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Characterizing humic substances from estuarine soils and sediments by excitation-emission matrix spectroscopy and parallel factor analysis

C. Santín · Y. Yamashita · X. L. Otero · M. Á. Álvarez · R. Jaffé

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Abstract The determination of optical properties of organic matter using spectroscopic techniques is a powerful tool for the characterization of humic substances (HS) in soils and sediments because of sensitivity, specificity and sample throughput. However, basic spectroscopic techniques have practical limitations because of the similarity in the optical properties of many HS. To improve resolution, the combination of excitation-emission matrix (EEM) fluorescence and parallel factor analysis (PARAFAC) was applied for characterizing fulvic acid (FA) and humic acid (HA) fractions from soils and sediments of two estuarine environments in Spain. Five fluorescent components were identified by EEM-PARAFAC and were found in both FA and HA fractions, consistent with the new paradigm of HS as supramolecular associations as well as the ubiquity of the HS components in the environment. Their contribution was, however, different between the FA and HA fractions. Two different, humic-like, fluorescent components were representative of FA and HA fractions, respectively. The spectral characteristics of these components were similar to previously reported PARAFAC components in dissolved organic matter (DOM) in a wide range of environments, suggesting their applicability in assessing OM quality and environmental dynamics. A microbial humic-like component was much more abundant in FA than in HA fractions. Furthermore, principal component analysis clarified that the two identified protein-like components, were enriched in sediment HA compared to soil HA, suggesting a larger contribution of refractory algaenan in sediment HA. The results of the present study demonstrate that EEM-PARAFAC is a useful technique for the biogeochemical characterization of soil and sedimentary HS.

Keywords EEM-PARAFAC · Estuary · Fulvic acids · Humic acids · Sediment · Soil

C. Santín · M. Á. Álvarez
INDUROT, Universidad de Oviedo, Campus de Mieres, 33600 Mieres, Spain

Y. Yamashita (✉) · R. Jaffé
Southeast Environmental Research Center and Department of Chemistry and Biochemistry, Florida International University, University Park, Miami, FL 33199, USA
e-mail: yamashiy@fiu.edu

X. L. Otero
Departamento de Edafología y Química Agrícola, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

Introduction

Humic substances (HS) are the most widespread natural organic materials in the environment. They represent up to 70–80% of organic carbon in mineral

soils and up to 60% of dissolved organic carbon in aquatic ecosystems (Stevenson 1994; Schlesinger 1997). HS play a variety of vital roles in carbon and nitrogen cycles (Zech et al. 1997), carbon sequestration (Hayes and Clapp 2001), mobilization and availability of nutrients (Stevenson 1994), metals (Wood 1996; Yamashita and Jaffé 2008) and pollutants (Senesi 1992), and even interactions with organisms (Steinberg et al. 2008). It is therefore clearly important to understand the nature, composition and environmental dynamics of HS. However, it is not an easy task to obtain such knowledge, as HS are complex supramolecular associations of heterogeneous molecules predominantly stabilized by weak dispersive forces and derived from the degradation and decomposition of a mixture of different biological materials (Piccolo 2001).

Excitation-emission matrix (EEM) fluorescence spectroscopy has become popular for characterizing organic materials from different environments. This technique provides an overall and complete view of all the features existing within a selected spectral range and thus provides an accurate picture of the excitation-emission wavelength pairs where the studied organic material has its maxima of fluorescence. EEM fluorescence spectroscopy has been widely used for the study of dissolved organic matter (DOM) in aquatic systems (Coble 1996; Del Castillo et al. 1999; Chen et al. 2003; Kowalczuk et al. 2003; Yamashita and Tanoue 2003; Wu et al. 2003; Fu et al. 2006; Maie et al. 2008). However, only few studies have used EEM fluorescence spectroscopy to characterize HS from soils and sediments (Mobed et al. 1996; Alberts and Takács 2004a, b; Sierra et al. 2005a) or their interactions with trace metals (Fukushima et al. 1997; Provenzano et al. 2004; Plaza et al. 2005) and pollutants (Sun et al. 2007).

In recent years, the interpretation of EEMs of natural organic matter by means of multivariate analysis, such as parallel factor analysis (PARAFAC) has undergone substantial improvement (Stedmon et al. 2003). This statistical modeling approach enables characterization and identification of individual fluorescent groups in the EEMs (Stedmon et al. 2003). Thus, it is particularly valuable in environmental applications where standards or reference components are not always available, or when EEMs represent complex mixtures of natural organic matter components. The combination of EEM and PARAFAC has been widely applied in the study of aquatic ecosystems, taking advantage of the natural fluorescence of chromophoric DOM. For instance, it has been used to characterize DOM in natural waters (Stedmon et al. 2003; Cory and Mcknight 2005; Holbrook et al. 2006), water soluble DOM in soil (Ohno and Bro 2006; Ohno et al. 2007; Fellman et al. 2008), to determine specific binding capacities of fluorescent components with trace metals (Ohno et al. 2008; Yamashita and Jaffé 2008), to classify water sources and their origin (Hall and Kenny 2007; Hua et al. 2007), and in studies of the DOM dynamics in oceanic as well as coastal environments (Stedmon and Markager 2005; Stedmon et al. 2007; Wedborg et al. 2007; Murphy et al. 2008; Yamashita et al. 2008).

To our knowledge, however, only one study has been carried out to characterize terrestrial HS by EEM fluorescence spectroscopy combined with PARAFAC (He et al. 2006). He et al. (2006) fitted a three component PARAFAC model to six standards obtained from the International Humic Substances Society (IHSS). One component, with an emission peak at a wavelength greater than 498 nm, was more abundant in the HA fraction than in the FA fraction. The difference was consistent with the findings of previous EEM studies (Mobed et al. 1996; Alberts and Takács 2004a; Sierra et al. 2005a).

Thus, EEM-PARAFAC is a potentially useful technique in the assessment of complex HS, that is, to evaluate the similarity/dissimilarity of compositions of fluorescent groups between fractions as well as among samples. Although this technique has not yet been applied to the characterization of HS in natural environments, this may provide additional insight into the optical characterization of HS as well their environmental dynamics. Therefore, in the present study, EEM-PARAFAC was used to characterize FA and HA fractions extracted from soils and sediments of two estuarine environments. The main objectives were (1) to evaluate the major representative fluorescent components in FA and HA fractions and to compare the fluorescent components in HS with previously reported PARAFAC components in natural environments; and (2) to test the usefulness of the technique for evaluating the similarity/dissimilarity of HS from different soils and sediments.

Materials and methods

Study sites and sampling

Two estuaries on the northwestern coast of the Iberian Peninsula were selected for sampling: the Urdaibai Estuary (43°24′N 2°41′W) and the Foz Estuary (43°33′N 7°15′W; Fig. 1A). The climate in the region is oceanic with mean annual temperatures of between 13 and 14°C and total annual precipitation between 1,000 and 1,200 mm.

