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Temporal variation in phytoplankton assemblages and pigment composition at a fixed station of the Ría of Pontevedra (NW Spain)

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Abstract

Phytoplankton composition and abundance were studied at a fixed station (P2, Ría of Pontevedra, NW Spain) weekly during a 2-year period (1999–2000). In addition to microscopic cell counts, a chemotaxonomic approach based on HPLC pigment analysis and CHEMTAX data processing was studied on two size classes. The contribution of the picoplankton fraction to the total chlorophyll (chl) *a* averaged 13 ± 10%. Pigment suites of the picoplankton fraction were mainly provided by picoeukaryotes. Chl *b* dominated in the picoplankton whereas chls *c* (c_2, c_1 and c_3) were the major accessory chlorophylls in the micro-nanoplankton. Despite this, fucoxanthin was by far the most abundant carotenoid in both size classes (often >70% of total carotenoids). Major 'pigment groups' in the picoplankton were 'prasinophytes' (with prasinoxanthin and carotenoids of the uriolide series) and 'chlorophytes', which contributed up to 60% total chl *a* during winter. 'Diatoms' and 'haptophytes' were other relevant picoplanktonic groups along the seasonal cycle. Micro-nanoplankton was dominated by 'diatoms I' (chl c_1 and chl c_2) and 'diatoms II' (chl c_3 and chl c_2), which contributed up to 70% of total chl *a* in spring. Chl *c* composition during diatom blooms exhibited higher chl c_1 : chl c_2 ratios in winter–spring and higher chl c_3 : chl c_2 ratios in summer–autumn.

Keywords: photosynthetic pigments; HPLC; size structure; picoplankton; Galician Rías; CHEMTAX

1. Introduction

Classical studies on phytoplankton succession in the Galician Rías (Margalef, Durán, & Saiz, 1955) describe a change from 'diatoms' to 'dinoflagellates', related to prevailing upwelling conditions mainly from May to October, due to the influence of southward winds (Fraga, 1981; Fraga, Mouriño, & Manríquez, 1982). Phytoplankton blooms in the Galician Rías occur mainly in spring and autumn, although the highest phytoplankton biomass is usually observed during summer due to the effect of upwelling and downwelling cycles (Varela, Díaz del Río, Álvarez-Osorio, & Costas, 1991). During upwelling, primary production and phytoplankton biomass in the Rías are dominated by large-sized cells, mainly diatoms (Bode, Casas, & Varela, 1994; Tilstone, Figueiras, Fermín, & Arbones, 1999). Once upwelling ceases, nutrients become exhausted in surface waters and the community becomes dominated by dinoflagellates and microflagellates (Pazos, Figueiras, Álvarez-Salgado, & Rosón, 1995). Superimposed on this cycle, there are shorter ecological successions which last an upwelling cycle (Blanco, Moroño, Pazos, Maneiro, & Mariño, 1998; Pazos et al., 1995; Tilstone, Figueiras, & Fraga, 1994). The high primary productivity of the Galician Rías (up to 3690 mg C m^{-2} d^{-1} in the Ría of Vigo; Tilstone et al., 1999) is attributed to nutrient enrichment from coastal upwelling (Álvarez-Salgado, Rosón, Pérez, Figueiras, & Pazos, 1996; Álvarez-Salgado, Rosón, Pérez, & Pazos,

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1993), regenerative processes inside the Rías and/or on the continental shelf (Varela, 1992) and run-off along the coast (Nogueira, Pérez, & Ríos, 1997).

In spite of this large body of knowledge of phytoplankton ecology in the Rías, very little is known about the composition of the small-sized phytoplankton (i.e. picoplankton) and its temporal patterns within the annual cycle. In this paper the temporal variability in composition and abundance within two phytoplankton size classes (micro-nanoplankton and picoplankton) is presented a fixed station (P2, Ría of Pontevedra) using HPLC pigment analysis. Pigment data were processed by means of CHEMTAX program to estimate 'pigment groups' in both size classes.

2. Materials and methods

2.1. Study site

A single sampling station located in the Ría of Pontevedra (station P2, 42°21.40′N, 8°46.42′W, see Fig. 1) was sampled weekly over a 2-year period (1999–2000) as part of the Galician HAB monitoring programme performed by the Centro do Control do Medio Mariño (CCMM; Mariño, Maneiro, & Blanco, 1998) on board *R.V. Jose Maria Navaz*. A CTD profiler (Sealogger, CTD Sea Bird 25) was employed to obtain conductivity and temperature data. Seawater samples from three depths were analysed in a semi continuous flow analytical system, Bran + Luebbe TRACCS 800, to obtain the concentration of nutrients. The upwelling index (I_w), which represents the flow of upwelled water by coastal kilometers, was calculated from the wind data, as described by Wooster, Bakun, and McClain (1976). Negative I_w values represent downwelling events.

2.2. Phytoplankton identification

Seawater samples for phytoplankton cell counts and spectrofluorometric pigment analysis were collected simultaneously from the water column using a PVC hose (Lindahl, 1986) divided in three sections: 0–5, 5–10, and 10–15 m. Cell count and identification were performed from an integrated water sample (0–15 m depth) by mixing equal volumes from each hose section. Samples were preserved in Lugol's iodine solution, and sedimented in Utermöhl's chambers (25 ml) for at least 12 h. Cell counts were obtained using an inverted microscope (Nikon Diaphot TMD). The whole bottom of the chamber was examined at 10× to identify the largest, and less abundant, organisms, but a single diameter at 20× and 40× to identify the smallest and usually more abundant organisms.

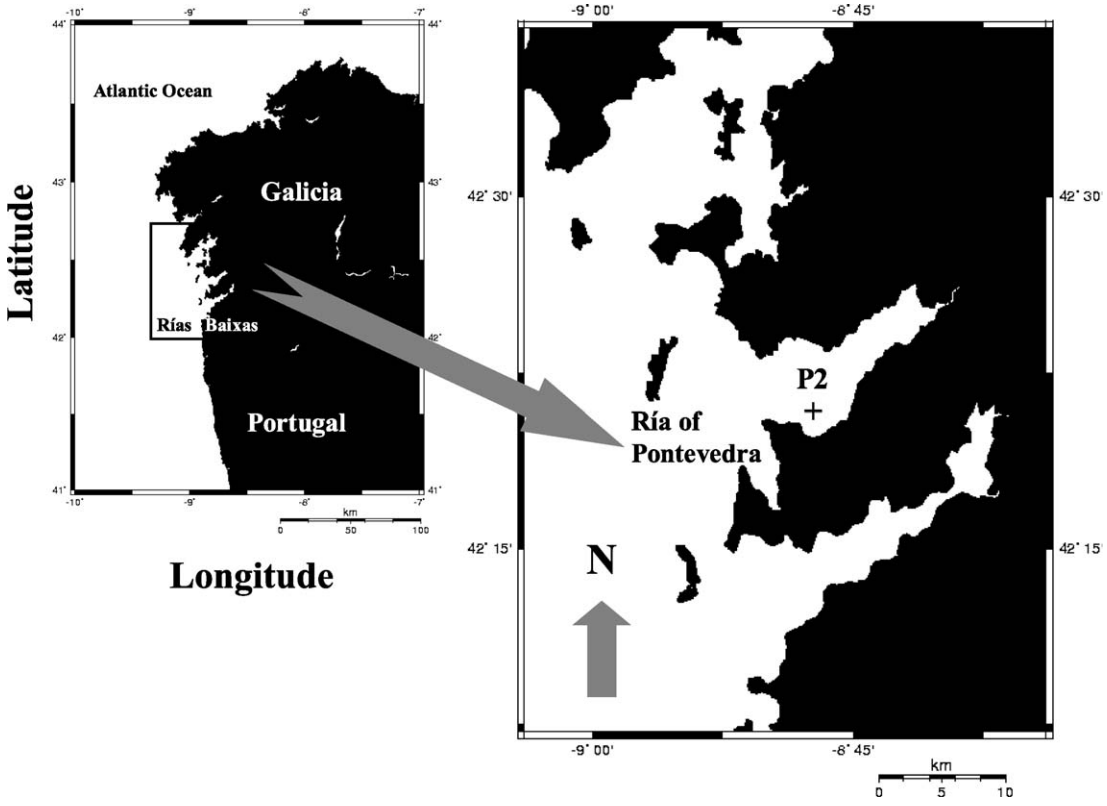

Fig. 1. Location of sampling station P2 in the Ría of Pontevedra (Galicia, NW Spain).

