

Monthly HPLC measurements of pigment concentration from an intertidal muddy sediment of Marennes-Oléron Bay, France

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ABSTRACT: Chloropigments and carotenoids were measured by HPLC in an intertidal muddy sediment of Marennes-Oléron Bay (France). Concentrations were determined as a function of sediment depth at low tide. The analyses were carried out at monthly intervals over 1 yr. Pigment analysis indicated that the microphytobenthic community was dominated by diatoms throughout the sampling year. Chlorophyll *b* was not encountered at any time or depth, indicating that no input from macrophytic detritus had occurred at the sampling site. There was a large pool of phaeopigments, of which phaeophorbides were the major forms (75%). A microphytobenthic bloom occurred between March and June during which phaeopigments significantly increased with a high proportion of phaeophorbides likely due to intense grazing activity of benthic invertebrates. Pigment concentrations were still high at 5 cm depth and changes occurred simultaneously in the aphotic (<5 mm sediment depth) and photic (>5 mm sediment depth) layers of the sediment, reflecting an impact of bioturbation and physical mixing (resuspension/redeposition) of the upper sediment layer. There was a decrease of the pigment content of the sediment between June and July. It was attributed to grazing and to a resuspension event. Overall, the pigment analysis of this intertidal sediment indicates a predominance of the diatom community, a close coupling between the dynamics of microphytobenthos biomass and the grazing activity of benthic invertebrates, and the influence of sediment mixing.

KEY WORDS: Pigment · HPLC · Intertidal mudflat · Microphytobenthos · Seasonal change

INTRODUCTION

Microphytobenthos is a major component of primary productivity on intertidal mudflats (Pinckney & Zingmark 1991, 1993, Blanchard 1994, Blanchard & Cariou-Le Gall 1994) and represents an important food source for benthic invertebrates (Montagna 1984, Montagna et al. 1995).

As mudflats are also frequently accumulation zones for allochthonous plant material, they can exhibit a high pigment diversity in the sediment so that chromatographic methods are necessary for pigment identification and quantification. High-performance liquid chromatography (HPLC) analysis is now commonly used in ecological studies concerning the sediment, which have largely focused on the problem of method intercalibration (usually between HPLC and the non-separative spectrophotometric and fluorometric meth-

ods) for chloropigment measurements (Brown et al. 1981, Daemen 1986, Barlow et al. 1990, Neveux et al. 1990, Plante-Cuny et al. 1993). Apart from these methodological works, investigations on sediment pigment measurements by HPLC have also been conducted to estimate algal pigment diversity in coastal sediments (Riaux-Gobin et al. 1987, Klein & Riaux-Gobin 1991), to trace the origin and the fate of plant material (Bianchi & Findlay 1990, Abele-Oeschger 1991, Levinton & McCartney 1991) or, more specifically, to follow the degradation of chlorophyll *a* through macrobenthic grazing (Bianchi et al. 1988, Abele-Oeschger & Theede 1991). Thus, by differentiating between autochthonous and allochthonous material as well as between living and degraded material (microphytobenthos vs phytodetritus) in sediments, pigment analysis increases insight into the dynamics of organic matter in coastal areas.

So far only few studies have dealt with seasonal changes of chloropigments and/or carotenoids (Abele-Oeschger 1991, Levinton & McCartney 1991, Bianchi et al. 1993), and none of them concerned intertidal mudflats. The present work aims at assessing temporal (over 1 yr) and spatial (as a function of depth) changes of chlorophylls and their degradation products as well as carotenoids from an intertidal muddy sediment of Marennes-Oléron Bay (France) to achieve a better understanding of microphytobenthos ecology of intertidal mudflats. The pigment content of the sediment was measured monthly at low tide.

MATERIALS AND METHODS

Study site. Marennes-Oléron Bay is located along the French Atlantic coast ($45^{\circ} 55' \text{ N}$, $1^{\circ} 10' \text{ W}$) and is separated from the Atlantic Ocean by Oléron Island (Fig. 1). Its area is approximately 170 km^2 , of which a large part is occupied by intertidal mudflats. The bay is bounded by the mouth of the Charente estuary and Aix Island to the north, and is very narrow in its southern part. Hydrobiological characteristics of the bay can be found in Héral et al. (1983). The study site (see Fig. 1) was in the upper part of the large eastern mudflat, about 100 m from the sand dune bordering it. Depend-

ing on the wind conditions, the site can remain exposed to the air for a few days during neap tide periods. Most of the mudflat lacks macrophytic vegetation so that benthic primary production is solely due to microphytobenthos. More than 95% of the particles in the top 1 cm of the sediment at the sampling site are smaller than $50 \mu\text{m}$.

