Effect of phytoplankton size classes on bio-optical properties of phytoplankton in the Western Iberian coast: application of models

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Abstract

Chlorophyll-*a* satellite products are routinely used in oceanography, providing a
synoptic and global view of phytoplankton abundance. However, these products lack
information on the community structure of the phytoplankton, which is crucial for
ecological modelling and ecosystem studies. To assess the usefulness of existing
methods to differentiate Phytoplankton Functional Types (PFT) or phytoplankton size
classes from satellite data, in-situ phytoplankton samples collected in the Western
Iberian coast, on the North-East Atlantic, were analysed for pigments and absorption

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spectra. Water samples were collected in five different locations, four of which were located near the shore and another in an open-ocean, seamount region. Three different modelling approaches for deriving phytoplankton size classes were applied to the *in situ* data. Approaches tested provide phytoplankton size class information based on the input of pigments data (Brewin et al., 2010), absorption spectra data (Ciotti et al., 2002) or both (Uitz et al., 2008).

Following Uitz et al. (2008), results revealed high variability in microphytoplankton chlorophyll-specific absorption coefficients, ranging from 0.01 to 0.09 m² (mg chl)⁻¹ between 400 and 500 nm. This spectral analysis suggested, in one of the regions, the existence of small cells (<20 µm) in the fraction of phytoplankton presumed to be microphytoplankton (based on diagnostic pigments). Ciotti et al. (2002) approach yielded the highest differences between modeled and measured absorption spectra for the locations where samples had high variability in community structure and cell size. The Brewin et al. (2010) pigment-based model was adjusted and a set of model coefficients are presented and recommended for future studies in offshore water of the Western Iberian coast.

Keywords: Remote sensing; Phytoplankton size classes; Ocean colour; Pigments; Chlorophyll-*a*; Western Iberian coast.

1. Introduction

Phytoplankton are a key component of the oceans and one of the most important primary producers in the pelagic system. They are responsible for approximately 50% of global net primary production (Field et al., 1998; Bowler et al., 2009). They also form a critical part of the biological pump, given that part of the carbon fixed by phytoplankton is exported to the deep ocean by the sinking of cells, thus becoming unavailable to the trophic chain in the upper layers (Anderson et al., 2005; Rudorff and Kampel, 2012). Phytoplankton also play a major role in global biogeochemical cycling of climatically-relevant elements such as silica and nitrogen, in addition to carbon.

The analysis of water leaving radiance from remote sensing has been used to derive estimates of phytoplankton chlorophyll-*a* (chl-*a*) in the oceans (O'Reilly et al., 1998; Siegel and Franz, 2010; Kahru et al., 2011). Satellite-derived chlorophyll-*a* products are one of the most widely-used ocean-colour products for biological oceanography. Chlorophyll-*a* maps from satellites have provided synoptic and global views of phytoplankton abundance for over a decade. However, they lack information on phytoplankton composition that is required to improve our understanding of biogeochemical cycling (Nair et al., 2008).

A taxonomically diverse phytoplankton community has, for practical purposes, been classified into several groups, organized by size-classes or by their specific ecological and biogeochemical roles in the system, the Phytoplankton Functional Types (PFT; Reynolds et al., 2002). According to their biogeochemical function, phytoplankton can be classified as calcifiers, silicifiers, dimethyl sulfide (DMS) producers or nitrogen fixers (Nair et al., 2008). The phytoplankton size classification (e.g. Sieburth et al., 1978) partitions them into three size classes: picophytoplankton (<2 μm), nanophytoplankton (2 to 20 μm) and microphytoplankton (> 20 μm).

The balance between phytoplanktonic groups is known to change with environmental forcing (Marinov et al., 2010). Factors such as nutrient enrichment, which can lead to eutrophication, or a global change in the climate are likely to affect the normal dynamics and dominance of phytoplankton species (e.g. Bricker et al., 2008; Tett et al.,

2008; Boyce et al., 2010). Ocean colour data could be a powerful tool to obtain a synoptic view of the phytoplankton community and to investigate its dynamics through time. However, differences in the optical signatures of the different phytoplankton communities are often small and hard to detect and to interpret. Phytoplankton are one of the primary elements that influence reflectance data in the open ocean, and their optical properties can be modified by the shape, size, pigment composition, or the physiological status of the cell (e.g. Bricaud et al., 1988). The size of cells is critical, as the absorption by large cells is normally influenced by intracellular shading, i.e. the packaging effect on pigments (Duysens, 1956; Sathyendranath et al. 1987; Bricaud et al., 1995; Baird et al., 2007). The light regime is also likely to provoke changes in the internal structures of cells and consequently the optical properties (Nair et al. 2008).

Several modelling approaches have been pursued during recent years to distinguish phytoplankton groups from satellite ocean-colour data. Some approaches differentiate cells according to their size class (Ciotti et al., 2002; Ciotti and Bricaud, 2006; Devred et al., 2006; Brewin et al., 2010) and others are designed to distinguish specific functional groups (Sathyendranath et al., 2004; Alvain et al., 2005). The model developed by Ciotti et al. (2002) and Ciotti and Bricaud (2006) is based on the shape of the phytoplankton absorption coefficient of pico- and microphytoplankton. Brewin et al. (2010) developed a model to distinguish three size classes. The approach of Brewin et al. (2010) is based on pigments and no optical data is used to parameterise the model, which can be an advantage in terms of its general use and implementation. It relies on an initial assessment of the size classes, which is performed following the method proposed by Vidussi et al. (2001) and Uitz et al. (2006), with some modifications. This method uses diagnostic pigments to obtain the fractions of pico, nano- and microphytoplankton (Uitz et al., 2006; Brewin et al., 2010). Sathyendranath et al. (2004) developed a model to distinguish populations dominated by diatoms from other mixed populations, based on their absorption characteristics. Uitz et al. (2008) developed a model to estimate the specific-absorption coefficient of three size classes using both diagnostic pigments (Uitz et al., 2006) and phytoplankton absorption data.

Models presented above have been previously tested against in-situ data. However, phytoplankton communities are influenced by the local specificities of their environment (e.g. hydrodynamics, light regime, etc.). Regional validation and adaptation is therefore important, especially for those models developed regionally such as that of Sathyendranth et al. (2004). For instance, Jackson et al. (2011) found that tuning of the diatom model to the waters off the Chilean coast was required. Brewin et al. (2012) also modified their model for the Indian Ocean, compared with what was previously proposed by Brewin et al. (2010). In addition, few studies have discussed bio-optical properties of phytoplankton communities off the Portuguese coast. Brotas et al. (2013) observed regional differences in the model of Brewin et al. (2010) when applied to North-East Atlantic. The study of Brotas et al. (2013) yielded a dominance of microphytoplankton contribution to chlorophyll-*a* concentrations when total chlorophyll-*a* was greater than 0.5 mg m^{-3} , rather than 1.3 mg m^{-3} as in the original model. Goela et al. (2013) has reported that size structure of phytoplankton has a major effect on the variability of phytoplankton absorption coefficient in the southwest coast of Portugal. Sá et al. (under review), in a validation exercise, compared different chlorophyll-*a* satellite products retrieving better results with the algorithm regionally developed with in situ radiometric data, reinforcing the need to account for regional specificities. Namely, it is likely that the phytoplankton communities have different properties according to region-specific environmental conditions (Mendes et al., 2012). Therefore, it is important to understand the biological and optical properties of phytoplankton communities in the Western Iberian coast, to identify an appropriate model for this region.

The successful application of ocean-colour data to evaluate how anthropogenic pressure, in the form of nutrient enrichment, affects the spatial and temporal distribution of PFT in the coastal areas of the North-East Atlantic would represent a considerable advance towards the understanding of the environmental quality of these waters, which is now mandatory under the Marine Strategy Framework Directive (MSFD; C.E.C., 2008). This study aims at supporting these goals by contributing to the understanding of the bio-optical properties of phytoplankton in the Western Iberian

coast, according to size class. This was done by: 1) analyzing phytoplankton community structure using pigment composition; 2) testing the usefulness of bio-optical models, based on absorption spectra and/or pigment data, to differentiate phytoplankton size groups; 3) assessing the most suitable approach for coastal and offshore waters around the Western Iberian Coast.