2.3. HPLC pigment analysis and in vivo fluorescence

Seawater samples (1.5 l) obtained from integrated profiles (0–15 m) were filtered through 47 mm diameter Whatman GF/D and GF/F filters (under vacuum pressure lower than 75 mmHg) and stored at −20 °C until analysis. An aliquot (20 ml) of each phytoplankton sample was employed to obtain in vivo fluorescence measurements (Turner Designs Fluorometer) on the initial sample and after the consecutive filtration steps through GF/D and GF/F. Samples were dark acclimated for almost 2 h before fluorescence measurements. Two phytoplankton size classes were operationally defined: (i) micro-nanoplankton, constituted by organisms retained onto a GF/D filter (2.7 μm nominal pore size) and (ii) picoplankton, constituted by organisms passing through a GF/D but retained onto GF/F filters (0.7 μm nominal pore size). Frozen filters were extracted in variable volumes (3.5–6 ml) of 95% methanol using a spatula for filter grinding and further sonication during 5 min at low temperature (~5 °C). Extracts were then filtered through Whatman GF/F filters to remove cell and filter debris. An aliquot (1 ml) of the methanol extract was mixed with 0.4 ml of Milli-Q water to avoid peak distortion (Zapata & Garrido, 1991). A volume of 200 μl was injected immediately after the water addition to avoid losses of pigments (Latasa et al., 2001). HPLC equipment was a Waters Alliance System consisting of a 2690 separations module and a 996 photodiode array detector interfaced with a 474 scanning fluorescence detector by a Sat/in analog interface. Pigment separation was performed by HPLC according to Zapata, Rodríguez, and Garrido (2000). The stationary phase was a C_8 column (Waters Symmetry 150 × 4.6 mm, 3.5 μm particle size, 100 Å pore size) thermostated at 25 °C by means of a refrigerated circulator water bath (Neslab RTE-200). Mobile phases were—A = methanol : acetonitrile : aqueous pyridine solution (0.25 M pyridine, pH adjusted to 5.0 with acetic acid) (50 : 25 : 25 v/v/v), and B = acetonitrile : methanol : acetone (60 : 20 : 20 v/v/v). A linear gradient from 0 to 40% B was pumped for 22 min, followed by an increase to 95% at 28 min and isocratic hold at 95% B for a further 12 min. Initial conditions were reestablished by reversed linear gradient. Flow rate was 1 ml min^{-1}. Chlorophylls and carotenoids were detected by diode-array spectroscopy (350–750 nm). Chlorophylls were also detected by fluorescence (excitation and emission wavelengths were 440 and 650 nm, respectively). Pigments were identified by co-chromatography with authentic standards (see Zapata et al., 2000) and by diode-array spectroscopy (wavelength range: 350–750 nm, spectral resolution: 1.2 nm). Each peak was checked for spectral homogeneity using the Waters Millennium^{32} software algorithms, and the absorption spectrum was compared with a spectral library previously created. Pigments were quantified by using external standards and extinction coefficients compiled by Jeffrey (1997).

2.4. CHEMTAX analysis

HPLC pigment data of each size-fraction were processed by means of Chemical Taxonomy program (CHEMTAX) developed by Mackey, Mackey, Higgins, and Wright (1996). Eight pigment groups were defined in the micro-nanoplankton fraction, and seven in the picoplankton fraction. These pigment groups were defined on base of pigment composition and pigment ratios normalized to chlorophyll *a* (chl *a*; pigment : chl *a*) of phytoplankton species listed in Table 1. It must be remembered that pigment groups do not match exactly with taxonomic phytoplankton classes. Therefore, in some cases a pigment group may be composed of several taxonomic classes.

3. Results

3.1. Hydrographic data

Hydrographic conditions in station P2 during 1999–2000 can be summarized as follows: between November and March vertical mixing in the water column and high nutrient concentrations (up to 15 μM $NO_3 l^{-1}$ and μ16 μM $SiO_3 l^{-1}$) was observed. In March–April thermal stratification developed followed by a decrease in nutrient concentrations. Successive upwelling and downwelling events were observed until October. The most intense upwelling episodes were detected between April and September. During April 1999 and 2000 negative I_w values, northward winds, high precipitations and a significant decrease of salinity in surface waters (weekly report from CCMM) were observed. This situation occurred before the upwelling in May, when southward winds, lower precipitations and increases in phytoplankton biomass (mainly diatoms) were detected.

3.2. Phytoplankton composition

3.2.1. Diatoms

The highest abundance of diatoms was observed between May and October (Fig. 2A), the most abundant species being *Chaetoceros socialis*. The spring bloom at the end of May 1999 (5.5×10^6 cells l^{-1}) was dominated by *C. socialis* and *Skeletonema costatum* (65 and 15% total diatom abundance, respectively). During the spring bloom in June 2000 (3.5×10^6 cells l^{-1}) *C. socialis* reached up to 96% of diatom abundance. The maxima densities in summer (July 1999 and September 2000) were contributed by *Leptocylindrus danicus* (up to 95%

Table 1
Initial and final pigment: chl *a* ratios calculated by CHEMTAX in the micro-nanoplankton and picoplankton fractions

	chl c_3	chl c_2	chl c_1	Perid	But-fuco	Fuco	Pras	Hex-fuco	Viola	Allo	Zea	chl *b*
Input matrix nano-microplankton												
Clorophytes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000		0.672
Cryptophytes	0.000	0.212	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.388		0.000
Diatoms	0.000	0.165	0.111	0.000	0.000	0.546	0.000	0.000	0.000	0.000		0.000
Diatoms II	0.116	0.299	0.000	0.000	0.000	0.777	0.000	0.000	0.000	0.000		0.000
Dinoflagellates	0.000	0.211	0.000	0.452	0.000	0.000	0.000	0.000	0.000	0.000		0.000
Haptophytes	0.086	0.208	0.000	0.000	0.000	0.194	0.000	0.546	0.000	0.000		0.000
Pelagophytes	0.153	0.316	0.000	0.000	0.761	0.162	0.000	0.000	0.000	0.000		0.000
Prasinophytes	0.000	0.000	0.000	0.000	0.000	0.000	0.216	0.000	0.000	0.000		0.445
Output matrix nano-microplankton												
Clorophytes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000		0.672
Cryptophytes	0.000	0.212	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.395		0.000
Diatoms	0.000	0.165	0.070	0.000	0.000	0.546	0.000	0.000	0.000	0.000		0.000
Diatoms II	0.267	0.375	0.000	0.000	0.000	0.933	0.000	0.000	0.000	0.000		0.000
Dinoflagellates	0.000	0.210	0.000	0.489	0.000	0.000	0.000	0.000	0.000	0.000		0.000
Haptophytes	0.086	0.208	0.000	0.000	0.000	0.190	0.000	0.546	0.000	0.000		0.000
Pelagophytes	0.143	0.305	0.000	0.000	0.729	0.151	0.000	0.000	0.000	0.000		0.000
Prasinophytes	0.000	0.000	0.000	0.000	0.000	0.000	0.201	0.000	0.000	0.000		0.464
Input matrix picoplankton												
Chlorophytes	0.000	0.000	0.000		0.000	0.000	0.000	0.000		0.000	0.000	0.843
Cyanobacteria	0.000	0.000	0.000		0.000	0.000	0.000	0.000		0.000	0.846	0.000
Cryptophytes	0.000	0.102	0.000		0.000	0.000	0.000	0.000		0.187	0.000	0.000
Diatoms	0.000	0.148	0.013		0.000	0.584	0.000	0.000		0.000	0.000	0.000
Haptophytes	0.123	0.170	0.000		0.058	0.398	0.000	0.384		0.000	0.000	0.000
Pelagophytes	0.154	0.316	0.000		0.725	0.162	0.000	0.000		0.000	0.000	0.000
Prasinophytes	0.000	0.000	0.000		0.000	0.000	0.174	0.000		0.000	0.432	0.657
Output matrix picoplankton												
Chlorophytes	0.000	0.000	0.000		0.000	0.000	0.000	0.000		0.000	0.000	0.843
Cyanobacteria	0.000	0.000	0.000		0.000	0.000	0.000	0.000		0.000	0.846	0.000
Cryptophytes	0.000	0.102	0.000		0.000	0.000	0.000	0.000		0.187	0.000	0.000
Diatoms	0.000	0.148	0.013		0.000	0.547	0.000	0.000		0.000	0.000	0.000
Haptophytes	0.123	0.170	0.000		0.058	0.398	0.000	0.384		0.000	0.000	0.000
Pelagophytes	0.149	0.289	0.000		0.231	0.162	0.000	0.000		0.000	0.000	0.000
Prasinophytes	0.000	0.000	0.000		0.000	0.000	0.188	0.000		0.000	0.072	0.678

total diatom abundance). Other secondary maxima were those of *Guinardia striata* (= *Rhizosolenia stolterfothii*) (June 1999), *Pseudo-nitzschia* g. *delicatissima* (transapical diameter <3 μm: *P. delicatissima, P. pseudodelicatissima, P. cuspidata;* Skov et al., 1999) (October 1999), *Chaetoceros* spp. (July 2000), and *Pseudo-nitzschia* spp. (transapical diameter >3 μm: *P. australis, P. fraudulenta*; Skov et al., 1999). (October 2000). The dominant species during winter were *Chaetoceros curvisetum*, *C. socialis*, *Nitzschia longissima*, *Thalassiosira rotula* and *S. costatum*.

3.2.2. Dinoflagellates

The highest densities of dinoflagellates in 1999 (Fig. 2B) were observed in June (2.5×10^3 cells l^{-1}, *Prorocentrum micans* and *Amphidinium Curvatum*) and August (4×10^4 cells l^{-1}, *Dinophysis* spp.). In 2000, three maxima were detected, the first in April–May (9×10^4 cells l^{-1}, dominated by *Ceratium lineatum*) and the two later in September (1×10^5 cells l^{-1}, dominated by *Gymnodinium* sp. and *Scrippsiella trochoidea*) and October (6.5×10^4 cells l^{-1}, *Scrippsiella trochoidea* and *Dinophysis* spp.).

