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# On the discrimination of multiple phytoplankton groups from light absorption spectra of assemblages with mixed taxonomic composition and variable light conditions

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According to recommendations of the international community of phytoplankton functional type algorithm developers, a set of experiments on marine algal cultures was conducted to (1) investigate uncertainties and limits in phytoplankton group discrimination from hyperspectral light absorption properties of assemblages with mixed taxonomic composition, and (2) evaluate the extent to which modifications of the absorption spectral features due to variable light conditions affect the optical discrimination of phytoplankton. Results showed that spectral absorption signatures of multiple species can be extracted from mixed assemblages, even at low relative contributions. Errors in retrieved pigment abundances are, however, influenced by the co-occurrence of species with similar spectral features. Plasticity of absorption spectra due to changes in light conditions weakly affects interspecific differences, with errors <21% for retrievals of pigment concentrations from mixed assemblages. © 2017 Optical Society of America

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## 1. INTRODUCTION

Large differences in taxonomic and size structures of algal communities influence many ecological and biogeochemical marine processes. The various phytoplankton groups have different roles in the biogeochemical cycles of elements [1], and they are responsible for different contributions to total primary production [2]. Diatoms can contribute to 40% of total marine primary production [2,3] and together with dinoflagellates export carbon to deep waters. Coccolithophores, such as the bloom-forming species *Emiliania huxleyi* [4], sequester large quantities of calcium carbonate to form their characteristic external plates (coccoliths), thus reducing seawater alkalinity. Various phytoplankton types also release dimethyl sulphide into the atmosphere [5–7], while others groups fix atmospheric nitrogen [8]. Hence, analysis of temporal and spatial variations of the phytoplankton community structure is of crucial importance to improve the understanding of biogeochemical fluxes in marine ecosystems, for instance, for modelling primary production and analyzing its climatic implications [7].

The synoptic detection and monitoring of changes in algal community structure can be pursued by the analysis of apparent and inherent optical properties derived from multispectral remote-sensing platforms [9–11]. Several bio-optical models were developed for the retrieval of products such as phytoplankton types, size classes, dominant size class, phytoplankton size distribution, or phytoplankton pigments [12,13]. In the perspective of the scheduled hyperspectral satellite missions (e.g., PACE and EnMAP missions), approaches based on *in situ* hyperspectral optical measurements were also successfully developed for the retrieval of pigment composition [14–16], size structure [17–20], or abundance of dominant species or groups [21–26].

Among the multispectral and hyperspectral approaches, the analysis of the spectral variations of the phytoplankton light absorption coefficients does not require any empirical relationship or assumption on the relationship between algal community composition and phytoplankton biomass [12,27]. The rationale is that the spectral characteristics of the phytoplankton light absorption coefficients are affected by pigment composition, concentration,

and packaging within the cell [17,28–30]. In particular, all algal pigments have defined absorption bands in the visible region of the electromagnetic spectrum [30–32], which influence the spectral shape of phytoplankton light absorption. Considering the fact that various phytoplankton groups are characterized by different pigment suites [33], the spectral signature of light absorption tends to have a similar shape within the same taxonomic group [29]. Despite these mechanistic considerations, there are sources of uncertainties affecting the performances of these spectral-response-based approaches which require investigation [12,27]. Some phytoplankton groups share similar pigments [33], which could yield similar optical signatures. Cell size influences the pigment packaging [28,30] and modifies the flattening of the light absorption spectra [17]. In addition, intracellular pigment concentration, packaging, and thus absorption signatures vary as a function of changes in growth factors such as light, temperature, and nutrient availability [34–42]. For example, high growth irradiances induce reduction of the cellular concentrations of chlorophyll *a*, as well as of other photosynthetic pigments, while the relative contribution of photoprotective pigments increases with respect to chlorophyll *a* [42]. As a consequence, cellular pigment packaging decreases while light absorption coefficients per unit of pigment increase [41] and spectra become sharper.

