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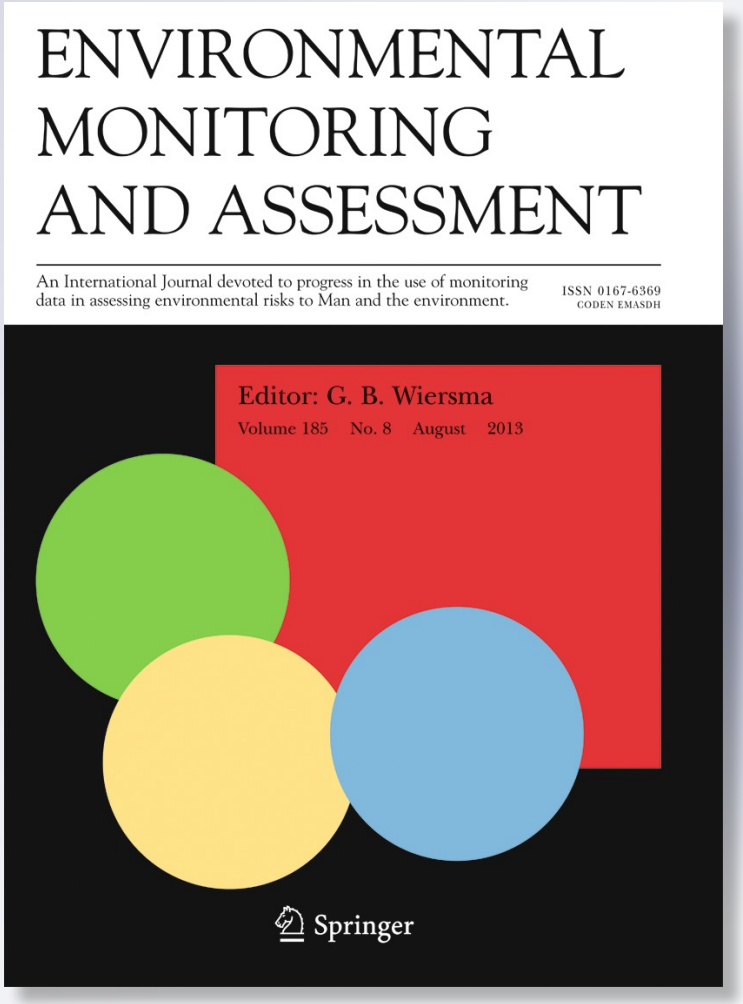

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Abstract Natural and contaminated waters of the final reaches of the Chubut River (Patagonia, Argentina) were studied to obtain information about river organic matter and effects of domestic and industrial discharges (fishery effluents and sewages). Fluorescence Excitation–Emission Matrices (EEMs) were obtained from samples only filtered (0.45 μm) and diluted, if necessary, to avoid the inner filter effect. In addition, physicochemical parameters were measured to know the quality of the water and the effluents. Results show that EEMs allow a rapid and simple control of the effluents from fisheries and domestic sewage in Chubut River estuary, necessary to take management decisions.

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Keywords Dissolved organic matter · Excitation–emission matrices · Chemometric analysis · Chubut River

Introduction

The unrestricted use of rivers and estuaries for the discharge of different types of effluents can generate adverse effects such as the phenomenon of eutrophication. Eutrophication caused by human activities causes: increased nitrogen and phosphorus concentrations, increased primary productivity, dissolved oxygen depletion, increased turbidity (decreased light penetration), and development of algal blooms detrimental to marine life (Gomoiu 1992; Aubert 1992).

In Chubut Province (Patagonia, Argentina), the Chubut River, the main freshwater source, is used for electric power generation (Florentino Ameghino Dam), irrigation, and drinking water supply of important cities such as Dolavon, Gaiman, Trelew, and Rawson, which also discharge their wastewater with diverse dissolved organic matter (DOM) contents.

Fluorescence spectroscopy is an important tool for DOM characterization. This methodology has increased its use with the development of excitation–emission matrices (EEMs), mainly in studies of complex systems (Baker and Spencer 2004; Cammack et al. 2004), where the emission spectrum may differ from the absorption one (De Souza-Sierra et al.

1994; Coble 1996) and the maximum emission wavelength is dependent on the excitation wavelength. EEMs show the pair of wavelengths, or range of wavelengths, where the excitation and emission are highest (Ex_{max}/Em_{max}). This is the fluorophore characteristic parameter and has been applied to DOM analysis of diverse aquatic systems (Galapate et al. 1998; Baker et al. 2003; Reynolds 2003).

In the Chubut River, only one study (Scapini et al. 2010) has applied EEMs in DOM research. However, this study was focused on extracted humic compounds and not on natural water samples without a previous concentration. Isolation, concentration, and purification operations involve laborious processing of large volumes of water. In contrast, the present work is centered on the direct analysis of the natural waters of the river and of the urban effluents with the aim of obtaining information about of the Chubut River DOM and the effects of the discharges by comparing the freshwater EEMs with those of the waters impacted by effluent inputs. It was also the aim of this work to examine the state of the Chubut River in its estuary with this analytical tool.