3.2.3. Other phytoplankton groups

The silicoflagellate *Dictyocha speculum* appeared almost only in spring (April–May) during both years studied. Low densities of euglenophyceans (*Eutreptiella* sp.) were also registered (maximum in summer 1999, 1.6×10^4 cells ml^{-1}). In September 1999–2000, the highest densities of the raphidophycean *Heterosigma akashiwo* ($\approx 0.7 \times 10^4$ cells ml^{-1}) were detected. The most abundant group was the unknown microflagellates (<5 μm), constituted by pico-nanoeukaryotes probably belonging to algal classes as 'chlorophytes', 'prasinophytes', prymnesiophytes and chrysophytes, among others. Microflagellates exhibited a maximum on 3 May 1999 (4×10^6 cells ml^{-1}) much higher than those densities observed along the study ($<2 \times 10^6$ cells ml^{-1}). On this date thermohaline stratification was observed in the upper part of the water column after a period without precipitation

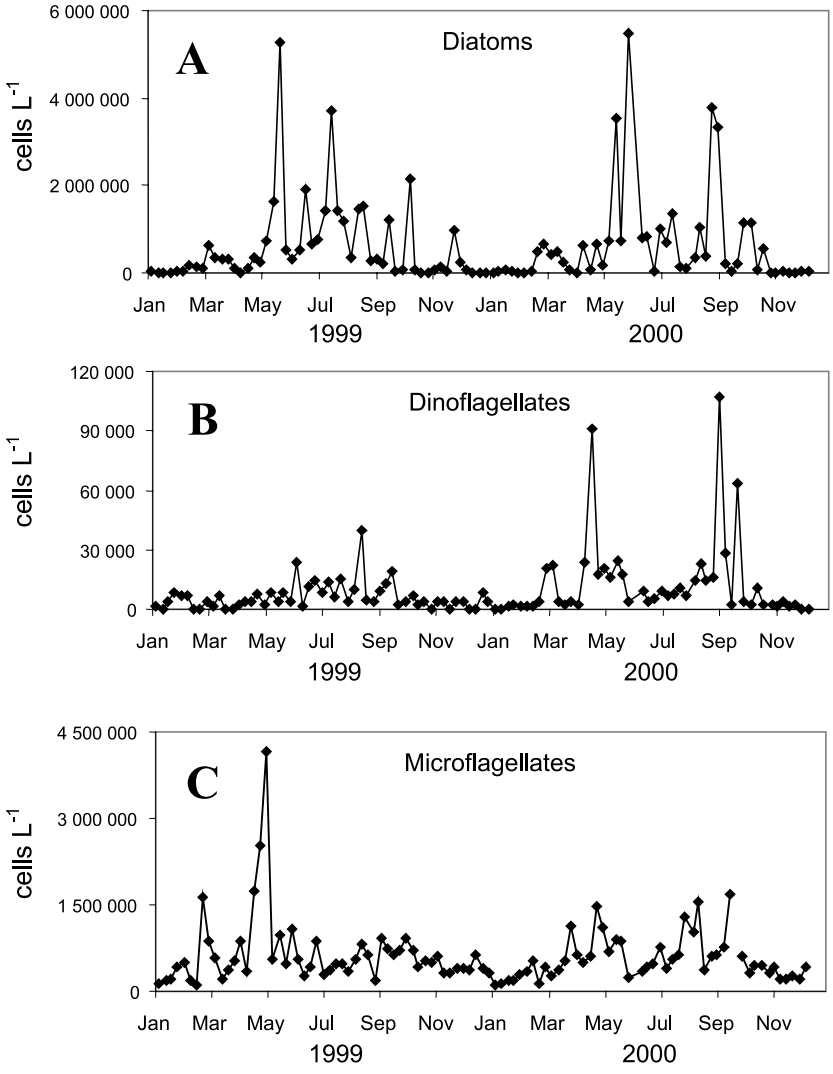

Fig. 2. Temporal distribution of abundance (cells l^{-1}) of (A) diatoms, (B) dinoflagellates and (C) microflagellates.

(CCMM weekly report data) and mixing conditions in the water column.

3.3. *Picoplankton: HPLC chl a vs.* in vivo *fluorescence*

The average contribution of picoplankton to total HPLC chl *a* during 1999–2000 was $13 \pm 10\%$, ranging from 0.5 to 56.5% throughout the period studied (Fig. 3A). The relative contribution of picoplankton shows a negative trend when plotted against increasing total chl *a* values (Fig. 3B). The highest contribution of picoplankton to total chl *a* was observed in winter (November–February: $23 \pm 11\%$), whereas the lowest values occurred in spring (March–June: $10 \pm 7\%$), and summer–autumn (July–October: $8 \pm 5\%$). During winter, total chl *a* (325 ± 300 ng l^{-1}) was markedly lower in comparison with spring (2300 ± 1250 ng l^{-1}) and summer–autumn (1140 ± 900 ng l^{-1}). On the other hand, in vivo fluorescence showed higher contribution of picoplankton in the annual average ($20 \pm 10\%$ total fluorescence), with maxima during winter ($30 \pm 10\%$) and minima in spring and summer–autumn seasons ($14 \pm 8\%$).

3.4. *HPLC pigment composition of micro-nanoplankton*

The pigment ratios chl *a*:*c* and chl *a*:*b* in the micro-nanoplankton were 3 and 20, respectively (Table 2). Polar chls *c* detected were: chl c_2, c_3, c_1, chl $c_{2\text{-like}}$ *P. gyrans-type* (Fawley, 1988) and traces of Mg-3,8-divinyl-pheoporphyrin a_5 monomethyl ester (MgDVP). In addition, two non-polar chl *c*-like pigments with chromatographic properties similar to chl c_2-MGDG [18:4/14:0] and chl c_2-MGDG [14:0/14:0] were detected. The main chl *a* derivative observed was chlorophyllide (chlide) *a*, mainly associated with diatom blooms (specially in autumn 1999 and spring

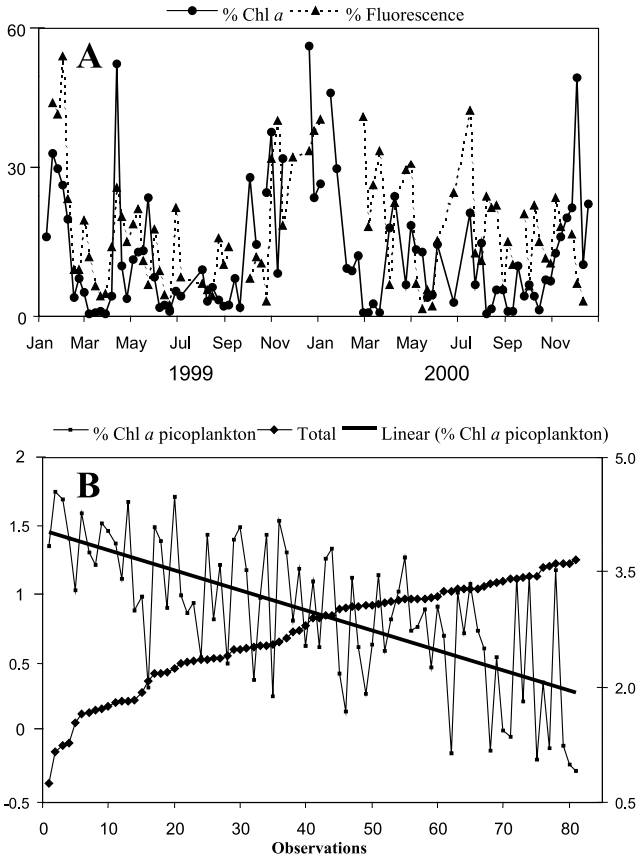

Fig. 3. Temporal variation of (A) % chl *a* in the picoplankton fraction determined by HPLC vs. % in vivo fluorescence contributed by picoplankton and (B) % chl *a* in the picoplankton fraction ($\log_{10}$) determined by HPLC against ordered ($\log_{10}$) total chl *a* values.

2000). The major carotenoid was fucoxanthin, whereas diadinoxanthin, 19′-hexanoyloxyfucoxanthin, peridinin and alloxanthin occurred in much lower amounts. The average ratio of chl *a* to fucoxanthin plus its derivatives was 2 : 1 (fucoxanthin contributed ca. 80% to the overall carotenoid pool).

3.5. Temporal variability of HPLC pigments in the micro-nanoplankton

The highest chl *a* value detected during the study period was 7073 ng l^{-1} in May 1999 (see Fig. 4A), which corresponded with a bloom of *Chaetoceros socialis* and other diatom species (*Navicula* spp., *Pseudo-nitzschia* spp.). Other chl *a* maxima were registered in summer and autumn 1999 associated with *Leptocylindrus danicus* (June–July) and *Pseudo-nitzschia* g. *delicatissima* and *Skeletonema costatum* (October). In 2000 successive chl *a* maxima between March and September were observed, increasing from 4000 to 6000 ng l^{-1}, respectively. Chls *c* values were higher during the 1999 spring bloom than in 2000 (Fig. 4A), and both maxima were correlated with diatom abundance (chl c_1: $r = 0.42$, $P < 0.001$, $n = 88$; chl c_2: $r = 0.47$, $P < 0.001$, $n = 88$; chl c_3: $r = 0.34$, $P < 0.005$, $n = 88$). The highest chl *b* levels were observed in August–October 2000, coinciding with increases in microflagellates. Chl *b* was correlated with violaxanthin ($r = 0.75$, $P < 0.001$, $n = 88$). Fucoxanthin values showed a temporal distribution similar to chl *a* (Fig. 4B), and were correlated with diatoms ($r = 0.48$, $P < 0.001$, $n = 88$), whereas 19′-hexanoyloxyfucoxanthin correlated with microflagellates ($r = 0.53$, $P < 0.001$, $n = 88$). Peridinin levels showed a maximum in spring associated with the dinoflagellate *Ceratium lineatum*. Alloxanthin concentration peaked on summer 2000 coinciding with higher abundance of diatoms (*Pseudo-nitzschia* spp., *S. costatum*), dinoflagellates (*Amphidinium flagellans*) and microflagellates. The highest values of prasinoxanthin and violaxanthin were detected in summer 2000 (>30 ng l^{-1}) associated with increases of microflagellates and chl *b* values.

