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Using CHEMTAX to evaluate seasonal and interannual dynamics of the phytoplankton community off the South-west coast of Portugal

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ABSTRACT

CHEMTAX was used to assess the relative contribution of the main phytoplankton classes to the total concentration of Chlorophyll *a* (Chl *a*) from the waters off SW coast of Portugal. Sampling campaigns were carried out during all seasons from 2008 to 2012, at three stations located 2, 10 and 18 km from the coast. Samples were taken from the surface, mid-Secchi and Secchi depth, for the determination of Chl *a* and other phytoplanktonic pigments by HPLC. Supporting data were also obtained including dissolved inorganic nutrients, salinity, transparency, temperature and upwelling indices. The CHEMTAX results were also related to microscopy counts and also spectral analysis of absorption of other samples from the same sampling campaigns. The pigment results showed that diatoms dominated from early spring to summer, coinciding with upwelling conditions, while cryptophytes, prymnesiophytes and prasinophytes dominated in autumn and winter, coinciding with seasonal stratification. Although the contribution of cyanobacteria to total Chl *a* was generally low, there were occasional sampling campaigns where it was exceptionally high, but these appeared not to be related to upwelling. Dinoflagellates and chrysophytes were minority groups although the pigment marker peridinin that was used to distinguish dinoflagellates was not adequate for distinguishing all the members of this group. CHEMTAX was particularly useful for discriminating between the smaller (0–20 μm) classes of the microplankton that could not be easily identified by microscopy.

1. Introduction

Phytoplankton is a fundamental component of the marine food web, and is largely responsible for primary productivity in oceanic environments. Continuous monitoring of the dynamics of phytoplankton assemblages is required because of its obvious impact on fisheries and aquaculture industries, and also for its link to climate change scenarios (Sathyendranath et al., 1991; Malin et al., 1992; Moisan et al., 2012).

Chlorophyll *a* (Chl *a*) has been used as the main indicator and proxy for phytoplankton biomass in coastal and oceanic systems for over four decades (Jeffrey and Mantoura, 1997). It has been classically measured from discrete samples by spectrophotometric (Jeffrey and Humphrey, 1975) and fluorometric (Holm-Hansen et al., 1965; Lorenzen, 1966) methods. Subsequently, the High-Performance Liquid Chromatography (HPLC) method has been developed and applied not only for the determination of Chl *a* but also for several other phytoplankton accessory pigments including other chlorophylls, xanthophylls and carotenoids (Mantoura and Llewellyn, 1983; Zapata et al., 1987). HPLC is accurate and rapid and has been selected for the validation of remote sensing data (Moisan et al., 2012).

Conventional light microscopy is the main tool for the identification and enumeration of phytoplankton, but it has limitations, particularly for the differentiation of small-sized phytoplankton groups, restricting its use in several regions of the world where small flagellates are dominant (Peterson et al., 1988; Rodriguez et al., 2002; Böttjer and Morales, 2007).

Some pigments are characteristic of specific phytoplankton groups (Gieskes and Kraay, 1983; Schlüter et al., 2000; Ediger et al., 2006) and can be used as diagnostic markers to classify phytoplankton assemblages. This approach has been termed

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phytoplankton chemotaxonomy, and it has contributed in the last fifteen years to a much better understanding of the distribution and composition of oceanic phytoplankton populations (Gibb et al., 2001).

CHEMTAX is one of the most robust methods for analyzing pigment markers (Mackey et al., 1996). It uses factor analysis and a steepest descent algorithm to identify the best fit to the data based on initial estimates of the most appropriate pigment ratio(s) for each phytoplankton class (Mackey et al., 1996). This analysis depends on a data matrix of pigment concentrations, and an initial estimate of the most appropriate ratios of pigment:Chl *a* for the phytoplankton classes that might be expected in the samples. The software modifies each positive element of the pigment:Chl *a* ratio by a specific factor (usually 10–20%), assessing the residual values that are obtained from the comparison between the estimated Chl *a* and the observed Chl *a* for each pigment. The variation that produces the greatest decrease in the residual values is retained to generate a new set of pigment:Chl *a* ratios. This process is continued until the residual values cannot be reduced further (Mackey et al., 1996; Higgins et al., 2011).

The overall objectives of CHEMTAX methodology are: (1) to determine the contribution of each individual phytoplankton class to total Chl *a*, and (2) to overcome the problem found in previous studies (e.g. Gieskes et al., 1988; Latelier et al., 1993) with differentiating between classes sharing the same pigment markers.

Successful applications of CHEMTAX to estimate phytoplankton dynamics are reported for different oceanic regions such as Antarctic (e.g. Rodriguez et al., 2002; Kozlowski et al., 2011), Atlantic (e.g. Gibb et al., 2001; Pan et al., 2011), Pacific (e.g. Mackey et al., 1998) or Indian Ocean (e.g Schlüter et al., 2011), but also in more restricted areas such as the North Sea (e.g. Muylaert et al., 2006), Black Sea (e.g. Eker-Develi et al., 2012), coastlines (e.g. Mendes et al., 2011) and in estuaries (e.g. Seoane et al., 2011).

In this current study, CHEMTAX has been applied to samples from three stations off Sagres on the South-western Coast of the Iberian Peninsula (Fig. 1). These samples have been collected for the validation of marine and coastal products from the MEdium Resolution Imaging Spectrometer (MERIS) sensor, onboard the European Space Agency (ESA) ENVISAT satellite (Cristina et al., 2009, 2014; Goela et al., 2013). The MERIS products include Algal Pigment Index 1 (API1) and Algal Pigment Index 2 (API2) which are validated according to the protocols for MERIS validation (Doerffer, 2002; Barker, 2011) by accurate measurement of chlorophyll and its derivatives from *in situ* samples using HPLC.

This region is subjected to seasonal coastal upwelling, usually from early spring to late summer, after favourable north and westerly winds (Wooster et al., 1976; Fiúza et al., 1982; Relvas and Barton, 2002; Ambar and Dias, 2008). There are no discharges from permanent rivers, so freshwater inputs flow mainly from torrential streams produced by episodic incidents of heavy rain (Peliz and Fiúza, 1999). There have been studies on the dynamics of the local phytoplankton assemblages from Sagres, relative to upwelling and non-upwelling conditions (Loureiro et al., 2005a, 2011) using microscopy and spectrophotometric techniques and, also, by the spectrophotometric determination of the absorption coefficients of phytoplankton and non-algal particles (Goela et al., 2013). This current study addresses three questions:

a) How useful is CHEMTAX for the evaluation of phytoplankton communities compared to other techniques such as microscopy counts, spectrophotometric analysis of pigments, and determination of the spectral features of the community?
b) Can CHEMTAX improve the analysis of the spatial distribution of phytoplankton community relative to each station and, also, relative to the Secchi depth within each station?