As recently highlighted by international committees of experts and algorithm developers [12,43,44], the extent to which the uncertainties introduced by the plasticity and/or similarity of spectral light absorption coefficients limit the optical detection of phytoplankton still needs to be addressed. In particular, among the various concerns raised by the dedicated international community, the following questions are of primary interest: (1) what is the effect of light-driven spectral modifications in the accuracy of phytoplankton retrieval from light absorption coefficients, and (2) how many phytoplankton groups can be discriminated from

the bulk spectral light absorption properties of marine algal communities characterized by mixed taxonomic composition.

Hence, the main objective of this study is to investigate these major questions in order to provide exploitable information and limits for development and application of methods and algorithms for the optical retrieval of phytoplankton community structure [12,43]. For this purpose, a set of laboratory experiments was carried out on marine algal cultures, representative of different taxonomic groups and covering a broad size range, grown in controlled conditions under various irradiance intensities. Considering the dataset of phytoplankton light absorption spectra and high performance liquid chromatography (HPLC) pigment concentrations provided by the experiments, we aimed at (1) assessing the influence of light growth conditions on the intra- and interspecific variability of the spectral shape of the phytoplankton light absorption coefficients and analyzing the effects on the optical classification; (2) extracting the absorption signature of a given species from the bulk light absorption properties of assemblages with mixed taxonomic composition and quantifying the species abundance; and (3) evaluating the errors in retrieving the abundance of a phytoplankton species within a mixed assemblage using reference light absorption spectra from populations adapted to different light regimes. No algorithm development and/or validation are here proposed.

## 2. MATERIALS AND METHODS

### A. Algal Cultures and Experimental Setup

Laboratory experiments were conducted on cultures of seven marine algal species representative of different taxonomic groups. The selected algal species covered a broad size range (0.6–23  $\mu\text{m}$ ) and were characterized by different suites of auxiliary and taxonomically significant pigments (see Table 1 for

**Table 1. Abbreviation, Names, Comments/Formulae for Phytoplankton Pigments and Pigment Sums: PS (Photosynthetic) and PP (Photoprotective) Pigments<sup>a</sup>**

Abbreviation	Pigment	Comment/formula	Taxonomic affiliation
Chl <i>a</i>	Chlorophyll <i>a</i> (plus allomers and epimers)	Phytoplankton biomass index, except for <i>Prochlorococcus</i> sp.	
Chl <i>b</i>	Chlorophyll <i>b</i>	PS in <i>Tetraselmis</i> sp.	<i>Tetraselmis</i> sp.
Chl $c_1 + c_2$	Chlorophyll $c_1 +$ Chlorophyll $c_2$	PS in <i>P. tricornutum</i> , <i>A. carterae</i> , <i>E. huxleyi</i> , <i>Cryptomonas</i> sp.	
Chl $c_3$	Chlorophyll $c_3$	PS in <i>E. huxleyi</i>	
Dv Chl <i>a</i>	Divinyl-chlorophyll <i>a</i>	Biomass index for <i>Prochlorococcus</i> sp.	<i>Prochlorococcus</i> sp.
Dv Chl <i>b</i>	Divinyl-chlorophyll <i>b</i>	PS in <i>Prochlorococcus</i> sp.	
Allo	Alloxanthin	PP in <i>Cryptomonas</i> sp.	<i>Cryptomonas</i> sp.
19'-BF	19'-Butanoyloxyfucoxanthin	PS in <i>E. huxleyi</i>	
Diad	Diadinoxanthin	PP in <i>P. tricornutum</i> , <i>A. carterae</i> , <i>E. huxleyi</i>	
Diato	Diatoxanthin	PP in <i>P. tricornutum</i> , <i>A. carterae</i> , <i>E. huxleyi</i>	
Fuco	Fucoxanthin	PS in <i>P. tricornutum</i> , <i>E. huxleyi</i>	<i>P. tricornutum</i>
Lute	Lutein	PP in <i>Tetraselmis</i> sp.	
19'-HF	19'-Hexanoyloxyfucoxanthin	PS in <i>E. huxleyi</i>	<i>E. huxleyi</i>
Perid	Peridinin	PS in <i>A. carterae</i>	<i>A. carterae</i>
Viola	Violaxanthin	PP in <i>Tetraselmis</i> sp.	
Zea	Zeaxanthin	PP in <i>Synechococcus</i> sp., <i>Prochlorococcus</i> sp.	<i>Synechococcus</i> sp.
Pigment sum		Formula	
TChl <i>a</i>	Total chlorophyll <i>a</i>	Chl <i>a</i> + Dv Chl <i>a</i>	
TP	Total pigments	Allo + 19' - BF + Fuco + 19' - HF + Perid + Zea + Chl <i>b</i> + Chl <i>a</i> + Dv Chl <i>b</i> + Dv Chl <i>a</i> + Chl $c_1 + c_2 +$ Chl $c_3 +$ Diadino + Diato + Lute + Viola	