3.6. HPLC pigment composition of picoplankton

Chl *b* and fucoxanthin were the most abundant accessory pigments in this size class (Table 2). The

Table 2
Average concentration (ng l^{-1}) of major chlorphylls and carotenoids detected in the micro-nanoplankton and picoplankton fractions, as well as other minor pigments detected in both size classes

	Chlorophylls (ng l^{-1})					Carotenoids (ng l^{-1})						
	chl *a*	chl *b*	chl c_1	chl c_2	chl c_3	Perid	Pras	But-fuco	Fuco	Hex-fuco	Allo	Zea
Nano-microplankton												
Average	1186	28	57	266	66	33	1	8	654	39	28	0.5
(Min–max)	(4–7073)	(1–285)	(0–1066)	(1–3475)	(0–1062)	(0–545)	(0–30)	(0–67)	(1–8629)	(0–289)	(0–541)	(0–8)
Other minor pigments	MgDVP, chl *c*-*P. gyrans*, chl c_2-MGDG [14 : 0/14 : 0], chl c_2-MGDG [14 : 0/18 : 4]					Diadino, Diato, Peridinol, P-457, Neo, Pras, Viola, 4-k-hex-fuco, Dino, Lut, Monado, Croco, $\beta\varepsilon$-Car, $\beta\beta$-Car						
Picoplankton												
Average	93	21	2	13	4	–	3	2	40	3	0.7	3
(Min–max)	(1–1750)	(0.6–153)	(0–96)	(0–307)	(0–32)		(0–24)	(0–28)	(0–1184)	(0–30)	(0–13)	(0–37)
Other minor pigments	MgDVP, chl c_2-MGDG [14 : 0/14 : 0], chl c_2-MGDG [18 : 4/14 : 0]					Diadino, Diato, Uriolide, Neo, Viola, Micromonol, Micromonal, Lut, Dhlut, lycopene, $\beta\varepsilon$-Car, $\beta\beta$-Car and unknown carotenoid from *Micromonas. pusilla*						

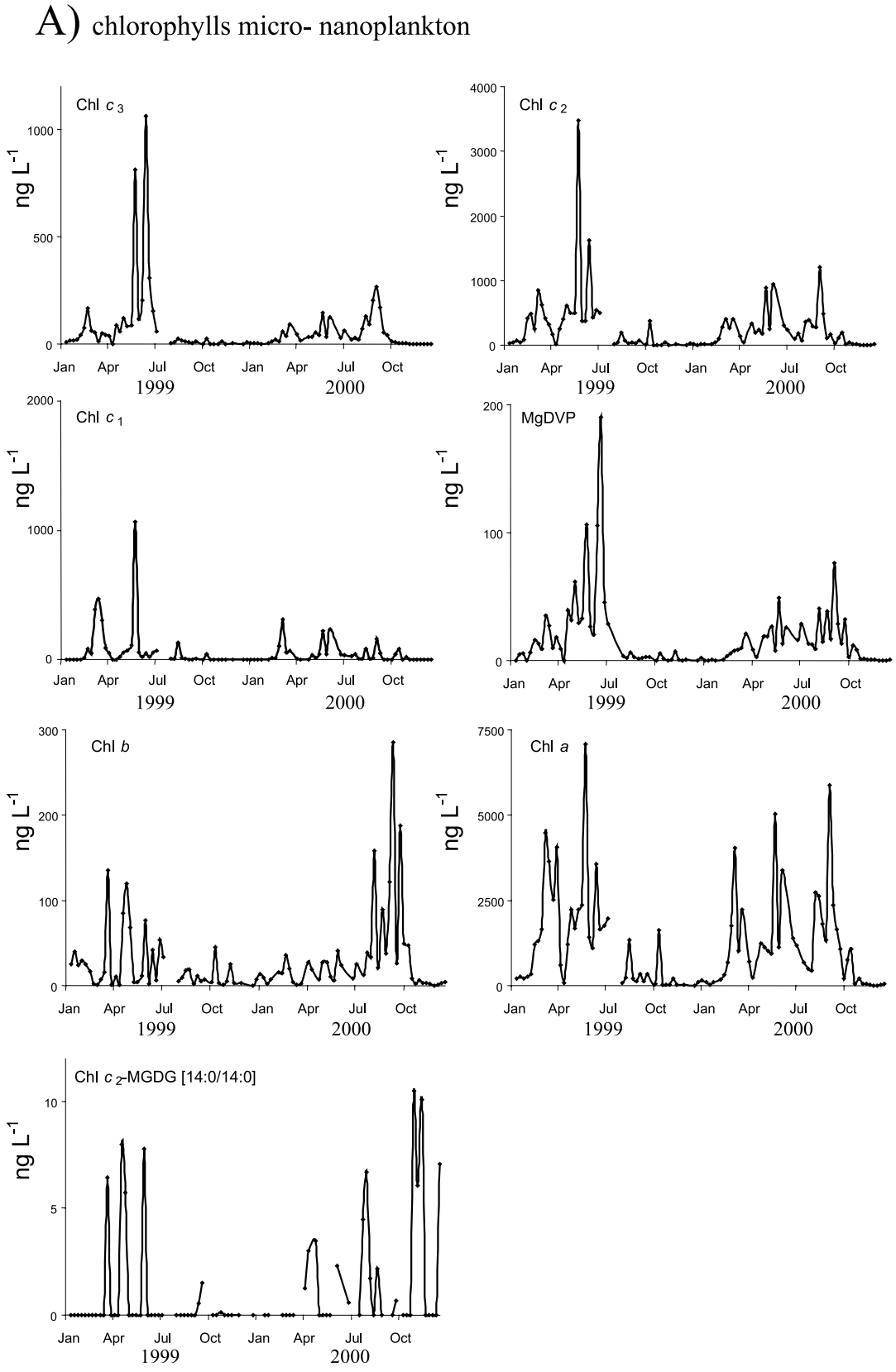

Fig. 4. Temporal distribution of main pigments ($ng\,l^{-1}$) detected in the micro-nanoplankton, (A) chlorophylls and (B) carotenoids.

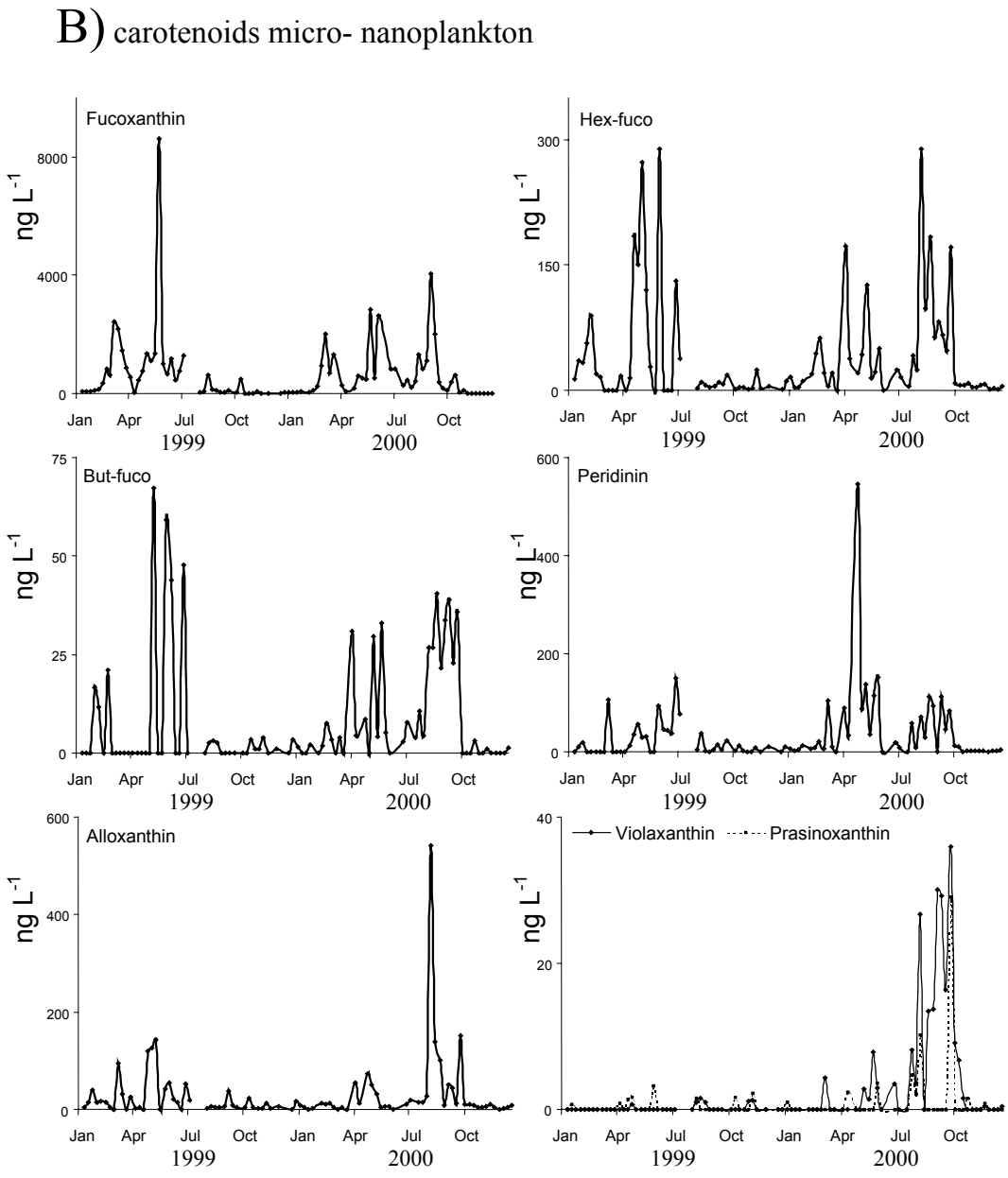

Fig. 4 (*continued*)

