Two-dimensional fluorescence as a fingerprinting tool for monitoring wastewater treatment systems

ARTICLE *in* JOURNAL OF CHEMICAL TECHNOLOGY & BIOTECHNOLOGY · JULY 2011

Impact Factor: 2.35 · DOI: 10.1002/jctb.2613

6 AUTHORS, INCLUDING:

Claudia F Galinha
New University of Lisbon
9 PUBLICATIONS **46** CITATIONS
SEE PROFILE

Gilda Carvalho
New University of Lisbon
56 PUBLICATIONS **1,159** CITATIONS
SEE PROFILE

Giuseppe Guglielmi
Università degli Studi di Trento
18 PUBLICATIONS **445** CITATIONS
SEE PROFILE

Maria A M Reis
New University of Lisbon
162 PUBLICATIONS **3,815** CITATIONS
SEE PROFILE

Research Article

Received: 10 November 2010 Revised: 19 January 2011 Accepted: 14 February 2011 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.2613

Two-dimensional fluorescence as a fingerprinting tool for monitoring wastewater treatment systems

Claudia F. Galinha,[a] Gilda Carvalho,[a,b] Carla A. M. Portugal,[a] Giuseppe Guglielmi,[c,d] Maria A. M. Reis[a] and João G. Crespo[a]*

Abstract

BACKGROUND: The use of two-dimensional (2D) fluorescence for monitoring complex biological systems requires careful assessment of the effect of chemical species present, which may be fluorescent and/or may interfere with the fluorescence response of target fluorophores. Given the complexity of fluorescence data (excitation emission matrices – EEMs), the challenge is how to recover the information embedded into those EEMs that can be related quantitatively with the observed performance of the biological processes under study.

RESULTS: This work shows clearly that interference effects (such as quenching and inner filter effects) occur due to the presence of multiple species in complex biological media, such as natural water matrices, wastewaters and activated sludge. A statistical multivariate analysis is proposed to recover quantitative information from 2D fluorescence data, correlating EEMs with the observed performance. A selected case study is discussed, where 2D fluorescence spectra obtained from the effluent of a membrane bioreactor were compressed using PARAFAC and successfully correlated with the effluent chemical oxygen demand, using projection to latent structures modelling.

CONCLUSION: This study demonstrates the potential of using 2D fluorescence spectroscopy as a status fingerprint. Additionally, it is shown how statistical multivariate data analysis can be used to correlate EEMs with selected performance parameters for monitoring of biological systems.

Keywords: 2D fluorescence spectroscopy; monitoring of biological processes; wastewater treatment; quenching and inner filter effects; multivariate statistical modelling

INTRODUCTION

Fluorescence spectroscopy is a highly sensitive and non-invasive technique that can be used *in situ* and on-line through an optical fibre probe. Biological systems contain many natural fluorophores, such as amino acids (tryptophan, tyrosine and phenylalanine), vitamins, coenzymes and aromatic organic matter in general that can be detected by fluorescence spectroscopy, regardless if they are intra- or extra-cellular. Therefore, this technique has great potential for real-time monitoring of biotechnological systems, as was previously demonstrated by Li *et al*. and Li and Humphrey.[1,2] In those studies, the fluorescence signal obtained at specific excitation wavelengths was used to measure key fluorophores in biological media. A broader approach consists of scanning a sample with simultaneous variation of the excitation and emission wavelengths, i.e. two-dimensional (2D) fluorescence. The resulting excitation-emission matrices (EEMs) capture information about the presence of multiple substrate components and microbial products, and thus can be seen as a fingerprint of the biological system.[3]

The 2D fluorescence signal is complex and subject to interferences due to the presence of multiple molecules in the system. Changes in the fluorescence signal (such as decrease in fluorescence intensity or change of the emission band shape) may be caused by quenching and/or by inner filter effects. Fluorescence quenching affects the fluorescence characteristics of target fluorophores present in the media, and results from the interaction of the fluorophore in the excited state with a (fluorescent or non-fluorescent) quencher, which interferes with the de-excitation process. The extent of the quenching effect will depend on the type and concentration of the quencher.[4] On the other hand, inner filter promoters interfere with the light pathway, e.g. by absorbing

* *Correspondence to: João G. Crespo, REQUIMTE/CQFB, Chemistry Department, Faculdade de Ciências e Tecnologia – Universidade Nova de Lisboa, 2829-516 Caparica, Portugal. E-mail: jgc@dq.fct.unl.pt*

a *REQUIMTE/CQFB, Chemistry Department, Faculdade de Ciências e Tecnologia – Universidade Nova de Lisboa, 2829-516 Caparica, Portugal*

b *Instituto de Biologia Experimental e Tecnológica (IBET), Av. República, Quinta do Marquês (EAN), 2784-505 Oeiras, Portugal*

c *Department of Civil and Environmental Engineering, University of Trento, Via Mesiano, 77-38123 Trento, Italy*

d *(current address): E.T.C. Engineering srl, Via Praga, 7-38121 Trento, Italy*

light either in the same absorbance region or the same emission region as the fluorophores.[4]

Although interference effects hamper the recovery of quantitative information about specific fluorophores, they are an extremely rich source of information if adequate mathematical tools are used to analyse the data. In fact, changes in spectra caused by interference effects can increase the richness of information contained in 2D fluorescence spectroscopy results, providing an overall fingerprint of the physiological state of a biological system. Additionally, EEMs are able to elicit information on the performance of bioreactors under specific process environments, since the fluorescence response is also sensitive to the environmental conditions (pH, ionic strength and salt composition). The challenge is how to recover and use all of this embedded information for systems monitoring and develop appropriate models that correlate input (the fluorescence EEMs) with output data (performance parameters), for process control.

