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Estuarine, Coastal and Shelf Science 66 (2006) 225–230
ESTUARINE COASTAL AND SHELF SCIENCE
www.elsevier.com/locate/ecss

Comparative study on microphytobenthic pigments of muddy and sandy intertidal sediments of the Tagus estuary

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Received 6 June 2005; accepted 30 August 2005
Available online 2 November 2005

Abstract

The abundance and distribution of microphytobenthic pigments determined by HPLC (chlorophylls and carotenoids) were compared between muddy and sandy sediments of the Tagus estuary (Portugal). In the two types of sediment, with similar periods of illuminated emersion, chlorophyll *a* concentrations on a per area basis (mg m^{-2}) were comparable (down to 2 mm). Pigment analysis also revealed similar microphytobenthic communities in terms of algal classes. Diatoms were the dominant microalgae, but cyanophytes, euglenophytes and phanerogam debris were also present. For both muddy and sandy sediments, microphytobenthic biomass showed a high level of variability both within and between two consecutive years. Microphytobenthos was highly stratified in the mud, with most of the chlorophyll *a* occurring in the top 500 μm. In the sand, relatively constant concentrations were found throughout the sediment profile down to 3 mm. This is probably related to deeper light penetration in sandy sediment and/or increased physical mixing caused by invertebrate activity or overlying currents, leading to the burial of an important fraction of the microphytobenthic cells. Differences observed in the intensity of sediment coloration of muddy and sandy sediments might have resulted from the different vertical distribution of benthic biomass.

Keywords: microphytobenthos; pigments; HPLC; chlorophylls; carotenoids; Tagus estuary

1. Introduction

Benthic microalgae of intertidal sediments show a high degree of spatial and temporal heterogeneity, and factors such as resuspension, nutrient and light availability, grazing, and desiccation have been suggested to control microphytobenthic biomass (see review by Underwood and Kromkamp, 1999). A major factor determining both the abundance and composition of microphytobenthic communities is the nature of the substratum. Some studies report higher biomass in muddy sediments (Riaux-Gobin et al., 1987; Riaux-Gobin and Bourgoin, 2002; Perkins et al., 2003), while others show higher chlorophyll *a* levels associated with sandier substrates (Cahoon et al., 1999; Cahoon and Safi, 2002).

Information regarding the distribution and abundance of benthic microalgae can be derived from methods like spectral reflectance (e.g. Paterson et al., 1998), chlorophyll *a* fluorescence (e.g. Serôdio et al., 2001) or high performance liquid chromatography (e.g. Brotas and Plante-Cuny, 1998). HPLC is a separative method that allows not only the determination of chlorophyll *a*, a reliable index of benthic microalgae biomass, but also sheds light over a complex pool of pigments and their degradation forms that occur in intertidal sediments (Cariou-Le Gall and Blanchard, 1995; Brotas and Plante-Cuny, 2003). The pigment composition determined by HPLC can be used for taxonomic purposes, since several pigments or pigment combinations are found only in certain algal classes (Riaux-Gobin et al., 1987; Klein and Riaux-Gobin, 1991; Brotas and Plante-Cuny, 1998). Furthermore, the presence of chlorophyll *a* degradation products has been related to grazing processes (Bianchi et al., 1988; Buffan-Dubau et al., 1996; Cartaxana et al., 2003).

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doi:10.1016/j.ecss.2005.08.011

The primary goal of this work was to compare the abundance and distribution of microphytobenthic pigments (chlorophylls and carotenoids) determined by HPLC in muddy and sandy sediments of the Tagus estuary (Portugal). Information derived from the optical properties of the sediment surface is increasingly being used in remote sensing studies (e.g. Méléder et al., 2003; Murphy et al., 2005) to quantify chlorophyll *a* in intertidal and subtidal benthic habitats. Furthermore, the surface coloration of the sediment is a general indicator of the nature of the surface assemblages (Paterson et al., 1998). From the observation of sediment coloration, it is hypothesised that muddy sediments, with similar periods of illuminated emersion to that of sandy sediments, are more favourable for benthic cells and are distinct in both the abundance and the composition of the microphytobenthos. The distribution of the microphytobenthic pigments with depth was also compared between the two types of sediment.

