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RESEARCH ARTICLE

Comparison of marine and river water humic substances in a Patagonian environment (Argentina)

María del Carmen Scapini · Víctor Hugo Conzonno · Vilma Teresa Balzaretti · Alicia Fernández Cirelli

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Abstract Structural aspects of humic substances (HSs) in marine waters of Engaño Bay (Atlantic Ocean) and water from the Chubut River (Patagonia, Argentina) were compared. The HSs were isolated, purified, and analyzed using a multiple-method approach for structural characterization: elemental analysis, nuclear magnetic resonance (^{13}C and ^{1}H NMR), infrared spectroscopy (FTIR), UV-visible absorption and fluorescence. Similarities between the marine and freshwater components were evaluated on the basis of N and O contents, H/C and C/N atomic ratios, infrared bands from nitrogen-containing and carboxylic groups, percentage of functional groups obtained from NMR spectra, spectral slope coefficient of absorption spectra, absorbance ratios at 250 and 365 nm (E_2/E_3), aromaticity, excitation–emission matrices and fluorescence quantum yield. Both the Engaño Bay and Chubut River waters have HSs that are mainly composed of fulvic acids (FAs); although they are very similar, they may be distinguished from one another. Similarities include the predominance of aliphatic carbon content (low aromatic content); however, the water bodies differed in their content of proteins and carboxylic acids. The Engaño Bay FAs have mainly an aquatic origin; although the Chubut River FAs suggest a similar aquagenic origin, there is also evidence of contributions from pedogenic FAs.

Keywords Fulvic acid · Humic substances · Excitation–emission matrices · Chubut River · Engaño Bay

M. C. Scapini (✉)
Facultad de Ciencias Naturales, Sede Trelew,
Universidad Nacional de la Patagonia San Juan Bosco,
Roca 115, CP 9100 Trelew, Chubut, Argentina
e-mail: mariadelcarmenscapini@yahoo.com.ar

V. H. Conzonno
Laboratorio de Investigación en Sistemas Ecológicos y Ambientales, Facultad de Ciencias Naturales y Museo,
CONICET, La Plata, Argentina

V. T. Balzaretti
Facultad de Ciencias Naturales, Sede Comodoro Rivadavia,
Universidad Nacional de la Patagonia San Juan Bosco,
Km. 4, Comodoro Rivadavia, Chubut, Argentina

A. F. Cirelli
Centro de Estudios Transdisciplinarios del Agua,
Facultad de Ciencias Veterinarias, Universidad de Buenos Aires,
CONICET, Avda. Chorroarín 280, Buenos Aires, Argentina

Introduction

This work summarizes systematic structural studies on aquatic humic substances (HSs) found in the Chubut River and Engaño Bay located in the Chubut Province of Argentina (Fig. 1). The river flows across the semi-arid Patagonian region (mean discharge 54 $m^3 s^{-1}$) and is dammed in its lower valley to give origin to the Florentino Ameghino Reservoir, which was built for hydroelectric generation and agricultural drinking water supplies. After about 140 km, the river reaches Engaño Bay, which is a typical estuarine environment.

Previous studies indicate that the river water is buffered mainly by sodium bicarbonate, has high dissolved oxygen concentrations (saturation values), and low concentrations of total dissolved solids. Santinelli and Esteves (1993) found a predominance of the diatoms *Odontella aurita* and *Aulacoseira granulata* in the phytoplankton composition of both the fresh and marine waters. They also found cells and quest of the dinoflagellate *Alexandrium excavatum,* which is responsible for red tides in the estuary. Some

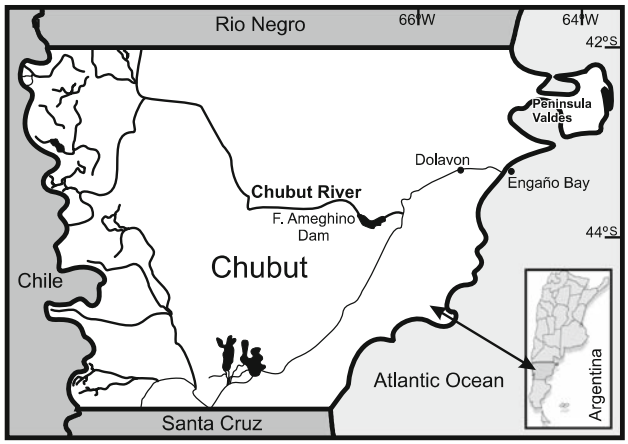

Fig. 1 Map of Chubut Province (Argentina) showing locations of sampling stations

Table 1 Physicochemical characteristics of waters (surface water; sampled station)

Parameter	Engaño Bay[a]	Chubut River[b]
Temperature (°C)	20	11
Salinity (g L^{-1})	32	0.208
Dissolved Oxygen (% S)	99	91
Chlorophyll (μg L^{-1})	6.5	2.9

[a] Summer (Esteves et al. 1997)

[b] Average; $n = 8$; 2 years (unpublished data)

physicochemical characteristics of the Chubut River and Engaño Bay are summarized in Table 1.

Research on aquatic HSs in Argentina is very limited, but began in the late 1980s with investigations on the chemical characteristics and factors influencing their distribution and dynamics in pampasic ponds (Conzonno and Fernández Cirelli 1987, 1988). The aim of the work reported here is to make a comparison between the structural characteristics of HSs present in freshwater (Chubut River) with that of the receiving marine environment (Engaño Bay) on the basis of elemental composition, IR, ^{13}C NMR, ^{1}H NMR, UV-visible absorption, fluorescence and other spectroscopic methods. This study also constitutes a first approach to better understand the role of HSs and associated problems that affect the use of these waters.