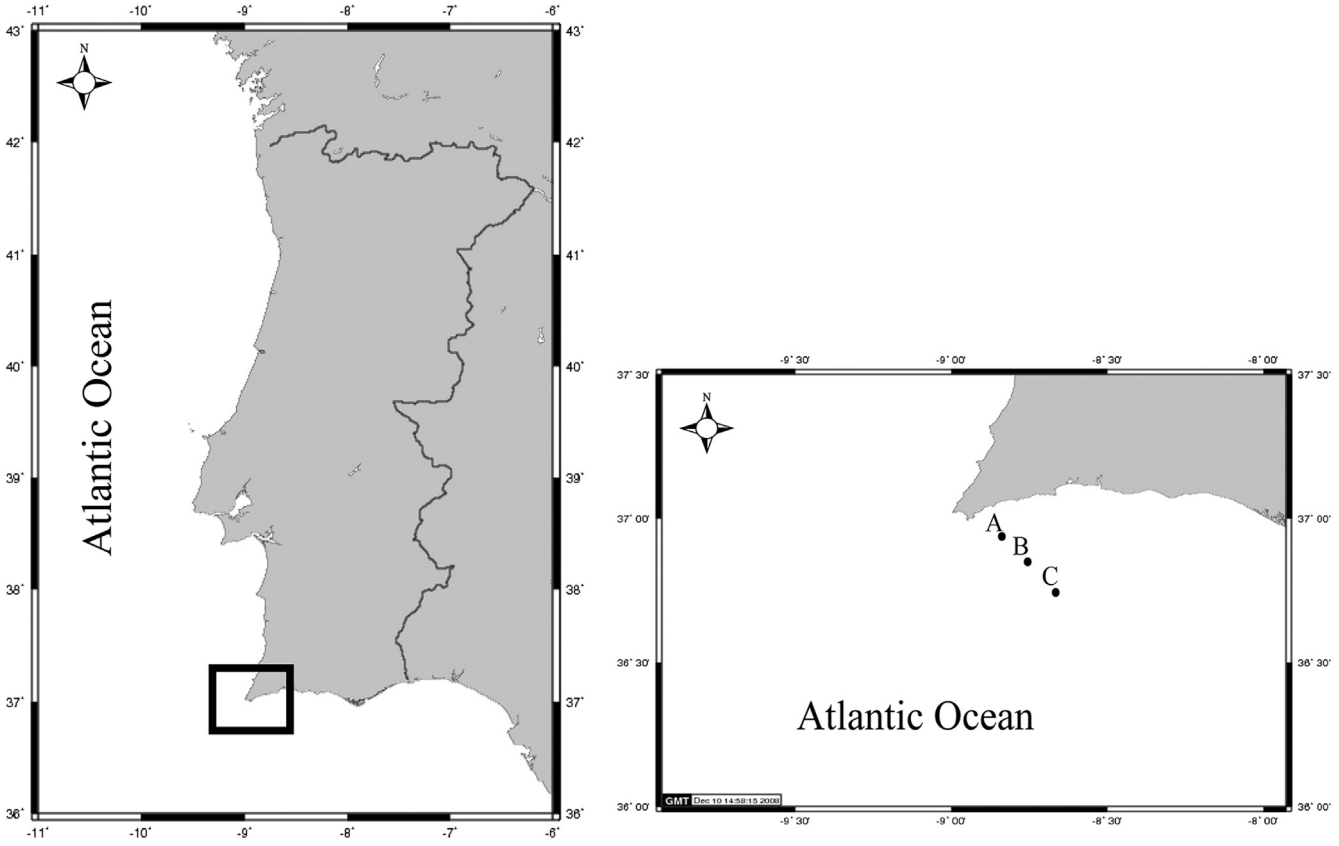

Fig. 1. Study area, showing the three sampling stations (A, B and C).

c) Can CHEMTAX improve the analysis of temporal differences in the phytoplankton community at each station?

2. Methodology

2.1. Sampling design

The field campaigns were from September 2008 until March 2012 and were generally restricted to days when there were clear skies and relatively calm sea conditions. The three sampling stations (A, B and C in Fig. 1) used for validation were at 2 (37°00′39″N and 8°53′58″W), 10 (36°56′06″N and 8°52′48″W) and 18 km (36°51′33″N and 8°50′16″W) off the coast from Cape Sagres, with approximate depths of 50, 100 and 150 m, respectively (Fig. 1). At each station, water transparency was estimated with a Secchi disk, and temperature and salinity were measured using a SEACAT SBE 19® CTD instrument. Water samples were taken from the surface, mid-Secchi and Secchi depths, using a Niskin bottle, and subsequently stored in 10 l Nalgene® containers where they were kept cool and dark for transfer to a field laboratory for further processing within 3 h of arrival onshore.

At the field laboratory, 2–4 l of water were filtered through Whatman® 47 mm glass-fibre filters (GF/F), of approximately 0.7 μm pore size for pigment determination. After filtration, the filters were wrapped in labelled aluminium foil and stored in liquid nitrogen. For nutrient analysis, 200 ml of sample was frozen at −19 °C for nutrients analysis. For microscopic analysis of phytoplankton, 300 ml of sample was filtered through a 200 μm mesh to remove larger organisms and preserved in Lugol iodine.

2.2. Supporting biological, chemical and physical variables

Microscopic identification and counts were performed with a Zeiss® Axiovert 15 inverted microscope following the Utermöhl (1931) methodology, modified by Evans (1972): a more detailed description is given in Goela et al. (2013). Flagellates belonging to prasinophytes, chrysophytes, prymnesiophytes and cryptophytes fractions that were not identified by microscopy and ranging from 2.5 μm up to 20 μm were grouped under the category 'small flagellates'. Coccolithophorides, *Phaeocystis* spp. (*Prymnesiophyceae*) and some cryptophytes were enumerated separately and presented under their respective categories. The nutrient concentrations of dissolved inorganic nitrogen (DIN), dissolved inorganic phosphate (DIP) and silicates were determined by molecular absorption spectrophotometry, as described in Grasshoff et al. (1999), using a Thermo Helius α® spectrophotometer. Upwelling indices (Q_x and Q_y) were calculated as described by Loureiro et al. (2005a), following Bakun (1973). Positive values of Q_x produce southward Ekman transport which are favourable for upwelling along the south coast, whilst negative values of Q_y produce westward Ekman transport which are favourable for upwelling along the west coast. The source for wind data were the 'Blended Daily Averaged 0.25-degree Sea Surface Winds at 10 m level' product provided by the National Oceanic and Atmospheric Administration (NOAA) and National Climatic Data Center (Zhang et al., 2006). Sources for satellite-derived sea surface temperature (SST) data were the NOAA Optimum Interpolation (OI) daily SST at 0.25-degree resolution (Reynolds et al., 2007), and the Multi-scale Ultra-high Resolution (MUR) SST daily analysis at nominal 0.011° resolution, produced by the Jet Propulsion Laboratory of the California University of Technology. All data were accessed via the NOAA Environmental Research Division Data Access Program (ERDDAP) at http://upwell.pfeg.noaa.gov/erddap/index.html (Simons, 2011).

2.3. Determination of phytoplankton pigments by HPLC

The analysis of the samples by HPLC followed the Scientific Committee Oceanic Research's (SCOR) procedures described in Wright and Jeffrey (1997). The phytoplankton pigments retained on the filters preserved in liquid nitrogen were extracted using 3–5 ml of 90% acetone. The process was assisted by triturating each filter with a glass rod followed by sonication for 20 s. After extraction for 4–6 h, the triturated filters were sonicated for a further 20 s and centrifuged to clarify the filtrate. Aliquots of 1 ml from each extract were transferred to HPLC vials where water was added to each one to improve peak shape (Zapata and Garrido, 1991) before the diluted extracts were injected into the HPLC. A Diode Array Detector (DAD), configured at 436 and 450 nm, was used to detect and identify chlorophylls and carotenoids, respectively. Standards for quantification were acquired from DHI® Labs.

Although the above methodology was followed for all samples, two different HPLC instruments were used over the sampling campaign. Samples from 8th September 2008 until 11th July 2009 were determined in a Waters® 600E HPLC system, equipped with DAD, and using a C18 Thermo® Hypersil-Keystone (ODS-2) column (25 mm of length, 4 mm of diameter and 5 μm of particle size). All the other samples were analyzed in an Agilent® HPLC-DAD equipment, using a C18 Alltech® Altima column (15 mm of length, 4.6 of diameter and 3 μm of particle size). The main procedural differences were: 1) in the first period, 5 ml of 90% acetone was used, while in the second, the extraction volume was 3 ml; 2) the aliquot was diluted with 0.1 ml of water, and this procedure was made automatically prior to the injection using the autosampler in the first period, while in the second period, 0.3 ml of water were manually added to each aliquot of 1 ml; 3) quantification of 19′hexanoyloxifucoxanthin and 19′butanoyloxifucoxanthin in the samples during the first period were made indirectly, using fucoxanthin as a reference, while in the second period, standards were available for more accurate quantification.

2.4. CHEMTAX design

Based on the main pigment markers and the microscopic counts for phytoplankton obtained in this study, as well as knowledge about phytoplankton community in the area from previous studies (e.g. Loureiro et al., 2005a, 2008; Goela et al., 2013), 7 different phytoplankton classes were uploaded for an initial configuration of CHEMTAX: chrysophytes, cryptophytes, cyanobacteria, diatoms, dinoflagellates, prasinophytes and prymnesiophytes. Chlorophytes were excluded from this study to avoid confusion with prasinophytes on the basis of Mendes et al. (2011) observations that the pigments shared between the two classes were significantly correlated with prasinoxanthin, which is an exclusive pigment from prasinophytes. The data matrix included concentrations of peridinin, 19′hexanoyloxifucoxanthin, fucoxanthin, 19′butanoyloxifucoxanthin, violaxanthin, alloxanthin, lutein, zeaxanthin, Chl *b*, Chl *c3* and Chl *a*.