2. Material and methods

2.1. Study site

The Tagus estuary (38°44′N, 09°08′W) is a shallow mesotidal estuary covering an area of 320 km^2. Tidal amplitude ranges from 1 to 4 m, and the intertidal area comprise 20–40% of the estuary in neap and spring tides, respectively. Sediments are typically sandier in zones where currents are more active and muddier in enclosed areas.

2.2. Sampling

From September 2002 to July 2004, sediment samples were collected at low tide during spring tides on two different intertidal sites. Both sites were exposed during each low tide for about 2.5–3 h (tidal height ±1 m), but had different sediment characteristics (Table 1). At each site, three different subplots separated by approximately 5 m were chosen for replicate sampling.

Sediment samples were collected using contact corers (Honeywill et al., 2002). The contact corer device, a small strip of metal plate with a cavity at the bottom side, was used to freeze the top 2 mm of the sediment. It was gently placed on the sediment surface so that the metal dish made contact. Liquid nitrogen (LN) was added on top, after which the sediment was allowed to freeze for up to 2 min (the freezing time was assessed for each site and varied according to factors such as weather conditions and sediment water content). Any sediment deeper than 2 mm was removed using an artist's palette knife leaving a flat disk of sediment. The sediment disc was wrapped in foil and stored in LN. In the laboratory, samples were weighed, transferred to −80 °C, then freeze-dried and re-weighed. Additional samples were taken for particle size, organic matter, and interstitial water salinity determination.

Another sampling technique employed a device called the cryolander (Wiltshire et al., 1997). The cryolander was gently placed on the sediment surface and a small amount of LN poured slowly onto the cotton wool in it. The LN vaporised and froze the immediate sediment surface without distortion even on a micrometer scale (Wiltshire et al., 1997). After the surface had frozen, the LN was poured onto it evenly through the cryolander mesh. The cryolanding process varied with environmental and site conditions and took up to 5 min. When a disk of sediment around the cryolander was frozen, the cryolander was lifted from the sediment surface, and a plunger used to remove the sediment disk. The disk was placed face down on a foil (so that the surface was always identifiable), wrapped, and stored in LN. In the laboratory, samples were transferred to −80 °C. The cryolander sediment blocks were cut into slices using a freezing microtome (Leiz Wetzlar Kryomat 1703). The sediment was sectioned into depth intervals of 0–180, 180–360, 360–540, 540–720, 720–1080, 1080–1500, 1500–1980, 1980–3000, and 3000–3480 μm. The sectioned sediment was removed from the microtome blade with a small piece of filter material (pre-weighed), then placed in a 1 ml Eppendorf (pre-weighed). The samples were freeze-dried and re-weighed prior to analysis.

Table 1
General characteristics (average ± standard deviation, $n = 36$) of muddy and sandy intertidal sediments of the Tagus estuary. Significant differences between sites are indicated: ***$p < 0.001$

	Sandy	Muddy
Water content (%)	27.8 ± 3.2	70.5 ± 3.0***
Organic matter (%)	1.3 ± 0.4	9.0 ± 0.5***
Sediment size fractions (%)		***
>1000 μm	5.4 ± 1.9	0.2 ± 0.2
1000–500 μm	31.2 ± 2.7	0.1 ± 0.06
500–250 μm	43.3 ± 3.6	0.8 ± 0.4
250–125 μm	10.4 ± 1.3	1.4 ± 0.8
125–63 μm	1.2 ± 0.4	0.9 ± 0.3
<63 μm	8.5 ± 3.3	96.7 ± 1.6
Wet bulk density (g cm^{-3})	2.04 ± 0.40	1.34 ± 0.17***
Salinity	21.8 ± 8.0	21.6 ± 10

2.3. HPLC analysis

Approximately 0.2 g of freeze-dried sediment from the contact cores were extracted for 15 min in 95% cold buffered methanol (2% ammonium acetate) with 30 s sonication (Cartaxana and Brotas, 2003). After filtration (0.2 μm Whatman membrane filters) extracts were immediately injected in a Shimadzu HPLC with a photodiode array (SPD-M10AVP) and a fluorescence detector (RF-10AXL). Chromatographic separation was carried out using a C18 column for reverse phase chromatography (Supelcosil; 25 cm long; 4.6 mm in diameter; 5 μm particles) and a 35 min elution programme. The solvent gradient followed Kraay et al. (1992) with a flow rate of 0.6 mL min^{-1} and an injection volume of 100 μL. Sediment samples from the cryolanders were extracted in acetone (48 h, dark, −20 °C) and analysed on a Waters system. Samples were injected in between two plugs of Milli-Q water (20 μl Milli-Q, 60 μl sample, 20 μl Milli-Q).