<sup>a</sup>Taxonomic affiliation of marker pigments is indicated for examined species.

126 details and symbols; [45,46]). The prymnesiophyte *Emiliana*  
127 *huxleyi* (RCC 904) and the two cyanobacteria *Synechococcus* sp.  
128 (Roscoff Culture Collection [RCC] 322) and *Prochlorococcus* sp.  
129 (Med4, ecotype High Light 1; RCC 151) were obtained from  
130 the Roscoff Culture Collection (France). The diatom  
131 *Phaeodactylum tricornutum* was provided by the Stazione  
132 Zoologica Anton Dornh (Naples, Italy). The dinoflagellate  
133 *Amphidinium carterae* and the cryptophyte *Cryptomonas* sp. were  
134 isolated from Ligurian and Tyrrhenian waters (Mediterranean  
135 Sea) and identified at the University of Florence (Italy) according  
136 to Steidinger and Tangen [47] and Butcher [48], respectively.  
137 The prasinophyte *Tetraselmis* sp. was isolated from a live food  
138 pack used for aquaculture and then identified following the de-  
139 scription reported by Throndsen [49].

140 Species were cultured in natural sterile seawater (Mediterranean  
141 Sea) with the addition of nutrients. The enriched seawater media  
142 were *f/2* medium [50,51] for *P. tricornutum*, *Cryptomonas* sp.,  
143 and *Tetraselmis* sp.; *f/2-Si* medium (modified from [51]) for  
144 *A. carterae*; K medium [52] for *E. huxleyi*; and PCR-S11 medium  
145 [53] for *Synechococcus* sp. and *Prochlorococcus* sp.

146 Prior to each experiment, species were precultured for at least  
147 six generations in an exponential growth phase in order to ensure  
148 the acclimation to given irradiances. Population growth rates and  
149 division times were measured daily according to Wood *et al.* [54],  
150 using chlorophyll *a* *in vivo* fluorescence (Perkin-Elmer LS-5B;  
151 SLIT 5/5; excitation/emission 440/685 nm). Inoculated cultures  
152 of exponentially growing cells precultured at a given light inten-  
153 sity were gently stirred at regular intervals during the growth to  
154 avoid cell sedimentation and to ensure a consistent level of light  
155 inside the vessel until sampling.

156 In a first experiment (hereafter “Experiment 1”), the seven  
157 species from different taxonomic classes were grown separately  
158 in batch cultures (300 mL) at  $22 \pm 2^\circ\text{C}$  under three different  
159 irradiance conditions (10, 100, and  $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ;  
160 **1** 12/12 h L/D cycle) classified respectively as low light (LL),  
161 medium light (ML), and high light (HL). Different growth  
162 irradiances (10, 25, and  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for  
163 *Prochlorococcus* sp. were chosen as a result of insufficient growth  
164 rate ( $<0.1 \text{ div/day}$ ) at  $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