Materials and methods

Samples

The materials used as samples were:

- Humic substances isolated from the Chubut River at Dolavon sampling site (43°21.1′S 65°43.9′W) by Scapini et al. (2004). This solid extract was re-analyzed by different techniques than previously performed in order to complement its structural characterization.
- Samples from the Engaño Bay coastal zone (43°17′56″S 65°01′26″W) were obtained under high tide conditions in order to avoid the influence of freshwater input from the river. The sampling was limited to 4 km from the mouth (Santinelli and Esteves 1993). In order to obtain the HS extract, aliquots (~75 L) of surface marine water were taken fortnightly, between December 2003 and March 2004, and processed immediately.
- The same analyses of humic acid presence and spectral slope determination were performed with filtered (0.45 μm) natural water.

Isolation of marine humic substances

The isolated and purified HSs were obtained using the method of Mantoura and Riley (1976). Each aliquot of marine water was grossly prefiltered (Whatman GF/C) and then filtered again through a 0.45 μm membrane filter (Sartorius 11106), previously washed to zero absorbance at 200–400 nm, 10 cm path cell. The absorption spectrum (200–900 nm; 10 cm path cell) was recorded to verify that the DOM quality was the same for all samples. Then, each filtered aliquot was acidified to pH 2 (HCl) and passed through the same Amberlite XAD-7 resin in order to retain all HSs together by adsorption. After passage of all aliquots (~580 L), the resin was rinsed with distilled water to remove salts (mainly chloride). The isolated HSs (~100 mg) were obtained by elution with MeOH–2 M NH_4OH (1:1); elimination of MeOH and NH_3 was achieved by vacuum distillation (35°C, rotary evaporator) and passage through an ion exchange resin (Amberlite IRA-120, H form) before final concentration (35°C, rotary evaporator) and lyophilization. This same procedure of isolation and purification was employed for the river water samples.

Analysis of humic acids

Before final concentration under vacuum and lyophilization, an aliquot of the solution eluted from the ion exchange resin was acidified to pH 1 (HCl 1 N) to determine the presence or absence of humic acids (HA). Also, another test was carried out with the method proposed by Dean (1972), treating 100 ml of the eluted solution with glacial acetic acid (5 ml) and isoamyl alcohol (15 ml). If HA is present, it precipitates at the interface. Both methods were also applied to samples (100 ml) of filtered (0.45 μm) natural water.

Elemental analysis and infrared spectroscopy

The elemental composition (C, H, N, O by difference) of the isolated and purified marine HSs was obtained using a Carlo Erba EA 1108 element analyzer, and FTIR spectra were recorded with a Nicolet Magna 550 instrument (KBr disc).

NMR spectroscopy

The ^{13}C and ^{1}H NMR spectra of marine and river FAs (80 mg ml^{-1} NaOH 0.5 N in D_2O) and the corresponding deconvolution were obtained with a Bruker Advance 300 Digital spectrometer at 75.475 and 300.13 MHz, respectively, using a 5 mm dual probe. Parameters for the ^{13}C NMR spectra were: spectral window (Sw) 300 ppm; pulse width (PW) 30°; RD 2 s, acquisition time (AQ) 1.44 s. The data were processed with LB 10, software zgpg30 and Lorentzian–Gaussian form for deconvolution. Parameters for the ^{1}H NMR spectra were: NS 128; Sw 16 ppm; Pw 30.0°; AQ 3.9997 s, with solvent suppression. The data were processed with LB 0.35, Standar Software Bruker and Lorentzian form for deconvolution.

Absorption and fluorescence spectroscopy

UV-Visible absorption spectra were obtained using a Metrolab 1700 UV-VIS spectrometer with SF 170 software and 10 cm path length quartz cells. To avoid self-quenching, lower concentrations of Chubut River HSs (3.2 mg L^{-1}) and Engaño Bay HSs (6.4 mg L^{-1}) in $NaHCO_3$ (150 mg L^{-1}) were employed in all fluorescence measurements. Conventional excitation and emission fluorescence spectra were obtained with a Shimadzu RF-5301PC spectrometer with quartz cells (1 cm path length).

An Aminco Bowman Series 2 with AB2 Software Luminescence Spectrometer Version 4.00 was used to obtain the excitation emission matrices (EEMs). Forty-seven emission spectra at excitation wavelengths 7 nm apart between 196 and 518 nm were run, with measurements of intensity at each nm of the spectrum obtained from 330 to 680 nm. Each EEM involves 16,497 fluorescence intensity measurements in arbitrary units. EEMs for each sample were corrected by subtracting the EEMs of solvent obtained under the same conditions. The acquired EEMs were analyzed by multivariate curve resolution alternating least squares (MCR-ALS) algorithms (Jaumot et al. 2005).

Quantum yields of fluorescence (Φ_F) were obtained by the comparative method that involves a reference standard with well-known Φ_F using the following equation:

$$\Phi_{FX} = \Phi_{FR}(A_R/A_X)\left(\int E_X d\lambda' \Big/ \int E_R d\lambda'\right)(\eta_X/\eta_R)^2$$

where A is the absorbance at the excitation wavelength, η is the refractive index of the solvent and $\int E_X d\lambda'$ is the area of the full emission spectrum (total photon number). The subscripts R and X denote reference standard and sample, respectively. Quinine sulfate in 0.1 N H_2SO_4 was selected as the reference standard because it fluoresces in the same emission range as the samples. The absorbance of the standard solutions and samples was adjusted to get similar results at the same excitation wavelength. Under these conditions, it may be assumed that both solutions absorb the same number of photons. These absorbance values were less than 0.05 (10 mm path length) to avoid self-quenching. The emission spectra of sample and reference standard solutions (recorded under identical conditions) were obtained after correction of instrumental response (applying a correction factor provided by the manufacturer) and subtraction of the emission spectrum of the solvent.

The ratio of the areas of the corrected emission spectra was used to calculate Φ_F. The emission range was always 400–600 nm, and the excitation wavelength was 325 nm to get Φ_{F325} (quantum yield at 325 nm as excitation wavelength) and 360 nm to find Φ_{F360} (quantum yield at 360 nm as excitation wavelength). Absorbance was measured with a Zeiss Spekol 1200 spectrometer.

