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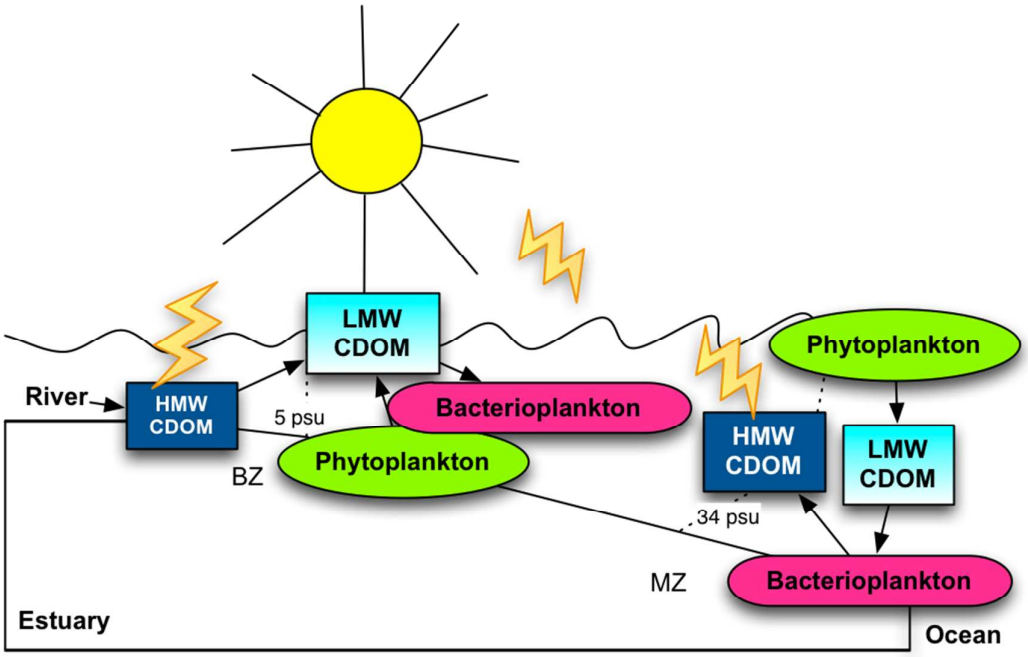

The coloured dissolved organic matter (CDOM) of marine (MZ) and brackish water (BZ) zones of Ria de Aveiro showed different spectral characteristics and susceptibility to photochemical alterations, reflecting the different amounts and prevailing sources of organic matter. HMW – High molecular height; LMW – Low molecular height;

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ARTICLE

Photochemical and microbial alterations of DOM spectroscopic properties in the estuarine system Ria de Aveiro

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,

Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Abstract

The influence of photochemical transformations of colored dissolved organic matter (CDOM) on microbial communities was evaluated in the estuarine system Ria de Aveiro. Two sites, representative of the marine and brackish water zones of the estuary were surveyed regularly in order to determine seasonal and vertical profiles of variation of CDOM properties. Optical parameters of CDOM indicative of aromaticity and molecular weight were used to establish CDOM sources, and microbial abundance and activity was characterized. Additionally, microcosm experiments were performed in order to simulate photochemical reaction of CDOM and to evaluate microbial responses to light-induced changes in CDOM composition. The CDOM of estuarine zones showed different spectral characteristics, with significant higher values of the specific ultra-violet absorbance at 254 nm ($SUVA_{254}$) (5.5 times) and of the absorption coefficient at 350 nm (a_{350}) (12 times) and lower ratio S_R ($S_{275-295}/S_{350-400}$) at brackish water compared with the marine zone, reflecting the different amounts and prevailing sources of organic matter, as well as distinct riverine and oceanic influences. At the marine zone, the abundance of bacteria and the activity of Leu-AMPase correlated with a_{350} and a_{254}, suggesting a microbial contribution to HMW CDOM pool. The irradiation of DOM resulted in a decrease of the values of a_{254} and a_{350} and in an increase of the slope $S_{275-295}$, and of the ratios E_2:E_3 (a_{250}/a_{365}) and S_R, which in turn increase its bioavailability. However, the extent of photoinduced transformations and microbial responses was dependent on the initial optical characteristics of CDOM. In Ria de Aveiro both photochemical and microbial processes yielded optical changes in CDOM and overall result of these combined processes determine the fate of CDOM in the estuarine system and have influence on local productivity and in adjacent coastal areas.

Introduction

The concentration, quality/composition of dissolved organic matter (DOM) and dissolved inorganic nutrients are key variables that influence the size, distribution and metabolism of bacterial communities in aquatic ecosystems[1-3], namely in estuaries[4-7]. The light-absorbing fraction of DOM, chromophoric dissolved organic matter (CDOM), from terrestrial and autochthonous origins, is the primary absorber of sunlight in aquatic ecosystems and plays an important role for most photochemically mediated processes in surface waters[8].

Natural solar radiation, especially ultraviolet radiation (UV-B [280–315 nm], UV-A [315–400 nm]), has been found to induce chemical transformations of CDOM with the production of a variety of photoproducts, including inorganic carbon[9-11], nitrogen[12-14] and phosphorus[15] compounds, and numerous low molecular weight (LMW) organic compounds[16-18], which in turn could stimulate bacterial metabolism[12, 19, 20]. The origin and chemical composition of DOM strongly influences its photoreactivity[21, 22] and photoproduction of dissolved inorganic carbon[23] and LMW organic compounds[24, 25] has been shown to correlate with the fraction of UV-absorbing

CDOM, measured by the absorbance. Moreover, the effect of irradiation on the ability of DOM to promote bacterial growth is dependent of their origin[26], and, therefore, the seasonal behavior of DOM sources in estuaries might impact its consumption and recycling, along the downwards transport. During its transits through the estuary, terrigenous DOM also experiments ionic-strength modifications and/or different trace-metal availability produced by salinity increases[27], that influences its sensitivity to photoremineralization and photobleaching by UV light. The spatial variations of DOM spectral characteristics along the estuarine transport have been the investigation target of numerous studies[27-29]. However, few had provided additional insights about seasonality[30, 31]. This study aimed to characterise seasonal profiles of variation of CDOM in Ria de Aveiro, as well as to evaluate the influence of photochemical and microbial processes in the dynamics of this light absorbing component of DOM in the estuarine system. In order to achieve that goal, selected CDOM optical properties, related with aromaticity and MW, were correlated with microbial parameters and photochemical induced transformations were experimentally simulated. Optical properties of CDOM have been used to characterise sources, composition and diagenic stage of DOM in a wide range of aquatic ecosystems[28, 29, 32-35], as well as induced photochemical transformations[28, 36, 37]. Based on the finding of Helms, et al. [28], and with a recent increasing application, the spectral slope of a narrow wavelength interval between 275 and 295 nm region ($S_{275-295}$), has been use to trace terrestrial DOM[38-40]. Additionally, the ratio between this and the slope between 350 and 400 nm region ($S_{350-400}$), S_R, provide information about DOM MW and photochemically induced alterations[28, 37].

Results

Water properties

Physicochemical parameters

The values of the physicochemical parameters determined in the water column at the marine (station N1) and brackish (station I6) water zones of Ria de Aveiro are summarized in Table I. Salinity showed a typical seasonal profile of variation, ranging from 10.1 to 36.5 at station N1 and from 0.2 to 36.5 at station I6. The highest and lowest values of water temperature were registered in summer and winter, respectively, varying between 12.5 and 19.6 ºC at station N1, and between 10.2 and 24.5 ºC at station I6. The concentration of nitrate plus nitrite (NO_3^- + NO_2^-) varied between 2.2 and 19.4 µmol L^{-1} at station N1 and between 4.2 and 92.3 µmol L^{-1} at station I6. Phosphate (PO_4^{3-}) concentration varied between 0.1 and 38.6 µmol L^{-1} at station N1 and between 0.4 and 50.4 µmol L^{-1} at station I6. The highest values of chlorophyll a concentration at station N1 were observed in July 2006, ranging from 1.7 to 6.0 µg L^{-1}. At station I6, the values of chlorophyll a ranged from 2.5 to 10.1 µg L^{-1} with maximum in May 2006. Suspended particulate matter (SPM) concentration varied between 26.1 and 178.1 mg L^{-1}, and between 28.5 and 83.1 mg L^{-1} at stations N1 and I6, respectively. The concentration of particulate organic matter (POM) ranged from 5.3 to 33.7 mg L^{-1}. The concentration of dissolved organic carbon (DOC) varied between 0.5 and 13.9 mg L^{-1} at station N1 and between 2.1 and 26.0 mg L^{-1} at station I6.

