THE DYNAMICS OF FLUORESCENT DISSOLVED ORGANIC MATTER IN THE PARANAGUÁ ESTUARINE SYSTEM, SOUTHERN BRAZIL

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A B S T R A C T

The aim of this study was to investigate the dynamics of the fluorescent dissolved organic matter (FDOM) in Paranaguá Estuarine System (PES) as to infer about the contribution of allochthonous FDOM to the estuarine waters in relation to tidal condition and seasons. Fluorescence spectroscopy was used for such purpose and DOM characterization through fluorescence emission was performed using excitation wavelengths of λex 350 nm and λex 450 nm, the two main fluorescence groups known to be present in natural DOM. Relations between emission wavelength (λem) and environmental variables, and the relevance of these variables to the different tides and seasons were identified by principal component analysis. The results showed that the first class of fluorophores (λex 350 nm) changed from the river (freshwater) towards the estuary, whilst the second class (λex 450 nm) has a more conservative nature and does not change as significantly as the first. Allochthonous DOM contribution to the estuarine system is intensified during the rainy season, especially in spring tides, whereas in the dry season the ratio of autochthonous DOM to total DOM in PES waters increased. We concluded that the variation of maximum λem of the first class of fluorophores (λex 350 nm) is mainly related to allochthonous contribution, whilst the maximum of emission for the second class of fluorophores (λex 450 nm) is dependent on the contribution of the different sources of organic matter (freshwater and marine water DOM contribution).

R E S U M O

O objetivo deste estudo foi investigar a dinâmica da matéria orgânica fluorescente (FMOD) no Complexo Estuarino de Paranaguá (CEP) para inferir sobre a contribuição da FMOD alóctone nas águas estuarinas em relação à condição de maré e estações do ano (seca e chuvosa). Empregou-se a técnica de espectroscopia de fluorescência, através da utilização de dois comprimentos de onda de excitação, os quais correspondem a duas classes conhecidas de fluoróforos, λex 350 nm e λex 450 nm, para desta forma determinar o comprimento de onda de máxima emissão (λem) da fluorescência da MOD. Relações entre λem e variáveis ambientais e a relevância das relações nas diferentes condições de maré (sizígia e quadratura) e estações do ano (seca e chuvosa) foram identificadas com o uso de análise de componentes principais. Os resultados demonstraram que a primeira classe de fluoróforos (λex 350 nm) foi alterada durante a transição rio - estuário, enquanto a segunda classe (λex 450 nm) apresentou um comportamento mais conservativo. A contribuição da MOD alóctone no estuário foi intensificada durante a estação chuvosa, especialmente durante as marés de sizígia, enquanto na estação seca a MOD autóctone é preponderante na composição da MOD total no CEP. Conclui-se que a variação nos λem da primeira classe de fluoróforos (λex 350 nm) é principalmente relacionada à contribuição alóctone, enquanto as diferenças nos λem da segunda classe (λex 450 nm) estão relacionadas com as flutuações nas contribuições das diferentes fontes de MOD no CEP.

Descriptors: Humic substances, Estuaries, FDOM's source, Tidal cycle, Estuarine turbidity maximum, Fluorescence spectroscopy.
Descritores: Substâncias húmicas, Estuários, Fonte de FMOD, Ciclos de maré, Máximo de turbidez estuarina, Espectroscopia de fluorescência.

INTRODUCTION

In estuaries the interactions between freshwater, saltwater, terrestrial and atmospheric systems occur with considerable intensity. They are dynamic ecosystems that respond to environmental forces with different time scales, such as semi-diurnal tides, spring and neap tides (fortnightly), riverine input variation (seasonal), and others. In addition, estuaries are among the most productive ecosystems known (UNDERWOOD; KROMKAMP, 1999) and great part of their production is supported by the terrestrial contribution of nutrients and organic materials such as humic substances.

Terrestrial humic substances are a major source of the fluorescent dissolved organic matter (FDOM) in estuarine waters (COBLE et al., 1998). This is the optically active portion of dissolved organic matter (DOM), being historically referred to as Gelbstoff or yellow substances. Besides the DOM of terrestrial origin, other contributions to the bulk of coastal and estuarine DOM are those from autochthonous sources, e.g. the DOM produced *in situ* by the biota (ROCHELLE-NEWALL; FISHER, 2002; MAYER et al., 1999), and also by processes such as the UV-induced polymerization of sugars, amino acids and other small molecules (HARVEY et al., 1983).

The characterization of DOM in estuaries is important for the understanding of its role in the functioning of the ecosystem (BENNER, 2002) since DOM dynamics interfere greatly in the carbon cycle. The DOM composition can affect, for example, microbial and plankton ecology (WILLIAMSON et al., 2001; CHOUERI et al., 2007), mobility of organic and inorganic contaminants (SANTOS et al., 2008), speciation and bioavailability of metals (NOGUEIRA et al., 2009; CHOUERI et al., 2009), toxicity of pollutants such as polycyclic aromatic hydrocarbons (DIAMOND et al., 2000), remineralization of nutrients, surface properties of minerals and carbon budget (HEDGES et al., 2002, and references therein). Different approaches are used to characterize FDOM in aquatic environments and many of them exploit its optical properties, i.e. light absorption and fluorescence emission. Because fluorescence can reveal important information about the FDOM's composition and biogeochemical cycling (BURDIGE et al., 2004), different approaches have been developed within the technique to study DOM fluorescence, such as Fluorescence Excitation and Emission Spectra, Fluorescence/Absorption Relation, Excitation-Emission Matrix Spectra, Synchronous Spectra, Fluorescence Quantum Yield (BLOUGH; DEL VECCHIO, 2002; COBLE, 2007).

Fluorescence Excitation and Emission Spectra has been largely used for the purpose of tracing the origin of the DOM in continental, estuarine and coastal ecosystems (DE- SOUZA-SIERRA et al., 1994, 1997; COBLE, 1996; LOMBARDI; JARDIM, 1999; CLARK et al., 2002; STEDMON et al., 2003). Considering that FDOM is a heterogeneous mixture of aliphatic and aromatic polymers, the rationale of this technique is based upon the characteristic of FDOM's components emitting fluorescence at different wavelength maxima (λ_{em}) when excited by specific wavelengths (λ_{ex}), depending on the nature and complexity of the FDOM's molecules.

Multivariate analysis methods have been used to aid in the interpretation of FDOM's fluorescence data. The application of multivariate analyses, such as Principal Component Analysis (PCA) (e.g. PERSSON; WEDBORG, 2001; BOEHME et al., 2004) and more recently Parallel Factor Analysis (PARAFAC) (e.g. STEDMON et al., 2003; STEDMON;MARKAGER, 2005), has led to new insights into the sources and concentrations of groups of fluorophores within the bulk of the FDOM. However, such techniques have been applied exclusively to Excitation-Emission matrices (EEM) data. To date, the integration of FDOM data and environmental variables through multivariate analysis has not been applied. Such an approach can help in the study of the dynamics of FDOM in estuaries, source tracking, and the assessment of the potential influence of the environment on FDOM dynamics.

The Paranaguá Estuarine System (PES) is an important site in terms of environmental protection. Nevertheless, it has been affected by anthropogenic pressures, such as port activities, fisheries and agriculture. A detailed characterization of the DOM in PES waters is of ecological and geochemical interest considering that the sources and transformations of such material can be traced and, consequently, insights may be provided into the fate of these substances in the ecosystem.