Results and discussion

Acid precipitation

The presence of HAs and FAs were analyzed taking into account the pH dependence on water solubility. After treatment of the sample with HCl and glacial acetic acid in isoamyl alcohol, no precipitation was observed suggesting the absence of HAs; only FAs, as defined operationally (Gagosian and Stuermer 1977), were present in the isolated marine HSs. These results are not surprising since it is well known that the proportion of lower molecular weight HSs predominate in surface seawater (e.g., Benner 1998).

Elemental composition

Data obtained for the HSs isolated from Engaño Bay and the Chubut River (Scapini et al. 2004) as well as marine waters and freshwaters of terrestrial origin for comparison, are shown in Table 2. These data indicate that the elemental composition of Engaño Bay FAs is similar to that of marine FAs, but different from those of freshwater or terrestrial origin, while the Chubut River FAs fall somewhere between the two.

Table 2 Elemental composition of water fulvic acids from different sources

Source of FA	Elemental composition[a]				Atomic ratio		
	C	H	O	N	H/C	O/C	C/N
Engaño Bay	56.1	7.1	32.1	4.7	1.5	0.43	14
Chubut River[b]	51.2	5.5	40.0	3.3	1.3	0.59	18
Marine water[c]	50.0	6.8	36.9	6.4	1.6	0.55	9
Freshwater terrestrial origin[d]	52.4	4.3	42.2	0.72	1.0	0.60	87

[a] Concentration: w/w dry ash free (Chubut River FA ash 3.2%, Engaño Bay FA ash 8.2%)

[b] Scapini et al. (2004)

[c] Buffle (1988)

[d] Suwannee River, 1S101F, IHSS, http://www.ihss.gatech.edu/

With regard to the C/N ratio, Engaño Bay FAs and Chubut River FAs are significantly smaller than those of terrestrial origin. These differences may be attributable to the phytoplankton degradation products (higher in proteins) found in the bay and river, while contributions from the degradation of the lignin in terrestrial vascular plants (lower in proteins) are more important in terrestrial ecosystems. Also, the differences between Engaño Bay FAs and Chubut River FAs may be associated with the contribution of terrestrial vascular plants, which for the river makes for a signal of mixed origin.

FT-IR spectroscopy

Infrared spectroscopy has mainly been used for qualitative determination of functional groups in HSs (Bloom and Leenheer 1989). The spectra have strong wide bands due to overlapping absorptions from these heterogeneous mixtures, thereby making exact assignments difficult. Typical HS spectral characteristics (Stevenson and Goh 1971; Stuermer and Payne 1976) are present in the Engaño Bay FA preparations, which we compared with the spectrum of the Chubut River FA (Fig. 2; Scapini et al. 2004).

The band at 3,200–3,600 cm^{-1}, assigned to O–H stretching in the phenols and alcohols and N–H stretching in amines and amides is present in both spectra. There is a strong and wide band beginning at 2,500 cm^{-1} in the river spectrum due to O–H stretching in carboxylic acids, which corresponds with the absorption near 1,400 and 1,200 cm^{-1} characteristic of acid groups. The marine spectrum has low carboxylic acid absorptions in both regions. This fact suggests that carboxylic groups are more important in the river FAs; perhaps such groups are in ester form in marine FAs. Buffle (1988) also points out that marine FAs have a low content of carboxylic acid residues.

The absorption at 2,900 cm^{-1}, assigned to aliphatic C–H stretching, is evident in both the marine (2,943 cm^{-1}) and river FA spectra (2,975 cm^{-1}). The presence of methyl and methylene groups is confirmed by C–H bending absorptions near 1,450 and 1,375 cm^{-1} (typical of CH_3).

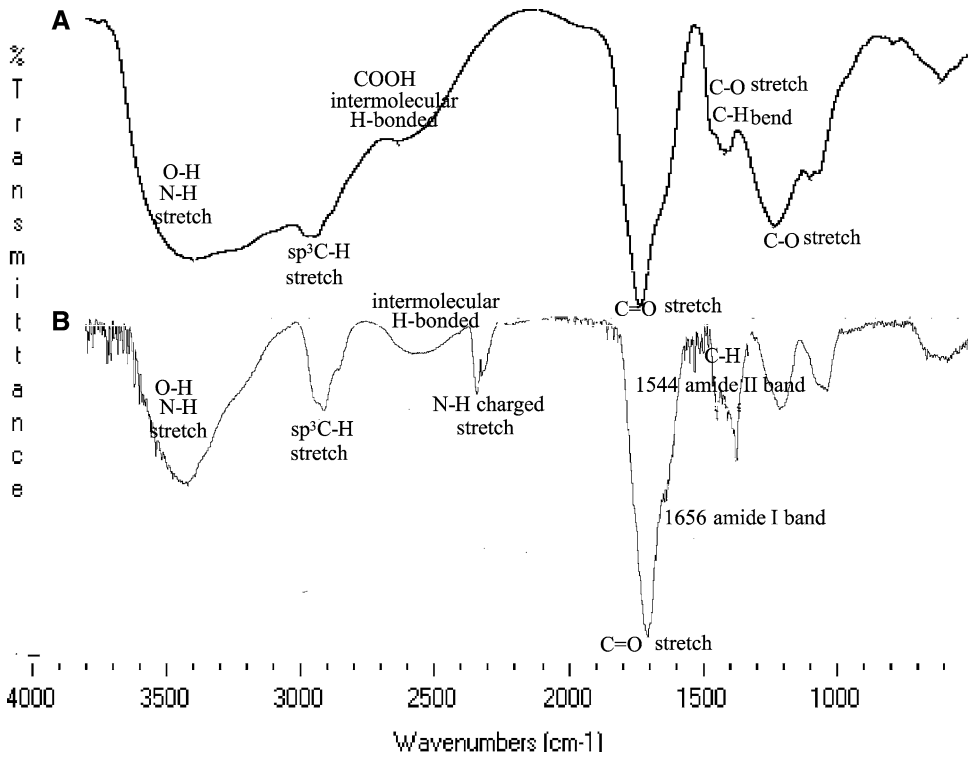

Fig. 2 FTIR spectra of fulvic acids: (**a**) Chubut River, (**b**) Engaño Bay

The C–H stretching absorption at 3,000–3,100 cm^{-1}, from alkenes and/or aromatic groups, is low in the marine FA spectrum and may overlap with other absorptions in the river FA spectrum. Both spectra exhibit a shoulder near 1,600 cm^{-1}, which may be related to C = C stretching. The low intensity near 2,300 cm^{-1} is possibly due to minor content of charged amine derivatives or amino acids (Flaig et al. 1975; Hesse et al. 1997; Silverstein and Webster 1998).

