Estuarine, Coastal and Shelf Science 79 (2008) 230–238

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss

Short-time scale variation of phytoplankton succession in Lisbon bay (Portugal) as revealed by microscopy cell counts and HPLC pigment analysis

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ARTICLE INFO

Article history:
Received 14 June 2007
Accepted 4 April 2008
Available online 12 April 2008

Keywords:
coastal phytoplankton succession
weekly sampling
HPLC
biomarker pigments

ABSTRACT

The phytoplankton distribution and composition in Lisbon bay was studied, at a short time scale based on a weekly sampling, during one year (April 2004 – May 2005), using microscopic examination and pigment analysis with high-performance liquid chromatography (HPLC). This work is a contribution to the knowledge on species succession and ecology of coastal communities. The frequency of the sampling permitted monitoring peak blooming and decaying, a process which frequently occurred within 1 –2 weeks.

Cell counts determined that the classes Dinophyceae, Bacillariophyceae and Prymnesiophyceae dominated the assemblages. Maxima abundances and diversity of phytoplankton were observed from spring to autumn. HPLC analysis reflected the major seasonal variations observed by the cell counts and in addition detected the presence of four small sized phytoplankton classes that were not identified by microscopy. Phytoplankton counts were essential to identify the main contributing species to total chlorophyll *a*. Fucoxantin, peridinin and 19′-hexanoyloxyfucoxanthin appeared as good indicators for diatoms, dinoflagellates and coccolithophores, respectively, with synchronized seasonal variations and significant positive correlations.

1. Introduction

Phytoplankton studies are crucial in studies of marine ecosystems as they play an important role in the structure and efficiency of the food web and thus contribute for the understanding of the organization and dynamics of these ecosystems. In classical studies, phytoplankton composition and abundance (cells l^{-1}) are determined from fixed samples observed under microscopy (Hasle, 1978). This technique allows a characterization to species level of the phytoplankton community. However, many species are difficult to identify and quantify by microscopy, because, in addition to their reduced size, are often fragile and not readily survive the various routine fixative and counting procedures used to enumerate cell abundances (Mackey et al., 1998; Havskum et al., 2004). An alternative method of characterizing phytoplankton relies on high performance liquid chromatography (HPLC) pigment analysis, which can provide complementary data to the direct cell counts. HPLC is used for estimating the quantitative contribution of phytoplankton groups to chlorophyll *a* (Chl *a*) using photosynthetic marker pigments (Gieskes and Kraay, 1983; Schlüter and Havskum, 1997; Ediger et al., 2006). Examples of carotenoid biomarkers for single algal class are alloxanthin for cryptophytes, prasinoxanthin for prasinophytes, peridinin for dinoflagellates and 19′-hexanoyloxyfucoxanthin for prymnesiophyceans. Less specific biomarkers are fucoxanthin for diatoms (also present in chrysophytes and prymnesiophyceans) and zeaxanthin for cyanobacteria (also present in green algae) (Jeffrey and Vesk, 1997). As many algal classes share pigments, a reliable interpretation of the data derived from pigment analysis should be supported by cell counts (Mackey et al., 1996; Jeffrey et al., 1999; Irigoien et al., 2004). The sole use of pigment signatures without a concurrent microscopic verification can sometimes be misleading (Millie et al., 1993). Thus a combination of both approaches has been recommended (Hallegraeff, 1981; Jeffrey and Hallegraeff, 1987), despite the tendency to rely mostly on pigment chemotaxonomy using HPLC analysis mainly because of shorter analysis time (Barlow et al., 1993; Peeken, 1997).

In the present study, the seasonal variability of the phytoplankton community in Lisbon bay will be described based on a weekly sampling. The major phytoplankton groups will be compared using the chemotaxonomic approach based on HPLC pigment analysis and cell counting by inverted microscopy. Cell counts are expected to corroborate the pigments identifications and variability and thus validate the use of marker pigments as indicators of the major phytoplankton groups. We intent to reinforce the utility and reliability of the HPLC as a monitoring tool for evaluating rapid and large scale changes in phytoplankton community.

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doi:10.1016/j.ecss.2008.04.004

2. Materials and methods

2.1. Study site

Phytoplankton composition and abundance was weekly studied at a fixed station in Cascais (located at 38° 41′ N and 09° 24′ W) (Fig. 1) during one year (April 2004 – May 2005). Surface seawater samples were collected with a Nansen bottle one hour before high tide, to avoid the direct influence of estuarine waters on the area. The water for nutrient determination was filtered through a Millipore filter of 0.45 μm and stored at − 4 °C for subsequent analysis. Ammonia (NH_4^+), nitrites and nitrates ($NO_2^- + NO_3^-$), phosphates (PO_4^{3-}) and silicates ($Si(OH)_4$) were determined using an autoanalyser "SKALAR" according to the methods of *Technicon Industrial Systems* (Grasshoff, 1983). The detection limit is 0.2 μM for ammonia and silicates and 0.05 μM for nitrites + nitrates and phosphates.

Temperature and salinity were determined *in situ* with a Quanta CTD. Data from Tagus flow were obtained from the "Water National Institute" in a public database (http://www.inag.pt) and a weekly average was calculated before each sampling date.

2.2. HPLC pigment analysis

Surface seawater samples (5 l) were filtered onto a Whatman GF/F filter (0.7 μm nominal pore size and 47 mm diameter), under vacuum pressure lower than 500 mbA. The filters were kept frozen at −80 °C before extraction. Photosynthetic pigments were extracted with 3 ml of 95% cold-buffered methanol (2% ammonium acetate) for 30 min at −20 °C, in the dark. Samples were sonicated for 30 s in the beginning of the extraction period. The samples were centrifuged at 3000 rpm for 15 min, at 4 °C. Extracts were filtered (Millipore membrane filters, 0.2 μm nominal pore size) immediately before injection in the HPLC to remove cell and filter debris. Each sample was diluted in 10% water (HPLC-grade), to prevent distortion of early eluting peaks (Zapata and Garrido, 1991). Pigment extracts were analyzed using a Shimadzu HPLC comprised of a solvent delivery module (LC-10ADVP) with system controller (SCL-10AVP) and a photodiode array (SPD-M10ADVP). The chromatographic separation of pigments was achieved using a C_8 column for reverse phase chromatography (Symmetry; 15 cm long; 4.6 mm diameter; 3.5 μm particles). The mobile phase used was: A = methanol:acetonitrile:aqueous pyridine solution (0.25 M pyridine, pH adjusted to 5.0 with acetic acid) in the proportions 50:25:25 (v/v/v), and B = acetonitrile:acetone (80:20 v/v). The solvent gradient followed Zapata et al. (2000) with a flow rate of 1 ml min^{-1}, an injection volume of 100 μl with duration of 40 min. Pigments were identified from absorbance spectra plus retention times and concentrations calculated from the signals in the photodiode array detector. Calibration of the HPLC peaks was performed using commercial standards, namely, chlorophyll *a* and chlorophyll *b* standards from Sigma, chlorophyll c_2, chlorophyll c_3, peridinin, fucoxanthin, diadinoxanthin, diatoxanthin, 19′-hexanoyloxyfucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, alloxanthin, 19′-butanoyloxyfucoxanthin and zeaxanthin standards from the DHI (Institute for Water and Environment, Denmark).