Sampling. Sediment was sampled monthly from March 1992 to February 1993 at low tide during spring tide periods (tidal coefficients between 75 and 112). On each sampling date, low tide occurred at midday and the site was exposed to the air for about 6 h. Sediment cores (5.3 cm^2 made with cut off syringes) were collected in triplicates at the beginning of the ebb tide, at slack water and at the end of the flood tide before the site was covered; a total of 9 sediment cores were thus sampled on each date, except in October 1992 when only 4 cores were sampled at slack water. Samples were immediately frozen in liquid nitrogen and wrapped in aluminum foil. In the laboratory, sediment cores were divided into 6 slices: 0–5, 5–10, 10–20, 20–30, 30–40 and 40–50 mm layers. Sediment samples were then freeze-dried and kept in the dark at -30°C until further analysis.

Analysis of the sediment pigment content. Dried sediment was ground and pigments were extracted using 5 ml of 90% acetone for 200 mg dry weight sediment. Extraction was performed at 4°C in the dark for 24 h on a shaking table. The sediment was then separated from the acetone extract by centrifugation, and the supernatant was filtered onto a Whatman GF/C filter.

Chloropigments and carotenoids were identified and quantified by means of reverse phase HPLC. Mantoura & Llewellyn's method (1983) was used with slight modifications. The mobile phase consisted of eluant A made up of solution P/distilled water/methanol (10/10/80, v/v), and eluant B made up of acetone/methanol (40/60, v/v). Solution P (ion-pairing reagent) was prepared from 1.5 g of tetrabutyl-ammonium acetate (Fluka, 95% purity) and 7.7 g of ammonium acetate made up to 100 ml with distilled water. For the analysis, $150 \mu\text{l}$ of solution P was added to $500 \mu\text{l}$ of acetone extract and samples allowed a stabilization period of 5 min prior to injection. Samples collected in March and July 1992 were analysed with a SFCC column ($150 \times 4.6 \text{ mm i.d.}$); other samples were analysed using an Alltech column ($100 \times 4.6 \text{ mm i.d.}$). Complete elution of all pigments with the SFCC column was achieved within about 17 min using a linear gradient from 20 to 100% B for 7 min followed by an isocratic elution with 100% B for 6 min then a return to the initial conditions in 4 min. Optimal separation with the Alltech column was achieved within about 16 min using a linear gradient from 20 to 100% B for 8 min fol-

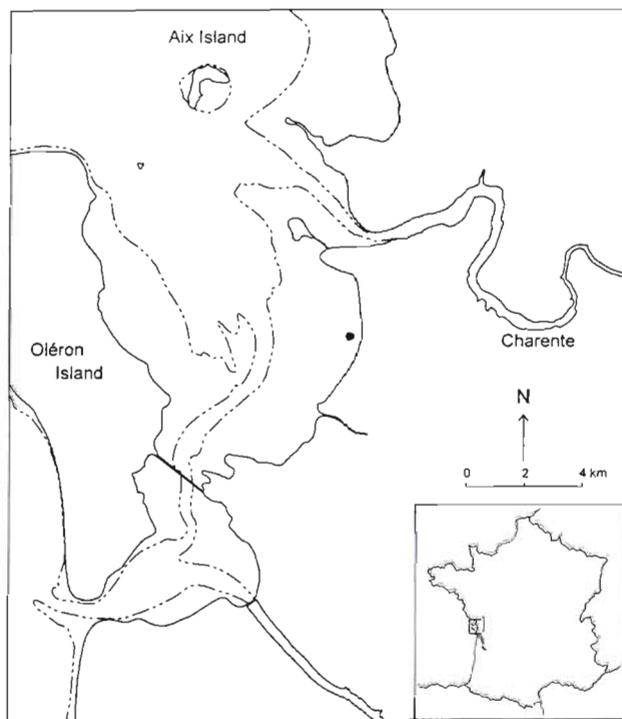


Fig. 1 Marennes-Oléron Bay, France. (●) Sampling site ($45^{\circ} 55' \text{ N}$, $1^{\circ} 10' \text{ W}$). Dashed lines indicate the lower limits of the intertidal zone

lowed by an isocratic hold of 4 min at 100% B and a return to 20% B in 4 min. Both solvents were filtered and degassed prior to use. HPLC equipment consisted of a double system pump (Kontron LC 414), an Altex injector equipped with a 100 μ l calibrated loop, a reverse-phase chromatographic column (hypersil, ODS, 3 μ m) with a C₁₈ direct-connect guard column. Chloropigment and carotenoid detection was performed with a fluorometer (Kontron, SFM 25; excitation wavelength of 430 nm and emission wavelength of 663 nm) and a spectrophotometer (Kontron Uvikon 722LC) set on 440 nm, respectively. A selection of samples was analysed with a spectrophotometer set on 660 nm and connected to a photodiode array detector.