The aim of this study was to investigate the dynamics of FDOM in the PES and identify the contribution of allochthonous FDOM to the estuarine waters in relation to tidal condition and seasons. For this purpose water samples were collected in the main incoming freshwater source (Nhundiaquara River) and in the estuarine turbidity maximum (ETM) zone of Paranaguá Bay during spring and neap tides during dry and rainy seasons. In the ETM, processes such as the flocculation of dissolved materials and resuspension of sediments, important for the characterization of the behavior of estuarine DOM, take place (MANN, 2000). In this study, FDOM was characterized through fluorescence spectroscopy based on emission spectra. Relationships between maximum fluorescence emission wavelengths at selected excitation wavelengths and environmental variables, such as salinity, chlorophyll a, seston, dissolved oxygen (D.O.), total nitrogen (total-N), total

phosphorous (total-P), nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_3^{2-}) were assessed through the application of a PCA. The associations between environmental variables, and the relevance of these associations for each season and tide, were also discussed in the light of the PCA. This is the first attempt to use fluorescence analysis as a tracer of the source, behavior, and transformations of FDOM in the Paranaguá Estuarine System; in addition, the application of multivariate analysis to integrate fluorescence and environmental data provided an ecological explanation for the FDOM results.

MATERIAL AND METHODS

Study Area

The Paranaguá Estuarine System (PES) is located at 25°16' - 25°34'S and 48°17' - 48°42'W (Brazil). This system is physically divided into 2 main bays: Paranaguá Bay (W-E direction) and Laranjeiras Bay (N-S direction), with a total area of 612 km² (Fig. 1). The climate is humid subtropical (Cfa), with an average annual rainfall of 2500 mm in the drainage basin. Two well-defined seasons dominate: rainy summer and dry winter (KNOPPERS et al., 2004).

The drainage area of the PES is 3361 km² and the average annual freshwater discharge is 200 m³ s⁻¹ (KNOPPERS et al., 2004). The PES encompasses different environments such as salt marshes, mangrove swamps, sandy beaches, coastal rocks, tidal channels, smaller estuaries formed by several streams and Atlantic rainforest. The site is part of the Cananéia-Iguape Estuarine-Lagoons System, an important area for environmental preservation that has been considered as part of the Ramsar's list of international protection wetlands since 2000 (IBAMA, 2008). Sixteen protected areas have been established and approximately 19% of the Atlantic rainforest remnants, considered "Biosphere Reserve" by UNESCO, are situated in this region. Besides the ecological importance of the PES, its area also harbors important economic activities, being distinguished by the presence of the major grain exporting port in South America, that of Paranaguá. Other products potentially hazardous to the environment, such as fertilizers, petroleum derivatives and minerals, are handled at the port. Additional environmental pressures in the PES are the unplanned urban development bordering the estuary and the consequent increasing discharge of untreated sewage, uncontrolled urban landfills and agricultural activities.
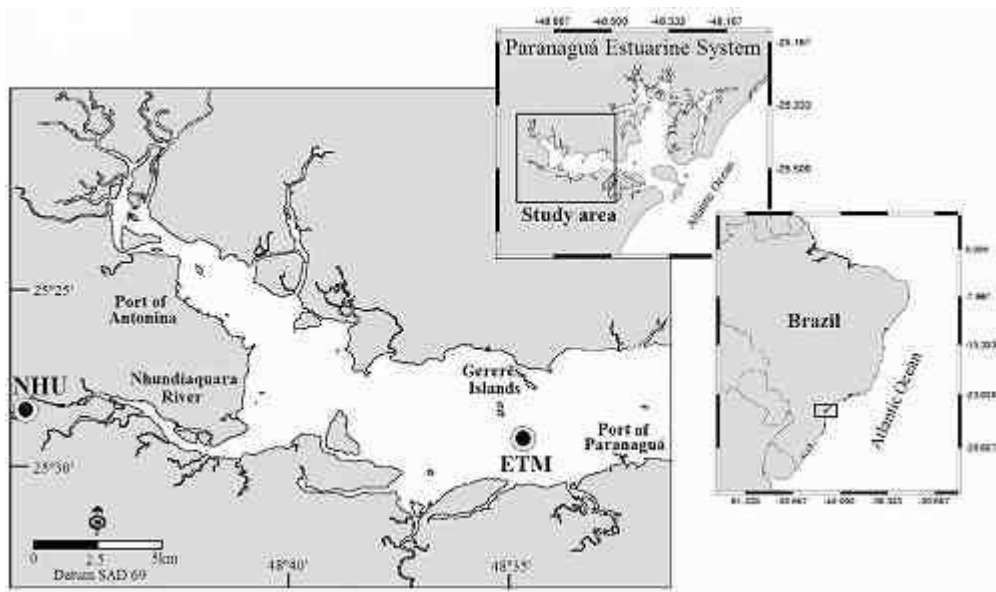

Fig. 1. Localization of the sampling sites in the Paranaguá Estuarine System. NHU is the station in the Nhundiaquara River; ETM is the station in the Estuarine Turbidity Maximum of Paranaguá Bay.

Sampling

Estuarine water samples were collected within the extension of the estuarine turbidity maximum (ETM) of Paranaguá Bay (Fig. 1) during spring and neap tides of rainy (December 2005) and dry season (August 2006). The mean rainfall in the three-month period before sampling in the rainy and dry season was 201.7±103.9mm and 49.8±24.2mm, respectively (data provided by the technological institute Simepar). Despite the ETM displacement into the bay along the tidal cycle (MANTOVANELLI et al., 2004), a fixed sampling station (25º29.26'S; 48º35.37'W) was set up at a place at which the field collections in both rainy and dry seasons could be undertaken within the ETM zone. The ETM's location was confirmed by *in situ* water turbidity measurements using an AP 2000 Policontrol turbidimeter. The depth at the sampling station varied between 6.8 and 7.0 m.

Surface and bottom waters were sampled with Niskin bottles every 2 hours over a period of 12 hours (a complete tidal cycle) during spring and neap tide events in both rainy and dry seasons. For D.O. analysis, glass bottles with glass stoppers were carefully filled to overflowing with water samples and immediately fixed to be later analyzed by Winkler's method. All D.O. samples were kept at water temperature, 29 to 31 ºC in the rainy season and 21 ºC in the dry season. The samples were kept in the dark for no longer than 8 h after sampling. The samples for nutrient analysis were placed in high density polyethylene bottles, kept under ice and in the dark during fieldwork. Onshore, nutrient samples were immediately filtered through calcined glass-fiber filters (Schleicher & Schuell GF-52C) and kept at 4 ºC for no longer than 48 h.

To define a reference fluorescence emission wavelength (λ_{em}) for the allochthonous FDOM, surface (0.33 to 0.48 m) freshwater samples (n=3) were collected in the Nhundiaquara River (point "NHU" in Figure 1, 25°25'40.6"S; 48°52'37.3"W), the main source of freshwater flowing into Paranaguá Bay. Sampling at the Nhundiaquara River was performed on one single occasion, since the λ_{em} "fingerprint" of allochthonous FDOM is not expected to change significantly with season as compared to the difference in λ_{em} that is applicable to this study, i.e. allowing for the distinction between continental and marine FDOM. The reference fluorescence (λ_{em}) value for the autochthonous FDOM was obtained from samples with higher marine influence taken at the ETM with salinity 29-30 (n=16).

Analytical Methods

Salinity was measured in the field with a hand refractometer (Atago) and pH measurements were made with a Hanna pH meter. Dissolved nutrients (total-N, total-P, NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}, SiO_3^{-2}) were analyzed by colorimetric methods in accordance with Grasshoff et al. (1983) after filtration of water samples as recommended by Head (1985) for particle-rich waters. Dissolved oxygen concentration was determined by Winkler's method (GRASSHOFF et al., 1983), with an automatic Metrohm Dosimat titration unit with a precision of 0.01 ml. For total seston quantification, water samples were filtered through pre-weighed and calcined glass fiber filters (Schleicher & Schüll GF-52C); the retained material being dried and quantified using the gravimetric method (STRICKLAND; PARSONS, 1972). Chlorophyll a was quantified by filtering water samples through Schleicher & Schüll GF-52C filters, followed by acetone extraction and fluorescence measurements (STRICKLAND; PARSONS, 1972).

