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Spatial and temporal patterns of microphytobenthic taxa of estuarine tidal flats in the Tagus Estuary (Portugal) using pigment analysis by HPLC

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ABSTRACT: Measurements of natural estuarine microphytobenthos communities were carried out in samples of intertidal sediments of the Tagus Estuary (Portugal) by means of pigment analysis by HPLC in order to identify microphytobenthos composition. The relative abundance of taxa was determined by microscopic observations. During spring 16 sites were sampled and 3 of them, differing in sediment grain size and tidal level, were visited fortnightly for 1 yr. At all sites throughout the year, diatoms were the dominant algal group, evidenced by the relatively high concentrations of chlorophylls c_1+c_2, fucoxanthin, neofucoxanthin, diadinoxanthin, diatoxanthin and β-carotene. Chl *b* and diatoxanthin were related with observed euglenophyte populations, zeaxanthin with cyanobacteria, lutein with macrophyte debris and alloxanthin with falling phytoplanktonic cells of cryptophytes. The spatial study allowed a distinction between sites regarding the presence of macrophyte debris, euglenophytes or only diatoms. The temporal survey showed the seasonal trends of diatoms and cyanobacteria and the main periods of plant debris deposition. Pheophytin *a* and chlorophyllide *a* concentrations were quite low. Degraded chloropigments were constituted mainly of pheophorbides *a*, 9 of which were quantified. The most abundant among them indicated intensive grazing by macrozoobenthos at the muddy stations.

KEY WORDS: Chloropigments · Carotenoids · HPLC · Estuarine mud flats · Tagus · Microphytobenthos · Taxonomic markers

INTRODUCTION

As in phytoplankton studies, chl *a* concentration in surface sediments gives overall information about the distribution of plant biomass. The HPLC technique, being essentially a separative method, makes it possible to isolate native chl *a* which can be used as a reliable index of this plant 'living mass' (biomass) of microbenthic algae (Riaux-Gobin et al. 1987, Barlow et al. 1990, Plante-Cuny et al. 1993, Pinckney et al. 1994).

But, microphytobenthos communities are known to present heterogeneous distributions in space (patchiness) as well as in time. Taxonomic composition is usually analysed just qualitatively, as microscopic cells are difficult to count on a large scale. The problem of how to evaluate the taxonomic composition of natural populations is now often handled by the utilization of HPLC pigment analysis in phytoplankton studies (Gieskes 1991, Millie et al. 1993, Quiblier et al. 1994, Andersen et al. 1996, Roy et al. 1996, Jeffrey & Vesk 1997). In microphytobenthos studies however, taxonomic markers are more difficult to identify, because autochthonous microphytobenthic chloropigments and carotenoids coexist with their degraded forms origi-

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nating from diagenic or biochemical processes (Keely & Brereton 1986, Bianchi et al. 1988, Buffan-Dubau et al. 1996, Pastoureaud et al. 1996). A vast number of carotenoids originating from macrophytodetritus before and after grazing have been also reported (Abele 1988, Bianchi & Findlay 1990, Levinton & McCartney 1991, Abele-Oeschger et al. 1992, Bianchi et al. 1993a and references therein).

Application of HPLC to characterize living sediment microalgal communities has been relatively recent (Riaux-Gobin et al. 1987, Klein & Riaux-Gobin 1991, Bianchi et al. 1993b, Barranguet et al. 1996) and particularly rare in the intertidal zone (Cariou-Le Gall & Blanchard 1995).

In a previous paper, Brotas et al. (1995) used chl *a* measured spectrophotometrically to describe spatial and temporal changes of microphytobenthic biomass in the Tagus Estuary (Portugal). The part played by the microphytobenthos in the estuarine ecosystem appeared to be of major importance. The aim of the present paper is to illustrate the use of the HPLC technique as a powerful tool to determine patterns of spatial and temporal distribution of microphytobenthic community taxa in a great variety of intertidal surface sediments. We present pigment signatures to identify the major taxonomic groups, which were determined by microscopic observations. The distribution of chlorophyll degradation by-products is also described and discussed.

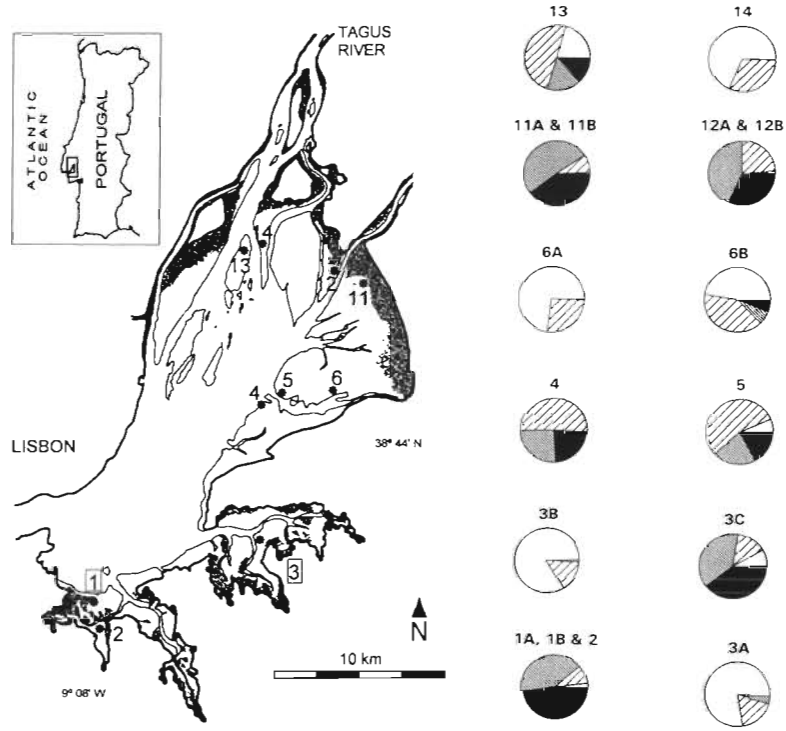

Fig. 1. Location of 10 stations (including 16 sites) in the Tagus Estuary, Portugal. The lower limit of the intertidal area is indicated by the thin line, and salt marsh zones are shown in grey. Pies represent sediment types of the 16 sites. Black: clay (<2 µm); fine hatching: silt (2 to 20 µm); large hatching: fine sand (0.02 to 0.2 mm) and white: coarse sand (0.2 to 2 mm). Identification code: number of the station plus A, B or C from the highest, middle and lowest tidal position. See Table 1

MATERIALS AND METHODS

Study stations. The Tagus Estuary (38° 44' N, 09° 08' W), which runs through the most populated area of Portugal, is a large (320 km^2) estuarine ecosystem (Fig. 1). It is a shallow (mean depth of 5 m) mesotidal estuary bordered mainly by mud flats. The tidal amplitude ranges from ca 1 to 4 m, so that the intertidal area comprises 20 to 40% of the total area in neap and spring tides, respectively. The upper littoral zone is occupied by a fringe of salt marsh vegetation, which covers ca 1300 ha (Catarino et al. 1985).

Sampling program. During April and May 1990, 16 sites from 10 stations (nos. 1 to 6 and 11 to 14) were sampled. Sites were chosen to cover the widest possible range of situations (salt marsh, muddy and sandy bottoms, different tidal heights) present in the estuarine intertidal area. In the stations which presented obvious spatial heterogeneity, 2 or 3 sites were considered (identification code: number of the station, i.e. 1, 2, 3, ..., plus site letter A, B or C, for the highest, middle and lowest tidal position respectively; Table 1, Fig. 1). Three sites (at Stns 1 and 3) were selected, each one representing a typical stratum of the ecosystem (Brotas et al. 1995): sites 1B (salt marsh mud), 3B (midshore sandy flat) and 3C (low tide mud flat), differing in sediment grain size and tidal level, were visited fortnightly from April 1991 to April 1992, to examine seasonal variations.