Pigments were identified by comparing their retention times with those of standard solutions (Sigma standards or unialgal culture extracts of known pigment composition) and by on-line spectra collection using the photodiode array detector. Chlorophyll (chl) *a* and *b* were obtained from Sigma and were converted to phaeophytin by acidification. Standards of chl *c*, fucoxanthin, diadinoxanthin and β -carotene were obtained from a culture of *Phaeodactylum tricorutum* whereas standards of violaxanthin and lutein were obtained from a culture of *Tetraselmis suecica*. Chlorophyllide *a* was prepared from cultures of the diatom *P. tricorutum* according to Barrett & Jeffrey (1971) and was converted to phaeophorbide *a* by acidification. All culture extracts were purified on a Merck chromatographic column (Lichoprep RP8, 40 to 63 μ m, 240 \times 10 mm i.d.). For each standard solution, a factor was calculated from the linear relationship between fluorescence or absorbance peak surfaces and pigment concentrations. Identified pigments in acetone extracts were quantified using the factors of the corresponding standard solutions. Units of pigment measurements are mg m⁻² mm⁻¹.

Those breakdown products of chl *a* which elute before chl *a* (because they are more polar) are called phaeophorbides, while those eluting after (because less polar) are called phaeophytins. Phaeophorbide *a* and phaeophytin *a* represent the standard phaeophorbide and phaeophytin, respectively. The pigments defined with the '-like' suffix are spectrally similar to the standards but they do not exhibit the same retention time when separated by a reverse-phase chromatographic column. Those compounds were numbered in the order of elution and were quantified using the response factor obtained with the standard solutions. For convenience, chl *a* concentration was calculated from the absorbance peak, detected at 440 nm (and including chl *a'*); we did not find any discrepancy between absorbance and fluorescence calculations.

Statistical analysis. All statistical tests were performed with Systat 5.0 software (Wilkinson et al. 1992).

The logarithmic transformation for pigment concentrations and the arcsine-square root transformation for proportions (pigment ratios) (Sokal & Rohlf 1981) were used to normalize the data.

RESULTS

Pigment identification

Chromatograms showed the same peaks throughout the year (see Fig. 2 for an example and Table 1 for pigment identification). Chl *a* was the major chlorophyllous pigment (peak 9) whereas we never detected any chl *b* (the detection limit was ca 40 μ g m⁻² mm⁻¹). The HPLC method used did not allow separation of chl *c*₁ and *c*₂ (peak 2). There were many breakdown products of chl *a*: 4 forms of phaeophorbide and 3 forms of phaeophytin were found. Over the year, phaeophorbide *a* (peak 5) represented 14.6% of total phaeopigments (only 20% of the pool of identified phaeophorbides) and phaeophytin *a* (peak 11) represent 16% (59.4% of the pool of identified phaeophytins). Phaeophorbide *a*-like₁ (peak 3) was more polar than phaeophorbide *a* whereas phaeophorbides *a*-like₂ and *a*-like₃ (peaks 6 & 7) were less polar, the 2 latter compounds being the major forms throughout the year and representing about 23.6 and 26.1% of total phaeopigments respectively (68% of total phaeophorbides). Phaeophytins *a*-like₁ and *a*-like₂ (peaks 12 & 13) were the least polar pigments that we found, the 2 breakdown products representing 2.4 and 8.6% of total phaeopigments respectively. Chlorophyllide *a* (peak 1) constituted a very small fraction of the breakdown products of chl *a* (about 0.5%). Peak 4 was identified as a breakdown product of chl *c*.

Concerning carotenoids, fucoxanthin (peak 14), diadinoxanthin (peak 15) and β -carotene (peak 16) were identified whereas many peaks remain unidentified (* in Fig 2). If we take into account the retention times and the absorption spectra, those peaks cannot correspond to chl *a* degradation products, nor to specific carotenoids such as peridinin, 19'-butanoyl- and 19'-hexanoyl-fucoxanthin. In fact, the absorbance spectra do not match those of most typical microalgal pigments, and the surface of most unidentified peaks increases with depth, suggesting that these compounds could be breakdown products of carotenoids. The last unidentified absorbance peak (# in Fig. 2) exhibits almost the same retention time as lutein and as zeaxanthin, which coelutes with lutein in the case of Mantoura & Llewellyn's method (1983). Its absorption spectrum does not correspond to the spectrum of lutein but might be that of zeaxanthin.