pigment ratio chl $a : b = 4$ and chl $a : c = 6$ were fivefold lower and twofold higher than in the micro-nanoplankton, respectively. The most abundant chls *c* were chl c_2 and chl c_3, with lower values of MgDVP, chl c_1 and the chls c_2-MGDG reported in the micro-nanoplankton. Divinyl forms of chl *a* or *b* (the marker pigments for the cyanobacterium *Prochlorococcus marinus*) were not detected during the sampling period. The main derivatives of chl *a* were chlide *a* and two non-fluorescent compounds close eluting chl c_3 and chl *b*, with a single absorption maximum at 430 nm. Fucoxanthin was the major carotenoid in the picoplankton. Minor compounds were 19′-hexanoyloxyfucoxanthin, zeaxanthin, diadinoxanthin, prasinoxanthin and 19′-butanoyloxyfucoxanthin (Table 2).

3.7. Temporal variability of HPLC pigments in the picoplankton

Chl *a* showed its maximum concentration in spring 1999 (1750 ng l^{-1}) coinciding with a chl *a* maximum in the micro-nanoplankton fraction (Fig. 5A). The marked difference between this chl *a* maximum and those values registered during the study was also observed in other pigments such as chl c_2, chl c_1 and fucoxanthin (Fig. 5A, B). This suggests the possibility that pigments belonging

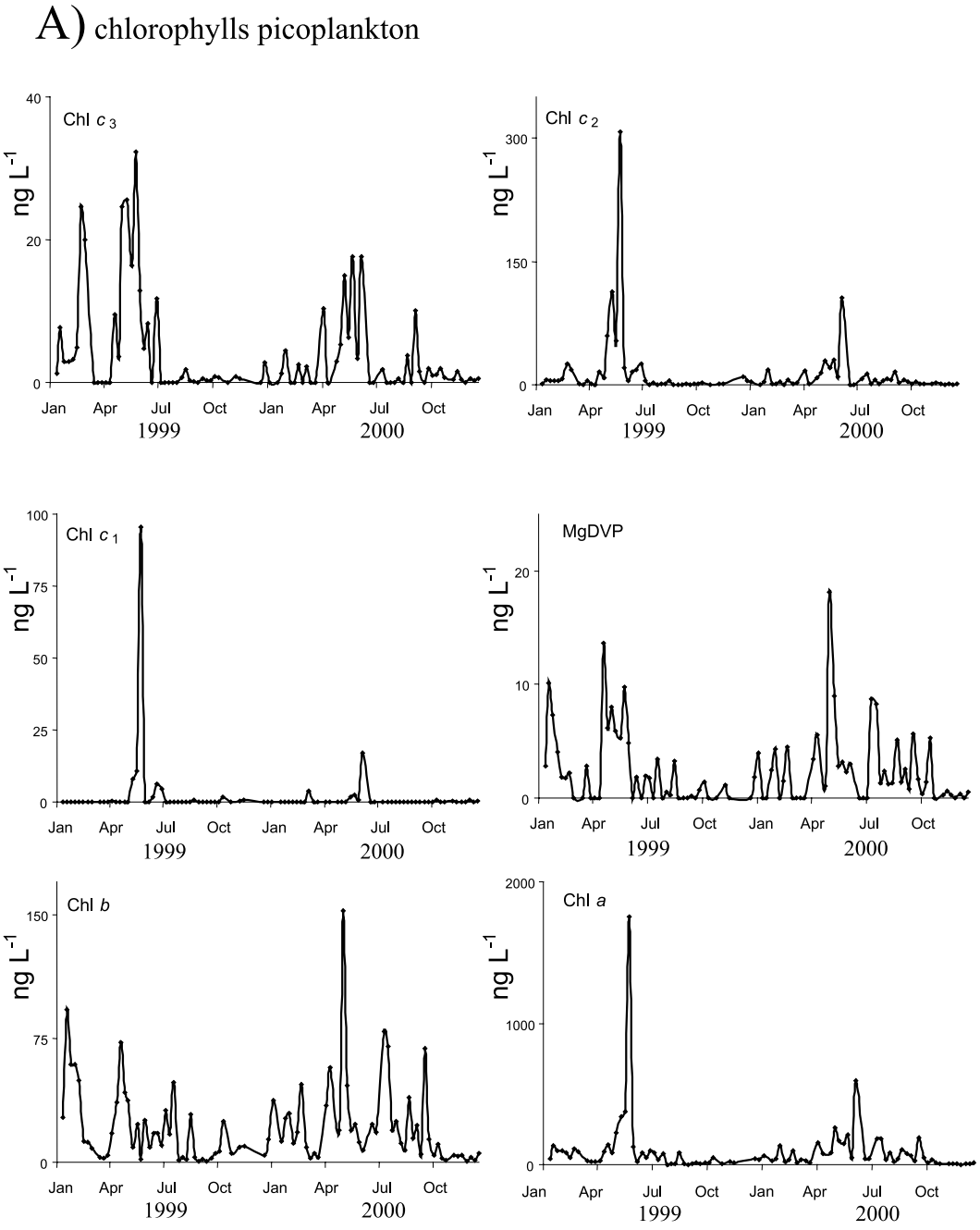

Fig. 5. Temporal distribution of main pigments (ng l^{-1}) detected in the picoplankton (A) chlorophylls and (B) carotenoids.

to the micro-nanoplankton fraction could have passed through the GF/D filter. Integrated (0–15 m) chl *a* concentration measured by spectrofluorometry (1789 ng chl *a* l^{-1}, data not shown) however, agree with the HPLC data, even though both data set were obtained employing different seawater samples taken at 0–5, 5–10 and 10–15 m depths.

The chl *a* values in the picoplankton were correlated with the same pigments in the micro-nanoplankton (chl c_1: $r = 0.94$, $P < 0.001$, $n = 88$; chl c_2: $r = 0.96$, $P < 0.001$, $n = 88$; chl c_3: $r = 0.67$, $P < 0.001$, $n = 88$ and fucoxanthin: $r = 0.97$, $P < 0.001$, $n = 88$), although, it did not display any significant relationships with environmental data. Chl c_3 was also correlated with microflagellate abundance ($r = 0.42$, $P < 0.001$, $n = 88$), as well as fucoxanthin and its derivatives (fucoxanthin: $r = 0.67$, $P < 0.001$, $n = 88$; 19′-butanoyloxyfucoxanthin: $r = 0.69$, $P < 0.001$, $n = 88$ and 19′-hexanoyloxyfucoxanthin: $r = 0.40$, $P < 0.001$, $n = 88$).

Chl *b* showed higher values in January 1999 (90 ng l^{-1}), and just before the diatom bloom in May 2000 (150 ng l^{-1}). Chl *b* was correlated with neoxanthin ($r = 0.83$, $P < 0.001$, $n = 88$) and prasinoxanthin ($r = 0.90, P < 0.001, n = 88$). Fucoxanthin showed its maxima