The Urdaibai Estuary covers an area of 1,200 ha and has been seriously affected by the reclamation and drainage of estuarine areas for farming, grazing and urban purposes (Gogeascoechea and Juaristi 1997). Salt marshes still occupy an important part of the estuary, although more than 75% of them are altered by human activities. The sampling area is located in the middle of the estuary, 4–5 km away from the estuary mouth. The selected transect (Fig. 1B) was: a natural salt marsh area (one sampling point in a tidal channel sediment and another in an adjacent high

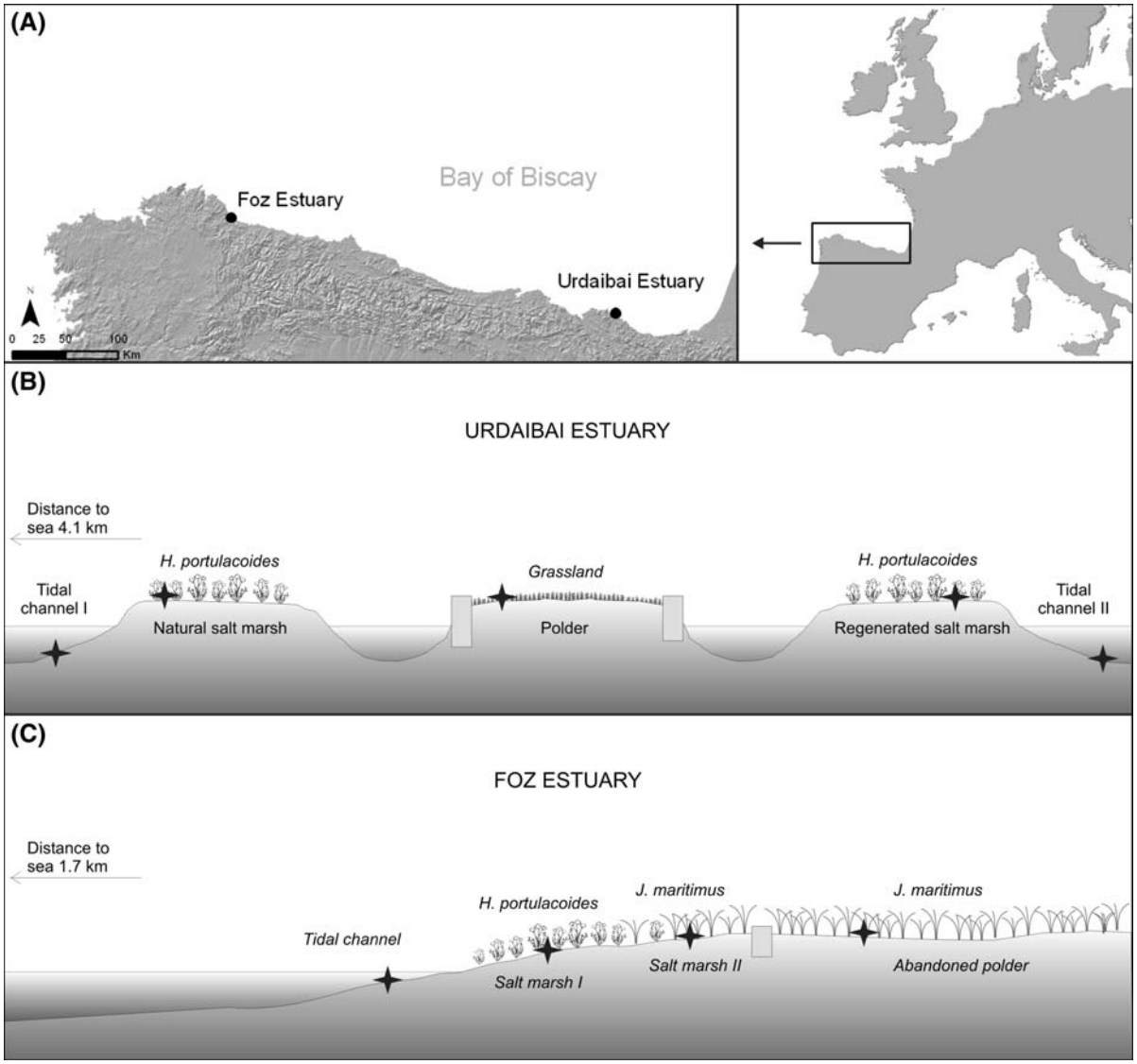

Fig. 1 **A** Locations of the sampling sites on the northwestern Coast of the Iberian Peninsula; **B** sample locations in the Urdaibai Estuary; **C** sample locations in the Foz Estuary. *Stars* represent sampling points

marsh soil covered by *Halimione portulacoides*); a regenerated salt marsh area (one sampling point in a tidal channel sediment and another in an adjacent high marsh soil covered by *H. portulacoides*); and a reclaimed salt marsh area (one sampling point in a polder grassland soil).

The Foz Estuary is small (460 ha) and scarcely affected by human activity, with only 6% of its surface reclaimed. The sampling area is located just 1.7 km from the estuary mouth, and the selected transect (Fig. 1C) was as follows: a sampling point in a tidal channel sediment; another in an adjacent salt marsh soil covered by *H. portulacoides*; another in a high marsh soil covered by *Juncus maritimus*; and another in an abandoned polder soil re-colonized by natural vegetation (*J. maritimus*).

Sampling was carried out in the summer of 2005. Each profile was collected with a PVC core (11 cm inner diameter and 60 cm in length). The cores were hermetically sealed, stored in an ice chest at 4°C, and transported in vertical position to the laboratory. The cores were then cut into 5-cm sections. From each profile three sections were selected for the analysis: the surface layer (0–5 cm), a subsurface layer (20–25 cm) and a deeper layer (40–60 cm). In the present study, the substrate colonized by vascular plants is denominated soil whereas the substrate non-colonized by rooted plants is considered as sediment (for further details see Ferreira et al. 2007).

Elemental analysis

Fresh samples were dried at 55°C and then sieved through a 2 mm mesh for further analysis. Total carbon (TC) and total nitrogen (TN) were measured in duplicate by dry combustion (975°C) in an Elementar Vario MAX CNS Analyzer. Organic carbon was eliminated from the bulk soil by combustion for 4 h at 450°C (Cambardella et al. 2001) and the remaining inorganic carbon (IC) was measured. Total organic carbon (TOC) was then estimated as the difference between TC and IC, after prior correction for weight loss.

Extraction of humic substances

The extraction of FA and HA fractions from soils and sediments was performed according to the NAGOYA method (Kuwatsuka et al. 1992). Samples containing approximately 100 mg TOC were placed in 100 ml plastic bottles, and 60 ml of 0.1 M NaOH were added. After purging with N_2, the bottles were capped and shaken continuously for 24 h at 180 rpm, at room temperature. Then, 1.8 g of Na_2SO_4 was added as coagulant, and the suspension was centrifuged at 3,000 rpm for 15 min. The supernatant was filtered through No. 6 filter paper, and the filter residue was washed twice with 60 ml of 0.1 M NaOH containing 1.8 g Na_2SO_4. The filtrate was centrifuged to obtain the HS extract. For separation of FA and HA fractions, HS extract were acidifying to pH 1 with 6 N H_2SO_4 and allowing it to stand overnight. The acid soluble FA fraction was separated from the precipitated HA fraction by centrifugation at 3,000 rpm for 15 min, and then, transferred to 250 ml low density polyethylene (LDPE) brown bottle. The residual HA fraction was washed twice with 30 ml of 0.1 N H_2SO_4 and the washings were added to the 250 ml LDPE brown bottle (FA fraction). The HA fraction was then re-dissolved in 0.1 N NaOH containing 30 g L^{-1} of Na_2SO_4 after neutralization with 0.2 N NaOH, and centrifuged at 3,000 rpm for 20 min to remove impurities. The HA fraction was re-precipitated by acidification to pH 1, recovered by centrifugation, and then re-dissolved in 0.1 N NaOH containing 30 g L^{-1} of Na_2SO_4 for further analysis.