Sampling and analysis protocol. Sediment cores, collected by hand with a Plexiglas corer (36 mm internal diam.), were transported to the laboratory in a cooling box and immediately frozen at -20°C until further processing. The first cm was sampled for water content, organic matter content (3 replicates) and grain size analyses. A superficial slice of 0 to 5 mm was sampled from 1 core and freeze-dried for HPLC pigment analysis: a quantity of 0.3 to 3 g of sediment was used. Some authors (Riaux-Gobin et al. 1987) considered that freeze-drying reduces some pigment concentrations by up to 90%. However, by means of a previ-

Table 1 Description of sampling sites. Physical and chemical characteristics of sediments. Sampling programs: *spring 1990, **spring 1990 and seasonal survey from April 1991 to April 1992. AFDW: ash free dry weight

Site description	Site number	Sampling program	Tidal height (m)	Annual emersion time (%)	Water content (%)	AFDW (%)
Salt marsh under *Spartina* canopy	1A	*	3.1	81	60	10.5
Salt marsh mud	1B	**	3.1	81	67	8.3
Salt marsh mud	2	*	2.0	44	65	10.4
Sand under *Spartina* canopy	3A	*	3.0	77	21	2.3
Muddy sandy flat	3B	**	2.0	44	20	1.0
Mud flat	3C	**	1.4	23	65	11.5
Mud flat	4	*	0.7	3	51	8.1
Mud flat	5	*	1.4	23	50	6.8
Muddy sandy flat	6A	*	1.6	31	20	0.6
Muddy sandy flat	6B	*	1.6	31	23	1.6
Salt marsh under *Spartina* canopy	11A	*	3.4	91	58	11.0
Salt marsh mud	11B	*	3.4	91	70	14.5
Salt marsh under *Spartina* canopy	12A	*	2.8	69	58	7.8
Salt marsh mud	12B	*	2.8	69	57	7.2
Muddy sandy flat	13	*	1.4	23	29	3.8
Muddy sandy flat	14	*	1.7	34	22	1.2

ous comparative study of pigment extraction from wet and freeze-dried sediments, we concluded that results obtained from wet samples were highly variable and non-reproducible (Plante-Cuny et al. 1993). Deep-freezing and subsequent thawing before extraction appears to be much more harmful than using freeze-dried samples. The latter method furthermore allows long-term conservation. Some other problems, also discussed by Plante-Cuny et al. (1993), may occur during extraction or when using the HPLC technique, especially through chlorophyllase action (Jeffrey & Hallegraeff 1987). Water content was determined as the percentage of water in relation to total fresh weight. Organic matter was measured as percentage of weight loss by ignition (500°C, 2 h) from the 100°C dried sediment (ash free dry weight; Table 1). A granulometric analysis was carried out to determine the relative abundance (% dry weight) of clay (<2 µm), silt, (2 to 20 µm), fine sand (0.02 to 0.2 mm) and coarse sand (0.2 to 2 mm) according to the Wentworth scale (Fig. 1).

HPLC analysis. A comprehensive description of HPLC procedures used in the present study (preparation of standards, identification and quantification of chloropigments and carotenoid pigments), adapted from Kraay et al. (1992), is given in Brotas & Plante-Cuny (1996). In short, pigment extraction was carried out using 10 ml of 95% buffered methanol (2% ammonium acetate) for 15 min at 5°C, in the dark. The extract was filtered onto Whatman GF/F on a Sweenex system. The apparatus used for pigment analysis consisted of 2 pumps (Altex 110A) driven by a gradient programmer (Altex 420), an injection valve with a 100 µl loop (Rheodyne 7010), a fluorescence detector (Schoeffel FS 720) and a UV-Vis variable wavelength detector (Beckman 165) connected to 3 peak integrators (Shimadzu). Chromatographic separation was carried out with a Bio-Sil C_{18} HL 90-5S column for reverse phase chromatography (Biorad, 150 mm long, 4.6 mm in diam., 5 µm particles). For chloropigments, fluorescence excitation was carried out at 430 nm (emission beyond 580 nm); absorbances (for chlorophylls and carotenoids) were set at 436 and 450 nm. The solvents for the HPLC gradient used were as follows: solvent A was 0.2 M ammonium acetate in methanol and water (80:20, v/v); solvent B was methanol and ethyl acetate (70:30, v/v). The flow rate was 1 ml min^{-1} and the gradient was linear. The program utilized was 34 min long. It started with 0% solvent B (100% solvent A), reached 100% solvent B in 20 min, stayed with 100% of solvent B for 7 min and returned to original conditions in 7 min.

Twenty pigments were quantified. Concentrations of chl *a*, chlorophyllide *a*, pheophytins *a* (peaks no. 29 and 31; Table 2), pheophorbides *a* (peaks no. 3, 5, 6, 9, 13 and 15; Table 2), chl *b*, chls c_1+c_2, fucoxanthin and β-carotene are expressed in µg g^{-1} of dry sediment, whereas several carotenoids (as respective standards were not available to us) are presented in peak area unit (PAU) per dry weight, namely: alloxanthin, diadinoxanthin, diatoxanthin, lutein, neofucoxanthin, violaxanthin and zeaxanthin.

Microscopic observations. Benthic microalgae are known to migrate vertically in response to a light stimulus. The fraction of the motile population was measured by the lens tissue method (Eaton & Moss 1966). Pieces of 2 × 2 cm of lens tissue were spread on the top of sediment cores and left undisturbed for 24 h. The sediment surface was illuminated artificially for 2 h before collection of lens tissues which were immediately put in 100 ml solution of acidic Lugol. A 10 ml

Table 2. Identification of peaks from chromatograms of methanol extracts of sediment samples from the Tagus Estuary. Detection by fluorescence (excitation at 430 nm) and absorbance at 436 and 450 nm (n.i.: not identified)

Pigments	Peak no.
Solvent front	0
Chlorophyllide *a*	1
Chlorophylls c_1+c_2	2
Pheophorbide *a*	3
Fucoxanthin	4
Pheophorbide *a*	5
Pheophorbide *a*	6
Neoxanthin	7
Carotenoid n.i.	8
Pheophorbide *a*	9
Violaxanthin	10
Pheoporphyrin c_2	11
Diadinoxanthin	12
Pheophorbide *a*	13
Neofucoxanthin	14
Pheophorbides *a*	15
Alloxanthin	16
Diatoxanthin	17
Carotenoid n.i.	18
Lutein	19
Zeaxanthin	20
n.i. peaks	21
Chlorophyll *b* allomers	22
Chlorophyll *b*	23
Chlorophyll *a* allomers	24
Chlorophyll *a* main allomer	25
Chlorophyll *a*	26
Chlorophyll *a* epimer	27
Pheophytins *b*	28
Pheophytin *a*	29
β-Carotene	30
Pheophytin *a*	31
β-Carotene	32
Phytol chl *c*	33

aliquot of this solution was used to identify and count cells in sedimentation chambers in a Wild inverted microscope. A total of 100 to 400 cells were counted (Frontier 1972, Venrick 1978), which enabled us to determine the relative abundance of dominant taxa. Diatoms were identified after cleaning the cells with 30% hydrogen peroxide. A definitive preparation was made by mounting them in resin (Hyrax). Observations and photography were carried out using an Olympus (BX 50) microscope at 400× and 1000×.

