	320-360/420-460	Humic-like, terrestrial
D	390/509	Humic-like, ubiquitous
Fluorescence index (FI)	370/470 370/520	Distinguishes microbial and terrestrial inputs
Biological index (BIX)	310/380 310/430	Contribution of biological transformations
Humification index (HIX)	$254/\int 435-480$ $254/\int 300-345 + 254/\int 435-480$	Ratio between protein- and humic-like fluorescence

Fluorescence regional integration (FRI) is a particularly popular technique in engineered systems by which integrals of wavelength regions in the EEM (Figure 5, yellow lines) are tracked across samples. The assignment of these regions is based on model compounds and natural environmental samples (Chen *et al*., 2003). According to Chen *et al*., (2003), the five regions as illustrated in Figure 5 are aromatic protein-like material (I + II), fulvic acid-like compounds (III), microbial by-product-like fluorescence (IV), and humic-like material (V). Subsequent to the publication of the FRI approach, multivariate modelling has indicated that underlying fluorescence spectra in regions I, II and IV, as well as III and V overlap spectrally and regional integrals are likely not as specific as the names above suggest (Stedmon *et al*., 2003).

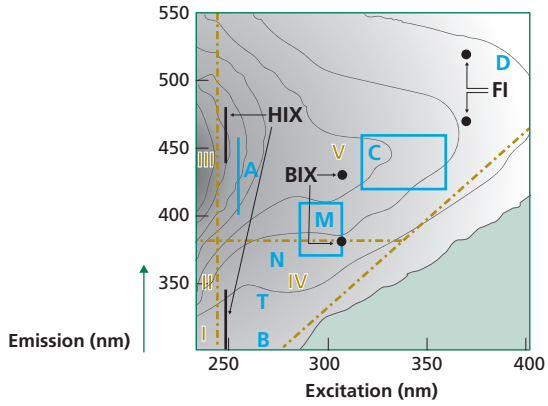

Figure 5 Emission-Excitation Matrix of a sample from a boreal river (Öre river, northern Sweden). Yellow lines and text refer to fluorescence regional integration areas (FRI). Black dots represent fluorescence indices: FI: Fluorescence Index; BIX: Biological Index; HIX: Humification Index). Blue lines and text refer to predefined peak locations.

Several fluorescence-based indices also find frequent application (Figure 5, thick black lines, Table 2). For example, the humification index (HIX) informs about the ratio between protein- and humic-like fluorescence and thus can help to understand qualitative shifts between samples (Ohno, 2002). The biological index (BIX) is a ratio between ultraviolet and visible fluorescence and can indicate the importance of recent biological transformations of material (Huguet *et al.*, 2009). Lastly, the fluorescence index (FI) distinguishes between microbial and terrestrial inputs in aquatic environments (Maie *et al.*, 2006; McKnight *et al.*, 2001). As with peak picking, one should be careful to extrapolate interpretations from studies performed on different sample types to membrane samples. The above-mentioned fluorescence indices were defined in studies of natural aquatic environments but can help to identify qualitative differences between samples.

12.7.1 PARAFAC

Amongst the multivariate analysis techniques, PARAFAC is the most popular technique in the analysis of DOM and this section will provide a short overview over this technique. For tutorials on MCR and PCA, we refer the reader to Bro and Smilde (2014), and de Juan *et al.*, (2014). PARAFAC is a particularly popular model for the decomposition since it naturally follows the analytical principals of fluorescence (Bro, 1997; Murphy *et al.*, 2013; Stedmon and Bro, 2008). Each analyte (termed 'component' in the model) is described as a product of an excitation and emission spectrum multiplied by a concentration factor. The entire EEM is the sum of the fluorescence arising from each of the components. PARAFAC is particularly attractive since it can distinguish spectrally overlapping components and thus allows insights into components that may not be distinguishable in the raw fluorescence data. Also, PARAFAC can isolate systematic signals in noisy measurements and thus help to improve the quality of the results. Moreover, the component spectra can be compared between studies and help to inform the chemical interpretation of the signals.

As a multivariate modelling approach, PARAFAC analyses work best if a number of criteria are met. For example, a minimum number of samples with meaningful compositional variability helps to identify meaningful and robust models. If two or more peaks covary perfectly, the approach may produce questionable models. Similarly, if a dataset consists of too few samples, the algorithm can struggle to identify the underlying components. As discussed above, the fluorescence occurring in each sample can be altered due to interferences. In such cases, it is most likely more fruitful to rely on peak picking and/or the interpretation of fluorescence indices.