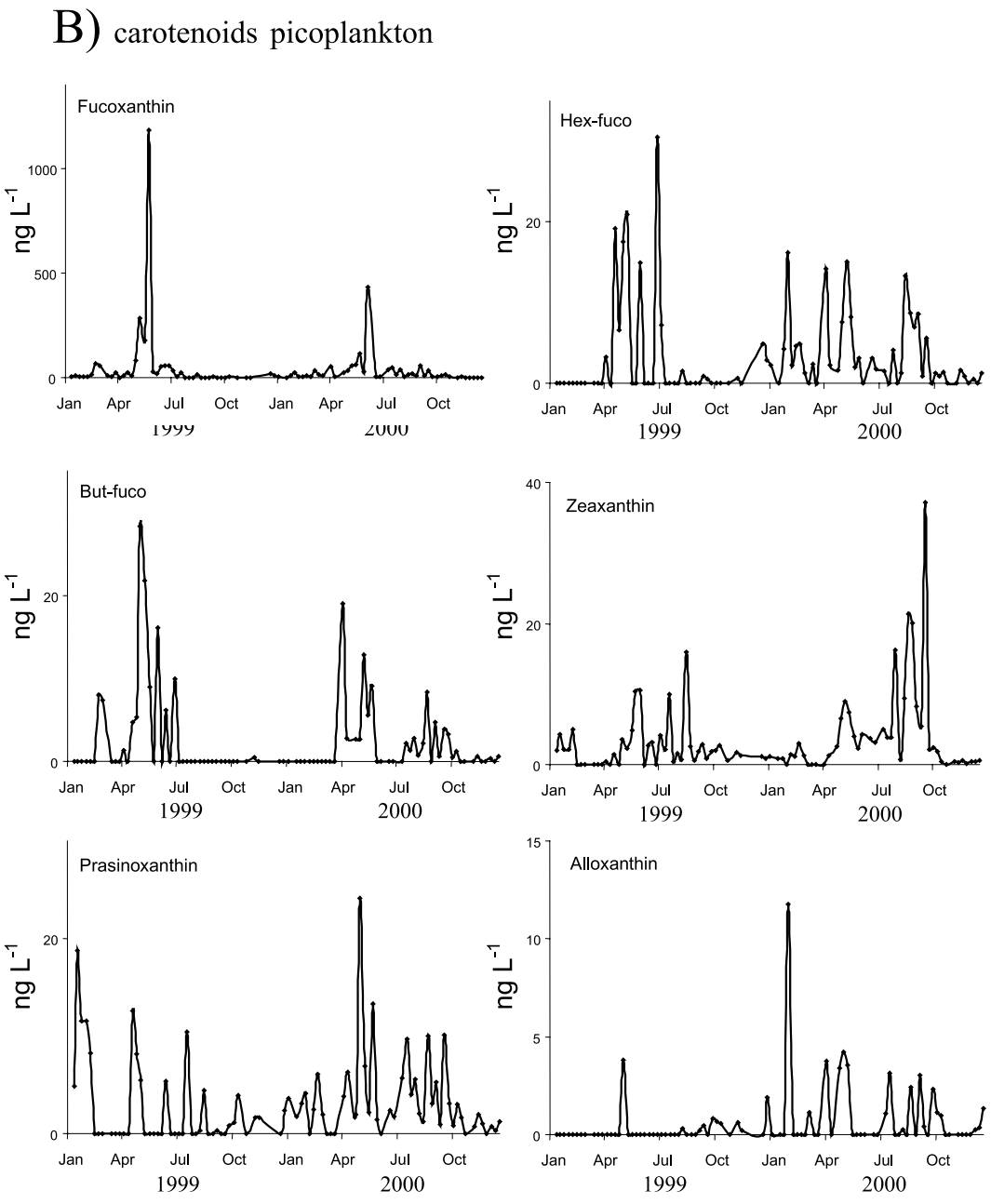

Fig. 5 (*continued*)

concentrations in May–June during both years (Fig. 5B), although the maximum in 1999 was much higher than those observed in the rest of the study. 19′-Butanoyloxyfucoxanthin and 19′-hexanoyloxyfucoxanthin showed similar trends, with higher values in spring 1999 and spring–autumn 2000. Zeaxanthin increased its concentration from spring to summer in both years, reaching its maximum in summer 2000 (40 ng l^{-1}). The most abundant carotenoid related with chl *b* was prasinoxanthin, which showed its maximum concentration in winter 1999 and during the spring bloom in 2000. Low alloxanthin concentrations were detected (<15 ng l^{-1}), the higher values during year 2000.

3.8. Pigment classes obtained by CHEMTAX analysis

3.8.1. Micro-nanoplankton

Table 1 lists the initial and output pigment ratios from the micro-nanoplankton fraction analyzed by CHEMTAX program. The most abundant pigment group in the micro-nanoplankton size class were diatoms. These were analyzed separately as two pigment groups ('diatoms I', with chl c_2 and chl c_1; and 'diatoms II', with chl c_2 and c_3) based on the observed chl *c* composition in characteristic diatom species isolated from the study area. Both pigment groups contributed 60–70% of total chl *a* in spring and summer (Fig. 6). During winter 'haptophytes'

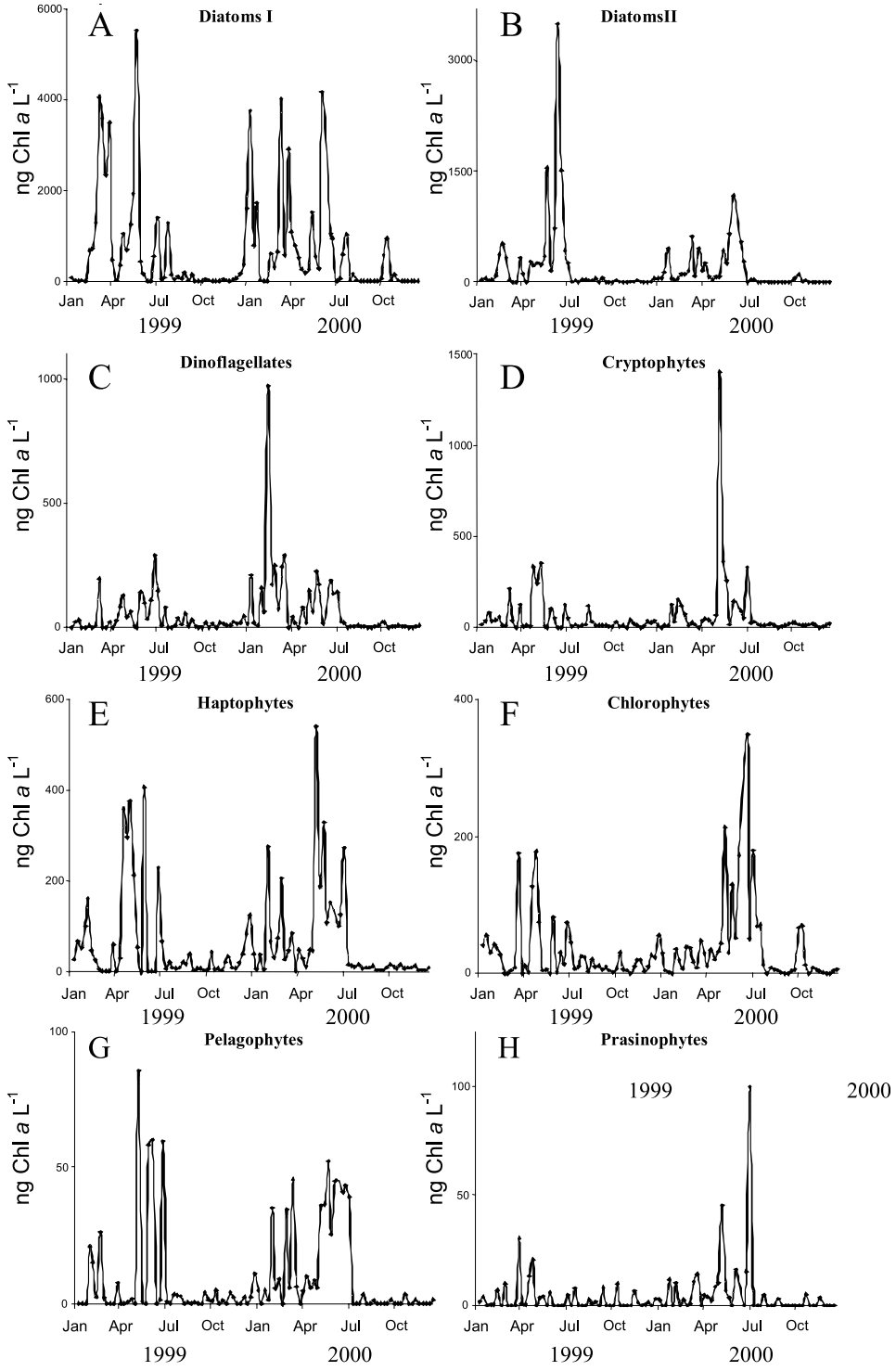

Fig. 6. Temporal distribution of chl *a* ($ng\,l^{-1}$) contributed by pigment groups in the micro-nanoplankton calculated by CHEMTAX program: (A) diatoms I, (B) diatoms II, (C) dinoflagellates, (D) cryptophytes, (E) haptophytes, (F) chlorophytes, (G) pelagophytes and (H) prasinophytes.

and 'cryptophytes' were relatively more abundant, representing 20 and 16% of total chl *a*. The rest of the groups contributed less than 15% to total chl *a* along the study.

Temporal distribution of 'diatoms I' (Fig. 6A) was similar to that of chl *a*, showing their maxima in 1999 at the end of winter and during the spring bloom of *Chaetoceros socialis*. In the year 2000 three chl *a* maxima were observed between February and September, associated mainly with winter diatom species, *C. socialis* and *Leptocylindrus danicus*, respectively. The distribution of 'diatoms II' (Fig. 6B) also shows its maxima in

1999 during the spring bloom, and in June, corresponding with the dominance of *Guinardia striata*. During the year 2000 their relative contribution was lower and their maxima coincided with those of 'diatoms I'. 'Dinoflagellates' (Fig. 6C) show its higher proportion to chl *a* during the *Ceratium lineatum* bloom (April 2000) and other minor maxima in May and August 2000 (*Scrippsiella trochoidea*, *Dinophysis* spp. and *Gymnodinium* sp.). *Cryptophytes* (Fig. 6D) show its maxima in August 2000, and as it was mentioned before, the absence of other alloxanthin-containing organisms seems to confirm that the alloxanthin concentrations registered belong to free-living cryptophytes. Haptophytes (Fig. 6E) presented their maxima contributions to chl *a* in spring 1999 and summer 2000, coinciding with maxima of microflagellates and cryptophytes, respectively. 'Chlorophytes' (Fig. 6F) showed higher values in spring 1999, associated with a maximum in microflagellate abundance, and in summer 2000 coinciding with the increase of cryptophytes and dinoflagellates. 'Pelagophytes' (Fig. 6G) showed its maxima in June 1999 coinciding with haptophytes, and in summer 2000 associated with several pigment groups as diatoms, haptophytes, cryptophytes and dinoflagellates. Prasinophytes (Fig. 6H) were less abundant than chlorophytes and their maxima contribution to chlorophyll *a* was observed in summer 2000.