Fluorescence Spectroscopy

Samples were filtered through 0.22 µm membrane filters (Sartorius) and stored in sterile amber glass flasks. The filters were previously soaked in 1 M HNO_3 for 24h to remove metallic elements that could cause quantitative and qualitative alterations, such as quenching, in the fluorescence signal (LOMBARDI; JARDIM, 1999). The whole filtering procedure was performed in a sterile laminar flow cabinet and samples were kept in the dark at 4ºC until analysis. This sample conservation procedure resulted in no pH variation in the samples (determined before and after storage), confirming that no microbial or light degradation had occurred.

Fluorescence determinations were performed using a commercial spectrofluorometer (JASCO, model FP 6500, Japan) equipped with a 150W xenon lamp as the light source. The equipment includes internal configuration to automatically correct for intensity variation and spectral characteristics of the light source and those of the excitation monochromator. This is important to keep the output currents from the monitoring photomultiplier tubes constant even if there be variations in the light source. Additionally, all samples were analyzed in the same period, therefore preventing any possible long term variations in lamp intensity and instrument setup. All spectra were recorded with a 10 nm slit-width for the emission monochromator and 5 nm for the excitation monochromator. Scan velocity was adjusted to 200 nm min^{-1} for both monochromators. Instrumental error, estimated as 0.16 %, was calculated by repeatedly measuring the fluorescence of a single sample and estimating the coefficient of variation of this series of data (n=10).

According to Bloom and Leenheer (1989), at least two important classes of fluorophores are present in the humic substances (or naturally occurring DOM). Following the authors, we have used two different

classes of fluorophores to characterize the FDOM in our samples: a first class of fluorophores, excitable within the range 310-390 nm, and a second class excitable within 410-470 nm. Within those ranges, a fixed excitation of 350 nm (λ_{ex} 350 nm) was defined empirically for the first class and 450 nm (λ_{ex} 450 nm) for the second class of fluorophores. All fluorescence emission spectra were recorded from 370 to 600 nm. Fluorescence intensity was recorded for both classes of fluorophores and reported in arbitrary units (A.U.). Raman peak corrections in the samples were made by subtracting the spectra obtained for the samples from those obtained for blank samples prepared with Milli-Q water. All fluorescence measurements were performed at 21 ± 1°C and samples were used with their natural pH that ranged within 6.0 - 7.5. According to Baker et al. (2007) such pH variation does not affect the fluorescence significantly, so pH was not adjusted and was kept as in the sampling. Self-shading effects were not expected since a quartz-cuvette of 1-cm lightpath was used, and the water samples were not concentrated prior to the fluorescence determinations.

Statistical Analysis

Univariate Methods

The mean values of environmental variables and λ_{em} for both λ_{ex} 350 nm and λ_{ex} 450 nm recorded for surface and bottom samples, in the spring and neap tides of dry and rainy seasons were tested for statistical differences. Firstly, the data series were tested for normality (Kolmogorov-Smirnov's test) and homoscedasticity (Bartlett's method). The statistical differences of mean values of each data series (n=6) which followed Analysis of Variance (ANOVA) assumptions were tested through ANOVA followed by post hoc Tukey test. Non-parametric statistical tests (Kruskal-Wallis test, with Dunn's multiple comparisons as post-test) were used to compare data series that violated ANOVA assumptions. Differences between surface and bottom samples were analyzed only within the same tide and season, either through ANOVA followed by Bonferroni's multiple comparisons test, or Kruskal-Wallis followed by Dunn's multiple comparisons test.

Least squares linear regression analyses were undertaken to examine the relationships between salinity and the maximum λ_{em} and intensity at λ_{ex} 350 nm and at λ_{ex} 450 nm fluorophore classes. This analysis helps in indentifying the variation of the fluorimetric characteristics of the DOM (λ_{em} and intensity) in relation to the contribution of autochthonous and allochthonous sources. The significance of linear regressions was assessed through ANOVA F-test. For all the statistical tests, an $\alpha = 0.05$ was considered.

Multivariate Analysis (Principal Component Analysis)

Principal Component Analysis (PCA) was used to highlight associations among the variables measured in this study. Basically, this technique establishes a new set of variables (principal components) that are linear combinations of the original variables, based on the correlation matrix of the original dataset. The new variables, although fewer in number, account for the inherent variation of the data to the maximum possible extent, clarifying the data relations with minimal loss of information. Associations between λ_{em} at λ_{ex} 350 nm and λ_{ex} 450 nm, chemical, and biological parameters (salinity, D.O., chlorophyll a, seston, total-N, total-P, NO_2^-, NO_3^-, NH_4^+, PO_4^{3-} and SiO_3^{2-}) were sought. The selected variables to be interpreted were those associated with the factors with a loading ≥ 0.45, as recommended by Tabachnic and Fidell (1996). The relevance of the observed correlations to each case (each data record of neap and spring tide of rainy and dry season), i.e. the 'factor score', was also quantified in the PCA. All the analyses were performed using the STATISTICA 8.0 software (StatSoft Inc., USA).

RESULTS

Mean values and standard deviation of the environmental variables analyzed in the ETM of the PES for a whole tidal cycle, in rainy and dry seasons, and spring and neap tides, as well as for those of Nhundiaquara River and samples with the highest marine influence (29 - 30 salinity) are shown in Table 1. No significant variation was obtained for most dissolved nutrients (PO_4^{-3}, NH_4^+, NO_3^-, SiO_3^{-2}), chlorophyll-a, and total-N and total-P (ANOVA, p>0.05) between Nhundiaquara river samples and those under marine influence. A different tendency were obtained for salinity, seston, and NO_2^- that were lower in the freshwater samples than in the marine samples (ANOVA, p<0.05), and D.O. that was higher in the lower than in the higher salinity samples (ANOVA, p<0.05).

Contrasting dry and rainy seasons, the results obtained for the environmental variables at the ETM showed a significant increase in dissolved NH_4^+, NO_2^-, and NO_3^- in the dry season as compared with the river values (ANOVA, p<0.05). In addition, the mean NH_4^+ concentration in the neap tide of the dry season was also higher than that obtained for the marine sample (ANOVA, p<0.05). In the rainy season, ETM results for the environmental variables showed that salinity and the concentrations of nitrogen derived compounds (NO_2, NO_3^-, total-N and, to a lesser extent, NH_4^+) were lower than the marine values. Although other significant differences were obtained, they could not be explained by any seasonal or tidal pattern.

Fluorescence Measurements (λ_{ex} 350 nm and λ_{ex} 450 nm)

Mean results and standard deviations of the maximum λ_{em} and fluorescence intensity for both λ_{ex} 350 nm and λ_{ex} 450 nm obtained in the ETM over a whole tidal cycle, both in rainy and dry seasons and spring and neap tides, are presented in Table 2, which also reports the fluorescence results for the allochthonous and autochthonous reference samples.

The maximum λ_{em} for λ_{ex} 350 nm and λ_{ex} 450 nm for surface and bottom samples in the spring and neap tides of the dry and rainy seasons are shown in Figure 2. The maximum λ_{em} of the first class of fluorophores (λ_{ex} 350 nm) for the Nhundiaquara river's FDOM (allochthonous FDOM reference) was different from that of the estuarine samples with the highest salinity (autochthonous FDOM reference) (Fig. 2a). Longer maximum λ_{em} was recorded for the allochthonous FDOM (λ_{em} 448.4 ±0.5 nm) than for the autochthonous FDOM (λ_{em} 441.9 ±1.5 nm) (Kruskal-Wallis; $p<0.05$). However, for the second class of fluorophores (λ_{ex} 450 nm), no difference for the maximum λ_{em} was obtained when contrasting allochthonous (516.2 ±1.8 nm) and autochthonous (516.0 ±3.7 nm) FDOM (Kruskal-Wallis; $p>0.05$) (Fig. 2b).