2. Methodology

2.1 Study Sites

Five locations were considered in this study, one oceanic, the Gorringe Seamount, two in the Northwestern Portuguese coast, Nazaré and Aveiro (i.e. HABSpot in Figure 1), one fixed point in Cascais bay, and several stations across the Southwestern Portuguese coast (CSA; Figure 1).

Gorringe seamount is located in the Atlantic Ocean, around 125-150 miles west of Portugal between Azores and the Strait of Gibraltar (Alteriis et al., 2003). It is considered to be an oligotrophic region with low nutrient and phytoplankton concentrations (Mendonça et al., 2012). Aveiro, in the Northern coast of Portugal is a region that is typically influenced by seasonal upwelling due to the northerly winds occurring during summer. It is characterized by a relatively large continental shelf, which extends from Aveiro to Nazaré, where there is an underwater canyon just off the coast of Nazaré. This is one of the largest underwater canyons in Europe (Mendes et al., 2011). Cascais is located in the vicinity of the mouth of the Tagus River, where upwelling events occur occasionally. It is important to note that all samples from Cascais were taken during the ebb/high tide period to reduce the river influence. The location considered as CSA in Figure 1 is characterized by weak upwelling episodes.

2.2 Phytoplankton data

2.2.1 Sampling

Water samples (minimum of 1 L) were collected using Niskin bottles during three cruises carried out in the location of Gorringe (June 2009), Nazaré (June-July 2010) and

Aveiro (September 2011; i.e. HABSpot in Figure 1). One continuous monitoring program was also carried out in Cascais and other occasional campaigns are described below. For the cruises, water samples were collected at the surface (5m) and at the deep chlorophyll maximum (DCM), which varied from 5 to 40 m. In the other campaigns only surface samples (~1m) were collected. During the Cascais monitoring program, samples were collected weekly at a fixed point in the bay of Cascais, from March 2011 to January 2012 (green in Figure 1). Several other samples were collected during 2010 and 2011, in the South and Western coast of Portugal (CSA in Figure 1), more specifically in the location of Cascais, Sines and Algarve.

All samples were kept in dark conditions under a refrigerated environment and filtered with Whatman glass microfiber filters (25mm Ø; 0.7 µm pore size) as soon as possible after collection. The filter containing the phytoplankton sample was then covered with aluminum foil and stored at -80°C. Two replicates of each sample were obtained, i.e. two filters per sample, one for phytoplankton pigments, which were analyzed by high performance liquid chromatography (HPLC; Zapata et al., 2000) and another for phytoplankton absorption spectra, which were analyzed following the method described in Tilstone et al. (2002).

2.2.2 Phytoplankton pigments and size class analysis

For pigment analysis, each filter was placed in a 10 cm³ centrifuge tube. Pigments were then extracted in the dark for 30 min at -20°C with 3 cm³ of 95% cold-buffered methanol (2% ammonium acetate) enriched with a known concentration of trans-beta-apo-8'-carotenal pigment (Sigma supplier). This pigment is used as an internal standard to evaluate and correct for the effects/errors due to processing of samples (e.g. differences in filter water content, evaporation and volume measurement errors). Samples were then sonicated (Bransonic model 1210, w:80, Hz:47) for 5 min and placed at -20°C for an additional period of extraction (30 min). After that period, samples were centrifuged at 4000 rpm for 15 min at 4°C. Extracts were filtered through a Fluoropore PTFE filter membrane (0.2 µm pore size) and immediately injected in the HPLC. Pigment extracts were analyzed through a Shimadzu HPLC

composed by a solvent delivery module (LC-10ADVP) with system controller (SCL-10AVP), a photodiode array (SPD-M10ADVP) and a fluorescence detector (RF-10AXL). Chromatographic separation of pigments was achieved using the method described in Zapata et al. (2000), which uses a monomeric OS C8 column and a mobile phase constituted by two solutions: methanol:acetonitrile:aqueous pyridine and methanol:acetonitrile:acetone; an injection volume of 100 μm^3 , a flow rate of 1 $\text{cm}^3 \cdot \text{min}^{-1}$ and a run duration of 40 min (see Hooker et al., 2012 for further details).

The limit of detection (LOD) and limit of quantification (LOQ) of this method were presented and discussed by Mendes et al. (2007). Pigments were identified by comparing the absorbance spectra and retention times with the HPLC calibration results carried out with pigment standards from DHI. Pigment concentrations were calculated from the signals in the photodiode array detector or fluorescence detector.

Table 1 – Number of samples (n) and average concentrations ($\text{mg} \cdot \text{m}^{-3}$) of the pigments needed for the different approaches used in this study. Two significant digits are presented.

Location	N	TChl- <i>a</i>	TChl- <i>b</i>	Perid	But	Hex	Allox	Zeax	Fuco	Diat	Diad	βcar
HABSpot	62	0.85	0.045	0.055	0.037	0.19	0.012	0.0082	0.28	< LOQ	0.024	< LOQ
Nazaré	62	0.32	0.071	0.021	0.010	0.078	0.015	0.0035	0.11	< LOQ	0.023	n.d.
Cascais	38	1.1	0.091	0.14	0.012	0.055	0.035	0.0089	0.55	0.010	0.10	0.013
Gorringe	22	0.15	0.020	0.010	0.025	0.056	n.d.	0.018	0.015	n.d.	0.012	n.d.
CSA	17	2.1	0.084	0.071	0.020	0.077	0.019	0.010	2.1	0.018	0.19	0.0077

TChl-*a* – Total chl-*a**

But - 19'-butanoyloxyfucoxanthin

Diat – diatoxanthin

LOQ – Limit of quantification

TChl-*b* – Total chl-*b*

Hex - 19'-hexanoyloxyfucoxanthin

Diad – diadinoxanthin

Perid – Peridinin

Zeax - zeaxanthin

βcar - β carotene

Allox – alloxanthin

Fuco – fucoxanthin

n.d. – not detected

* For simplicity TChl-*a* is referred to as chl-*a* in the text.

The final step consisted of a quality control application to the dataset. All samples with an incomplete set of pigments or with the majority of pigments below the quantification limit were rejected. The procedure for quality assurance of pigments

presented by Aiken et al. (2009) was conducted. In addition, all samples below the euphotic depth were also eliminated. This criterion was applied as approaches used in this study were specifically developed for the upper part of the water column (euphotic zone). Table 1 presents a summary of pigment composition for the five cases presented: HABSpot, Nazaré and Gorringe cruises, the Cascais fixed point and CSA stations. Pigments presented are the ones used throughout this study for: 1) the phytoplankton size class assessment, calculated according to Uitz et al. (2006), and as described below; and 2) the analysis of the photoprotective (PPC) and photosynthetic (PSC) carotenoids. In this study PPC were calculated as alloxanthin + zeaxanthin + diatoxanthin + diadinoxanthin + β carotene. PSC were determined as peridinin + 19'-butanoyloxyfucoxanthin + 19'-hexanoyloxyfucoxanthin + fucoxanthin. Note that the dataset was also reduced after applying quality control to spectral absorption data, as described in the following section. Only the samples with both quality controlled pigment and spectral data were retained for the analyses. Finally, a total of 201 samples were used in this study (Table 1).