2D fluorescence has been applied for monitoring surface water composition and wastewater systems.[5–9] However, most of those studies did not use fluorescence on-line; the samples were extracted and/or fractioned before fluorescence analysis, and the interferences existing in a complex media were not considered. Recently, Kobbero *et al.*[10] assessed the quenching effects observed in activated sludge samples, and concluded that individual components could not be quantified by direct observation of 2D fluorescence peak intensity. However, no attempt to deconvolute the matrices through modelling techniques was made.

This study aims at demonstrating the applicability of 2D fluorescence for real-time monitoring of biological wastewater treatment systems, by regarding the EEMs as fingerprints that may be mathematically correlated with representative performance parameters. Similar approaches have been previously employed by Scheper and co-workers[3,11–13] as well as by our research group,[14–16] to monitor other biotechnological systems with better defined media and/or biological composition.

The sensitivity of this technique to water and wastewater compositions was assessed in the present study, as well as the occurrence of fluorescence interferences in these media. Multivariate statistical modelling was used to correlate the interlinked information contained in EEMs from a wastewater treatment system with a selected performance indicator (chemical oxygen demand of the treated effluent). Data from a membrane bioreactor (MBR) for wastewater treatment were selected as a case study to illustrate this approach and exemplify how the information obtained by 2D fluorescence spectroscopy can be correlated with performance parameters.

The modelling approach described in this study could be used in the development of expert-control systems. Such control systems may be operated together with on-line 2D fluorescence monitoring, using optical fibres located in defined positions of the biological process, without the need to sample the system periodically and run a high number of time-consuming and laborious analytical procedures. Therefore, real-time control and operator decision-making may be ensured.

MATERIALS AND METHODS

Water and wastewater samples and aqueous solutions used for 2D fluorescence analysis

Surface water was collected from the river Tagus, in Portugal, spring water was collected in Alenquer, Portugal, and domestic wastewater was collected at the entrance of the wastewater treatment plant (WWTP) of Mutela, located in the region of Lisbon (Almada), Portugal. All samples were collected within a 5 week period during autumn. The wastewater sample was previously centrifuged at 10 000g for 10 min at 10 °C. The samples were filtered using a hydrophilic, surfactant-free cellulose acetate filter with 0.20 μm pore diameter (Minisart®, from Sartorius Stedim) to remove microorganisms before fluorescence scanning.

Solutions of commercial humic acids (Sigma, USA) in deionized water were used to confirm the excitation/emission regions typical from these compounds. Excitation-emission maps of a model protein, bovine serum albumin (BSA, Fluka, Switzerland), were obtained in the presence and absence of commercial humic acids (10 mg L^{-1}), in order to test fluorescence interferences caused by the simultaneous presence of humic acids and proteins in media. BSA was employed at concentrations of 10 and 50 mg L^{-1} for these tests. Subsequently, 2D fluorescence maps were obtained for different water samples with and without addition of BSA at the same final concentrations (10 and 50 mg L^{-1}) to assess the interference of natural humic acids in protein fluorescence.

Table 1. Range values of wastewater quality parameters feed to the MBR during operation time

Parameters	Range
Total chemical oxygen demand	151–1862 mg COD L^{-1}
Soluble chemical oxygen demand	17–410 mg COD L^{-1}
Chemical oxygen demand after flocculation	46–467 mg COD L^{-1}
Ammonia	9.1–193 mg N L^{-1}
Nitrite	0.01–9.98 mg N L^{-1}
Nitrate	0.1–26.8 mg N L^{-1}
Organic nitrogen	0.1–118 mg N L^{-1}
Phosphate	0.1–7.35 mg P L^{-1}
Total phosphorus	1.2–33 mg P L^{-1}
Total suspended solids	50–2100 mg L^{-1}
Volatile suspended solids	30–2071 mg L^{-1}

Membrane bioreactor set-up and operation

Pre-screened wastewater, activated sludge and permeate samples were collected from a pilot MBR located in the WWTP of Lavis (Trento), in Italy. The system consisted of an anoxic/aerobic compartment (4.7 m^3 denitrification, 8.7 m^3 nitrification) and a separate membrane tank (1.5 m^3), in which a hydrophilized PVDF ultrafiltration membrane was immersed (GE Zenon ZW500d hollow fibre module; 0.04 μm; 100 m^2). The plant was operated at an average solids retention time of ∼22 days; the mixed liquor suspended solids (MLSS) in the biological tank was around 7.5 kg m^{-3} whereas the MLSS content in the membrane compartment ranged between 10 and 11 kg m^{-3}, with a recirculation ratio of ∼2.5 from the membrane tank to the anoxic one. The range values of wastewater quality characteristics during the MBR operation are shown in Table 1. Samples were taken simultaneously from the feed, the activated sludge recirculation and the permeate, and were immediately analyzed by 2D fluorescence spectroscopy by immersion of an optical fibre probe in a stirred beaker. In order to avoid sedimentation of suspended solids present in wastewater and activated sludge samples, identical stirring conditions were applied to all samples (wastewater, activated sludge and permeate) during the acquisition of fluorescence spectra. In addition, chemical oxygen demand (COD) was determined in the permeate samples according to APHA Standard Methods. COD was selected as a

performance indicator since it is the principal parameter used to characterize effluent quality in WWTPs used for carbon removal. All analyses were carried out in duplicate.