The pigment:Chl *a* ratios which constituted the initial matrix were obtained from Schlüter et al. (2000) for prasinophytes and prymnesiophytes (Chl *c3*:Chl *a* for Secchi depth bins) and from Gibb et al. (2001) for all other classes. To avoid potentially unreliable initial pigment:Chl *a* ratios, sixty ratio matrices were generated by adjusting each of the pigment ratios according to a random function described in Wright et al. (2009). The best 10% of the outputs, based on lower Root Mean Square (RMS) errors, were selected as starting matrices to determine the contribution of each class to the total Chl *a* concentration, but only if they were realistic for the area. Final ratio matrices with a high percentage of change relative to the initial ratio were discarded.

Table 1
Pigment:Chl *a* matrices used in CHEMTAX analysis of pigment data: (a) initial matrix uploaded, (b) output averaged matrix obtained for the autumn samples, (c) output averaged matrix obtained for the winter samples, (d) output averaged matrix obtained for the spring samples and (e) output averaged matrix obtained for the summer samples. In each pigment:Chl *a* ratio, when 3 different values are presented, they correspond to the ratios used and obtained for the three different depths (Surface/mid-Secchi/Secchi).

Class/pigment	Perid	19′BF	Fuco	19′HF	Viola	Allo	Lut	Zea	Chl *b*	Chl *c3*
(a) Initial										
Prasinophytes	0	0	0	0	0.138	0	0.018	0.079	0.679	0
Dinoflagellates	1.06	0	0	0	0	0	0	0	0	0
Cryptophytes	0	0	0	0	0	0.23	0	0	0	0
Cyanobacteria	0	0	0	0	0	0	0	0.59	0	0
Diatoms	0	0	0.76	0	0	0	0	0	0	0
Crysophytes	0	1.56	0.97	0	0	0	0	0	0	0.25
Prymnesiophytes	0	0.02	1.21	1.36	0	0	0	0	0	0.17/0.17/0.21
(b) Output autumn										
Prasinophytes	0	0	0	0	0.126/0.122/0.140	0	0.020/0.020/0.018	0.090/0.082/0.075	0.834/0.749/0.934	0
Dinoflagellates	1.10/0.90/1.06	0	0	0	0	0	0	0	0	0
Cryptophytes	0	0	0	0	0	0.12/0.15/0.10	0	0	0	0
Cyanobacteria	0	0	0	0	0	0	0	0.71/0.47/0.56	0	0
Diatoms	0	0	0.95/1.13/1.20	0	0	0	0	0	0	0
Crysophytes	0	1.61/0.83/1.64	0.80/0.84/0.89	0	0	0	0	0	0	0.24/0.36/0.22
Prymnesiophytes	0	0.02/0.02/0.02	0.14/0.28/0.44	0.95/1.37/1.40	0	0	0	0	0	0.24/0.20/0.40
(c) Output winter										
Prasinophytes	0	0	0	0	0.136/0.187/0.103	0	0.010/0.003/0.010	0.077/0.086/0.052	0.805/0.874/0.712	0
Dinoflagellates	1.18/0.95/0.97	0	0	0	0	0	0	0	0	0
Cryptophytes	0	0	0	0	0	0.14/0.12/0.10	0	0	0	0
Cyanobacteria	0	0	0	0	0	0	0	0.59/0.60/0.63	0	0
Diatoms	0	0	0.92/0.76/0.85	0	0	0	0	0	0	0
Crysophytes	0	1.95/1.42/1.18	0.76/1.06/0.82	0	0	0	0	0	0	0.17/0.29/0.41
Prymnesiophytes	0	0.02/0.02/0.03	0.34/0.30/0.58	1.19/1.42/2.79	0	0	0	0	0	0.20/0.21/0.36
(d) Output spring										
Prasinophytes	0	0	0	0	0.132/0.139/0.135	0	0.018/0.016/0.020	0.091/0.076/0.091	0.770/0.752/0.782	0
Dinoflagellates	1.10/1.10/1.02	0	0	0	0	0	0	0	0	0
Cryptophytes	0	0	0	0	0	0.05/0.04/0.09	0	0	0	0
Cyanobacteria	0	0	0	0	0	0	0	0.64/0.69/0.62	0	0
Diatoms	0	0	0.96/0.95/0.87	0	0	0	0	0	0	0
Crysophytes	0	1.24/1.60/1.59	0.89/0.93/0.93	0	0	0	0	0	0	0.30/0.23/0.19
Prymnesiophytes	0	0.04/0.02/0.02	0.24/0.70/0.35	2.66/1.38/0.98	0	0	0	0	0	0.33/0.26/0.40
(e) Output summer										
Prasinophytes	0	0	0	0	0.114/0.124/0.161	0	0.019/0.020/0.019	0.080/0.075/0.075	0.757/0.667/0.593	0
Dinoflagellates	1.09/0.99/1.04	0	0	0	0	0	0	0	0	0
Cryptophytes	0	0	0	0	0	0.19/0.18/0.19	0	0	0	0
Cyanobacteria	0	0	0	0	0	0	0	0.52/0.30/0.32	0	0
Diatoms	0	0	0.93/0.77/0.74	0	0	0	0	0	0	0
Crysophytes	0	1.96/1.53/1.50	0.77/0.89/0.96	0	0	0	0	0	0	0.21/0.22/0.19
Prymnesiophytes	0	0.02/0.02/0.02	0.57/0.56/0.78	0.68/1.10/0.57	0	0	0	0	0	0.23/0.19/0.41

Data were split into 12 different bins, matching each season and depth, and analyzed separately using CHEMTAX v.1.95. This procedure assured the homogeneity of the pigment:Chl *a* ratios within all samples from the same bin, since these ratios are known to vary with light availability and seasonality (Schlüter et al., 2000; Higgins et al., 2011). The final reorganisation of the data was only taken after multiple CHEMTAX trials with data divided into periods of 1 year where shown to contribute no advantage to the analysis (i.e. improve the RMS). After the data were reorganised into seasonal and depth periods, each pigment data bin was inspected for the presence of outliers (abnormal pigment:Chl *a* ratios), which were then excluded from the analysis before each run of the software. Table 1 shows the initial and final pigment matrices obtained in this study. The seasons were defined as December to February for winter samples, March to May for spring samples, June to August for summer samples, and September to November for autumn samples.

2.5. Data treatment

The Statistica® 10 (Stat. Soft Inc.) package was used for the statistical analysis including measures of means, standard deviations, maxima and minima. A non-parametric Spearman correlation analysis evaluated the degree of correlation between the study variables. Main-effects Analysis of Variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) tests were used to compare concentrations of nutrients and Chl *a*, as well as the contribution of individual phytoplankton classes to the means for total Chl *a*, using stations (A, B, C), depth code (surface = 1, mid-Secchi = 2 and Secchi depth = 3), and seasons (spring, summer, autumn and winter) as categorical predictors. The level of significance for all these analyses was $\alpha \leq 0.05$.

3. Results

3.1. Nutrient dynamics

A total of 253 nutrient samples have been collected and analyzed during this study. The means and standard deviations for DIN, DIP and silicates are 2 ± 3, 0.11 ± 0.09 and 1 ± 2 μM, respectively, with maxima for these nutrients of 11.22, 0.57 and 1.12 μM, respectively. Fig. 2 shows box and whisker plots for nutrient conditions separated on the basis of seasons over three sampling periods. Outliers are removed from the data wherever there are inconsistencies within the depth and sampling day; remaining outliers are considered and presented in the figure. The greatest variability in the nutrient concentration data occurs in winter for DIN and silicates, and in summer for DIP. Based on the observation of Spearman correlation coefficients between nutrient concentrations and sampling depth, there are no significant vertical patterns observed within the water column.

The comparison and tests for the differences in the means of nutrient concentrations between stations, depth code and seasons, from the ANOVA, shows that the effect of the stations and depth is not statistically significant. Meanwhile, seasonality does have a statistically significant effect on the concentration of nutrients (Wilks test, $F = 21.15$, p-value < 0.05). *Post hoc* comparisons using LSD tests indicate that winter means ($M = 7.40$ μM, SD = 3.24 μM) for DIN are significantly higher than for the other seasons, followed by autumn ($M = 1.89$ μM, SD = 2.05 μM) and spring ($M = 1.82$ μM, SD = 1.94 μM) concentrations, which are not significantly different from each other, and finally summer, with significantly lower concentrations than autumn and winter ($M = 0.80$ μM, SD = 0.84 μM). Winter shows a significantly higher mean DIP ($M = 0.19$, SD = 0.08), followed by summer ($M = 0.14$ μM, SD = 0.11 μM), autumn ($M = 0.10$ μM, SD = 0.10 μM), and finally spring ($M = 0.07$ μM, SD = 0.05 μM). In the case of silicates, seasonality has a smaller effect on the concentrations, but winter shows a significantly higher mean ($M = 2.40$ μM, SD = 0.69 μM) than for the other seasons. Summer ($M = 0.69$ μM, SD = 1.93 μM), spring ($M = 1.04$ μM, SD = 1.56 μM) and autumn ($M = 1.30$ μM, SD = 1.77 μM) show no significant differences between each other.