As mentioned, after quality control, phytoplankton size structure and community were then determined following the method proposed by Uitz et al. (2006), who developed a statistical model to differentiate the fractions of micro-, nano- and picophytoplankton in the total chlorophyll-*a* (chl-*a*) concentration, based on a large pigment (HPLC) database. The method involved the use of a multiple regression analysis between chl-*a* and diagnostic pigments, obtaining a set of coefficients for each diagnostic pigment used (see Vidussi et al., 2001; Uitz et al., 2006 and Brewin et al., 2010 for further details). The diagnostic pigments considered were fucoxanthin, peridinin, 19'-hexanoyloxyfucoxanthin, 19'-butanoyloxyfucoxanthin, alloxanthin, total chlorophyll-*b* (mv-chlorophyllb + divinyl chlorophyll-*b*) and zeaxanthin. According to this approach, chl-*a* concentration (*C*) can be obtained from the set of coefficients (*W*) and the pigments (*P*), as follows:

$$C = \sum_{i=1}^7 W_i P_i \quad 1)$$

where [*W*] = {1.41; 1.41; 1.27; 0.35; 0.6; 1.01; 0.86} and [*P*] = {fucoxanthin; peridinin; 19'-hexanoyloxyfucoxanthin; 19'-butanoyloxyfucoxanthin; alloxanthin; chlorophyll-*b*

and divinyl chlorophyll-*b* (TChl-*b*); zeaxanthin}. Following Uitz *et al.* (2006) the fractions (F) of each size class can be calculated according to:

$$F_3 = \frac{\sum_{i=1}^2 W_i P_i}{C} \quad 2)$$

$$F_2 = \frac{\sum_{i=3}^5 W_i P_i}{C} \quad 3)$$

$$F_1 = \frac{\sum_{i=6}^7 W_i P_i}{C} \quad 4)$$

where subscripts of 1, 2 and 3 represent pico, nano and microphytoplankton respectively. The chl-*a* concentration associated with each size class can then be calculated as follows:

$$C_3 = F_3 \cdot C \quad 5)$$

$$C_2 = F_2 \cdot C \quad 6)$$

$$C_1 = F_1 \cdot C \quad 7)$$

2.2.3 Analysis of size index

Bricaud *et al.* (2004) proposed a method to provide an approximation of the average cell size of each sample, the size index (SI). This index has the advantage of providing a single continuous parameter to characterize in-situ samples. It is calculated by attributing a central size to each class (1 µm, 5 µm and 50 µm to picophytoplankton, nanophytoplankton, and microphytoplankton, respectively) and weighting this value by the biomass proportion of the corresponding class. Thus:

$$SI = 1.F_1 + 5.F_2 + 50.F_3 \quad 8)$$

2.2.4 Analysis of spectral absorption characteristics of phytoplankton

274

275 The phytoplankton absorption spectra were analysed on a Shimadzu dual-beam
276 spectrophotometer. The baseline and spectral noise were documented using air-air
277 scans to check instrument performance. The frozen filters containing the
278 phytoplankton sample were placed in a petri dish on filtered water to hydrate and
279 thaw for at least 5 min before absorption measurement was made, from 400 to 800nm
280 with a 1nm resolution. After measuring the absorption spectrum of the sample, filters
281 were bleached using approximately 5 cm³ of 0.1% NaClO solution for 10 min (Tassan
282 and Ferrari, 1995; Tilstone et al., 2002), except for samples from the Gorringe cruise,
283 which were bleached with hot methanol according to the method described in Mitchell
284 et al. (2000). This procedure is carried out to remove the phytoplanktonic pigments in
285 the sample. The filters were then rinsed with 0.2µm filtered artificial seawater, to
286 remove any residual NaClO (or methanol), and measured against a bleached blank.

287

288 Phytoplankton absorption data were then corrected for differential scattering, filtered
289 volume, area of the filter and pathlength amplification (Ciotti et al., 2002). The residual
290 scattering was corrected by subtracting the mean absorption between 730 and 750nm
291 from the initial absorption data (see Tassan et al., 2000). The theoretical pathlength
292 amplification correction (beta factor) of Roesler (1998) was then applied.
293 Phytoplankton absorption was finally computed, after the corrections described
294 above, as the difference between the absorption spectra, before (total particulate) and
295 after (detritus) extraction.

296 Quality assurance criteria were applied to the dataset, and samples that did not meet
297 the required conditions were eliminated (both absorption and HPLC data, as indicated
298 before). The absorption data were normalized using chlorophyll-*a* data to obtain the
299 specific absorption coefficient and data variance was analyzed to check for outliers.
300 Additionally, the relationships between phytoplankton absorption coefficient and chl-*a*
301 were also investigated at 412, 443, 490, 510, 555, 620 and 670nm. This was also done
302 to check for outliers.

303

304 **2.3 Models implemented**

To assess the usefulness of different methods to differentiate PFT or phytoplankton size classes and to understand the bio-optical properties of the phytoplankton community in the Western Iberian waters, three different modelling approaches were applied to our in-situ data. One of these approaches is based on the shape of the phytoplankton absorption coefficient (Ciotti et al, 2002). Another approach is based on both the absorption and pigment data (Uitz et al., 2008). The latter approach implemented here is a simplified version of what was proposed by Uitz et al. (2008) since they dealt with three size classes and only two are considered here. As implemented here, the chlorophyll-*a* concentrations of two size classes are first derived from pigment data (Uitz et al. 2006; Equations 1-7), then the chlorophyll-specific phytoplankton absorption coefficients of each size are derived assuming the total phytoplankton absorption coefficient is an additive property of the two size classes. The third approach (Brewin et al., 2010) uses exclusively pigment data to determine the chlorophyll-*a* concentrations of three size classes (based on Uitz et al. 2006; described previously), which are then used to fit a conceptual model (Sathyendranath et al. 2001) that describes how the chlorophyll-*a* concentrations of each size class vary as a function of total chlorophyll-*a*.

The Uitz et al. (2008) and the Brewin et al. (2010) approaches are similar in that they both use pigment data. In the development phase, these two approaches require chlorophyll-*a*, as well as diagnostic pigments data to distinguish phytoplankton size classes. In the implementation phase, however, the Brewin et al. (2010) approach relies on the fitted model to estimate the size classes from total chlorophyll-*a* (Figure 2), while the Uitz et al. (2008) approach makes use of the chlorophyll-*a* concentration in the three size classes (estimated using HPLC pigments) and the total phytoplankton absorption coefficient to derive the chlorophyll-specific phytoplankton absorption coefficient of each size class. In this study, the chlorophyll-specific absorption coefficients for only two size classes are derived for the study region using the regional data described above. The models of Brewin et al. (2010) and Uitz et al. (2008) can provide information on three size classes (pico-, nano- and microphytoplankton) or on two size classes, by combining pico- and nanophytoplankton. The approach of Ciotti et al. (2002) is not dependent on chlorophyll-*a*. This model uses the spectral shape of

phytoplankton absorption to distinguish between phytoplankton size classes, both in the development and in the implementation phases. Therefore, this approach has the potential to distinguish different phytoplankton classes independently of knowledge on the total chlorophyll-*a* concentration, provided spectral phytoplankton absorption data are available.

2.3.1 Methods based on phytoplankton absorption spectra

2.3.1.1 Method based on the specific absorption spectra of phytoplankton – Uitz et al. (2008)

The phytoplankton absorption coefficient (a_{ph}) is an additive property, which can be expressed as the sum of the absorption coefficients of three size classes. The absorption by each size class can be expressed as the product of the specific absorption coefficients (a^* ; $m^2(mg\ chl)^{-1}$) and the chl-*a* concentration of that size class. Furthermore, Uitz et al. (2008) assumed that the specific absorption of the three size classes would vary as a function of the optical depth of the sample, leading to the following equation:

$$a^*(\lambda) = \left(\frac{1}{C}\right) \sum_{i=1}^3 C_i a_i^*(\lambda) \exp\left(-R_i \frac{z}{Z_p}\right) \quad 9)$$

where $i = \{\text{pico-, nano- and microphytoplankton}\}$, and R_i represents the slopes describing variations in a^* along the vertical z/Z_p axis, where z = depth and Z_p euphotic depth. Considering that the present study is limited to surface and near-surface samples, the variation of a^* along the vertical axis was ignored, and the chl-*a* concentrations of pico- and nanophytoplankton were combined, because of lack of sufficient resolution in the data to analyse these two size classes independently, such that the above equation was simplified to:

$$\frac{a_{ph}(\lambda)}{C} = \left(\frac{1}{C}\right) \cdot (a_{1,2}^*(\lambda)C_{1,2} + a_3^*(\lambda)C_3) \quad 10)$$

where the subscripts of 1,2 and 3 represent combined-pico-nanophytoplankton and microphytoplankton respectively, so that $C = C_{1,2} + C_3$. The chlorophyll-*a* concentrations for each size class were first obtained using the Uitz *et al.* (2006) diagnostic pigment approach described previously. Once these were obtained, the spectral specific absorption coefficients of each group were derived from fitting the Equation 10 using a standard nonlinear least squares method (Levenberg-Marquardt), with phytoplankton absorption (a_{ph}) and chlorophyll-*a* concentrations for each size class as input. The values of the specific absorption coefficient were constrained to be greater than zero. The specific absorption spectra derived by Uitz *et al.* (2008) for a global application were used along with $a_{ph}(\lambda)$ of individual samples to derive C_1 , C_2 and C_3 according to Equation 10. According to this method, the abundances of the two size classes are estimated according to their chlorophyll-*a* concentrations. This fitting procedure was also based in the Levenberg-Marquardt algorithm, which is a combination of the Gauss-Newton and the steepest descent algorithms. No wavelength-dependent weights were attributed and no additional criteria to select samples were applied. The default value of 10^{-6} was used as convergence criterion, both for the function and the coefficients, with a maximum value of 400 iterations allowed.