12.8 APPLICATION IN MEMBRANE SYSTEMS

As outlined above, there are myriad ways in which fluorescence measurements can be applied to aquatic systems that utilise some form of membrane. Organic matter is a common source of membrane fouling, and it follows that fluorescence measurement of organic matter has gained traction in the analysis and investigation of how organic fouling occurs in these systems. Whether it is microfiltration, ultrafiltration, nanofiltration, or reverse and forward osmosis, using flat sheet, hollow fiber, tubular, or spiral wound constructions, there is nearly always a way in which fluorescence measurements can be, and has been applied.

These methods can assist in evaluating the membrane structure, the process performance in general, and of course the level and character of fouling. However, the construction, matrix, and analytical targets for each of these specific applications will determine how fluorescence EEM spectroscopy can, and cannot, be used in each case.

Section 11.3 has highlighted two physical application differences: (1) front-face fluorescence spectroscopy; and (2) right-angle fluorescence spectroscopy. Both methods can be used to assess and/or predict organic fouling, though in different ways and typically for different applications. Front-face fluorescence EEM spectroscopy currently finds its most common (membrane-related) use for systems such as MBRs (membrane bioreactors), or direct measurement from fouled membrane surfaces. Regardless of whether front-face or right-angle fluorescence is used, the organic character of any fouling will be dependent on both the feed, and the physical and/or chemical characteristics of the membrane in use.

The direct, *in situ* fluorescence analysis of membranes has to date been carried out in different ways in order to understand the main organic components behind the organic fouling of membrane surfaces. For example, Yamamura *et al.,* (2019) used in situ front-face EEM spectroscopy to investigate the organic fouling of PVDF membranes from secondary treated wastewater, in a bench-scale study. They observed increasing intensities with time over three main peaks, and by combining intermittent backwashing with EEM analysis, the authors observed peaks which were predominantly associated with reversible and/or irreversible fouling. Yu *et al.,* (2019), also utilised front-face fluorescence to detect and quantify model foulants on UF membranes, and concluded that it was a better method than liquid right-angle fluorescence in this particular study due to lower standard deviations observed between repeated measurements (see Figure 6). Pawlowski *et al.,* (2016) used front-face fluorescence to monitor fouling deposition on ion-exchange membranes. In this study, the authors found it to be a useful tool in evaluating reverse electrodialysis processes, and particularly for increasing membrane cleaning efficiencies.

Similar front-face techniques have also been used to investigate activated sludge systems (Huaorng, 2022), to quantify biomass and bioactivity amongst other parameters. From a practical spectroscopy perspective, these can be likened to the sludge and mixed liquor components of MBRs. The fluorescence character of the sludge over time can be linked to the evolution of fouling on the membranes, as the organic matter in particular EPS (extracellular polymeric substances) has been found to be closely related to TMP (Chen *et al.*, 2018). Various iterations of front-face spectroscopy have been used for assessing both the sludge and membrane components of MBR systems *in situ* (Galinha and Crespo, 2022).

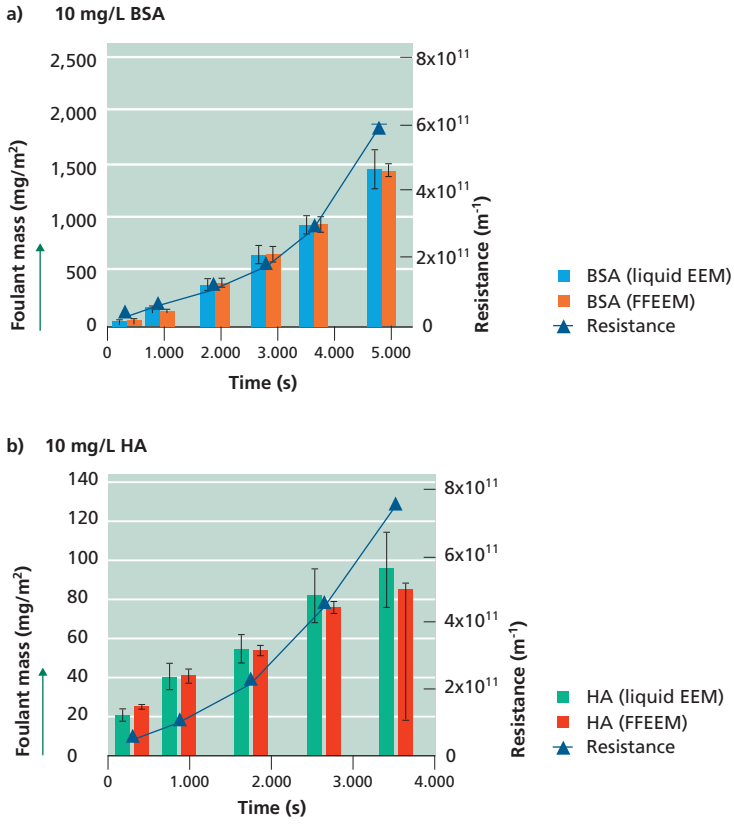

Figure 6 Comparison of fouling by 10 mg/L of (a) Bovine Serum Albumin and (b) Humic Acid measured by liquid right-angle EEM and front-faced fluorescence EEM measurements (adapted from Yu *et al.* (2019)).