3.2. Phytoplankton pigments and biomass

Fig. 3 shows the variation in the total concentration of Chl *a*, the main proxy indicator for phytoplankton biomass, with an average concentration of 0.80 μg dm^{-3} (SD = 0.86), reaching maximum values of about 7 μg dm^{-3} and minima below the limit of detection. Peak concentrations of Chl *a* were found on 3rd and 22nd April 2009, 11th July 2009, 19th June 2011 and 12th March 2012.

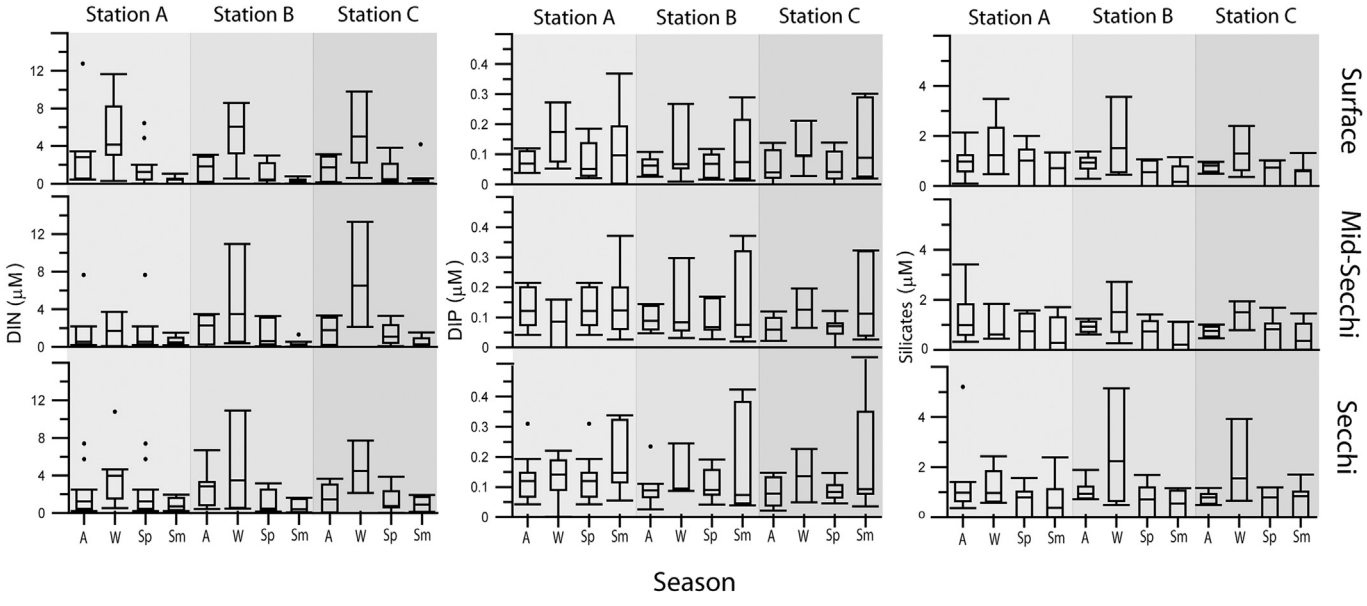

Fig. 2. Seasonal dynamics of dissolved inorganic nitrogen (DIN), dissolved inorganic phosphate (DIP) and silicate concentrations (box and whisker plots) at stations A, B and C from three depths (surface, mid-Secchi and Secchi-depths).

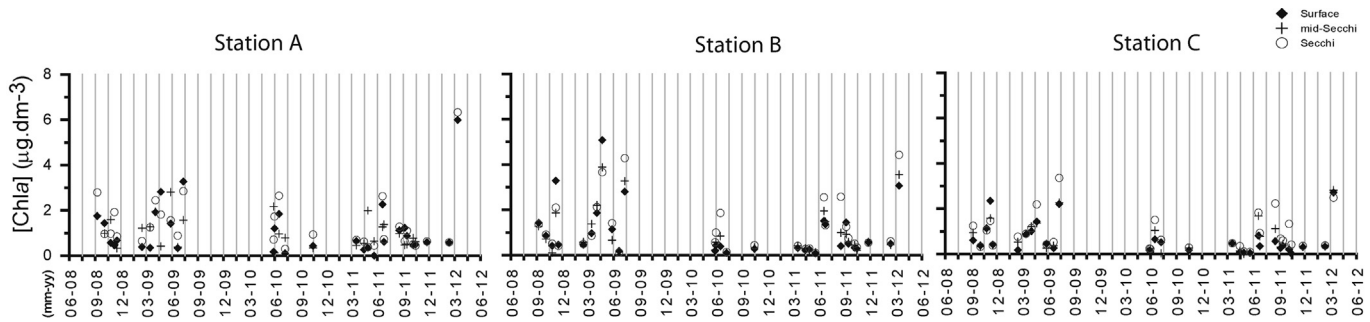

Fig. 3. The concentration of total Chl *a* (TChl *a*) along the duration of the study for stations A, B, C. The TChl *a* values for the three different depths are represented by ◆ (surface), + (mid-Secchi) and ○ (Secchi).

Conversely, 14th February 2009, 8th July 2010 and 20th May 2011 are sampling dates when Chl *a* is low. The Spearman correlation matrix reveals relations between Chl *a* and physico-chemical variables. Significant positive correlations are found with DIN and DIP ($r_s = 0.26$ and $r_s = 0.30$, respectively) and significant negative correlations with Secchi depth and temperature ($r_s = -0.72$ and $r_s = -0.48$, respectively).

In common with nutrients, seasonality has a significant effect on the Chl *a* concentration (main effects ANOVA, $F = 3.33$, *p*-value = 0.01). LSD *post hoc* test shows that winter and autumn have significantly lower means of Chl *a* concentration ($M = 0.54$ µg dm^{-3}, SD = 0.23 µg dm^{-3} and $M = 0.82$ µg dm^{-3}, SD = 0.59 µg dm^{-3}, respectively) than spring and summer, which are not significantly different from each other ($M = 1.24$ µg dm^{-3}, SD = 1.46 µg dm^{-3} and $M = 1.34$ µg dm^{-3}, SD = 1.07 µg dm^{-3}, respectively). Also in common with nutrients, the categorical predictors stations and depth do not show any statistically significant effects on the concentration of Chl *a*.

The HPLC-DAD technique enables the detection and quantification of a total of 20 different pigments in the samples collected during the study. The concentrations of divinil-chlorophyll *a* (DVChl *a*) have only been correctly determined for the samples after 2009. Similarly, neoxanthin (Neo), prasinoxanthin (Pras) and carotenes (α-Car and β-Car) have only been determined after 18th August 2011. Consequently, these pigments have not been processed by CHEMTAX for interannual and seasonal variations; despite this, they provide important additional information on which phytoplankton classes should be uploaded to the initial CHEMTAX configuration. For example, the chlorophytes class is excluded for the same reason mentioned in Mendes et al. (2011), since the concentration of Pras, which is exclusively from prasinophytes, is highly correlated with chlorophyll *b* (Chl *b*) ($R^2 = 0.77$, *p*-value < 0.05), β-Car ($R^2 = 0.35$, *p*-value < 0.05), Neo ($R^2 = 0.70$, *p*-value < 0.05) and Viola ($R^2 = 0.61$, *p*-value < 0.05), which are all pigments present in both chlorophytes and prasinophytes.

Chl *a*, fucoxanthin (Fuco), 19′-hexanoyloxifucoxanthin (19′HF), diadinoxanthin (DD), β-Car and chlorophyll *c2* (Chl *c2*) are detected in 98% of the samples. Pras, Neo, α-Car and DD are also regularly detected in 90–95% of the samples. Chlorophyll *c3* (Chl *c3*), Chl *b*, chlorophyllide *a* (Chlide *a*), 19′-butanoyloxifucoxanthin (19′BF), Zea, Viola, alloxanthin (Allo) and diatoxanthin (DT) are present in 60–85% of the samples. Peridinin and lutein are the rarest pigments with occurrence in only in 45 and 20% of the samples, respectively.