RESULTS

Qualitative pigment composition

During the spatial and temporal surveys, a total of 40 peaks were detected, from which 29 pigments were identified, including 14 chlorophyll derivatives. As an example, 4 chromatograms obtained from the salt marsh site sediment (1B), during 3 seasons, are presented in Fig. 2. Fluorescence detection (Fig. 2A) allowed the separation of chloropigments, namely chl *a* and allomers, chlorophyllide *a*, pheophorbides *a* (9 peaks), pheophytins *a* (2 peaks), chl *b*, pheophytins *b* (2 peaks), chls c_1+c_2, pheoporphyrin c_2 (Table 2). Fluorescence detection is particularly useful for sediment sample analysis because some chlorophyll breakdown products co-elute or interfere with carotenoids. Since the latter are not fluorescent, absorbance detection makes it possible to highlight the carotenoids (Fig. 2B, C, D, Table 2). Then chloropigments may be distinguished from carotenoid ones, by comparing Fig. 2A & B. For example, peaks no. 11, 13, and the group 15 (4 peaks) in Fig. 2A correspond to pheophorbides which co-elute partly with carotenoids no. 10, 12 and 14 (violaxanthin, diadinoxanthin, neofucoxanthin).

The chromatograms B, C, and D (Fig. 2) concern 3 dates (25 September 1991, 6 November 1991 and 19 February 1992) and seasonal changes of qualitative composition in the carotenoid stock are obvious. Table 3 links the pigments identified in the Tagus samples to microalgal groups or macrophyte debris, generally observed in our intertidal surface sediments. The following pigments were taken as diagnostic markers ('fingerprints') for taxonomic microalgal groups and macrophytic detrital material: fucoxanthin for diatoms (bacillariophytes), chl *b* for euglenophytes, chlorophytes and/or phanerogam debris, lutein for chlorophytes and/or phanerogam debris, zeaxanthin for cyanobacteria and alloxanthin for cryptophytes. But, it should be pointed out that the presence of one isolated pigment is not enough to identify an algal group; rather, a certain set of pigments is needed (Table 3). For example, for diatoms, fucoxanthin appears associated with chls c_1+c_2 and in diverse proportions with diadinoxanthin, diatoxanthin and neofucoxanthin. Thus, we observed in September (Fig 2B) and February (Fig. 2D) the simultaneous importance of chls c_1+c_2 (peak 2), fucoxanthin (peak 4), neofucoxanthin (peak 14), characteristic pigments of diatoms, whereas in November, the detection of a prominent diadinoxanthin peak (no. 12) and zeaxanthin peak (no. 20) indicates the presence of euglenophytes and cyanobacteria, respectively. This was ascertained by microscopic observations. A combination of chl *b*, violaxanthin, neoxanthin and lutein is associated with the chlorophytes, but the particular abundance of lutein will indicate the accumulation of plant detritus in the vicinity of the halophyte community. During our study, microchlorophytes were rarely observed. Dinophytes were not counted, nor was their characteristic pigment, peridinin.

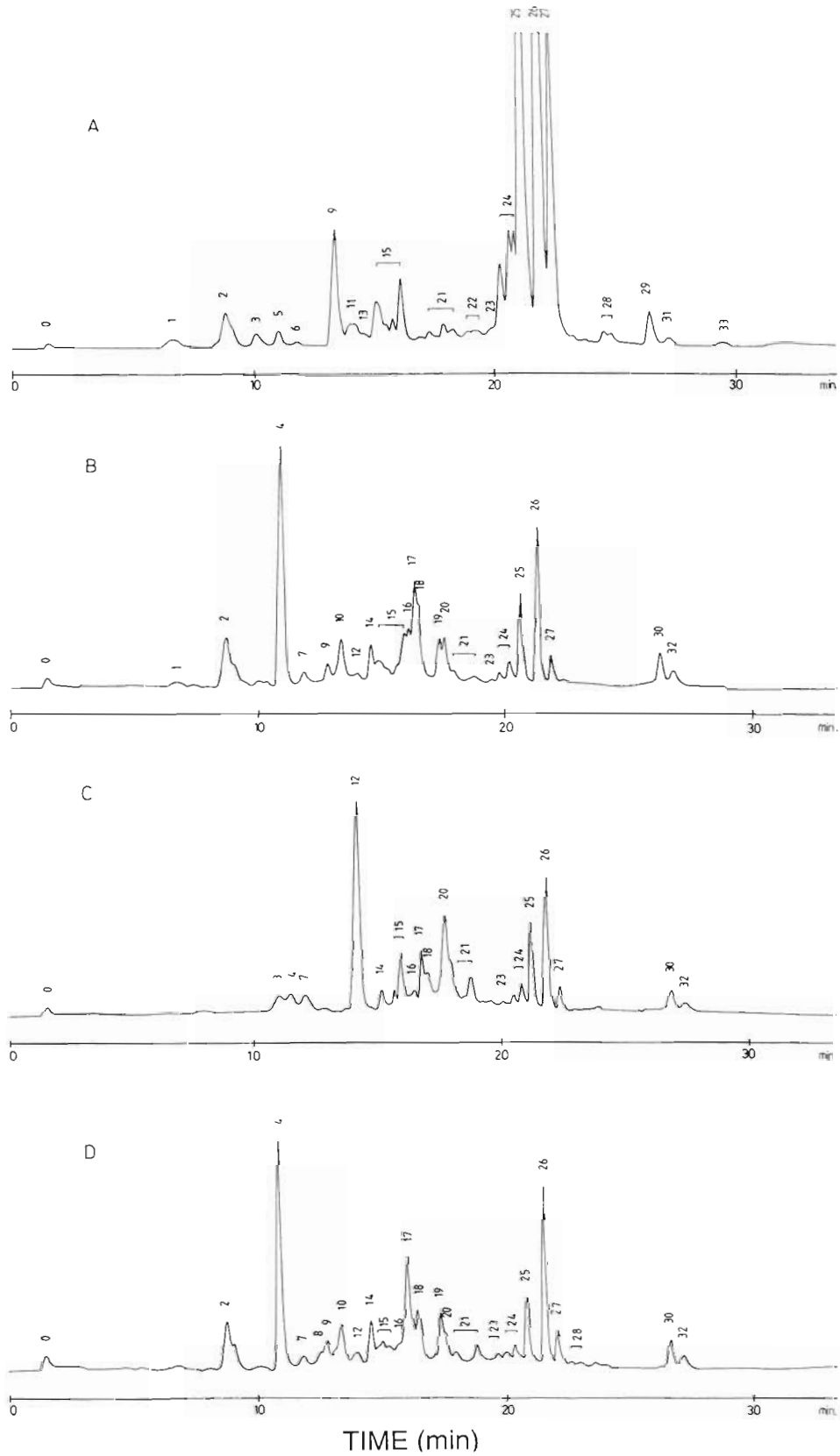

Fig. 2. Chromatograms of the (A) fluorescence and (B, C, D) absorbance at 450 nm obtained from site 1B (salt marsh mud) in the Tagus Estuary. (A, B) 25 September 1991 (sample dry weight: 0.7 g), (C) 6 November 1991 (0.6 g), (D) 19 February 1992 (0.4 g)

Table 3. Distribution of pigments identified among microphytobenthic algal groups or macrophytic debris in Tagus Estuary intertidal surface sediments. H: high concentration; L: low concentration (after Rowan 1989)