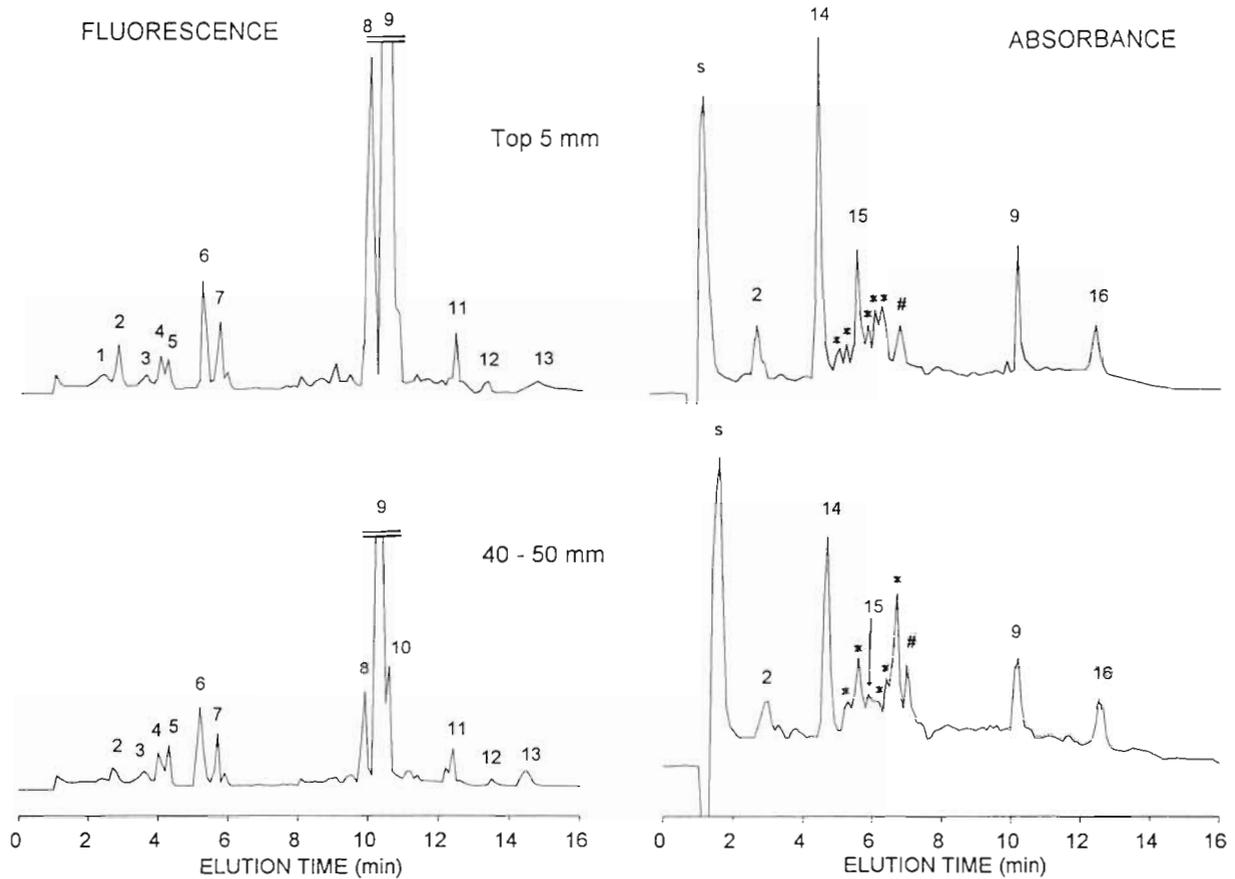


Fig. 2. Fluorescence (excitation: 430 nm, emission: 663 nm) and absorbance (440 nm) chromatograms of the intertidal mud of Marennes-Oléron Bay (top 5 mm and the 40–50 mm layers; for peak identification see Table 1). S: solvent

Table 1. Identification of fluorescence (excitation: 430 nm, emission: 663 nm) and absorbance (440 nm) peaks from chromatograms of acetonic extracts obtained from sediment samples collected during low tide on an intertidal mudflat in Marennes-Oléron Bay

