Distribution of phytoplankton groups within the deep chlorophyll maximum

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Abstract

The fine vertical distribution of phytoplankton groups within the deep chlorophyll maximum (DCM) was studied in the NE Atlantic during summer stratification. A simple but unconventional sampling strategy allowed examining the vertical structure with ca. 2 m resolution. The distribution of Prochlorococcus, Synechococcus, chlorophytes, pelagophytes, small prymnesiophytes, coccolithophores, diatoms, and dinoflagellates was investigated with a combination of pigment-markers, flow cytometry and optical and FISH microscopy. All groups presented minimum abundances at the surface and a maximum in the DCM layer. The cell distribution was not vertically symmetrical around the DCM peak and cells tended to accumulate in the upper part of the DCM layer. The more symmetrical distribution of chlorophyll than cells around the DCM peak was due to the increase of pigment per cell with depth. We found a vertical alignment of phytoplankton groups within the DCM indicating preferences for different ecological niches in a layer with strong gradients of light and nutrients. Prochlorococcus occupied the shallowest and diatoms the deepest layers. Dinoflagellates, Synechococcus and small prymnesiophytes preferred shallow DCM layers, and coccolithophores, chlorophytes and pelagophytes showed a preference for deep layers. Cell size within groups changed with depth in a pattern related to their mean size: the cell volume of the smallest group increased the most with depth while the cell volume of the largest group decreased the most. The vertical alignment of phytoplankton groups confirms that the DCM is not a homogeneous entity and indicates groups’ preferences for different ecological niches within this layer.

The deep chlorophyll maximum (DCM) is a subsurface layer enriched in chlorophyll (Chl) typical of stratified marine and freshwater bodies. It might be the result of diverse processes and may present different characteristics (Cullen 1982, 2015). In temperate areas, the DCM disappears in winter when mixing takes place and reappears after the spring bloom, when stratification is established. The dynamics of this kind of DCM have been described in the literature (Estrada et al. 1993; Mignot et al. 2014). Briefly, winter surface waters replete with nutrients start stratifying after the air-water heat balance becomes negative (Sverdrup 1953; Taylor and Ferrari 2011) producing the spring bloom in temperate areas (although this classical approach has been challenged by Behrenfeld 2010). At this moment, the abundant phytoplankton accumulates very close to the surface, limiting the irradiance immediately below and inducing the synthesis of pigments by the deeper phytoplankton and the formation of a shallow DCM that might not correspond to a biomass maximum. However, this is a very dynamic situation because phytoplankton quickly (days-weeks) consume the nutrients from the well illuminated layers becoming less abundant at surface and concentrating at depth. This process originates a deeper DCM, coinciding with the nutricline and usually corresponding to a phytoplankton biomass maximum. Unless other vertical processes take place (Vaillancourt et al. 2003; Liccardo et al. 2013), phytoplankton keeps depleting the nutrients in the euphotic zone and thus deepen the DCM. In conclusion, during a relatively long period of the year, the DCM corresponds to a deep biomass maximum (DBM) in temperate areas.
It has been recognized that phytoplankton (Venrick 1988, 1999; Estrada et al. 2016) and heterotrophic bacterioplankton (Sunagawa et al. 2015) communities at the DCM and in the overlying mixed layer can be different, reflecting the different environmental characteristics of these two major layers and indicating that tens of meters in the vertical scale may make a stronger difference than tens of km in the horizontal scale. The DCM has routinely been considered a single entity and most research does not increase the sampling effort at the DCM. Under this view, the starting hypothesis would say that the DCM is a homogeneous layer where all phytoplankton groups are equally distributed. However, because the DCM layer is located in strong and opposite gradients of nutrients and irradiance, the main resources for phytoplankton growth, it could be expected that different groups are better adapted to the distinctive environmental conditions within the DCM layer if the conditions persist. Few works deal with the fine-scale structure of the DCM probably because of the difficulties to obtain samples with high vertical resolution. Special procedures have been designed to sample small-scale vertical structures of thin layers (Lunven et al. 2005) but they have not been yet extended and adapted to study the fine structure of the open ocean DCM.

Here, we used a simple CTD procedure to obtain samples every 2–3 m and tested the null hypothesis that the DCM is a single, homogeneous ecological entity in which phytoplankton groups are equally distributed. To that aim, we studied the vertical distribution of phytoplankton taxa within the DCM layer in offshore waters of the north and northwestern Iberian peninsula, belonging to the biogeographic North Atlantic Drift Province (Longhurst 1998). In this area, winter mixing reaches ca. 200 m (Somavilla et al. 2011), well below the nutricline, and the posterior re-stratification beginning around March produces a marked spring bloom. Summer is characterized by a progressive strengthening of stratification, following the typical temporal pattern of the North Atlantic (Lindemann and St. John 2014). Our study was performed during the summer period of pronounced thermal stratification characterized by the presence of a marked DCM located between 22 m and 65 m depth at the different profiles.

Material and methods

Samples were obtained during the two legs of the cruise Indemares0710 at Avilés Canyon (AC) and Galicia Bank (GB), in the north and northwestern waters of the Iberian Peninsula, respectively, in summer 2010 on board the R/V Thalassa (Fig. 1). Cast location, sample parameters and characteristics of the DCM layer are described in Table 1. The AC sampling stations were located on the continental shelf, the slope, and the abyssal plane. The GB stations were all located in an open ocean area. The sampling equipment consisted of a CTD profiler, a fluorometer (WET labs, ECO-AFL) and a 24-bottle Niskin rosette. To obtain detailed profiles of the DCM (deep chlorophyll maximum) the Niskin bottles were closed every 8–12 s during the upcast at a constant ascent rate of 0.25 m s⁻¹, i.e., every 2–3 m. The first sample below the DCM was chosen on the basis of the downcast fluorescence profile. An additional surface water sample was collected. In the GB also an intermediate sample was collected in the middle of the mixed layer. Because sampling was always performed at night, irradiance measurements were not available. To reconstruct the irradiance profiles we assumed that the depth of the DCM peak (Z_{DCM}) was located at the 1% surface irradiance (Navarro and Ruiz 2013; Mignot et al. 2014). An attenuation coefficient ($K_{PAR}$) for each profile was obtained as $K_{PAR} = 2/Z_{DCM}$ and the vertical irradiance profile (as percentage of surface) estimated from $E_z = E_0 \times 10^{-K_{PAR} z}$, where $Z_{DCM}$ is the depth of the DCM, $E_0$ and $E_z$ are the percent irradiances at surface (100%) and at depth $z$, respectively.