Table 1. Mean values and standard deviation of the environmental variables analyzed in the Estuarine Turbidity Maximum in the Paranaguá Estuarine System for a whole tidal cycle, in rainy and dry seasons, and spring and neap tides. These values are also presented for Nhundiaquara River and marine samples.

Season	Tide	Sample	Salinity	Chl a	Seston	D.O.	PO_4^{-3}	NH_4^+	NO_2^-	NO_3^-	SiO_3^{-2}	Total-P	Total-N
			(%)	($\mu g\ L^{-1}$)	($mg\ L^{-1}$)	($mg\ L^{-1}$)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)
Rainy	Neap	Surface	22±3	1.24±0.28	44.11±28.79	6.78±0.6	1.47±0.91	0.55±0.66	0.29±0.36	3.37±1.65	16.69±3.49	0.99±0.18	19.62±3.4
Rainy	Neap	Bottom	24±3	2.16±1.45	38.05±13.73	6.00±0.5	1.36±0.58	0.49±0.31	0.49±0.35	5.86±5.9	15.40±5.57	1.03±0.31	18.19±2.37
Rainy	Spring	Surface	20±2	2.40±1.34	21.81±5.42	7.30±0.23	0.39±0.08	0.76±0.65	0.05±0.04	0.09±0.03	56.89±7.01	0.47±0.11	13.11±5.03
Rainy	Spring	Bottom	24±1	5.29±1.56	41.36±31.71	6.66±0.29	0.42±0.05	0.75±0.34	0.06±0.03	0.14±0.05	54.29±11.22	0.52±0.14	11.99±3.44
Dry	Neap	Surface	28±1	0.40±0.35	33.41±8.52	7.17±0.31	0.79±0.13	4.58±0.88	0.82±0.03	18.68±8.14	55.71±7.64	0.96±0.24	23.53±3.24
Dry	Neap	Bottom	30±0.5	1.04±1.1	48.83±11.83	6.71±0.14	0.58±0.16	3.88±0.7	0.48±0.07	18.65±6.66	19.76±3.34	1.18±0.24	24.73±2.91
Dry	Spring	Surface	28±1	0.78±0.44	23.31±3.56	7.14±0.52	0.83±0.23	6.45±1.27	0.69±0.06	24.96±5.46	30.67±8.14	1.43±0.64	21.17±3.31
Dry	Spring	Bottom	29±0.5	1.12±0.89	53.68±27.31	7.09±009	0.67±0.12	5.44±1.02	0.61±0.05	28.87±7.50	24.96±4.27	3.24±1.54	24.14±2.71
Nhundiaquara River			3±1	0.44±0.18	2.95±0.78	8.40±0.14	0.28±0.04	2.10±0.85	0.16±0.04	10.65±0.21	32.05±9.12	3.03±0.04	19.00±1.41
Marine samples[1]			29±0.5	1.29±1.64	49.87±27.09	7.02±0.4	0.64±0.15	3.65±2.92	0.60±0.13	21.80±8.60	29.27±15.13	1.87±1.43	23.65±2.92

[1] Samples collected in the ETM with higher marine influence (higher salinity)

Table 2. Mean results and standard deviations of λ_{em} and fluorescence intensity for both λ_{ex} 350 nm and λ_{ex} 450 nm in the Estuarine Turbidity Maximum of Paranaguá Estuarine System for a whole tidal cycle, in rainy and dry seasons, and spring and neap tides. Mean results and standard deviations of λ_{em} and fluorescence intensity for allochthonous and autochthonous references are also presented.

| Season	Tide	Sample	$\lambda_{em}/\lambda_{ex}350$	$\lambda_{em}/\lambda_{ex}450$	Intensity $\lambda_{ex}350$	Intensity $\lambda_{ex}450$
			(nm)	(nm)	(A.U.)	(A.U.)
Rainy	Neap	Surface	443.85±2.02	514.68±1.59	145.35±34.12	275.60±26.55
Rainy	Neap	Bottom	444.18±0.87	513.92±1.57	121.65±30.78	238.75±59.28
Rainy	Spring	Surface	445.48±031	514.28±0.69	103.25±8.1	275.17±24.64
Rainy	Spring	Bottom	445.92±0.46	513.88±1.32	96.62±3.71	257.50±7.08
Dry	Neap	Surface	443.53±1.37	517.03±3.04	53.83±13.12	139.77±13.11
Dry	Neap	Bottom	441.20/±1.54	513.27±4.14	85.62±15.97	220.05±26.39
Dry	Spring	Surface	441.85±2.10	519.97±4.31	77.98±24.5	186.00±35.23
Dry	Spring	Bottom	442.20±1.34	517.47±1.64	63.95±12.12	198.22±47.28
-	-	Allochthonous reference	448.40±0.21	516.20±0.60	107.81±1.45	353.55±12.83
-	-	Autochthonous reference	442.08±1.71	515.99±3.69	64.64±18.71	177.62±46.07
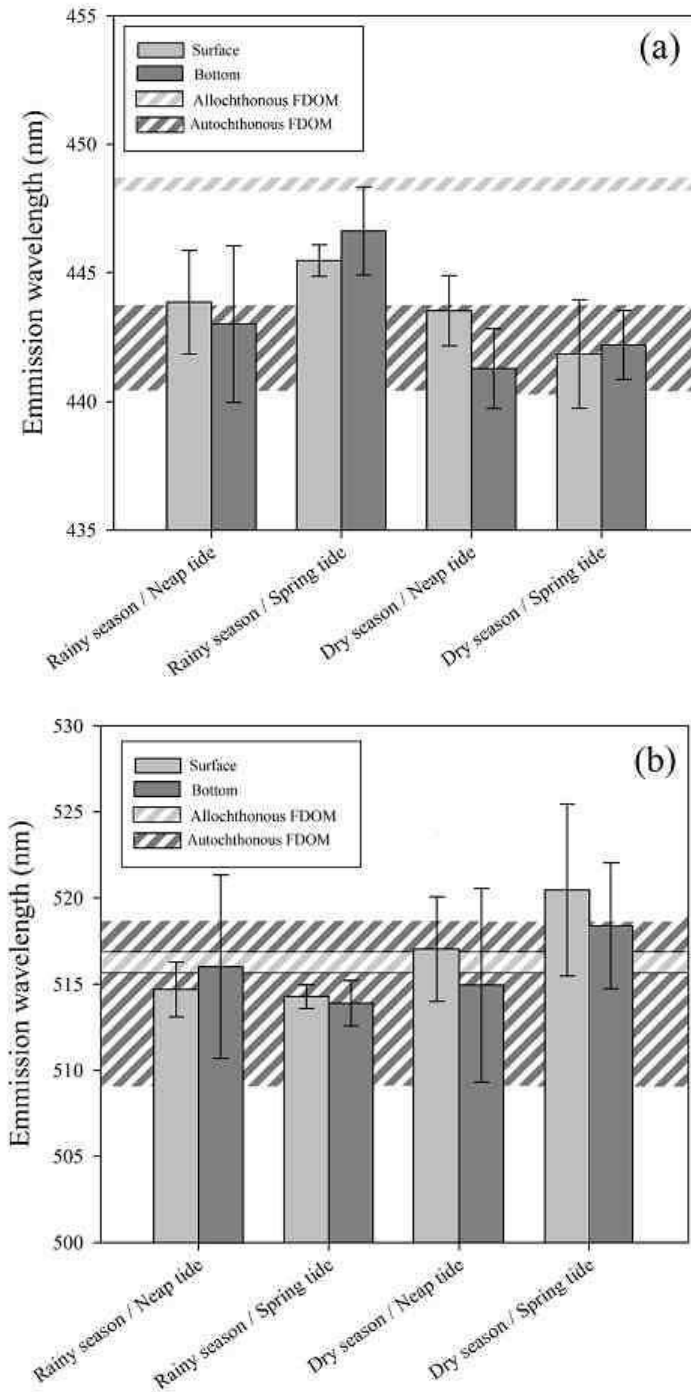
Fig. 2. Variation of λ_{em} of surface and bottom samples, in the spring and neap tide of the dry and rainy seasons, obtained by: (2a) λ_{ex} of 350 nm and (2b) λ_{ex} of 450 nm. Error bars represent standard deviation (SD) of n = 6. Gray patterned stripes in the back represent the range of variation (mean ± SD) for allochthonous (light gray) and autochthonous (dark gray) λ_{em} reference values.