3.8.2. Picoplankton

The composition of pigment groups shows higher temporal variability in this size class than in the micro-nanoplankton (Fig. 7). In spring, the relative contribution of diatoms is the highest (40% of total chl *a* in diatoms and 30% in chlorophytes + prasinophytes) while 'pelagophytes' and haptophytes reached up to 25% of total chl *a*. By contrast, in winter, chlorophytes and prasinophytes account for 60% of total chl *a*, while diatoms and haptophytes sum up to 25%. During summer, chlorophytes account for 40% of total chl *a* and 'diatoms' only 20%, while 'cyanobacteria' contributed up to 15% of total chl *a*. 'Cryptophytes' were a minor group in the picoplankton fraction, contributing less than 10% to total chl *a* during the studied period.

The temporal distribution of pigment groups shows that diatoms (Fig. 7A) reached its maxima in May 1999 and June 2000, being much higher in the former. Prasinophytes (Fig. 7B) showed its maxima in winter and spring 1999, as well as in spring and summer 2000. 'Chlorophytes' (Fig. 7C) presented a similar temporal distribution, coinciding with the maximum of prasinophytes in spring 2000 and an additional maximum in July 2000. 'Pelagophytes' (Fig. 7D) reached a maximum in May 1999 coinciding with high abundance of microflagellates, and other secondary maxima in April–May 2000 associated with increases in microflagellates. Their maxima were usually associated with decreases in chlorophytes and haptophytes. The latter showed their higher contribution in 1999, corresponding with the maximum of microflagellates in May and after the diatom bloom in June. 'Cyanobacteria' (Fig. 7E) appeared in increasing amounts from spring to summer. 'Cryptophytes' (Fig. 7F) were a minor group and their maxima were observed in winter and spring 2000.

3.9. Chlorophyll c composition during diatom blooms

The CHEMTAX analysis showed that diatoms were the major component in the micro-nanoplankton, i.e. to the overall phytoplankton community as picoplankton averaged $13 \pm 10\%$ annual chl *a*. Selected samples from diatom blooms (defined as those with chl *a* values $>1500\,\mathrm{ng\,l^{-1}}$) showed a pigment composition characterized by chls c_1, c_2, c_3 and fucoxanthin. If these samples are split into three periods, i.e. winter (February–March), spring (April–June) and summer (July–September), the average contribution of 'diatoms I' and 'diatoms II' to total chl *a* is 95, 82 and 75%, respectively (always higher than 60% excepting 7 August 2000, which has not been included as it was dominated by cryptophytes).

In Fig. 8 the pigment ratios chl $c_1 : c_2$ and chl $c_3 : c_2$ in the above mentioned periods are shown. In this figure a significant change can be observed in the chl *c* composition along the year as chl $c_1 : c_2$ decreases 80% (winter, 0.46; summer, 0.10) and chl $c_3 : c_2$ increases twofold (winter, 0.12; summer, 0.30). The highest chl $c_1 : c_2$ ratios were observed in March 1999 and 2000 due to different species from genera *Chaetoceros*, *Nitzschia*, *Thalassiosira* and *Skeletonema*, and maxima chl $c_3 : c_2$ values were registered in June 1999 (*Guinardia striata* and *Leptocylindrus danicus*) and September 2000 (*Proboscia alata* and *L. danicus*).

4. Discussion

The pigment-based interpretation of phytoplankton in separate size classes (Latasa & Bidigare, 1998) allows a description of algal communities which differ in their taxonomic composition, metabolism and ecological function in the planktonic ecosystem (Teira, Serret, & Fernández, 2001). Particularly, the picoplankton has been recently shown to be composed of very diverse phylogenetic groups (Moon-van der Staay et al., 2001), and among them, the picoeukaryotes have been much less studied in comparison with the prokaryotes *Prochlorococcus marinus* and *Synechococcus* spp. In this work, the pigment suites of the picoplankton fraction were mainly contributed by picoeukaryotes. Dominant groups varied from chl *b*-containing organisms (prasinophytes and chlorophytes) throughout summer–winter,

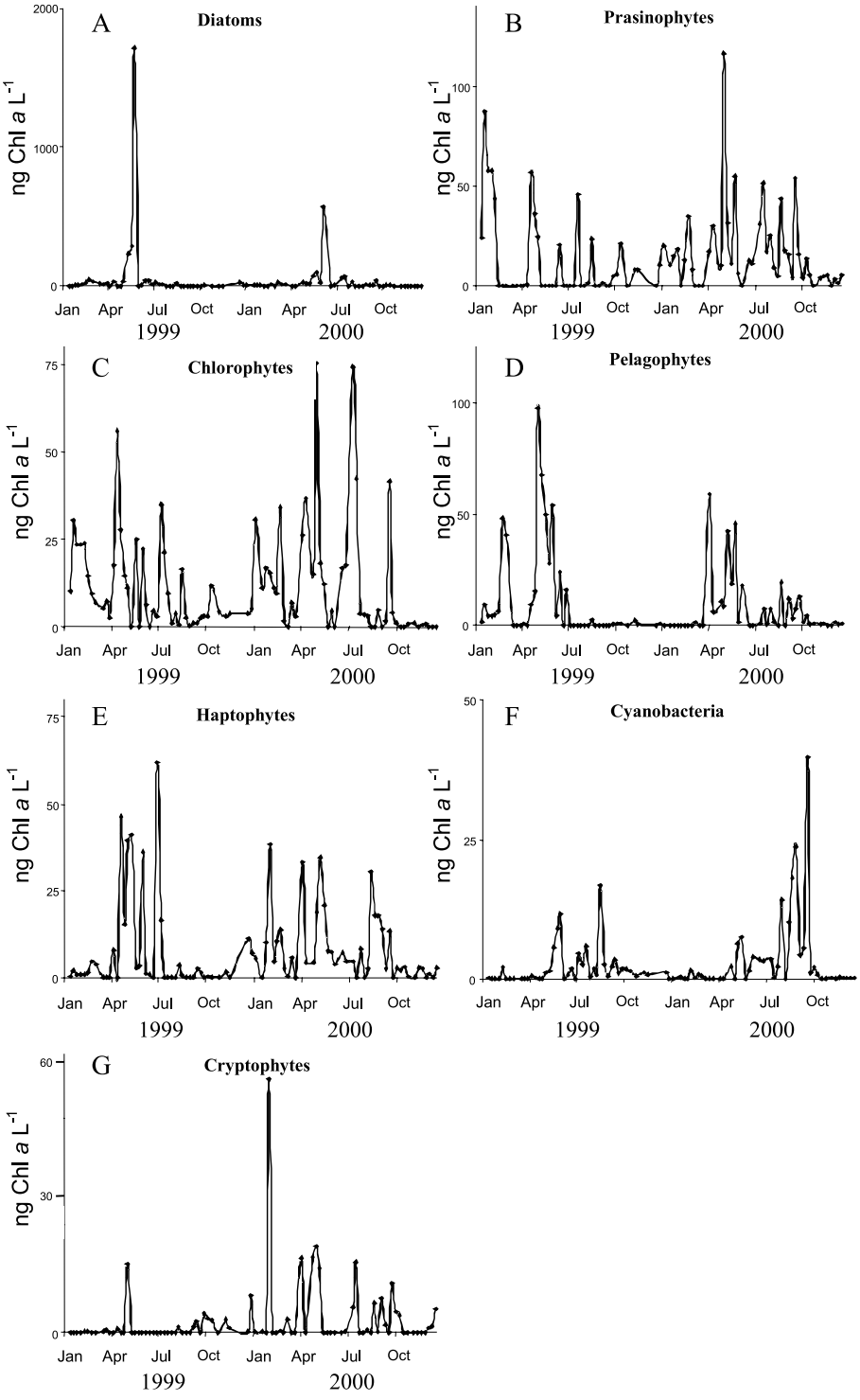

Fig. 7. Temporal distribution of chl *a* ($ng\,l^{-1}$) contributed by pigment groups in the picoplankton calculated by CHEMTAX program: (A) diatoms, (B) prasinophytes, (C) chlorophytes, (D) pelagophytes, (E) haptophytes, (F) cyanobacteria and (G) cryptophytes.

to chl *c*-containing groups in spring. By contrast, in the micro-nanoplankton fraction diatoms (Types I and II) were the most abundant pigment groups throughout the year.