Variables analyzed

For nutrient analysis, 8 mL of unfiltered sample were frozen and kept at −20°C immediately after sampling. Inorganic nutrients were analyzed by standard nutrient techniques with a SKALAR San Plus Autoanalyzer. Detection limits for the different molecules were, in mmol m⁻³: 0.02 (NO₃), 0.01 (NO₂), 0.013 (PO₄), and 0.018 (SiO₄).

For flow cytometry analysis, 1.8 mL of sample were fixed with 1% paraformaldehyde plus 0.05% glutaraldehyde, flash frozen in liquid nitrogen and kept at −80°C. The analysis was performed with a FACSCalibur flow cytometer (BD Biosciences) equipped with a laser emitting at 488 nm. The flow rate was calibrated daily by weight to estimate cell concentrations. A solution of 1 μm fluorescent latex beads (ref. F-13081, Molecular Probes) was added as an internal standard so that all cellular variables were related to fluorescent beads values. Prochlorococcus and Synechococcus flow cytometry cell counts were converted to carbon (C) after estimating their cell volume from the relative side scatter signal as described in Calvo-Díaz and Morán (2006), and using the average value of 237 fg C μm⁻³ (Worden et al. 2004).

For CARD-FISH analysis of target groups of photosynthetic picocyanobacteria, 45 mL of sample were prefiltered by 20 μm, fixed with 37% formaldehyde (4% final concentration), filtered on 0.6 μm pore size Nuclepore filters (25 mm diameter) and kept frozen. Group-specific oligonucleotide probes were applied to target chlorophytes (CHL002), small prymnesiophytes (PRYM02) and pelagophytes (PELA01 and PELA1035). Concentrations were estimated from cell counts under an epifluorescence microscope (Olympus BX61) at 1000X under UV (DAPI signal), blue light (Alexa 488 signal), or green light (Alexa 594 signal) excitations. Cells were classified under the microscope in <2, 2-3, 3-4 and 4-5 μm diameter bins. Cell volumes were converted to C from cell volume estimates (Menden-Deuer and Lessard 2000). For details on the CARD-FISH assay, counting process and biomass estimates see Cabello et al. (2016).
Samples for large (> 5 μm) phytoplankton community composition were collected in brown glass bottles, preserved with a hexamine buffered 19% formaldehyde solution (0.6% final concentration) and kept in the dark until analysis under an inverted microscope (Nikon Eclipse TE2000-S without epifluorescence accessories) using the Utermühle technique (Utermühle 1958). The 95% confidence limit of the cell counts was ±20% (Lund et al. 1958; Sournia 1978). Composition and abundance of coccolithophores, diatoms and dinoflagellates were quantified. C from coccolithophores was estimated from a value of 11 pg cell−1, the C content of the dominating Emiliania huxleyi species (Riebesell et al. 2000). E. huxleyi and cf. Emiliania made up 90–99.8% of the coccolithophores counts, with an average contribution of 97.5%.

For HPLC analysis of pigments, 2 L of samples were vacuum (< 0.03 bar) filtered through 25 mm diameter Whatman GF/F glass fiber filters. Filters were folded, blotted dry and frozen in liquid nitrogen. After the cruise, samples were transferred to a −20°C freezer. For pigment extraction, filters were placed in 10 mL polypropylene tubes with 2.5 mL of 90% acetone containing trans-β-apo-8’-carotenal (Sigma) as internal standard and stored at −20°C. After 24 h, the individual 10 mL tubes were placed in a beaker filled with crunched ice and sonicated with a Bandelin GM2070 sonicator equipped with a MS73 tip. The tip was slightly

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Table 1. Sample location, parameters analyzed and main characteristics of the DCM layer. Casts 48-73 are from AC (Avilés Canyon) and casts 75-101 are from GB (Galicia Bank).