Classification of humic acid fraction

A classification diagram of HA fraction using two humification indices, i.e., ΔlogK and RF, originally proposed by Kumada et al. (1967) was used for the present study. The ΔlogK and RF are indexes of absorbance characteristics and relative absorbance in organic carbon for the HA fraction, respectively. They were calculated according to Kumada et al. (1967) and Ikeya and Watanabe (2003) as follows: $\Delta\log K = \log (A_{400}/A_{600})$

$$RF = \frac{A_{600}}{OC} \times \frac{1}{0.0648}$$

where A_{400} and A_{600} are the absorbance at 400 nm and 600 nm, respectively, and OC is the organic carbon concentration in FA and HA fractions (gC L^{-1}). RF was originally calculated as $(A_{600}/b) \times 1000$, where b is the volume (mL) of 0.02 M $KMnO_4$ consumed by 30 ml HA solution (Kumada et al. 1967). 0.0648 is the

conversion factor from *b* to OC (Ikeya and Watanabe 2003). The OC in the FA and HA fractions was determined by a colorimetric method with a potassium dichromate-sulfuric acid solution as reagent (Tatsukawa 1966). For colorimetric OC determination as well as estimation of A_{400} and A_{600}, absorbance measurements were made in a 1 cm quartz cell, in a Varian Cary 50-Bio UV–visible spectrophotometer at room temperature (25 ± 3°C). The absorbance of a blank solution, which was obtained using the FA and HA extraction procedure without soil/sediment samples, was subtracted from those of the samples.

Optical analysis for fulvic and humic acid fractions

Because absorbance spectra of the HA and FA fractions were over the range for the spectrophotometer in the UV region, both fractions were diluted with blank solution to an absorbance below 1 at 240 nm, and then, the pH was adjusted to 8. Using these diluted samples, absorbance spectra (200–800 nm) and fluorescence measurements were conducted with a Varian Cary 50-Bio UV–visible spectrophotometer and a Horiba Jovin Yvon SPEX fluoromax-3 fluorometer, respectively, at room temperature (25 ± 3°C). The fluorometer was equipped with a 150 W continuous output xenon arc lamp.

Analytical procedures for determining EEMs have been reported elsewhere (Maie et al. 2006; Yamashita and Jaffé 2008). Briefly, forty-four emission scans were acquired at excitation wavelengths (λ_{ex}) between 240 and 455 nm at 5 nm intervals. The emission wavelengths were scanned from λ_{ex} + 10 nm to λ_{ex} + 250 nm at 2 nm intervals. Bandpass was set at 5.7 nm and 2 nm for excitation and emission, respectively. Fluorescence spectra were scanned with 0.25 s of integration time and acquired in ratio mode.

For correction of EEMs, inner-filter correction was carried out by use of absorbance spectra (McKnight et al. 2001), and the EEMs of Milli-Q water were then subtracted from the sample EEMs. The specific instrument components were also corrected with excitation and emission correction files supplied by the manufacturers. Fluorescence intensities in EEMs were corrected to the peak area of the water Raman scatter (382–412 nm emission ranges at 350 nm excitation) analyzed daily (Cory and McKnight 2005), and then converted to quinine sulfate units (QSU) (Yamashita and Tanoue 2003). The EEMs of HA and FA blank solutions were corrected using the same procedures as for the samples, and then subtracted from corrected sample EEMs.

PARAFAC

The PARAFAC modeling for EEMs of natural organic mater has been described in detail elsewhere (Stedmon et al. 2003; Ohno and Bro 2006). The range of wavelengths used for the PARAFAC model were 260–455 nm and 290–500 nm for excitation and emission, respectively. The analysis was carried out in MATLAB with the "N-way toolbox for MATLAB" (Andersson and Bro 2000). A non-negatively constraint was applied to the parameters. Two to eight components were modeled for all FA and HA EEMs ($n = 56$, Table 1) which were diluted, as mentioned above. Determination of the correct number of components was assessed by applying the core consistency diagnostic score and residual analysis (Bro and Kiers 2003; Ohno and Bro 2006). A five component EEM-PARAFAC model was selected as most suitable for this dataset, since core consistency was 83.6% and it decreased dramatically for higher numbers of components. In addition the model explained 99.7% of the variability in the dataset. The relative abundance of each of the five components were presented as CX(%) and calculated as follows (e.g., component 1, C1%);

$$\mathrm{C1}(\%) = \frac{\text{fluorescence intensity of C1}}{\text{fluorescence intensities of C1} + \mathrm{C2} + \mathrm{C3} + \mathrm{C4} + \mathrm{C5}} \times 100$$

Table 1 Description of sampling sites, pH, total organic carbon (%), total nitrogen (%) and TOC/TN ratio of the studied soils and sediments and RF and Δlog K values for fulvic and humic acids

	Site description	Vegetation	Depth (cm)	pH	TOC (%)	TN (%)	TOC/TN	*Fulvic Acids*		*Humic Acids*	
								RF	Δlog K	RF	Δlog K
Urdaibai Esturay (43°24′N 2°41′W)	Tidal channel I (sediments)	Seaweeds	0–5	8.2	1.4	0.13	10.8	0.4	1.3	5.2	1.0
			20–25	8.0	1.9	0.17	11.5	0.7	1.2	10.9	0.8
			40–50	7.9	1.0	0.12	8.4	0.7	1.3	9.0	0.9
	Tidal channel II (sediments)	Seaweeds	0–5	7.6	1.2	0.11	10.9	0.4	1.3	4.2	1.0
			20–25	8.0	1.0	0.12	8.5	1.2	1.2	16.4	0.8
			50–60	7.3	0.9	0.09	9.9	1.4	1.2	15.0	0.8
	Natural salt marsh	*H. portulacoides*	0–5	6.4	6.7	0.49	13.6	0.5	1.2	5.1	1.2
			20–25	6.4	2.3	0.26	8.9	0.5	1.2	9.8	0.9
			45–55	6.5	1.2	0.14	8.9	0.6	1.2	12.1	0.8
	Regenerated salt marsh	*H. portulacoides*	0–5	6.7	3.1	0.30	10.3	0.7	1.2	6.2	1.1
			20–25	6.6	2.7	0.25	10.7	0.9	1.1	9.1	0.9
			40–50	6.8	1.6	0.18	8.7	1.0	1.0	14.0	0.8
	Polder (reclaimed salt marsh)	Grassland	0–5	5.5	6.6	0.59	11.2	1.1	1.0	11.8	0.8
			20–25	7.1	1.2	0.15	8.4	0.5	1.2	6.9	1.0
			40–50	6.9	1.4	0.16	9.1	0.8	1.3	10.3	0.9
			55–70	4.3	1.3	0.13	10.1	0.8	1.5	11.1	0.9
Foz Estuary (43°33′N 7°15′W)	Tidal channel (sediments)	Seaweeds	0–5	7.8	2.8	0.21	13.4	0.7	1.3	4.0	0.9
			20–25	7.8	2.8	0.29	9.5	0.4	1.3	4.9	0.9
			50–55	7.8	1.7	0.15	11.4	0.9	1.3	8.7	0.9
	Salt marsh I	*H. portulacoides*	0–5	4.8	15.0	0.97	15.4	0.2	1.4	4.9	1.2
			20–25	6.4	5.6	0.47	11.9	0.7	1.3	8.5	1.0
			45–50	7.1	1.8	0.18	10.4	0.6	1.4	10.9	0.9
	Salt marsh II	*J. maritimus*	0–5	6.2	21.3	1.45	14.7	0.8	1.2	6.4	1.1
			20–25	6.0	9.3	0.60	15.6	1.0	1.2	7.0	1.0
			40–50	6.2	4.6	0.38	12.2	0.9	1.3	8.4	0.9
	Abandoned polder	*J. maritimus*	0–5	6.2	14.1	1.02	13.9	0.8	1.3	8.0	1.0
			20–25	6.5	3.5	0.30	11.4	0.8	1.3	10.9	0.9
			40–50	6.5	0.9	0.10	8.9	0.5	1.4	10.8	0.9