3.3. Dynamics of the phytoplankton community based on CHEMTAX results

Fig. 4 shows the relative contribution of the different classes of the phytoplankton community to total Chl *a* between 2008 and 2012. Cryptophytes and diatoms make the greatest contribution to total Chl *a* with relative contributions of 30% and 28%, respectively, followed by prymnesiophytes and prasinophytes with 14% and 13% relative contribution to total Chl *a*, respectively. In contrast, the classes which seem to contribute least to total biomass are cyanobacteria, with an average of 9% of relative contribution to Chl *a*, followed by chrysophytes and dinoflagellates, with relative contributions of about 3% and 2%, respectively. The combined contributions of the generally small-sized cell classes including cryptophytes, prasinophytes and prymnesiophytes (Jeffrey and Vesk, 1997), contribute up to 60% of the total Chl *a* in over half the samples analyzed by CHEMTAX.

3.3.1. Seasonality

ANOVA confirms that seasonality has a significant effect in all phytoplankton classes (Wilks test, $F = 13.04$, $p < 0.05$). According to *post-hoc* LSD comparisons, diatom dominance occurs in spring ($M = 36.23\%$, SD = 27.16%) and summer ($M = 41.37\%$, SD = 31.26%), rather than autumn and winter. Prymnesiophytes present markedly higher contributions in summer ($M = 18.63\%$, SD = 14.14%), but with respect to the other seasons, there are no significant differences ($M_{\text{autumn, winter, spring}} \approx 12\%$). In contrast, for cryptophytes, the higher contributions to Chl *a* are reached in winter ($M = 45.14\%$, SD = 4.79) with the lower ones in summer ($M = 20.64\%$, SD = 20.93%).

In common with cryptophytes, prasinophytes attain higher contributions in winter ($M = 28.76\%$, SD = 4.42%), and lower ones in summer and spring ($M \approx 9\%$). Similarly, the chrysophytes contribution to total Chl *a* is also higher in autumn and winter ($M \approx 5\%$), with a decreasing contribution in spring ($M = 2.85\%$, SD = 3.66%) and summer ($M = 1.32\%$, SD = 2.14%). However, cyanobacteria and dinoflagellates show lower seasonal differences, with only higher contributions to total Chl *a* in autumn ($M = 13.83\%$, SD = 1.57% and $M = 4.85\%$, SD = 6.05%, respectively), and no significant differences for the other seasons.

3.3.2. Spatial conditions

The main-effects ANOVA using depth code and stations as categorical predictors shows that sampling depth has no statistically significant effects on any of the phytoplankton classes. However the stations variable does have significant effects on the contribution of the different classes to total Chl *a* estimated by CHEMTAX. The *post-hoc* LSD tests show the relative contributions of diatoms and dinoflagellates decrease from station A to station C whereas the contribution of prasinophytes, chrysophytes and prymnesiophytes to total Chl *a* increase from inshore to offshore. There are no significant differences in cryptophytes and cyanobacteria between the three stations.

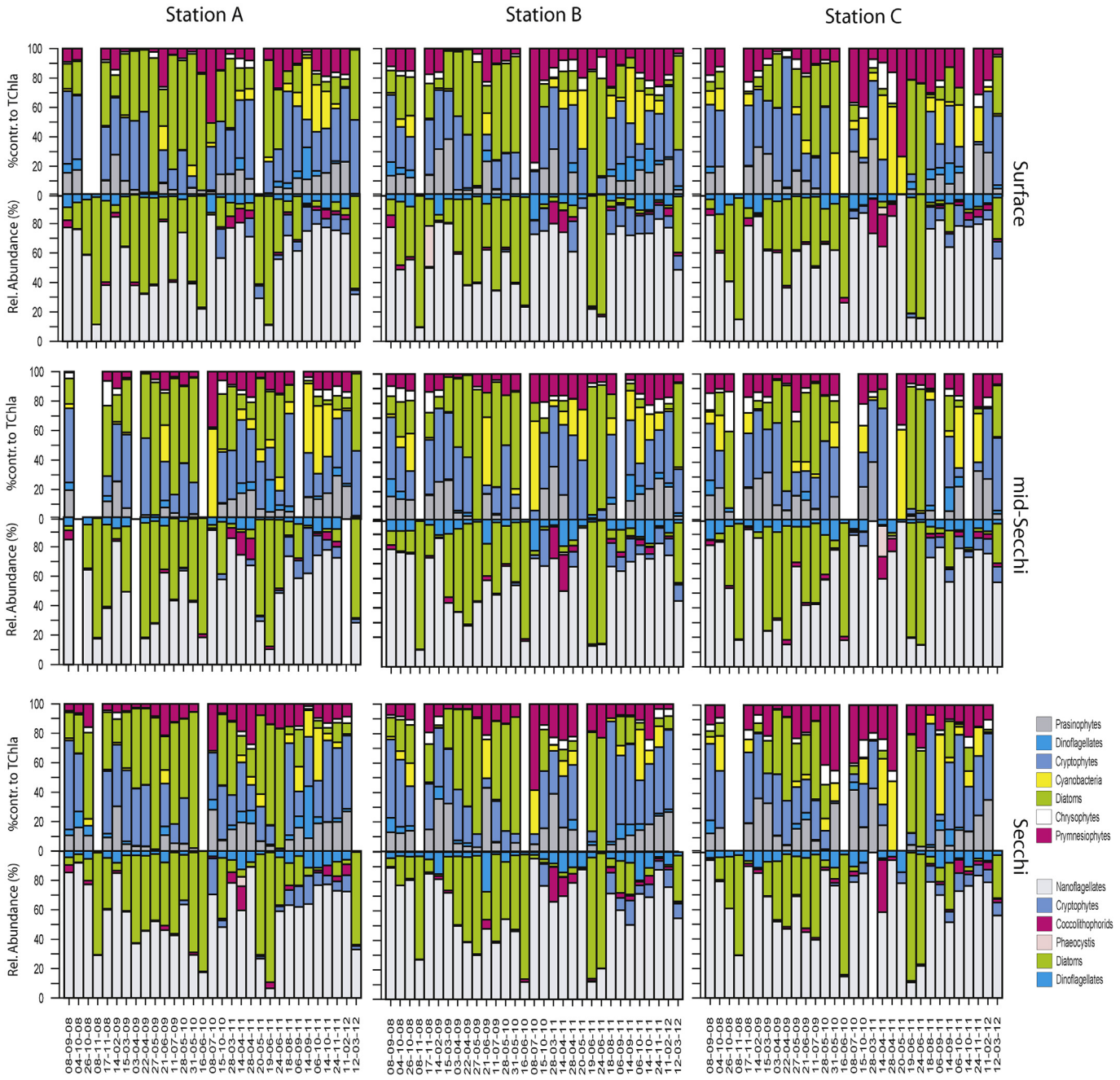

Fig. 4. The relative contribution of the different phytoplankton classes to Chl *a*, determined by CHEMTAX (% contr.to TChl *a*) and to total abundance, determined by microscopy (Rel. Abundance (%)), with the different classes represented by different colours. Each sampling date is shown along the *x* axis for the panels representing stations A, B and C, at the three different depths (Surface, mid-Secchi and Secchi depths).

3.3.3. Oceanographic conditions

In order to understand the influence of oceanographic factors on the community dynamics, a Spearman correlation analysis is presented in Table 2. Diatoms, cyanobacteria, cryptophytes, dinoflagellates and prymnesiophytes present are affected by temperature regimes, with positive correlations for cyanobacteria ($r_s = 0.76$, p-value < 0.05), dinoflagellates ($r_s = 0.37$, p-value < 0.05) and prymnesiophytes ($r_s = 0.32$, p-value < 0.05) and negative correlations with diatoms ($r_s = -0.36$, p-value < 0.05) and cryptophytes ($r_s = -0.24$, p-value < 0.05).