<table>
<thead>
<tr>
<th>Cast</th>
<th>Coordinates (W, N)</th>
<th>Date</th>
<th>Parameters analyzed</th>
<th>DCM depth (m)</th>
<th>Chl a max (mg m⁻³)</th>
<th>DCM width (m)</th>
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<tbody>
<tr>
<td>48</td>
<td>06.2745, 43.7565</td>
<td>29 Jul FCM, HPLC, microscopy</td>
<td>32</td>
<td>1.59</td>
<td>11</td>
<td></td>
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<tr>
<td>52</td>
<td>06.2693, 44.0028</td>
<td>30 Jul FCM, HPLC, nutrients, FISH, microscopy</td>
<td>54</td>
<td>0.628</td>
<td>39</td>
<td></td>
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<tr>
<td>55</td>
<td>06.3852, 43.7555</td>
<td>31 Jul FCM, HPLC, nutrients</td>
<td>22</td>
<td>1.87</td>
<td>17</td>
<td></td>
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<tr>
<td>66</td>
<td>06.2718, 44.1720</td>
<td>02 Aug FCM, HPLC, nutrients</td>
<td>59</td>
<td>0.411</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>05.7192, 43.8695</td>
<td>03 Aug FCM, HPLC, nutrients</td>
<td>41</td>
<td>1.38</td>
<td>8</td>
<td></td>
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<tr>
<td>73</td>
<td>05.6977, 44.0425</td>
<td>04 Aug FCM, HPLC, nutrients, FISH, microscopy</td>
<td>42</td>
<td>1.22</td>
<td>13</td>
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<tr>
<td>75</td>
<td>11.2572, 43.1665</td>
<td>07 Aug FCM, HPLC</td>
<td>54</td>
<td>0.417</td>
<td>32</td>
<td></td>
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<tr>
<td>80</td>
<td>12.3895, 43.1682</td>
<td>08 Aug FCM, HPLC, nutrients, FISH, microscopy</td>
<td>56</td>
<td>0.449</td>
<td>36</td>
<td></td>
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<tr>
<td>83</td>
<td>11.7078, 42.9993</td>
<td>09 Aug FCM, HPLC, nutrients, FISH, microscopy</td>
<td>52</td>
<td>0.507</td>
<td>27</td>
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<td>88</td>
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<td>0.616</td>
<td>26</td>
<td></td>
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<tr>
<td>92</td>
<td>11.4838, 42.8332</td>
<td>11 Aug FCM, HPLC</td>
<td>38</td>
<td>0.487</td>
<td>24</td>
<td></td>
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<tr>
<td>101</td>
<td>12.1630, 42.6675</td>
<td>13 Aug FCM, HPLC, nutrients</td>
<td>58</td>
<td>0.474</td>
<td>30</td>
<td></td>
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</table>
introduced into the extract. The sonicator was set at 50% power for 30 s with 8:2 on: off intervals. Before analysis, extracts were cleared by filtration through Whatman GF/F glass fiber filters by centrifugation at 3000 r.p.m. during 3–4 min, following the procedures of Wright and Mantoura (1997). HPLC analysis was based on the method of Zapata et al. (2000) with the following modifications in the gradient: 0' 100%B, 2' 10%B, 4' 20%B, 17' 38%B, 24' 80%B, 36' 100%B. Solvent A was pyridine (0.05 mM, pH = 5.0) : methanol : acetonitrile (25 : 50 : 25) and solvent B was methanol : acetonitrile : acetone (60 : 20 : 20). Organic solvents were from Carlo-Erba (HPLC grade), pyridine was from Carlo-Erba (analysis grade) and water was 0.2 μm filtered Milli-Q water (Millipore). The Agilent (Waldborn, Germany) series 1200 chromatographic system used consisted of a G1311A quaternary pump, a G1367C autosampler with a 100 μL capillary loop, a G1316B column thermostat, and a G1315C diode array detector. An additional 2 mL loop with 0.5 mm internal diameter was placed in the autosampler between the needle seat and the Rhodyne changing valve. The system was governed by Agilent Chemstation v. B.04.03 software. A 150 × 4.6 mm, 3.5 um particle, Symmetry C8 (Waters) column was used for analysis. A large volume (800 μL) injection procedure was performed to improve the detection and quantification of pigments (Latasa 2014). The first injection of the day was always discarded. Acetone with internal standard was injected to correct for mistakes in water content of the filter or extract evaporation. Coefficient of variation of the acetone with internal standard was 1.8%.

Data treatment
All the data treatments are based on cell concentrations. We gained statistical power deducing cell count profiles from the distribution of pigment markers for those casts where pigment concentration but not cell counts were available. We compared the cell counts of casts obtained by epifluorescence (casts 52, 73, 80 and 83) and light microscopy (cast 48, 52, 73, 80, and 83, Table 1) against the corresponding pigment profiles analyzed by HPLC to obtain the pigment per cell of each group at the different depths, adjusting the changes in pigment per cell with irradiance. Using these relationships we estimated cell counts from pigment distributions after correcting for photoacclimation for casts where counts were not available. Photoacclimation was estimated as the change in pigment marker per cell or per cell carbon with depth or irradiance according to the equation:

\[ \text{Pigment per cell or C} = (P_{\text{max}} - P_{\text{min}})e^{(-k_{\text{acc}}E_z)} + P_{\text{min}}, \]

where \( P_{\text{max}} \) and \( P_{\text{min}} \) were the adjusted maximum and minimum concentration of pigment, \( k_{\text{acc}} \) is the coefficient of photoacclimation and \( E_z \) is the percent of surface irradiance at depth \( z \). Then, cell vertical distribution was estimated dividing pigment concentration by pigment per cell at each irradiance (depth) as:

\[ \text{Cell concentration} = \text{Pigment concentration} \times \left( \frac{P_{\text{max}} - P_{\text{min}}}{e^{(-k_{\text{acc}}E_z)} + P_{\text{min}}} \right)^{-1}. \]

Photoacclimation was taken into account for dinoflagellates, small prymnesiophytes, and pelagophytes but for chlorophytes the average pigment content was used (which is the same as using the pigment profiles directly converted to cell counts) because the violaxanthin content per cell did not change significantly with irradiance. Viola-xanthin is not a light harvesting pigment and at the depths sampled it is likely that it did not react to the variations of irradiance at those levels. In summary, we obtained direct cell counts for Synechococcus, Prochlorococcus, coccolithophores and diatoms and cell counts were complemented with cell concentrations derived from pigment profiles for the rest of the groups.

The weighted mean depth (WMD)
We called the weighted mean depth (WMD) of a variable the average depth weighted by the distribution of that variable. Thus the WMD of Chl \( a = \Sigma \) (Depth \( i \) × Chl \( ai \))/ΣChl \( ai \), where Chl \( ai \) is the Chl \( a \) concentration at depth \( i \). Likewise, we defined the WMD of each taxonomic group as WMD of Group \( A = \Sigma \) (Depth \( i \) × Group \( Aj \))/ΣGroup \( Aj \) - WMD Chl \( a \) where Group \( Aj \) is the cell concentration at depth \( i \). Because the DCM was located at different depths in the 12 vertical profiles sampled, the term WMD Chl \( a \) is subtracted to make the distribution of the groups relative to that of Chl \( a \). We estimated the WMD for each group within the DCM layer, which excluded the surface sample and an intermediate sample collected in the GB sampling.