165 During a second experiment (hereafter “Experiment 2”), the  
166 species *P. tricornutum*, *A. carterae*, *E. huxleyi*, *Synechococcus* sp.,  
167 and *Prochlorococcus* sp. were selected to simulate algal assem-  
168 blages with mixed taxonomic composition. These species were  
169 chosen because of the broad size range they represented and  
170 because they are representative of major algal groups and phyto-  
171 **2** plankton functional types (i.e., silicifiers, calcifiers and DMS  
172 producers [1]) that can be encountered and coexist in various  
173 locations of the world’s open oceans [55–57]. In order to avoid  
174 interspecific competition for nutrients and light, the species  
175 were grown separately in batch cultures (3 L) at  $22 \pm 2^\circ\text{C}$   
176 under a photon flux density of  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$   
177 (12/12 h L/D cycle). Then, just before sampling, the cultures  
178 were mixed to obtain 26 mixed assemblages (300 mL) with  
179 exact taxonomic structure. Desired taxonomic structures  
180 were achieved by varying the contribution to total chlorophyll  
181 *a* of each species, from 0% to 100% (increments of 20%), with  
182 the contribution of the other species decreasing at the  
183 same rate.

## B. Bio-optical Analyses

184 Spectral light absorption coefficients (350–750 nm; resolution  
185 of 1 nm) were measured on filters using the transmittance–  
186 reflectance (T-R) method [58]. Culture samples (2–12 mL)  
187 of exponentially growing cells were filtered under low vacuum  
188 on glass-fiber filters (Whatman GF/F;  $\varnothing 25 \text{ mm}$ ) and immedi-  
189 ately stored at  $-80^\circ\text{C}$ . Small volumes were sampled to avoid  
190 high optical densities ( $>0.3$ ), outside the range where the cor-  
191 rection for the path-length amplification factor ( $\beta$ ) (see later)  
192 was established. Three replicates of each culture were analyzed  
193 using a LI-COR LI1800 spectroradiometer equipped with a LI-  
194 COR LI1800-12S integrating sphere, a LICOR LI1800-10  
195 quartz fiber optic probe, and a halogen light source [59] (regu-  
196 larly calibrated and maintained). T-R measurements were car-  
197 ried out outside the sphere, before and after pigment extraction  
198 in methanol at  $4^\circ\text{C}$  for 24 h [60]. Optical densities were com-  
199 puted following Tassan and Ferrari [61]. Correction for the  
200 path-length amplification factor ( $\beta$ ) was carried out according  
201 to Bricaud and Stramski [62]. New protocols have been re-  
202 cently proposed to decrease the uncertainty related to the  
203  $\beta$ -factor correction [63,64] by using a specific instrument con-  
204 figuration that was not available at the time of measurements.  
205 However, the T-R method has been shown to address such an  
206 issue [64]. Therefore, it can be used as an alternative despite  
207 being a more laborious and time-consuming technique [64].  
208 Optical densities were then converted into total [ $a_p(\lambda)$ ]  
209 and nonpigmented particle [ $a_{\text{NAP}}(\lambda)$ ] coefficients, and light ab-  
210 sorption spectra of phytoplankton [ $a_{\text{ph}}(\lambda)$ ] were finally deter-  
211 mined by subtraction of  $a_{\text{NAP}}(\lambda)$  from  $a_p(\lambda)$ .  
212