Peak no.	Pigment identification
1	Chlorophyllide a
2	Chlorophyll c
3	Phaeophorbide a-like ₁
4	Phaeopigment c
5	Phaeophorbide a
6	Phaeophorbide a-like ₂
7	Phaeophorbide a-like ₃
8	Allomerized chlorophyll a
9	Chlorophyll a
10	Chlorophyll a'
11	Phaeophytin a
12	Phaeophytin a-like ₁
13	Phaeophytin a-like ₂
14	Fucoxanthin
15	Diadinoxanthin
*	Unidentified carotenoids
#	Zeaxanthin?
16	β-Carotene

Spatial and temporal variations of sediment pigment content

Vertical profiles of chl *a* and total phaeopigments *a* changed throughout the year (Fig. 3). There were significant increases of chl *a* and total phaeopigment contents in the top 5 mm between March and June, followed by a significant decrease in July (1-way ANOVA, $p < 0.001$); concentrations then remained low until January 1993. In February 1993, there was a significant increase in chl *a* concentration at the surface resembling the profile found in March 1992, whereas phaeopigment content did not increase significantly. Beneath the surface (10 to 50 mm), there were also significant changes in chl *a* and total phaeopigment contents over the year ($p < 0.01$). There was a significant increase in chl *a* concentration between March and June, followed by a decrease in July, then an increase between July and September and another from September 1992 to February 1993. Total phaeopigment content was lowest during winter (from October 1992 to February 1993) and reached a peak in April–May.

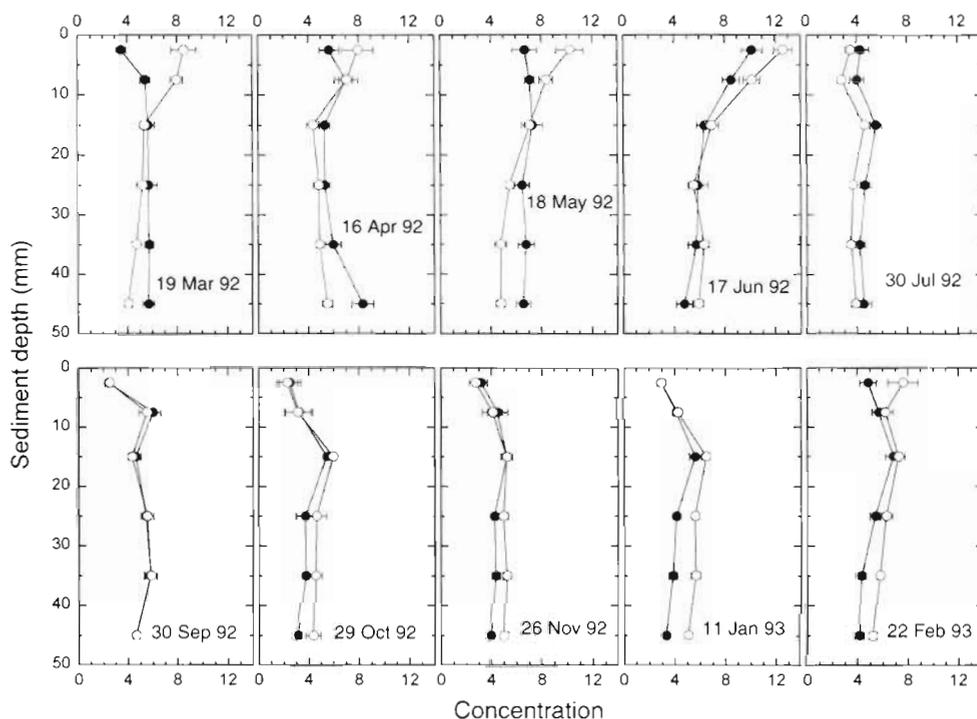


Fig. 3. Chl a (○) and total phaeopigments a (●) averaged concentration (mg m⁻² mm⁻¹) as a function of sediment depth (0 to 50 mm) during the sampling period. Horizontal bars denote SE

Between March and June 1992 and in February 1993, chl a concentration was higher (about 2-fold) in the top 1 cm than deeper in the sediment. From July 1992 to January 1993 there was an inversion of the concentration gradient: chl a content was lower at the surface than deeper in the sediment. Phaeopigments exhibited the same profile as chl a except during spring (March to May 1992) and in February 1993 when phaeopig-

ment concentrations were lower at the surface than deeper in the sediment.