2.3. Phytoplankton microscopic identification

Phytoplankton samples were preserved with hexamethylenetetramine buffered formalin to a final concentration of 2% (Throndsen, 1978). Subsamples of 50 ml were allowed to settle for 36 h (Margalef, 1969 in Hasle, 1978). Cells were identified and counted by the Utermöhl technique using a Zeiss IM35 inverted microscope with phase contrast and bright field illumination (Hasle, 1978). A magnification of 160× and 400× was used to identify and enumerate the phytoplankton assemblage with a detection limit of 40 cells l^{-1} and 2000 cells l^{-1}, respectively. When possible, the cells were identified to species level according to Hasle and Syvertsen (1996) and Dodge (1982). Small-sized phytoplankton with morphological features difficult to recognize were placed into the category of "others". This group would likely include different algal classes: criptophyceae, chlorophyceae, prasinophyceae, cyanobacteria and other not identified small algae. Coccolithophores were separately identified following Young et al. (2003) and counted, from an area of 2.2 mm^2 of a nitrate cellulose membrane (Whatman, 47 mm with a 0.45 μm nominal pore size) at a maximum of 300 cells (Fatela and Taborda, 2002) with a Zeiss optical microscope under cross-polarized light at a magnification of 1250×.

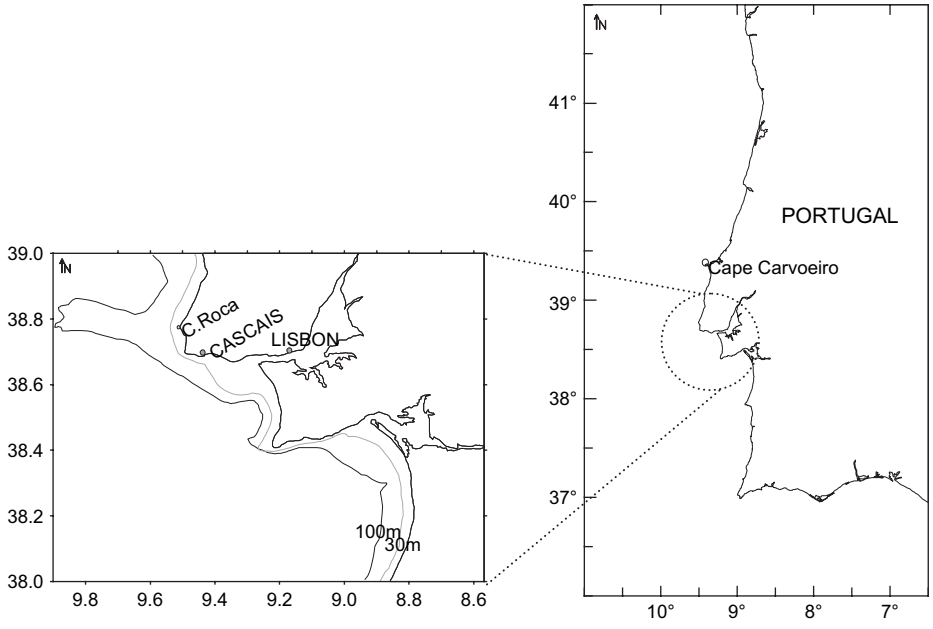

Fig. 1. Location of the sampling site 38° 41′ N and 09° 24′ W – Cascais Bay.

3. Results

3.1. Hydrographic data

Sea surface temperature (Fig. 2) was characterized by minima and maxima values of 12.2 °C and 20.5 °C recorded in February 2005 and August 2004. The lower values were observed from mid-December 2004 until Mars 2005 while during the rest of the year temperatures were always above 14 °C.

Surface salinity (Fig. 2) was measured using the Practical Salinity Scale and remained constant (34.5– 35.5) through the year, except during autumn 2004 when the lowest salinities (31.7) were