The absorption at 1,720 cm^{-1} is due to C = O stretching of the carbonyl group in carboxylic acids and/or esters because there are neither quinones nor ketones present (see NMR data below). The presence of bands assigned as C = O stretching of the amide I band at 1,656 cm^{-1} and C–N stretching of the amide II band at 1,544 cm^{-1}, agrees with the N content in the marine FAs and is another important difference that may be attributed to peptides or protein-like components. The peak at 1,050 cm^{-1} is assigned at C–O stretching in alcohols and may be attributed to polysaccharide or polysaccharide-like components.

The IR analysis indicates that the Engaño Bay FAs may have aliphatic, alcoholic, ester, amide and amine groups present. This agrees with the literature that points to a generally significant presence of aliphatic components such as fatty acids, polysaccharides and amino sugars in marine FAs (Buffle 1988).

^{13}C and ^{1}H NMR spectra

The importance of NMR spectroscopy for determining the chemical structure of HSs is well known (Benner 2002; Abbt-Braun et al. 2004). Early on, Hatcher et al. (1980) also pointed out the utility of the ^{1}H NMR to complement the results obtained from using ^{13}C NMR. We obtained both ^{1}H and ^{13}C NMR spectra of the marine and river water FAs (Fig. 3).

We observed the typically broad and poorly resolved bands attributable to extremely complex and non-repeating structural sub-units (Stuermer and Payne 1976; Wilson et al. 1981; Thorn et al. 1989). In addition, we acquired the

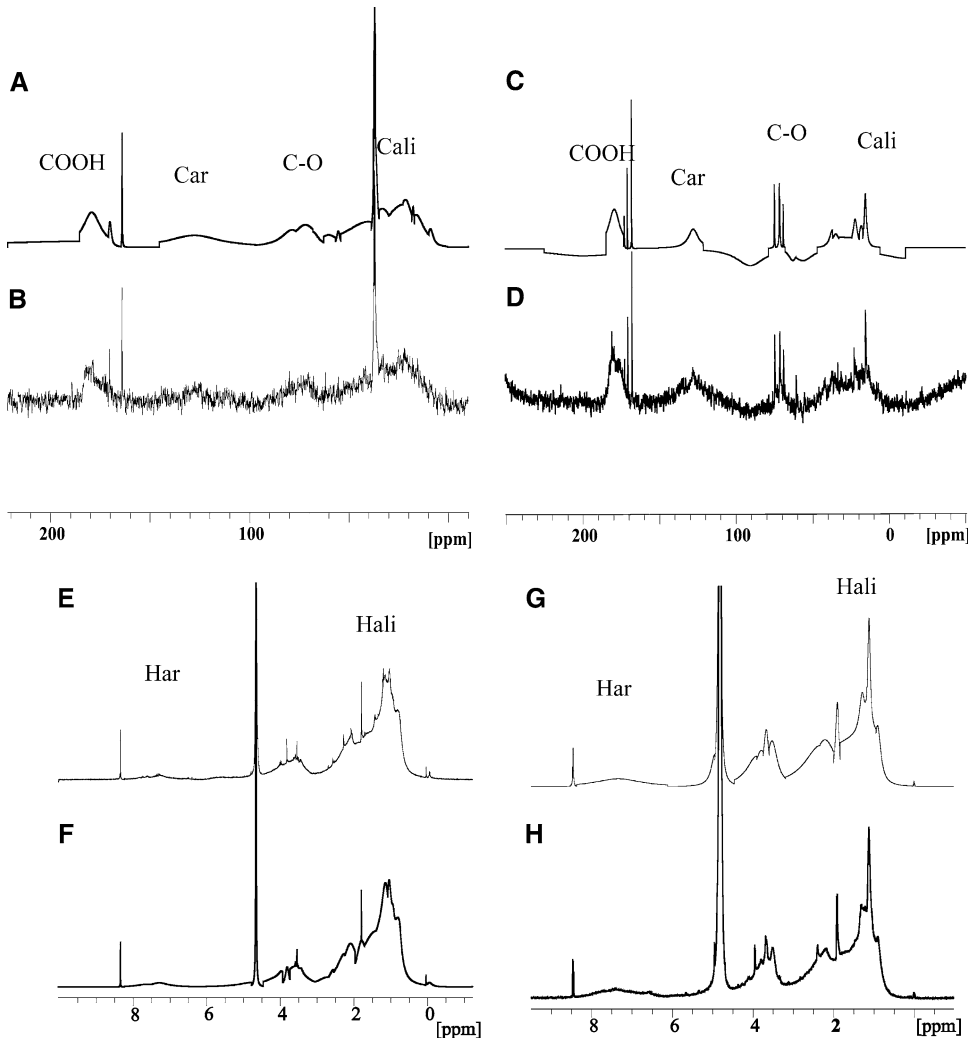

Fig. 3 ^{13}C NMR spectra of fulvic acids: (**a**) Engaño Bay, (**c**) Chubut River, and their deconvolutions (**b**) Engaño Bay, (**d**) Chubut River. ^{1}H NMR spectra of fulvic acids of (**e**) Engaño Bay, (**g**) Chubut River and their deconvolutions (**f**) Engaño Bay, (**h**) Chubut River (*Cali* aliphatic carbon, *Car* aromatic carbon, *Hali* hydrogen bounded to aliphatic carbon, *Har* hydrogen bounded to aromatic carbon)

deconvoluted spectra (Fig. 3) to calculate the C and H atomic percentages of functional groups from electronically integrated peak areas (Table 3). The ^{13}C spectra of the two FAs studied showed some similarities and some differences. The band from aliphatic groups (0–60 ppm), due primarily to sp^3 hybridized carbons bonded to other carbons, is predominant in both spectra and is suggestive of their aliphatic nature.