Results
Water column
The physical conditions were more heterogeneous in the AC (Avilés Canyon) than in the GB (Galicia Bank) since stations from the AC included shelf, slope, and open ocean areas, while the GB samples were exclusively from the open
ocean (Fig. 1). In addition, the more heterogeneous stations
of the AC could be also due to the presence of hydrographic
placement of the isopycnals because of tides and internal
waves, especially at stations close to the canyon (Sánchez
et al. 2014). In spite of the existence of these potentially dis-
ruptive mechanisms, especially in the AC area, the water
-column was always highly stratified but with a deeper, slightly
colder and saltier mixing layer in the GB (Fig. 2). There was
a marked DCM located at the upper part of the nitracline
(Fig. 2). Oxygen profiles displayed a maximum some meters
above the Chl a maximum in all DCMs examined in the AC,
and at the same depth of the Chl a maximum in all DCMs
from the GB (data not shown). Cell profiles indicated that
the DCM was also a deep biomass maximum (Fig. 3). Profiles
of CTD fluorescence and extracted Chl a agreed well (Fig. 2,
r = 0.88). The depths of the DCM peaks were located
between 22 m and 59 m in the AC, and 38 m and 65 m in
the GB (Table 1). The Chl a maxima ranged between 0.4 mg
m⁻³ and 1.9 mg m⁻³ in the AC, and 0.4 mg m⁻³ and
0.6 mg m⁻³ in the GB. The width of the DCM was defined
as the depth interval where Chl a concentration was more
than half the difference between the maximum and mini-
mum values in the upper 100 m. The width ranged between
8 m and 32 m in the AC, except for a value of 65 m for cast
66, and 24 m and 36 m in the GB. There were significant
negative correlations between maximum Chl a concen-
tration and depth (r = −0.84) and width (r = −0.70) and a posi-
tive correlation between depth and width (r = 0.63).

Criteria for data selection
An initial analysis of our database directed the selection
of the samples considered in our study. PRYM02 is a FISH
probe specific for all Haptophyta, including pavlovophytes.
The latter group does not contain 19’hexanoyloxyfucoxan-
thin and their presence could have distorted our compar-
ison between FISH- and pigments-based estimates of prymnesio-
phytes. However, pavlovophytes contain significant amounts
of chlorophyll c₁ (van Lenning et al. 2003) which was always
below detection, and we conclude that pavlovophytes were
not present or detectable in our samples. Phaeocystis colonies
were not detected in the samples. Thus, prymnesiophytes
were counted as coccolithophores (> 5 μm, optical microsco-
py) and small prymnesiophytes (< 5 μm, FISH microscopy).
Both types share the pigment marker 19’hex-fuco and any
estimate of cell distribution from 19’hex-fuco cell concentra-
tion should include both types of prymnesiophytes. Howev-
er, the pattern of 19’hex-fuco content was very different in
the four vertical profiles where all prymnesiophytes (coccoli-
thophores plus small prymnesiophytes) were counted side by
side (Supporting Information Fig. S1). In casts 52 and 73
(AC), 19’hex-fuco per cell increased from the deepest sam-
ple to the DCM depth (1% irradiance) or slightly above,
and then decreased toward the surface. In casts 80 and 83
(GB), however, the expected continuous decrease of pigment
content with increasing light toward the surface was
observed. The main difference between these two different
patterns is the contribution of coccolithophores to the total
biomass of prymnesiophytes (Table 2, Supporting Informa-
tion Fig. S1). It appears that coccolithophores produced an
unexpected response in the pigment content with depth for
which we cannot provide an explanation. Because of the sig-
nificant and variable contribution of coccolithophores to the
prymnesiophytes in the AC samples, we decided to use only
the GB samples plus casts 52 and 73 from the AC, where
samples were counted directly, to investigate the depth dis-
tribution of the small prymnesiophytes. Coccolithophores
and diatom distribution was studied from microscopy counts
in casts 48, 52, 73, 80, and 83. Diatoms in cast 52 were
numerically dominated by a single group with a vertical dis-
tribution different from any other phytoplankton group
(Fig. 3). Because of the peculiarity of the profile and our
inability to relate it to any other parameter measured, the
diatoms from this profile were not considered in this study.
Fucoxanthin, the marker pigment of diatoms, was dominant
only in casts 48, 55, and 73 in the AC (Supporting Informa-
tion Fig. S2). On the other hand, fucoxanthin is also present
in prymnesiophytes and pelagophytes, which pigments were
more abundant than fucoxanthin in the rest of the casts.
This fact precludes the use of the pigment profiles to deduce
the diatom concentrations in the rest of the casts as made
for dinoflagellates, chlorophytes, pelagophytes, and prymne-
siophytes. Thus, the number of profiles to study the depth
distribution of diatoms was limited to the direct cell counts
from casts 48 and 73 from the AC and 80 and 83 from the
GB. Dinoflagellates counts could not distinguish heterotro-
phic and autotrophic forms. Peridinin concentration divided
per total dinoflagellates concentration increased with
decreasing irradiance, as expected for a light-harvesting pig-
ment as peridinin. However, this pattern only occurred
down to 0.5% surface irradiance. Below that depth the ratio
peridinin to dinoflagellates decreased (Fig. 4). We hypothe-
size that this pattern, different from any other observed for
the rest of the light harvesting pigments, was due to an
increase in the heterotrophic to autotrophic ratio of dinoflag-
gelettes below that depth and therefore those data were not
included in the photoacclimation estimations.