Materials and methods

Study area: sampling sites

Table 1 and Fig. 1 show the geographic locations of the sampling sites obtained by Global Positioning System (GPS) in the Chubut River area, with their corresponding names. A first sampling site (drinking water treatment plant of Trelew City, WTP), located upstream of all the discharges studied, was taken as reference. Two types of effluents were sampled previously to their discharges in the river: the effluent of a fishery (FE sampling site) and the treated effluent of the urban sewage treatment plant of Rawson City (SE sampling site). The river waters were also sampled in three sites: two placed immediately downstream of the mentioned discharges, called Chubut River-fishery effluent (CHR-FE) and Chubut River-sewage effluent (CHR-SE), and one placed immediately upstream of the sewage discharge, called Chubut River-New Bridge.

Discrete samples of each effluent before their discharge and composed samples in transects crossing the river (right, center, and left) were taken at a depth not less than 60 cm. All samples and their duplicates were immediately transferred (protected from light and kept at 4 °C) to the laboratory and processed immediately.

Physical and chemical parameters

Temperature (T, graduated 0.5 °C mercury thermometer), the flow of each discharge (by chronometer and volume measures) were determined in situ. Conductivity (χ) 25 °C determinations were made using a WTW LF90 conductimeter; salinity with a Plessey salinometer; pH with a Hanna HI 8519N pH meter, using an I1332 electrode; and suspended solids (SS) and settled solids (SeS) were measured according to the Standard Methods 2540D and 2540F (APHA-AWWA-WEF 1998), respectively, in unfiltered samples. Chemical Oxygen Demand (COD) determinations were made using DR2010 spectrophotometrically after closed digestion with potassium dichromate (Hach, Method 8000).

Aliquots of samples were grossly filtered through a glass fiber filter (Whatman GF/C) and then through a Millipore nitrocellulose membrane with a pore diameter of 0.45 μm, previously washed with 100 ml portions of distilled water until no significant absorbance between 200 and 900 nm (10 cm quartz cell) was observed. These aliquots were used to obtain the absorbance at 250 nm (A_{250}) as an organic matter indicator (Conzonno and Fernández Cirelli 1987/8; Bloom and Leenheer 1989; Liebezeit 2000) with a Metrolab 1700 UV–VIS spectrometer with SF 170 software and quartz cells of 1 cm of path length for the analysis of effluent samples and of 10 cm of path length for the other samples. Statistical analysis was performed by means of Excel 2003 and Sigma Plot 8 programs.

Excitation–emission matrices

The EEMs were obtained from filtered aliquots (Whatman GF/C and Millipore nitrocellulose membrane of 0.45 μm) as detailed below. Samples with high absorbance were diluted (the sewage effluent was diluted 1/10 and the fishery effluent 1/20) with distilled water so that absorbance was reduced to 0.02 to avoid inner filter effects in fluorescence measurements.

EEMs were obtained, in a first step, from concatenating emission spectra (between 280 and 700 nm)

Table 1 Geographic location and physicochemical parameters (*COD* Chemical Oxygen Demand, A_{250} absorbance at 250 nm with 1 cm of path length, χ conductivity, *SeS* settled solids, *SS* suspended solids, *T* temperature, *Q* flow) of the sampling sites (*WTP* water treatment plant, *FE* fish effluent, *CHR-FE* Chubut River-fish effluent, *CHR-NB* Chubut River-New Bridge, *SE* sewage effluent, *CHR-SE* Chubut River-sewage effluent) and bibliographic data of sewage effluent (Metcalf and Eddy 1995), fish effluent (Morry et al. 2003), and Chubut River (Scapini et al. 2011)

Sampling site	COD (mgL^{-1})	A_{250}	χ 25 °C ($\mu S\ cm^{-1}$)	pH	SeS (mlL^{-1})	SS (gL^{-1})	*T* (°C)	*Q* (m^3h^{-1})	Geographic location
WTP	4	0.068	310	7.9		0.073	22		43° 16′ 33″ S 65° 16′ 25″ W
FE	1,180	0.625	1,558	6.9	0.8	0.210	27	2.9	43° 20′60″ S 65° 15′33″ W
CHR-FE	7	0.069	335	7.5		0.328	21		43° 32′08″ S 65° 11′45″ W
CHR-NB	5	0.099	323	7.6		0.320	23		43° 17′54″ S 65° 06′17″ W
SE	65	0.200	915	7.5	0.2	0.193	25	72	43° 18′69″ S 65° 04′56″ W
CHR-SE	6	0.071	525	7.9		0.372	23		43° 20′45″ S 65° 10′35″W
Sewage effluent[a]	500	Wi	wi	7–8	10	220–500	<45		
Fish effluent[b]	999	Wi	800	8	3.82	Wi	<45		
Chubut River[c]	8.9	0.539	260	7.7		14	11.2	54[d]	

wi without information,

[a] Metcalf and Eddy (1995)

[b] Morry et al. (2003)

[c] Scapini et al. (2011)

[d] $m^3\ s^{-1}$

recorded at different excitation wavelengths (220 to 520 nm) separated by 1 nm in both the excitation and emission dimensions. From these spectra, matrices were constructed by processing them with the Sigma Plot software version 8. Each matrix was corrected by subtracting a blank matrix recorded for distilled water and obtained under the same conditions. A Shimadzu RFPC-530 spectrofluorometer with quartz cells of 1 cm path length was used for these preliminary measurements. Final EEMs were obtained using a Varian Eclipse spectroluminometer, using quartz cuvettes of 1 cm in the excitation range from 200 nm to 482 nm and an emission range from 280 to 700 nm every 3 and 5 nm for excitation and emission, respectively.