Statistical analyses

Principal component analysis (PCA) was carried out using the relative abundance (%) of the five PARAFAC components for all FA and HA fractions ($n = 56$). PCA was performed via the correlation matrix and according to the least squares criterion with the Statistica 8.0 software. The Kaiser criterion was used for selecting the appropriate number of factors (eigenvalues greater than 1).

Furthermore, one way analysis of variance (ANOVA) was applied to test differences in the average relative abundances of PARAFAC components between FA and HA fractions as well as average PCA scores among different groups of samples. Before each ANOVA, the assumption of normality was checked by the Shapiro–Wilk statistic and the variance equality among the compared groups of samples was tested by the Levene statistic. When possible, the non-normal variables were transformed, if not the Kruskal–Wallis test was performed as a nonparametric alternative to the ANOVA. Furthermore, in case of heteroskedasticity the Brown–Forsythe test was used instead of the

ANOVA. These statistical analyses were carried out with the SPSS statistical package (version 15.0, 2006).

Results and discussion

General characterization of soils and sediments

The general parameters analyzed for the studied soil and sediment profiles are shown in Table 1. The sediments were slightly basic (pH 8.2–7.3) whereas the soils were neutral or acidic (pH 7.1–4.3). Interestingly, the most acidic conditions (pH 4.3) were observed in the reclaimed salt marsh at depth (55–70 cm), which may be due to oxidation of iron sulfides in this soil layer because of the drainage of the marsh (Van Breemen 1982).

Accumulation of TOC was greater in soils than in sediments, with the highest values at the surface layers and decreasing with depth in all the profiles (Table 1). Furthermore, the Foz soil profiles were more TOC enriched compared to the Urdaibai soil profiles, especially at the surface layers. This may be related to a higher tidal influence in the sampled soils of the Foz Estuary than in those of the Urdaibai Estuary. A longer period of flooding would lead to an oxygen poor geochemical environment, which implies a slower decomposition rate of the organic matter (D'Angelo and Reddy 1999) and therefore greater accumulation of TOC in the soil.

Total nitrogen (TN) was well correlated with TOC ($r = 0.99$, $n = 28$, $p < 0.01$), indicating the organic origin of most of the nitrogen. Regarding the TOC/TN ratio, the highest values were found at the surface. Furthermore, this ratio tended to decrease with depth in most of the studied profiles (Table 1). Several authors have reported a decrease in the TOC/TN ratio on litter and soil organic matter during the decomposition of marsh vegetation such as *Juncus* sp. and *Halimione* sp. (Newell et al. 1984; Buth and de Wolf 1985; Kuehn et al. 2000; Chabbi and Rumpel 2004).

Table 1 also shows the Δlog K and RF indexes, calculated for FA and HA fractions from the UV–visible measurements. The Δlog K value decreases with the development of conjugation systems in the organic molecules, whereas the RF value is influenced by the proportion of non- or less colored moieties, and decreases as the proportion of these non-colored molecules increases (Ikeya and Watanabe 2003). Thus, high RF values and low Δlog K values will be related to higher humification of HS and vice versa. The classification diagram for HA proposed by Kumada et al. (1967) is shown in Fig. 2. In this classification, HA are grouped into five main types: A, B, R_p, P_0 and P_1, according to their position on the graph of RF plotted against Δlog K. Type A includes HA with the highest degree of humification, rich in aromatic carbon, and carboxylic groups; whereas types B, P_0 and P_1 are transitory forms of HA between type R_p, with a high concentration of alkyl carbon and low degree of humification, and type A end members. Even though the lowest RF values and highest Δlog K values were found at the surface layers with exception of the grassland (polder soil; Table 1; Fig. 2), all of the HA fractions under study were included in Type R_p (immature character), indicating that further optical analysis are necessary for better characterization of HA fractions for the present study. Since this classification was created exclusively for the HA, the FA fractions were not included in the diagram. However, it is interesting to note that all the FA fractions presented much lower RF values and much higher Δlog K values than the HA fractions (Table 1), suggesting a lower degree of humification of the FA fractions compared to their HA counterparts.

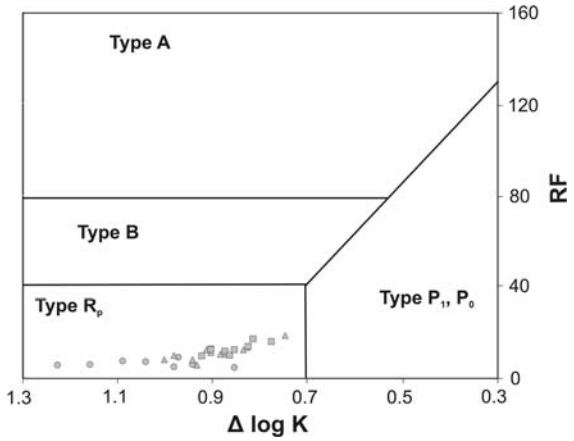

Fig. 2 Classification of humic acids according to Kumada et al. (1967). Humic acid fractions from the studied soils and sediments profiles are represented as follows: *circles* (surfaces layers); *triangles* (subsurface layers) and *squares* (bottom layers)

Assignment of fluorescent components

Fluorescence characteristics of the five components obtained by PARAFAC are shown in Fig. 3 and their average relative abundances for both the FA and the HA fractions are shown in Fig. 4. Table 2 shows the Ex_{max} and Em_{max} of each PARAFAC component found in the present study with comparison to traditional peak assignments (Coble 1996), three PARAFAC components found for IHSS HS (He et al. 2006), and comparable PARAFAC components of DOM found in a wide range of aquatic environments (Cory and McKnight 2005; Yamashita and Jaffé 2008).