Considering the first class of fluorophores (λ_{ex} 350 nm), the maximum λ_{em} for the ETM was always shorter than the allochthonous reference FDOM. It was only during the spring tide of the rainy season that the ETM had longer maximum λ_{em} for both surface and bottom waters (Kruskal-Wallis; $p<0.05$). In the dry season during neap and spring tides for surface and bottom waters, ETM maximum λ_{em} fluorescence was similar to the autochthonous reference λ_{em}. In contrast, comparing ETM surface waters within both tides and seasons, longer λ_{em} in the rainy season as compared with those of the dry season in spring tides (ANOVA; $p<0.05$) were obtained. For ETM bottom waters, the spring tide of the rainy season showed significantly longer λ_{em} than any other tide or season (ANOVA; $p<0.05$).

Regarding the second class of fluorophores (λ_{ex} 450 nm), the maximum λ_{em} for allochthonous and autochthonous FDOM were similar, even considering different tides and seasons. Samples of bottom water did not show any difference between tides or seasons (Kruskal-Wallis; $p>0.05$), whereas surface water showed significantly longer λ_{em} in the dry than in the rainy season for the spring tides (Kruskal-Wallis, $p<0.05$). No statistical differences were found between surface and bottom samples, in the same tide and season, for either first or second-class fluorophores (ANOVA; $p>0.05$).

The fluorescence emission spectra for the first class of fluorophores of estuarine samples ranged from 47 A.U., occurring at $\lambda_{em} = 439$ nm, and 206 A.U. occurring at $\lambda_{em} = 446$ nm. For the second class of fluorophores, the values ranged from the 127 A.U. obtained at $\lambda_{em} = 511$ nm and a maximum intensity of 312 A.U. recorded at maximum $\lambda_{em} = 516$ nm.

<div align="center">

Linear Correlations: Maximum λ_{em} vs. Salinity, and Intensity vs. Salinity

</div>

Figure 3 shows the plots of maximum λ_{em} (λ_{ex} 350 nm - Fig. 3a, and λ_{ex} 450 nm - Fig. 3b) as a function of salinity. The maximum λ_{em} of the first class of fluorophores shows a negative and significant linear correlation ($r=0.63$; $p<0.05$) with salinity, whereas λ_{em} of the second class of fluorophores is positively and significantly correlated with salinity ($r=0.32$; $p<0.05$).

The plots of fluorescence intensity (A.U.) at λ_{ex} 350 nm (Fig. 4a) and λ_{ex} 450 nm (Fig. 4b) against salinity are shown in Figure 4. For both classes of fluorophores, fluorescence intensity was negatively correlated with salinity ($r=0.68$ for λ_{ex} 350 nm; $r=0.75$ for λ_{ex} 450 nm; $p<0.05$ for both).

<div align="center">

Principal Component Analysis

</div>

Within the scope of this study, the factors relevant for analysis are those in which the variables of wavelength of maximum fluorescence (λ_{em}) at λ_{ex} 350 nm and/or λ_{ex} 450 nm weighed significantly. These were the first two factors of the PCA (F1 and F2) which together represented 54.5% of the total variance in the data matrix. The first factor (F1) accounted for 41.0% of the total variance in the dataset. The λ_{ex} 350 nm and chlorophyll a were negatively associated with F1 with significant loadings (higher than the pre-established loading cut-off), whereas another group of variables (λ_{ex} 450 nm, salinity, total-P, total-N, NO_2^-, NO_3^-, and NH_4^+) was positively associated with F1. The second factor (F2) explained an additional 13.5% of the variance in the original dataset and grouped, with significant loadings, λ_{ex} 450 nm, salinity, dissolved oxygen, and SiO_3^{2-}. Orthophosphate (PO_4^{3-}) was negatively associated with these variables with a significant loading.

Figure 5 shows the projection of variables (a) and cases (b) on the factorial plane, formed by factor 1 (horizontal axis) and factor 2 (vertical axis). In Figure 5a, the associations between variables detailed in the above paragraph can be distinguished. In Figure 5b, three groups of cases can be defined: one for spring tide/rainy season (white triangles and light gray diamonds) with a strong negative association with F1; a second group of cases for the dry season (neap and spring tide together - dark gray squares, dark gray triangles, black circles, and black diamonds) with a strong positive association with F1; and lastly, a group for neap tide/rainy season (white squares and light gray circles) negatively associated with F2, as well as a fairly negative association with F1.