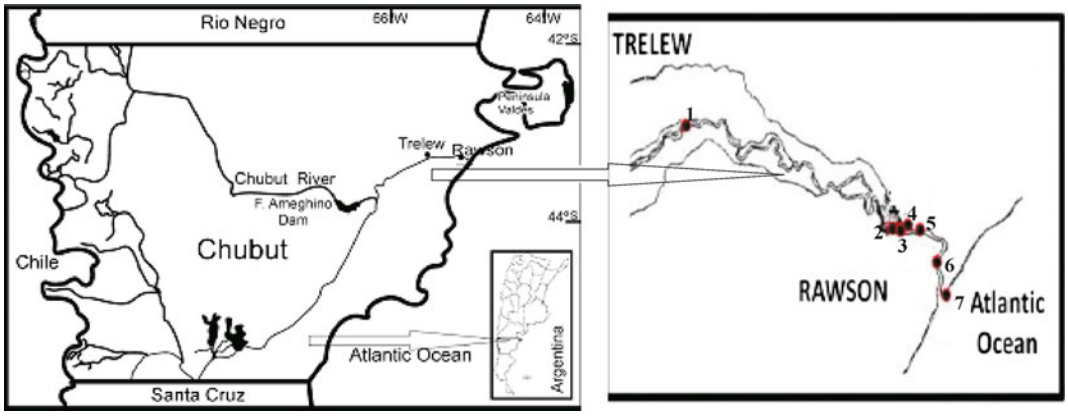

Fig. 1 Map of Chubut Province (Argentina) and the restricted study area, showing the locations of the sampling sites: *1* Trelew City treatment plant for drinking water (reference sampling site), *2* fishery effluent, *3* Chubut River downstream of fishery effluent input, *4* Chubut River in New Bridge upstream of sewage effluent input, *5* Rawson City sewage effluent, *6* Chubut River downstream of sewage effluent input

These matrices were then processed with Sigma Plot version 8. Excitation and emission wavelengths at maximum fluorescence intensities (Ex_{max}/Em_{max}) were obtained from the contour plots of the emission wavelength vs. excitation wavelength.

EEMs deconvolution

The multivariate curve resolution-alternating least squares (MCR-ALS) algorithm was used to decompose each single EEM into excitation and emission spectra for the detected components (Tauler et al. 1995). If a given EEM (D) is of size $J \times K$, where J is the number of data points in the excitation mode and K is the number of data points in the emission mode, the bilinear decomposition of the matrix is performed according to the expression:

$$D = S_{exc} S_{em}{}^{T} + E \quad (1)$$

where the columns of S_{exc} contain the spectral excitation profiles of the intervening species, the columns of S_{em} their related emission spectra, and E is a matrix of residuals not fitted by the model.

The iterative ALS procedure aims at minimizing the Frobenius norm of E and is initialized using an initial estimation of the spectral or concentration profiles for each intervening species. If the initial estimations are the spectral profiles in the emission dimension, the unconstrained least-squares solution for the concentration profiles can be calculated from the expression:

$$S_{exc} = D\left(S_{em}{}^{T}\right)^{+} \quad (2)$$

where $(S_{em}{}^{T})^{+}$ is the pseudoinverse of the spectral matrix $S_{em}{}^{T}$, which is equal to $[S_{em}(S_{em}{}^{T}S_{em})^{-1}]$ when $S_{em}{}^{T}$ is full rank. If the initial estimations were the concentration profiles, the unconstrained least-squares solution for the spectra can be calculated from the expression:

$$S_{em}{}^{T} = S_{exc}{}^{+} D \quad (3)$$

where $S_{exc}{}^{+}$ is the pseudoinverse of S_{exc} $[S_{exc}{+}= (S_{exc}{}^{T}S_{exc})^{-1}S_{exc}{}^{T}]$, when S_{exc} is full rank. Both steps can be implemented in an alternating least-squares cycle, so that in each iteration, new S_{exc} and $S_{em}{}^{T}$ matrices are obtained.