Pigment Algal type, material or process		Source
Chlorophylls and their breakdown products		
Chlorophyll *a*	Cyanobacteria, bacillariophytes, euglenophytes, chlorophytes, cryptophytes, macrophytic debris	1, 2, 3, 4
Chlorophyllide *a*	Chlorophyllase containing cells, senescent cells	4, 5
Pheophytins *a*	Microbial degradation process	6
Pheophorbides *a*	Plant detritic material, grazed material	7, 8
Pheophorbide *a* (peak no. 9)	Grazed material, fecal pellets	9, 10
Chlorophyll *b*	Euglenophytes, chlorophytes, phanerogam debris	1, 2, 3, 4
Pheophytins *b*	Detrital material from eugl., chloro., phanerogams	6
Chlorophylls $c_1 + c_2$	Bacillariophytes	1, 2, 3, 11
Chlorophyll c_2	Cryptophytes	1, 11
Pheoporphyrin c_2	Detrital bacillariophytes (grazing), phaeophyte debris	10
Carotenoids		
Alloxanthin	Cryptophytes (H)	1, 2, 12, 13, 14, 15
β-Carotene	Cyanobacteria (H), bacillariophytes (H), euglenophytes (L), chlorophytes (H), macrophyte debris	1, 2, 15, 16, 17, 18
Diadinoxanthin	Euglenophytes (H), bacillariophytes (H)	2, 12, 15, 18
Diatoxanthin	Euglenophytes (H), bacillariophytes, phaeophyte debris (occasional occurrence)	2, 12, 15, 18
Fucoxanthin	Bacillariophytes (H), phaeophyte debris	2, 3, 4, 12, 15, 16
Lutein	Chlorophytes (H), phanerogam debris	1, 2, 3, 4, 15,16,18
Neofucoxanthin	Bacillariophytes (occasional occurrences, i.e. *Cylindrotheca closterium*)	4, 18, 19
Neoxanthin	Cyanobacteria (L), euglenophytes (L), chlorophytes (L), bacillariophytes (traces amount), phanerogam debris	2, 15
Violaxanthin	Bacillariophytes (L), chlorophytes (L), phaeophyte debris	2, 4, 12, 16, 18
Zeaxanthin	Cyanobacteria (H), chlorophytes (L), phanerogam debris	1, 2, 15, 16, 17, 20

1, Cohen (1986)
2, Rowan (1989)
3, Wright et al. (1991)
4, Levinton & McCartney (1991)
5, Jeffrey (1974)
6, Bianchi et al. (1991)
7, Downs (1989)
8, Bianchi et al. (1988)
9, Vernet & Lorenzen (1987)
10, Plante-Cuny et al. (1993)
11, Jeffrey (1989)
12, Bjornland & Liaaen-Jensen (1989)
13, Pennington et al. (1985)
14, Gieskes & Kraay (1983)
15, Bianchi et al. (1993b)
16, Abele-Oeschger (1991)
17, Villanueva et al. (1994)
18, Klein & Riaux-Gobin (1991)
19, Stauber & Jeffrey (1988)
20, Gieskes et al. (1988)

Quantitative pigment analysis

Spatial variations

Data from chl *a* values and the ratio of 'total pheopigments *a*' (pheophytins *a* + pheophorbides *a* + chlorophyllide *a*) to total chl *a* + pheopigments *a* (pheos *a*/ chl *a* + pheos *a*) of the 16 sites (10 stations) are given in Fig 3A. The chl *a* values ranged from 4 to 38 µg g^{-1} of dry sediment, while total pheopigments *a* ranged from 3 to 81 µg g^{-1}. The percentage of pheopigments was generally greater in salt marshes and low tidal mud flats than in sandier sites. Fig. 3B, C & D also shows chl *b*, chls c_1+c_2 and fucoxanthin values obtained at the same sites. Highest values for chl *b* were observed at sites 1A, 1B (salt marsh mud flats), and 3C (mud flat). Chl *b* was not detected at sites 6A and 6B (sand). According to the literature (Table 3), chl *b* might originate both from phanerogam debris or some benthic microalgae. In our case, and according to our microscopic observations, chl *b* concentration at sites 1A and 3A was mainly due to *Spartina* debris, whereas at the 3 other sites, it seems to have originated mainly from euglenophytes, as microscopic chlorophytes were seldom seen. The general patterns of chls c_1+c_2 and fucoxanthin were similar, and differed from that of chl *b*. Chls c_1+c_2 exhibited high values at sandy sites (1A, 3A, 3B, 6A), indicating the presence of diatoms. High concentrations of fucoxanthin were observed at sites exhibiting the highest chl *a* values (e.g. 12A and 3C). Stn 3 (sites A, B and C) was simultaneously rich in chl *b*, chls c_1+c_2 and fucoxanthin, suggesting a highly diverse taxonomic composition.

A linear correlation study among 7 pigments (Table 4) shows that chl *a* concentrations were positively and significantly correlated with chls c_1+c_2, fucoxanthin,

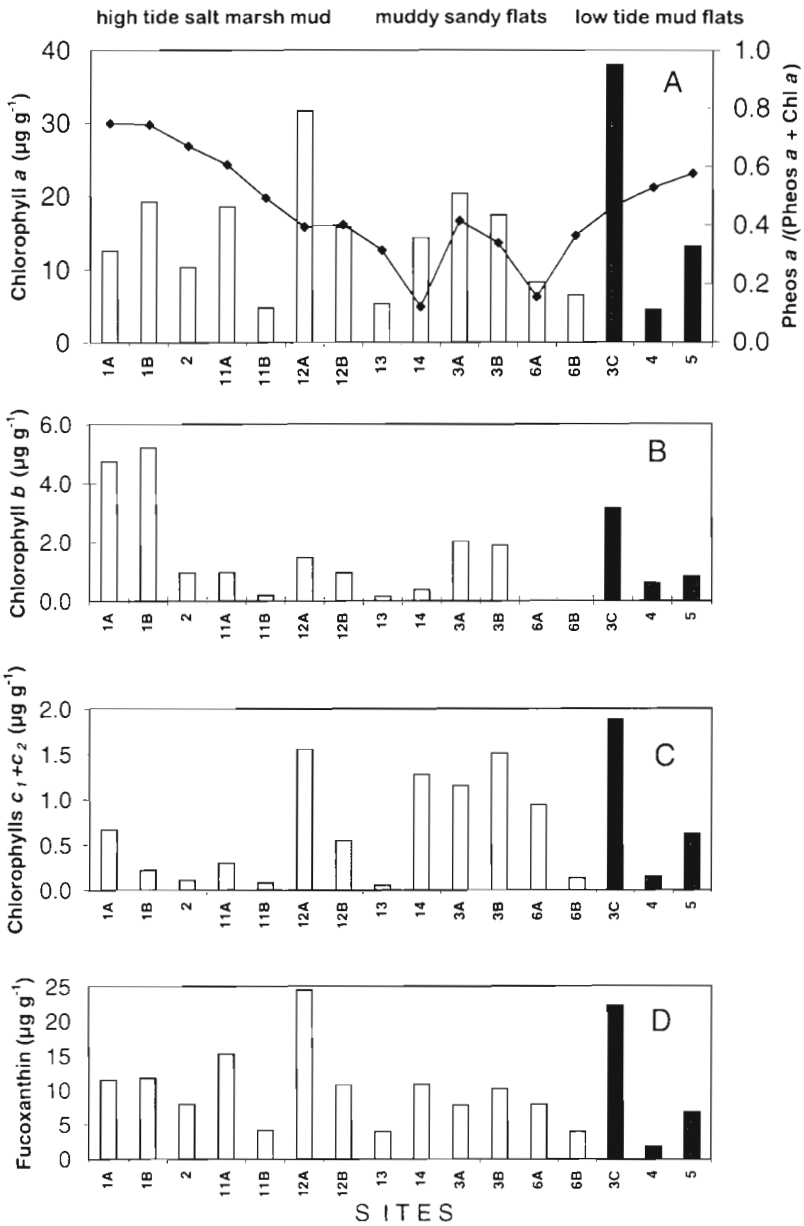

Fig. 3. Spatial variations of the concentrations of (A) chl *a* (bars) and 'total pheopigments *a*/chl *a* + total pheopigments *a*' ratio (solid line), (B) chl *b*, (C) chls c_1+c_2, and (D) fucoxanthin in the top 5 mm of sediment sampled at 16 sites in spring 1990

and neofucoxanthin. These results indicate that diatoms constitute the major contributing group to the total chl *a* pool. The very high positive correlation between chl *b* and diatoxanthin (r = 0.92) suggests that the main source of diatoxanthin is euglenophyte cells.