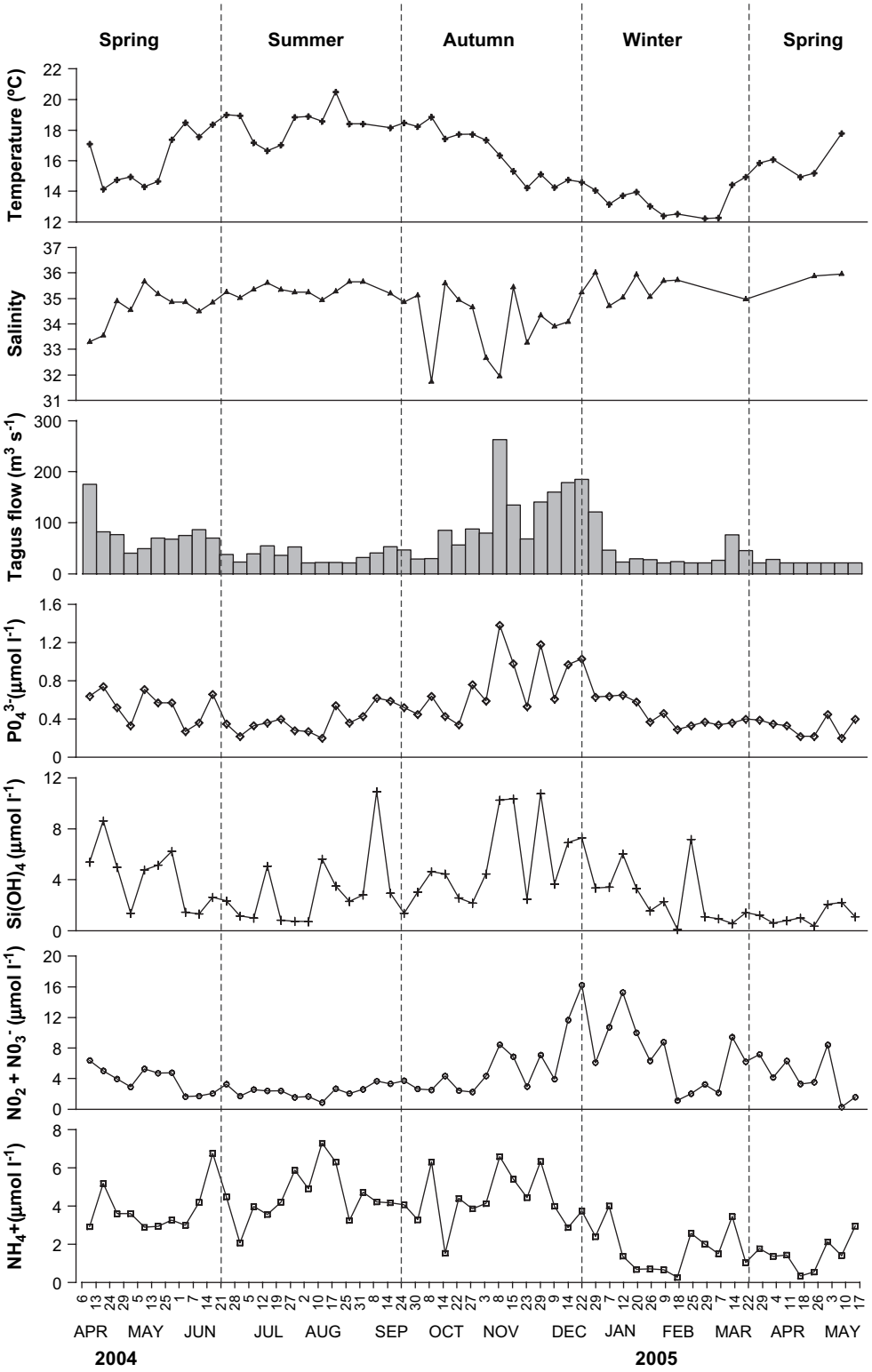

Fig. 2. Weekly distribution of sea surface temperature, surface salinity, Tagus flow, phosphates, silicates, nitrites + nitrates and ammonia, during the sampling period (April 2004 – May 2005). The day of each sampling is represented on the abscis axis.

observed, coincident with rainy periods. Tagus river flow (Fig. 2) also showed a major increase during this period, reaching 263 $m^3 s^{-1}$ in November 2004. The lower runoff values (21 $m^3 s^{-1}$) were recorded during summer 2004 and from winter 2005 until the end of the sampling. Winter 2005 corresponded to a drought period, reflected in the low river flow values registered, well below average annual value of 400 $m^3 s^{-1}$.

Nutrient concentrations (Fig. 2) changed along the year. The measured phosphate varied between 0.20 and 1.38 μmol l^{-1} (August 04 – November 04), silicates ranged between 0.11 and 10.91 μmol l^{-1} (February 05 – August 04), nitrate + nitrite between 0.29 and 16.23 μmol l^{-1} (May 05 – December 04) and ammonia values were between 0.28 and 7.30 μmol l^{-1} (February 05 – August 04). Phosphates and nitrite + nitrate had minimum values during spring and summer and maxima during autumn – winter. Positive significant correlations were found between Tagus runoff and phosphates ($r^2 = 0.6$, $p < 0.001$) as well as with silicates ($r^2 = 0.3$, $p < 0.05$). Ammonia values were generally lower during 2005, in accordance to reduced runoff.

Concerning nutrient stoichiometry, from the 57 sampling occasions, it was observed that half of N:P ratios were lower than 16 (during spring and autumn 2004) whilst 95% of the Si:N values were lower than 1 (Fig. 3).

3.2. Seasonal succession: HPLC pigment analysis versus species quantification

Chromatographic analysis revealed the presence of a wide range of pigments, exhibiting a clear temporal variability. Chlorophyll *a* and fucoxanthin (a proxy for diatoms) were the two most abundant pigments, present in all samples. Relatively high concentrations of two other accessory pigments were also observed: peridinin and 19′-hexanoyloxyfucoxanthin, which are the major carotenoids of dinoflagellates and prymnesiophyceans, respectively. The only prymnesiophyceans identified by microscopy were the coccolithophores. In addition to these pigments, chlorophyll *b*, chlorophyll $c_1 + c_2$ and c_3, diadinoxanthin, diatoxanthin, violaxanthin, neoxanthin, zeaxanthin, prasinoxanthin, 19′-butanoyloxyfucoxanthin and alloxanthin concentrations also were quantified (Table 1). The abundance of phytoplankton classes contributing to total Chl *a* can be estimated from the concentrations of biomarker pigments using a Chemical Taxonomy software, known as Chemtax (Mackey et al., 1996). This chemotaxonomic approach was attempted but it did not provide any additional relevant information than the simple regression analysis between cell counts of a given class and its most characteristic pigment (Figs. 4 and 5 represent the statistical correlations obtained), hence we chose to present the raw data concerning pigment concentrations, as we found to be more useful for other authors studying coastal systems. The index of phytoplankton biomass, Chl *a*, evidenced a good correlation with cell counts ($r^2 = 0.37$; $p < 0.01$; Fig. 4). The seasonal variation of Chl *a* was coincident with the seasonality of total phytoplankton (Fig. 6) with maxima occurring through all seasons and reflecting the highest concentrations of the dinoflagellates, diatoms and coccolithophores. Additionally, the major Chl *a* peaks matched the peaks of diatoms. The highest Chl *a* value observed was 0.916 μg l^{-1} in October 2004.

The most abundant pigment detected, fucoxanthin, evidenced a very good correlation with diatoms cell counting ($r^2 = 0.81$; $p < 0.01$; Fig. 5a). The seasonal variation of this carotenoid was coincident with the diatoms distribution along the year, with maximum values of 3.142 μg l^{-1} in October 2004 and 1.116 μg l^{-1} in February 2005 (Fig. 6). Significant correlation ($p < 0.01$) was found between the concentration of peridinin and the density of dinoflagellates ($r^2 = 0.54$; Fig. 5b) as well as between coccolithophores abundance and 19′-hexanoyloxyfucoxanthin ($r^2 = 0.56$; Fig. 5c). The annual variation of these two carotenoids accompanied dinoflagellates and coccolithophores seasonality, respectively (Fig. 6). The carotenoid 19′- butanoyloxyfucoxanthin, a trace pigment in some chrysophytes and prymnesiophytes (but a major pigment in *Phaeocystis*), according to Jeffrey et al. (1997), occurred only four times (Table 1), without any relation to cell countings from these two divisions.