During the iterative recalculations of S_{exc} and $S_{em}{}^{T}$, a series of constraints are applied to improve these solutions, to give them a physical meaning, and to limit their possible number for the same data fitting, i.e., to avoid the so-called rotationally ambiguous solutions. The MCR-ALS package permits the following restrictions during optimization: (1) non-negativitiy in either data mode, which was applied in the present case to the spectral profiles in both modes because the fluorescence spectra of the chemical species are always positive values or zero; (2) unimodality, which ensures the presence of a single maximum and is useful for chromatograms (this restriction was not applied in the present case); and (3) closure, which is regularly applied when two or more components are species involved in an acid–base process or in a chemical reaction, i.e., they form a closed system (this restriction was not applied either to the present problem). In sum, with non-negativity restrictions and a good initialization procedure, the MCR-ALS solutions for the present case were satisfactory. Iterations continue until an optimal solution is obtained that fulfills the postulated constraints and the established convergence criteria.

Results and discussion

Environmental quality parameters

Physicochemical parameters were measured for each sampling site in order to know the quality of river waters and wastewaters. Table 1 shows the obtained results and literature data for comparison. Chubut River natural waters are neutral or slightly alkaline, slightly mineralized, low in organic matter and SS (Scapini et al. 2011). The river water in the sites sampled showed similar characteristics with the exception of total solids and SS, which were increased at the reference site and downstream, indicating the existence of previous erosion phenomena of particulate material.

The COD of the sewage effluent from the treatment plant of Rawson City was lower than expected, showing a good level of treatment. The plant is a biological secondary treatment plant (activated sludge) composed of gross and fine screenings, aeration tanks, secondary clarifiers, and disinfection units (chlorination). The sewage effluent is composed of a heterogeneous mixture of

compounds, including fulvic acids, proteins, carbohydrates, lipids, organic surfactants, nucleic acids, and fatty acids (Ahmad and Reynolds 1995).

In contrast, the fishery effluent showed a high COD value that agrees with the A_{250} values, the high settled, suspended, and dissolved solids (estimated from conductivity) but a lower flow than the urban effluent and then a lesser impact on the receiving waters. This can be concluded from the low growth of the river COD and A_{250} downstream of this discharge (Table 1). Fish plant effluents are generally untreated except for fine screening prior to the discharge from the plant, as the fishery effluent studied here. In addition, they are characterized by large amounts of organic matter and suspended particulate, high levels of organic nitrogen and phosphorus, high COD, and a characteristic grayish color. Organic pollutants consist of carbohydrates, proteins, detergents, high fat content, and a fraction of tryptophan product of biodegradable materials (Morry et al. 2003).

Fluorescence analysis

The DOM from different sources can be distinguished by fluorescence EEMs (Coble 1996; Baker. 2002; Clark et al. 2002). For example, the fluorescence of amino acids (primarily tryptophan and tyrosine) has been used to detect the presence of proteins in aquatic systems. In these environments, distinct classes of fluorophores are generally considered: Coble (1996), for example, referred to two humiclike fluorophores and two proteinlike fluorophores (tyrosinelike and tryptophanlike fluorophores). Peaks of humic compounds have the same range of emission maxima (420–450 nm) but differ in the excitation maxima because one peak is stimulated at 230–260 nm and the other at 320–350 nm. The first peak is called A and the second one C. Proteinlike fluorophores are B and T. These fluorophores are designed by letters because, as stated by Komada et al. (2002), 'the true nature of the fluorophores responsible for DOM fluorescence in natural waters is unknown.'

Table 2 shows the values of Ex_{max}/Em_{max} obtained from our EEMs and those from the literature for comparison, using Coble's (1996) nomenclature. The contour plots of the EEMs obtained for each sampling site are presented in Fig. 2 with black circles that show the Ex_{max}/Em_{max} of fluorophores according to Coble (1996).

The reference site showed Ex_{max} /Em_{max} coincident with that of peak A (Fig. 2a). This is in agreement with a previous work (Scapini et al. 2010), where we reported peak A as the principal component responsible for the fluorescence of nonimpacted waters in Chubut River. Also, in agreement with our previous findings, the SS values obtained in the waters of this site were higher than the mean. The fluorescent DOM of freshwaters contains mainly fluorophores A and C of humiclike substances (Katsuyama and Nobuhito 2002).

The sewage effluent showed greater intensity of fluorophore C (Fig. 2e), which is the peak mainly related to humic substances (HSs) of allochthonous origin (Chen et al. 2004). In contrast, the fishery effluent presented larger intensities of fluorophores B and T (Fig. 2b), corresponding to the proteinlike substances, and lower intensities in areas of peaks A and C, corresponding to humiclike substances. During EEMs studies conducted in untreated sewage, a fraction of humic acids and protein fractions, corresponding to the amino acids tyrosine and tryptophan, usually appear (Baker and Spencer 2004).

The EEMs of waters downstream of the fishery and sewage discharges into the receiving body were similar to those of the reference site, except that the proteinlike fluorophores were more intense (Fig. 2c and f). This seems not only to indicate that the DOM of the impacted water of the Chubut River contains mainly humic compounds but also that the discharges of the type studied can be visualized by using fluorescence EEMs. The presence of these amino acids is common in waters with anthropogenic influence, such as bays, estuaries, coastal areas with high primary productivity, bacterial activity in water, and effluent discharge areas, as in our working area (Yamashita and Tanoue 2003). Modifications from natural, humic-rich upstream waters to 'peak-T'-rich downstream waters with growing anthropogenic inputs have been reported (Baker and Spencer 2004).