The resonances in the range (60–90 ppm), characteristic of C singly bonded to oxygen (ether, alcohol, carbohydrate carbons), are low in both FAs. This band, in spite being small, is second in predominance in the marine FAs. This means that the aforementioned functional groups are the most important among the minor components of the seawater FAs. The low resonances at 90–110 ppm, typical of the anomeric C of carbohydrates, suggest an infrequent presence of these compounds and/or a singular structure of these particular FAs.

The major differences observed are in resonances in the range 110–165 ppm, characteristic of aromatic and olefinic carbons, and the resonances at 165–190 ppm, characteristic of carboxylic carbons (acid, ester, and amide). These resonances produce a noticeable band in the river FA spectrum, which is, in fact, the second most abundant in the spectrum. On the other hand, this band is lower in the marine FA spectrum than the river one, which is in agreement with the IR results. The minor marine FA aromaticity agrees with the findings of Hatcher et al. (1980) and reflects the lack of lignin components. The river FAs have an aromaticity similar to that of Suwannee River FAs (Thorn et al. 1989), but differ in other regions of the ^{13}C–NMR spectra. Typical resonances of C=O from aldehydes and ketones in the 190–230 ppm range (Thorn et al. 1989) are not evident in our spectra of Engaño Bay and Chubut River FAs. This suggests that carbonyl groups, in coincidence with the IR spectra, might be mostly in the form of carboxylate groups and/or their derivatives (esters and/or amides).

Table 3 Percentage of functional groups of fulvic acids from deconvoluted ^{13}C and ^{1}H NMR spectra of Engaño Bay and Chubut River

Functional group (chemical shift in ppm)	Percentage of integrated peak area	
	Engaño Bay	Chubut River
^{13}C-NMR		
Aliphatic (0–60)	74	51
Hetero-aliphatic (60–90)	12	6
Aromatic (110–165)	6	16
Carboxyl (165–190)	9	27
^{1}H-NMR		
Aliphatic (0–3.0)	87	79
Hetero-aliphatic (3.0–4.2)	10	16
Olefinic (4.7–6.0)	1	2
Aromatic (6.0–9.0)	2	3

Results obtained from the ^{1}H NMR spectra support those obtained from the ^{13}C spectra. The bands from aliphatic protons are predominant in both spectra. Marine FAs exhibit an insignificant signal in the range for aromatic protons. Early on, Harvey et al. (1983) found an absence of aromaticity in the NMR spectrum of marine FAs. The 6.8 ppm peak (aromatic H adjacent to phenol and methoxy groups), which is indicative of lignin and tannin residues (Spitzy and Leenheer 1988) is not present in our ^{1}H NMR spectra.

The spectra of both FAs are similar at those of Big Soda Lake (Spitzy and Leenheer 1988), which is characterized by a high aliphatic carbon content, low aromatic carbon content and negligible ketone content. In contrast, the Suwannee River fulvic acid has a significant ketone content and high aromatic carbon content. The FA of Big Soda Lake has an aquagenic origin, being a product of phytoplankton degradation, while the Suwannee River FA has pedogenic origin, being a product of higher plant decomposition. These comparisons suggest that both our FAs have an aquagenic origin. In this context, it is known that the principal source of seawater HSs is planktonic/bacterial excretion and degradation (aquagenic origin). For freshwater HSs, Spitzy and Leenheer (1988) maintain that an autochthonous origin in rivers which flow from great lakes to reservoirs can be expected.

UV-Visible absorption spectroscopy

The UV-Visible spectrum is generally divided into three parts: the vacuum UV (100–200 nm), the UV (200–400 nm) and the visible (400–800 nm). The vacuum UV range is not useful for the study of HSs because water and most other solvents used with HS absorb strongly in this region (Bloom and Leenheer 1989).

We found that both the marine and river water FAs absorb significantly, but only in the UV range. Inorganic chemicals typically present in natural freshwaters do not absorb light significantly at wavelengths greater than 230 nm (Korshin et al. 1997). These facts restrained our useful wavelength range to 230–400 nm. The two absorption spectra obtained (Fig. 4) exhibit the typical characteristics of HS: broad and featureless with absorption increasing at lower wavelengths. Though they are similar, absorbance is greater in the Chubut River spectrum than that of Engaño Bay at all wavelengths; in addition, the light shoulder at 280 nm is more evident in the former.

The absorption of HSs in the UV region is due to the excitation of π electrons in aromatic rings with different degrees and types of substitution. The number of