213 HPLC analysis provided concentrations of 16 pigments in-  
214 cluding chlorophyll *a*, auxiliary chlorophylls, and carotenoids  
215 (Table 1). Up to three samples (2–25 mL) of each culture were  
216 filtered under low vacuum on glass-fiber Whatman GF/F filters  
217 ( $\varnothing 25 \text{ mm}$ ) and immediately stored at  $-80^\circ\text{C}$ . Pigment extrac-  
218 tion was performed in 90% acetone at  $4^\circ\text{C}$  for 24 h. HPLC  
219 analysis was performed by a Class VP system (SHIMAZDU)  
220 equipped with a reverse-phase Shandon Hypersil MOS RP-C8  
221 column, capable of resolving divinyl-chlorophyll *a* from chloro-  
222 phyll *a*. The analysis was performed according to Vidussi *et al.*  
223 [65] and Barlow *et al.* [66] using the internal standard  $\beta 8$  APO  
224 CAROTENAL (Fluka). Pigment concentrations were computed  
225 according to Mantoura and Repeta [67]. The sum chlorophyll  
226 *a* + divinyl-chlorophyll *a* concentration is referred to as TChl *a*,  
227 and total pigment (TP) is defined as the sum of all chlorophylls  
228 and carotenoids (Table 1).

229 Cell counts were performed using a light microscope  
230 Optiphot (Nikon) equipped with an Hg lamp for fluorescence.  
231 Culture samples (50 mL) were collected in dark glass flasks and  
232 immediately fixed with neutralized formalin to the final concen-  
233 tration of 1%. Cell numbers of micro- and nanoplanktonic spe-  
234 cies were counted using a Burkner hemacytometer with a  $20 \times$   
235 objective, according to the manipulation, filling, and counting  
236 practices described in Guillard and Sieracki [68]. Cell numbers  
237 of *Synechococcus* sp. were counted by epifluorescence microscopy.  
238 Culture samples (25–150  $\mu\text{L}$ ) were filtered under low vacuum  
239 on Nuclepore black polycarbonate filters ( $0.2 \mu\text{m}$ ,  $\varnothing 25 \text{ mm}$ ).  
240 Details on sample preparation and counting ( $100 \times$  objective)  
241 are described in Guillard and Sieracki [68]. An average of three  
242 counts was used to estimate cell abundance for each batch

243 culture. Cell biovolume was calculated for each species (at least  
244 on 20 individuals) according to their geometrical shapes [69] and  
245 used to calculate the diameter of a sphere equivalent to cell vol-  
246 ume. No count and biovolume calculation were performed for  
247 *Prochlorococcus* sp. Cell counting was performed only in  
248 Experiment 1 and used for calculation of cellular pigment  
249 content.

### 250 C. Statistical Analysis

251 The dataset produced with Experiment 1 was used to evaluate  
252 the intra- and interspecific spectral variability of the phyto-  
253 plankton light absorption coefficients among the examined spe-  
254 cies as induced by different light growth conditions. Firstly, the  
255 **3** one-way ANOVA test (factor: light; levels: LL, ML, HL) was  
256 used to test the significance of intraspecific  $a_{\text{ph}}(\lambda)$  variability at  
257 selected wavelengths. Since a small number of samples ( $n = 3$ )  
258 was analyzed within each level of the examined factor, F values  
259 of the ANOVA tests could be seriously affected by random vari-  
260 ations; therefore, the nonparametric Kruskal–Wallis test [70]  
261 was used in parallel with the ANOVA. Levene’s test (absolute  
262 deviations;  $\alpha = 0.05$ ; [71]) of variance homogeneity was per-  
263 formed to test the assumptions of the ANOVA test. In very few  
264 cases the data variance failed to satisfy the homogeneity cri-  
265 terion; therefore the nonparametric Kruskal–Wallis test was  
266 used instead of the one-way ANOVA. Then the application  
267 of a hierarchical cluster analysis (HCA) to spectral absorption  
268 data (400–700 nm) was used to classify the light absorption  
269 spectra. The cluster trees (i.e., dendrograms) were obtained us-  
270 ing the unweighted pair-group average linkage algorithm [72],  
271 which joined the clusters according to the average distance be-  
272 tween all members. The cosine distance was chosen as criterion  
273 for evaluating the similarity level (from 0, i.e., no similarity, to  
274 1, i.e., highest similarity) between each pair of objects following  
275 Torrecilla *et al.* [15]. The cophenetic correlation coefficient  
276 [73] was calculated to assess how faithfully the dendrogram pre-  
277 served the pairwise distances between the examined samples.  
278 Cluster analysis was carried out by the free statistical software  
279 PAST version 3.04 [74].