Acquisition of 2D fluorescence spectra

The 2D fluorescence spectra of humic and BSA solutions, surface water, spring water and domestic wastewater from Mutela were obtained with a fluorometer computer-interface Perkin-Elmer LS 50B. The excitation source was a pulsed Xenon UV lamp, and the detector was a gated photomultiplier. Reflection grating monochromators were used on both excitation and emission sides. The scanning speed was 1500 nm min^{-1}; excitation and emission slits were 10 nm. Fluorescence spectra were generated in the range 200 to 600 nm (excitation) and 225 to 625 nm (emission), with an excitation wavelength incrementing step of 10 nm.

The 2D fluorescence spectra obtained from the MBR in the WWTP of Lavis were acquired with a fluorescence spectrophotometer Varian Cary Eclipse equipped with excitation and emission monochromators and coupled to a fluorescence optical fibre bundle probe. The optical fibre bundle contains 294 randomized optical fibres (147 excitation and 147 emission) each with a diameter of 200 μm and a length of 2 m. Fluorescence spectra were generated in an excitation wavelength range 250 to 700 nm and an emission wavelength range between 260 and 710 nm, with an excitation wavelength incrementing step of 10 nm. Fluorescence spectra were obtained using excitation and emission slits of 10 nm and a scan speed of 3000 nm min^{-1}.

Mathematical data deconvolution

The 2D fluorescence spectroscopy measurements result in excitation-emission matrices where each value of fluorescence intensity corresponds to each pair of excitation/emission wavelengths, giving more than 5000 model input variables. In order to reduce the number of variables to introduce in mathematical models, EEMs were vectorized and compacted. Compression was done to decrease the number of variables, co-linearity and noise using PARAFAC (parallel factor analysis), a decomposition method for multi-way data (such as EEMs) that can be considered an extension of the well-known principal component analysis (PCA).[17] PARAFAC was applied using three components, corresponding to >99.5% of variance captured. Projection to latent structures (PLS) were then used to maximize the co-variance between the compacted fluorescence maps of permeate, measured during operation of the pilot MBR, and COD in the permeate. The PLS model was developed using 146 different measurements of 2D fluorescence spectroscopy and COD in the MBR permeate effluent, obtained throughout 10 months of operation. These 146 observations were divided randomly into a training set (75% of the observations, which were used to calibrate the model) and a validation set (25% of the observations, which were used to validate the final model). A model was thus obtained for prediction of the permeate COD through linear correlation of the three new fluorescence input variables (PC1, PC2 and PC3) resulting from EEM compression.

RESULTS AND DISCUSSION

Discriminating water samples of different nature with 2D fluorescence spectroscopy

Three different types of real water samples were analyzed by 2D fluorescence spectroscopy: spring water, surface water, and domestic wastewater. Their spectra showed significant differences between them (Fig. 1). The spring water sample had few components in solution, which is reflected in the low complexity of the EEM, displaying no detectable fluorescence peaks of protein or humic-like compounds in their corresponding regions of the contour maps (at Ex/Em of 280/320–350 nm and Ex/Em of 320–340/420–455 nm regions, respectively). The surface water sample had a fluorescence signal in the humic compounds region, but no detectable proteins. It is also interesting to note the similarity between the surface water fingerprint (Fig. 1(b)) and the humic acid aqueous solution fingerprint (see Fig. 4(b)), which reflects the presence of humic compounds in this natural water, as could be anticipated. Domestic wastewater showed high fluorescence peaks in the protein and the humic-like compounds regions, as expected from such a complex biological matrix. These results clearly show that it is possible to qualitatively distinguish water samples with different nature by comparing the profile and intensities of their fluorescence matrices.

In addition, samples collected from different sampling points of an MBR system (influent wastewater, activated sludge and permeate effluent) were also analyzed by 2D fluorescence spectroscopy. Figures 2 and 3 show the results obtained for the three sampling points for two different days of operation. The EEMs obtained show significant differences between the three types of samples, corresponding to their different characteristics, which confirms the capability of this technique to qualitatively distinguish samples collected in different treatment phases of a WWTP. Although fluorescence spectra have a consistent profile for each sampling point, small differences can be observed between different days. In Figs 2 and 3 these differences are probably related to changes observed in the wastewater composition (e.g. total COD in wastewater changed from 970 mg L^{-1} on day 7 (Fig. 2) to 377 mg L^{-1} on day 23 (Fig. 3)) and operating conditions, such as hydraulic retention time (11.6 and 7.9 h, respectively) and average temperature (16.2 and 13.1 °C, respectively), which affect the system conditions and process performance. From these results it can be seen that 2D fluorescence measurements of influent wastewater, activated sludge and effluent permeate can be used to monitor changes occurring through MBR operation.