Concerning nutrient conditions, dinoflagellate and cyanobacteria communities seem to be favoured by low DIN concentration (significant negative correlations for the contribution of both classes to total Chl *a* and DIN). The contribution to total Chl *a* estimated by CHEMTAX for prasinophytes is positively correlated with higher concentrations of DIN ($r_s = 0.31$, p-value < 0.05) and moderately correlated with silicates ($r_s = 0.36$, p-value < 0.05). Also considering the contribution to total Chl *a*, diatoms are negatively correlated with silicates ($r_s = -0.30$, p-value < 0.05), whilst cryptophytes are positively correlated with silicates ($r_s = 0.39$, p-value < 0.05).

There is a significant negative correlation between the contribution of diatoms to total Chl *a* and the upwelling indices from the west coast (Q_y) ($r_s = -0.43$, p-value < 0.05). There are also

Table 2
Spearman correlation matrix between the study parameters: distance from coast (km) (Dist. coast), Secchi depth (m), water temperature (°C) (Temp), salinity (Sal), dissolved inorganic nitrogen (μmol dm^{-3}) ([DIN]), dissolved inorganic phosphate (μmol dm^{-3}) ([DIP]), silicates (μmol dm^{-3}) ($[SiO_4]$), southward (Q_x, $m^3\ s^{-1}\ km^{-1}$) and westward (Q_y, $m^3\ s^{-1}\ km^{-1}$) Ekman transport component, Sampling depth (m) (Samp. depth), and contribution of Prasinophytes (Pras), Dinoflagellates (Dino), Cryptophytes (Crypt), Cyanobacteria (Cyano), Diatoms (Diat), Chrysophytes (Chrys) and Prymnesiophytes (Prymn) to [Chl *a*]. Significant correlations (p-value < 0.05) are shown in bold.

	Dist. coast	Secchi depth	Temp	Sal	Pras	Dino	Crypt	Cyano	Diat	Chrys	Prymn	[DIN]	[DIP]	$[SiO_4]$	[Chl *a*]	Q_x	Q_y	Samp. depth
Dist. coast	1.000																	
Secchi depth	**0.386**	1.000																
Temp	−0.042	**0.207**	1.000															
Salinity	0.065	**0.209**	**0.508**	1.000														
Pras	**0.136**	**0.326**	0.090	**0.228**	1.000													
Dino	**−0.159**	**−0.229**	**0.373**	−0.136	0.053	1.000												
Crypt	−0.072	0.113	**−0.241**	**−0.237**	**0.210**	**0.130**	1.000											
Cyano	**0.134**	**0.408**	**0.755**	**0.332**	**0.247**	**0.186**	−0.080	1.000										
Diat	**−0.253**	**−0.566**	**−0.362**	−0.154	**−0.536**	−0.018	**−0.312**	**−0.647**	1.000									
Chrys	**0.227**	**0.389**	0.132	**0.239**	**0.495**	−0.009	−0.008	**0.271**	**−0.399**	1.000								
Prymn	**0.270**	**0.463**	**0.325**	**0.261**	**0.239**	−0.118	**−0.306**	**0.389**	**−0.462**	**0.217**	1.000							
[DIN]	−0.071	**−0.183**	**−0.525**	−0.128	**0.311**	**−0.273**	**0.318**	**−0.309**	0.021	**0.142**	**−0.241**	1.000						
[DIP]	**−0.151**	**−0.219**	**−0.241**	**−0.344**	**0.160**	**0.158**	**0.161**	**−0.147**	−0.006	−0.045	0.019	**0.216**	1.000					
$[SiO_4]$	−0.091	0.010	**−0.225**	−0.156	**0.362**	0.052	**0.393**	0.092	**−0.284**	**0.162**	−0.105	**0.424**	**0.190**	1.000				
[Chl *a*]	**−0.231**	**−0.718**	**−0.483**	**−0.394**	**−0.250**	**0.234**	0.095	**−0.537**	**0.529**	**−0.360**	**−0.593**	**0.260**	**0.299**	0.070	1.000			
Q_x	0.005	**0.168**	−0.047	**−0.171**	**−0.229**	0.028	0.044	−0.028	−0.031	**−0.283**	**0.137**	**−0.260**	0.015	**−0.204**	**−0.126**	1.000		
Q_y	−0.002	0.072	**0.204**	**0.177**	**0.243**	**0.192**	**0.270**	**0.343**	**−0.429**	**0.142**	**0.145**	**0.159**	−0.031	**0.255**	**−0.215**	−0.005	1.000	
Samp. depth	**0.127**	**0.317**	−0.046	0.082	**0.193**	−0.056	0.056	0.065	**−0.215**	0.035	**0.154**	0.024	**0.148**	0.035	−0.080	0.058	0.008	1.000

significant positive correlations between Q_y and prasinophyte, dinoflagellate, cyanobacteria and cryptophyte contributions to total Chl *a* (Table 2).

3.4. Microscopic counts compared to CHEMTAX estimations

Microscopic identification of the microphytoplankton (>20 μm) component from coastal stations shows 31 species of diatoms and 101 species of dinoflagellates (Table 3), out of a total of 87% and 81%, respectively. Although the smaller nanoplankton (2–20 μm) classes were counted in 2011–2012, only the *Phaeocystis* spp. (prymnesiophyte), *Dictyocha fibula* (chrysophyte) and *Myrionecta rubra* (ciliate with symbiotic cryptophytes) were identified to genus/species level.

Fig. 4 shows the relative contribution of different phytoplankton groups both to total Chl *a* estimated by CHEMTAX (upper panels)

Table 3
List of diatoms and dinoflagellates identified to genus or species in all stations of Sagres site.

Diatoms	Dinoflagellates	
Asterionella gracialis	*Alexandrium* spp.	*Oxytoxum scolopax*
Coscinodiscus spp.	*Amphidinium* sp.	*Oxytoxum variabile*
Detonula pumila	*Amphidoma* sp.	*Phalacroma* sp.
Diploneis sp.	*Blepharocysta* sp.	*Phalacroma rotundatum*
Biddulphia sp.	*Ceratium* spp.	*Podolampas palmipes*
Chaetoceros spp.	*Ceratium azoricum*	*Polykrikos* sp.
Cylindrotheca closterium	*Ceratium declinatum*	*Pronoctiluca* sp.
	Ceratium candelabrum	*Prorocentrum compressum*
Ditylum brightwellii	*Ceratium contrarium*	*Prorocentrum triestinum*
Eucampia sp.	*Ceratium extensum*	*Prorocentrum scutellum*
Guinardia sp.	*Ceratium furca*	*Prorocentrum micans*
Guinardia delicatula	*Ceratium fusus*	*Prorocentrum ovatum*
Guinardia flaccida	*Ceratium hexacanthum*	*Prorocentrum* spp.
Guinardia striata	*Ceratium horridum*	*Protoceratium* sp.
Hemiaulus sinensis	*Ceratium incisum*	*Protoperidinium cerasus*
Leptocylindrus danicus	*Ceratium inflatum*	*Protoperidinium brevipes*
	Ceratium lineatum	*Protoperidinium divergens*
Leptocylindrus mediterraneus	*Ceratium longipes*	*Protoperidinium conicoides*
	Ceratium longirostrum	*Protoperidinium conicum*
Leptocylindrus minimus	*Ceratium macroceros*	*Protoperidinium curtipes*
	Ceratium massiliense	*Protoperidinium depressum*
Meuniera membranacea	*Ceratium pentagonum*	*Protoperidinium diabolum*
	Ceratium teres	*Protoperidinium excentricum*
Navicula spp.	*Ceratium trichoceros*	*Protoperidinium bipes*
Nitzschia acicularis	*Ceratium tripos*	*Protoperidinium grande*
Nitzschia spp.	*Ceratocorys horrida*	*Protoperidinium granii*
Paralia sulcata	*Cochlodinium* spp.	*Protoperidinium islandicum*
Skeletonema costatum	*Corythodinium* spp.	*Protoperidinium leonis*
Thalassionema sp.	*Dinophysis acuminata*	*Protoperidinium minutum*
Thalassiosira sp.	*Dinophysis acuta*	*Protoperidinium mite*
Proboscia spp.	*Dinophysis caudata*	*Protoperidinium oblongum*
Pleurosigma spp.	*Dinophysis fortii*	*Protoperidinium oceanicum*
Pseudo-nitzschia spp.	*Dinophysis ovum*	*Protoperidinium ovatum*
Pseudo-nitzschia delicatissima	*Dinophysis* spp.	*Protoperidinium pallidum*
	Diplopsalis spp.	*Protoperidinium pellucidum*
Rhizosolenia spp.	*Diplopsalis lenticula*	*Protoperidinium pentagonum*
Rhizosolenia styleformis	*Dissodinium* sp.	*Protoperidinium pyriforme*
	Dissodium asimmetricum	*Protoperidinium steinii*
	Gonyaulax spp.	*Protoperidinium* spp.
	Gonyaulax digitale	*Pyrophacus* sp.
	Gonyaulax grindleyi	*Pyrophacus horologium*
	Gonyaulax polygramma	*Scrippsiella* spp.
	Gonyaulax spinifera	*Scrippsiella trochoidea*
	Gonyaulax verior	*Torodinium* sp.
	Gymnodinium spp.	*Torodinium robustum*
	Gymnodinium catenatum	*Triadinium* sp.
	Gyrodinium spp.	*Triadinium polyedricus*
	Heterocapsa spp.	*Nematodinium* spp.
	Katodinium glaucum	*Warnowia polyphemus*
	Lingulodinium polyedrum	*Warnowia* spp.
	Oxytoxum spp.	