Spectral characteristics of CDOM

The values of the different spectral characteristics of CDOM determined in the water column at the station N1 and station I6 are shown in Figure 1 and 2, respectively. All of determined DOM spectral parameters were significantly different (Mann-Whitney test, $p<0.05$) at the marine and brackish waters zones of the estuary and similar (Kruskal-Wallis test, $p>0.05$) at the different sampling depths in the water column of both sites. The values of $SUVA_{254}$ ranged from 0.31 to 62.4 L mg^{-1} C m^{-1} and seasonal differences were only statistically significant (Friedman test, $p<0.05$) at station I6, with larger deviations between cold and hot seasons. The median values of the absorption coefficients at 350 nm (a_{350}) were 0.91 m^{-1} at the marine zone and 7.42 m^{-1} at brackish water zone, varying between 0.16 and 41.30 m^{-1}. The a_{350} showed a seasonal pattern similar to $SUVA_{25}$ (Friedman test, $p<0.05$) with significant deviations among cold and hot seasons. At station N1, the median value of $S_{275-295}$ was 7.52 µm^{-1} and at station I6 was 6.71 µm^{-1}. The median values of $S_{350-400}$ at the marine 6.60 µm^{-1} and brackish water 7.71 µm^{-1} zones were different (range 1.9 - 10.2 µm^{-1},). The median value of E_2:E_3 ratio was 6.25 at station N1 and 5.62 at station I6 (range 1.9 - to 8.9). The E_2:E_3 ratio and $S_{275-295}$ showed similar seasonal profile at both stations (Friedman test, $p<0.05$) with significant differences between cold and hot seasons. The ratio SR ranged from 0.77 to 4.19 (median 1.14) at station N1 and from 0.71 to 1.28 (median - 0.90) at the station I6. The seasonal differences were statistically significant (Friedman test, $p<0.05$), with larger deviations between April 07 and February, December and May 2006 at station N1 and between December 2006 and January, May and July 2006 at station I6.

Bacterioplankton: abundance, biomass productivity and extracellular enzymatic activity

The seasonal profile of variation of total bacterial number (TBN), bacterial biomass production (BBP) and, the activity rates of the enzymes aminopepetidase (Leu-AMP) and ß-glucosidase (ß-GlCase) determined in the water column for each sampling events at station N1 and station I6 are presented in Figure 3. The maximum values of TBN were registered in late spring/summer and the minimum in winter/early spring, showing a typical seasonal profile of variation (range 0.5 - 12.6 x 10^9 cells L^{-1}).

Table I. Water column physicochemical characteristics in the different sampling events at the marine (station N1) and brackish water (station I6) zones of the estuarine system Ria de Aveiro.

Year	2006						2007	
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr
Marine zone [N1]								
Salinity	33.4±0.4	28.1±5.7	34.6±0.5	36.4±0.1	35.5±0.1	22.2±13	28.6±6.0	34.1±0.3
Temp [ºC]	12.6±0.1	15.1±0.2	18.0±0.2	17.7±0.4	19.4±0.2	14.4±1.6	14.1±0.1	16.8±1.5
NO_3^- + NO_2^- [µmol L^{-1}]	10.4±1.7	7.5±2.1	4.9±0.9	7.4±2.2	6.6±1.8	12.4±6.4	3.4±0.8	5.2±0.8
PO_4^{3-} [µmol L^{-1}]	1.3±0.2	1.0±0.1	1.0±0.1	1.6±0.6	2.2±0.5	26.9±9.6	1.7±1.1	0.2±0.1
Chl a [µg L^{-1}]	2.1±0.2	2.6±0.7	3.5±0.5	5.7±0.3	2.0±0.2	2.6±1.3	4.0±0.5	4.9±0.6
SPM [mg L^{-1}]	46.5±1.7	44.5±8.2	61.1±4.2	72.3±3.8	59.2±4.5	83.8±71	56.6±19	64.4±17
POM [mg L^{-1}]	12.0±0.5	11.8±2.3	14.8±1.3	20.9±1.8	17.0±0.9	17.3±12	15.3±4.3	12.0±1.8
DOC [mg L^{-1}]	2.4±0.7	7.0±3.8	2.4±0.8	9.2±3.2	N.D.	1.0±0.4	4.1±2.5	4.2±2.3
Brackish water zone [I6]								
Salinity	20.7±0.2	7.8±0.3	27.1±0.1	36.5±0.1	31.6±0.8	0.3±0.1	3.9±0.1	23.2±0.1
Temp [ºC]	10.9±0.2	16.6±0.1	23.5±0.4	24.3±0.2	21.9±0.2	10.2±0.1	13.5±0.1	20.3±0.2
NO_3^- + NO_2^- [µmol L^{-1}]	47.7±5.5	75.6±18	25.7±5.3	12.8±3.6	19.5±6.9	13.7±7.0	58.9±27	25.3±2.1
PO_4^{3-} [µmol L^{-1}]	0.9±0.3	1.7±0.3	1.6±0.5	2.9±0.3	4.2±0.9	40.4±12	3.6±0.5	0.6±0.2
Chl a [µg L^{-1}]	2.8±0.3	4.3±0.4	9.7±0.3	6.3±1.1	3.5±0.2	3.2±0.2	5.7±0.6	7.9±0.3
SPM [mg L^{-1}]	30.7±1.3	37.5±2.3	71.1±2.1	74.0±4.3	59.9±4.3	29.7±0.8	49.2±1.2	70.5±17
POM [mg L^{-1}]	8.0±0.6	7.5±0.3	14.2±1.1	19.1±1.2	18.0±1.6	5.4±0.2	10.8±0.6	13.6±2.8
DOC [mg L^{-1}]	11.6±2.9	14.7±3.0	8.5±1.9	22.7±3.4	N.D.	4.3±2.3	4.8±2.2	10.1±2.2

Average of the water column±standard deviation (*n*=4); Sal-salinity; Temp-Temperature; Chl a-Chlorophyll a; SPM–Suspended particulate matter; POM-Particulate organic matter; DOC-Dissolved organic carbon; N.D.–Not determined;

The seasonal differences were statistically significant (Friedman test, $p<0.05$), with larger deviations between May and July 2006 and between the two sampling events that took place in 2007. The median value was significantly different

(Mann-Whitney test, $p<0.05$) between the two stations. BBP was significantly different at station N1 (median 5.31) compared with I6 (median 8.49) (Mann-Whitney test, $p<0.05$), ranging from 0.6 to 32.1 µg C L^{-1} h^{-1}. The seasonal differences were statistically significant (Friedman test, p<0.05). At station N1, the deviations were significant between January 2006 and, September 2006 and April 2007 and, at station I6, between January 2006 and April 2007.