Temporal variations

Seasonal variations in pigment concentration were studied at sites 1B (salt marsh mud), 3B (mid shore sand flat) and 3C (low tide mud flat).

Chl a and breakdown products. Chl *a* values varied seasonally from 3 to 98 µg g^{-1}, considering data from all sites (Fig. 4). Chlorophyllide *a* and pheophytins *a* concentrations were almost negligible compared to chl *a* values. In contrast, pheophorbides *a* values were 2 to 4 times higher than chl *a* values. On average, pheophorbides *a* represented 90% of total pheopigments *a*. On the whole, chl *a* concentrations were 4-fold and pheophorbides *a* 5- to 7-fold higher at muddy sites (1B and 3C) than at the sandy site (3B).

At the salt marsh site 1B, annual mean values of chl *a* and pheophorbides *a* were 52 and 118 µg g^{-1}, respectively. In summer, autumn and late winter, chl *a* peaks were accompanied by pheophorbide increases, suggesting an accumulation of detrital material from autochthonous chl *a*. At the sandy site 3B, the distribution of chl *a* was uniform throughout the year (annual mean value of 14 µg chl *a* g^{-1}), while pheophorbides *a* increased slightly in autumn and early spring (annual mean = 24 µg g^{-1}). In contrast to the muddy sites (1B, 3C),

Table 4. Linear correlation between pigment concentration for the 16 sites studied during spring 1990. Significance: $^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.001$

	Chl *a*	Chl *b*	Chls c_1+c_2	β-Carotene	Diatoxanthin	Fucoxanthin
Chl *a*	–					
Chl *b*	0.49	–				
Chls c_1+c_2	0.77$^{\bullet\bullet}$	0.23	–			
β-Carotene	0.48	0.45	0.62$^{\bullet}$	–		
Diatoxanthin	0.39	0.92$^{\bullet\bullet}$	0.11	0.33	–	
Fucoxanthin	0.92$^{\bullet\bullet}$	0.43	0.69$^{\bullet}$	0.25	0.36	–
Neofucoxanthin	0.89$^{\bullet\bullet}$	0.21	0.74$^{\bullet\bullet}$	0.21	0.19	0.95$^{\bullet\bullet}$

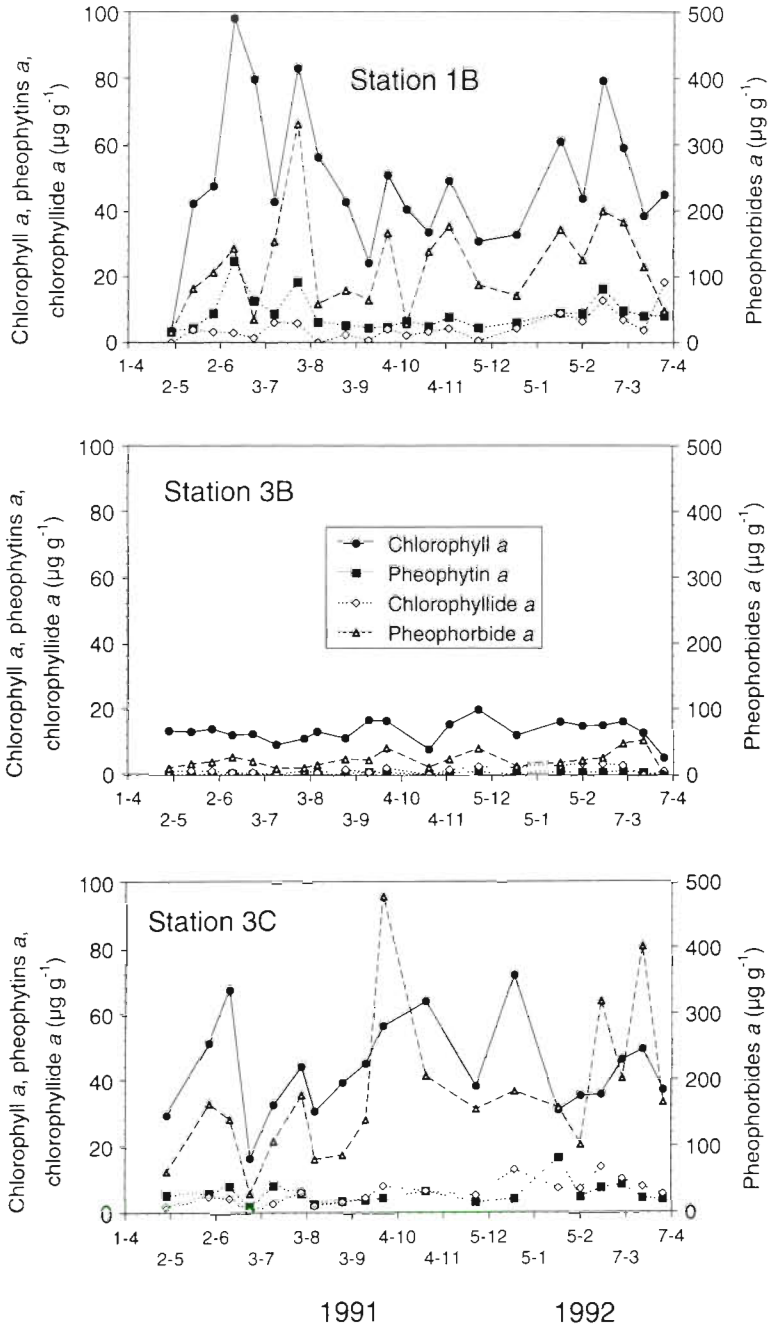

Fig. 4. Temporal variations of the concentrations of chlorophyll pigments (chl *a* + chlorophyllide *a* + pheophytins *a*, left *y*-axis; and pheophorbides *a*, right *y*-axis) during 12 mo (1991–1992) at sites 1B, 3B and 3C. *x*-axis units represent day-month

there was also an extremely low concentration of pheophytin *a* (annual mean = 0.6 µg g^{-1}), lower than chlorophyllide *a* concentration (annual mean = 1.8 µg g^{-1}). At site 3C, seasonal variations in chl *a* and pheopigments *a* seemed to vary independently of one another, particularly from September onward. Annual mean values of chl *a* and pheophorbides *a* were respectively 44 and 179 µg g^{-1}. Interestingly, pheophorbides reached high values (>400 µg g^{-1}) in 2 distinct periods, September and March. On the whole, seasonal chl *a* maxima were seen in June, July and late winter in site 1B, whilst at site 3C, during June and again in December. No peak occurred at site 3B.

Biomarker pigments. The seasonal variations of 5 'fingerprints' for sites 1B (salt marsh mud) and 3C (low tide mud flat) from April 1991 to April 1992 are presented in Fig. 5. As far as site 3B (muddy sandy tidal flat) is concerned, only fucoxanthin and chl *b* are shown, as the other diagnostic pigments were present in very low concentrations (Fig. 6).

At the salt marsh site (1B), high values of fucoxanthin (annual mean 32 µg g^{-1}) matched with the abundance of diatom cells (Fig. 7A, B, C, J & K shows some characteristic diatom species from this site). Relatively high levels of chl *b* (annual mean 9.6 µg g^{-1}) were due to the abundance of halophyte detritus and euglenophytes, particularly *Euglena acusformis* Schiller (Fig. 7E). As chlorophytes were rarely observed, the high lutein concentration indicates that a significant part of chl *b* could originate from plant detritus. June, July, October and February lutein peaks coincided with those of pheophorbides *a* (Fig. 4, site 1B), attesting to the degradation process of autochthonous vegetation. Zeaxanthin concentrations were sometimes nondetectable (Fig. 5) but sometimes high (for example Fig. 2C, 6 November 1991, peak no. 20), corresponding then to populations of cyanobacteria filaments (Fig. 7H). Distinct peaks of alloxanthin appeared in 1991-92 (Fig. 5 and also Fig. 2B, 25 September 1991, peak no. 16) but cryptophyte cells, the usual source of alloxanthin, were not observed in sediment samples. In short, in summer and spring, chl *a* peaks (Fig. 5, dotted line) correspond to the presence of diatoms and plant debris as evidenced by fucoxanthin and lutein peaks. Diatom populations decreased sharply in November, when they were temporarily replaced by cyanobacteria (zeaxanthin peak). These in turn virtually disappeared from 5 February onward.