Peridinin reached a maximum concentration of 2.341 μg l^{-1} in October 2004 (Fig. 6). Maxima of 19′-hexanoyloxyfucoxanthin were during summer and autumn with values of 0.11 μg l^{-1} in September and October 2004 (Fig. 6).

Prasinoxanthin was present in lower concentrations throughout the year with a maximum abundance of 0.05 μg l^{-1} in October 2004 (Fig. 7). This carotenoid is exclusive of prasinophytes, a group not identified under the microscope during the sampling period. Another phytoplankton group not recognized during cell counts was the cryptophytes, however, relevant concentrations of alloxanthin (exclusive pigment of this group) were detected by HPLC

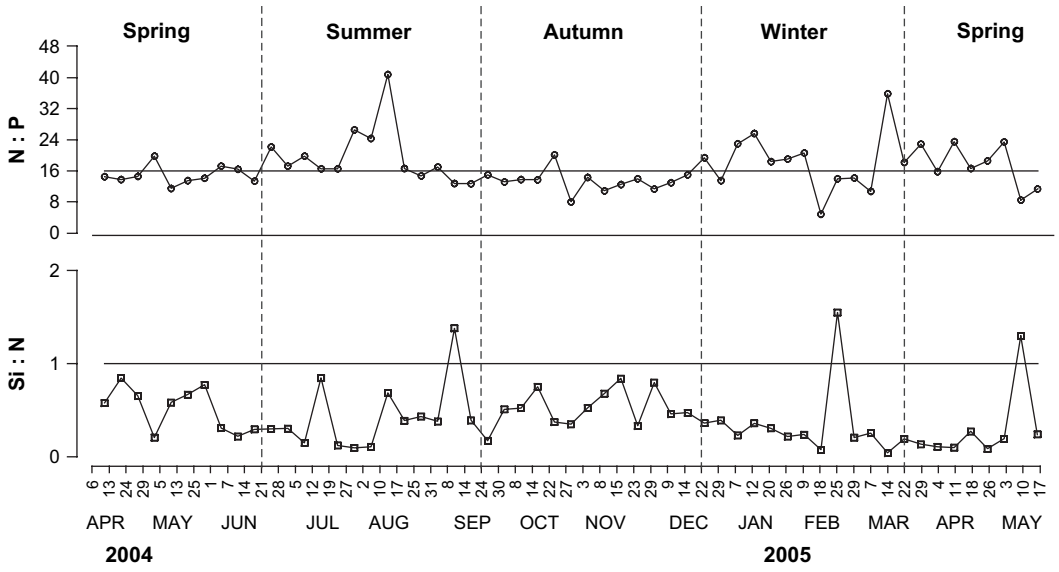

Fig. 3. N:P and Si:N ratios during the sampling period (April 2004 – May 2005). The day of each sampling is represented on the abscis axis.

Table 1
HPLC photopigments concentration registered (annual average and range) and their associated phytoplankton classes (Jeffrey et al. 1997; Gibb et al. 2001)

Pigments	Concentration ($\mu g\ l^{-1}$)	%	Occurrence
Chlorophyll *a*	0.260 (0.005–0.916)	49.1	A proxy of total algae biomass
Chlorophyll c_1, c_2	0.199 (0.000–2.546)	37.5	Diatoms, prymnesiophytes, crysophytes, dinoflagellates
Chlorophyll c_3	0.040 (0.000–0.233)	7.5	Crysophytes, prymnesiophytes
Chlorophyll *b*	0.031 (0.000–0.118)	5.8	Chlorophytes, euglenophytes, prasinophytes
Total chlorophylls	0.530 (0.005–3.813)	100	
Fucoxanthin	0.349 (0.021–3.142)	54.4	Diatoms, prymnesiophytes, crysophytes
Peridinin	0.121 (0.000–2.341)	18.8	Dinoflagellates
Diadinoxanthin	0.081 (0.000–0.995)	12.6	Diatoms, prymnesiophytes, crysophytes, dinoflagellates
19′-hexanoyloxyfucoxanthin	0.024 (0.000–0.113)	3.7	Prymnesiophytes
Alloxanthin	0.024 (0.000–0.171)	3.7	Cryptophytes
Violaxanthin	0.016 (0.000–0.496)	2.5	Chlorophytes, prasinophytes
Prasinoxanthin	0.008 (0.000–0.055)	1.2	Prasinophytes
Diatoxanthin	0.008 (0.000–0.094)	1.2	Diatoms, prymnesiophytes, crysophytes, dinoflagellates
Neoxanthin	0.005 (0.000–0.077)	0.8	Chlorophytes, prasinophytes
Zeaxanthin	0.004 (0.000–0.037)	0.6	Cyanobacteria, chlorophytes
19′-butanoyloxyfucoxanthin	0.002 (0.000–0.035)	0.3	Crysophytes, prymnesiophytes
Total carotenoids	0.642 (0.021–7.556)	100	

with maxima during summer and autumn (0.171 $\mu g\ l^{-1}$ in September 2004; Fig. 7). Several minor pigments were also detected by chromatography, such as Chl *b*, zeaxanthin, violaxanthin and neoxanthin, which we considered as representing an assembly of euglenophytes, chlorophytes and cyanobacteria. Cells from the last two divisions were not identified by microscopy. This set of pigments had maximum concentrations during summer and autumn (0.647 $\mu g\ l^{-1}$ in October 2004; Fig. 7).

3.3. Phytoplankton species composition

The 129 phytoplankton species observed were grouped into four classes and one extra group with different contributions to total abundance: 43% of dinophyceae (dinoflagellates), 41% of bacillariophyceae (diatoms), 9% of prymnesiophyceae (coccolithophores), 2% of euglenophyceae and 5% of the extra group designated as "others" (not identified small algae). A species richness index (SR) was determined, as it is the simplest measure of diversity, representing the total number of different species in a given area (Kevin and Spicer, 2004). It ranged from 11 to 44 species identified per sample. Species diversity increased from spring to summer, attaining its maximum, and decreased towards the winter to values three times lower (Table 2).