The influent wastewater samples had high fluorescence intensity in the excitation/emission regions of humic-like compounds and proteins, as observed for the wastewater analyzed in the first assay (Fig. 1). Permeate samples had lower fluorescence intensity in both regions when compared with the influent wastewater, particularly in the protein region. This observation suggests that small molecules like small peptides, amino acids, and small humic compounds can cross the membranes while the larger molecules (such as proteins) are preferentially retained, which agrees with previously published results.[18] Activated sludge samples showed peaks both in the protein and humic compounds region; however, when compared with wastewater and permeate fluorescence peaks, their intensity was lower than expected, especially in a media with such a high cell concentration and consequently with high amounts of fluorophores. The relatively low fluorescence intensity in activated sludge samples was probably due to the high cell density in the media. High concentrations of cells and other suspended solids (high turbidity), and the colour of the liquid phase itself, increase light scattering and media absorbance. Highly concentrated complex multi-component matrices, such as in the present study, can also lead to inner filter effects. Indeed, other substances present in the mixture may compete with the fluorophores for the incident light or reabsorb emitted light.[4] The overall contribution of these effects explains the reduced fluorescence intensity

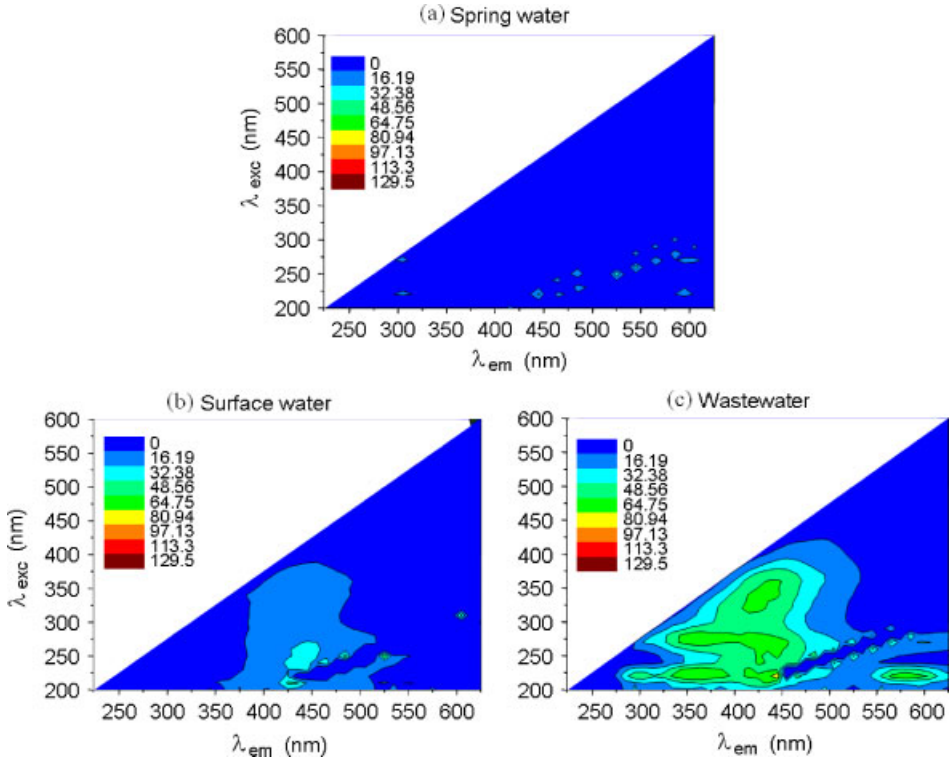

Figure 1. 2D fluorescence spectra of (a) spring water collected in Alenquer, Portugal; (b) surface water collected from the river Tagus, Portugal; and (c) domestic wastewater collected at the entrance of a WWTP in Almada, Portugal.