2.3.1.2 Method based on spectral shapes of phytoplankton absorption – Ciotti *et al.* (2002)

Ciotti *et al.* (2002) assessed the dominant cell size of phytoplankton through a variety of techniques, which included physical separation of chlorophyll-*a* into size fraction and subsequent measurement by Turner fluorometry. When size fractionated data were not available, the dominant cell size was established through microscopy and flow cytometry. The shape of the phytoplankton absorption spectra was also analyzed. The absorption spectra ($a_{ph}(\lambda)$, m^{-1}) was normalized using the mean absorption (a_{ph} , m^{-1}) computed between 400 to 700 nm, with a wavelength step of 1 nm (Ciotti *et al.*, 2002).

In their work, four phytoplankton groups: pico- (<2 μm), ultra- (2-5 μm), nano (5-20 μm) and microphytoplankton (> 20 μm) were considered. The model developed using mean absorption spectra for each of the size classes was able to explain more than

80% of the variability for most wavelengths from 400 to 700nm. An additional step was introduced to characterize the contributions to absorption by the smallest and the largest cells in the database, i.e. pico or microphytoplankton, using a coefficient (S_f) which is allowed to vary between 0 (100% microphytoplankton) and 1 (100% picophytoplankton). The intermediate values represent all possible combinations of these two communities. The value of S_f is found by performing a least-square routine, using phytoplankton absorption spectra obtained from field campaigns normalized with the mean absorption computed between 400 and 700nm, and the normalized absorption spectra for the smallest and largest cells, as compiled by Ciotti et al. (2002). Thus:

$$\hat{a}_{ph}(\lambda) = [S_f \cdot \bar{a}_{pico}(\lambda)] + [(1 - S_f) \cdot \bar{a}_{micro}(\lambda)] \quad 11)$$

where $\bar{a}_{pico}(\lambda)$ and $\bar{a}_{micro}(\lambda)$ are the basis vectors (the normalized absorption spectra for the smallest and largest cells) taken from Ciotti et al. (2002) and S_f is the size parameter, which varies between zero and one. In this study, Equation 11 was used to derive the size parameter for each sample. Note that the size parameter provides a measure of the relative contributions of the two size classes (pico- and microphytoplankton) to the total phytoplankton absorption. The measure of phytoplankton abundance in this model is absorption, unlike the method based on Uitz et al. (2008), in which the measure is chlorophyll-*a* concentration.

2.3.2 Pigment-based method – Brewin et al. (2010)

Brewin et al. (2010) extended the two-component model of Sathyendranath et al. (2001) to a three-size-class model of phytoplankton. For this approach only pigment (HPLC) data are used, which distinguish it from the other models. The total chlorophyll-*a* concentration (C) is expressed as the sum of pico- (1), nano- (2) and microphytoplankton (3), such that

$$C = C_1 + C_2 + C_3 \quad 12)$$

The chlorophyll-*a* concentrations in the three size classes are first computed using HPLC data. For this step, we used the method of Uitz et al. (2006) without the adjustment by Brewin et al. (2010), so that methods are consistent with each other in this respect. Once the chlorophyll-*a* concentrations of picophytoplankton (C_1) and combined pico-nanophytoplankton ($C_{1,2}$) are known, in addition to the total chlorophyll-*a* (C), the following equations were fitted to derive the unknown model parameters:

$$C_{1,2} = C_{1,2}^{max} [1 - \exp(-S_{1,2}C)] \quad 13)$$

$$C_1 = C_1^{max} [1 - \exp(-S_1C)] \quad 14)$$

These equations assume that the phytoplankton size structure varies with chlorophyll-*a* concentration in a predictable manner, as presented in Figure 2. The chlorophyll-*a* associated with other components can then be derived:

$$C_3 = C - C_{1,2} \quad 15)$$

$$C_2 = C_{1,2} - C_1 \quad 16)$$

The unknown parameters of equations 13 and 14 were derived using a standard nonlinear least squares method (Levenberg-Marquardt). The fitting procedure was implemented in MATLAB 10 software. This method allows the differentiation of size classes from an initial estimate of chlorophyll-*a* concentration, and has been applied to ocean colour products obtained from satellite imagery on a global scales (as in Brewin et al., 2010) or regionally, after validation with in situ data (as in Brotas et al., 2013).

2.4 Statistical tools used

The spectral absorption (first and second) and pigment (third) approaches involved the use of a standard nonlinear least squares method (Levenberg-Marquardt) to fit model parameters. For the first approach, 95% confidence intervals for the coefficients retrieved were computed through a bootstrapping procedure with 10,000 sub-

sampled populations (Efron and Tibshirani, 1993). The unbiased root mean square error (URMSE) and the bias were determined following the method presented in Friedrichs et al. (2009) and Brewin et al. (2012).

3. Results

3.1 Estimation of size classes from pigment data

Chlorophyll-*a* concentrations for the three size classes (pico-, nano- and microphytoplankton) obtained using the method proposed by Uitz et al. (2006) are presented in Figure 3. Considering all available data (Figure 3-A), the phytoplankton community seemed to be dominated by microphytoplankton for chl-*a* concentrations higher than 0.5 mg.m⁻³. Figures 3-B to G present the community structure for each location, except Figures 3-D and E, which represent the same location (Aveiro – HABSpot cruise) at surface (D) and at the deep chlorophyll maximum (DCM – E). Data from this cruise were analysed separately for the two depths because the concentrations of several marker pigments (Fuco, Perid, TChl-*b*, Hex and Zeax) relative to chl-*a* indicated the presence of two communities (Figure 4), with several nanophytoplankton groups and diatoms dominating at the surface and Prymnesiophytes (component of the nanophytoplankton) at the DCM. In general, microphytoplankton dominated at surface samples for chl-*a* concentrations higher than 0.5 mg.m⁻³ and nanophytoplankton dominated at the DCM samples for chl-*a* concentrations up to 1 mg.m⁻³. Note that in Gorringe (Figure 3-F), chlorophyll-*a* concentrations were always lower than 0.5 mg.m⁻³.

The class dominances (in percentage) were plotted per station for each location (Figure 5). Microphytoplankton fraction is found to dominate most samples in Cascais (Figure 5-A), Nazaré (Figure 5-B) and CSA (Figure 5-F). Gorringe (Figure 5-E) was clearly dominated by nanophytoplankton. Samples from Aveiro (HABSpot) were dominated either by microphytoplankton or by nanophytoplankton (Figure 5-C and D).

The highest ratios of PPC/PSC were obtained in samples from Gorringe and Cascais (≥ 0.25). The lowest ratio was obtained in samples from the HABSpot cruise (located at

the DCM; < 0.1). Ratios of PPC to chl-*a* (i.e. TChl *a*) were always lower than those of PSC (Table 2).

Table 2 – Concentrations (mg.m⁻³) of Photosynthetic (PSC – Fuco+Hex+But+Perid) and Photoprotective (PPC – Allox+Diat+Diad+Zeax+ β car) carotenoids in the five location of this study. Ratios between PPC and PSC and PPC and total chlorophyll-*a* (chl-*a*) are also provided. Values are represented by the average $\pm$ standard deviation.