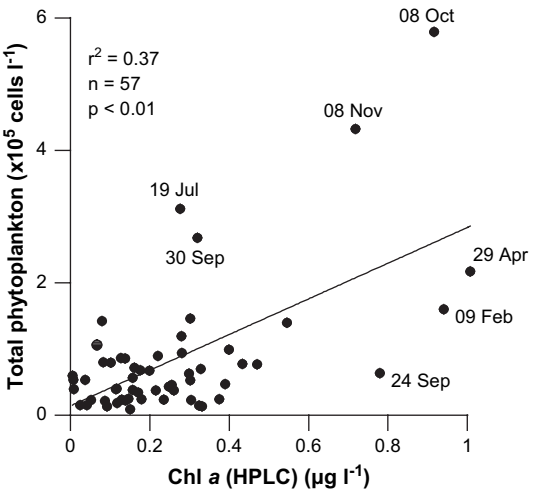

Fig. 4. Total phytoplankton measured by the Utermöhl technique in relation to chlorophyll *a* measured by HPLC.

The majority of the taxa were dinoflagellates, with 56 identified species. *Protoperidinium*, with 11 species and *Ceratium* with 10 species, were the two most represented genus, followed by *Dinophysis* and *Prorocetrum* with 6 species each. Nonetheless, the dinoflagellate *Scripsiella* cf. *trochoidea* was the dominant species from this group, being responsible for all the four maximum values. Dinoflagellates contribution to total biomass ranged between 0.2 and 86% (Table 2) reaching a maximum abundance of 2.5×10^5 cells l^{-1} in July 2004 (Fig. 6).

Diatoms were the second largest group with 53 identified species. The most representative diatom genera were *Thalassiosira* and *Guinardia* both with 4 species identified, but the major abundances belonged to chain forming species like *Thalassiosira* spp., *Chaetoceros* spp., *Asterionelopsis glacialis*, *Skeletonema costatum, Pseudo-nitzschia* spp., *Detonula pumila, Lauderia annulata* and *Leptocylindrus danicus.* The contribution of diatoms to total abundance varied between 0.3 and 90% (Table 2) and this group reached a maximum concentration of 3.7×10^5 cells l^{-1} in October 2004 (Fig. 6), achieving 65% of phytoplankton abundance. From the four maxima recorded (Fig. 6) just the peak observed in April 2004 was dominated by *Pseudo-nitzschia* spp. (Table 2), the others were mainly composed by *Thalassiosira* spp. (October and November 2004 and February 2005).

Seven species of coccolithophores were identified, *Emiliania huxleyi* and *Gephyrocapsa* spp. being the main components of this community with a regular occurrence throughout the study. The contribution of coccolithophores to total biomass was between 1 and 86% (Table 2) and a maximum abundance of 1×10^5 cells l^{-1} was observed in September 2004 (Fig. 6) constituted by *E. huxleyi.* From the end of July 2004 until mid August 2004, *Syracosphaera pulchra* dominated the coccolithophore assemblage reaching 0.3×10^5 cells l^{-1} in August 2004 (Table 2).

The class of euglenophyceae reached a maximum concentration of 5×10^3 cells l^{-1} in April 2005 and the category of "others" achieved 3×10^4 cells l^{-1} in June 2004 (Table 2).

4. Discussion

Dinoflagellates, diatoms and coccolithophores dominated the phytoplankton assemblage in terms of abundance and community dynamics as showed both by microscopic observations and pigment analysis. Maxima concentrations of total phytoplankton were observed in autumn, although short-time peaks were registered throughout all seasons. The correlations obtained between biomarker pigment concentration and the corresponding taxon

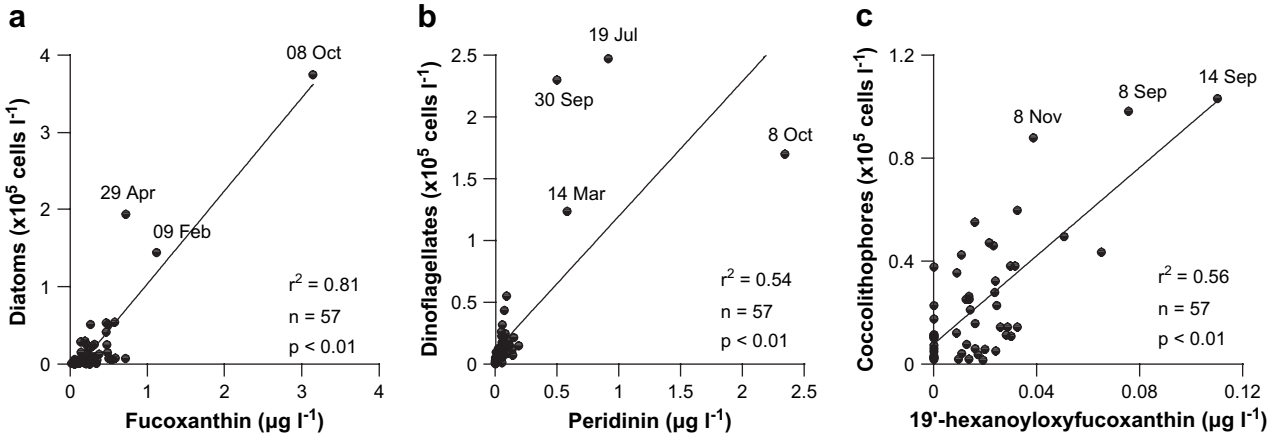

Fig. 5. Relationship between (a) fucoxanthin concentration and diatoms density, (b) peridinin and dinoflagellates density and (c) 19′-hexanoyloxyfucoxanthin and coccolithophores density.

specific cell number, constitute interesting results and are a relevant contribution of the present paper to coastal phytoplankton studies. The microscopic analysis showed that the outliers of these correlations (Fig. 4) were coincident with maximum concentrations (cells l^{-1}) of each phytoplankton group, evidencing the need of microscopic observations to fully characterize peak events. Specifically, diatoms presented three outliers, which corresponded to peaks of chain forming species such as: *Pseudo-nitzschia* spp.,