It is also important to emphasize that the results obtained in this study agree with those obtained by some authors who believe that tryptophan fundamentally occurs with high intensity in the EEMs because it is the result of anthropogenic material in raw water and untreated effluent or with primary treatment

Table 2 Ex_{max}/Em_{max} obtained from each sampling site (*WTP* water treatment plant, *FE* fish effluent, *CHR-FE* Chubut River-fish effluent, *CHR-NB* Chubut River-New Bridge, *SE* sewage effluent, *CHR-SE* Chubut River-sewage effluent) with that of literature (Coble 1996) for comparison and deconvolution results

Sampling site/ type of fluorophores	Peak	Ex_{max} (nm)/Em_{max} (nm)	MCR-ALS	
			Component–peak	Explained variance (%)
WTP	A	237–260/400–500	1–A	98
FE	A, B, C, and T	220–300/250–500	1–T	74.9
			2–A	9.3
CHR-FE	A and T	237–260/400–500	1–A	86.1
			2–T	0.3
CHR-NB	A	237–260/400–500	1–A	97
SE	A, B, C, and T	220–380/250–500	1–C	98.7
			2–T	1.2
CHR-SE	A	237–260/400–500	1–A	99.8
			2–T	0.12
Humiclike	A[a] UV	250–260/380–480		
	C[a] Visible	330–350/420–480		
Proteinlike	B[a] Tyrosinelike	270–280/300–320		
	T[a] Tryptophanlike	270–280/320–350		

[a]Coble (1996)

(Galapate et al. 1998; Baker and Spencer 2004). Other authors associate the presence of this amino acid with the growth of bacterial communities (Elliott et al. 2006). The fluorescence of proteins in aquatic environments is the result of a mixture of autochthonous and allochthonous sources. These amino acids were expected in fishery effluents.

All river waters showed fluorophore A and a scarcely visible fluorophore C. It must be stressed that these two fluorophores correspond to humiclike substances. HSs have properties with significant environmental effects such as: forming complexes with metal ions (Spitzy and Leenheer 1988; Kevin et al. 1997); influencing the cycle, transport, and bioavailability of metals; associating with organic pollutants (Mastrangelo et al. 2005) such as pesticides and polynuclear aromatic hydrocarbons; affecting the growth of algae and bacteria (Conzonno and Fernández Cirelli 1988); generating carcinogenic disinfection products in the chlorination of drinking water (Fujii et al. 1998); and affecting the stability of colloids (Stuermer and Payne 1976).

The last three properties are important for the waters of the discharge area. Since this area has potential recreational use but is not used as a source of drinking water, its use should be restricted to navigation, and swimming should be completely prevented.

Regarding the relationship between HSs and the growth of algae in the Chubut River, there are studies that show the inappropriate growth (Chiarandini and Santinelli 2004) of a particular algal species (*Aulacoseira granulata*) and species of dinoflagellates, both responsible for harmful algal blooms (Sastre et al. 1994).

As to the formation of carcinogenic disinfection products, previous studies have found that the levels of trihalomethanes are below the limits accepted by the United States Environment Protection Agency (Chiarandini, personal communication), which in turn, coincide with those established by the Trelew Regulator Office. However, further studies are necessary because of the significance of this issue on the quality of raw water.

Multivariate curve resolution-alternating least squares algorithm

The MCR-ALS model allows differentiating components present in natural complex samples (Esteves Da Silva et al. 2006). Fluorophores A and C, which have the same emission maxima but