Phytoplankton biomass distribution
Similar to the physical environment, Chl a concentra-
tions were also more variable in the AC than in the GB area, with
the former showing the highest (coastal cast 55) and lowest
(oceanic cast 66) values (Table 1). Pigment distribution
revealed a dominant presence of diatoms only in casts 48,
55, and 73 from the AC (Supporting Information Fig. S2).
Optical microscopy revealed that dinoflagellates and coccoli-
thophores dominated the large phytoplankton (> 5 μm) in
the GB (data not shown). Detailed results from FISH micros-
copy of small (<5 μm) eukaryotic phytoplankton are
Fig. 2. Vertical profiles of temperature (continuous line), salinity (dashed line), Chl $a$ (ng L$^{-1}$, filled circles), and NO$_3$ ($\mu$mol L$^{-1}$, empty circles). For visual clarity, only NO$_3$ is represented among the four nutrients analyzed. PO$_4$ and SiO$_4$ presented a very strong correlation with NO$_3$ while NO$_2$ presented the typical vertical structure with a maximum beneath the DCM (not shown). Note the changes in scales for casts 48-73 and 75-101. Fluorescence profiles are presented as gray areas for reference in relative units with the same scale. (Continued on next page).
Fig. 2.
Fig. 3. Vertical profiles of pigments (filled circles, bottom x-axis, ng L\(^{-1}\)) and cell counts (empty circles and triangles, top x-axes, cell mL\(^{-1}\)) measured by flow cytometry, FISH, and light microscopy. Per: peridinin, Fuco: fucoxanthin, 19’hex: 19’hexanoyloxyfucoxanthin, 19’but: 19’butanoyloxyfucoxanthin, Viol: violaxanthin, Zea: zeaxanthin, DVa: divinyl chlorophyll a, Chl a: chlorophyll a, Syn: Synechococcus, Dino: dinoflagellates, Diat: diatoms, Cocco: coccolithophores, Prym: small (< 5 \(\mu m\)) prymnesiophytes, Pelag: pelagophytes, Chloro: chlorophytes, Pro: Prochlorococcus. Note the changes in scales. Fluorescence profiles are presented as gray areas for reference without scale to show the shape of the profile. In the 19’hex, coco and Prym plots, the top x-axis corresponds to cell counts of coccolithophores, and the offset top x-axis to cell concentrations of small prymnesiophytes. (Continued on next page).
Fig. 3. (Continued on next page).

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Fig. 3. (Continued on next page).
Fig. 3. Distribution of phytoplankton groups in the DCM
Table 2. Average C abundance (mg m\(^{-3}\)) of phytoplankton groups in the DCM layer of each cast. Numbers in italics were estimated from pigment concentrations (n.d., not detected).

<table>
<thead>
<tr>
<th></th>
<th>48</th>
<th>52</th>
<th>55</th>
<th>66</th>
<th>68</th>
<th>73</th>
<th>75</th>
<th>80</th>
<th>83</th>
<th>88</th>
<th>92</th>
<th>101</th>
<th>Mean ± SD</th>
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<tr>
<td>Prochlorococcus</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.28</td>
<td>3.68</td>
<td>5.66</td>
<td>3.71</td>
<td>4.11</td>
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<td>6.17</td>
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<td>2.68</td>
<td>1.25</td>
<td>1.95</td>
<td>2.08</td>
<td>1.17</td>
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<td>1.72</td>
<td>1.47</td>
<td>1.59</td>
<td>1.69 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Small prymnesiophytes</td>
<td>2.46</td>
<td>0.88</td>
<td>2.88</td>
<td>1.25</td>
<td>3.39</td>
<td>1.98</td>
<td>1.52</td>
<td>2.14</td>
<td>3.02</td>
<td>1.90</td>
<td>1.54</td>
<td>1.90 ± 0.75</td>
<td></td>
</tr>
<tr>
<td>Coccolithophores</td>
<td>1.38</td>
<td>0.90</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.99</td>
<td>–</td>
<td>0.56</td>
<td>0.70</td>
<td>–</td>
<td>–</td>
<td>0.91 ± 0.31</td>
<td></td>
</tr>
</tbody>
</table>

Photosynthesis

Changes in pigment per cell or C with depth were observed for most groups (Fig. 4, Supporting Information Fig. S3). How those changes affected each group is described next. In Prochlorococcus, the photoacclimation parameters indicated that DVChl \(a\) per Prochlorococcus was 0.3–4.27 fg cell\(^{-1}\) (Table 3), similar to the 0.44–5.14 reported by Veldhuis and Kraay (1990). These values are equivalent to C : DVChl \(a\) ratios of 37–80. In Synechococcus zeaxanthin per cell was 0.63 fg cell\(^{-1}\), a value well below the 1.8 fg cell\(^{-1}\) reported by Kana et al. (1988), the 2.9–6.8 reported by Biddigare et al. (1989) and the three reported by Moore et al. (1995). However, in terms of pigment per biomass, the mean value of 4.9 mg g\(^{-1}\) is in the 2–5 range provided by Liu et al. (1999), suggesting that Synechococcus cells in our samples were small. There are no reports for direct pigment content in pelagophytes, neither for the non-calcifying small prymnesiophytes. Andersen et al. (1996), however, reported correlations between Chl \(a\) ascribed to pelagophytes and pelagophytes cells and Chl \(a\) ascribed to prymnesiophytes and small prymnesiophytes cells. A rough exercise could be made but with some caution because the intercepts of these correlations, although small, were not zero. The slopes of Chl \(a\) vs. cell concentrations reported were 180 and 373 (fg cell\(^{-1}\)) for pelagophytes and small prymnesiophytes, respectively. To obtain the content of 19’but-fuco and 19’hex-fuco per cell those Chl \(a\) values should be divided by the 19’but-fuco to Chl \(a\) ratio for pelagophytes [which was 0.9 according to Andersen et al. (1996)] and by the 19’hex-fuco to Chl \(a\) ratio for prymnesiophytes (1.3). Thus values of 200 fg cell\(^{-1}\) of 19’but-fuco for pelagophytes and 287 fg cell\(^{-1}\) of 19’hex-fuco for prymnesiophytes are obtained from Andersen et al. (1996). Considering the larger size of prymnesiophytes (around two times, Table 3), the pigment per cell values estimated in our study (82 and 194 fg cell\(^{-1}\), or 43 and 48 fg C\(^{-1}\), respectively, Supporting Information Table S1) appear to depart less from the average content of pigment per C in the cells of both groups. It should be noted that there is a contribution of coccolithophores (around 20% in the GB samples, Table 2; Supporting Information Fig. S1) to the 19’hex-fuco attributed...
Fig. 4. Changes in the pigment marker content per cell as a function of the irradiance. Empty symbols in the peridinin vs. irradiance plot are excluded from the curve fitting data (see text for details).
to small prymnesiophytes not accounted for in Supporting Information Table S1. In relation to chlorophytes, to our knowledge, this is the first time that violaxanthin is used as the specific pigment marker of this group. A mean value of 0.071 violaxanthin to Chl *a* has been reported for Prasinophyceae (Latasa et al. 2004), a group belonging to chlorophytes and probably dominating this class in coastal and open ocean (Not et al. 2004, 2008). Dividing that ratio by the violaxanthin content (violaxanthin: cell C = 1.81 × 10⁻³, Table 3) a mean value of 39 (w : w) C to Chl *a* would result for chlorophytes in the DCM.