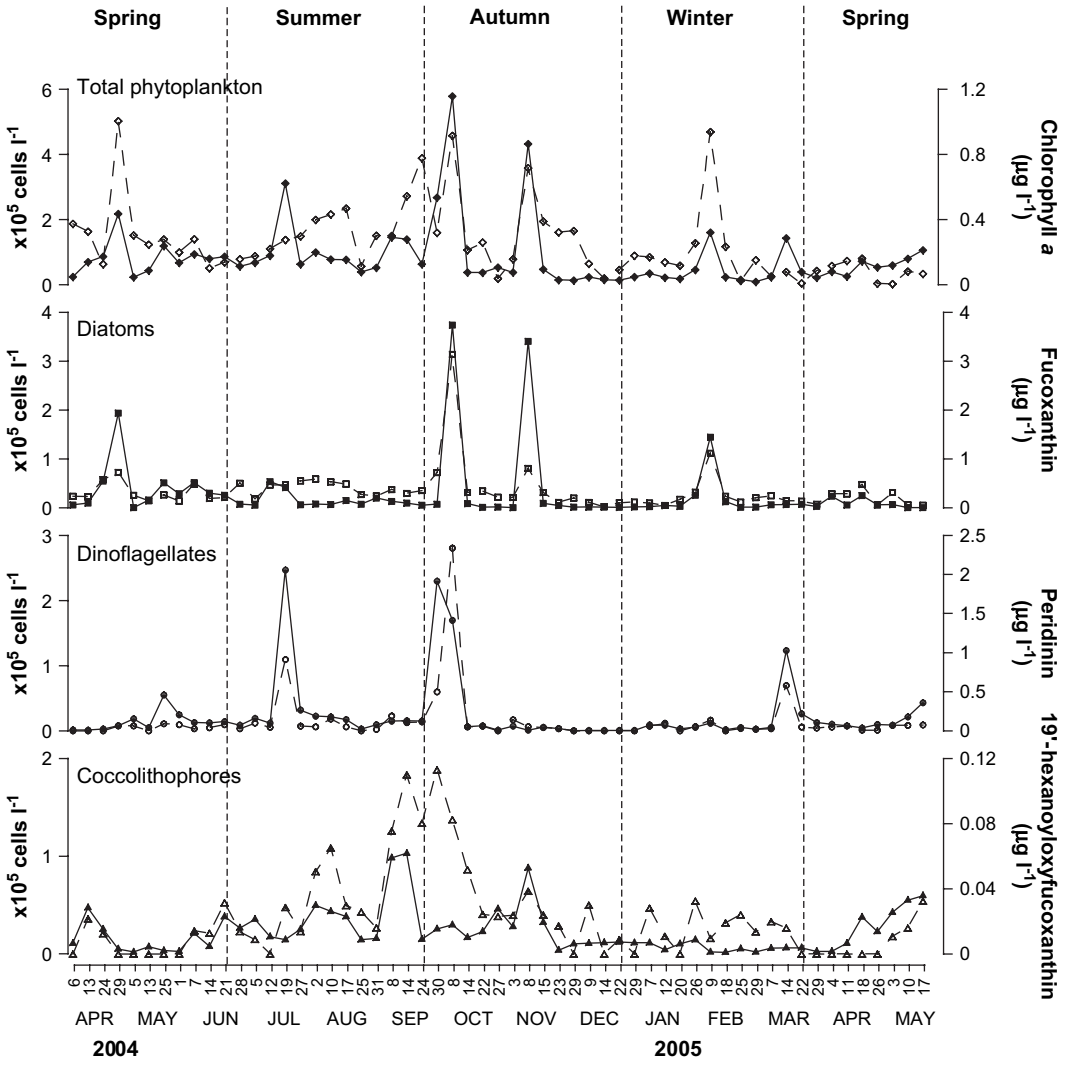

Fig. 6. Weekly surface distribution of total phytoplankton and chlorophyll *a* and of the dominant phytoplankton groups with the respective marker pigments, during the sampling period (April 2004 – May 2005). Diatoms and fucoxanthin, dinoflagellates and peridinin, coccolithophores and 19′-hexanoyloxyfucoxanthin. Cell counts and pigments are represented by solid and dotted lines, respectively. The day of each sampling is represented on the abscis axis.

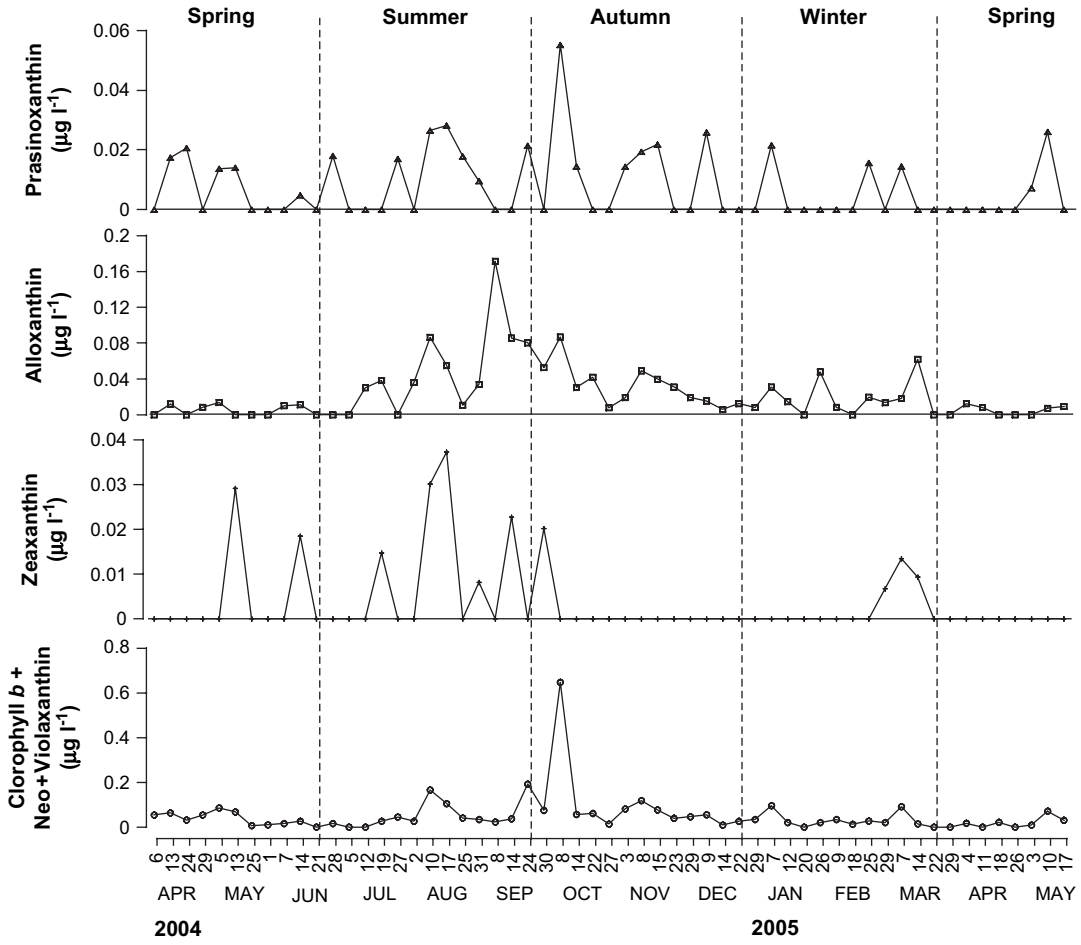

Fig. 7. Weekly surface distribution of marker pigments during the sampling period (April 2004 – May 2005): prasinoxanthin, alloxanthin, zeaxanthin and chlorophyll *b* + neoxanthin + violaxanthin. The day of each sampling is represented on the abscissa axis.