The results of PCA confirmed the significant negative association between maximum λ_{em} of the first class of fluorophores (λ_{ex} 350 nm) and salinity. Observation of Figure 5a and 5b allows the identification of associations between variables that are represented by vectors in Figure 5a, and cases, represented by points in Figure 5b. Thus Figure 5 shows that the rainy season has notably influenced both the λ_{em} of the first class of fluorophores and chlorophyll a concentration towards higher values, since the vectors for these variables (Fig. 5a) are pointing in the same direction as the rainy season points are placed in the factor space F1 x F2 (Fig. 5b). Conversely, the vectors for salinity and some dissolved nutrients (total-P, total-N, NO_2^-, NO_3^-, NH_4^+) are pointing in the opposite direction to the rainy season points, which implies that these variables presented lower values in this season.
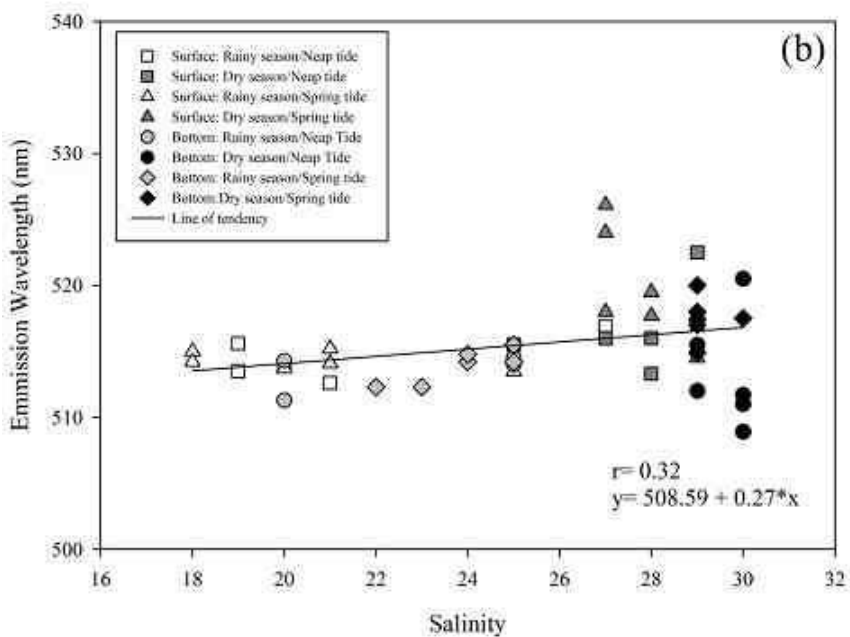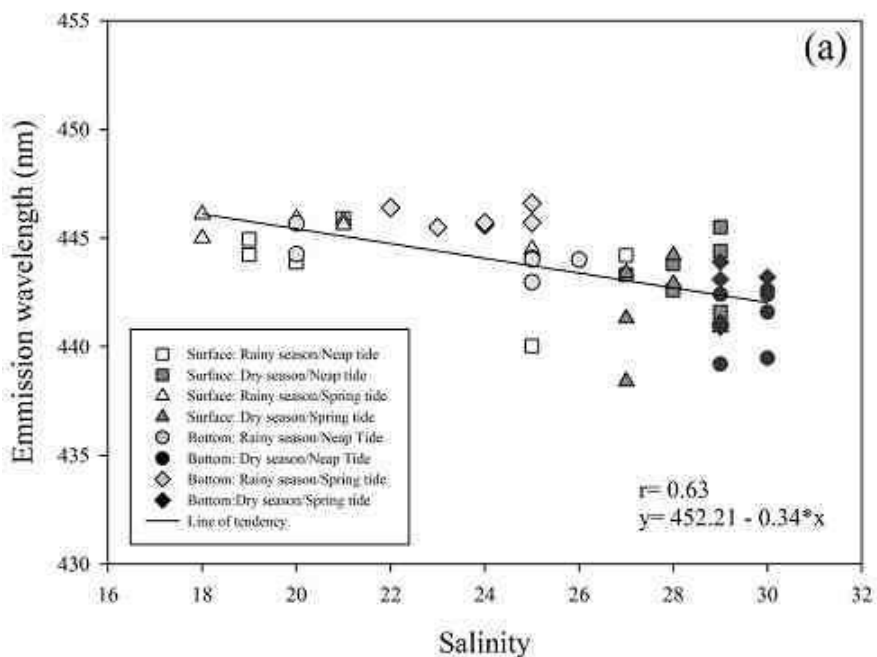
Figs 3a and 3b. Wavelength of maximum emission fluorescence (λ_{em}) for λ_{ex} 350 nm (3a) and λ_{ex} 450 nm (3b) plotted against salinity. The fit line represents the least squares linear regression.
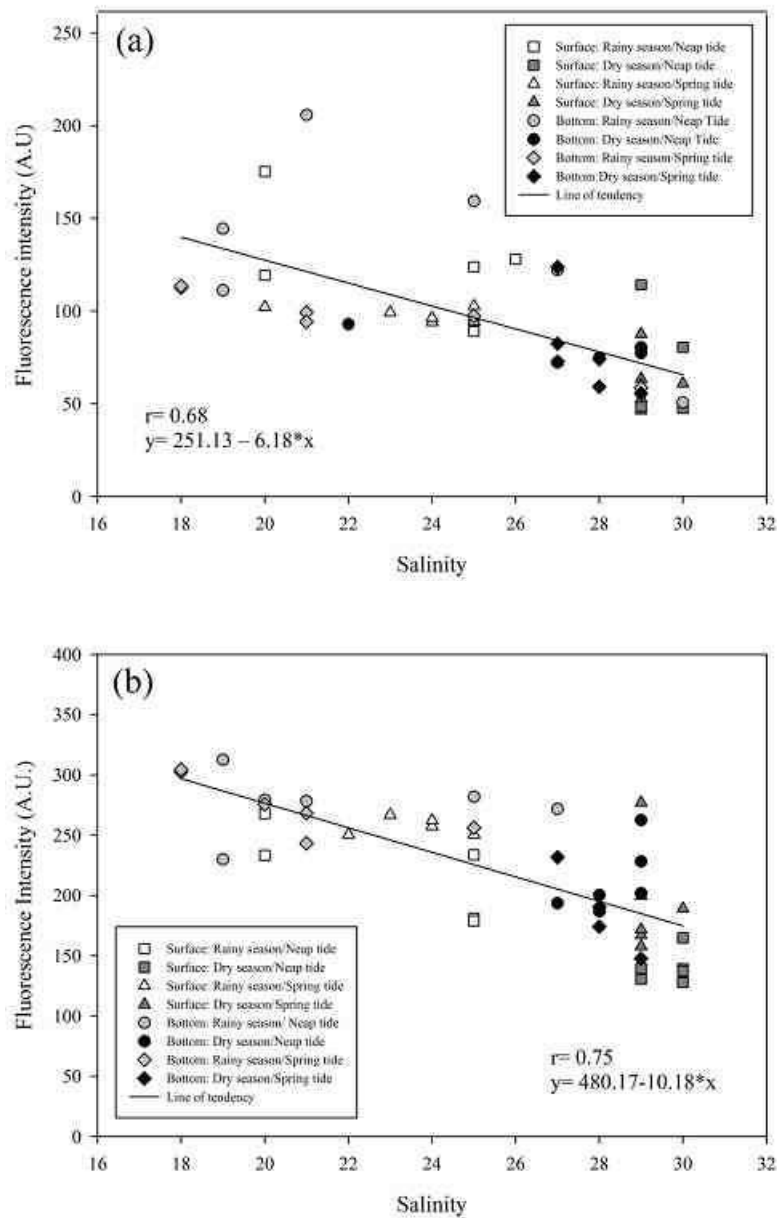
Figs 4a and 4b. Data of fluorescence intensity at λ_{ex} 350 nm (4a) and λ_{ex} 450 nm (4b) plotted against salinity. The fit line represents the least squares linear regression.
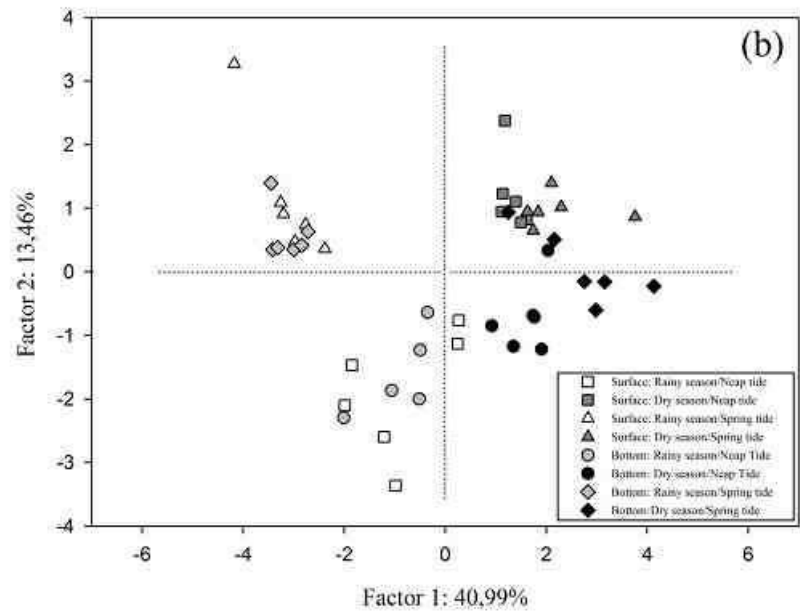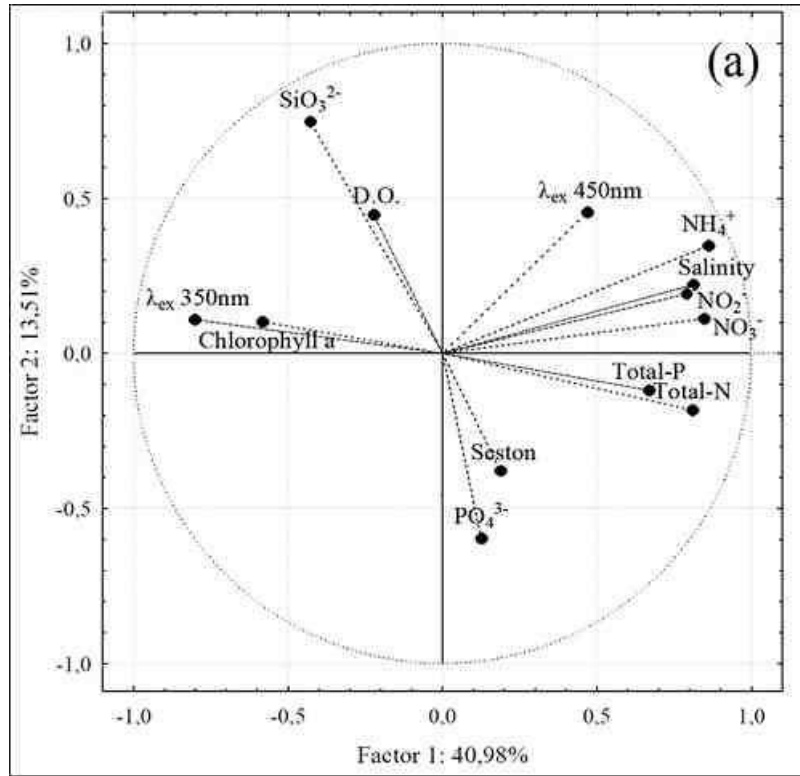
Fig. 5. Projection of variables (Fig. 5a) and cases (Fig. 5b) on the F1xF2 factorial plane of the PCA.