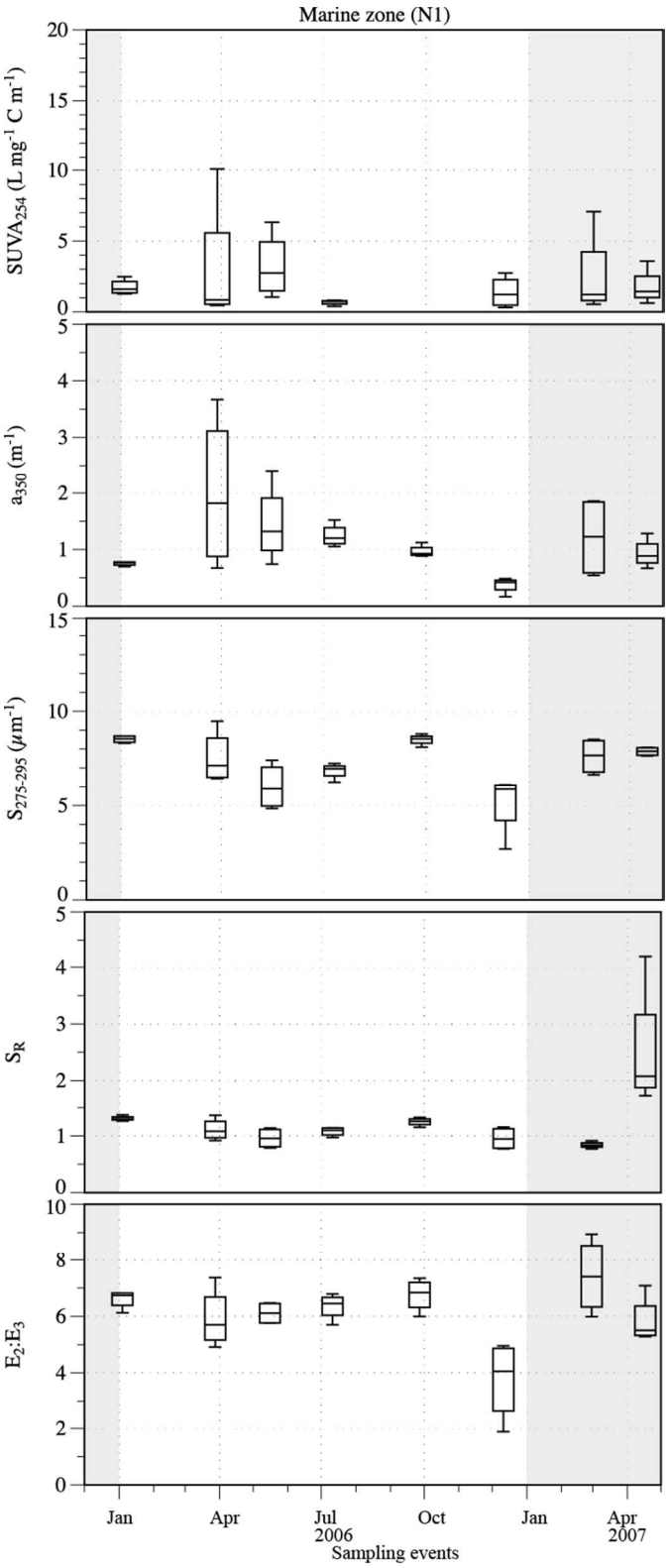

Figure 1. Box plots of spectral properties of dissolved organic matter (DOM) in the water column for each of the different sampling events at the marine zone (N1) of the estuarine system Ria de Aveiro.

The median values of Leu-AMPase Hm were significantly different (Mann-Whitney test, $p<0.05$) at the station N1 (1214.3 nmol L^{-1} h^{-1}) and station I6 (2383.8 nmol L^{-1} h^{-1}). The seasonal differences were statistically significant (Friedman test, p<0.05) between warm and cold seasons samples. Hm of ß-GlCase values ranged from 0.9 to 2373.3 nmol L^{-1} h^{-1} and were statistically different at the station N1 (median 69.30 nmol L^{-1} h^{-1}) compared with the station I6 (median 171.90 nmol L^{-1} h^{-1}). The values of the microbiological descriptors were similar (Kruskal-Wallis test, $p>0.05$) at the different sampling depths in the water column at the two zones. The seasonal differences were statistically significant (Friedman test, p<0.05). At station N1, the deviations were significant within July 2006 and February 2007. At station I6 the differences were significant within January 2006 and December and July 2006 and, within March and December 2006.

Correlations of CDOM spectral properties with environmental characteristics

The Spearman correlations between spectral parameters of CDOM and the different physicochemical parameters in the water column of the station N1 and station I6 are shown in Table II. All of the investigated spectral properties showed higher number of correlations with the environmental variables in the water column at the station I6 compared with station N1. The parameter $SUVA_{254}$ was negatively correlated with the concentrations of DOM and POM at both estuarine zones. At the brackish water zone, the $SUVA_{254}$ and the absorption coefficient at 350 nm (a_{350}) showed a strong negative correlation with salinity and temperature. The a_{350} at station N1 only showed a strong correlation with the absorption coefficient at 254. However, at the station I6, this specific coefficient correlated negatively also with POM and DOC concentrations. At station N1, the ratios E_2:E_3 and S_R and, the slope $S_{275-295}$ did not correlate with any of the physicochemical parameters. However, these spectral characteristics at the station I6 were correlated positively with the concentrations of POM and chlorophyll a and with temperature and salinity. The slope $S_{275-295}$ correlated positively with the ratio E2:E3 at both estuarine zones.

Correlations between microbial parameters and physicochemical and spectral properties

The values of the spearman correlation coefficient for microbial parameters and different physicochemical parameters and CDOM spectral characteristics are presented in Table III. Bacterial abundance only correlated with Leu-AMPase activity at the station N1. At station I6, BBP showed a negative correlation with nitrate plus nitrite. The activity of

Leu-AMP correlated with temperature at both estuarine stations, and with a high number of CDOM spectral characteristics at station I6. The activity of ß-GlCase correlated negatively with the ratio E_2:E_3 and the slope $S_{275-295}$ at station N1 and with nitrate plus nitrite concentration at station I6.

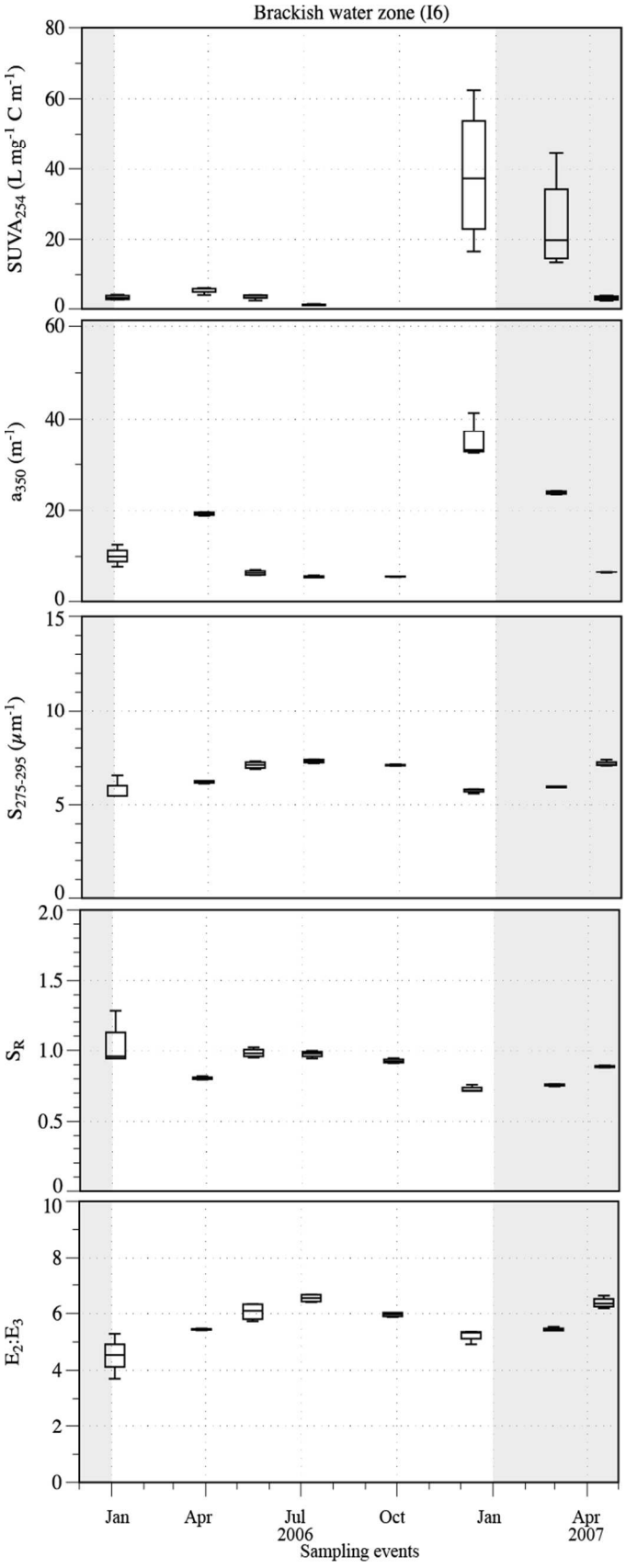

Figure 2. Box plots of spectral properties of dissolved organic matter (DOM) in the water column for each of the different sampling events at the brackish water zone (I6) of the estuarine system Ria de Aveiro.