Looking beyond front-face fluorescence EEM spectroscopy, the more common method of analysing membrane performance has historically been by a direct measurement of the different liquid streams with right-angle fluorescence. This is due to the higher signal and higher sensitivity that is achieved by this method, though front-face fluorescence spectroscopy has been described and utilised for high absorbance liquid samples for nearly half a century (Eisinger and Flores, 1979). In the case of membrane systems, using the right-angle fluorescence method typically means a comparison of one or more out of the feed, permeate, and concentrate streams. Whether some, or all, of these streams can be analysed with right-angle fluorescence will depend on whether the target matrix adheres to the requirements set out in section 11.4 Quality Assurance. Primarily, the liquid matrix must exhibit low absorbance values, which either are below the threshold where they can be considered negligible, or within the mathematically correctable range. In the latter case, an absorbance measurement must accompany the fluorescence measurement to guarantee accurate values.

For example, Poojamnong (2020) used right-angle fluorescence to compare the feed and permeate EEMs of an MBR treating pulp and paper wastewater. This study found protein-like fluorescence to be the most reduced region from the feed to the permeate, which correlated to the main component of the fouling formed on the UF membranes. A comparison of feed and permeates from RO processes of water recycling plants have also shown that right-angle fluorescence EEM could be used to monitor organics rejection and membrane integrity (Pype *et al*., 2013; Singh *et al*., 2009; Singh *et al*., 2015). Bagastyo *et al.,* (2011) identified humic and fulvic-like organics, as well as soluble microbial products as the main constituents of the concentrate stream within an RO system treating secondary wastewater effluent.

A direct analysis of already fouled membranes can also be performed with right-angle fluorescence EEM spectroscopy. Stripping off individual foulant layers by, for example, backwashing, or acid/base washing, has been used to understand differences in the formation of membrane foulant layers. In 2004, Kimura *et al.* (2004) showed that alkaline cleaning removed primarily protein-like fluorescence from membranes fouled by surface water. Henderson *et al.* (2011) also found that protein-like fluorescence was the predominant fluorescence region that was removed through a three-step UF membrane cleaning procedure. More specifically, in this case a low UV 'tyrosine-like' fluorescence component found within 5 different sources of wastewater, was found to be highly correlated to membrane fouling potential. In a study that investigated the role of DOM in the fouling of membrane bioreactors (MBR) treating wastewater, Tang *et al.* (2010) also found that two protein-like fluorescence components were most correlated to membrane fouling. These and other similar studies highlight how EEM spectroscopy can be used effectively to gain insight into how different foulant layers form, and therefore how to minimise their formation.

Further to the direct observation of various fluorescent membrane fouling components, studies have also investigated different pre-treatment steps to remove these components and hence minimise organic fouling. For example, Wang *et al.* (2017) utilised liquid EEM analysis to compare the performance and effect of 8 different types of powder activated carbon (PAC), as a pre-treatment to UF membrane treatment. They showed that whilst initial fouling was linked to the humic-like fluorescence region, ultimately the ability of PAC to minimise irreversible fouling was linked to how well it absorbed protein-like fluorescence. Aftab *et al.* (2020) applied combinations of different pre-treatment processes to change the FDOM character of landfill leachate, in order to compare how the resulting organics character ultimately affected NF fouling. This study concluded that both fulvic-like and protein-like fluorescence was more linked to irreversible fouling, than was humic-like fluorescence. In slight contrast to this, Xu *et al.* (2022) concluded that both proteins and humics contributed to the initial pore blocking stage, though the study was conducted on synthetic mixtures and model foulant compounds. Through a combination of size exclusion chromatography and liquid fluorescence EEM analysis, Haberkamp *et al.* (2011) also showed that protein-like fluorescence correlated with the extent of hydraulically irreversible fouling of UF membranes by secondary effluents. In this case, the authors also showed that chemical coagulation and biological sand filtration as pre-treatment were both able to significantly reduce membrane fouling. Furthermore, in a study that investigated dissolved air flotation as a potential pre-treatment for membrane desalination, Shutova *et al.* (2016) showed

that DAF treatment removed higher proportions of humic-like fluorescence than protein-like fluorescence, yet it still was able to remove between 28% and 58% of protein like fluorescence from real water samples.