Chaetoceros spp., *Thalassiosira* spp, *Skeletonema costatum* and *Asterionellopsis glacialis*. The four maxima abundances of dinoflagellates were coincident with the outliers present in the correlation and were constituted by *Scripsiella* cf. *trochoidea*. Finally, the same picture was found for coccolithophores: the three outliers corresponded to maxima of *Emiliania huxleyi* (Fig. 3, Table 2). Therefore, the variations between the three main phytoplankton groups, dinoflagellates, diatoms and coccolithophores were reflected by the peridinin, fucoxanthin, and 19′-hexanoyloxyfucoxanthin concentrations, respectively, although the last two can not be considered truly fingerprint pigments as they are present in other phytoplankton classes. However, the good agreement between fucoxanthin and diatoms concentration ($r^2 = 0.81$) indicate that this group is the most important carrier of this pigment for our samples. Hence, in spite of being present also in haptophytes (Jeffrey and Vesk, 1997), fucoxanthin can be used to trace diatoms, providing a solid proxy for monitoring seasonal variations, in this region.

As far as coccolithophores are concerned, the positive correlation ($p < 0.01$) between the concentrations of 19′-hexanoyloxyfucoxanthin and the density of coccolithophores ($r^2 = 0.54$) but not with fucoxanthin, point out this carotenoid as biomarker of coccolithophores in our waters. The same conclusion was achieved by Ediger et al. (2006), who found a good correlation between *Emiliania huxleyi* and 19′-hexanoyloxyfucoxanthin, but not with fucoxanthin. Furthermore, Stolte et al. (2000), indicate 19′-hexanoyloxyfucoxanthin as the major light harvesting carotenoid in all Atlantic strains for this species.

The seasonal distribution of total phytoplankton biomass was generally higher in spring and summer, however, it did not evidence the typical pattern of temperate phytoplankton seasonal evolution. The relevant biomass peaks were registered in autumn, with a major bloom of diatoms, dinoflagellates, prasinophytes and other chlorophyll *b* containing groups on 8 October 2004, followed by a second one in 8 November, dominated by diatoms and cocolitophores. In both occasions, salinity attained its minimum values due to heavy rainfall.

Phytoplankton growth is dependent on light and nutrients availability. In Cascais Bay, nutrients seem to be mostly from riverine origin, the transport of silicates and phosphates from Tagus estuary was clearly proved by the correlations obtained between each of these nutrients and runoff. For dissolved inorganic nitrogen, a statistical valid correlation was not found however, ammonia values diminished considerable on drier year 2005, whereas nitrates + nitrites increase in December/January as a response to the higher runoff in November/December. In order to assess nutrient limitation, the obtained results were discussed following Dortch and Whitledge (1992). Phosphates were only limiting on 10 August 2004 (with $PO_4^{3-} \leq 0.2$, N/P > 30 and Si/P > 3), where the community was dominated by the coccolithophore *Syracopshaera pulchra* and the dinoflagellate *Ceratium fusus*. Nitrates were limiting during a major bloom of the diatom *Asterionellopsis glacialis* at 18 February 2005, as DIN was 1 µmol l^{-1}, N:P ratio 5 (< 10), and Si:N lower than 1 (0.08). Availability of silicates clearly diminished from February 2005 onwards, most probably due to decreased river flow, potentially limiting conditions, with $SiO_4^{4-} < 2$ µmol l^{-1},

Table 2
Weekly phytoplankton relative distribution (%), species richness (SR) and dominant phytoplankton species. Diat. – diatoms; Dino. – dinoflagellates; Cocc. – coccolithophores; Eugl. – euglenophytes