Microcosm experiments

Photochemical alterations of CDOM spectral characteristics

The variations of the different spectroscopic parameters of CDOM induced by natural sunlight irradiation in the long-term assays are presented in Table IV. After 168h there was reduction in the absorption coefficients at 254 (a_{254}) and 350 (a_{350}) nm and increment of the values of the $S_{275-295}$, and of the ratios E_2:E_3 and S_R. Compared with the initial value, a_{254} in the natural sunlight treatment decreased 29 % and 9 % after 168 h in the assay 1 and 2, respectively. Contrastingly, in the dark control, the value of a_{254} increased 11 and 75 % after 168 h in the assay 1 and 2, respectively. The value of a_{350} nm showed similar pattern of variation, with higher decreases (68% - assay 1 and 60 % - assay 2) in the natural sunlight treatment and increases in the dark control (18% - assay 1 and 117 % - assay 2). The values of the spectral slope between 275 and 295 nm ($S_{275-295}$) of sunlight exposed-CDOM increased 75 % and 100 % in the assay 1 and 2, respectively, whereas in the dark control the values remain stable.

Table II. Spearman correlations between dissolved organic matter (DOM) spectroscopic parameters and the different physicochemical parameters at the marine and brackish water zones of the estuary Ria de Aveiro.

	Marine zone [N1]	Brackish water zone [I6]
$SUVA_{254}$	DOC r_s =-0.649** (n=28)	DOC r_s =-0.803** (n=28)
	POM r_s =-0.636** (n=32)	POM r_s =-0.725** (n=32)
		Sal r_s =-0.902** (n=32)
		Temp r_s =-0.704** (n=32)
		a_{350} r_s =0.908** (n=32)
		E_2:E_3 r_s =-0.566** (n=32)
		$S_{275-295}$ r_s =-0.650** (n=32)
		S_R r_s =-0.813** (n=32)
a_{350}	a_{254} r_s =0.982** (n=32)	POM r_s =-0.852** (n=32)
		DOC r_s =-0.638** (n =28)
		Sal r_s =-0.962** (n =32)

		Temp r_s =-0.853** (*n*=32)
		$SUVA_{254}$ r_s =0.908** (*n*=28)
		$E_2:E_3$ r_s =-0.772** (*n*=32
		$S_{275-295}$ r_s =-0.826** (*n*=32)
		S_R r_s =-0.730* (*n*=32)
$E_2:E_3$	$S_{275-295}$ r_s =0.717** (*n*=32)	Chl a r_s =0.732** (*n*=32)
		POM r_s =0.759** (*n*=32)
		Sal r_s =0.748** (*n*=32)
		Temp r_s =0.880** (*n*=32)
		$SUVA_{254}$ r_s =-0.566** (*n*=28)
		a_{350} r_s =-0.772** (*n*=32)
		$S_{275-295}$ r_s =0.950** (*n*=32)
$S_{275-295}$	$E_2:E_3$ r_s =0.717** (*n*=32)	Chl a r_s =0.672** (*n*=32)
	S_R r_s =0.602* (*n*=32)	POM r_s =0.738** (*n*=32)
		Sal r_s =0.801** (*n*=32)
		Temp r_s =0.870** (*n*=32)
		$SUVA_{254}$ r_s =-0.650** (*n*=28)
		a_{350} r_s =-0.826** (*n*=32)
		$E_2:E_3$ r_s =0.950** (*n*=32)
S_R	$S_{275-295}$ p=0.602* (*n*=32)	POM p=0.582** (*n*=32)
		DOC p=0.586** (*n*=28)
		Sal p=0.792** (*n*=32)
		Temp p=0.638** (*n*=32)
		$SUVA_{254}$ p=-0.813** (*n*=28)
		a_{350} p=-0.730** (*n*=32)

**Correlation is significant at the 0.01 level (two-tailed); *Correlation is significant at the 0.05 level (two-tailed); Sal–salinity; Temp-temperature; Chl a-Chlorophyll a; SPM–suspended particulate matter; POM-particulate organic matter; DOC-dissolved organic carbon;

The ratios $E_2:E_3$ and S_R increased considerably in the natural sunlight treatment. The exposure of CDOM to natural sunlight resulted in the increment of 140 and 125 % of the ratio $E_2:E_3$ in the assay 1 and 2, respectively. In the assays 1 and 2, the S_R ratio also increased 104 and 130 % in the natural sunlight treatment, respectively. In the dark control, the ratios showed $E_2:E_3$ and S_R slightly variations during the assays. Photochemical alterations of the different spectroscopic parameters of CDOM induced by natural sunlight exposure during the short-term assay are shown in Table VII. The exposure of DOM to natural sunlight during a short-term period (12 h) resulted in the increase of a_{254} and a_{350} values. Compared with the initial value, a_{254} increased approximately 90 % in both natural sunlight and dark control treatments. a_{350} nm showed similar pattern of variation, increasing 66±2.7 % in the natural sunlight treatment and 107±2.5 % in the dark control. The values of the spectral slope between 275 and 295 nm ($S_{275-295}$) of sunlight exposed-CDOM increased 14 % in the natural sunlight treatment and 1.4 % in the dark control compared with the initial values. The ratios $E_2:E_3$ and S_R also showed slightly increases in the natural sunlight treatment, 13.5 and 17.6 % respectively.

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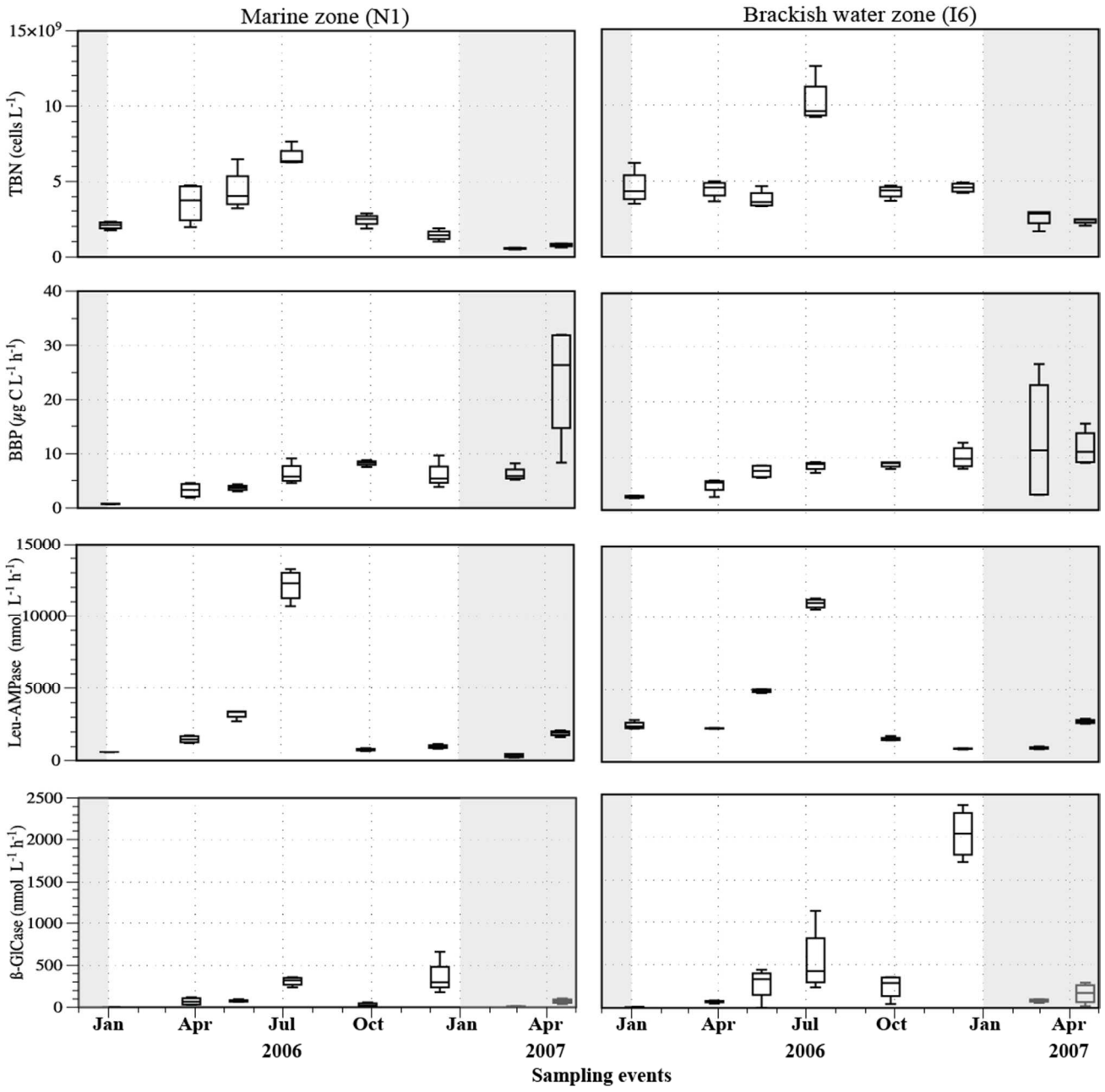

Figure 3. Box plots of total bacterial number (TBN), bacterial biomass production (BBP) and hydrolysis rates of the enzymes aminopeptidade (Leu-AMPase) and ß-glucosidase (ß-GlCase) in the water column for each sampling event at the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro.