and to the total abundance of phytoplankton enumerated by microscopy (lower panels). In general, there is good agreement between the CHEMTAX-derived taxa contribution to total Chl *a* and the proportion of cells to total cell numbers of respective taxa for diatoms (in all seasons) and in some cases prasinophytes (e.g. 31st May 2010, 27th May 2009 and 12th March 2012) and cryptophytes (summer campaigns and 11th February 2012) with nanoflagellates, and prymnesiophytes with coccolithophorids (4th October 2008). However, in the case of dinoflagellates there is no clear association.

4. Discussion

The low nutrient status of these coastal sites for most samples can be attributed to the limited supply of nutrients from terrestrial and riverine sources off the SW Iberian coast (Peliz and Fiúza, 1999; Loureiro et al., 2008). Days when nutrient concentrations were high could be attributed to upwelling processes, similar to observations from other studies (Sousa and Bricaud, 1992; Loureiro et al., 2008). As there are only limited anthropogenic pressures from shore, this would explain why there are no significant differences in nutrient concentrations between the inner and outer sampling stations.

The seasonality observed both in nutrient and in Chl *a* data may also be attributed to upwelling events. According to Fiúza et al. (1982) and Loureiro et al. (2005a, 2011), the upwelling season in the area is from early spring to late summer. In this study, observations show that high nutrient and Chl *a* episodes combine with favourable upwelling indices which start in early spring. Chl *a* in the water column is related to higher nutrient conditions that are also associated with lower water temperatures (Table 2), which suggests that productivity dynamics is significantly influenced by the upwelling regime. Regarding the vertical distribution of Chl *a*, the results show that there is no trend related with sampling depth (Fig. 3, Table 2), consistent with a well mixed water column, and consistent with the observations by Goela et al. (2013).

The dominance of diatoms in spring with their positively correlated contribution to total Chl *a* and negative correlation with temperature corroborate the study by Loureiro et al. (2011) who suggest that the dominance of diatoms is caused by the cold and nutrient rich water mass supplied by upwelling: other studies report the same trend based on the upwelling conditions along Portuguese coast (Moita, 2001; Silva et al., 2008; Mendes et al., 2011). Indeed, mesocosm experiments using waters from the Algarve coast showed that when primary production is stimulated, diatoms proved to be the class most sensitive to nutrient enrichment (Edwards et al., 2005; Loureiro et al., 2005b, 2008).

Loureiro et al. (2005a) have reported that collapses in diatom blooms are related to the decrease in favourable upwelling conditions combined with intrusion of the warm counter current originating in the Gulf of Cadiz. However, in this study, diatom abundance decreases even with the persistence of favourable upwelling conditions through the summer ($Q_x > 0$, $Q_y < 0$). One example is the decline from 24th June 2011 (more than 70%) to 18th August 2011 (less than 10%). During this transition, DIN and DIP concentrations remain close to the average values, but silicates are very low or below the limit of detection. Indeed, there is a moderate negative correlation between diatoms contribution to Chl *a* and concentration of silicates ($r_s = -0.44$, $p < 0.05$). This suggests that silicates are acting as a limiting nutrient, resulting in the termination of diatom blooms, since silicon is essential for diatom growth (Lewin, 1962).

There are many samples from this study dominated by classes composed of nanoplankton cells (Jeffrey and Vesk, 1997), which are difficult to discriminate taxonomically by conventional inverted microscopy. Thus, the decision on what phytoplankton classes should be uploaded to CHEMTAX was not only based on the microscopy results and historical knowledge, but also strongly supported by the pigments found in the samples. Microscopy does indicate the presence of coccolithophorids, *Phaeocystis* spp. (prymnesiophytes) and *Myrionecta rubra* (ciliate with cryptophytes). Weissbach et al. (2011) have also observed cryptophytes in the Sagres area whilst Edwards et al. (2005) have reported cyanobacteria from the same area. In terms of phytoplankton pigments, the occurrence of violaxanthin, prasinoxanthin, zeaxanthin and Chl *b* indicate the presence of both prasinophytes and cyanobacteria, whilst concentrations of 19′BF, 19′HF and Chl *c3* indicate the presence of chrysophytes and prymnesiophytes. Alloxanthin is also a common pigment in the samples, providing the basis for uploading cryptophytes into the initial configuration for CHEMTAX.

Both from their contribution to total Chl *a*, and from their contribution to biomass using abundance as a proxy, it is evident that classes belonging to nanoplankton category predominate in autumn and winter. Apart from dinoflagellates, it is also evident that these other phytoplankton classes are significantly negatively correlated with diatoms, especially during relaxation periods for upwelling. Böttjer and Morales (2007) observed a similar succession from the larger sized diatom community to the smaller nanoflagellates in an upwelling region off Concepción, in central Chile. According to Loureiro et al. (2011), the phytoplankton community at Sagres area has demonstrated nanoflagellate dominance, whereas in an earlier study which was limited to only the summer months, diatoms are clearly the prevalent community (Loureiro et al., 2005a). Several studies have documented this phenomenon as the typical succession pattern of phytoplankton in upwelling systems, whereby there is interchange between diatom domination with upwelling water, followed by flagellates as the upwelling relaxes and stratification occurs: the flagellates are better adapted to low-mixing conditions (Smith et al., 1983; Mann, 1993; Tilstone et al., 2000; Mendes et al., 2011). Most of these studies also note that fluctuations in nutrient regimes are related to this succession pattern, with phosphorus limitation favouring flagellate dominance. Indeed, in this study, the DIN:DIP ratio was extremely high on several occasions where nanoflagellates dominated diatoms (e.g. 17th November, 2008 and 14th October 2011).

4.1. Minor phytoplankton classes

Although the domination by dinoflagellates, chrysophytes and cyanobacteria classes has been rarely seen during this study, it is important to analyze their dynamics and seasonality as there are several species from these classes that have been observed at Sagres that are capable of producing harmful algal blooms (Loureiro et al., 2005a, 2008, 2011). Both CHEMTAX and microscopy have demonstrated that dinoflagellates and cyanobacteria are minority classes.

This study has shown discrepancies between estimates for dinoflagellates from CHEMTAX or microscopy. In some cases CHEMTAX did not detect dinoflagellates contribution to Chl *a*, while microscope counts of the same samples found that dinoflagellates can comprise up to 27% of the total cell count for plankton. These differences may arise from the uses of abundances as opposed to biovolumes: as larger organisms will make a greater contribution to pigment concentrations and biomass than a smaller individuals. Also, errors may arise from the difficulty of identification and enumeration of the smaller dinoflagellates, such as the gymnodinioid group. However, the greatest source of underestimation by the CHEMTAX method is probably limitations in the detection of heterotrophic dinoflagellates, as the method uses peridinin as a diagnostic pigment that only occurs in some of the auto- or mixotrophic species of dinoflagellates (Throndsen, 1997). All other pigmented species have acquired their chloroplasts and their

pigments from other taxa, and as a result heterotrophic dinoflagellates may be dominant, but will only be detected by the pigment analysis from what they have consumed (Higgins et al., 2011).

In this study, there is a significant positive correlation between dinoflagellates and temperature which also corroborates the observation by Loureiro et al. (2005a) that an increase in dinoflagellates is related with the warmer waters of the Sagres counter current.