At the low tide mud flat site (3C), the fucoxanthin and chl *a* seasonal patterns were similar (Fig. 5). The diatom populations was noticeably less abundant in

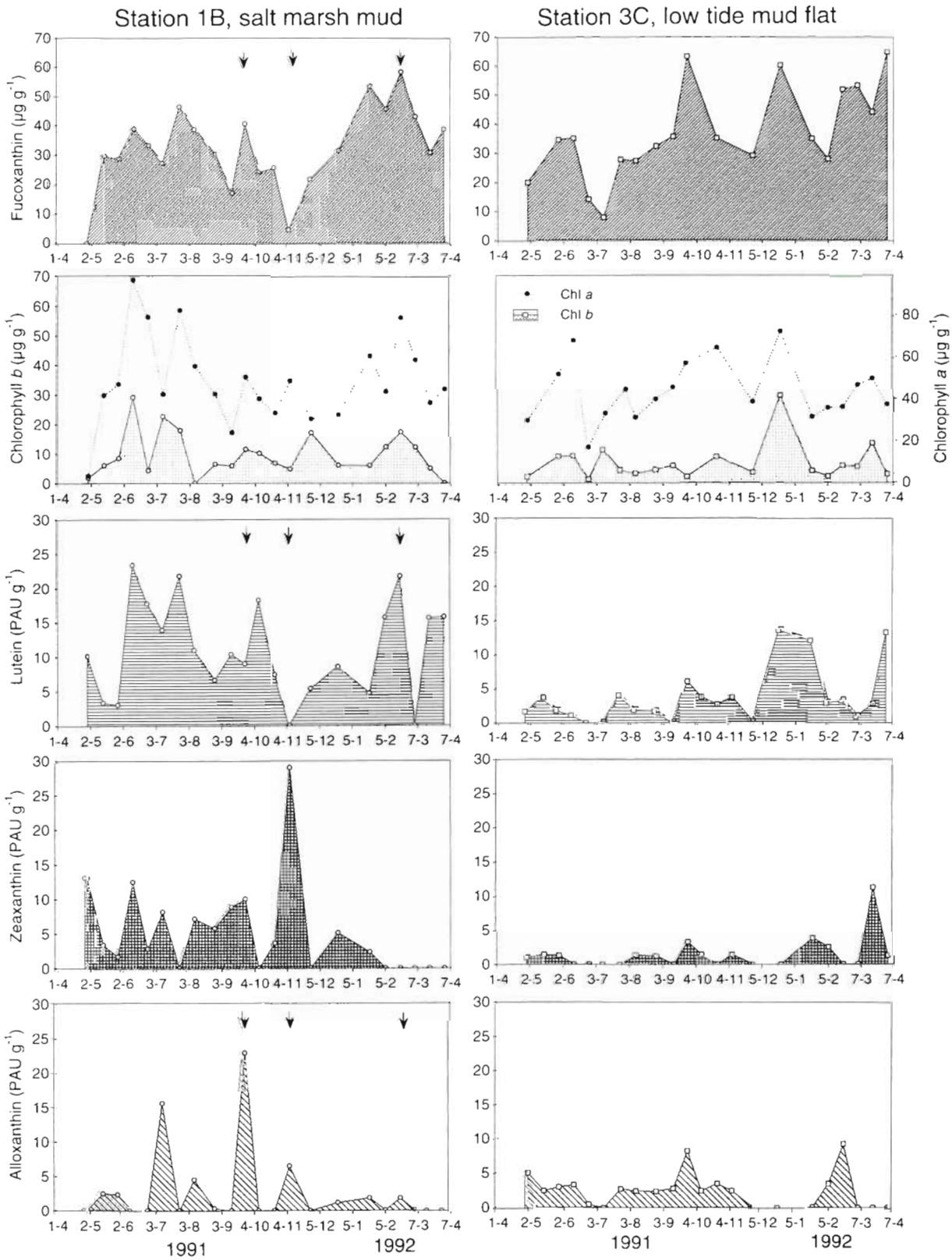

Fig. 5. Pigment marker concentrations in the sediment surface (0 to 5 mm) of sites 1B (high tide salt marsh mud) and 3C (low tide mud flat) between April 1991 and April 1992. Dotted line: chl *a* concentration. Arrows indicate sampling dates corresponding to Fig. 2 chromatograms. PAU: peak area units

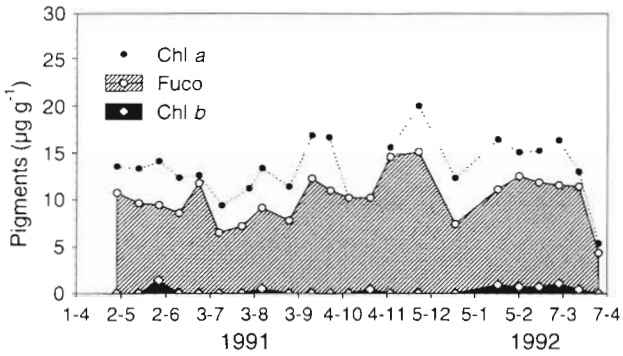

Fig. 6. Seasonal variations in the concentration of chl *a*, chl *b* and fucoxanthin in the sediments at site 3B (mid-shore sandy flat). The concentrations of lutein, zeaxanthin and alloxanthin were very low

summer than during the rest of the year. Typical mud flat diatom species are shown in Fig. 7A, C, D & G. Chl *b* concentration (annual mean of 5 µg g^{-1}), although lower than at site 1B, was still relatively high. However, lutein was much less abundant, which suggests that euglenophytes were here the important source of chl *b*, particularly in December, when they contributed to the chl *a* peak. Zeaxanthin concentration, which matched the observation of a small cyanobacteria species, was also less abundant than at site 1B, although it presented a peak in March. Alloxanthin was observed throughout the year, but did not display such abrupt peaks as at site 1B.

At the sandy site 3B (Fig. 6), fucoxanthin concentration (annual mean of 10 µg g^{-1}), as well as that of chl *a* (Fig. 4), remained fairly constant throughout the year, except for a slight increase in winter. Diatoms dominated the microphytic population (95 to 99% of all cells), and *Cylindrotheca closterium* (Ehrenb.) Reimer & Lewin (Fig. 7I) was the dominant species. Chl *b* was merely present and may be related to the presence of some euglenophytes and filamentous chlorophytes.

Considering data from the 3 sites, diatom microscopic counts (motile cells only) were positively correlated with fucoxanthin concentration ($r = 0.53$, $n = 48$, $p < 0.001$). For euglenophytes and cyanobacteria, the number of individuals counted was relatively low; there was thus no significant relationship with the diagnostic pigment results, although species observations corresponded to pigment concentrations.

Diatoms dominated at the 3 sites examined. *Cylindrotheca closterium* (Fig. 7I) and *Navicula cryptocephala* Kützing (Fig. 7J) were the dominant species. Euglenophytes were observed at all sites, but more rarely at the mid-shore sandy flat site (3B), and cyanobacteria were much more conspicuous in the salt marsh mud site 1B. Microchlorophyte cells were also observed, but more rarely.