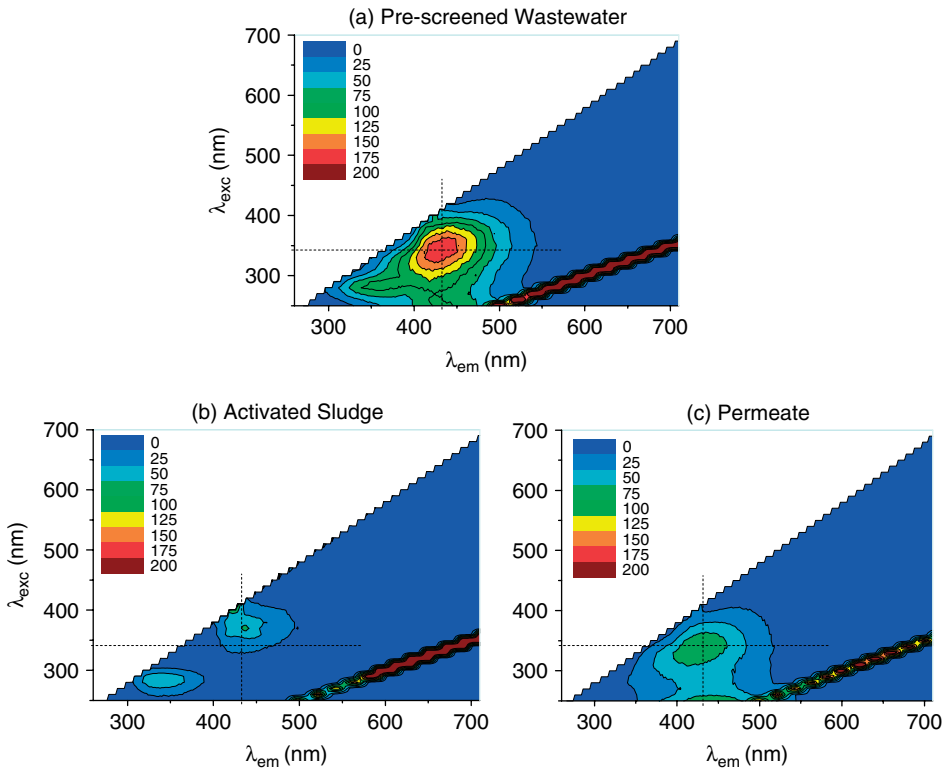

Figure 2. 2D fluorescence spectra of (a) influent pre-screened wastewater, (b) activated sludge, and (c) effluent permeate collected in the WWTP of Lavis in Italy on the 7th day of operation.

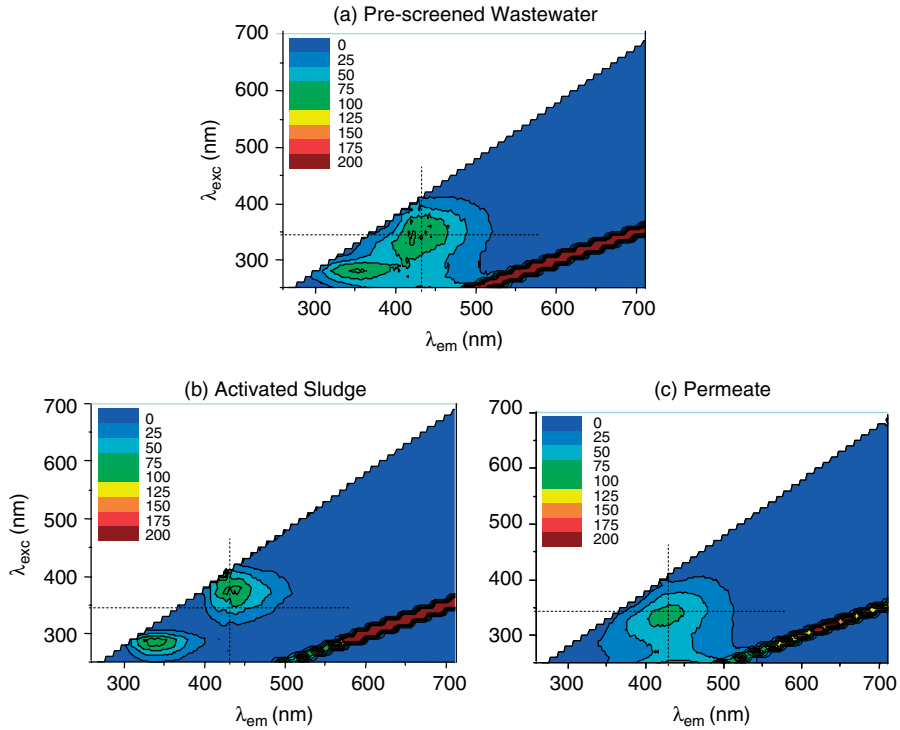

Figure 3. 2D fluorescence spectra of (a) influent pre-screened wastewater, (b) activated sludge, and (c) effluent permeate collected in the WWTP of Lavis in Italy on the 23rd day of operation.

observed for activated sludge samples. Moreover, the humic-like compounds peak revealed a shift in the maximum excitation wavelength (compare Figs 2 and 3 with Fig. 4). This apparent peak shift could be due to a change in composition of the humic compounds fraction, since organic compounds present in the wastewater are degraded, whereas other organics resulting from biological activity are generated. Additionally, fluorescence interference effects may also have a role on this peak shift, since quenching and inner filters effects can interfere with fluorescence intensity in this specific region of the spectra, masking part of the humic substances peak.

The analysis carried out in this study demonstrates the high sensitivity of the 2D fluorescence technique for monitoring water and wastewater treatment processes.

Assessment of interference effects on protein fluorescence spectra

Proteins are one of the key compounds that can be monitored using fluorescence techniques in biological processes such as wastewater treatment systems. However, these systems also possess high amounts of numerous organic and inorganic compounds (e.g. the humic compounds present in the wastewater, oxygen, nitrates) that have been shown to interfere with protein fluorescence spectra.[10,19,20] In the present study, quenching and inner filter effects on protein fluorescence spectra were investigated using a commercial humic acid and a standard protein, bovine serum albumin (BSA). Figure 4a and b show the 2D fluorescence spectra obtained with two independent solutions of BSA and humic acid, both at a concentration of 10 mg L^{-1} (Fig. 4(a) and (b)), displaying the expectable peaks in the protein and in the humic-like compounds regions, respectively. However, when humic acids and BSA were both combined at the same final concentration of 10 mg L^{-1} in a mixed solution, the protein peak was no longer visible (Fig. 4(c)). Protein and humic acid peaks do not overlap in their emission spectra and consequently this cannot be the cause for protein signal disappearance. To investigate the reason for this fact, a UV/visible light absorption spectrum of the humic acids was obtained using a spectrophotometer (data not shown), which showed that humic compounds absorb in a range of wavelengths that includes the region of protein absorption (competing with proteins by excitation light) and in the region of protein emission, absorbing the light emitted by proteins. These effects may explain the disappearance of the protein peak in Fig. 4(c).