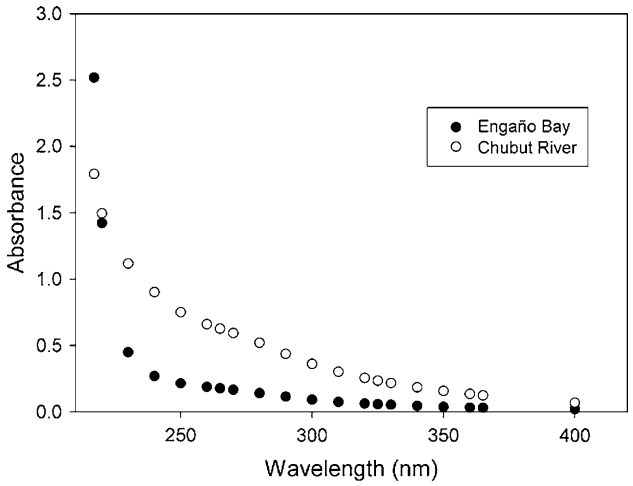

Fig. 4 UV-Visible absorption spectra of fulvic acids from Engaño Bay and Chubut River

chromophore types is high; neither has an easily distinguishable absorption spectrum, and the respective concentrations are unknown (Leenheer and Pecroué 2003). These facts make difficult the deconvolution of UV spectra of HSs (Korshin et al. 1997). Absorption measurements were frequently made at a single wavelength, e.g., 254 nm (Mrkva 1983; Martin-Mousset et al. 1997; Lepane et al. 2003), despite the featureless spectra. Others researchers (e.g., Green and Blough 1994) have used the fact that light absorption by HSs decreases approximately in an exponential way:

$$a(\lambda) = a(r)\exp[S(r-\lambda)] \text{ and } a(\lambda) = 2.303\,A(\lambda)/l$$

where r = reference wavelength and l = path length (m).

The spectral slope coefficient (S) is useful in making comparisons under different environmental conditions. It is a parameter that expresses light absorption efficiency as a function of wavelength. It is related to the nature of the chromophore (Blough and Del Vecchio 2002) and is indicative of changes in it. Here, S was determined from the UV spectra of FAs of natural waters from Engaño Bay, previously filtered (0.45 μm), and was compared with those of Chubut River FAs. We plotted ln (a) versus λ from 250 to 400 nm, and S was determined from regression analysis. The result obtained, 18×10^{-3} nm^{-1} with a standard deviation of 0.3×10^{-3} nm^{-1}, is very similar to the S determined for Chubut River FAs (i.e., 16×10^{-3} nm^{-1} with standard deviation 0.3×10^{-3} nm^{-1}; Scapini et al. 2008).

With regard to the relationship between S and HS structure, Blough and Del Vecchio (2002) point out that the S of FAs is higher than for HAs and that S increases with decreasing molecular weight and aromaticity due to the absence of extended aromatic systems. Hence, the S values obtained in this study suggest low molecular weights for both of the studied FAs and no detectable extended aromatic system. Also, they indicate a very low contribution of pedogenic FAs in the Chubut River. This contribution is even minor in the Engaño Bay FAs.

Peuravuori and Pihlaja (1997) have related the quotient, E_2/E_3 (absorbance at 250 and 365 nm) to the aromaticity (percentage aromatic carbon) of humic solutes in natural surface waters from the following relationship: aromaticity = $52.509 - 6.78\,E_2/E_3$. The aromaticity of the Engaño Bay FAs, calculated from the UV spectrum of unaltered water by applying this equation, was 5.5 ($E_2/E_3 = 6.9$). This value is lower than that for the Chubut River FAs (aromaticity 21, $E_2/E_3 = 4.7$) obtained previously (Scapini et al. 2008) by a similar approach. Both values of aromaticity agree with the percentage of aromatic carbon obtained from the ^{13}C NMR spectrum. It is clear that the results obtained from absorption spectroscopy support the conclusions derived from other methods applied in this study.

Fluorescence spectroscopy

Several studies have investigated the intrinsic fluorescence spectral properties of HSs in order to obtain information about molecular structure, especially because of the high sensitivity and non-destructive nature of the technique. The fluorescence is attributable to aromatic compounds, but aliphatics, palmitates and stearates also fluoresce (Bloom and Leenheer 1989). In this study, the investigated fluorescence properties, included excitation emission matrices and quantum yield measurements.

Pure simple compounds have excitation spectra very similar to their absorption spectra, and the emission fluorescence maximum is independent of the excitation wavelength. This is not valid, however, for HSs because their absorption spectra differ from their excitation spectra, and the wavelength of maximum emission is dependent on the excitation wavelength. The EEMs are a better approach than conventional spectroscopy for obtaining information about the existence and classes of the fluorophores because they are wavelength-independent measurements (Coble 1996).

We acquired the EEMs of the HSs isolated from both Engaño Bay and the Chubut River. The wavelength-independent maximum fluorescence (Ex_{max}/Em_{max}) values obtained from the contour plots of the emission wavelength versus excitation wavelength (Fig. 5) were compared. Marine and riverine FAs show one region of maximum fluorescence intensity at Ex_{max}/Em_{max} = 220 − 260 nm/400 − 450 nm for the Chubut River FAs and Ex_{max}/Em_{max} = 260 − 280 nm/420 − 450 nm for the Engaño Bay FAs. Both FAs exhibit a similar range of emission wavelengths and only minor differences in their range of