The chromatography technique was otherwise similar for both contact core and cryolander sediment. Identification and calibration of the HPLC peaks were done with chlorophyll *a* and β-carotene standards from Sigma and chlorophyll c_2, fucoxanthin, diadinoxanthin, diatoxanthin, and pheophytin *a* standards from DHI. Pheophorbide *a* standard was prepared from the macroalgae *Enteromorpha intestinalis* following the procedure described by Brotas and Plante-Cuny (1996).

Pigments were identified from absorbance spectra and retention times and concentrations calculated from signals in the photodiode array (chlorophylls *a* and *c*, and carotenoids) or fluorescence detector (Ex. 430 nm; Em. 670 nm) (chlorophyllide, pheophorbides and pheophytins *a*). Concentrations in the contact cores were expressed on an areal basis (mg m^{-2}), by accounting for the density of each sediment sample. For the cryolanders, data were converted to volumetric units (μg chl *a* cm^{-3}) using mean dry bulk densities of 1.46 g cm^{-3} and 0.40 g cm^{-3} for sandy and muddy sediments, respectively (average values for sediments collected during the studied period).

2.4. Statistical analysis

The existence of significant differences between data was tested using two- and one-way analysis of variance (ANOVA) for effects of sampling date and sediment type. Multiple comparisons among pairs of means were performed using the T-method (Tukey's honestly significance difference method) when a significant ANOVA result occurred. Homogeneity of variances was tested using the Bartlett's test. Data were logarithmically transformed when necessary to comply with the assumptions of ANOVA.

3. Results

Two different intertidal sediments were analysed. Based on the classification of Folk (1954), one site was composed of sand and the other of mud. The sandy sediment had an average of 43% of particles between 250 and 500 μm, while the muddy sediment had 96.7% of particles smaller than 63 μm (Table 1). The sediments were also significantly different for water content, organic matter and wet bulk density (Table 1). No significant differences were found for interstitial water salinity.

Pigments (chlorophylls, carotenoids and degradation products) present in the sediment extracts were identified and quantified by HPLC. As an example, an HPLC chromatogram obtained for the muddy sediment is shown in Fig. 1. Fluorescence detection was particularly useful in the detection and quantification of pheophorbides *a* because these chlorophyll degradation products co-elute and interfere with some carotenoids.

The most abundant pigments were chlorophylls *a*, c_1 and c_2 (the method did not allow the separation of the two chlorophylls *c*), fucoxanthin, diadinoxanthin, diatoxanthin, β-carotene and degradation products like pheophorbides and pheophytins *a* (Fig. 1; Table 2). Sediment chlorophyll *a* concentrations ranged between 28.5 and 101 mg m^{-2}, with an average concentration of 60 mg m^{-2} for all samples analysed. High concentrations of fucoxanthin were also found in all samples analysed (9.9–41.6 mg m^{-2}). Zeaxanthin was present in all samples analysed but concentrations were significantly lower than the more abundant pigments, ranging from 0.06 to 0.44 mg m^{-2}. Chlorophyll *b* was present in most sediment samples but in even smaller proportions (0–0.28 mg m^{-2}).