**Depth distribution**

The weighted mean depth of each phytoplankton group indicated a preference for a different position within the DCM, hinting a preference for different ecological niches within the DCM. A pattern of vertical alignment can be identified (Fig. 5). Overall, *Prochlorococcus* occupied preferentially the shallowest parts of the DCM layer and diatoms the deepest; dinoflagellates, *Synechococcus*, and small prymnesiophytes were the next shallowest groups, and coccolithophores, chlorophytes and pelagophytes showed a preference for deeper layers. Statistical differences (Tukey–Kramer multiple comparison test for unequal sample sizes, *p* < 0.05) were only significant between clearly distant groups (Fig. 5). Thus, *Prochlorococcus* were located distinctly above small prymnesiophytes, coccolithophores, chlorophytes, pelagophytes, and diatoms. Dinoflagellates, the next shallowest group, were significantly above chlorophytes, pelagophytes, and diatoms. Pelagophytes and diatoms, the deepest groups, were significantly deeper than the groups occupying the shallowest layers of the DCM: *Prochlorococcus*, dinoflagellates, *Synechococcus*, and small prymnesiophytes. The high variability of diatoms adds uncertainty to the distribution of this group. The counting error might contribute to this variability, but the consistent patterns in their vertical distribution (no strong spikes, Fig. 3) provide confidence on the results and indicate a considerable degree of variability highlighted by the low number of samples (*n* = 4).

**Discussion**

The new sampling strategy proposed here allows investigating the vertical structure in the water column with ca. 2 m resolution. Vertical sampling of the water column has followed two strategies representing different objectives that require different methodologies. The study of thin layers focuses on narrow structures of up to a few meters thick (< 5 m) with mechanisms of formation and characteristics different from typical DCM (Hodges and Fratantoni 2009; Durham and Stocker 2012). Sampling is performed with specific devices capable of resolving scales of cm without affecting those structures (Lunven et al. 2005). On the other hand, the most usual oceanic studies perform bottle sampling with intervals in the order of several meters (5–10 and more). However, shallow pycnoclines, thermoclines, nutriclines, and DCM structures encompass depth intervals around 10–40 m (Fig. 2; Table 1). These structures are not adequately sampled with the two strategies specified above because thin layers devices would become unmanageably large, and the 5–10 m intervals usually used when sampling with bottles miss the resolution needed to adequately describe those structures. We have shown that with a 0.25 m/s speed to carry a 24 bottle rosette up and closing the bottles without stopping could satisfactorily resolve the nutricline and the different phytoplankton ecological niches within the 10–40 m depth interval embraced by most of the DCM layers of our study (Table 1). As a cautionary note, the small-scale vertical structure is undoubtedly disturbed and smeared by the equipment, thus preventing the study of proper thin layers with this procedure.

The combination of group pigment-markers, flow cytometry, optical microscopy, and FISH allowed the study of the

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**Table 3. Parameters of photoacclimation for pigment cell⁻¹ and pigment C⁻¹ vs. percent irradiance.** See text for equation. Violaxanthin per chlorophytes vs. irradiance failed to adjust. Zeaxanthin per C⁻¹ in *Synechococcus* failed to adjust. Changes in pigment per C content were not calculated for dinoflagellates and diatoms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Value (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVChl/PerPro</td>
<td>fg cell⁻¹</td>
<td>4.2734 ± 0.3264</td>
</tr>
<tr>
<td>Zeax per Syn*</td>
<td>fg cell⁻¹</td>
<td>0.7118 ± 0.4856</td>
</tr>
<tr>
<td>Viol per chloro*</td>
<td>fg cell⁻¹</td>
<td>*</td>
</tr>
<tr>
<td>19′but-fuco per pelago</td>
<td>fg cell⁻¹</td>
<td>119.98 ± 62.77</td>
</tr>
<tr>
<td>19′hex-fuco per prym</td>
<td>fg cell⁻¹</td>
<td>301.6 ± 67.1</td>
</tr>
<tr>
<td>Per per dino</td>
<td>fg cell⁻¹</td>
<td>397.68 ± 62.58</td>
</tr>
</tbody>
</table>
Phytoplankton groups aligned vertically in a consistent way under the summer stratification conditions in our study area, suggesting that they have certain ecological preferences for different niches within the DCM (Fig. 5), where vertical gradients of nutrients and light are strongest. Thus we falsified our null hypothesis that the DCM is a homogeneous layer. Although our study was neither intended nor designed to explain why the groups were distributed the way we found them, in the following we investigate the possible reasons for the differential vertical distribution according to the most common explanation for the existence of a deep biomass maximum: differences in losses to predators, growth and sinking or migration among the different groups of phytoplankton (Cullen 2015).