The relative abundance (%) of C1 ($Ex_{max} < 260$ (305), Em_{max} 439) was greater in the FA fraction compared to the HA fraction (Fig. 4), and thus, C1 was considered as a representative fluorescent component for FA fractions (fulvic acid-type component, Table 2). The spectral characteristics of C1 were similar to PARAFAC component obtained for IHSS HS (Table 2) and were also similar to terrestrial humic-like components of DOM in aquatic environments (Table 2, Stedmon and Markager 2005; Banaitis et al. 2006; Holbrook et al. 2006; Hall and Kenny 2007). On the other hand, C2 [Ex_{max} 265 (385), $Em_{max} > 500$] could be considered as a representative fluorescent component for HA fractions (humic acid-type component) from the relative abundances in FA and HA fractions (Fig. 4). PARAFAC components similar to C2 have also been found in IHSS HS and DOM in aquatic environments (Table 2; Stedmon and Markager 2005; Yamashita et al. 2008).

Similarly to C1, the relative abundance of C3 was higher in the FA fraction than in the HA fraction (Fig. 4). Component C3 (Ex_{max} 320, Em_{max} 388), however, was not reported for the previous IHSS HS PARAFAC model (Table 2). This component is comparable to peak M traditionally defined in DOM (Table 2), and also to PARAFAC component of microbial origin (Table 2; Yamashita et al. 2008).

Spectral characteristics of C4 (Ex_{max} 275, Em_{max} 304) and C5 ($Ex_{max} < 260$, Em_{max} 365) were similar to tyrosine-like and tryptophan-like fluorophores, respectively (Table 2). However, the spectral characteristics of C5 were also similar to those of gallic acid, a major constituent of hydrolysable tannins (Fukushima et al. 1997; Maie et al. 2007), but were not similar to those of tannins (Maie et al. 2007). In the present study, C5 is assigned to a tryptophan-like component because it is unlikely that a monomer of gallic acid occurs in HS. In addition, PCA analysis in the present study showed that C4 and C5 clustered closely (see below), suggesting that sources and transformations of C4 and C5 in HS were similar.

Distribution of fluorescent components in humic and fulvic acid fractions

The relative abundance of the five components in all FA and HA fractions are summarized in Table 3. Interestingly, fulvic acid-type (C1), humic acid-type (C2), microbial humic-like (C3), and tryptophan-like (C5) components were found in all of the fractions, irrespective of site differences and in both FA and HA fractions (Table 3). He et al. (2006) and Ohno and Bro (2006) also found that all three modeled PARAFAC components were shared in their 6 HS samples (2 IHSS FA and 4 IHSS HA samples) and indicated consistency with the new paradigm for the structure of soil HS, that is, soil/sediments HS are composed of relatively low molecular weight components that interact strongly with each other by hydrophobic interactions and hydrogen bonds, and cannot be separated effectively by chemical or physical methods (Sutton and Sposito 2005).

Despite the presence of all the PARAFAC components in both FA and HA fractions, statistically significant differences between the relative abundance of each of these five components to both fractions were observed (Table 4). As mentioned above, the fulvic acid-type C1, was the dominant fluorescence component in the FA fraction whereas the predominant component in the HA fraction was the humic acid-type C2 (Fig. 4). This trend was consistent throughout the sample set, regardless of sample type (Table 2).

Such compositional differences in fluorescent components between FA and HA fractions (Fig. 4) are consistent with those found in previous EEM studies, i.e., a red shift in fluorescence maxima in the HA fraction compared with the FA fraction (Mobed et al. 1996; Alberts and Takács 2004a; Sierra et al. 2005a). A red shift in the fluorescence maxima has been considered to be due to the presence of high molecular weight fractions, electron-withdrawing substitutes, and a higher degree of conjugation (Mobed et al. 1996). Thus, significant differences in

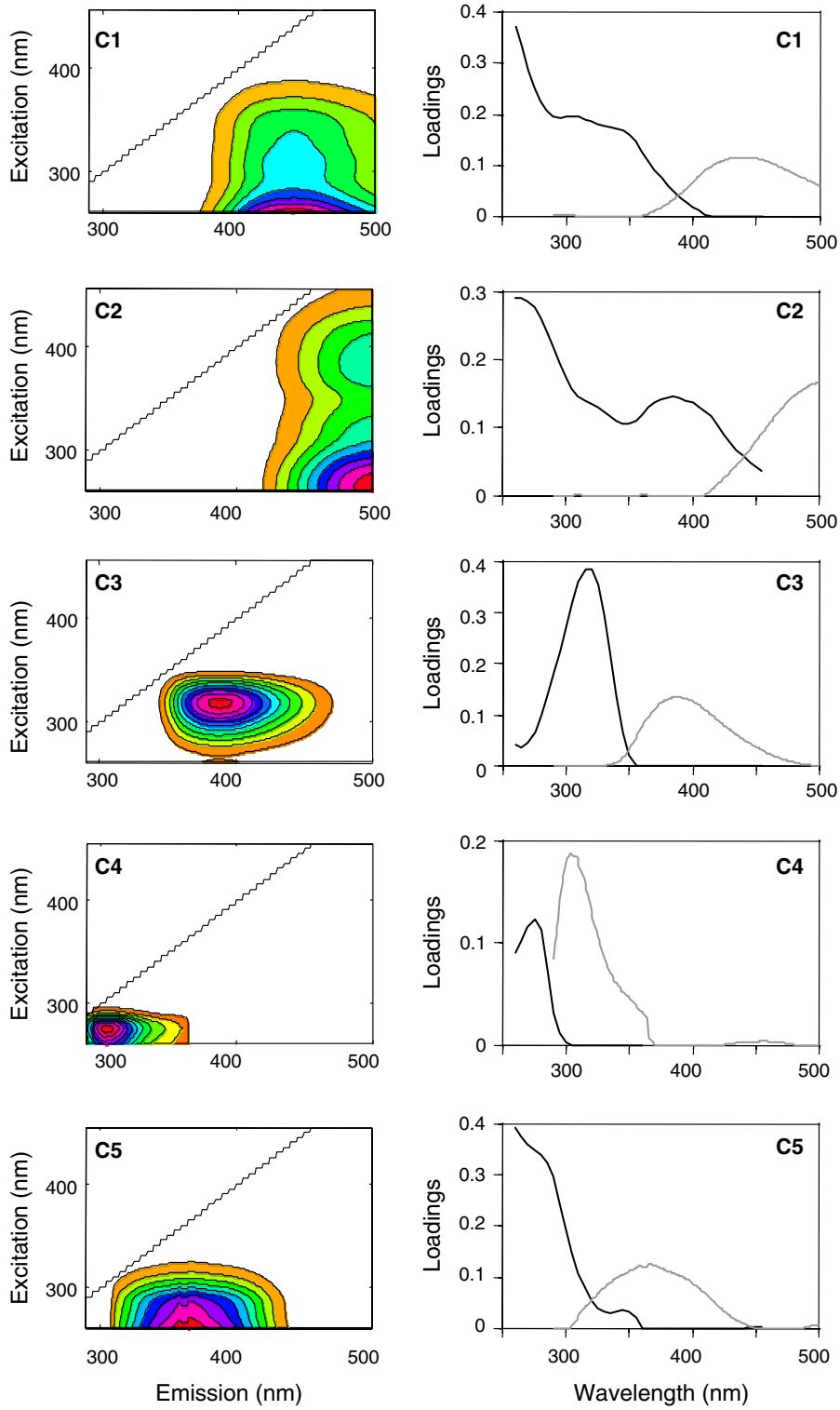

Fig. 3 Contour plots (*left*) and excitation and emission loadings (*right*) of the five fluorescent components obtained by PARAFAC. *Black* and *grey* lines in *right* figures are excitation loadings and emission loadings, respectively