The ratio of total phaeopigments a to chl a + total phaeopigments a significantly increased with depth (Fig. 4A) from 0.45 in the top 5 mm to 0.51 in the 40 to 50 mm layer ($p < 0.001$). There was also a change in the composition of phaeopigments (Fig. 4B): the proportion of phaeophorbides significantly decreased from

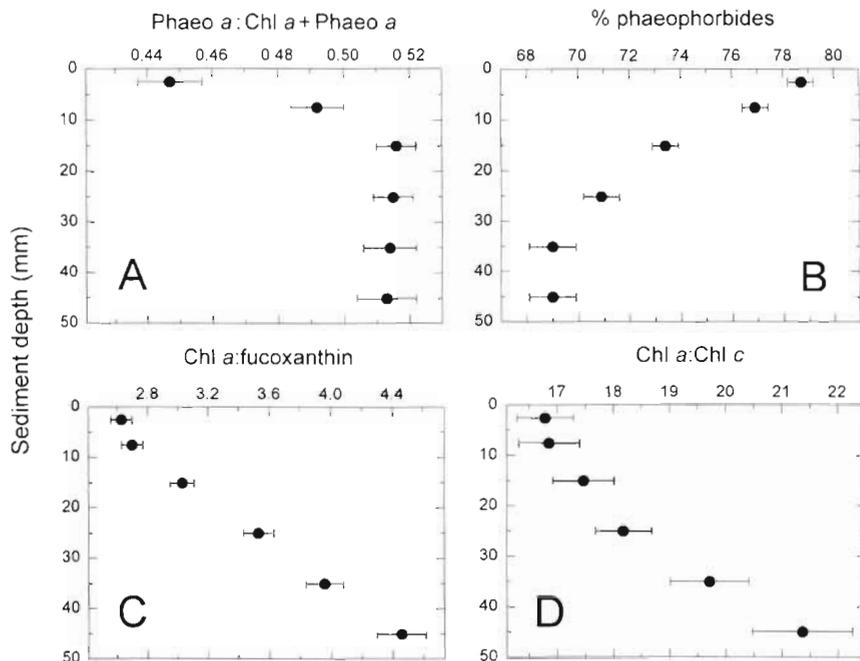


Fig. 4. Vertical profiles (averages over the sampling year) of: (A) total phaeopigments a:chl a + total phaeopigments a ratio; (B) % of phaeophorbides within total phaeopigments a; (C) chl a:fucoxanthin ratio; (D) chl a:chl c ratio. Horizontal bars denote SE

79% in the top 5 mm to 69% in the deepest layer ($p < 0.001$). The chl *a*:fucoxanthin ratio (Fig. 4C) significantly increased from 2.6 in surface sediment to 4.5 at 5 cm deep ($p < 0.001$), and the chl *a*:chl *c* ratio (Fig. 4D) exhibited the same trend with a significant increase from 16.8 at the surface to 21.4 in the deepest layer ($p < 0.001$). There was a strong correlation between chl *a* and chl *c* ($r = 0.818$; $p < 0.001$) as well as between chl *a* and fucoxanthin ($r = 0.819$; $p < 0.001$).

From a temporal standpoint, in the top 5 mm of the sediment column which includes the entire photic layer, monthly variations show statistically significant differences (1-way ANOVA, $p < 0.001$) in the total phaeopigments *a*:chl *a* + total phaeopigments *a* ratio. There was an increase from March to July, then a decrease between November 1992 and January 1993 and between January 1993 and February 1993 (Fig. 5A). The composition of phaeopigments also changed as a function of time in the photic sediment (Fig. 5B): the proportion of phaeophorbides increased significantly between March and September ($p < 0.001$) while that of phaeophytins decreased ($p < 0.001$).