Location	PSC	PPC	PPC/PSC	PPC/chl- <i>a</i>
Cascais	0.75 $\pm$ 0.57	0.17 $\pm$ 0.16	0.25 $\pm$ 0.16	0.16 $\pm$ 0.070
Nazaré	0.22 $\pm$ 0.087	0.041 $\pm$ 0.025	0.19 $\pm$ 0.025	0.13 $\pm$ 0.038
HABSpot S	0.42 $\pm$ 0.60	0.038 $\pm$ 0.039	0.15 $\pm$ 0.11	0.045 $\pm$ 0.033
HABSpot DCM	0.66 $\pm$ 0.50	0.053 $\pm$ 0.054	0.077 $\pm$ 0.049	0.062 $\pm$ 0.024
Gorringe	0.10 $\pm$ 0.071	0.031 $\pm$ 0.016	0.34 $\pm$ 0.091	0.23 $\pm$ 0.047
CSA	2.1 $\pm$ 1.6	0.25 $\pm$ 0.19	0.12 $\pm$ 0.083	0.11 $\pm$ 0.050

3.2 Modelling methods: absorption vs pigment methods

3.2.1 Results obtained applying the model of Uitz et al. (2008)

Table 3 – Coefficient of determination (r^2) obtained by performing a linear regression of absorption coefficient at a specific wavelength against chlorophyll-*a* data. The corresponding values of the slope, i.e. specific absorption coefficients, are also presented.

Wavelengths (nm)	r^2 from linear regression					
	All	Cascais	Nazaré	HABSpot	Gorringe	CSA
412	0.69	0.30	0.54	0.74	0.90	0.76
443	0.71	0.37	0.75	0.78	0.91	0.78
490	0.72	0.38	0.77	0.78	0.91	0.80
510	0.74	0.34	0.75	0.81	0.89	0.81
555	0.75	0.28	0.55	0.81	0.78	0.79
620	0.78	0.34	0.78	0.89	0.85	0.85
670	0.76	0.44	0.89	0.90	0.96	0.85
	Slope					
	All	Cascais	Nazaré	HABSpot	Gorringe	CSA
412	0.081	0.031	0.051	0.044	0.077	0.12
443	0.081	0.034	0.074	0.044	0.094	0.12
490	0.056	0.023	0.052	0.032	0.074	0.079
510	0.044	0.016	0.032	0.026	0.045	0.062
555	0.022	0.0079	0.012	0.014	0.018	0.031
620	0.021	0.0081	0.013	0.013	0.018	0.029
670	0.046	0.020	0.040	0.026	0.047	0.066

A clear linear relationship between the absorption coefficients at 443nm and the chl-*a* concentrations was found for all study sites. Chlorophyll *a* has its main absorption coefficient peak around the 443nm wavelength. Samples collected in all locations, except Cascais, yielded a coefficient of determination higher than 0.50. In fact, for the wavelengths of 443, 490, 510, 620 and 670nm, the coefficient of determination observed was always higher than 0.70 (Table 3). The slopes of these linear relationships represent the phytoplankton specific absorption coefficients, which vary between 0.034 and 0.12 m².mg chl⁻¹ for the 443nm wavelength.

The specific absorption coefficients for pico+nano- and microphytoplankton are presented in Figure 6. Fits were performed for each wavelength and uncertainties for each wavelength were computed using a bootstrapping procedure, which re-sampled the database 10,000 times, randomly. Considering all the available data, the resultant specific absorption coefficient for microphytoplankton was approximately 0.018 m².mg chl⁻¹ at 430nm, and the corresponding value for the nano-picophytoplankton fraction was approximately 0.18 m².mg chl⁻¹ (Figure 6-A). The clear separation between the 95% confidence intervals indicates statistically-significant differences between spectra of micro- and nano+picophytoplankton fractions.

Equation 10 was also fitted to each location individually to investigate the optical properties of phytoplanktonic communities in these regions. No statistical differences were found between the spectra of the two specific absorption coefficients (micro- and nano+picophytoplankton), as 95% confidence intervals overlap, except for the HABSpot stations (Figure 6-D and E). This is perhaps a consequence of the small number of samples in each dataset and/or the lack of a gradient from micro- to picophytoplankton (Table 1 and Figure 3). At Gorringe, microphytoplankton yielded a specific absorption coefficient higher than that for the nano+picophytoplankton fraction (Figure 6-F). This pattern is the opposite of what was expected, and found for the other regions. It is important to note that this result for Gorringe is likely to be an artifact due to the small amount of samples used, in which very low fucoxanthin concentration was present, indicating that samples were not dominated by microphytoplankton. Excluding Gorringe, the highest specific absorption coefficient for microphytoplankton were found in Nazaré and CSA and reached approximately 0.09

$\text{m}^2.\text{mg chl}^{-1}$ at $\sim 430\text{nm}$. Microphytoplankton coefficients for HABSpot were lower, only reaching $0.018 \text{ m}^2.\text{mg chl}^{-1}$. The highest nano+picophytoplankton coefficients were found in the region of HABSpot ($0.17 \text{ m}^2.\text{mg chl}^{-1}$) and CSA ($0.55 \text{ m}^2.\text{mg chl}^{-1}$) also at $\sim 430\text{nm}$. The coefficients of determination (r^2) were found to be lower than 0.15 for Cascais and CSA and higher than 0.15 for HABSpot and Nazaré.

The specific absorption spectra (for pico-, nano- and microphytoplankton) taken from Appendix I of Uitz et al. (2008) were used to feed Equation 10 and derive the chl- a concentrations associated with each size class (Figure 7). Results were then compared with the chl- a concentrations of each phytoplankton size class derived directly from pigments, i.e. following Uitz et al. (2006). Cascais and HABSpot were the locations where the best agreement between model results and pigment data was obtained, especially for nano- and microphytoplankton. Please note that even in these cases, although r^2 values are reasonable, the slopes from linear regression showed deviations from the 1:1 line. Generally, results were poor for picophytoplankton.

3.2.2 Results from the model of Ciotti et al. (2002)

The absorption data from this study were compared with the basis vectors or shapes of the absorption spectra proposed by Ciotti et al. (2002) and the size parameters were computed and plotted against the total chlorophyll- a concentrations (Figure 8-A). All fits resulting in the S_f values presented in Figure 8-A had a coefficient of determination (r^2) higher than 0.9. Samples with chlorophyll- a concentration higher than 1 mg.m^{-3} yielded S_f values between 0.3 and ~ 0 , which is indicative of microphytoplankton dominance. On the other hand, samples with chlorophyll- a concentration lower than 0.5 mg.m^{-3} were characterized by S_f values which varied between 0 and 1, with samples from Gorringe and HABSpot-surface being associated with the picophytoplankton (S_f between 0.6 and 1). The values of S_f for samples from Nazaré were mostly between 0.3 and 0.6, except for 11 samples which yielded S_f values below 0.3. For chlorophyll- a values between 0.5 and 1 mg.m^{-3} , values of S_f were mainly

between 0 and 0.6. Samples from Cascais and CSA were dominated by microphytoplankton.

The size index (SI) analysis, which is based on pigments, also revealed similar trends. Samples from Cascais and CSA yielded SI values mainly between 30 and 50 μm , which indicate the presence of large cells, mainly microphytoplankton (Figure 8-B). Samples from Gorringe and HABSpot were the ones with the lowest SI values, which is also in agreement with S_f . It is important to note that the presence of microphytoplankton cells, even in low densities, strongly affects the final SI value (Equation 8). Thus, the identification of pico- and nanophytoplankton dominance is difficult and should be carefully evaluated.

The general agreement between S_f and SI, which are two independent methods, was reasonably good for these locations, nevertheless, some points are worth noting. Samples dominated by microphytoplankton found at CSA and Cascais presented SI values higher than 30 μm and S_f values lower than 0.3-0.4. They are located in the lower right corner of Figure 8-C. Samples from HABSpot S and Gorringe are located in the upper left corner of Figure 8-C, with S_f values higher than 0.5-0.6 and SI values lower than 20 μm . Although a high number of samples from HABSpot S and Gorringe have S_f values higher than 0.8, i.e. near 1, which indicates picophytoplankton dominance, they were dominated by nanophytoplankton cells (Figures 5 and 8-C) according to the pigment data. Samples from Nazaré yielded S_f values between 0.2 and 0.6, which are intermediate values.