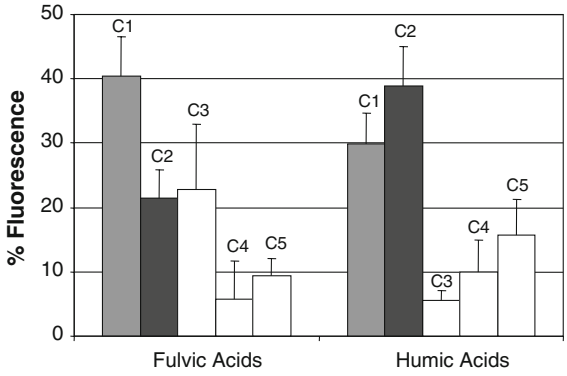

Fig. 4 Average relative abundances (%) of each of the five PARAFAC components for fulvic and humic acid fractions. Standard errors across all samples are shown

relative abundances of C1 and C2 between FA and HA fractions were also consistent with differences in molecular weight distribution between FA and HA fractions (Sutton and Sposito 2005).

The microbial humic-like C3 was much more abundant in the FA than in the HA fraction (Fig. 4). It presented the highest variability among the studied group of FA samples (Fig. 4). The C3 was the least abundant component in the HA fraction (Fig. 4) and presented a relatively narrow range in all the HA samples (Table 3). With regards to the variability in the composition of the FA samples, 78.6% of them (n = 22/28, Table 3) were dominated by C1, and the remaining 21.4% of the samples (n = 6/28), were dominated by C3. Previous EEM studies of soil/sediment HS, however, did not specifically identify such a microbial humic-like component (Mobed et al. 1996; Alberts and Takács 2004a; Sierra et al. 2005a). Sierra et al. (2005a) observed a shoulder of microbial humic-like component in soil/sediment HA fractions, but not in their FA fractions and indicated that the absence of the microbial humic-like component in the FA fractions may be due to (1) solubility characteristics and (2) overlapping with the intense fulvic acid-like fluorophore in the FA fraction.

In marine environments where the contribution of terrestrial HS is small, a marine (microbial) humic-like fluorophore, similar to C3, is known to predominate in the EEM (Coble 1996; Del Castillo et al. 1999; Yamashita and Tanoue 2003; Maie et al. 2006) and to be a major PARAFAC component (Murphy et al. 2008; Yamashita et al. 2008). In the present study, EEM-PARAFAC was able to resolve the microbial humic-like components from its fulvic acid-type counterpart and suggests that microbial humic-like components are rich in the FA fraction, but not in the HA fraction. In addition, the differences in relative abundances of the microbial humic-like component (C3) in the FA and HA fractions provided insights for source characteristics of HS, i.e., stronger microbial sources in the FA fraction than in the HA fraction. The enrichment of microbial derived organic

Table 2 Characteristics of the five PARAFAC components obtained in the present study compared to those previously identified

Present study			Traditional assignment[a]	HS (He et al. 2006)		DOM (Cory and McKnight 2005)		DOM (Yamashita and Jaffé 2008)	
Comp.	Ex_{max}/Em_{max}	Assingment		Comp.	Ex_{max}/Em_{max}	Comp.	Assignment	Comp.	Ex_{max}/Em_{max}
C1	<260 (305)/439	Fulvic acid-type	C/A	Comp. 1	<240 (318)/438	C10	Terrestrial unknown	1 (Terrestrial humic)	305 (<260)/428
C2	256 (385)/> 500	Humic acid-type	–	Comp. 2	252/> 498	SQ1	Terrestrial reduced quinone	2 (Terrestrial humic)	<260 (340, 405)/> 500
C3	320/388	Microbial humic-like	M	–		C3	Microbial unknown	4 (Microbial humic)	305 (<260)/378
C4	275/304	Tyrosine-like	B	–		Tyr-like	Protein-like	8 (Tyrosine-like)	275/324
C5	<260/365	Tryptophan-like	T	Comp. 3	<240/390	Trp-like	Protein-like	7 (Trptophan-like)	295/340

Secondary excitation maxima are shown in brackets

[a] Traditional assignment were carried out based on Coble (1996)

Table 3 Relative concentrations (%) of the five identified fluorophores in the PARAFAC model for all the studied samples

	Site description	Depth (cm)	Fulvic Acids					Humic Acids				
			C1 (%)	C2 (%)	C3 (%)	C4 (%)	C5 (%)	C1 (%)	C2 (%)	C3 (%)	C4 (%)	C5 (%)
Urdaibai Esturay (43°24′ N 2°41′W)	Tidal channel I (sediments)	0–5	47	20	16	6	12	26	30	5	14	26
		20–25	44	21	16	7	12	31	41	10	7	11
		40–50	38	24	30	1	7	29	41	6	10	15
	Tidal channel II (sediments)	0–5	31	18	36	4	10	23	27	4	18	29
		20–25	46	28	19	1	6	29	39	6	12	14
		50–60	46	29	19	0	6	31	45	5	6	13
	Natural salt marsh	0–5	34	15	39	5	7	31	50	5	4	10
		20–25	35	19	39	0	7	34	39	5	8	14
		45–55	43	27	23	0	7	32	42	5	8	14
	Regenerated salt marsh	0–5	31	18	40	2	8	28	35	6	12	18
		20–25	39	21	28	5	7	30	36	6	10	18
		40–50	41	22	28	2	8	31	41	6	10	13
	Polder (reclaimed salt marsh)	0–5	45	18	22	6	9	35	34	7	10	14
		20–25	43	23	26	0	8	37	42	5	4	12
		40–50	44	25	8	8	15	35	45	5	4	11
		55–70	50	29	13	0	8	37	46	6	2	9
Foz Estuary (43°33′N 7°15′W)	Tidal channel (sediments)	0–5	36	23	15	13	13	20	35	6	18	22
		20–25	27	16	38	9	9	18	24	3	22	32
		50–55	40	21	21	9	8	24	34	5	17	20
	Salt marsh I	0–5	37	15	16	18	13	26	44	4	12	13
		20–25	37	15	13	26	9	27	36	6	14	17
		45–50	43	25	23	3	6	32	46	4	6	11
	Salt marsh II	0–5	31	16	37	3	11	33	37	9	7	14
		20–25	45	21	17	7	10	30	37	7	11	14
		40–50	36	22	25	10	6	27	34	7	15	17
	Abandoned polder	0–5	48	16	14	8	14	34	36	7	9	13
		20–25	47	23	10	7	13	34	41	4	8	13
		40–50	47	29	9	4	12	34	50	4	2	10

matter in FA fractions compared to HA fractions has been previously reported (Hajje and Jaffé 2006).