In order to assess fluorescence interference effects in natural water sources and wastewater samples, BSA solutions were prepared using domestic wastewater, as well as surface and spring water. Two different concentrations of BSA (10 and 50 mg L^{-1}) were tested in order to understand if the interference effects are constant for each type of water, independently of the concentration of protein. BSA solutions with the same concentration, prepared in deionized water, were used for comparison purposes. The results showed that the presence of other components in water and wastewater samples reduced the protein fluorescence emission of the EEMs obtained, as exemplified in Fig. 5. The fluorescence intensity values at the 280/345 nm excitation/emission pair in these tests are shown in Table 2. The degree of interference of water/wastewater samples on the BSA fluorescence emission was lower than a concentrated humic acids solution, but still induced a decrease in the protein peak intensity of 31–58% (despite the additional contribution of the proteins present in the wastewater to this peak). These interferences were probably due to the presence of humic-like compounds in the water matrices, as revealed by their fluorescence spectra (Fig. 1). The extent of interference varied in these different matrices (Table 2), which was probably due to differences in the concentration and composition

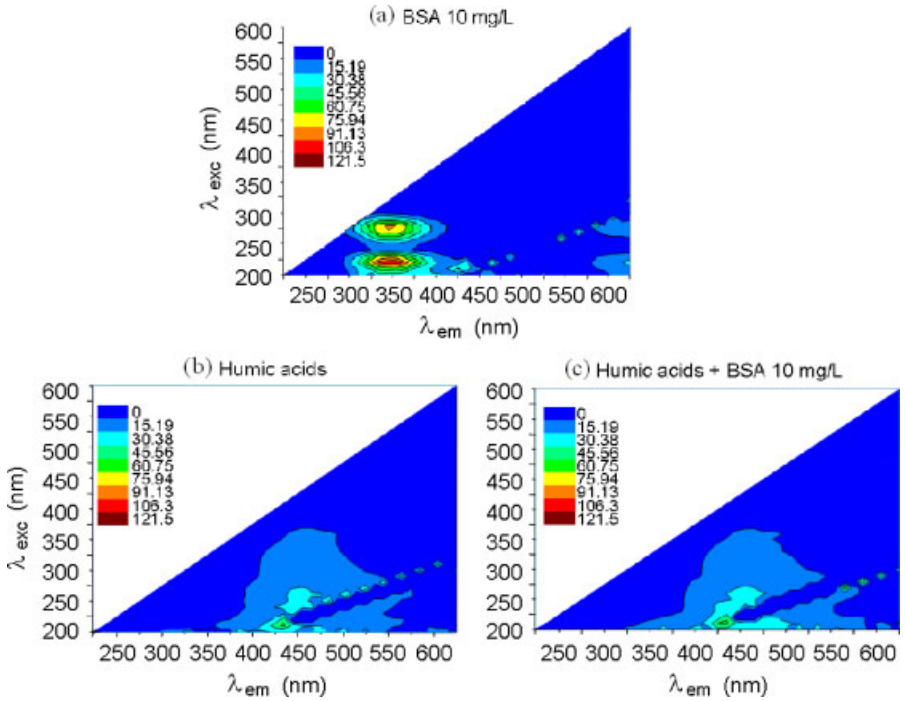

Figure 4. 2D fluorescence spectra of (a) a BSA solution with a concentration of 10 mg L^{-1}, (b) a commercial humic acid solution (10 mg L^{-1}), and (c) a mixed solution of humic acid and BSA, both at 10 mg L^{-1}.

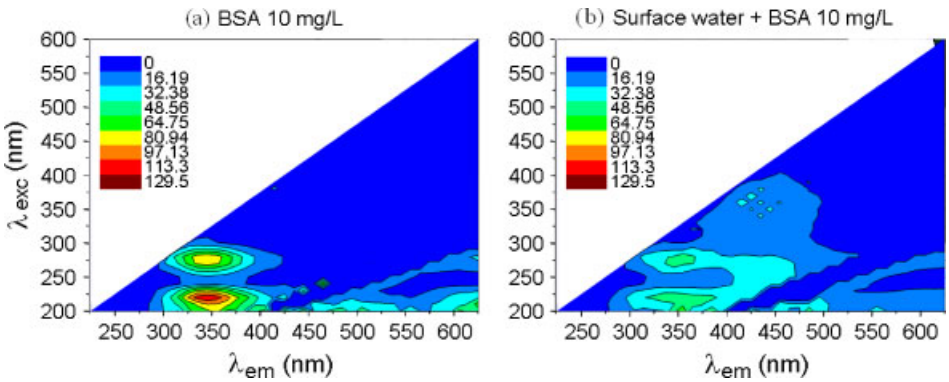

Figure 5. Example of fluorescence interference effects in surface water: EEM of a BSA solution (10 mg L^{-1}) in (a) deionized water and (b) prepared with surface water.

of the humic-like compounds. Indeed, the surface water spectrum showed higher fluorescence intensity than the spring water spectrum in the humic compounds region, and a different contour shape from the wastewater spectrum (Fig. 1). Correspondingly, different interference levels were observed for all three water types (Table 2). Moreover, the results obtained with wastewater showed that the percentage fluorescence decrease was dependent on the protein concentration, reflecting the high complexity of the interference phenomena occurring in such media.