Table 5. Linear correlation between chlorophyll *a* and fucoxanthin concentrations and the other pigments recorded during the seasonal survey at sites 1B and 3C. Significance: $^{*}p < 0.05$, $^{**}p < 0.001$

	Site 1B, salt marsh mud		Site 3C, low tide mud flat	
	Chl *a*	Fucoxanthin	Chl *a*	Fucoxanthin
Chlorophyllide *a*	0.18	0.56*	0.17	0.21
Pheophytin *a*	0.92**	0.51*	0.05	0.03
Chl *b*	0.60*	0.30	0.65*	0.28
Chls c_1+c_2	0.43*	0.60*	0.18	0.35
Alloxanthin	−0.04	0.02	0.10	0.19
β-Carotene	0.90**	0.36	−0.03	0.54*
Diadinoxanthin	0.12	−0.38	0.09	0.39
Diatoxanthin	0.19	0.40	0.16	0.59*
Fucoxanthin	0.56*	–	0.52*	–
Lutein	0.48*	0.31	0.22	0.62*
Neofucoxanthin	0.79**	0.79**	0.25	0.71**
Violaxanthin	0.62*	0.19	0.25	0.50*
Zeaxanthin	0.00	−0.52*	0.02	0.18

Examination of the linear correlation matrix among 14 pigment concentrations (Table 5) gives rise to several remarks. At site 1B, positive and significant correlations between chl *a* and chls c_1+c_2, fucoxanthin, and neofucoxanthin, as well as between fucoxanthin and chls c_1+c_2 and especially neofucoxanthin, were observed. Chl *a* is also well correlated with chl *b*, β-carotene, lutein and violaxanthin, showing the importance of plant debris at this salt marsh muddy site. The negative correlation between fucoxanthin and zeaxanthin ($r = -0.52$) confirms that diatom and cyanobacteria populations seem to succeed one another in time. At site 3C (mud flat), chl *a* was correlated with fucoxanthin and chl *b* only. This supports the hypothesis that diatoms and euglenophytes contribute most, and together, to the chl *a* pool. Fucoxanthin was related to neofucoxanthin, diatoxanthin (which is common both in diatoms and euglenophytes) and to violaxanthin, lutein and β-carotene, which could indicate the presence of macrophyte debris (Table 3). In the vicinity of sites 3B and 3C there are populations of *Fucus vesiculosus* (phaeophyte), which might contribute to the carotenoid pool.

DISCUSSION

Pigment signatures, biomarkers

In the benthic field, studies using pigments to determine the origin of organic matter are more frequent than those analysing microphytobenthos communities from a taxonomic standpoint. Papers dealing with live microphytic populations have been rare, due to the

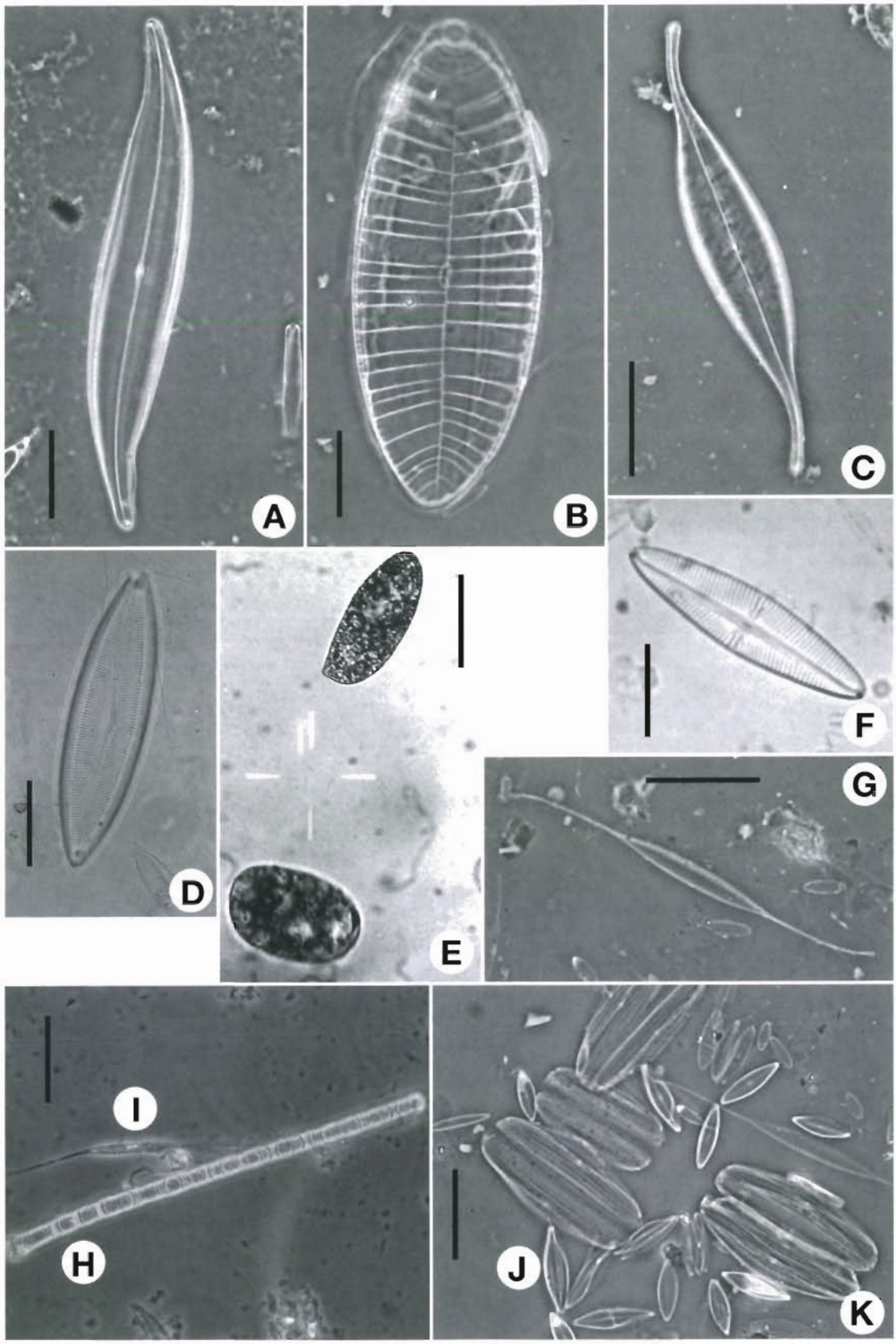

Fig. 7. Microphytic species observed on the different type of sediments. Diatoms (bacillariophytes): (A) *Gyrosigma acuminatum* Grün., (B) *Surirella gemma* (Ehrenb.) Kützing, (C) *Gyrosigma fasciola* (Ehrenb.) Cleve, (D) *Caloneis westii* (Wm. Smith) Hendey, (F) *Navicula digito-radiata* (Gregory) Ralfs, (G) *Nitzschia longissima* (Brébisson) Ralfs, (I) *Cylindrotheca closterium* (Ehrenb.) Reimer, (J) *Navicula cryptocephala* Kützing, (K) *Amphora hyalina* Kützing. Euglenophyte: (E) *Euglena acusformis* Schiller (H) Cyanobacteria. Scale bars = 20 µm

complexity prevailing in the sediment surface, where pigments from several taxonomic groups of microalgae are in a pool with degraded chlorophylls and carotenoids originating from senescent cells, macroalgae and phanerogams.

In the present work, both pigment results and microscopic observations testified that diatoms are the major taxonomic group present at all sites and almost throughout the year. Most authors, in various sediment types, found the same results, i.e. high concentration of fucoxanthin and chls c_1+c_2, corresponding to the dominance of diatom populations (Riaux-Gobin et al. 1987, Abele-Oeschger & Schramm 1989, Levinton & McCartney 1991, Bianchi et al. 1993b, Barranguet & Alliot 1995, Cariou-LeGall & Blanchard 1995, Barranguet et al. 1996). Moreover, Klein & Riaux-Gobin (1991), in coastal sediments from sub-antarctic islands, linked the microscopic observation of diatoms with the detection of 'chl *c*', fucoxanthin, neofucoxanthin, diadinoxanthin, diatoxanthin and β-carotene, as we have. We have reported the importance of neofucoxanthin in our samples. This xanthophyll pigment is particularly found in cultures of the diatom *Cylindrotheca closterium* (Stauber & Jeffrey 1988). Accordingly, *C. closterium* is a very abundant species in all our samples, often the dominant one.