DISCUSSION

In general, the results for the environmental variables showed little variation between freshwater and marine samples, as confirmed by the analysis of variance. This suggests that the input of dissolved nutrients from the Nhundiaquara River into the estuary can be significant. However, our results also suggest that the Nhundiaquara River is not the main source of seston to the ETM. The low concentration of seston in the river may be related to the location of its source and great part of its course, which flows through areas of rocky outcrops (BIGARELLA et al., 1978) with high resistance to erosion (LICHT et al., 1997). Since approximately 70% of the coastal watershed of the State of Paraná flows into the PES (MANTOVANELLI, 1999), other rivers are probably more important sources of particulate matter for the ETM of the PES than the Nhundiaquara River. The analysis of variance also revealed a seasonality of some environmental variables, among them salinity (lower in the rainy season) and some dissolved nutrients (NH_4^+, NO_2^-, and NO_3^-) (increased in the dry season and decreased in the rainy season); this pattern will be further discussed in the light of the results of the PCA.

The results of maximum λ_{em} obtained in this study are in accordance with those of the literature for both the first (λ_{ex} 350 nm) and second classes of fluorophores (λ_{ex} 450 nm) (DE-SOUZA-SIERRA et al., 1994; COBLE, 1996; LOMBARDI; JARDIM, 1999; BLOUGH; DEL VECCHIO, 2002). In agreement with the data given in the literature, our results showed that the fluorescence emission maximum is dependent on the excitation wavelength, which is the result of multiple fluorophores, jointly referred to as classes of fluorophores (DE-SOUZA-SIERRA et al., 1994; LOMBARDI; JARDIM, 1999). In agreement with the findings of Coble (1990), who noticed a red shift in the emission maximum as the excitation wavelength was increased, our results showed that the second class of fluorophores is red-shifted (longer λ_{em}) compared with the first class.

The excitation wavelength from 320 to 360 nm is commonly used to register the fluorescence spectra of natural waters and to identify peaks related to humic-like substances (COBLE, 1996). Using λ_{ex} 350 nm, so defining the first class of fluorophores, we obtained maximum λ_{em} ranging from 437 to 448 nm, which is within the range for fluorescence emission maxima obtained in several investigations that focused on various natural water samples (fresh, estuarine, and marine). Several authors have reported fluorescence emission maxima ranging from 420 to 480 nm (DE-SOUZA-SIERRA et al., 1994; LOMBARDI; JARDIM, 1999; BAKER; SPENCER, 2004; LUCIANI et al., 2008).

The second class of fluorophores (λ_{ex} 450 nm) has previously been described by Lombardi and Jardim (1999) who studied the fluorescence of marine and terrestrial organic materials and found $\lambda_{ex}/\lambda_{em}$ of 450/522 and 450/524 nm, respectively. Also, Hemmingsen and McGown (1997) reported a λ_{em} of 520 nm for commercial humic acids excited at 460 nm. Thus the present results of λ_{em} ranging from 510 to 527 nm are in agreement with the data previously given in the literature.

The general pattern of continental DOM fluorescence emission at longer wavelengths as compared with marine DOM fluorescence for the first class of fluorophores obtained in the present study is in accordance with other results given in the literature. Donard et al. (1989) studied the fluorescence emission of DOM from the Mediterranean Sea and obtained longer λ_{em} for coastal as compared with marine samples when λ_{ex} 313 nm was used. Also studying the fluorescence of marine DOM obtained from the Mediterranean Sea, Lombardi and Jardim (1999) reported shorter λ_{em} for marine DOM (λ_{em} 437 nm) than for a soil fulvic acid (λ_{em} 452 nm) when fixed λ_{ex} 350 nm was used. Note that λ_{ex} 350 nm is the same as that used in the present study and we obtained λ_{em} 442 nm for the estuarine samples. De-Souza-Sierra et al. (1994) showed variations in the fluorescent properties of marine DOM, including progressive shifts of up to 40 nm towards longer wavelengths as seawater mixed with river waters. In addition, Coble (1996) and Del-Castillo et al. (1999), using the excitation-emission matrix spectroscopy technique, reported shorter λ_{em} towards higher salinity at λ_{ex} 330 to 350 nm.

The FDOM's blue shift (shorter λ_{em}) towards higher salinity for the λ_{ex} 350 nm that is observed in Figure 2(a) (i.e. the difference between autochthonous and allochthonous FDOM) of the present study supports the negative linear correlation between $\lambda_{em}/\lambda_{ex}$350 nm and salinity. This difference in λ_{em} between allochthonous and autochthonous FDOM can be attributed to differences in molecular mass and aromaticity of the FDOM present in the two different environments. According to Baker and Spencer (2004), the more red-shifted is the peak, the higher the molecular mass and aromaticity of the FDOM; conversely, the more blue-shifted it is, the lower the molecular mass and aromaticity of the FDOM.

The differences in freshwater and estuarine FDOM obtained by the present authors can be correlated to the lower complexity of FDOM in the samples of ETM. This indicates that the continent-originated organic matter is subjected to modifications during its transportation and residence in the estuary. In fact, it has been shown that different physical, chemical and biological processes affect DOM structure and results in loss of aromaticity and molecular mass (COBLE, 1996), as well as in its

fluorescence efficiency (MOPPER et al., 1991; ZIEGLER; BENNER, 2000). According to data given in the literature, processes such as microbial activity (BOYD; OSBURN, 2004), photodegradation (COBLE et al., 1998), and autochthonous production of organic material (RAYMOND; BAUER, 2001) can account for the blue-shifted characteristic of FDOM in estuarine and marine waters compared with terrestrial FDOM. In addition, particular characteristics of ETM can boost some of these processes. Boyd and Osburn (2004) observed that allochthonous DOM was preferentially biodegraded over autochthonous DOM resulting in a wavelength shift. These authors hypothesized that this could be the result of conformational changes taking place in allochthonous DOM while it is subjected to different ionic strength from freshwater to estuarine waters. Furthermore, the authors propose that this process may facilitate further microbial degradation of the allochthonous DOM. It has also been shown that the increasing salinity gradient from rivers to estuaries may increase DOM flocculation and particle settling, eventually removing selectively the DOM responsible for fluorescence at longer wavelengths from the water column (DE-SOUZA-SIERRA et al., 1997).