3.2 Results from the model of Brewin et al. (2010).

The model fit obtained using the approach of Brewin et al. (2010) applied to all available data was significant for all phytoplankton components (Figure 9-A to H; Table 4). The coefficients obtained from fitting Equations 13 and 14 in log space are provided in Table 4. Note that 95% confidence intervals, obtained through a bootstrapping analysis with 10,000 runs, are also provided in parenthesis. The best result was

obtained for microphytoplankton (Figure 9- A to D), for which the model explained most of the observed variability (Table 5). Lower coefficients of determination and higher URMSE were found for all the other fractions, however the biases were lower for the smaller size classes (Table 5).

Table 4 – Model coefficients obtained for the three-component model ($C^{\max}$ and S from Equations 13 and 14). 10,000 bootstrapping analysis was performed to obtain the median and the 2.5% and 97.5% confidence intervals (provided between brackets).

	Size	$C^{\max}$ (mg.m ⁻³)	S (Initial slope)
All data	Pico	0.087 (0.071 - 0.11)	1.9 (1.4 - 2.6)
	Pico+Nano	0.26 (0.23 - 0.31)	3.3 (2.6 - 4.1)
HABSpot, Nazaré & Gorringe	Pico	0.11 (0.077-0.19)	1.5 (0.79-2.3)
	Pico+Nano	0.50 (0.38-0.75)	1.5 (0.94-2.1)

The model (Figure 9 A) represents well all the data for the microphytoplankton fraction derived from diagnostic pigments ($r(3)^2=0.48$). Although the fits were also reasonable for the other fractions (i.e. pico, pico+nano and nanophytoplankton) for all the data as a whole, some locations seem to exhibit variations in patterns: for example in the case of Cascais and CSA (Figure 9 – B to D), the model overestimated the chlorophyll-*a* concentrations associated with the various size classes. (Figures 9 – B and C), leading to lower r^2 values ($r(1)^2= 0.16$, $r(1,2)^2=0.48$ and $r(2)^2=0.45$ for pico, pico+nano and nanophytoplankton respectively). For chlorophyll-*a* concentrations lower than 0.1 mg.m⁻³, an overestimation of chlorophyll-*a* concentrations was found for Cascais and CSA data. For chlorophyll-*a* concentrations higher than 0.1 mg.m⁻³, an underestimation of chlorophyll-*a* concentrations was found, especially in the region of HABSpot and Gorringe, as represented by the data points below the 1:1 plot (Figure 9 – B and C). Note that the latter is masked by HABSpot data points.

Table 5 – Statistical results for the model fitting procedure. Coefficient of determination (r^2), the unbiased RMSE (denoted URMSE, an index of precision in data) and the bias (index of accuracy in data) are provided. Statistics are performed in log10 space.

	Size	r^2	URMSE	Bias
All data	Pico	0.56	0.28	0.001
	Pico+Nano	0.49	0.23	0.000
	Nano	0.30	0.28	0.033
	Micro	0.89	0.20	0.059
HABSpot, Nazaré & Gorringe	Pico	0.64	0.25	0.002
	Pico+Nano	0.80	0.14	0.001
	Nano	0.72	0.17	0.019
	Micro	0.84	0.20	0.051
Individual cruises (mean)	Pico	0.70	0.23	0.003
	Pico+Nano	0.74	0.16	-0.001
	Nano	0.72	0.17	0.010
	Micro	0.96	0.12	0.023

Applying the regional model parameterised using all data to a local scale may result in an under or overestimation. This is especially important when using remote sensing data to investigate phytoplankton community structure at the local scale. Therefore, additional fits were performed using only data from Nazaré, HABSpot and Gorringe, which were the most oceanic stations and yielded similar results in the initial analysis (Figure 9). The model output was significantly correlated with data for all phytoplankton components, and as before, the highest determination coefficient was obtained for microphytoplankton ($r(3)^2 = 0.84$; Table 5). The agreement of the fit with the combined nano and picophytoplankton components was also high ($r(1,2)^2 = 0.80$). The fits for pico and nanophytoplankton presented coefficients of determination between 0.64 and 0.72, respectively.

The same exercise was carried out for each individual dataset. Fits were performed and averaged statistical results are also presented in Table 5. Under these conditions

the model yielded a better agreement with individual cruises than considering all data together.

4. Discussion

4.1 Methods based on phytoplankton absorption

Values of the specific absorption coefficient obtained are within the range of coefficients reported by Goela et al. (2013) for southern Portugal, which ranged from 0.02 to 0.12 m².mg chl⁻¹ (440nm) at surface (0-5m). The highest slopes, i.e. specific absorption coefficients for phytoplankton, were found at 443nm, as expected (e.g. Bricaud et al., 1995). Spatially, the highest specific absorption coefficients were found in samples from CSA, Gorringe and Nazaré. These high values for Gorringe were in accordance with the dominance by non-microphytoplankton cells, as inferred from the pigment analysis (Uitz et al., 2006). However, Nazaré was composed of a relatively high proportion of microphytoplankton cells and CSA was dominated by microphytoplankton, according to pigment analysis. Samples from CSA presented the highest absorption coefficients, reaching up to 0.6 m⁻¹ at 443nm, with chl-*a* concentrations up to 4.8 mg.m⁻³, resulting in a specific absorption coefficient of approximately 0.12 m².mg chl⁻¹. Generally, the specific absorption coefficients of microphytoplankton in the literature are much lower, varying between 0.005 and 0.03 m².mg chl⁻¹ (Sathyendranath et al., 2001; 2004; Devred et al., 2006; Alvain et al., 2012).

Equation 10 of the Uitz et al. (2008) method, as implemented here, allowed distinguishing two different phytoplankton populations. Considering all the available data, the specific absorption coefficient for nano+picophytoplankton yielded high values, with $a_{1,2}^* > 0.1 \text{ m}^2.\text{mg chl}^{-1}$ and $a_3^* < 0.02 \text{ m}^2.\text{mg chl}^{-1}$ at 440nm, which is in accordance with several studies (e.g. Ciotti et al., 2002; Devred et al., 2006; Uitz et al., 2008). The model confirmed the high specific absorption coefficients for CSA and Gorringe, as obtained through the linear regressions presented above.

The analysis of the absorption spectra in Nazaré showed low variability in the specific absorption coefficient obtained for the secondary absorption peak of phytoplankton in the red, from 650 to 700nm. Differences were more pronounced for the other locations. Absorption in this wavelength range has been used to estimate size class (Roy et al., 2011). At these wavelengths, the absorption is dominated by chlorophyll-*a* and the influence of other pigments is low. Therefore, it is easier to study the packaging effect, using just one pigment, the chl-*a*. Consequently, a size-classification method based on spectral properties in the red could perhaps be further developed.

The model of Ciotti et al. (2002) compares the absorption spectra obtained in field samples with the signature shapes defined for pico- and microphytoplankton. Each sample is then classified according to its similarity to the pico and microphytoplankton spectra. Most of the samples from Cascais and part of the samples from CSA were classified as microphytoplankton-dominated according to this method. Samples from HABSpot Surface and part of the samples from Gorringe were classified as picophytoplankton. The samples from other locations yielded intermediate S_f values. The comparison between mean measured and modeled normalized absorption spectra yielded the highest differences for samples from Nazaré and HABSpot (data not shown). These samples had high variability in community structure and cell sizes, which was probably responsible for the higher differences. Slight differences were also found in Gorringe.

4.2 The pigment-based methods

The pigment-based model of Brewin et al. (2010) estimates the fractions of micro-, nano- and picophytoplankton, which can then be applied to remote sensing data. Fits were significant for all groups and errors were comparable with those of Brewin et al. (2010; 2011a) and Brotas et al. (2013).