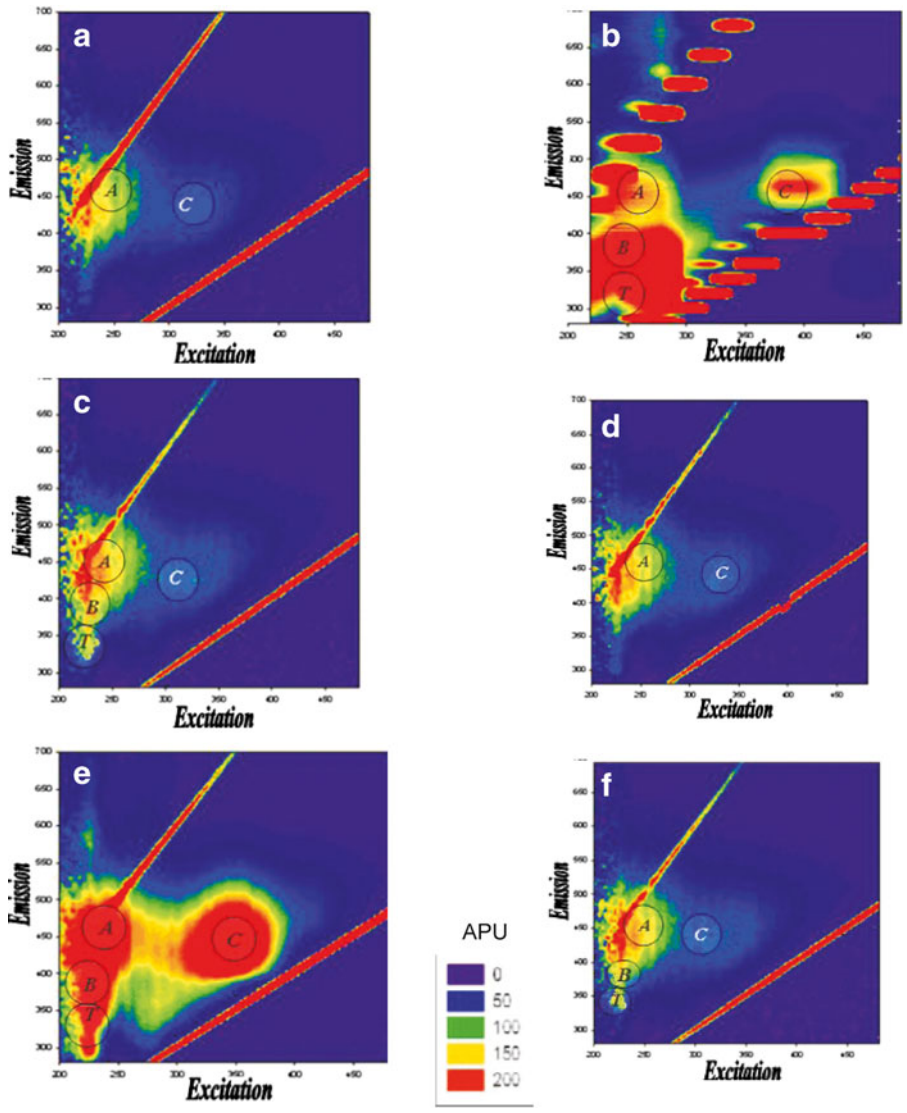

Fig. 2 EEM contour plot of: **a** water treatment plant of Trelew City (reference sampling site), **b** fishery effluent discharge, **c** Chubut River downstream of fishery effluent discharge, **d** Chubut River in New Bridge upstream of sewage effluent, **e** sewage effluent discharge, **f** Chubut River downstream of sewage effluent discharge. *AFU* arbitrary fluorescence units

different excitation maxima, are recognized by the MCR-ALS model as a single component. The same applies to fluorophores B and T, which differ only in their emission spectra. However, by inspection of the excitation (or emission) spectra of that single component, it was possible to observe the characteristics of each fluorophore (either A/C or B/T), which are present in the form of a linear combination, with a greater proportion of the component that produces the greatest signal (see Figs. 3 and 4).The percentage of variance, which is explained by the MCR-ALS model, was satisfactory in most samples, except in those where the fluorescence signal was too low in comparison with the noise and with other spectral artifacts such as the Rayleigh (first- and second-order) and Raman dispersions. Table 2 shows the principal component in each EEM obtained and the variance explained. In Chubut River-New Bridge (upstream of the sewage discharge) and in the water treatment plant (upstream of the fish effluent discharge), the deconvolution demonstrates the larger amounts of HSs over bio-based materials. The MCR-ALS model yields 97.8 % and 98 % of the explained variance in the latter and the former, respectively, considering only one main component. This was attributed to the fluorophore type A based on the graphic analysis (contour plot). Taking into account that MCR-ALS cannot differentiate fluorophores that have the same emission spectra but different excitation spectra, the