Table III. Spearman correlations between total bacterial number (TBN), bacterial biomass production (BBP), aminopeptidase (Leu-AMPase) and ß-glucosidade (ß-GlCase) hydrolysis rates, and the different physicochemical parameters at the marine and brackish water zones of the estuary Ria de Aveiro.

	Marine zone [N1]	Brackish water zone [I6]
TBN	Leu_AMPase r_s =0.673** (n=32)	
BBP		NO_3^-+NO_2^- r_s =-0.612** (n=32)
Leu_AMPase	TBN r_s =0.673** (n=32)	Chl a r_s =0.559** (n=32)
	ß-GlCase r_s =0.623** (n=32)	POM r_s =0.532** (n=32)
	Temp r_s =0.536** (n=32)	DOC r_s =0.672** (n=28)
		Sal r_s =0.728** (n=32)
		Temp r_s =0.743** (n=32)
		$SUVA_{254}$ r_s =-0.893** (n=28)
		a_{350} r_s =-0.686** (n=32)
		E_2:E_3 r_s =0.633** (n=32)
		$S_{275-295}$ r_s =0.654** (n=32)
		S_R r_s =0.791** (n=32)
ß-GlCase	E_2:E_3 r_s =-0.635** (n=28)	NO_3^-+NO_2^- r_s =-0.651** (n=32)
	$S_{275-295}$ r_s =-0.662** (n=28)	

**Correlation is significant at the 0.01 level (two-tailed); *Correlation is significant at the 0.05 level (two-tailed); Sal – salinity; Temp-temperature; Chl a- Chlorophyll a; SPM – suspended particulate matter; POM-particulate organic matter; DOC-dissolved organic carbon;

Response of bacteria to irradiated CDOM

Long-term assay: The incubation of bacteria with DOM irradiated during different times resulted in considerable higher values of biomass production values in the natural sunlight treatments compared with dark controls, in both assays and, in both 72 and 168 h of irradiation (Figure 4). Bacteria inoculated with non-irradiated DOM (0 h) increased their biomass production with the incubation time from 0.07 to 5.43 µg C L^{-1} h^{-1} in assay 1, and from 0.01 to 18.57 µg C L^{-1} h^{-1} in assay 2. Bacteria inoculated with 12 h-irradiated DOM increased their biomass production from 0.43 to 19.15 µg C L^{-1} h^{-1} in sunlight treatment and, from 0.43 to 9.55 µg C L^{-1} h^{-1} in the dark control in the assay 1. In the assay 2, bacterial biomass production increased from 0.45 to 22.41 µg C L^{-1} h^{-1} in the natural sunlight treatment and from 0.49 to 7.97 µg C L^{-1} h^{-1} in the dark control. Along the incubation time, the response of bacteria to 168 h-irradiated DOM was higher in the natural sunlight treatment than in the dark control, in both assays. However, in assay 2 the response was faster compared than in assay 1, and the highest value (27.68 µg C L^{-1} h^{-1}) was observed in the natural sunlight treatment, at the 72 h.

Short-term assay: With the exception of Leu-AMPase, for which Hm values were higher in the natural sunlight treatment (range 324 - 2015 nmol L^{-1} h^{-1}) than in dark controls (range 357 - 1094 nmol L^{-1} h^{-1}), bacterial abundance, biomass production and ß-GlCase activity did not show a different response to sunlight irradiated DOM or to dark treatment (Figure 5). TBN (range 1.5 – 11.4 x10^8 cells L^{-1}) and BBP (range 0.36 – 23.3 µg C L^{-1} h^{-1}) showed a similar pattern, increasing in the first 72 h and decreasing after. The activity of ß-GlCase (range 12.2 - 76.0 nmol L^{-1} h^{-1}) showed a decreasing trend along of the incubation time, with both irradiated and non-irradiated DOM.

Table III. Variation of DOM spectroscopic characteristics (SC) in long-term experiments of surface water samples irradiated with natural sunlight compared with dark controls. Water samples were collected at the marine zone (N1) of the estuary Ria de Aveiro in two different dates, May 2006 (assay 1) and June 2006 (assay 2).

SC	Irradiation time (h)	0		12		168	
	Assay	1	2	1	2	1	2
a_{254} (m^{-1})	Sunlight	4.98	3.31	4.37	3.86	3.53	3.01
	Dark control			6.59	4.93	5.53	5.80
a_{350} (m^{-1})	Sunlight	1.17	0.66	0.71	0.54	0.37	0.26
	Dark control			1.60	1.25	1.38	1.43
E_2:E_3	Sunlight	5.84	6.54	8.09	9.78	14.01	14.7
	Dark control			5.24	4.99	5.22	5.08
$S_{275-295}$ (μm^{-1})	Sunlight	7.63	8.44	9.94	11.6	13.5	16.4
	Dark control			7.50	7.64	7.42	7.54
S_R	Sunlight	1.21	1.40	1.67	1.80	2.47	3.22
	Dark control			1.39	1.54	1.30	1.48

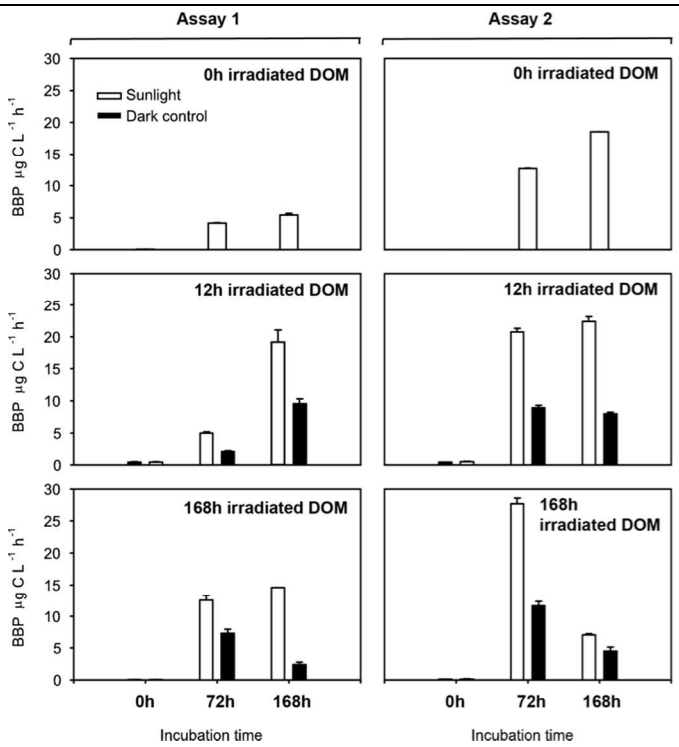

Figure 4. Variation of bacterial biomass production (BBP) along of the incubation assays with DOM irradiated to different times of natural sunlight (long-term assays). Bars and respective errors represent the average and standard deviation of the 3 replicates, respectively.

Table V. Variation of DOM spectroscopic characteristics of surface water samples rooftop irradiated with natural sunlight compared with dark controls during a short period of irradiation (12 h). Water sub-samples were collected at the marine zone (N1) of the estuary Ria de Aveiro in March 2006.