280 The dataset produced with Experiment 2 was used to assess  
281 the feasibility to discriminate the contribution of a given species  
282 from bulk light absorption properties of assemblages with  
283 mixed taxonomic composition. For this purpose, the spectral  
284 similarity analysis introduced by Millie *et al.* [21] was used  
285 to extract the spectral signature of a species from a mixed  
286 assemblage. This method calculates the degree of similarity  
287 between two absorption spectra (i.e., similarity index, SI) by  
288 computing the cosine of the angle between two vectors such  
289 that [21]

$$290 \quad \text{SI} = \frac{A_b \cdot A_c}{|A_b| \times |A_c|}, \quad (1)$$

291 where  $A_b$  is the absorption spectrum of a mixed assemblage and  
292  $A_c$  is the absorption spectrum of a given species used as a refer-  
293 ence. The cross operator ( $\times$ ) is the vector product. The SI cal-  
294 culation, performed within the range 400–700 nm, yielded a  
295 number from 0 (i.e., no similarity between spectra) to 1 (i.e.,  
296 highest similarity between spectra). Because the cosine distance  
297 was chosen as a criterion of similarity in both hierarchical cluster  
and spectral similarity analyses, the results and interpretation of

Experiment 1 can be extended to Experiment 2. Then, model I  
regression type was used to relate SI values to the relative abun-  
dance of a given species and the respective concentrations of  
marker pigments (MP) within mixed assemblages. A Student’s  
 $t$ -test was performed to check the significance of the regression  
models. Then, the error in quantifying the MP concentrations  
from a range of representative SI values obtained from regression  
models was estimated using the percentage root mean square  
error (RMSE%) such that [75]

$$\text{RMSE\%} = 100 * \left( \sum_{i=1}^n \frac{(\bar{x}_i - x_i)^2}{n} \right)^{1/2}, \quad (2)$$

where  $\bar{x}_i$  and  $x_i$  were the estimated and measured MP concen-  
trations, respectively.

Before applying both hierarchical cluster and spectral sim-  
ilarity analyses, each phytoplankton absorption spectrum  
(400–700 nm) was first smoothed using a simple moving average  
filter ( $\Delta\lambda = 9$  nm [18]), then transformed by a normalized-ratio  
method (i.e., each data pair was divided by the largest of the pair  
[21]), and finally the corresponding fourth-derivative spectrum  
was computed by a finite approximation method assessing  
changes in curvature of a given spectrum over a sampling interval  
of 7 nm. The rationale of using the normalized-ratio transforma-  
tion is twofold. First, it reduces the influence of broad peaks in  
the blue and red portions of the absorption spectra (due to  
chlorophyll  $a$ ), which have similar traits in all algal species  
[21]. Second, it improves the sensitivity and linearity of the sim-  
ilarity index [21,24]. The fourth-derivative estimation enables a  
better separation of absorption bands and quantification of  
pigments [76].

## 3. RESULTS AND DISCUSSION

### A. Intraspecific and Interspecific Variability of Light Absorption Spectra as Induced by Light Growth Conditions

In the following sections we present results and analysis for  
Experiment 1. Relationships between environmental factors  
(i.e., light, nutrients, and temperature) and bio-optical proper-  
ties of various marine algal species and taxonomic groups have  
been reported and discussed by several studies both for natural  
(e.g., [36,77,78]) and controlled (e.g., [37–42]) conditions.  
Here we focus on the intracellular pigment contents and light  
absorption spectral characteristics of the seven marine algal spe-  
cies, useful to discuss the influence of different growth irradi-  
ances on their optical classification.