The range of variability of chl *a* in the picoplankton was lower than that observed in the micro-nanoplankton (excepting the maximum of $1.75\,\mu g\,l^{-1}$ in spring 1999, discussed later). Though it shows a similar seasonal distribution (as it suggests the correlation between chl *a* in both size classes), picoplankton does not present a significant relationship with any environmental variable in this study (temperature, salinity and

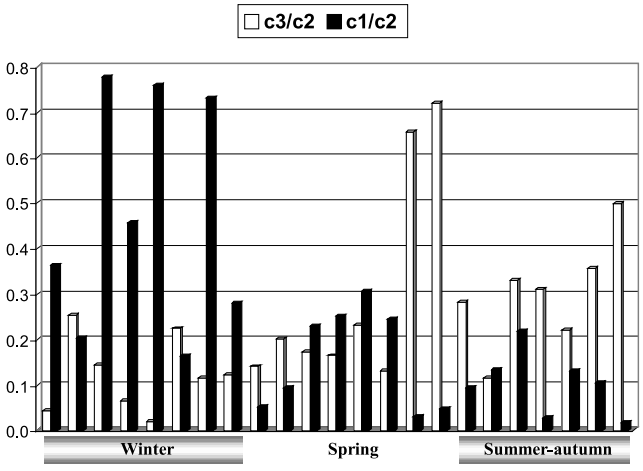

Fig. 8. Chl $c_3 : c_2$ and chl $c_1 : c_2$ ratios observed during proliferations of diatoms (chl *a* values higher than $1.5\,\mu g\,l^{-1}$, and dominance of diatoms estimated by light microscopy and CHEMTAX program), ordered in three seasonal periods (winter 1999–2000, spring 1999–2000 and summer–autumn 1999–2000).

nutrients). In some phytoplankton blooms (May and June 1999–2000) simultaneous maxima of chl *a* in picoplankton and micro-nanoplankton occurred, but in March 1999–2000 and August–September 2000 maxima of picoplankton were registered immediately before and after the maximum of micro-nanoplankton. This latter trend can be due to a faster photosynthetic response of the smaller organisms (pico-nanoplankton) during upwelling episodes (Tilstone et al., 1999).

Agawin, Duarte, and Agustí (2000) analyzed data from different oceanic and coastal regions concluding that the percentage of chl *a* in the picoplankton varies between 10% in high chl *a* areas ($>5\,\mu g\,l^{-1}$) and 50% in oligotrophic areas (chl $a < 0.3\,\mu g\,l^{-1}$). Thus, the range of chl *a* contributed by the picoplankton in this study (average $13 \pm 10\%$ of total chl *a*, ranging from 0.5 to 56%) includes the limits of regions with extreme degrees of productivity. The minimum values of chl *a* were registered during winter, due to lower light intensity and intense vertical mixing which prevents higher growth of phytoplankton (Figueiras & Niell, 1987; Figueiras, Niell, & Zapata, 1985). During this period the highest picoplankton contributions, averaging 25% of total chl *a*, were observed. This agrees with previous results obtained in the coastal shelf off the Rías (Bode et al., 1994) where a larger proportion of nanoplankton (0.8–12 μm) has been reported relative to netplankton (>12 μm) in the winter and summer stratified period. Although, higher contributions of picoplankton during summer relative to those in spring and autumn have not been detected in this study (only two samples in summer 2000 with 18 and 27% of chl *a* in the picoplankton).

A particularly interesting feature, referred to the picoplankton distribution, is the maximum of $1.75\,\mu g$ chl $a\,l^{-1}$ in May 1999, 2 weeks before the diatom bloom of *Chaetoceros socialis*. In the last 5 years (1997–2001) picoplankton at station P2 espectrofluorometric or HPLC values $>1\,\mu g$ chl $a\,l^{-1}$ were not detected. In an extensive review from data series in several oceanographic provinces, Chisholm (1992) determined the upper limits of chl *a* related with phytoplankton size. These values were $0.5\,\mu g$ chl $a\,l^{-1}$ for cells <1 μm, and $1\,\mu g$ chl $a\,l^{-1}$ for cells <3 μm. If this latter value is compared with those of picoplankton at station P2, the maximum of $1.75\,\mu g$ chl $a\,l^{-1}$ is nearly two times higher than that estimate. However, it cannot be discarded that cell debris from larger organisms and/or sexual forms from diatoms could have passed the GF/D filter during the size-fractionation. This last explanation would agree with the similar pigment composition detected in the picoplankton and micro-nanoplankton during the maximum in spring 1999.

Changes observed in chl *c* pigment composition during diatom proliferations suggested that in summer–autumn the pigment pattern interpreted by CHEMTAX as diatoms would include other fucoxanthin-containing groups such as haptophytes, pelagophytes and silicoflagellates which increase the overall chl c_3 to c_1 proportion. Although, it is also hypothesized that in summer–autumn dominant species, as *Guinardia striata* and *Leptocylindrus danicus*, the chl *c* composition would include chl c_3. This hypothesis was confirmed at least in *L. danicus* once a culture was successfully established. In this sense, several authors have also reported the presence of chl c_3 in other diatom species (Stauber & Jeffrey, 1988: *Rhizosolenia setigera*; Richardson, Ciotti, Cullen, & Villareal, 1996: *Rhizosolenia formosa*).

In the micro-nanoplankton both non-polar chls *c* characterized previously in *Emiliania huxleyi* (Garrido, Otero, Maestro, & Zapata, 2000), and the genus *Chrysochromulina* (Zapata, Edvardsen, Rodríguez, Maestro, & Garrido, 2001), respectively, were also detected. This last genus includes some ichtyotoxic species responsible for toxic episodes in Scandinavian coastal regions (*Chrysochromulina polylepis* and *Chrysochromulina leadbeateri*; Edvardsen & Paasche, 1998; Johnsen et al., 1999) but those episodes have not been reported till date in the Galician Rías. A chl *c*-like pigment with retention time close to chl c_3 and absorption spectrum similar to the chl $c_{2\text{-like }P.\ gyrans\text{-type}}$ (Fawley, 1988) was commonly detected at low concentration. Although, the occurrence of this pigment was associated with higher abundance of diatoms and it has been detected previously in cultures of *Pseudo-nitzschia* species (Zapata, Freire, & Garrido, 1998) as well as in *Chaetoceros socialis* and *Leptocylindrus danicus* isolated from the Rías (Rodríguez, personal communication).

Among the chl *b*-containing picoplankton, prasinoxanthin was always associated with carotenoids from the uriolide series (uriolide, micromonol and micromonal), which corresponds with the pigment Type III defined by

Egeland, Guillard, and Liaaen-Jensen (1997). This carotenoid composition has been mainly found in the order Mamiellales (*Micromonas pusilla*, *Mantoniella squamata*, *Bathycoccus prasinos* and the unidentified strain Arousa 2; Egeland et al., 1995, 1997). Thus, species belonging to this order were probably a significant component of the chl *b*-containing picoplankton at station P2. On the other hand, CHEMTAX results and temporal distribution of pigments show that, mainly in the micro-nanoplankton fraction, there is a significant bulk of chl *b*-containing organisms which lack prasinoxanthin. Chlorophytes accounted for these typical chlorophytes with violaxanthin as its major carotenoid, prasinophytes lacking prasinoxanthin and minor groups as euglenophytes, detected in cell counts, with diadinoxanthin and diatoxanthin as main carotenoids.

Fucoxanthin was the dominant carotenoid in both size classes, as it is expected especially in the micro-nanoplankton fraction, where diatoms were the dominant group in the microscopic counts. Although, the presence of chl *c* and fucoxanthin in the picoplankton does not mean the exclusive presence of diatoms and these pigments could be contributed by haptophytes, chrysophytes, pelagophytes, or even bolidophytes. Varela (1992) describes the nanoflagellates (<10 μm) in the coastal shelf of the Rías as mainly constituted by haptophytes (*Isochrysis*, *Diacronema*), chrysophytes (*Ochromonas*), cryptophytes (*Hillea*) and prasinophytes (*Micromonas*). Among the three former groups are included species as *Isochrysis galbana*, *Diacronema vlkianum* and *Ochromonas moestrupii*, whose pigment composition is indistinguishable from diatoms (chl c_1, chl c_2, fucoxanthin, diadinoxanthin and diatoxanthin). Moreover, the lower carotenoid to chl *a* ratios usually found in green algae (Graham & Wilcox, 2000) are also a likely explanation to this dominance of fucoxanthin among the picoplankton fraction characterized by chl *b*-containing groups. All these features show the necessity of more cultured species from picoplankton, characterized by electron microscopy and molecular techniques, in order to improve the chemotaxonomical approach to study the picoplankton composition.

The detection of alloxanthin in the picoplankton fraction may indicate the presence of cryptophytes in this size range, although, to our knowledge, they have not been described (Jeffrey & Vesk, 1997). However, some of the cryptophyte species identified in the Galician coast, as the genus *Hillea* (Varela, 1992), with a lower limit of cellular diameter about 2.5 μm (Tomas, 1993), could have been included in the picoplankton fraction. On the other hand, a possible explanation of alloxanthin in the picoplankton are free chloroplasts from broken cryptophyte cells.

The same differences described in the micro-nanoplankton composition from coastal and oceanic regions can be probably applied to the picoplankton fraction. In the open ocean there are abundant chl *b*-containing groups (as it is also observed in station P2), but unlike in our samples, the dominant carotenoids are generally 19′-butanoyloxyfucoxanthin and 19′-hexanoyloxyfucoxanthin (Letelier et al., 1993; Simon, Barlow, Marie, Partensky, & Vaulot, 1994), while diatoms or pigment diatom-like patterns have not been reported to contribute significantly in these open ocean regions (Guillou, Moon-van der Staay, Claustre, Partensky, & Vaulot, 1999). From this, it is clear that more studies are needed to determine the taxonomic composition of picoplankton among different oceanic and coastal zones.

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