Irradiation time		0h			12h		
SC	Subsample	1	2	3	1	2	3
a_{254} (m^{-1})	Sunlight	2.44	2.38	2.38	4.51	4.54	4.50
	Dark control				4.82	4.82	4.81
a_{350} (m^{-1})	Sunlight	0.48	0.49	0.49	0.81	0.81	0.80
	Dark control				1.01	1.01	1.00
E_2:E_3	Sunlight	6.67	6.65	6.72	7.55	7.60	7.59
	Dark control				6.58	6.51	6.53
$S_{275-295}$ (μm^{-1})	Sunlight	7.21	7.22	7.24	8.23	8.16	8.23
	Dark control				7.31	7.30	7.31
S_R	Sunlight	0.85	0.85	0.86	1.00	1.00	1.01
	Dark control				0.89	0.80	0.81

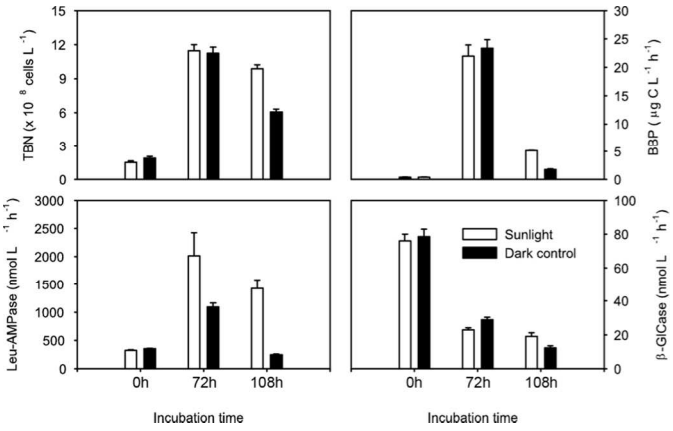

Figure 5. Variation of total bacterial number (TBN), bacterial biomass production (BBP) and, Hm of the enzymes aminopetidase (Leu-AMPase) and ß-glucosidase (ß-GlCase) along of the incubation assay with DOM irradiated to 12 h of natural sunlight (short-term assay). Bars and respective errors represent the average and standard deviation of the 3 sub-samples, respectively.

Discussion

CDOM optical properties at the two estuarine sites

The CDOM of the marine and brackish water zones showed different spectral characteristics, reflecting the different amounts and prevailing sources of organic matter, as well as distinct riverine and oceanic influences. The CDOM at the brackish water zone showed significantly higher values of the $SUVA_{254}$ and a_{350} and lower SR ratios compared with the marine zone, indicating that, at this estuarine zone, DOM is

composed by higher proportion of land derived materials [41], with higher molecular weight (MW) [28] and higher aromatic content [42]. The absorption coefficient at 350 nm has been used to trace land originated DOM inputs in coastal [41] and estuarine systems [34] due to its strong correlation with the dissolved lignin content [41]. In the present study, a strong negative correlation of both $SUVA_{254}$ and a_{350} parameters with salinity was observed at station I6, suggesting a riverine transport of this land-derived CDOM. This estuarine site is influenced by River Boco discharges at the end of the Ílhavo channel and, notwithstanding its relative low contribution to the global freshwater inputs in Ria de Aveiro, its importance increases after periods of heavy precipitation [43], reducing water residence time[44]. A seasonal pattern of variation of these spectroscopic parameters was highlighted by their negative correlation with temperature, indicating higher relative amounts of allochthonous sources in DOM pool during the cold season, in comparison to the warm season.

The higher values of the ratios S_R and E_2:E_3 at the marine zone indicated that the DOM pool consists of lower MW compounds than in the brackish water zone. An increasing trend of S_R and E_2:E_3 along an estuarine salinity-increasing gradient has been reported by other authors, indicating a decrease in color as well as in the average MW of the DOM pool [28, 29, 45]. However, a lack of correlation with primary producers and/or the abiotic factors considered in the present work suggests that other factors contribute to the proportion of LMW compounds in the DOM pool, at this estuarine area (N1 station). Contrastingly, at the brackish water zone, these ratios correlated positively with chlorophyll, POM and DOC concentrations, as well as with salinity and temperature. The typical increase of phytoplankton biomass at this estuarine section during the warm season, might contribute to increase the relative proportion of autochthonous DOC and POM, containing a higher proportion of LMW compounds [46].

Relation between bacterial abundance, activity and CDOM properties

The abundance of bacteria and Leu-AMPase activity in the water column of the marine zone showed positive correlations with the parameters a_{254} and a_{350}. The increase of abundance and proteolytic activity of bacterial communities with salinity and temperature during the low riverine influence (warm season), suggest other contributions besides than land-derived DOM inputs for the absorbance of CDOM at 254 and 350 nm.

The increase of UV absorbance and of the humification index values of DOM after incubation with bacteria, were found to be more pronounced for algal- and plant-derived DOM compared with other sources [47]. Moreover, algal-derived DOM causes a substantial increase in the average MW value, whereas little changes or even decreases in the MW values were observed for terrestrial sources of DOM [48]. Guillemette and del Giorgio [49] also observed a production of humic-like fractions during incubations of bacteria with DOM, with rates vary in function of bacterial growth efficiency and concentration of inorganic nutrients. At this estuarine area, bacterial abundance and proteolytic activity have a typical seasonal pattern of variation, related with a strong increase of primary production during the warm season [50, 51]. A selective consumption of LMW algal-derived and a simultaneous production of HMW DOM might increase the UV absorbance of CDOM, explaining the relation with a_{254} and a_{350} parameters.

At the brackish water zone, bacteria abundance was significantly related with the concentrations of DOC and inorganic nitrogen, and Leu-AMPase activity correlated with phytoplankton biomass and both particulate and dissolved organic concentrations. At this estuarine area, Leu-AMPase activity also showed a positive correlation with E_2:E_3 and S_R ratios, supporting a phytoplankton production of proteic-rich DOM [52], with LMW and aromaticity. At the marine zone, ß-GlCase correlated negatively with S_R and E_2:E_3 ratios, suggesting stimulation by HMW DOM, probably supplied by freshwater inputs, indicated by the positive correlation with inorganic nitrogen.

Bacterial responses to photochemical changes in CDOM

In aquatic systems, the absorption of light by CDOM results in significant changes on its optical properties [28, 37]. In the present study we observed pronounced changes on CDOM optical properties during the long-term assays but only slight variations in the short-term assay. The first explanation would be the different times of DOM sunlight-irradiation, but the selected spectral parameters showed considerable changes after 12 h in the long-term assays, the same time of sunlight irradiation in the short-term assay. Therefore, other factors, rather than light dose, may underlie the distinct photoreactivity of CDOM. The sunlight-irradiation of DOM can reduce or increase the bioavailability of the exposed DOM, depending of the initial content of labile or refractory DOM [21, 22]. The comparison of the initial values of the selected spectral parameters in the short and long-term assays showed that water samples used in the long-term assays setup had higher aromatic content (a_{254}, a_{350}) and MW (lower E_2:E_3) suggesting a more refractory nature, and therefore susceptible of higher photochemical alterations [53]. A different seasonal photochemical reactivity of CDOM was observed previously at this estuarine system (Pinto et al, unpublished data).

The decrease of the values of the absorption coefficients at 254 (a_{254}) and 350 (a_{350}) nm and in the increase of the values of the $S_{275\text{-}295}$, and of the ratios E_2:E_3 and S_R indicate that CDOM suffered significant transformations during the irradiation with sunlight in the long-term assays. The slope ratio SR increased

in the photochemical process, and therefore could be used as an indicator of photobleaching and composition change of CDOM [28, 37]. A photochemical degradation and decrease of DOM MW was also indicated by the increase of ratio E_2:E_3 during sunlight exposure. Changes in MW are inversely correlated with the ratio of the absorbance at 250 nm to that at 365 nm (E_2:E_3) [54]. The exposure of DOM to sunlight increased its bioavailability, stimulating bacterial activity, probably due to reduction of MW. Bacterial biomass production was twice as high at light treatment, compared with dark control in the long-term assays.