In addition to the humic-like compounds, there are other substances with quenching properties that can be present in wastewater systems, such as dissolved oxygen and heavy metals,[19] which were not investigated in this study. However, it is clear that the complexity of interferences on the fluorescence signal limits the simple and direct quantitative measurement of specific fluorophores in complex biological systems, such as wastewater treatment systems. Nevertheless, fluorescence EEMs may be used as fingerprints, which can be regarded as extremely rich, although complex, data sources. Actually, since fluorescence spectroscopy is highly sensitive to the composition of biological media and to the environmental conditions (*T*, pH, ionic strength), these effects should not be regarded as a problem but, on the contrary, as a source of information. The challenge is to develop methodologies that allow the extraction of this embedded information, correlating 2D fluorescence data with selected performance indicators.

Use of multivariate statistical analysis to extract quantitative information from EEMs

To extract deeper, quantitative information from 2D fluorescence spectra, previous studies used either an approach based on pattern recognition, i.e. artificial neural networks (ANN)[14,15] or PLS modelling[21] to deconvolute EEMs from mixed culture bioreactors. In the present study, PLS modelling was chosen over ANN to describe the data analyzed since the multivariate linear PLS models are less complex than non-linear ANN models, and therefore

Table 2. Decrease in fluorescence intensity at $\lambda_{exc} = 280$ nm and $\lambda_{em} = 345$ nm (maximum emission of BSA) of a standard BSA solution in comparison with solutions prepared with either commercial humic acids (at 10 mg L^{-1}), or real water/wastewater samples

	Fluorescence intensity decrease, %	
	BSA 10 mg L^{-1}	BSA 50 mg L^{-1}
Humic acids	95	96
Surface water	52	57
Spring water	33	31
Wastewater	37	58

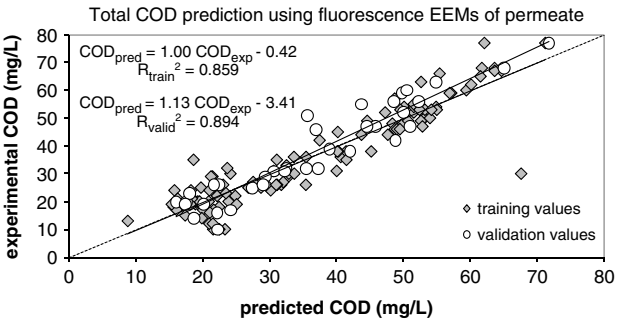

Figure 6. Observed values of COD in the permeate represented vs the corresponding values predicted by a PLS model developed with permeate EEM data.

minimize over-fitting of the data points.[22] Previous compression of fluorescence spectra with PARAFAC is an important step, since it translates fluorescence EEM information into a lower number of new orthogonal variables. These new variables describe data contained in the spectra while eliminating noise, and are each a combination of the information present in different regions of the EEMs.

2D fluorescence data of a MBR for municipal wastewater treatment was selected to illustrate this approach. 2D fluorescence spectroscopy has previously been applied for monitoring MBRs[9,18] aiming at the detection of single specific compounds. In this study, the complete EEMs obtained in a MBR were used as fingerprints, which were correlated with a selected performance indicator (permeate COD) through multivariate statistical analysis.

A PLS model was developed to predict COD in the permeate, as a function of the compressed permeate EEMs (PC1, PC2 and PC3).

$$[\mathrm{COD}] = -0.198\,\mathrm{PC1} - 0.887\,\mathrm{PC2} + 0.198\,\mathrm{PC3} \qquad (1)$$

The model obtained (Equation (1)) shows good fitting for both training and validation sets of data, as can be seen from Fig. 6 where experimentally observed values are plotted against predicted values. Regression coefficients (R^2) were 0.86 and 0.89, respectively, for training and validation, with slopes of approximately 1, and a root mean square error of prediction (RMSEP) of 6.0 mg COD L^{-1}, which is approximately 8% of the maximum measured COD. The COD variance captured by the three compressed inputs was 86%. This value shows that the model was able to predict the experimental COD values while excluding noise.

This example demonstrates that multivariate statistical models can adequately correlate EEM information to predict physical parameters associated with the system performance.

CONCLUSIONS

2D fluorescence can be applied for monitoring biological systems because of the ability of this technique to distinguish matrices with different compositions. 2D fluorescence data can be acquired in multiple locations of the system (off-line or on-line, according to specific needs), and time-programmed with the help of an optical 'switch-box'. Considering that the acquisition of a complete fluorescence map takes only a few minutes (depending on the number of data points), this tool can be used as an on-line, non-invasive, real-time monitoring technique. The challenge, however, remains on the ability of integrating this information in quantitative models, where fluorescence data are related with relevant process performance parameters determined independently.

To solve this problem, the EEMs can be vectorized and introduced as input information in multivariate statistical models, namely using PLS tools. Through this procedure, 2D fluorescence input data (that characterize influent, effluent and biological media) can be correlated with relevant output variables (e.g. effluent quality).

The approach presented in this paper has high potential for several reasons: (1) 2D fluorescence monitoring of biological systems is simple and fast; other traditional characterization methods, which are laborious and slow, may be totally replaced by this technique; (2) the multivariate statistical model developed may be continuously improved with new data; (3) this modelling approach could be implemented within expert-control systems; the data captured by the fluorescence fingerprints could then be used to rapidly evaluate the system status and support a real-time adjustment of the operating conditions.