Another factor that can contribute to the shortening of λ_{em} of estuarine FDOM in relation to continental FDOM is the input of marine FDOM in the ETM. Previous studies have shown that marine DOM molecules are typically less aromatic (BENNER, 1998; CHEN et al., 2003) compared to continental DOM, which is rich in tannins and lignins (STEDMON et al., 2003). In addition, marine humic like organic materials are less aromatic, have lower C/N ratios and lower levels of conjugated chromatophores, contain more carboxylic groups and sugars than do terrestrial organic materials. These characteristics are consistent with observations that marine humics have a blue-shifted fluorescence relative to terrestrial humics (COBLE, 2007). As discussed above, in this present study it was also observed that in water samples with higher salinities FDOM's λ_{em} is shorter than that of river samples for the first-class fluorophores.

In relation to the second class of fluorophores (λ_{ex} 450 nm), the similarity we obtained for the reference values of FDOM from freshwater and estuarine waters is in agreement with other data given in then literature (DE- SOUZA-SIERRA et al., 1994; LOMBARDI; JARDIM, 1999). According to Clark et al. (2002), although there are differences between riverine FDOM and marine FDOM, some of the fluorophores are similar. Boyd and Osburn (2004) stated that the second class of fluorophores has more conservative characteristics during the mixing of fresh and estuarine waters. According to the authors this is due to a resistance of these substances to microbial

degradation. In the present study, the linear regression with positive slope between λ_{ex} 450 nm (second class of fluorophores) and salinity may be influenced by seasonal differences in the relative contribution of the sources of the second-class fluorophores rather than be related to a marine contribution, since λ_{ex} 450 nm did not vary between continental and marine sources of FDOM.

The intensity of DOM's fluorescence at a certain wavelength is given by a number of different factors, including absorptivity and concentration. A linear relationship is expected between fluorescence intensity and organic carbon concentration (e.g. SMART et al., 1976; BURDIGE et al., 2004). Similarly to the present results (Figs 4a and 4b), it has been reported that in most estuarine waters, fluorescence intensity decreases linearly with salinity (DORSCH; BIDLEMAN, 1982; HAYASE et al., 1987; LAANE; KRAMER, 1990; DE-SOUZA-SIERRA et al., 1997). This linear variation has been assigned to conservative behavior on the part of the organic matter contained in freshwater upon its mixing with sea water (COBLE, 2007; BOWERS; BRETT, 2008).

The environmental interpretation based on the PCA analysis is that during the rainy season, the ETM zone of the PES presented lower salinity, higher λ_{em} of FDOM (λ_{ex} 350 nm, first-class fluorophores), increased levels of chlorophyll a, and lower concentrations of nutrients. Higher λ_{em} at λ_{ex} 350 nm indicates that the FDOM detected during the rainy season in the ETM has higher complexity, i.e. it is mostly composed of allochthonous FDOM, with a lower contribution of marine FDOM. The low salinity observed confirms a higher contribution of freshwater during the rainy season. This is corroborated by the results of Mantovanelli (1999) who reported a freshwater contribution to the PES four times higher in rainy (182 $m^3 s^{-1}$) than in dry (41 $m^3 s^{-1}$) seasons.

Although the red-shifted characteristic of FDOM (λ_{ex} 350 nm) in the ETM during the rainy season was detected in both neap and spring tides, it was more clearly present in the spring tide. This may be due to the higher amplitude of spring tides, in which the estuarine waters reach continental areas and transport allochthonous organic matter more frequently and intensively than during neap tides, therefore contributing to an increase in the allochthonous FDOM in the estuary. This is supported by the results of Marone and Jamiyanaa (1997), who reported average tidal amplitudes of 1.4 m for neap tides and 1.7 m for spring tides as characteristic of the PES. Thus the 0.3 m difference is enough to wash out larger portions of the coastal areas.

Another important finding obtained from the present results regards the rainy season, when higher levels of chlorophyll a associated with lower levels of

inorganic nutrients were observed. This indicates enhanced primary production in the estuary at this time of the year, which is to be expected as the higher water temperature and solar radiation in the rainy season (summer) stimulate primary production. According to Coble et al. (1998) and Siegel and Michaels (1996), phytoplankton growth contributes to the pool of marine FDOM and, consequently, in situations of higher phytoplankton activity, FDOM emission would show a blue shift. However, an association has been detected between the red-shifted emission of the first class of fluorophores and the high phytoplankton activity of the rainy season. This leads to the reasoning - and reinforces it - that, during spring tides in the rainy season the allochthonous contribution is more significant than the autochthonous phytoplankton contribution to the pool of estuarine FDOM in the PES. In addition, previous studies have proposed that phytoplankton do not directly produce FDOM, it rather acts as a source of organic materials that are converted into FDOM through processes mediated by bacteria (ROCHELLE-NEWALL; FISHER, 2002; TRANVIK; BERTILSSON, 2001). Consequently, a time-lag between high phytoplankton activity and the production of typical marine FDOM can be expected.

The PCA also revealed that during the dry season (winter), in both spring and neap tides, salinity was higher and the wavelength of maximum emission for the FDOM at λ_{ex} 350 nm was shorter, indicating a reduced contribution of freshwater in the estuary, as well as a lower input of allochthonous FDOM. Consequently, a higher ratio of marine FDOM to the ETM of the Paranaguá Estuarine System was observed. In addition, Figure 5 shows inorganic nutrients as the principal components of the dry season samples; chlorophyll-a, also representative, is negatively correlated. The lower levels of chlorophyll-a and the higher concentrations of inorganic nutrients indicate a reduction of primary production during the dry season - winter in the PES.

PCA applied to our data set showed that the fluorescence for second-class fluorophores (λ_{ex} 450 nm) in the dry season occurred mainly at longer wavelengths than did that of the rainy season (Fig. 5). This indicates a red shift in the fluorescence of second-class fluorophores during the dry season in relation to that of the rainy season. Since the λ_{ex} 450 nm of continental and estuarine sources are similar, as shown in this study and corroborated by previous ones (DE-SOUZA-SIERRA et al., 1994; LOMBARDI; JARDIM, 1999), the seasonal variation of λ_{em} of the FDOM excitable at 450 nm may be due to an oscillation in the contribution of the different sources of organic matter in the PES over the year.

CONCLUSIONS

In the PES, the environmental variables showed little variation between the Nhundiaquara River and the ETM, suggesting that the input of dissolved nutrients from the river into the estuary may be significant. However, the seston in the ETM probably derives from sources other than the Nhundiaquara River.

Some environmental variables showed a seasonal pattern in the ETM of the PES, among them being salinity (lower in the rainy season) and some dissolved nutrients (NH_4^+, NO_2^-, and NO_3^-) (increased in the dry season and decreased in the rainy season). An increase in the freshwater input leads to a red-shift in the first-class FDOM maxima λ_{em} in the ETM. However, for the second-class of fluorophores, λ_{em} was similar for both freshwater and estuarine environments, indicating that at least part of the Nhundiaquara's riverine FDOM is similar to the estuarine fluorophores.

The seasonality observed in the hydrological processes in the PES modulates the FDOM composition and dynamics in the ETM. In the rainy season, the higher rainfall leads to an increase in the contribution of allochthonous first-class FDOM in the ETM, with a lower contribution of marine FDOM in this season. Consequently, the fluorescence of FDOM (λ_{ex} 350 nm) is red-shifted in the rainy season in relation to the dry season, as supported by the PCA. In contrast, in the dry season the input of allochthonous first-class FDOM into the ETM is diminished, following a reduction in the contribution of freshwater in the estuary, which results in the blue shift observed in the dry season.

The second-class FDOM also showed a seasonal variation in the maxima λ_{em} in the ETM of the PES. This is related to seasonal changes in FDOM sources other than those related to the Nhundiaquara River and the sea, since λ_{ex} 450 nm did not vary between continental and estuarine sources of FDOM.

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