Figure 9 shows that the data are organized in two separate groups, one composed of the regions of CSA and Cascais, where the model overestimates chl-*a* concentrations, and the other, composed of the regions of HABSpot, Nazaré and Gorringe where the model underestimates chl-*a* concentrations. These deviations are clear for total

chlorophyll-*a* concentrations higher than 0.2 mg chl m⁻³ and are likely to be part of the natural variability between regions. However, it is important to note that special care has to be taken when applying such general models to specific geographic locations. For example, if the model is applied to offshore waters in Northern Portugal, i.e. HABSpot and Nazaré regions, the results would underestimate the nano and picophytoplankton components. It is also important to consider that temporal variation is likely to contribute significantly to the natural variability of phytoplankton data. For studies in the North-Western coast of Iberia, it would be best to use the model parameters fitted for the most offshore locations, i.e. HABSpot, Nazaré and Gorringe, as presented in Table 4. This region is influenced by strong upwelling events due to the northerly winds (Fiúza, 1983; Moita, 2001), that supply nutrients and turbulence that favour the development of nano- and microphytoplankton blooms in coastal waters (Margalef, 1978). Therefore, this would be a very useful method to study phytoplankton blooms in this location.

Spatial scales are also very important when using these approaches, as the Brewin et al. (2010) model tends to become more precise and effective when tuned for specific regions. Specifically, the C_{max} and slope parameters estimated here for pico+nanophytoplankton (Table 4), are nearer the ones estimated by Brotas et al. (2013) for a large region in North East Atlantic including Portuguese Coast, then the ones presented by Brewin et al. (2010, ie. C_{max} and slope for surface waters in Brotas et al. (2013) were 0.280 mg m⁻³ and 3.4, respectively, values close to All Data from our Table 4). However, it is important to ensure a good temporal coverage, to include a broad range of pigment concentrations, and to use the model only within the range of chlorophyll-*a* concentrations for which it has been tuned.

One of the most striking differences between the offshore and inshore waters is the asymptotic maximum values (C^{max}). For individual cruises, higher C^{max} values were found for nano and picophytoplankton in the offshore locations and lower values were found in the inshore waters (Cascais and CSA). This suggests microphytoplankton dominance sets in at different total chlorophyll-*a* concentrations in different regions, as was evident from pigment data. Moreover, it is interesting that chlorophyll-*a* concentrations of nano+pico and nanophytoplankton fractions seem to reach maximum at total chlorophyll-*a* concentrations of 0.3 mg chl.m⁻³, which is different

from what is presented in Figure 2 (closer to 1 mg chl.m^{-3}), highlighting the importance of regionally tuning such algorithms.

4.3 Comparison between model results and diagnostic pigments

Individual specific absorption coefficient spectra were classified as dominated by pico-, nano- and microphytoplankton using diagnostic pigments. Average spectra for nano- and microphytoplankton dominated samples from HABSpot were found to be different from each other. No significant differences were found between the average spectra for pico-, nano- and microphytoplankton dominated samples from Cascais, CSA, Nazaré and Gorringe (Figure 6). However, samples from Nazaré and CSA yielded high values for a_3^* when using the Uitz et al. (2008) method, suggesting that the presumed microphytoplankton community was actually composed of small cells, though it is difficult to assign any confidence to these results, since the differences in the absorption properties of the two communities in these regions were not extracted with high statistical significance. But it does leave a suggestion that, for those field samples, the size classes inferred from the specific absorption coefficients are not in full agreement with those based on pigment data.

No reasonable agreement was found between chlorophyll-*a* concentrations associated with the different phytoplankton size classes, derived using the spectral model (Uitz et al., 2008) and in-situ data (from diagnostic pigments), except for a few cases. A general overestimation of chlorophyll-*a* concentrations was found, especially for microphytoplankton, revealing the need of a regional adjustment with a more comprehensive database.

The output of the Ciotti et al. (2002) optical model (S_f) was found to be in general agreement with the Size Index (SI) analysis, particularly for Cascais and CSA. However, some differences were found. Samples from HABSpot S and Gorringe were found to have S_f values near 1, i.e. generally assigned to picophytoplankton, whereas diagnostic pigments (Figure 5) and SI values suggested a nanophytoplankton dominance of the community. It should be considered that HPLC method, in comparison with other methods such as size-fractionated filtration, has been recently reported to

overestimate nanophytoplankton and underestimate picophytoplankton fractions (Brewin et al., 2014). Moreover, samples from Nazaré yielded intermediate S_f values, whereas SI values, as well as diagnostic pigments, indicated microphytoplankton dominance. Nevertheless, it is important to note that differences between the results of optical and pigment approaches are not unexpected, since one (i.e. Ciotti et al. 2002) estimates dominance based on relative contributions of the different size classes to total phytoplankton absorption, and the other is based on pigment information (i.e. Uitz et al. 2006).

Results obtained after the re-parameterization of the Brewin et al. (2010) model were much improved compared with the ones obtained using previously-published global parameters. In Figure 9, the data are organized into two groups: one composed of the regions of CSA and Cascais samples, where the model overestimates chl- a concentrations, and the other, which is composed of the regions of HABSpot, Nazaré and Gorringe where the model underestimates chl- a concentrations. These deviations are clear for total chlorophyll- a concentrations higher than 0.2 mg chl m⁻³ and represent the natural variability between regions. These results reinforce the need for a regional and a seasonal adjustment when using such empirical models, as already noted.

4.4 Considerations regarding cell size

As discussed in section 4.1, no statistical differences were found between the specific absorption coefficients of nano+picophytoplankton and microphytoplankton in the Nazaré region, and the a_3^* values obtained were high (≈ 0.08 m².mg chl⁻¹ at ~ 430 nm), compared with other regions. Several studies (e.g. Ciotti et al., 2002; Devred et al., 2006; Uitz et al., 2008; Brewin et al., 2011b; Devred et al., 2011) have presented $a_3^* < 0.03$ m².mg chl⁻¹ at 440nm for microphytoplankton in a variety of geographical locations. However, Goela et al. (2013) presented values similar to ours for samples that were dominated by microphytoplankton according to diagnostic pigments, in southern Portugal. The phytoplankton size classes estimated from pigments may not

always indicate their true size structure (Uitz et al., 2006). It is possible that the high specific absorption coefficients obtained here for diatoms and dinoflagellates, that are taken to be microphytoplankton, may be composed of small cells, which would have higher specific absorption coefficients because of lower packaging effect (Sathyendranath et al., 1987; Stuart et al., 1998; Lohrenz et al., 2003). The packaging of pigments within phytoplankton cells leads to a decreased absorption compared with the potential absorption of the same amount of pigment solution (Baird et al., 2007). This results in a flattened absorption spectrum and decreases the absorption coefficients for large cells (Fujiki and Taguchi, 2002; Baird et al., 2007). Some samples from Nazaré were analyzed under the microscope. These preliminary results seem to suggest that the apparently 'microphytoplankton' fraction according to the pigments, was in fact composed of species characterised by relatively small cells, with sizes equal to, or less than 20 μm (data not shown). For example, some of the most abundant genera were the diatoms *Chaetoceros* and *Paralia*, and the dinoflagellates *Amphidinium* and *Gymnodinium*. Cells from the non-chain-forming species from *Gymnodinium* genus (cell size $\leq 20 \mu\text{m}$) constituted an important part of the samples. They represented on average 36% to the pool consisting of diatoms and dinoflagellates and the proportion in individual samples were as high as 87%. However, further experiments need to be performed to confirm these results. In the absence of concrete evidence to the contrary, the classification of 'microphytoplankton' based on pigments is retained throughout this discussion.

Note that small concentrations of peridinin and higher concentrations of fucoxanthin are observed in our samples from Nazaré. However, microscopic analysis revealed that most samples have similar proportions of diatoms and dinoflagellates, with some exceptions. These results also point towards the importance of dinoflagellates, such as *Gymnodinium*, which may not have peridinin and possess fucoxanthin and fucoxanthin derivatives instead (Roy et al., 2012).