In the short-term assay, the small changes of CDOM properties only promote significant different responses in sunlight treatment when compared with dark controls in the activity of Leu-AMPase. Smith and Benner [13] observed that bacterial carbon metabolism of photo-altered DOM is coupled to an enhanced demand for inorganic nutrients, which may induce an N-limitation in sunlight treatment, and consequent stimulation of proteolytic activity. A positive correlation between aminopeptidase activity and N limitation has been observed in this estuary and inferred as an indication of organic N-sources utilization by bacteria [7]. In this study, the concentration of inorganic nutrients was not determined, and therefore we can only speculate about the possible occurrence of N-limitation during the experiments. Furthers investigations are needed to clarify a relation between nutrients and responses of estuarine bacteria to photochemical transformations.

Methods

Sampling strategy and laboratory simulations

In a first stage, two estuarine sites (stations N1 and I6; Figure 6) were sampled regularly, in order to identify the main sources and characterize DOM in the Ria de Aveiro, as well as the temporal and spatial profiles of variation. The two sites display distinct levels of microbial activity[55-57], amounts of organic matter and are differently impacted by river discharges and oceanic influence[44]. Station N1, located near the mouth of estuary, is highly exposed to oceanic influence, whereas, station I6, located at inner section of the Ílhavo channel, the narrower and shorter of the main channels [58], is directly influenced by river Boco discharge. The two sites have also different water columns dimensions and transparency and are currently referred as the marine zone and brackish water zones, respectively [55, 56]. In a second stage, in order to understand the influence of photochemical processes in the estuarine system, particularly in the deep and transparent areas, which are highly impacted by solar radiation, water samples from the station N1 were submitted to long-term (168 h) sunlight irradiation. The response of estuarine bacteria to irradiated DOM was evaluated by the determination of bacterial biomass production. Additionally, these experiments allowed the determination of the most convenient timeframe to assess measurable photochemical alterations of DOM induced by sunlight irradiation and bacterial growth. Therefore, in a third stage, short-term experiments (12h) were performed and the response of bacteria to irradiated DOM was characterized in detail.

Study site and sampling

Ria de Aveiro (40º 38'N, 8º 45'W; Figure 6) is a shallow tidal lagoon [59] situated on the Northwest Atlantic coast of Portugal, separated from the sea by a sand bar. The lagoon covers an area ranging from 66 at low tide to 83 km^2 at high tide. It exchanges with the sea a volume of water of 137 Mm^3 for maximum spring tide and 35 Mm^3 for minimum neap tide [59]. The lagoon has a complex topography, with four main channels spreading from the mouth: S. Jacinto, Espinheiro, Mira and Ílhavo. Due to their unique characteristics, each channel could be considered as an independent estuary connected to a common inlet [58]. Freshwater is supplied to lagoon mainly by rivers Vouga, Antuã, Caster, Gonde and Boco, which discharge an average water input of 1.8 Mm^3 during a tidal cycle [43]. Of these rivers, the major contributor is Vouga River which discharges more than 66% of the incoming freshwater [60] and is connected to the Atlantic Ocean by the Espinheiro Channel. Sampling was conducted at low tide, approximately every two months, between January 2006 and April 2007, at station N1 and I6. Water samples were collected with a horizontal Van Dorn bottle at the fixed depths of 20 cm, 50 cm, mid-depth and 50 cm above the sediment surface. Samples were kept at 4 ºC during the transport to the laboratory and processed within 2-3 hours after collection.

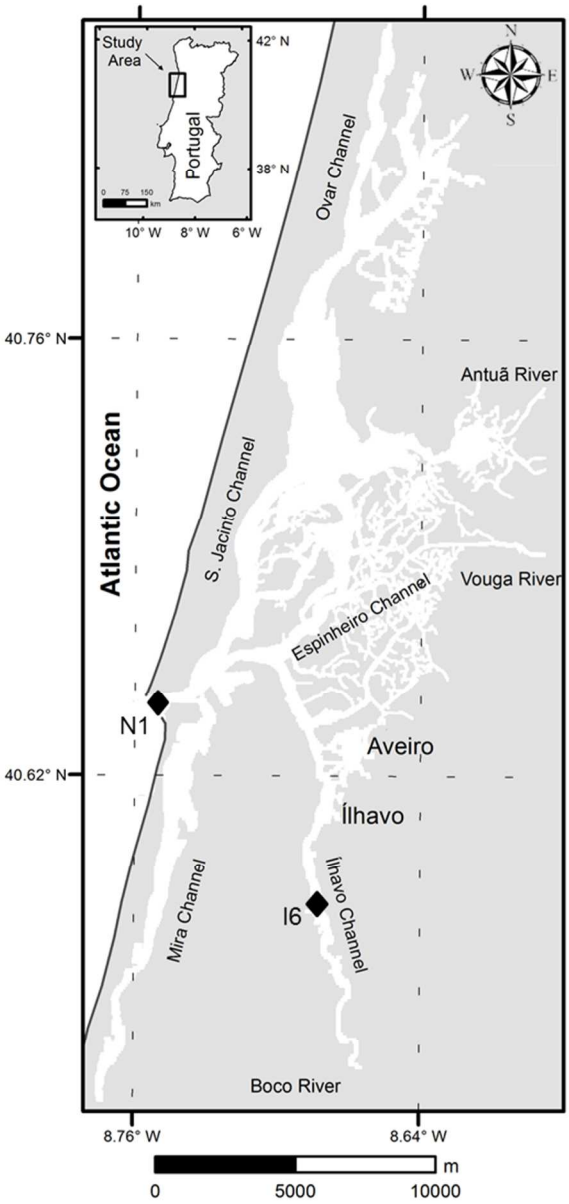

Figure6. The estuarine system Ria de Aveiro with indication of sampling stations. Station N1 in Canal de Navegação represents the marine zone, and station I6, in Canal de Ílhavo, represents the brackish water zone.

Water column properties

Water temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter (Wissenschaftlich Technische Werkstätten). The total depth of the water column was estimated with a Sonar probe (Hondex PS-7 LCD Digital Sounder). Chlorophyll a (Chl a) was estimated fluorimetrically [61] after filtration of 0.5 L triplicate subsamples through Whatman GF/F filters and overnight cold extraction in 90% (v/v) acetone. Suspended particulate matter (SPM) concentration was determined after filtration of triplicate 0.5 L water aliquots through pre-weighted and pre-combusted Whatman GF/F filters. The filters were dried at 60 ºC for 24 h, and SPM was calculated as the increase in dry weight. Particulate organic matter (POM) was determined from loss of weight after 4 h incineration of SPM at 550°C[62]. For nutrient analysis, water subsamples were filtered through MSI acetate membranes (GE Osmonics) with 0.45 µm pore size and stored at -20 °C in acid-cleaned polyethylene flasks until determination. Orthophosphate and nitrite were quantified using methods described by Hansen and Koroleff [63]. Nitrate was assayed using an adaptation of the spongy cadmium reduction technique [64], with the nitrite value subtracted from the total. The concentrations of total carbon (TC) and inorganic carbon (IC) were determined with a Shimadzu TOC-5000A analyzer. The dissolved organic carbon (DOC) content of the samples was calculated as the difference TC − IC. For TC quantification, standards were prepared from reagent grade potassium hydrogen phthalate (Fluka) in ultrapure water in the range of 0.5 to 2 mg C L^{-1}. For IC measurements, standards were made from reagents grade sodium hydrogenocarbonate (Fluka) plus sodium carbonate (Fluka) in ultrapure water, also in the range of 0.5 to 2 mg C L^{-1}. Control standards were generally within 5% agreement in terms of TC and IC content. For each sample, three replicates were analyzed for determining the DOC content.

Microcosm experiments

Experimental set-up and treatments

Water samples for the short (12 h irradiation) and long-term (168 h irradiation) assays experimental set-up treatments were collected at the N1 station, at low tide, with a horizontal Van Dorn bottle at 20 cm depth, in May and June 2006, for long-term assays, and in May 2007, for the short-term assay. Water samples were filtered sequentially through pre-combusted (550°C, 4 h) Whatman GF/F filters (0.7 µm) and then through 0.2 µm PVDF filters (Pall Corporation) in order to remove POM and bacteria. Filtered water was stored at 4°C in the dark for 12 h prior to the incubation.