In the spatial survey, the ratio chl *a*:fucoxanthin varied between 1 and 2.6 (g/g); the highest value was obtained in a sandy site under a *Spartina* canopy (site 3A), where an important chl *a* source is the phanerogam debris. The mean values from the seasonal study were 1.38 at site 1B, 1.36 at site 3B and 1.26 at site 3C. In a temperate intertidal muddy sediment (west coast of France), Cariou-LeGall & Blanchard (1995) found a mean value of 2.6. Barranguet et al. (1996) report ratios between 0.72 and 2 in Mediterranean sediments (0.5 to 13 m depth). Burford et al. (1994) obtained a mean value of 0.35 in an Australian tropical bay (0 to 60 m depth), attributing this low value to the abundance of detritic fucoxanthin. The ratios we obtained in intertidal estuarine sediments can be qualified as low, taking into account that only a part of chl *a* originates from diatoms. We therefore put forward 2 hypotheses to attempt to explain this fact. The first is the decrease in chl *a* concentration which occurs together with the increase of carotenoids as photoprotectors under high light intensities. During low tide periods in the Tagus Estuary, the surface sediment is submitted to high light intensities, particularly in spring and summer (Brotas & Catarino, 1995). The second hypothesis is that part of the fucoxanthin could be the result of digestion of infauna. In fact, Abele-Oeschger & Theede (1991), analyzing the digestion of the gastropod *Littorina littorea*, confirmed that chl *a* was degraded into pheophytins *a* and pheophorbides *a*, whereas fucoxanthin was digested in negligible quantities.

The range of the ratio chl *a*:chl *b* (g/g) in the 16 sites varied from 2.6 to 39; the minimum values came from the salt marsh site, and the maxima from sandy flats where no euglenophytes or plant debris were present. In fact, the pigment ratio values confirm that sandy stations have a community constituted exclusively of diatoms (see Figs. 1 & 3), whereas in muddy sites, the taxonomic diversity is higher. A wide range is also reported by Barranguet et al. (1996), who found values between 1.2 to 36.8 in different sites between 0.5 and 13 m.

During the seasonal study, chl *b* was measured in appreciable quantities (annual mean value of 9.6 µg g^{-1} at site 1B and 5 µg g^{-1} at site 3C). Klein & Riaux-Gobin (1991) measured high concentrations of chl *b* (6.38 µg g^{-1}) and diadinoxanthin (3.54 µg g^{-1}), related to euglenoid and/or green algae populations, whereas Cariou-LeGall & Blanchard (1995) were unable to detect chl *b*. In most papers dealing with sediment, high concentrations of chl *b* and lutein are often linked to the presence of plant debris (Levinton & McCartney 1991, Barranguet & Alliot 1995) as well as chl *b*, lutein and β-carotene (Abele-Oeschger 1991, Bianchi et al. 1993b). The important peaks of alloxanthin observed in July and September 1991 (site 1B) could originate from ephemeral cryptophyte planktonic blooms. In fact, Cabeçadas (in press) observed blooms of cryptophytes in the water column at this site (up to 80% of total cells) which appeared and disappeared in 1 wk.

The difficulty in characterizing a microphytic community is increased by the possible presence of non-degraded carotenoids from macrophytes, and the reactions designated as 'xanthophylls cycles' (Stransky & Hager 1970), where low light intensities promote epoxidation and high intensities stimulate de-epoxidation (Rowan 1989, Demmig-Adams 1990, Young 1991). Thus, as a response to stress by high light intensities, the diadinoxanthin-diatoxanthin equilibrium is shifted towards diatoxanthin (Demers et al. 1991, Brunet et al. 1993, Johnsen et al. 1994), and zeaxanthin is produced from violaxanthin transformation (Demmig-Adams 1990). Hence, high diatoxanthin concentrations may be caused by an excess of light in the Tagus Estuary during low tide. The predominance of diadinoxanthin in the euglenophytes in sediments near sub-antarctic islands where the light exposure may be low (Klein & Riaux-Gobin 1991) is also in agreement with this hypothesis.

We should point out that previous results of chl *a* measurements in parallel samples with 6 replicates (Brotas et al. 1995) indicated a mean coefficient of variation of 22% for sandy sites and 33% in muddy ones. Therefore analysis of seasonal patterns of Fig. 5 must

be limited by the assumption of the same order of variability for the various pigments. Brotas et al. (1995) also concluded that there was no definite seasonal pattern. Biomass at the upper littoral muddy site (1B) was more influenced by cyclic climatic parameters (e.g. light intensities higher in spring or summer) whereas at the lower littoral site (3C) a decrease in resuspension (derived from the action of tides) caused an increase in biomass in winter. Present data apparently corroborate this idea. Concerning site 1B, chl *a*, fucoxanthin, chl *b* and lutein showed higher values in June, July and early spring. At site 3C, chl *a* has peaks in June and December, fucoxanthin values are clearly higher in winter than in summer, and chl *b* and lutein present distinct peaks in December. Regarding sandy site 3B, there is an uniform distribution of pigment content, with a slight increase in winter as well.

Degraded chloropigments

The interpretation of degraded chloropigments is particularly complex in sediment samples. It is noticeable that we found very low concentrations of degraded chl *b* and chls c_1+c_2, but found a large number of various pheophorbides *a* (9 peaks of unequal importance; Fig. 2A). Pheophorbides *a* are considered by several authors as an index of planktonic or benthic grazing activities (Klein & Sournia 1987, Vernet & Lorenzen 1987, Bianchi et al. 1988, Abele-Oeschger & Theede 1991, Bianchi et al. 1991, Plante-Cuny et al. 1993, Buffan-Dubau et al. 1996) whereas pheophytins *a* are generally linked to microbial activity (Bianchi et al. 1991). Our results consistently showed that the pheophorbide *a* no. 9 (Fig. 2, Table 2), which is less polar than pheophorbides *a* no. 3, 5 or 6 and than fucoxanthin, always presented the highest concentrations. It was particularly abundant in September-October and in March-April at the low tide mud flat site. This fact could be related to the abundance in that zone of the fish *Liza ramada* (Almeida et al. 1993), which is known to feed on the surface microfilm sediment, its faeces accumulating in the surface sediment (Odum 1970). Buffan-Dubau et al. (1996) found a similar pheophorbide *a* in the digestive tract of benthic harpacticoid copepods. In the Mediterranean Sea (France), Plante-Cuny et al. (1993) found the most important pheophorbide *a* peak (corresponding to no. 9 from the Tagus Estuary) in mussel faeces and under mussel mud extracts, attributing its origin to phytoplankton digestion. In oyster farms, Pastoureaud et al. (1996) found 6 peaks of pheophorbides *a* in the natural seawater and 8 peaks in the faeces of the oyster *Crassostrea gigas*. The 2 most important peaks correspond to our peak no. 9 (less polar than the former group). Therefore, chl *a* appears to be subjected to weaker degradation processes in molluscs and sediment grazing fish than in copepods.

In conclusion, the HPLC analysis of chloropigments and carotenoids associated with the study of convenient pigment ratios and correlations appears a powerful tool to discriminate microphytic communities, both in spatial heterogeneity distribution and temporal succession, although controls by microscopic observations are still needed, as demonstrated for phytoplankton communities by Roy et al. (1996). Further studies of the various pheophorbides from chl *a* are apparently a promising way to distinguish the origin of organic matter degraded by the various functional groups of grazers.

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