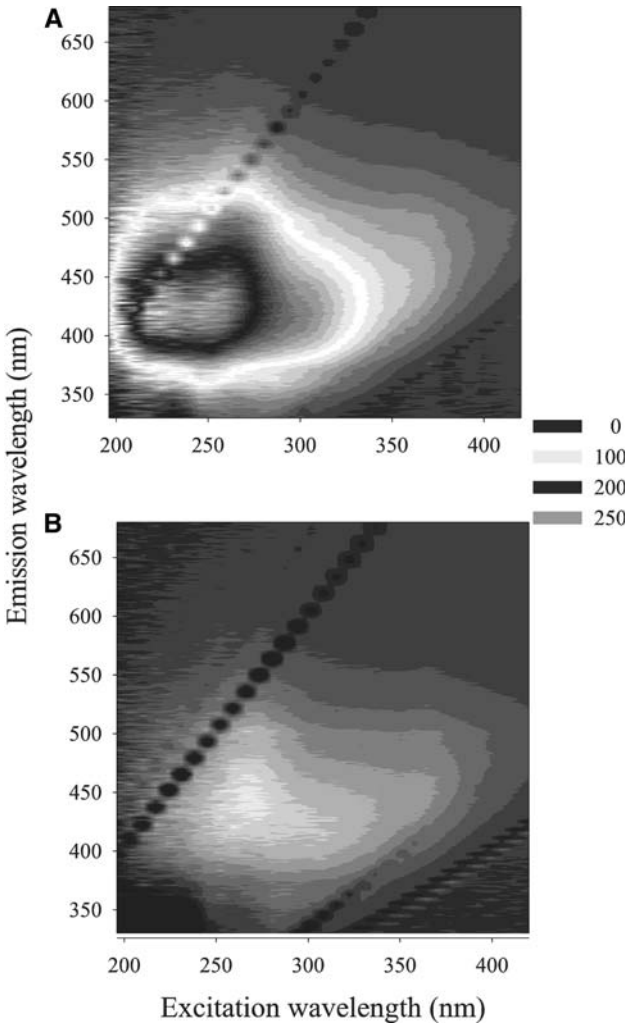

Fig. 5 Contour plots of excitation emission matrices of fulvic acids from (**a**) Chubut River and (**b**) Engaño Bay (intensity in arbitrary units)

excitation wavelengths. The marine FAs have a small shoulder with a slight shift toward greater wavelength and low intensity of fluorescence. The latter is expected due at their low absorption in the UV-visible range.

Distinct classes of fluorophores are generally considered in the literature. For example, Coble (1996) and De Souza Sierra et al. (2005) referred to two humic-like fluorophores and two protein-like fluorophores (tyrosine and tryptophan-like). They also described one specific marine humic-like fluorophore. Table 4 shows excitation/emission wavelength ranges at maximum fluorescence intensities for our FAs and that of the same peak obtained from the literature for comparison using Coble's (1996) designations.

In spite of their different intensities, both marine and river FAs have values of Ex_{max}/Em_{max} similar to that of peak A (Ex_{max}/Em_{max} = 250–260 nm/380–480 nm). Visual observations suggest that peak A is the principal type of fluorophore present in both river and marine FAs. In order to verify the presence and/or importance of other fluorophores, the EEMs acquired were analyzed by the multivariate curve resolution-alternating least-squares (MCR-ALS) algorithm (Jaumot et al. 2005). For the marine FAs, a single component explains 94.6% of the overall spectral variance (with a fitting error of 23.5% with respect to the average recorded signal), while two components explain 96.4% of the variance (fitting error = 19.5%). For the FAs of the Chubut River, a single component explains 98.1% of the variance (fitting error = 13.5%) and two components explain 98.9% of the variance (fitting error = 10.2%). These results suggest that it is feasible to describe both FAs on the basis of two components: a major one whose contribution is about 98% and a minor one with 1–2%. In both FAs, the Ex_{max}/Em_{max} of the major component falls in the range of peak A, although the marine

Table 4 Names and Excitation/Emission wavelength range at maxima fluorescence intensity of peaks from different sources

Fluorophore type	Peak	Wavelength range (nm)	
		Excitation	Emission
	Engaño Bay FAs	260–280	420–450
	Engaño Bay FAs (major component)	240–325	390–460
	Engaño Bay FAs (minor component)	Two maxima: 270 and 375	430–525
	Chubut River FAs	220–260	400–450
	Chubut River FAs (major component)	220–260 and 300–310 (shoulder)	390–460
	Chubut River FAs (minor component)	Two maxima: 270 and 375	430–525
Visible Marine humiclike	M[a]	310–320	380–420
UV Humiclike	A[a]	250–260	380–480
Visible Humiclike	C[a]	330–350	420–480
Tyrosine-like, proteinlike	B[a]	270–280	300–320
Tryptophan-like, proteinlike	T[a]	270–280	320–350

[a] Coble (1996)

FAs have an excitation range with a small shoulder that is shifted slightly to the red and has a smaller emission intensity. The characteristics of the minor component are similar in both FAs: they present signals of peaks A and C (slightly shifted to longer wavelengths). The precedent analysis supports the predominance of fluorophores of type A.

In an extensive study of the EEMs of FAs and HAs from natural sources and references, De Souza Sierra et al. (2005) found that the A and C peaks are ever present regardless of HS source and that the first and more intense region of fluorescence intensity for FAs, is located at around Ex_{max}/Em_{max} = 260 nm/460 nm and the second, less intense, at Ex_{max}/Em_{max} = 310 nm/440 nm. For HAs, these two regions are at Ex_{max}/Em_{max} = 260 nm/525 nm and Ex_{max}/Em_{max} = 360 nm/520 nm. Our data are similar to that FA. This is an additional element that supports the fulvic nature of these HSs, in agreement with the conclusions of other applied methods.

In our EEMs, the contribution of peak C is very small and so its contribution appears to be negligible. The C peak is believed related to lignin-derived contributions (De Souza Sierra et al. 1997; Coble 1996) which are very low in our HSs (see absorption spectroscopy).

EEMs in which peak C does not appear to be clearly distinguishable have been reported by Coble (1996) in the Gulf of Maine and by Coble et al. (1998) in highly irradiated oligotrophic marine environments. The authors attribute these findings to photodegradation in surface waters with low organic matter content and in regions of high solar irradiation that produce the remaining fluorophores that are only stimulated at wavelengths lower than 300 nm. The visible marine humic-like peak M is not noticeable in Engaño Bay FAs. This is another coincidence with the pattern described by Coble for highly irradiated oligotrophic marine environments as mentioned above. These facts allow us to formulate the hypothesis, to be confirmed in future work, that radiation significantly affected the FAs in our water samples.