Differential sinking has been considered in modeling studies as a main factor influencing the formation and maintenance of a DCM (Hodges and Rudnick 2004; Dijkstra 2011). Not aggregated phytoplankton increased sinking speeds have been related to poor nutritional status (Bienfang and Harrison 1984; Waite et al. 1997; Gerecht et al. 2015) and size (Smayda 1970; Waite et al. 1997; Richardson and Jackson 2007). Size is considered a good proxy for gravitational sinking because of two reasons: sinking speed is directly related to the square of the particle radius according to Stokes’ law, and nutrient uptake at low concentrations penalizes cells with high volume to surface ratios (large particles). In our study, cell size within groups changed with depth with a quite surprising but consistent pattern related to their mean size (Figs. 6, 7, Supporting Information Fig. S4; Supporting Information Table S1). Prochlorococcus, the smallest cells, increased their C content about 8 times from surface to deepest sample. The increase in Synechococcus, the next smallest group, was about 2–3 times. However, pelagophytes and small (5 μm) prymnesiophytes reversed this trend and decreased their C content with depth. Chlorophytes, the smallest picoeukaryotes of our study did not show statistically significant changes in C content. The strongest change in size for all groups occurred between 0.3% and 3% irradiance level (Supporting Information Fig. S4; Supporting Information Table S1). Prochlorococcus, the smallest cells, increased their C content about 8 times from surface to deepest sample. The increase in Synechococcus, the next smallest group, was about 2–3 times. However, pelagophytes and small (5 μm) prymnesiophytes reversed this trend and decreased their C content with depth. Chlorophytes, the smallest picoeukaryotes of our study did not show statistically significant changes in C content. The strongest change in size for all groups occurred between 0.3% and 3% irradiance level (Supporting Information Fig. S4; Supporting Information Table S1). In summary, changes in cell volume with depth within classes followed a continuous trend related to their overall size: the smallest cells increased their volume with depth the most, while the largest cells decreased their volume the most. In relative terms this decrease was similar for all picoeukaryote groups (Fig. 7; Supporting Information Table S1). We currently do not have an explanation for this clear and consistent pattern also reported by Hickman et al. (2009) and Casey et al. (2013). These latter authors suggested that the increase with depth in Prochlorococcus is due to the increase in photosynthetic structures under low irradiance. However, the photoacclimation response was the same for all groups while the size response was not, leaving the decrease in volume of pelagophytes and small prymnesiophytes with depth unexplained.

Fig. 5. Weighted mean depth of phytoplankton groups referred to the weighted depth distribution of Chl a. Significant differences among groups (Tukey-Kramer multiple comparison test) are represented by different letters. Error bars indicate 95% confidence intervals, the middle horizontal bar indicates the mean.

Fine vertical distribution of Prochlorococcus, Synechococcus, chlorophytes, pelagophytes, small prymnesiophytes, coccolithophores, diatoms, and dinoflagellates. Prochlorococcus were absent in the Avilés Canyon, a result already reported for this period of the year (Calvo-Díaz et al. 2008). All groups presented minimum cell abundances at the surface and a maximum close to the DCM (Fig. 3). Therefore, the DCM was also a deep biomass maximum (DBML sensu Cullen 2015) and as such will be considered throughout this discussion. It is interesting to note that the cell distribution is not vertically symmetrical around the DCM (Fig. 5). This result indicates that, in general, cells tend to accumulate preferentially in the upper part of the DCM layer, different from a more symmetrical vertical distribution of Chl a. This difference was a result of the increase of Chl a per cell with depth due to photoacclimation processes. Also, overlooking the differential distribution could lead to some errors when describing the abundances of phytoplankton groups at the DCM. We have estimated roughly this error for the extreme case of Prochlorococcus with the shallowest distribution with a very simple calculation. Cell concentrations were considered in three samples: above, below and at the DCM peak, comprising about 8–10 m of the DCM layer, which is a typical resolution in oceanographic sampling. In average, we would count only 0.54 (± 0.22) times the number of Prochlorococcus at the DCM peak if we had sampled two depths (4–5 m) below, and 1.47 (± 0.43) times if sampled two depths above.
In theory, deeper samples should experience higher nutrient supply. Mourino-Carballido et al. (2016) found a negative relationship between nutrient supply (deeper samples) and cell size of Prochlorococcus, Synechococcus and small picoeukaryotes, a result consistent with ours in the case of the picoeukaryotes but not for the prokaryotes. Finally, Falkowski and Owens (1980) reported a clear decrease in size with light limitation in two large species of phytoplankton. Therefore, it is not clear whether nutrient or irradiance was responsible for the cell size changes with depth.

In spite of all this variability, the average size of phytoplankton groups distinguished here did not overlap at any given depth. Only the size range of Prochlorococcus and Synechococcus intersected but, because they followed the same trend (increased size with depth), the size of the cells of these two groups never overlapped at any given depth. Cell size went from smallest to largest in the following order: Prochlorococcus, Synechococcus, chlorophytes, pelagophytes, small prymnesiophytes, coccolithophores, and dinoflagellates/diatoms (the last three groups examined by optical microscopy and without a systematic size classification). Although there are some similarities, this size distribution does not match the vertical distribution observed for these groups (Fig. 5).

The higher than expected position of dinoflagellates could be explained by their swimming ability to search for well-lit areas—laden waters of the DCM layer. The wide distribution of these groups never overlapped at any given depth. Cell size went from smallest to largest in the following order: Prochlorococcus, Synechococcus, chlorophytes, pelagophytes, small prymnesiophytes, coccolithophores, and dinoflagellates/diatoms (the last three groups examined by optical microscopy and without a systematic size classification). Although there are some similarities, this size distribution does not match the vertical distribution observed for these groups (Fig. 5).

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Another point to explore is a group-selective depth-dependent grazing. We do not find specific reasons to assume a differential top-down control to produce the presented profiles (Figs. 3), Predator avoidance has been described by swimming phytoplankton for anoxic lakes (Pedrós-Alió et al. 1995) but such an extreme environment for heterotrophic feeding cannot be envisioned here (Fig. 2).

Also, toxin-producing phytoplankton might reduce ingestion rates of predators thus producing a biomass increase of such phytoplankton (Turner and Tester 1997; Boyd et al. 2000). The predominant large phytoplankton (> 5 μm) in our samples corresponded to the genus Chaetoceros sg. Hyalochaete, Leptocylindrus, Proboscia, Rhizosolenia, Pseudo-nitzschia group delicatissima, Nitzschia bicapitata-type and some fusiform pennates (diatoms), Gymnodinium and Ceratium (dinoflagellates) and Emiliania huxleyi (coccolithophores), which do not belong to the most typical toxin-producing groups, thus invalidating the hypothesis. Finally, Strom (2001) described digestibility of phytoplankton ingested by microzooplankton to be aided by higher irradiance levels, implying higher grazing rates in the upmost part of the DCM layer than in the deeper parts. This last mechanism could explain the increase of phytoplankton at the DCM but not a differential grazing. Thus, it appears that there are no satisfactory reasons to sustain a top-down control for the existence of specific phytoplankton niches in the DCM.