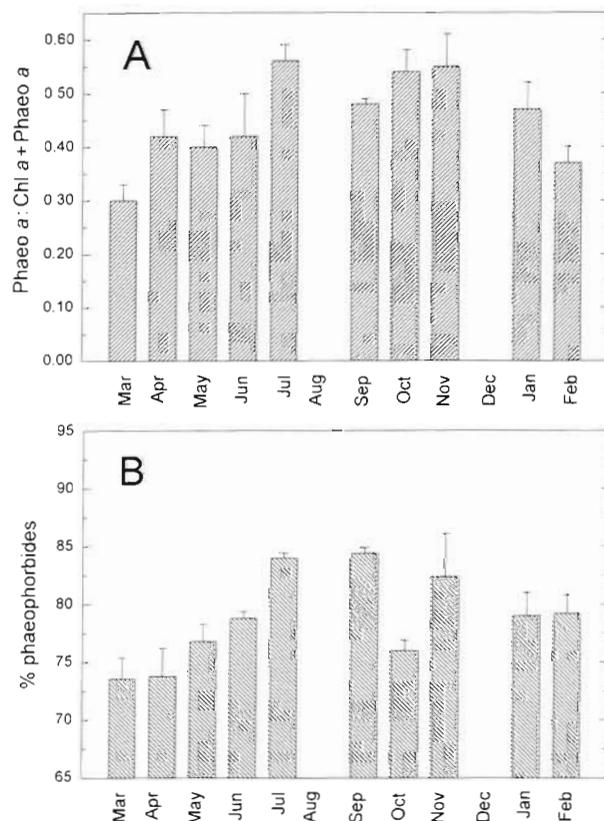


Fig. 5. Monthly variations (during the sampling period) in the top 5 mm of: (A) total phaeopigments *a*:chl *a* + total phaeopigments *a* ratio; (B) % of phaeophorbides within total phaeopigments *a*. Vertical bars denote SE

DISCUSSION

Qualitative analysis

The pigment composition of the sediment did not alter throughout the sampling period (March 1992 to February 1993), reflecting a constant composition of the algal community. It also indicates the dominance of diatoms in the microphytobenthic community (confirmed by microscopic observations), which is now well established (Colijn & de Jonge 1984, Riaux-Gobin et al. 1987, Barlow et al. 1990). The carotenoids identified — fucoxanthin, diadionoxanthin and β -carotene — are all present in diatoms [see Stauber & Jeffrey (1988) concerning 51 diatom species]. Other chl *c*-containing algal classes were absent from the community. Indeed the pigment analysis revealed the absence of peridinin and fucoxanthin derivatives which characterize Dinophyceae (Jeffrey 1974), Prymnesiophyceae and Chrysophyceae (Vesk & Jeffrey 1987, Wright & Jeffrey 1987, Bjornland et al. 1989), respectively. Therefore, the presence of chl *c* was solely due to diatoms. The complete absence of chl *b* in our samples excludes the possibility that Chlorophyceae and/or Euglenophyceae contributed to the microphytobenthic community of this intertidal mudflat. This is an important characteristic since chl *b* has been frequently detected in coastal sediments (Riaux-Gobin et al. 1987, Klein & Riaux-Gobin 1991), but our observations corroborate those of Barlow et al. (1990), who studied sediments of salt marshes in the same area ($46^{\circ} 10' N$, $1^{\circ} 15' W$). Lutein, which is associated with chl *b* (Jeffrey 1974), was not detected in our samples whereas 1 unidentified peak might be zeaxanthin, which has been proposed as a taxonomic marker of Cyanophyceae (Guillard et al. 1985, Gieskes et al. 1988). These microalgae were observed only in a few samples, suggesting that zeaxanthin rather originated from diatoms.

The concomitant absence of chl *b*, phaeopigments *b* and lutein further indicates that our sampling site was not an accumulation zone for terrestrial plant detritus (Abele-Oeschger 1991, Levinton & McCartney 1991), at least during the sampling period. There were nevertheless large amounts of degraded pigments which probably derived from the breakdown of autochthonous material. We have indeed detected many forms of phaeopigment *a*, which is consistent with findings of other studies concerning sediment (Brown et al. 1981, Riaux-Gobin et al. 1987, Klein et al. Riaux-Gobin 1991, Yacobi et al. 1991). The 4 phaeophorbides, which represented about 75% of total phaeopigments, are markers for macrobenthic deposit-feeding processes (Bianchi et al. 1988, 1993) and more generally for metazoan grazing activity (Brown et al. 1981, Welschmeyer & Lorenzen 1985, Abele-Oeschger et al. 1992).

Based on their order of elution and retention times, the 2 major phaeophorbides *a*-like₂ and *a*-like₃ (accounting for about 50% of total phaeophorbides) correspond very likely to the phaeophorbides *a*-like in Hawkins et al. (1986) study and to phaeophorbides *a*₂ and *a*₃ reported by Plante-Cuny et al. (1993). In these studies, the 2 degraded pigments are reported to result from chl *a* digestion by mussels. Mussel beds are areas of intense deposition and are not far away from our sampling site. It is, however, more likely that these phaeopigments were produced *in situ* by macrofauna grazing (Bianchi et al. 1988) since high densities of the amphipod *Corophium volutator* and meiofauna were present at our sampling site.

Chlorophyllide *a* was always present at very low concentration levels (0.5% of chl *a*). As this pigment characterizes senescent cells (Jeffrey 1974) and species containing chlorophyllase (Jeffrey & Hallegraeff 1987), the low concentrations that were detected suggest a good physiological state of the microphytobenthic community. This also confirms Plante-Cuny et al.'s study (1993) which suggests that chlorophyllide *a* may be considered as negligible in marine coastal sediments (about 1% of chl *a*).