Long-term experiments: For long-term experiments, 3 L of bacteria-free filtrate (0.2 µm filtrate) was distributed by 150 mL quartz tubes (natural sunlight-irradiated sample) and 1L aluminum foil-wrapped borosilicate bottles (dark control). Both quartz tubes and dark controls were irradiated with natural sunlight at environment temperature conditions during 168 h. Replicates for DOM spectral characterization (UV/Vis spectroscopy) and bioassays were collected at 0, 12 and 168 h. Bioassays were initiated by addition of a natural bacterial inoculum that was obtained from the same study site (0.7 µm filtrate) diluted 10-fold. Incubations were conducted in the dark at room temperature (22°C) during 168 h, with gentle agitation, and subsamples were collected at 0, 72 and 168 h in order to assess the response of bacterial biomass production to the different periods of DOM irradiation with sunlight. DOM characterization and determinations of bacterial production were performed immediately.

Short-term experiments: For the short-term experiments, 3 sub-samples with 2.5 L of bacterial free filtrate (0.2 µm filtrate)

were placed in 3L Pyrex trays (natural sunlight treatment). The trays were wrapped in polypropylene foil, which screens out approximately 20% of PAR, 25 % of UVR-A and 30 % of UVR-B, and 3L borosilicate bottles were wrapped in aluminum foil representing dark controls. Pyrex trays and dark controls were irradiated with natural sunlight and environment temperature conditions during 12 h, in a cloudless day. Optical characteristics of DOM (UV/Vis spectroscopy) were determined before and after exposition to natural sunlight. Bioassays were initiated by adding a natural bacterial inoculum (0.7 µm filtrate) obtained from the same study site and diluted 10-fold. Incubations were conducted in the dark at room temperature (22°C) during 108 h, with gentle agitation. Subsamples were collected at 0, 72 and 108 h to assess the response of bacteria to irradiated DOM. In order to evaluate a possible regrowth, bacteria were enumerated in both sunlight-exposed and dark control samples for the times 0 and 12h in the case of short-term experiments and, for times 0, 72 and 168 h in the case of long-term experiments. The results (data not shown) showed that, in all experiments, filtration removed more than 90% of bacteria. In short-term experiments, the regrowth of bacteria was imperceptible (less than 3% of the initial counts), whereas in long-term experiments occurred an unimportant regrowth of bacteria, about 8% of the original bacterial population at time 72 and 14% at time 168h. Nonetheless, the regrowth was similar in the light-exposed and the dark control samples, which suggests that the effect of photodegradation was not masked.

CDOM spectroscopic characteristics

UV–Vis spectroscopy was performed on a Shimadzu Model UV 210PC spectrophotometer using 1 and 10 cm quartz cuvettes (as required depending on sample absorbances) for the range 200–700 nm. The absorption coefficients (a_λ, m^{-1}) at each wavelength (λ) were calculated as $a_\lambda = 2.303\ A_\lambda/l$, where A_λ is the absorbance reading at wavelength λ and l (m) is the optical path length [65]. Ultrapure water was used as reference and the absorbance of the sample at the wavelength 700 nm was considered as the zero absorbance and was subtracted from each absorbance value in the 200–700 nm range.

The absorption coefficients (a) were used in the determination of the spectral characteristics listed below. The ratio of spectral slopes, S_R, is the ratio of S values for wavelengths 275–295 nm ($S_{275–295}$) and 350–400 nm ($S_{350–400}$) where the values for these S sections were determined by plotting ln(a) versus wavelength [28]. The E_2:E_3 ratio is calculated as the ratio of a_{250} to a_{365} and is inversely correlated with molecular size [45]. Specific ultra-violet absorbance at 254 nm ($SUVA_{254}$) was calculated by dividing a_{254} by the DOC concentration in mg L^{-1}. $SUVA_{254}$ is indicative of the amount of humification or aromaticity within the sample [42].

Total bacterial number

Total bacterial number (TBN) was determined by epifluorescence microscopy using a Leica DMLS microscope equipped with a I 2/3 filter for blue light. Three replicates for each sample were filtered through 0.2 µm black polycarbonate membranes (GE Osmonics) and stained with 0.03 % acridine orange [66]. At least 200 cells or 20 microscope fields were counted for each replicate measurement.

Bacterial biomass production

Bacterial biomass production (BBP) was determined in 10-ml triplicate plus a control that was fixed by addition of formaldehyde (2% final concentration). The samples were incubated at a saturating concentration (121.6 nmol L^{-1}) of ^{3}H-leucine (Amersham, specific activity - 2.55 TBq $mmol^{-1}$) for 1 h, at *in situ* temperature, in the dark. After incubation, replicates were fixed with 2% (v/v) formaldehyde. Protein was precipitated by the addition of 1 ml of 20% (w/v) ice-cold TCA followed by incubation for 15 min on ice. The 10-ml triplicate and the control were then filtered through 0.2 µm polycarbonate membranes (GE Osmonics) and rinsed with 2 ml of 5% (w/v) ice-cold TCA and 5 ml of 90% (v/v) ice-cold ethanol. Membranes were then placed into 5 mL scintillation vials and 4.5 mL of scintillation cocktail UniverSol (ICN Biomedicals, USA) was added. Radioactivity was measured after a period of 3 days in a Beckman LS 6000 IC liquid scintillation counter. BBP was calculated from leucine incorporation rates using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073[67].

Extracellular enzymatic activity

Extracellular enzymatic activity **(**EEA) was determined fluorimetrically (Jasco FP-777 fluorometer) as the maximum hydrolysis rate (Hm) of model substrates [68]. The substrate L-leucine-7-amido-4-methyl-coumarin hydrochloride (Fluka) was used for leucine aminopetidase (E.C. 3.4.11.1) [69] and the 4-methylumbelliferyl-β-glucopyranoside (Fluka) for β-glucosidase (E.C. 3.2.1.21) [70]. Both substrates were added at saturating concentrations (10 mM). Wavelengths for excitation and emission were 380 to 440 nm for MCA (7-amino-4-methylcoumarine) and 360 to 450 nm for MUF (4-methylumbelliferone). Measurements were made in 3 replicates for each sample after 2 h, for MCA, and 18 h for MUF. Incubations were made at *in situ* temperature. Calibration was performed by adding a series of 6 to 8 concentrations of the fluorescent products (0 to 500 nmol L^{-1} for MUF and 0 to 6 µmol L^{-1} for MCA) to a pool of water from the 2 sampling stations.

Data analysis

The statistical analysis of data was performed with the IBM SPSS Statistics 22.0 software. Normal distribution was assessed by the Kolmogorov-Smirnov test and homogeneity of variances by the Levene test. The significance of differences in microbial

and spectral parameters between the two estuarine zones was determined by the Mann-Whitney test and between the different depths by the Kruskall-Wallis test. To test the significance of differences within sampling events was used the Friedman's ANOVA followed by *post-hoc* multiple comparisons[71]. The relations between the different parameters were examined using a Spearman correlation. The values were considered significantly different after applying the Bonferroni´s correction to α=0.05.

Conclusions

In estuarine system Ria de Aveiro, both photochemical and microbial processes yielded optical changes in CDOM. The magnitude of photochemical and microbial induced CDOM changes is dependent on the initial characteristics of DOM pool. A more "labile" DOM pool might undergo lower photochemical alterations and stimulate the production of refractory compounds by bacteria, whereas, a more "refractory" DOM pool might experience substantial photochemical changes, increasing its bioavailability and recycling. Considering the seasonal and hydrological influences in the relative proportions of the "labile" and "refractory" fractions in the DOM pool, the importance of these processes might vary as well annually and inter-annually. The overall result of these combined photochemical and microbial effects determine the bioavailability and fate of CDOM in the estuarine system and have influence on local productivity and in adjacent coastal areas. A further investigation, incorporating the experimental simulation of photochemical and microbial processes in the shallow and turbid areas of the estuary will allow obtain a more comprehensive knowledge of these processes in this estuary and in other similar aquatic ecosystems.

Acknowledgements

This work was supported by FEDER funding through COMPETE - Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia (Foundation for Science and Technology), within the research projects COMPETE: FCOMP-01-0124-FEDER-029428 (PTDC/MAR - EST/2314/2012), PTDC/AAC-AMB/121191/2010 and DyEPlume PTDC/MAR/107939/2008. Thanks are also to Centre for Environmental and Marine Studies, University of Aveiro (CESAM, project Pest C/MAR/LA0017/2011) and to Department of Biology of University of Aveiro. Financial support to Luísa Santos was provided by FCT in the form of a PhD grant (SFRH/BD/31827/2006).

Notes and references

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