The last point to explore looking for a reason for the different vertical preferences of phytoplankton groups is the possibility of differential growth of phytoplankton groups at different depths in the DCM, a bottom-up approach. Growth rates of phytoplankton are driven by temperature, irradiance, and nutrient availability. All three factors change abruptly in the DCM layer in an almost synchroneous and opposite way: light and temperature conditions become less favorable with depth (darker and colder) while nutrient concentrations increase. With the data at hand it is not possible to disentangle the main factors that might influence a potential difference in growth rates of the different phytoplankton groups, but we could try to compare described traits from the groups with the variable conditions of the DCM layer and make some reasonable speculations about the alignment found.

Since comparative studies among phytoplankton groups addressing the combined effects of the strongly depth-covarying variables light, nutrients and temperature are not available, we used the information from related studies to explain the reasons for the vertical distribution of the groups. In theory, all groups should prefer shallower, better illuminated waters. Prochlorococcus were capable to stay the shallowest, above the nutrient rich depths, indicating their well-known ability to cope in low nutrient conditions (Parmentsky et al. 1999; Latasa et al. 2010). Dinoflagellates were the eukaryotes with the shallowest distribution by far. Sampling was performed always at night, thus the usual explanation that vertical migrations by dinoflagellates are performed to obtain nutrients at depth at night followed by swimming toward the surface during the day, does not explain the results found here. The shallow distribution of dinoflagellates has been attributed to the presence of mixotrophic and heterotrophic forms (Estrada 1991). If this was the case, the described population would be formed by mixotrophic forms since their abundance was based in part on their pigment marker concentration. Synechococcus also remained in the more illuminated waters of the DCM layer. The wide distribution of Synechococcus hints their preference for mesotrophic conditions (Parmentsky et al. 1999; Latasa et al. 2010) but also their plasticity to survive in low nutrient environments (Paerl et al. 2011). The small prymnesiophytes considered here do not include coccolithophores but belong to the non-calcifying populations described by pigment (Andersen et al. 1996; Not et al. 2008) and molecular (Liu et al. 2009) approaches. The shallow position of small prymnesiophytes in relation to other picoeukaryotic algae agrees with other results (Dupont et al. 2015; Cabello et al. 2016). They appear to have mixotrophic abilities (Unrein et al. 2007, 2014; Zubkov and Tarran 2008) therefore presenting an argument supporting their relatively shallow position away from the nutrient richest conditions at depth.

The middle position of chlorophytes should be attributed to their broad vertical distribution with a not very
Fig. 6. Changes in C content per cell as a function of the irradiance.
pronounced peak at the DCM and sometimes two maxima separated by several meters within this layer (Fig. 3, Cabello et al. 2016). These special distribution hints the presence of two vertically segregated populations of chlorophytes in the DCM layer. As concluded by Cabello et al. (2016) the coarse taxonomic resolution at the class level, although it provides some valuable information, does not unveil the complete physiological diversity of phytoplankton. The deep position of pelagophytes, a small (2–3 μm) eukaryotic phytoplankton, should not be surprising. There are increasing evidences that pelagophytes can use the low-light high-nutrient conditions typical of the DCM. Physiological experiments showed their low-light photosynthetic characteristics (Dimier et al. 2009; Kulk et al. 2012) and genetic analysis confirmed a very high number of genes involved in the formation of light-harvesting complexes (Gobler et al. 2011) and in the assimilation of nitrogen (Dupont et al. 2015). Finally, diatoms presented a very broad distribution, which could be explained observing the details of the database and appealing to the theory that large cells require high nutrient concentrations. Our explanation is that the position of diatoms is due to their ability to capture nutrient pulses from below. The two shallowest points of the weighted mean depth of diatoms (Fig. 5) belonged to the AC area (Fig. 3). The canyon is an area of internal tides and waves activity that produce isopycnal displacements of c.a. 30 m near the surface (Sánchez et al. 2014) bringing nutrients to well-lit layers. Diatoms are well capable to rapidly uptake and store nutrients that will be used later on (Lomas and Gilbert 2000; Tozzi et al. 2004). Nutrient pulses are often invoked in the DCM layer as a result of internal waves and mesoscale eddies (McGillicuddy et al. 1998; Uz et al. 2001). Thus, diatoms should be well adapted to thrive in the pulsing environment of the AC (Goldman and McGillicuddy 2003) and could manage to stay above their theoretical depth if just constant nutrient diffusion was taking place. The GB is an area farther away from the coast (Fig. 1) where physical processes bringing nutrient to the euphotic zone should be more occasional and thus forcing the nutrient-seeking diatoms to deeper layers.

In summary, we found a vertical alignment of phytoplankton groups within the DCM layer. We have explored the main habitual reasons for the presence of a DCM (differential losses to predators, growth rates, and sinking) to explain the reason for such an alignment. The most likely explanation is that the different groups present different ecological needs and traits adapted to the potential different microenvironments in this layer of strong abiotic gradients. Nutrients and irradiance are the most evident environmental variables with an impact for the presence of phytoplankton gradients, a bottom-up approach already investigated to explain the distribution of *Synechococcus* and picoeukaryotes in the DCM (Hickman et al. 2009). Our results are coherent with known ecophysiological responses of the studied groups and thus we hypothesize that the vertical distribution described here is reproducible in DCM layers with similar light and nutrient patterns. Further investigations on the fine structure of the DCM will help to better identify the ecological traits of phytoplankton groups. It is clear that the vertical alignment of phytoplankton groups preclude considering the DCM as a homogeneous layer.

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Distribution of phytoplankton groups in the DCM


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Conflict of Interest

None declared.

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Distribution of phytoplankton groups in the DCM