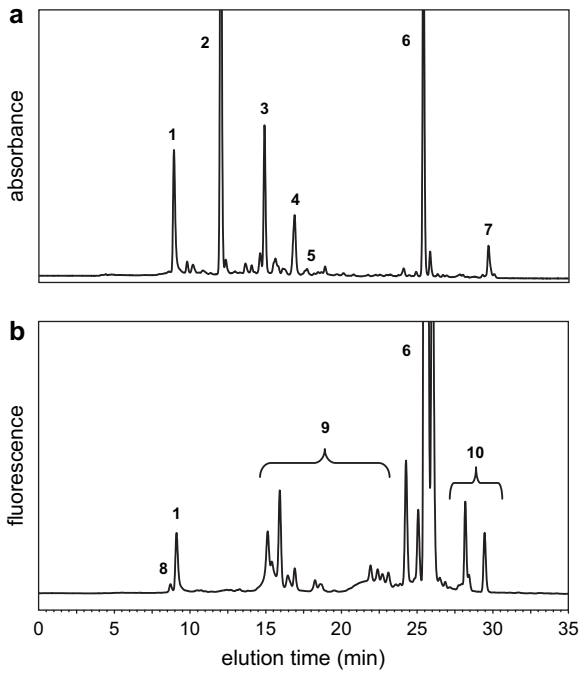

Fig. 1. HPLC chromatograms of absorbance at 440 nm (a) and fluorescence (Ex. 430 nm; Em. 670 nm) (b) obtained for a Tagus estuary intertidal muddy sediment. Peak identification: 1 – chlorophyll $c_1 + c_2$, 2 – fucoxanthin, 3 – diadinoxanthin, 4 – diatoxanthin, 5 – zeaxanthin, 6 – chlorophyll *a*, 7 – β-carotene, 8 – chlorophillide *a*, 9 – pheophorbides *a*, 10 – pheophytins *a*.

No significant differences were obtained for total concentrations (per unit area of sediment) of ubiquitous pigments like chlorophyll *a* and β-carotene between muddy and sandy sediments (Table 2). Significant higher concentrations were found in the mud for chlorophyll *b*, diadinoxanthin, diatoxanthin, pheophorbides and pheophytins *a*. Higher chlorophyllide *a* concentrations were observed in the sand (Table 2).

Table 2
Average (±standard deviation, $n = 36$) pigment concentrations (mg m^{-2}) of muddy and sandy intertidal sediments of the Tagus estuary. Significant differences between sites are indicated: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

	Sandy	Muddy
Chlorophyllide *a*	1.52 ± 1.59	0.82 ± 0.77*
Chlorophyll $c_1 + c_2$	5.21 ± 2.05	4.45 ± 1.72
Fucoxanthin	22.1 ± 7.7	24.8 ± 8.2
Diadinoxanthin	3.77 ± 1.62	5.00 ± 1.76**
Diatoxanthin	1.11 ± 0.43	1.65 ± 0.76**
Zeaxanthin	0.19 ± 0.12	0.20 ± 0.08
Pheophorbides *a*	2.04 ± 0.96	6.55 ± 2.04***
Chlorophyll *b*	0.020 ± 0.064	0.080 ± 0.080**
Chlorophyll *a*	59.2 ± 18.3	60.7 ± 19.7
Pheophytins *a*	2.10 ± 0.98	3.48 ± 0.75***
β,β-Carotene	1.35 ± 0.50	1.49 ± 0.49

Chlorophyll *a* concentrations varied throughout the two studied years, although with no clear seasonal pattern (Fig. 2). In the sandy sediment, concentrations were generally higher in later winter and spring and lower in summer. In the muddy sediment, there was a less consistent pattern, with peak concentrations followed by sharp decreases in chlorophyll *a* concentrations (Fig. 2). Chlorophyll *a* concentrations were highly correlated to fucoxanthin ($r = 0.98$; $p < 0.001$) and other pigments present in diatoms like chlorophyll *c* ($r = 0.87$; $p < 0.001$) and diadinoxanthin ($r = 0.85$; $p < 0.001$), and to a lesser extent to zeaxanthin ($r = 0.28$; $p < 0.05$). Chlorophyll *a* was not correlated to chlorophyll *b*. The latter pigment was only significantly correlated to diadinoxanthin ($r = 0.26$; $p < 0.05$), diatoxanthin ($r = 0.30$; $p < 0.01$) and pheopigments *a* ($r = 0.27$; $p < 0.05$).

Muddy and sandy sediments differed in the typical sediment profile (Fig. 3). In the mud, chlorophyll *a* concentrations decreased from the surface down to about 500 μm at which point concentrations remained constant down to 3 mm. Percentage of chlorophyll *a* in the first 500 μm averaged 76% of the total in the upper 2 mm. In the sand, relatively constant concentrations were found throughout the sediment profile (Fig. 3). Percentage of chlorophyll *a* in the first 500 μm averaged 30% of the total in the upper 2 mm.