Regarding the protein-like components, the tryptophan-like component (C5) was evident in all FA and HA fractions (Table 3). On the other hand, the tyrosine-like component (C4) was not found in 5 of the 28 FA samples (Table 3). Protein-like fluorophores have been reported in EEMs of relatively poorly humified sediment/soil HA and FA fractions (Sierra et al. 2005a). Thus, the presence of protein-like components in HA and FA fractions could be due to the low humification of the studied samples (Fig. 2). This is consistent with the findings in similar coastal environments, of a predominance of labile and low humified compounds in FA and HA fractions (Santín et al. 2008, 2009). Interestingly, both protein-like components were generally more abundant in HA than in FA fractions (Table 3; Fig. 4). This result differs from that obtained by Sierra et al. (2005a) who reported better visualization of the protein signal in the EEM from FA fractions than in the EEM from HA fractions. On the other hand, Sierra et al. (2005b) studied HS from subtropical coastal environments and observed a higher nitrogen content in HA fractions than in FA fractions, most of which was attributed to proteinaceous materials. Furthermore,

Table 4 Results of statistical analyses performed for different groups of samples

Varible	Factor	Factor levels	*n*	*p*-values		
				Shapiro–Wilk	Levene	ANOVA
EEM C1	HS fractions	FA fraction	28	0.184	0.067	*0.000*
		HA fraction	28	0.188		
EEM C2	HS fractions	FA fraction	28	0.105	0.119	*0.000*
		HA fraction	28	0.666		
EEM C3	HS fractions	FA fraction	28	0.061	*0.000*	*0.000*[b]
		HA fraction	28	0.016		
EEM C4[a]	HS fractions	FA fraction	28	0.051	*0.003*	*0.001*[b]
		HA fraction	28	0.184		
EEM C5[a]	HS fractions	FA fraction	28	0.054	0.853	*0.000*
		HA fraction	28	0.037		
PC1	HS fractions	FA fraction	28	*0.008*	0.353	*0.000*[c]
		HA fraction	28	0.018		
PC2	HS fractions	FA fraction	28	0.780	0.353	*0.002*
		HA fraction	28	0.420		
PC1	HS fractions; depth	FA surface	9	0.080	0.833	0.712
		FA midle	9	0.518		
		FA bottom	10	0.110		
		HA surface	9	0.067	0.481	0.390
		HA midle	9	0.019		
		HA bottom	10	0.824		
PC2	HS fractions; depth	FA surface	9	0.712	0.425	*0.004*[d]
		FA midle	9	0.451		
		FA bottom	10	0.835		
		HA surface	9	0.709	0.902	0.158
		HA midle	9	0.049		
		HA bottom	10	0.497		
PC1	HS fractions; estuary	FA Urdaibai	16	*0.002*	0.227	*0.001*[c]
		FA Foz	12	0.372		
		HA Urdaibai	16	0.148	0.268	0.208
		HA Foz	12	0.149		
PC2	HS fractions; estuary	FA Urdaibai	16	0.726	0.617	0.085
		FA Foz	12	0.736		
		HA Urdaibai	16	0.457	0.474	0.271
		HA Foz	12	0.974		
PC1	HS fractions; sustrate	FA soil	9	0.026	0.312	0.873
		FA sediment	19	0.162		
		HA soil	9	0.696	*0.002*	*0.010*[c]
		HA sediment	19	0.347		
PC2	HS fractions; sustrate	FA soil	9	0.983	0.962	0.850
		FA sediment	19	0.727		
		HA soil	9	0.480	0.047	*0.010*
		HA sediment	19	0.690		

Italic type indicates significance at the $p \leq 0.01$ level

[a] Transformed variables (Ln transformation)

[b] As result of heteroskedasticity the Brown–Forsythe test was used instead of the ANOVA

[c] As result of no normality the nonparametric Kruskal Wallis test was used instead of the ANOVA

[d] According to the Turkey test the significant differences are between the FA surface and the FA bottom

relative abundances of tryptophan-like component were generally greater than those of tyrosine-like component in both FA and HA fractions (Table 3; Fig. 4). This may be indicative of a low decomposition of the proteinaceous materials, since tryptophan-like fluorescence appears to be indicative of less degraded peptide material and tyrosine-like fluorescence of more degraded peptide material (Yamashita and Tanoue 2003, 2004). This data suggest that labile protein-like components may occur in FA and HA fractions through their interactions with other organic molecules, and as such could be protected from microbial degradation (Knicker 2004; Sutton and Sposito 2005). Such interactions were more strongly evident for HA fractions compared to FA fractions from the soils and sediments studied here.

Distribution of variability in fluorescence among samples

Although differences in the fluorescence characteristics between FA and HA fractions were apparent (section "Distribution of fluorescent components in humic and fulvic acid fractions"), spatial and vertical variability in the contribution of each of the fluorophores among samples were not as clear (Table 3). Such results may be due to similar character of all HA fractions examined (Type R_p; Fig. 2) and variable sources of organic matter in the study area. To clarify such similarity/dissimilarity, PCA was carried out using the relative abundance of each EEM-PARAFAC component for all HA and FA fractions.

Figure 5A shows the projection of the PARAFAC components onto the plane formed by the first two factor coefficients of the PCA. Principle factors 1 (PC1) and 2 (PC2) explained 57% and 26% of the total variance, respectively. The PC1 coefficient could discriminate samples mainly according to the type of HS, i.e., FA or HA fractions. C1 and C3 contributed equally positively to PC1 and they were the predominant components in the FA fraction whereas C2, C4, and C5 contributed negatively and their relative abundances were greater for the HA than for the FA fractions (Fig. 4).

With regards to the PC2 coefficient, C3, C4, and C5 contributed positively to it, whereas C1 and C2 had a strong negative effect in this axis, indicating that PC2 was likely to be related to the contribution of fresh organic matter (protein-like C4 and C5) or degraded organic matter (fulvic acid-type C1 and humic acid-type C2). Interestingly, microbial humic-like C3 showed positive value on the PC2 coefficient, suggesting that microbial humic-like materials might be derived from microbial activity associated to fresh organic matter.

Figure 5B shows all the samples projected onto the plane PC1 × PC2 scores. As expected from differences in fluorophore compositions between FA and HA fractions, the HA fractions clustered statistically separate from the FA fractions for PC1 and PC2 scores (Table 4). Moreover, the PCA highlights the degree of sample heterogeneity. The plots in HA fraction were linearly distributed (Fig. 5B), indicating that the composition of HA fraction was basically controlled by two end-members, namely, protein-like components (C4 and C5) and the humic acid-type component (C2; Fig. 5A). On the other hand, the plots in FA fraction were scattered for both PC1 and PC2 scores (Fig. 5B), indicating that variability in the fluorophore composition was due to the variation in all five components.