ACKNOWLEDGEMENTS

The authors thank Rui Oliveira for his advice in modelling strategies.

This work was funded by the EUROMBRA research project (Contract No. 018480), under the 6th Framework Programme of the European Commission. FCT (Portugal) is thankfully acknowledged for the project PTDC/EBB-EBI/098862/2008 and grants SFRH/BD/30253/2006 and SFRH/BPD/30800/ 2006.

REFERENCES

1 Li JK, Asali EC, Humphrey AE and Horvath JJ, Monitoring cell concentration and activity by multiple excitation fluorometry. *Biotechnol Prog* **7**:21–27 (1991).
2 Li JK and Humphrey AE, Use of fluorometry for monitoring and control of a bioreactor. *Biotechnol Bioeng* **37**:1043–1049 (1991).
3 Marose S, Lindemann C and Scheper T, Two-dimensional fluorescence spectroscopy: a new tool for on-line bioprocess monitoring. *Biotechnol Prog* **14**:63–74 (1998).
4 Valeur B, *Molecular Fluorescence: Principles and Applications*. Wiley-VCH, Germany (2002).
5 Her N, Amy G, McKnight D, Sohn J and Yoon Y, Characterization of DOM as a function of MW by fluorescence EEM and HPLC-SEC using UVA, DOC, and fluorescence detection. *Water Res* **37**:4295–4303 (2003).
6 Lee N, Amy G and Croué JP, Low-pressure membrane (MF/UF) fouling associated with allochthonous versus autochthonous natural organic matter. *Water Res* **40**:2357–2368 (2006).
7 Wang Z, Wu Z and Tang S, Extracellular polymeric substances (EPS) properties and their effects on membrane fouling in a submerged membrane bioreactor. *Water Res* **43**:2504–2512 (2009).
8 Kimura K, Naruse T and Watanabe Y, Changes in characteristics of soluble microbial products in membrane bioreactors associated with different solid retention times: Relation to membrane fouling. *Water Res* **43**:1033–1039 (2009).

9 Tang S, Wang Z, Wu Z and Zhou Q, Role of dissolved organic matters (DOM) in membrane fouling of membrane bioreactors for municipal wastewater treatment. *J Hazard Mater* **178**:377–384 (2010).
10 Kobbero C, Keiding K, Larsen KL and Nielsen PH, Quenching effects in the application of multi-channel fluorescence in activated sludge suspended solids. *Water Res* **42**:2449–2456 (2008).
11 Boehl D, Solle D, Hitzmann B and Scheper T, Chemometric modelling with two-dimensional fluorescence data for *Claviceps purpurea* bioprocess characterization. *J Biotechnol* **105**:179–188 (2003).
12 Surribas A, Geissler D, Gierse A, Scheper T, Hitzmann B, Montesinos JL, *et al*, State variables monitoring by *in situ* multi-wavelength fluorescence spectroscopy in heterologous protein production by *Pichia pastoris*. *J Biotechnol* **124**:412–419 (2006).
13 Ganzlin M, Marose S, Lu X, Hitzmann B, Scheper T and Rinas U, *In situ* multi-wavelength fluorescence spectroscopy as effective tool to simultaneously monitor spore germination, metabolic activity and quantitative protein production in recombinant *Aspergillus niger* fed-batch cultures. *J Biotechnol* **132**:461–468 (2007).
14 Wolf G, Almeida JS, Pinheiro C, Correia V, Rodrigues C, Reis MAM, *et al*, Two-dimensional fluorometry coupled with artificial neural networks: a novel method for on-line monitoring of complex biological processes. *Biotechnol Bioeng* **72**:297–306 (2001).
15 Wolf G, Almeida JS, Crespo JG and Reis MA, An improved method for two-dimensional fluorescence monitoring of complex bioreactors. *J Biotechnol* **128**:801–12 (2007).
16 Teixeira AP, Portugal CAM, Carinhas N, Dias JML, Crespo JP, Alves PM, *et al*, *In situ* 2D fluorometry and chemometric monitoring of mammalian cell cultures. *Biotechnol Bioeng* **102**:1098–1106 (2009).
17 Bro R, PARAFAC. Tutorial and applications. *Chemometr Intell Lab* **38**:149–171 (1997).
18 Teychene B, Guigui C, Cabassud C and Amy G, Toward a better identification of foulant species in MBR processes. *Desalination* **231**:27–34 (2008).
19 Lakowicz J R, *Principles of Fluorescence Spectroscopy*. Plenum Press, New York (1983).
20 Ricci RW and Nesta JM, Inter- and intramolecular quenching of indole fluorescence by carbonyl compounds. *J Phys Chem* **80**:974–980 (1976).
21 Morel E, Santamaria K, Perrier M, Guiot SR and Tartakovsky B, Application of multi-wavelength fluorometry for on-line monitoring of an anaerobic digestion process. *Water Res* **38**:3287–3296 (2004).
22 Chan ZSH, Ngan HW, Rad AB, David AK and Kasabov N, Short-term ANN load forecasting from limited data using generalization learning strategies. *Neurocomputing* **70**:409–419 (2006).