4. Discussion

Intertidal muddy and sandy sediments with similar periods of illuminated emersion supported similar chlorophyll *a* levels on a per area basis. However, if data were expressed per unit mass, muddy sediments had significantly higher chlorophyll content (77 μg g^{-1} compared to 21 μg g^{-1} of sandy sediments). This is due to the higher water content of muddy sediments, which averaged 70%, as compared to 28% of sandy sediments and is probably one of the main reasons that different studies show distinct sediment grain size relationships with sediment chlorophyll *a* levels. According to Perkins et al. (2003), chlorophyll *a* concentrations associated with sandy sediments are typically lower compared to muddy sediments, as wetter sediment areas are more favourable for the cells in terms of nutrient supply, gas exchange, and avoidance of desiccation. On the other hand, Cahoon and Safi (2002) report lower microalgal biomass associated with muddier sediments. According to these authors, reduced interstitial space volumes, nutrient fluxes, and light penetration in muddier sediments are factors that may account for this pattern. It is not surprising that the relationship between benthic microalgal biomass and sediment grain size is not simple, and is likely to be strongly influenced by a complex set of interacting factors (Cahoon et al., 1999).

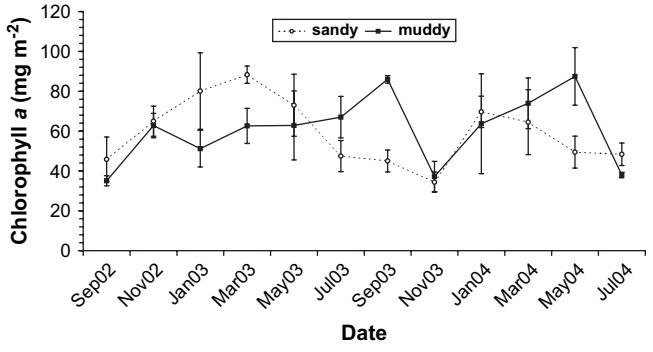

Fig. 2. Seasonal variation of chlorophyll *a* concentrations per unit area of sediment (mg m^{-2}) for muddy and sandy intertidal sediments (0–2 mm) of the Tagus estuary. Bars indicate standard deviation ($n = 3$).