The PCA also revealed differences in fluorophore composition of HS obtained between the two estuaries, between sediments and soils, and with depth (Table 4). The scores for FA fractions distinguished between surface and bottom samples by the PC2, although differences were not significant between surface and mid-depth samples or between mid-depth and bottom samples (Table 4). On the other hand, no significant differences were found with depth for the HA fractions (Table 4). The PC2 scores in surface FA fractions tended to show more positive values than that in bottom FA fractions (Fig. 5B), suggesting that relative abundances of protein-like and microbial humic-like components tended to decrease with depth, probably due to increased humification. A decrease in the contribution of protein-like fluorophores with depth was also found in the FA fractions extracted from mangrove sediments (Sierra et al. 2005a), suggesting that such compositional changes in FA fraction during humification processes are likely to be ubiquitous in these transitional environments.

Comparing the FA fractions corresponding to both estuaries presented significant differences (Table 4), with the FA fractions from the Foz estuary showing more negative values for PC1 scores than those corresponding to the Urdaibai estuary (Fig. 5B). Such results were due to greater richness in protein-like

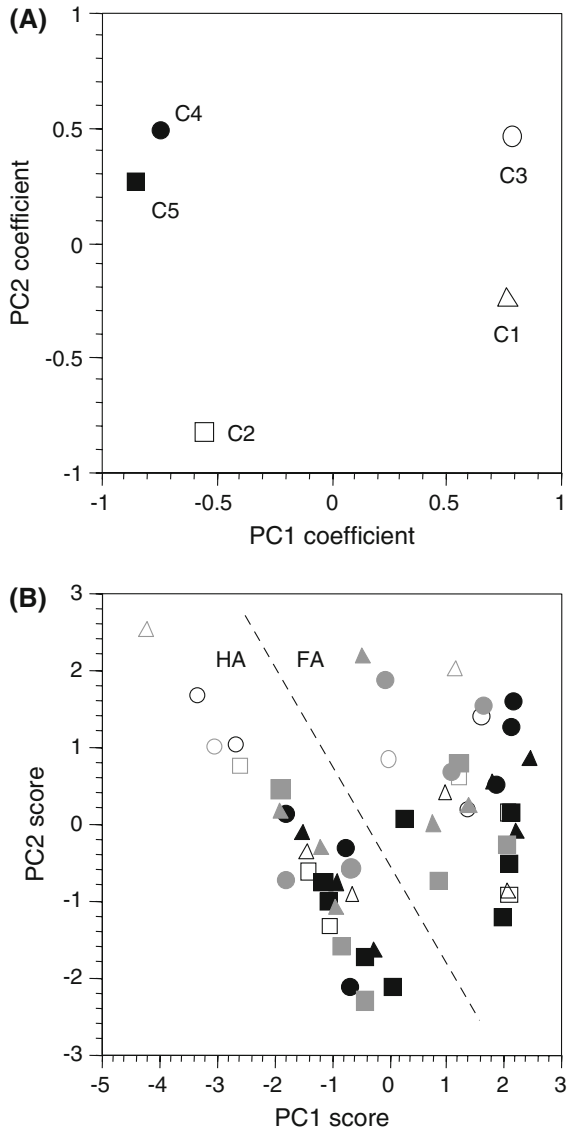

Fig. 5 Results of principal component analysis (PCA) of the five PARAFAC components. **A** Coefficient plots for PC1 and PC2 of the five PARAFAC components; **B** Score plots for PC1 and PC2 of all fulvic and humic acid fractions. The *dotted line* was arbitrarily described. *Black* and *grey* symbols indicate samples from the Urdaibai and Foz estuaries, respectively. *Open* and *closed* symbols indicate sediment and soil samples, respectively. *Circles*, *triangles*, and *squares* indicate samples from surface, subsurface, and bottom, respectively

components, especially tyrosine-like components (poor in fulvic acid- and microbial humic-like components) in the Foz FA fractions compared to the Urdaibai FA fractions (Table 3). Differences between the two estuaries were, however, not significant for the HA fraction (Table 4). The compositional difference in FA fractions between the estuaries may be the result of better OM preservation as consistent with differences in TOC contents between two estuaries (Table 1) and/or a greater marine influence in the Foz estuary than in the Urdaibai estuary (see section "General characterization of soils and sediments").

Significant differences between sediment and soil samples, were also found in the PC1 and PC2 scores in the HA fraction (Table 4). Such differences in HA fraction were the result of a large contributions of protein-like components in sediment samples compared with those in soil samples (Fig. 5B). Such a large contribution of protein components observed in the tidal channel sediments may be the result of marine inputs rich in proteinaceous materials and to the contribution of algaenan. Algaenan is a typical refractory component in algae, a highly paraffinic biopolymer which also contains proteinaceous materials in its structure (Knicker 2004). The mechanism whereby peptides in algaenan are protected against microbial degradation has been explained by the encapsulation pathway, whereby bio-labile proteinaceous material covalently bind to recalcitrant biopolymers (Knicker and Hatcher 1997, 2001). This view is also consistent with the new paradigm of HS (Sutton and Sposito 2005) as mentioned above. The non-significant differences in the composition of HA fractions with depth and between the two estuaries suggest that algaenan may be distributed randomly at depths and between the two estuaries. It should be noted that differences in PCA between sediment and soils samples were not significant in the FA fraction (Table 4). Further studies are necessary to clarify the factors controlling potential algaenan distribution in estuarine environments.

Conclusions

The FA and HA fractions extracted from estuarine sediments and soils were characterized by EEM-PARAFAC and five components were identified in the present study. All five components were found in both FA and HA fractions, however, significant compositional differences between FA and HA fractions were observed. Components C1 and C2 were representative for the FA and HA fractions (Table 3, Fig. 4) and assigned as fulvic acid-type and humic acid-type components, respectively. Spectral

characteristics of fulvic acid-type and humic acid-type components were similar to those found in DOM in soils and in water soluble fractions from soils (Ohno and Bro 2006; Ohno et al. 2007; Hunt et al. 2008; Fellman et al. 2008) and DOM in a wide range of aquatic environments (Table 2, Stedmon and Markager 2005; Yamashita et al. 2008), indicating that these components will be useful for better understanding of DOM quality, sources, and environmental dynamics.

Spectral characteristics of C3 were similar to microbial humic-like components previously found in DOM (Table 2). Component C3 was enriched in the FA fraction compared to the HA fraction (Table 3; Fig. 4), suggesting a greater microbial contribution to the FA fraction compared to the HA fraction. Protein-like components (C4 and C5) were found in almost all FA and HA fractions, and their relative abundances were generally higher in HA fractions compared to FA fractions. Such distributional patterns suggest that interactions of these components with other organic molecules may protect labile protein-like components from microbial degradation, and such interactions are likely more strongly developed for HA fractions compared to FA fractions.

Even though all of the HA fractions studied here were characterized traditionally as poorly humified, the results of PCA with EEM-PARAFAC components revealed significant differences between FA and HA fractions, between sediments and soils, and among depths, indicating that EEM-PARAFAC is a reliable technique for the characterization of HS in soil and sedimentary environments.

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