Table 3 An overview of publications which have utilised fluorescence within membranerelated treatment studies. N.B. MF (Microfiltration); UF (Ultrafiltration); RED (Reverse electrodialysis); CE (cation exchange); AE (anion exchange); MBR (membrane bioreactor); RO (reverse osmosis); AS (Activated sludge); FF (front-face), RA (right angle), PP (peak picking); PARAFAC (Parallel factor analysis); FRI (fluorescence regional integration); FI (fluorescence indices); PCA (principal components analysis); and PLS (partial least squares)

Reference	Application	Method	Highlights or main findings
Kimura *et al.* (2004)	UF	RA + PP	Polysaccharide-like organic matter, Fe and Mn in surface water responsible for irreversible fouling.
Singh *et al.* (2009)	RO	RA + PP	Humic-like fluorescence most appropriate for distinguishing between stage 1 and stage 2 RO.
Tang *et al.* (2010)	MBR	RA + PP	Protein-like fluorescence correlated positively with membrane fouling.
Henderson *et al.* (2011)	UF	RA + PP/ PARAFAC	Tyrosine-like fluorescence monitoring could be used as an indicator of fouling potential from domestic wastewater.
Bagastyo *et al.* (2011)	RO	RA + PP	Advanced oxidation of RO concentrates more efficient than coagulation & MIEX adsorption.
Haberkamp *et al.* (2011)	UF	RA + PP	Removal of protein-like substances by sand filtration or coagulation resulted in reduced irreversible fouling.
Galinha *et al*. (2011)	MBR	RA/FF + PLS	3 fluorescence components could be used to predict COD concentration in MBR permeate.
Pype *et al.* (2013)	RO	RA + FRI	Fluorescence proposed as surrogate for pathogen removal in RO systems.
Singh *et al.* (2015)	RO	RA + PP	Peak C linked to TMP/fouling.
Shutova *et al.* (2016)	RO	RA + PARAFAC	Humics concentration used to determine optimal coagulant dose.
Pawlowski *et al.* (2016)	RED - CE/AE	FF / RA + PCA	Fluorescence able to monitor fouling development of ion-exchange membrane surfaces.
Vera *et al*. (2017)	UF/RO	RA + PARAFAC	Quantified OM removals through treatment plant, monitoring FDOM composition can optimise treatment conditions due to seasonal variation.
Wang *et al.* (2017)	UF	RA + PP	Humic-like substances contributed to initial membrane fouling, protein-like correlated with irreversible fouling.
Cai *et al*. (2017)	MBR	RA + PARAFAC	Protein-like substances more readily biodegradable than humic-like substances.

Experimental Methods for Membrane Applications

Reference	Application	Method	Highlights or main findings
Jacquin *et al*. (2017)	MBR	RA + FRI	Correlations established between LC-OCD-OND and EEM data to quantify protein-like and humic-like substances.
Xiao *et al*. (2018)	MBR	RA + FRI	Identified correlations between characteristic EEM wavelength regions and hydrophobic/hydrophilic DOM components.
Yamamura *et al.* (2019)	MF	FF / RA + PP	Proteinaceous substances responsible for reversible and irreversible fouling, gels mainly contributed to irreversible fouling.
Yu *et al.* (2019)	UF	FF/RA + PARAFAC	FF-EEM method superior to RA-EEM coupled with mass balance for UF foulant determination.
Aftab *et al.* (2020)	NF	RA + PARAFAC	Different pre-treatment led to different quantities and qualities of membrane foulants .
Poojamnong *et al.* (2020)	MBR	RA + FRI	Irreversible foulants mainly comprised of protein-like substances.
Yu *et al*. (2021)	MBR	RA + PP/FI	Combination of protein-like fluorescence and UV280 used to predict fouling MBR potentials.
Xu *et al.* (2022)	UF	RA + PP	Proteins and humics mainly participate in initial pore blocking, polysaccharides mainly participate in later gel/cake layer stage
Yu *et al.* (2022)	AS	FF + PARAFAC	Protein-like substances, NADH, and humic-like substances correlated with MLVSS, intracellular NADH, and humic-like substances in SMP, respectively.
Cifuentes-Cabezas *et al*. (2023)	NF	RA/FF + PP	Fluorescence showed different fouling development between different NF membrane products.

The studies that have been mentioned in this section are but a small proportion of the many research applications of fluorescence EEM spectroscopy within membrane treatment systems to date. While they highlight the method as a clear and practical use for measuring organic fouling on membranes, it is nevertheless imperative to keep in mind that only a fraction of OM is fluorescent. Although the fluorescent fraction is typically considered to be representative of OM as a whole, a better overall picture of OM and OM-based fouling will nearly always be attained when it is applied in combination with other analytical tools.

Within the near future, further advances in optical technology and computer processing power look to be the main impetus' behind further development and wider application of fluorescence sensors in membrane systems. As optical sensors become cheaper and more sensitive, and light sources become more stable with higher output and tighter bandwidths (particularly at lower wavelengths) fluorescence spectroscopy will become even more practical and accessible for both research and industry applications.

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