Cyanobacteria are also a minority group in terms of average contribution to total Chl *a*, but again in a few samples their contribution can attain maxima of 50% and 60% (e.g. 6th and 14th September 2011 at station C), indicating that there are periods during the year when this group dominates in the area. A short study on cyanobacteria using epifluorescence microscopy made simultaneously from 8th September 2008 to 11th July 2009 of this study, showed a reasonable correlation between our results and abundance of cyanobacteria ($R^2 = 0.68$, $p < 0.05$) (Anna Gladkikh, *personal communication*). The contribution of this class to total Chl *a* was higher in autumn and also was not favoured by upwelling conditions (positive correlation with Q_y and temperature). There is very little information on the cyanobacteria dynamics in similar upwelling zones, but several studies have documented that regulation of cyanobacteria is by biological factors such as grazing by dinoflagellates (Verity et al., 1993; Schumann et al., 1994; Jeong et al., 2005; Böttjer and Morales, 2007), rather than by hydrological factors such as upwelling.

Although the sampling was extensive, it was limited to calm periods (see Section 2.1) which could bias the results, particularly when considering minor classes such as dinoflagellates and cyanobacteria. These classes may make notable contributions to the phytoplankton community, but only during limited periods in the year.

4.2. Integrating CHEMTAX results with other techniques

CHEMTAX, as well as other techniques and studies applied to the same area (e.g. inverted microscopy by Loureiro et al., 2011), have shown that the Sagres region is generally dominated by classes from the nanoplankton (Jeffrey and Vesk, 1997).

Goela et al. (2013) studied the spectral features of phytoplankton during the first period of study (2008–2009) and found that both the specific phytoplankton absorption coefficient spectra and blue to red ratios showed shapes and ranges of values typical for small-sized cell phytoplankton community in several samples (e.g. 17th November 2008 and 14th February 2009). Moreover, using another chemotaxonomic method (Uitz et al., 2006) for the determination of community size structure for the same samples, it was found that *nano* and *pico* fractions of phytoplankton also dominated the community, which is in agreement with the CHEMTAX results in this current study.

With respect to phytoplankton size structure, the three methods: spectral, CHEMTAX and microscopy are in agreement, but at the taxonomic level there discrepancies between CHEMTAX and microscopy especially with the more minor classes such as dinoflagellates (see also Section 4.1). Conversely, the more dominant diatoms show very similar results for all three techniques (Goela et al., 2013, e.g. 15th March 2009, 3rd April 2009 and 22nd April 2009). Despite this, CHEMTAX is the only one of these techniques that can readily discriminate between the small-sized phytoplankton classes such as prymnesiophytes, cryptophytes and prasinophytes, which are dominant on a significant number of days at Sagres. There is a specific requirement for *in situ* data to validate remote sensing algorithms for the detection of different phytoplankton communities (Platt et al., 2006; Brewin et al., 2011), where the most effective approach is indeed with chemotaxonomic methods (Moisan et al., 2012). With regard to validation, the knowledge of optical and functional role of the pigments is more relevant than the structural composition of the community. However, reliance on CHEMTAX methodology alone is not advised for the overall characterization of the phytoplankton dynamics in this area. There are limitations to CHEMTAX in this current study: a) results obtained from a large temporal window increase the possibility of occasional events causing significant fluctuations in the pigment:Chl *a* ratios (Higgins et al., 2011) within the same bin (e.g. occasional unialgal blooms, nutrient limitation, etc); b) the initial pigment:Chl *a* ratios have not been obtained from cultures collected in Sagres, as it is known to be the best practice (Laza-Martinez et al., 2007), but from literature values, which can cause some degree of bias in the results; c) there are also other limitations related to specific classes of phytoplanktons, particularly dinoflagellates (Section 4.1).

In contrast, there are also problems with the inverted microscopy technique: a) the misidentification or overlooking of small-sized classes; b) inadequate preservation of cells prior to analysis; c) the reliance of the method on good taxonomical skills (Gieskes and Kraay, 1983; Simon et al., 1994; Higgins et al., 2011). Despite this, the taxonomic results from the microscopy are critical in the decision of what major algal classes should be uploaded for the initial configuration of CHEMTAX (e.g. this study, Irigoien et al., 2004; Higgins et al., 2011). Both methods are able to detect development of diatom-dominated communities; moreover, microscopic data were useful in revealing the species composition of diatoms and dinoflagellates. The CHEMTAX method is capable of providing information on the smaller nanoplankton size class which is difficult to identify by microscopy. The discrepancies between CHEMTAX estimation of prymnesiophytes contribution to total Chl *a* with coccolithophorids abundance suggest that non-colonial prymnesiophytes could be present in the unidentified nanoflagellates group. However, there are dates where there is an observable correspondence (4th October 2008), suggesting that Chl *a* from prymnesiophytes in those days has been contributed mainly by coccolithophorids. Regarding cryptophytes, CHEMTAX has shown that this is one of the main classes of phytoplankton contributing pigments, despite the relatively low abundance shown by microscopy (Fig. 4). It is probable that cryptophytes are not readily identified by microscopy and are only included in the overall estimates for nanoflagellates; indeed, during 2008 and 2009 cryptophytes were not counted separately.

4.3. Comparison with other chemotaxonomic studies

The Sagres study has demonstrated by CHEMTAX that diatoms and cryptophytes are the main phytoplankton classes contributing to total Chl *a*, followed closely by prymnesiophytes and prasinophytes. However, the high density of cryptophytes is not a universal observation. For example, Mendes et al. (2011), found that in the north of Sagres, the major groups were prymnesiophytes at offshore sites and diatoms at coastal stations, whilst cryptophytes made only a minor contribution. Gibb et al. (2001) presented results for a northeastern Atlantic transect whose southern end is at a latitude (19.0°W) similar to Sagres, where the results also confirmed a high predominance of prymnesiophytes and cyanobacteria, with low densities of cryptophytes. However, Pereira et al. (2007) observed that cryptophytes were the second most abundant group in the Ria Formosa Lagoon, which is located 100 km east of Sagres and is highly influenced by the coastal water (Loureiro et al., 2005b). Similar patterns to Sagres have been observed in a Peruvian upwelling system with the dominance of cryptophytes at several stations and it is possible that in most coastal regions with upwelling cycles, cryptophytes are dominant at specific stages of the cycle.

5. Conclusions

In answer to the questions posed in the Introduction:

a) How useful is CHEMTAX for the evaluation of phytoplankton communities compared to other techniques? The comparison between CHEMTAX and other techniques used at Sagres has shown that for larger phytoplankton (>20 μm) the results agree with those for other techniques especially for diatoms. However CHEMTAX has been poor for identifying dinoflagellates compared to microscopy, probably as peridinin, which is the pigment marker used to distinguish dinoflagellates in this study, does not occur in most of the species identified for this group at Sagres. However, with the smaller phytoplankton (<20 μm), CHEMTAX has been particularly useful for identifying the contribution of cryptophytes, prasinophytes, prymnesiophytes and chrysophytes to the abundance of the flagellate community and, also, cyanobacteria. None of these smaller organisms are easily identified by microscopy, nor readily separated into classes by the spectrophotometric determination of the absorption coefficients.

b) Can CHEMTAX improve the analysis spatial distribution of the phytoplankton community? CHEMTAX has shown that the spatial distribution of the Chl *a* at the three stations at Sagres is remarkably uniform. In relation to Secchi depth, there is also no trend, which is consistent with a well mixed water column. Regarding the distribution of the phytoplankton classes, CHEMTAX has shown that the contribution of diatoms and dinoflagellates to total Chl *a* decreases from inshore to offshore, as opposed to the contribution from prasinophytes, chrysophytes and prymnesiophytes, which increases further offshore.

c) Can CHEMTAX improve the analysis of the temporal differences in the phytoplankton at each station? CHEMTAX has been especially useful for identifying the seasonal changes in the phytoplankton community at Sagres. Diatoms alternate in dominance with the flagellate classes, in which cryptophytes, followed by prymnesiophytes and prasinophytes play important roles. Diatoms tend to dominate early spring to summer, and the flagellate classes in autumn and winter. These observations could be mostly attributed to seasonal upwelling effects, with relaxation of upwelling conditions and silicon limitation suggested as possible factors contributing to the collapse of the diatom bloom. Seasonal patterns are less evident for cyanobacteria and dinoflagellates.

Given these findings, it is evident that this study, using a combination of CHEMTAX and inverted microscopy has been the best approach for understanding the dynamics of the phytoplankton community at Sagres. In this study, the smaller nanoplankton community has only been discriminated with CHEMTAX, whilst the dinoflagellate community has only been represented accurately with microscopy.

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