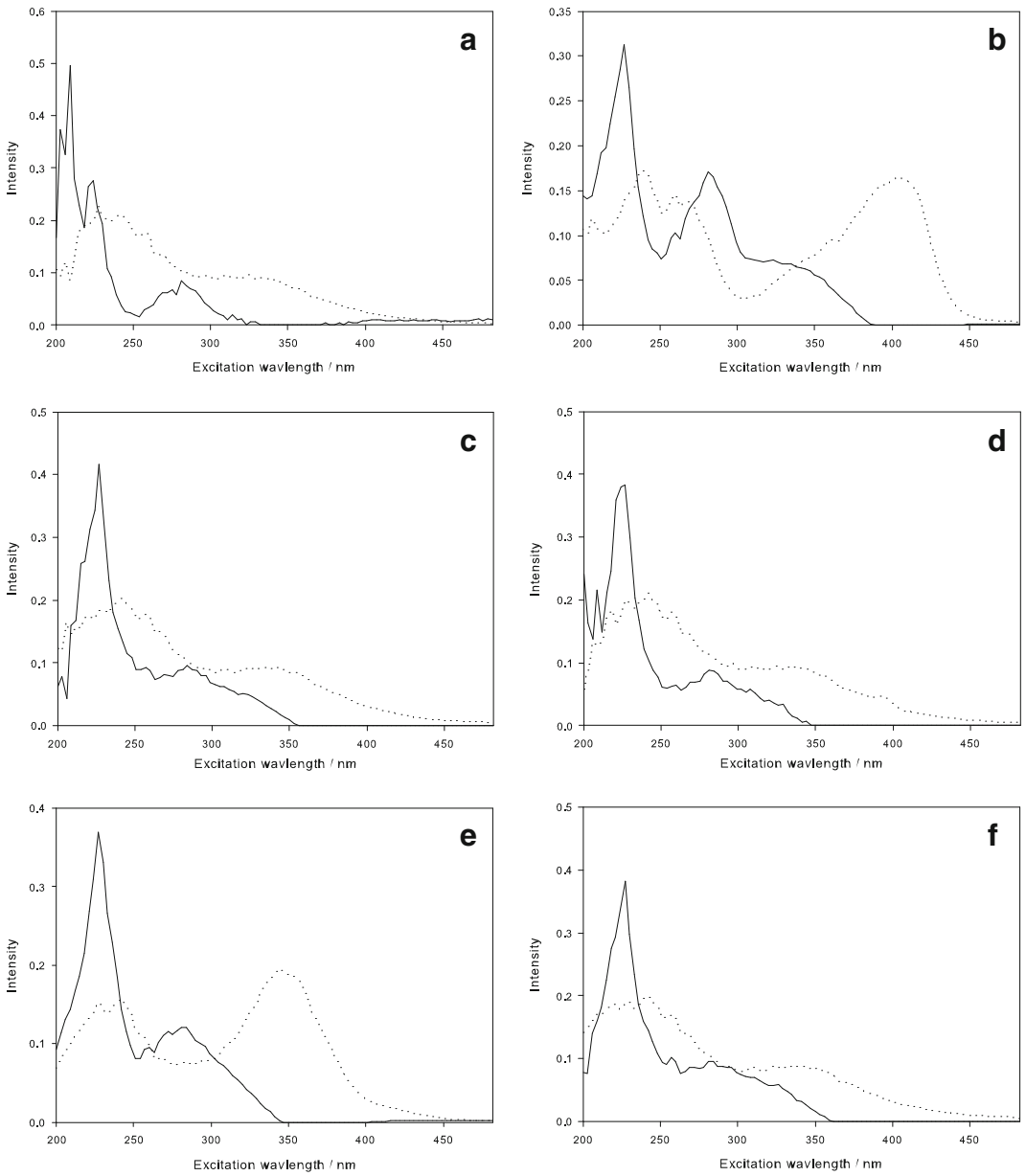

Fig. 3 Excitation spectra of the principal components (MCR-ALS deconvolution) for: **a** water treatment plant of Trelew City (reference sampling site), **b** fishery effluent discharge, **c** Chubut River downstream of fishery effluent discharge, **d** Chubut River in New Bridge upstream of sewage effluent, **e** sewage effluent discharge, **f** Chubut River downstream of sewage effluent discharge

presence of fluorophores A and C cannot be distinguished but the contour plot of EEMs allowed these assignments, which agree with the spectra obtained by MCR-ALS (Figs. 3a and d and 4a and d).

In relation to the fishery and sewage effluents, where the EEM showed a mixture of fluorophores (A, B, C, and T), the MCR-ALS model explained a variance of 99.9 % with two main components for the sewage effluent, with 98.7 % for component 1 (fluorophore A or C) and 1.2 % for component 2 (fluorophore T or B) (Fig. 4e). Similarly, for the fishery effluent, the explained variance was 84.2 % with two