None of FAs studied exhibit signals of protein-like fluorophores. These signals have been related to tyrosine and/or tryptophan and their aromaticity (Coble 1996). These results lend support to our conclusion that the protein compounds detected by elemental composition and infrared spectroscopy in Engaño Bay FAs are predominantly aliphatic. Although individual compounds, such as hydroxybenzoic acids and other substituted phenolics, have been studied to better understand the origin of the humic fluorescence (Senesi 1990), the assignment of structures responsible for the fluorescence is still an open question (Abbt-Braun et al. 2004). The literature assigns fluorophores emitting at longer wavelengths as degraded or aged to be mostly responsible for the fluorescence, and the increase of linear conjugation or addition of certain functional groups such as carboxyl, nitro, and hydroxyl groups to aromatic compounds. In addition, short wavelength-emitting fluorophores have been attributed to the smallest structures (De Souza Sierra et al. 1997); however, various authors emphasize that 'humic-like fluorescence is derived from a mixture of fluorophores and that the number of fluorophores present and their individual characteristics may never be known' (Coble 1996; Coble et al. 1998). Furthermore, that 'the true nature of the fluorophores responsible for dissolved organic matter fluorescence in natural waters is unknown' (Komada et al. 2002) and 'the question of the fluorophores responsible for HS, principally HA fluorescence, remains unanswered" (De Souza Sierra et al. 2005).

We determined the quantum yield (Φ) of the marine FAs at two different excitation wavelengths and compared it with the Φ of the Chubut River FAs. The Φ of the former at 320 nm was $0.30 \times 10^{-2} \pm 0.02 \times 10^{-2}$ and at 360 nm was $0.72 \times 10^{-2} \pm 0.02 \times 10^{-2}$. These values agree with those found for HSs from different aquatic environments (Green and Blough 1994), but are lower than the Chubut River FAs, which at 320 nm was $0.40 \times 10^{-2} \pm 0.02 \times 10^{-2}$ and at 360 nm was $0.87 \times 10^{-2} \pm 0.05 \times 10^{-2}$. These results were expected because of the lower absorbance of the marine FAs. The analysis of data suggests that both FAs are mixtures of compounds, many of which may absorb without fluorescing.

The Φ of both FAs increased with the excitation wavelength, which is in agreement with the findings of Del Vecchio and Blough (2004). They also found that Φ decreases at excitation wavelengths greater than 350 nm. This is one of the results of an extensive study that supports an interaction model on the basis of the optical properties of HSs. The authors propose transfer interactions between hydroxy-aromatic donors and quinonoid acceptors. How much the Engaño Bay and Chubut River FAs, which have low aromaticity and undetectable quinones, agree with this model is out of the scope of this present study.

Structural characteristic and environmental effects

The different analytical methods applied in this work were coincident in that both studied FAs have similar, but distinguishable, structures. The most important similitude is that both are highly aliphatic and contain oxygenated aliphatic functional groups. Buffle (1988) pointed out that the properties of aquagenic freshwater HSs are similar to those of the ocean because both have originated from phytoplankton degradation. Thus, the similarity between the Chubut River and Engaño Bay FAs support the aquagenic origin of the former and conclusions of our previous work (Scapini et al. 2004).

On the basis of the characteristics mentioned above, it is possible to offer some speculations about the environmental effects that are expected with regard to the principal problems that affect the use of these waters; namely, the plugging of filters during drinking water treatment and poisoning of shellfish by dinoflagellates.

Humic substances are precursors of potentially carcinogenic trihalomethanes (THMs) formed during disinfection by chlorination in water treatment processes. Early studies established that aromatic structures (such as resorcinol) and other, such as β diketone, both frequently present in HSs, are the most reactive (Rook 1977; Reckhow et al. 1990). It is expected, therefore, that the Chubut River FAs, which have low aromaticity and lack ketones, present a low trihalomethane formation potential (THMFP). This has been verified in our laboratory (Chiarandini, personal communication) as well as in the Trelew Treatment Plant Laboratory; however, organic carbon aromaticity alone cannot fully explain or predict THM precursor content (Fujii et al. 1998; Fleck et al. 2004). UV measurements are good indicators of aromatic compounds, but are less informative about β diketone structures (Uyguner et al. 2004). Additional investigations will be needed for better identification of THMs precursors (Chow et al. 2006).

Humic substances increase the biomass yield and the growth rate of marine dinoflagellates (Granéli et al. 1985; Granéli and Moreira 1990; Carlsson and Granéli 1998; Doblin et al. 2000). Several early studies (Prakash and Rashid 1968; Prakash et al. 1973) indicated that growth enhancement of marine dinoflagellates and diatoms is dependent on HS concentration. This stimulatory effect is apparently independent of nutrient concentration and can be attributed to chelation processes as well as to stimulation of algal cell metabolism. These studies also conclude that the growth response to HAs is favored by dinoflagellates, while FAs are stimulatory to diatoms. These results are advantageous for the marine waters of Engaño Bay where the humic substances are mainly FAs.

Additional and more specific futures studies will be carried out in order to verify these hypotheses especially under extreme climatic conditions. In this work, waters were sampled during dried periods. Events like stormwater runoff can bring HAs to river and marine coastal waters and may change the composition, characteristics and effects of humic substances.

Finally, this is the first study of marine humic acids in Argentina, and the conclusions provide new information about the structural properties of humic substances in these aquatic systems. They also support and complement the results obtained in previous work and contribute to our general knowledge of humic substances.

Conclusions

Comparative analysis of humic substances isolated from Engaño Bay marine water (coastal zone) and from the Chubut River, using a multiple method approach, indicates that both are composed of fulvic acids with similar, but distinguishable, structures. The principal difference is the lower amounts of aromatic and carboxylic groups and the correspondingly high amounts of aliphatic structures in the seawater FAs that have nitrogen functional groups among their minor components. Theses differences suggest that marine FAs have more protein-like compounds and a notable deficiency in lignin components compared to the Chubut River FAs. The most important similarity is that the FAs from both water types are highly aliphatic and contain oxygenated aliphatic functional groups. The similarity between Chubut River FAs and Engaño Bay FAs support the aquagenic origin of the former. On the basis of the characteristics mentioned above, it is reasonable to expect that the HSs of the Chubut River and Engaño Bay present stimulatory effects on the growth of dinoflagellates and diatoms (lower in the former and larger in the latter) and low trihalomethane formation potential.

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