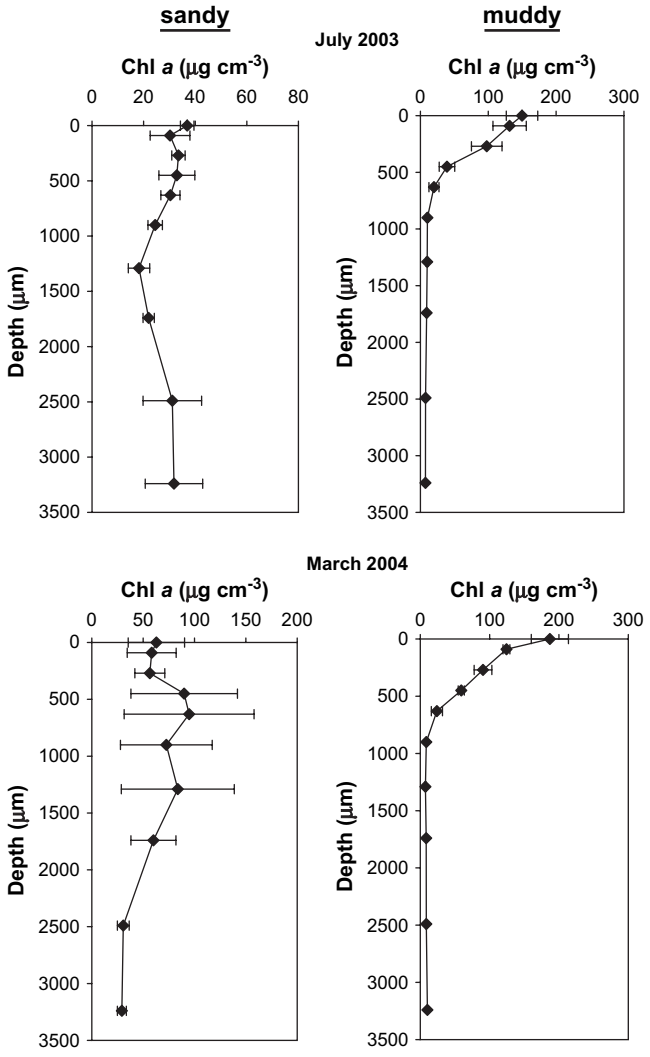

Fig. 3. Depth profiles of chlorophyll *a* concentration (μg cm^{-3}) for muddy and sandy intertidal sediments of the Tagus estuary. Bars indicate standard error ($n = 3$).

Pigment composition determined by HPLC did not reveal major differences in the abundance of the algal classes present in muddy and sandy sediments. The most abundant pigments in the samples analysed (chlorophylls *a* and *c*, fucoxanthin, diadinoxanthin, diatoxanthin and β-carotene) indicate that the microphytobenthic assemblages of the two studied sites were strongly dominated by diatoms. The presence of zeaxanthin and the absence of lutein indicate the existence of cyanophytes in both microphytobenthic communities. Chlorophyll *b* was also detected, but even in smaller concentrations. The absence of lutein and the positive correlations between chlorophyll *b*

and diadinoxanthin, diatoxanthin and pheopigments *a* suggest that the sources of chlorophyll *b* are euglenophyte cells and phanerogam debris, more abundant in the studied muddy sediments.

Microphytobenthic biomass showed a high level of variability both within and between two consecutive years. In an exhaustive literature review on microphytobenthos, MacIntyre et al. (1996) concluded that most populations of microphytobenthos show a biomass peak in spring or summer, although this was not always the case. Brotas et al. (1995) and Cabrita and Brotas (2000) have reported a lack of a clear seasonal trend for intertidal microphytobenthos biomass of the Tagus estuary. Several authors (e.g. Pinckney et al., 1994; Kromkamp et al., 1998; Serôdio et al., 2001) have demonstrated the importance of short-term variability in the biomass of intertidal microphytobenthos. Therefore, the significance of seasonal variations cannot be assessed without being compared to the variability taking place on shorter time scales. Serôdio and Catarino (2000) predicted a predominance of fortnightly over seasonal variability in the microphytobenthic productivity for the Tagus estuary. In our study, the sampling was carried out regularly at low tide during spring tides to reduce the influence of short-term variability, although a short-term trend superimposed on the seasonal trend cannot be excluded.

The major difference found between muddy and sandy sediments was the vertical distribution of pigments in the sediment profile. In the mud, microphytobenthos was highly stratified with most of the chlorophyll *a* occurring in the top 500 μm. Wiltshire (2000) obtained similar results for three European estuaries and referred the importance of thin sediment slicing in order to correctly estimate algal biomass in intertidal sediments. Serôdio et al. (1997), studying the migratory rhythms of benthic microalgae of intertidal muddy sediments of the Tagus estuary, estimated the photic depth to reach only 270 μm. In the sand, relatively constant chlorophyll *a* concentrations were found throughout the sediment profile down to 3 mm. Since light penetration is deeper with increased sediment particle size (Yallop et al., 1994; Kühl et al., 1994), microphytobenthic communities of sandy sediments may distribute throughout the deeper photic zone found in this type of sediment. Furthermore, physical mixing caused by invertebrate activity or overlying currents, more pronounced at the sandy site, can lead to the burial of an important fraction of the microphytobenthic cells.

5. Conclusions

The hypothesis that muddy sediments of the Tagus estuary supported higher microphytobenthic biomass was not confirmed. Both muddy and sandy sediments, with similar periods of illuminated emersion, had comparable overall chlorophyll *a* concentrations (on a per area basis and down to 2 mm), as well as similar microphytobenthic assemblages in terms of algal classes. Microscopic identification is being carried out to compare the species composition of these two types of sediment. It is suggested that differences observed in the intensity of sediment coloration of the studied muddy and sandy sediments resulted from the different vertical distribution of benthic biomass.

Acknowledgements

This work was funded by project HIMOM (contract EVK3-2001-00043) and FCT/POCTI.

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