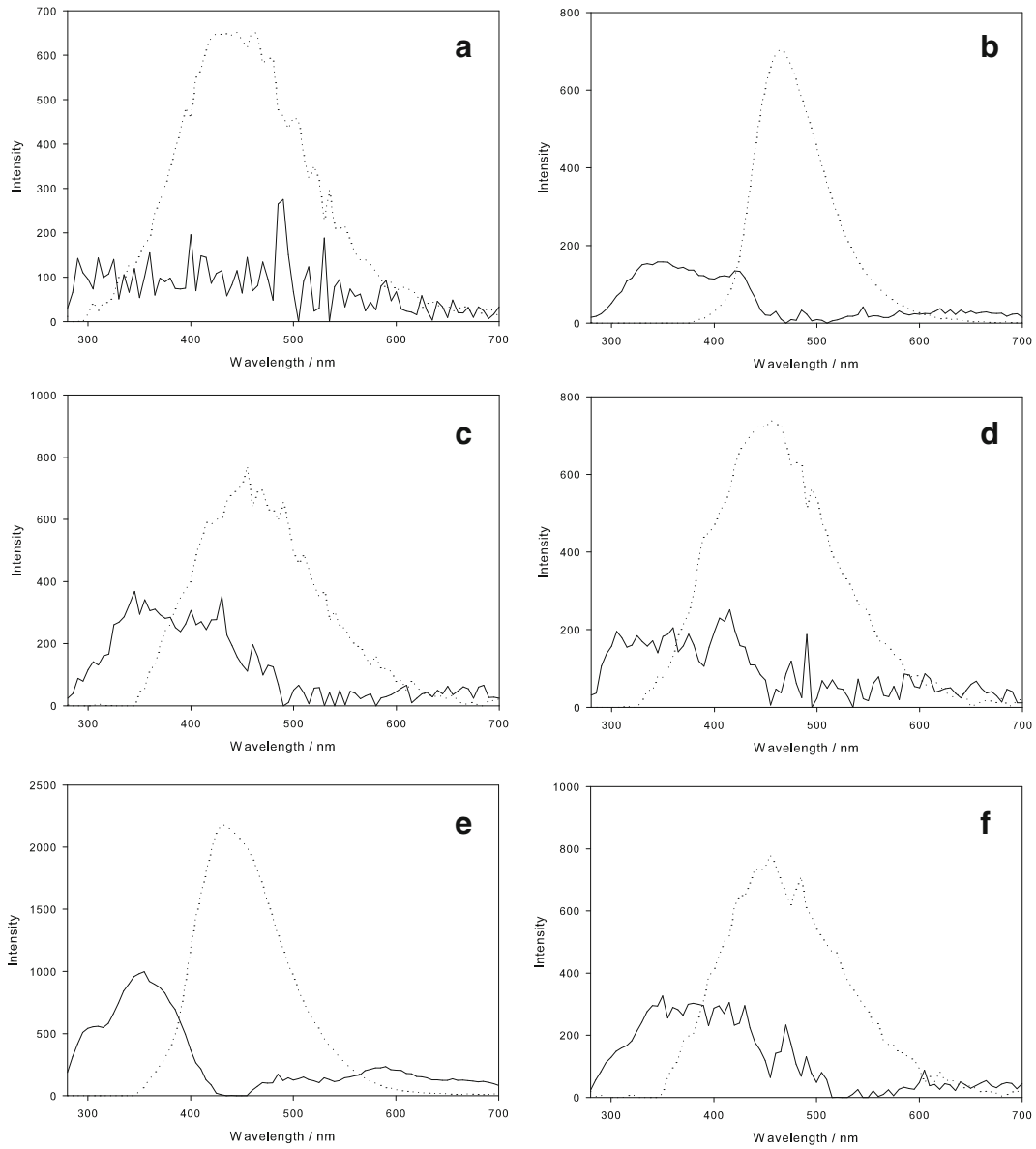

Fig. 4 Emission spectra of the principal components (MCR-ALS deconvolution) for: **a** water treatment plant of Trelew City (reference sampling site), **b** fishery effluent discharge, **c** Chubut River downstream of fishery effluent discharge, **d** Chubut River in New Bridge upstream of sewage effluent, **e** sewage effluent discharge, **f** Chubut River downstream of sewage effluent discharge

components, consisting of 74.9 % for component 1 (fluorophore T or B) and 9.3 % for component 2 (fluorophore A or C) (Fig. 4b). It is important to remark that the model does not allow the distinction between fluorophores A and C, both of which have very high fluorescence intensity. Although the deconvolution results indicate the presence of a single major component, due to the intrinsic characteristic of the sample, this major component should correspond to C as shown in Fig. 3e, which represents HSs of allochthonous origin linked to contact with these sludge treatment plants in the waters of the liquid sewage. Regarding fluorophore T, it is important to note that it is present as a main component for both types of effluents. In the case of the sample corresponding to the fishery effluent, it is the first component, a situation

that seems to be logical given that it represents tryptophan-rich protein amino acid, the fundamental basis of the raw material used in the manufacture process of this industry.

As can be seen in Figs. 3c and f and 4c and f, for the discharge of sewage and fishery effluents on the receiving body (Chubut River), the MCR-ALS model yields an explained variance of 99.9 % with two main components for the case of sewage discharge: component 1 with 99.8 % (fluorophore A) in agreement with the HSs, which are the priority for the MOD fraction of the river, and component 2 with 0.12 % (fluorophore T) characteristic of sewage. For the fishery effluent, MCR-ALS provides 86.4 % of the explained variance also with two main components: component 1 with 86.1 % (fluorophore A) and component 2 with 0.3 % (fluorophore T). The results are consistent with the above discussion, bearing in mind the low flow of this effluent compared to the sewage effluent, which could indicate a lower impact of these effluents on the waters of the Chubut River.

It can be concluded that EEMs are useful to study the DOM of the Chubut River in natural and anthropogenic-impacted waters; to characterize the natural organic matter present in waters, either as allochthonous or autochthonous sources; to track pollution or contaminated areas; and to detect anthropogenic activity in certain areas in relation to other areas in their natural state.

Conclusions

Excitation–emission matrix fluorescence spectroscopy can be considered as a good indicator of the environmental changes under the influence of external factors such as the discharge of industrial effluents from fisheries or domestic sewage. It is also a good tool for the characterization of industrial effluents or sewage through a methodology that is sensitive, simple, and rapid. Finally, using these matrices through fluorescence enabled a rapid and specific analysis, giving the possibility to evaluate the type of problem and ensure the monitoring of contaminated areas, allowing the distinction between the impacted areas and those that are taken as the reference area.

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