Quantitative analysis

The significant increase of chl *a* (Fig. 3) between March and June 1992 in the photic zone of the sediment (top 5 mm) ($p < 0.01$) indicates the occurrence of a microphytobenthic bloom as the photoperiod, the light intensity and the temperature increased. The concentration of total phaeopigments *a* also increased between March and June 1992 ($p < 0.01$), but at a higher rate than chl *a* so that the phaeopigments *a*:chl *a* + phaeopigments *a* ratio significantly increased ($p < 0.01$; Fig. 5A) during the same period. As there was also a significantly higher proportion of phaeophorbides within total phaeopigments *a* in June than in March ($p < 0.01$; Fig. 5B), there was likely an intense grazing activity by meio- and macrofauna at the surface of the sediment during summer (because animals were more active and more abundant).

Between June and July 1992, there was a sharp decrease of the concentration of both chl *a* and total phaeopigments *a* which was probably due to resuspension of the upper sediment column since windy conditions occurred before sampling in July (Germaneau pers. obs.). Although we cannot quantify the presumed effect of this physical process, it might have had an impact on the pigment content of the sediment by resuspending a fraction of the algal cells and detritus into the water column during successive high tides. In addition, the increase between June and July of

both the phaeopigments *a*:chl *a* + phaeopigments *a* ratio ($p < 0.01$; Fig. 5A) and the proportion of phaeophorbides within total phaeopigments ($p < 0.01$; Fig. 5B) suggests that pigment degradation by grazing also occurred. Chl *a* was converted to phaeopigments and coprophagy could have converted phaeopigments to colourless residues (Gieskes et al. 1991) which cannot be detected either spectrophotometrically or fluorometrically. It has to be noted that a spring bloom followed by a summer depression of microphytobenthic biomass has also been recorded by Cadée & Hegeman (1974) and Colijn & Dijkema (1981). Riaux-Gobin et al. (1993) even showed that macrofaunal grazing can stimulate primary production during summertime while decreasing the biomass level on an intertidal mudflat. Productivity measurements performed at our site (Blanchard & Cariou-Le Gall 1994) confirm these observations.

There was very likely a sediment mixing of the top 5 cm of the sediment by bioturbation and physical processes (resuspension/redeposition) since pigment changes in deep sediment (10 to 50 mm) were similar to those in surface sediment (top 5 mm; Fig. 3). This also explains the presence of considerable amounts of chl *a* at several cm sediment depth, which has already been reported in the literature (Fenchel & Straarup 1971, Cadée & Hegeman 1974, Nienhuis & de Bree 1984).

The analysis of vertical profiles, averaged over the whole year (Fig. 4C, D), shows significant increases of the chl *a*:fucoxanthin ($p < 0.01$) and chl *a*:chl *c* ($p < 0.01$) ratios as a function of depth, suggesting higher degradation rates of fucoxanthin and chl *c* than of chl *a*. Compared to chl *a*, we observed a 42% decrease of fucoxanthin and a 22% decrease of chl *c*. This further shows that fucoxanthin was degraded at a faster rate than chl *c*. This is consistent with previous studies: Gillan & Johns (1980) reported that chl *a* was more stable than chl *c* in a temperate, sandy intertidal sediment (Australia), and Klein & Riaux-Gobin (1991) found that fucoxanthin was degraded much faster than chl *a* and chl *c* in a subtidal sediment (Kerguelen Islands). So, relative stabilities of these 3 pigments present in diatoms are as follows: chl *a* > chl *c* > fucoxanthin. The apparent loss of chl *c* and fucoxanthin with sediment depth suggests that part of the deep chl *a* is not associated with intact cells.

Throughout the entire year, the total phaeopigments *a*:chl *a* + total phaeopigments *a* ratio (Fig. 4A) was significantly lower ($p < 0.01$) in the top 5 mm of the sediment (0.45) than deeper (0.5 at 10 to 50 mm sediment depth). This was due to microphytobenthos autotrophic activity. This activity also probably enhanced grazing of meio- and macrobenthos in surface sediment since the proportion of phaeophorbides in total

phaeopigments *a* (Fig. 4B) was significantly higher in the top 5 mm than deeper in the sediment ($p < 0.01$).

Overall, the pigment analysis of this intertidal sediment indicates a predominance of the diatom community, with a spring bloom and a close coupling between microphytobenthos dynamics and the grazing activity of benthic invertebrates, as well as the influence of physical forcing.

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