The effects of light availability are also of importance. Waters off Iberia have high light levels due to the large values of incident energy (per unit of area) on average, compared with higher latitude regions. In addition, low values of the diffuse attenuation coefficient (e.g. Mucho et al., 2013) and suspended particulate matter

(e.g. Oliveira et al., 1999; Santos-Echeandía et al., 2012) have also been reported for specific locations of the Portuguese coast. Cells experiencing high light levels tend to have less chlorophyll-*a* molecules per cell, compared with low-light adapted cells. Therefore they are likely to have a smaller packaging effect, with direct implications for the specific absorption coefficients (Lohrenz et al., 2003). In addition, it is also important to keep in mind that pigment composition may also change within the same group, as cells may be adapted to different light regimes and have higher or lower concentrations of photoprotective pigments, such as zeaxanthin (Ras et al., 2008). PPC/PSC values in Nazaré were higher at the surface and lower at the DCM, pointing to the importance of photoprotective carotenoids for light absorption by phytoplankton at the surface. The highest PPC/PSC ratios were observed in the Gorringe location. High PPC/PSC ratios have been associated with high light levels, low nutrient concentrations and stratified waters (Eisner and Cowles, 2005; Barlow et al., 2007), which is in agreement with the general conditions observed in the Gorringe seamount. Low values of PPC/PSC ratios, as well as PPC/chl-*a*, have been reported in upwelling areas with mixed water columns, such as Nazaré and Aveiro (where HABSpot sampling was conducted). Highly productive regions are generally characterised by low proportions of photoprotective to photosynthetic pigments (Barlow et al., 2007; Ras et al., 2008). Eisner et al. (2003) and Eisner and Cowles (2005) have also discussed the association of high PPC/PSC ratios with small cell size (see also Sathyendranath et al., 2005).

5. Conclusions

The development of an effective model to differentiate phytoplankton size classes and phytoplankton functional types using satellite imagery, at a regional scale, is crucial to understand the responses of the phytoplankton community to climate variability or change, as well as the potential impacts at higher trophic levels. Furthermore, these approaches can be used as an important tool to evaluate the environmental quality of coastal waters following the environmental directives in effect, such as the EU Marine Strategy Framework Directive. Designed for large-scale application, the global models tested were unable to capture the variability in local in-situ datasets, unless regional

adjustments were performed. Our results, based on the spectral properties of phytoplankton in the Western Iberian coast, showed high variability in the chlorophyll-specific absorption coefficients of the phytoplankton fraction identified as microphytoplankton on the basis of their diagnostic pigments, with some high values suggesting that small cell size diatoms or dinoflagellates (<20 µm) were misclassified as microphytoplankton fraction for one region. The model based on Brewin et al. (2010) was adjusted to our in-situ data and was used to estimate the contribution of chlorophyll-*a* concentration in each phytoplankton fraction. The parameterization of the proportions of the three size classes with change in total chlorophyll-*a* concentration schematised in Figure 2 was initially developed and validated for Case-1 waters (Brewin et al., 2010), whereas, according to the method proposed by Lee and Hu (2006), some of our locations, Cascais and CSA are Case-2 waters. This study contributes towards the implementation of phytoplankton models at regional scales, where there is still a considerable lack of understanding of micro- and meso-scale biological processes. The Western Iberian coast is a very productive region, with strong upwelling events and frequent episodes of harmful algal blooms, which have significant impacts in socio-economic activities. The use of combined spectral and pigment methods should be further investigated in future studies and may improve the discrimination power within each phytoplankton size class.

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Figure caption

Figure 1 – Map of the Western Iberian coast with sampling stations identified in the locations of Nazaré, Sines and Cascais/Lisbon Bay.

Figure 2 – Scheme of the change in percentage (A) and concentrations (B) of the three size classes of phytoplankton as a function of the total chlorophyll-*a* concentration. Note that the x-axes of both schemes and the y-axis of the second scheme are in logarithmic scale. Shifts in the phytoplankton community dominance are indicated. Adapted from Brewin et al. (2010).

Figure 3 – Chlorophyll-*a* concentrations per size fraction (mg.m^{-3} ; micro-, nano- and picophytoplankton), as calculated by Uitz et al. (2006) method, versus total chlorophyll-*a* data (mg.m^{-3}) for: all data (A), Cascais (B), Nazaré (C), HABSpot surface (D), HABSpot DCM (E), Gorringe (F) and CSA (G). Symbols indicate size fractions.

Figure 4 – Ratios between pigments (Fuco, Perid, TChl-*b*, Hex and Zeax) and chl-*a*, for each location.

Figure 5 – Relative dominance (in %) of the three phytoplankton classes in the locations of: Cascais (A), Nazaré (B), HABSpot (Surface-C; DCM-D), Gorringe (E) and CSA (F). Fractions obtained following the method of Uitz et al. (2006).

Figure 6 – Phytoplankton specific absorption coefficients for micro- (a_m) and nano+picophytoplankton (a_{np}) derived from the first approach (Uitz et al., 2008). Values were normalized to the chl- a concentration ($m^2 \cdot mg\ chl^{-1}$). Solid lines represent the specific absorption coefficient of microphytoplankton and dashed lines represent the nano+picophytoplankton. The 95% confidence interval is also represented in grey.

Figure 7 – Modelled chlorophyll- a concentrations ($mg \cdot m^{-3}$) obtained using the approach of Uitz et al. (2008), i.e. Equation 10 for 2 and 3 size fractions, plotted against the chlorophyll- a concentrations (C_i ; $mg \cdot m^{-3}$) derived using Uitz et al. (2006) approach, considered as data. First column represents all data together and the following columns represent different locations. Results were obtained by applying the specific absorption spectra available for three size classes from Appendix I in Uitz et al. (2008).

Figure 8 – Size parameter S_f (A) and Size index SI (B) against the total chlorophyll- a concentrations ($mg \cdot m^{-3}$) of each sample obtained at Cascais, Nazaré, Aveiro (HABSpot - Surface and DCM), Gorringe and CSA locations. SI is also presented against S_f (C).

Figure 9 – Three component model for each phytoplankton component (3-micro; 2-nano; and 1-picophytoplankton), using all available data, plotted against the total chlorophyll- a concentration (A to D). The fit to the chlorophyll concentrations of each fraction is also illustrated. The correspondent fractions of each component are also represented against the total chl- a concentration (E to H). Legend of data: green $\diamond$ – Cascais; black + – Nazaré; light blue x – HABSpot(Surface); dark blue $\square$ – HABSpot(DCM); magenta * – Gorringe; red Δ – CSA.

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Figure 6 – Phytoplankton specific absorption coefficients for micro- (am) and nano+picophytoplankton (anp) derived from the first approach (Uitz et al., 2008). Values were normalized to the chl-*a* concentration ($\text{m}^2 \cdot \text{mg chl}^{-1}$). Solid lines represent the specific absorption coefficient of microphytoplankton and dashed lines represent the nano+picophytoplankton. The 95% confidence interval is also represented in grey.

Figure 7 – Modelled chlorophyll-*a* concentrations (mg.m^{-3}) obtained using the approach of Uitz et al. (2008), i.e. Equation 10 for 2 and 3 size fractions, plotted against the chlorophyll-*a* concentrations (C_i ; mg.m^{-3}) derived using Uitz et al. (2006) approach, considered as data. First column represents all data together and the following columns represent different locations. Results were obtained by applying the specific absorption spectra available for three size classes from Appendix I in Uitz et al. (2008).

Figure 8 – Size parameter S_f (A) and Size index SI (B) against the total chlorophyll- a concentrations (mg.m^{-3}) of each sample obtained at Cascais, Nazaré, Aveiro (HABSpot - Surface and DCM), Gorringe and CSA locations. SI is also presented against S_f (C).

Figure 9 – Three component model for each phytoplankton component (3-micro; 2-nano; and 1-picophytoplankton), using all available data, plotted against the total chlorophyll-*a* concentration (A to D). The fit to the chlorophyll concentrations of each fraction is also illustrated. The correspondent fractions of each component are also represented against the total chl-*a* concentration (E to H). Legend of data: green ◇ – Cascais; black + – Nazaré; light blue x – HABSpot(Surface); dark blue □ – HABSpot(DCM); magenta * – Gorrige; red Δ – CSA.