		Day	Phytoplankton groups (%)					SR	Dominant phytoplankton species
			Diat.	Dino.	Cocc.	Eugl.	Others		
2004	APR	6	23.9	5.0	45.1	0.2	25.9	24	*Pseudonitzschia* spp.; *Emiliania huxleyi*
		13	13.5	1.5	67.7	0.9	16.4	31	*Pseudonitzschia* spp.; *Emiliania huxleyi*; *Gephyrocapsa* spp.
		24	62.8	3.0	28.9	0.5	4.9	27	*Pseudonitzschia* spp.; *Emiliania huxleyi*; *Gephyrocapsa* spp.
		29	**89.0**	3.6	2.2	0.2	5.0	32	*Pseudonitzschia* spp.; *Chaetoceros* spp.
	MAY	5	1.6	**79.6**	7.8	2.4	8.7	16	*Scripsiella* cf. *trochoidea*
		13	35.7	11.2	16.4	7.1	29.6	30	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
		25	43.0	46.0	2.6	0.3	8.1	32	*Detonula pumila*; *Scripsiella* cf. *trochoidea*
	JUN	1	42.5	36.5	3.5	2.5	15.0	33	*Detonula pumila*; *Scripsiella* cf. *trochoidea*
		7	54.5	13.5	22.4	0.0	9.6	27	*Detonula pumila*; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
		14	37.3	15.2	9.5	0.2	37.8	34	*Detonula pumila*; *Scripsiella* cf. *trochoidea*
		21	29.5	16.4	44.1	0.1	9.9	44	*Detonula pumila*; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
		28	13.1	14.9	**46.4**	0.1	25.6	40	*Thalassiosira* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
	JUL	5	7.5	**28.2**	52.1	0.0	12.2	43	*Ceratiumfusus*; *Ceratium furca*; *Gephyrocapsa* spp.
		12	59.5	13.0	19.4	0.2	7.8	30	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
		19	13.3	79.3	4.6	0.2	2.7	43	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*
		27	9.5	50.4	39.8	0.0	0.3	38	*Scripsiella* cf. *trochoidea*; *Syracopshaera pulchra*
	AUG	2	7.7	22.7	**50.0**	0.0	19.7	38	*Scripsiella* cf. *trochoidea*; *Syracopshaera pulchra*
		10	8.4	27.5	**56.0**	0.0	8.1	38	*Ceratiumfusus*; *Syracopshaera pulchra*
		17	19.1	22.4	49.5	0.3	8.7	38	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
		25	18.8	6.5	36.5	0.0	38.2	30	*Pseudonitzschia* spp.; *Gephyrocapsa* spp.; *Syracopshaera pulchra*
		31	37.2	17.2	29.7	0.2	15.7	29	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
	SEP	8	8.9	10.1	67.3	0.8	12.8	34	*Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.; *Emiliania huxleyi*
		14	6.5	11.0	**73.6**	0.1	8.7	30	*Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		24	7.9	23.6	23.3	1.6	43.5	29	*Protoperidinium* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		30	2.7	**85.9**	9.5	0.4	1.6	28	*Scripsiella* cf. *trochoidea*
	OCT	8	**64.7**	29.3	5.1	0.2	0.7	31	*Thalassiosira* spp.; *Skeletonema costatum*; *Scripsiella* cf. *trochoidea*
		14	22.3	16.4	44.2	0.3	16.7	35	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		22	2.0	20.1	60.5	0.9	16.5	24	*Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		27	2.5	2.0	86.3	1.3	8.0	21	*Emiliania huxleyi*; *Gephyrocapsa* spp.
	NOV	3	1.1	18.2	72.9	2.3	5.6	17	*Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		8	**78.9**	0.2	**20.3**	0.1	0.5	21	*Thalassiosira* spp.; *Chaetoceros* spp.; *Emiliania huxleyi*
		15	18.9	11.6	68.7	0.6	0.2	20	*Thalassiosira* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		23	30.1	22.6	23.8	9.6	13.8	21	*Chaetoceros* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		29	8.0	1.2	74.9	0.0	15.9	15	*Gephyrocapsa* spp.
	DEC	9	5.6	2.2	45.6	3.4	43.2	15	*Emiliania huxleyi*; *Gephyrocapsa* spp.
		14	7.4	2.7	73.7	0.8	15.4	17	*Emiliania huxleyi*; *Gephyrocapsa* spp.
		22	4.8	3.0	85.5	4.5	2.1	12	*Emiliania huxleyi*; *Gephyrocapsa* spp.
		29	7.5	1.5	46.6	2.3	42.1	17	*Emiliania huxleyi*; *Gephyrocapsa* spp.
2005	JAN	7	6.2	25.5	32.2	12.1	24.0	21	*Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		12	21.2	38.3	18.3	3.8	18.3	17	*Thalassiosira* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		20	15.8	16.3	55.8	0.7	11.4	21	*Paralia sulcata*; *Protoperidinium* spp.; *Gephyrocapsa* spp.
		26	55.1	11.9	31.1	1.6	0.3	26	*Thalassiosira* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
	FEB	9	**90.1**	7.1	1.1	0.2	1.4	28	*Thalassiosira* spp.; *Asterionellopsis glacialis*
		18	52.5	6.6	6.1	0.9	33.9	18	*Asterionellopsis glacialis*
		25	6.2	32.0	32.4	13.4	16.0	19	*Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		29	11.6	26.2	19.2	0.0	43.0	15	*Thalassiosira* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
	MAR	7	25.9	23.1	24.9	6.4	19.6	31	*Lauderia annulata*; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		14	4.6	**86.4**	4.2	0.5	4.3	31	*Scripsiella* cf. *trochoidea*
		22	17.7	66.4	15.2	0.5	0.2	15	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
		29	11.8	58.4	11.0	0.4	18.4	17	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
	APR	4	58.9	25.4	6.0	9.7	0.0	20	*Pseudonitzschia* spp.; *Scripsiella* cf. *trochoidea*
		11	22.8	30.8	42.8	2.4	1.3	21	*Leptocylindrus danicus*; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
		18	34.7	6.1	52.3	0.2	6.7	20	*Leptocylindrus danicus*; *Gephyrocapsa* spp.
		26	10.7	17.9	42.3	9.7	19.3	29	*Chaetoceros* spp.; *Scripsiella* cf. *trochoidea*; *Gephyrocapsa* spp.
	MAY	3	10.6	14.4	70.9	0.6	3.4	26	*Chaetoceros* spp.; *Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		10	0.3	26.6	68.9	1.4	2.7	23	*Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*
		17	0.4	40.7	**56.3**	0.6	2.0	20	*Scripsiella* cf. *trochoidea*; *Emiliania huxleyi*

Si:N < 1 and Si:P < 3, occurred a dozen times, in summer 2004 and spring 2005, however, according to the authors op cit, caution must be applied when discussing silicate limitation in marine environments. Tagus river flow seems to be a strong influence on phytoplankton temporal distribution however, the action of upwelling waters in this region can not be discarded.

The other phytoplankton groups not identified under the microscope as well as euglenophytes seemed to prefer more stable situations, especially during summer, when the higher abundances were recorded. Within Tagus estuary, Gameiro et al. (2007), registered higher abundances of euglenophytes during this season.

5. Conclusions

The pigments detected under the HPLC showed a good correlation with phytoplankton identifications with maxima ($\mu g\ l^{-1}$) coincident with the higher phytoplankton cell counts. Fucoxantin, peridinin and 19′-hexanoyloxyfucoxanthin appeared as good indicators, for diatoms, dinoflagellates and coccolithophores, respectively, with synchronized seasonal variations and significant positive correlations. Furthermore, the chemotaxonomic analysis had the capacity of quantifying concentrations of biomarker pigments and recognizing the presence of phytoplankton taxa that were difficult to identify and enumerate by microscopy such as cryptophytes, prasinophytes, chlorophytes and cyanobacteria. These groups face problems mainly concerned with their small size making the HPLC approach an accurate tool to access and describe the total phytoplankton biomass. The pigment methodology was a helpful and faster way of analyze larger changes of the phytoplankton community with relatively much less effort compared to microscopic studies. However, these studies revealed changes within phytoplankton groups and allowed us to recognize small scale variations on species succession and an accurate characterization of total biomass and species composition. Microscopic analyses are crucial to an exact assignment of marker pigments to phytoplankton taxa and thus permit a reliable study of phytoplankton community structure and dynamics.

Acknowledgements

This work was supported by projects QCAIII- POPesca MARE "Caracterização ecológica da zona costeira – Plataforma Continental" and PROFIT – "Interdisciplinary study of oceanographic processes underlying the phytoplankton dynamics in the Portuguese upwelling system", PDCTE/CTA/50386/2003. C.R. Mendes grant and the HPLC analyses were supported by INTAGUS (POCTI/MAR/58022/2004). We thank M. Falcão for performing all nutrient analysis. We also thank to A. Morais, T. Quental and F. Quintela for all the assistance during the sampling period. We are grateful to T. Moita for the helpful comments to the manuscript.

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