#### 1. Influence of Light on the Intracellular Pigment Content

The algal pigment concentrations measured for the examined  
species varied with the three chosen light growth conditions  
(LL, ML, and HL; Table 2). According to previous studies  
[41,42], analysis of pigment modifications evidenced a  
common behavior among species, that is, the increase of the  
cellular total pigment and chlorophyll  $a$  contents as a conse-  
quence of the long-term acclimation to low irradiances.  
Recall also that all species were cultured under an excess of nu-  
trients and, in synergy with limiting growth irradiances, this  
may cause an enhanced production of photosynthetic pigments

**Table 2. Cellular Pigment Contents (pg cell<sup>-1</sup>; fg cell<sup>-1</sup> for *Synechococcus* sp.) of Species Grown at 22°C under Three Irradiances (E, μmol photons m<sup>-2</sup> s<sup>-1</sup>)<sup>a</sup>**

Species	E	Chl										TP	d				
		Chl e <sub>3</sub> (PS)	e <sub>1</sub> + e <sub>2</sub> (PS)	Perid (PS)	19'-BF (PS)	Fuco (PS)	19'-HF (PS)	Viola (PP)	Diadino (PP)	Allo (PP)	Diato (PP)			Zea (PP)	Lute (PP)	Chl b' (PS)	Chl a' (PS)
<i>P. tricornutum</i>	10		0.06			0.30		0.03			0				0.58	0.97	6.24 ± 0.54
	100		0.05			0.25		0.06		0.002					0.27	0.64	6.50 ± 0.41
	300		0.03			0.15		0.07		0.01					0.16	0.42	[5.26 ± 1.13]
<i>A. carterae</i>	10		2.69	6.00				1.34		0					8.19	18.22	9.94 ± 0.80
	100		1.23	2.77				1.52		0.05					3.71	9.27	[12.5 ± 0.70]
	300		0.66	1.37				1.22		0.07					2.09	5.42	9.48 ± 0.65
<i>E. huxleyi</i>	10		0.08		0.007	0.005	0.41	0.02		0.007					0.41	1.05	3.44 ± 0.15
	100		0.04		0.007	0.01	0.22	0.03		0.01					0.26	0.62	3.51 ± 0.11
	300		0.04		0.004	0.02	0.22	0.09		0.02					0.26	0.71	[4.67 ± 1.20]
<i>Cryptomonas</i> sp.	10		0.13							0.31					1.29	1.73	8.04 ± 0.40
	100		0.05							0.25					0.77	1.07	7.99 ± 0.51
	300		0.02							0.17					0.43	0.62	8.06 ± 0.37
<i>Tetraselmis</i> sp.	10								0.32						4.81	8.92	8.16 ± 0.51
	100								0.81		0.23	0.23	0.59	3.14	6.89	12.77	[9.44 ± 0.87]
	300								0.27		0.42	0.62	0.42	1.76	2.86	5.65	8.39 ± 0.77
<i>Synechococcus</i> sp.	10														1.97	2.59	[1.04 ± 0.11]
	100														1.63	3.24	1.14 ± 0.14
	300														1.30	2.63	1.14 ± 0.14
<i>Prochlorococcus</i> sp.	10														0.71	1	
	25														0.56	1	
	100														0.46	1	

<sup>a</sup>Pigment contents for *Prochlorococcus* sp. are calculated as ratio (dimensionless) to the total pigment concentration (TP) because of cell count unavailability. See Table 1 for pigment abbreviations, comments, and formulas. The average diameter (d', in μm) of a sphere equivalent to cell volume is reported together with the standard deviation for each species, except for *Prochlorococcus* sp.; brackets indicate growth conditions with significant changes in cell size (one-way ANOVA test, p < 0.01).

<sup>b</sup>Dv Chl b and Dv Chl a for *Prochlorococcus* sp., respectively.

[35,79]. TP cellular concentration of HL acclimated cultures was 0.68 (in *E. huxleyi*) to 0.26 (in *A. carterae*) times the cellular content observed in LL acclimated cultures (Table 2). Similarly, the Chl *a* per cell content of the HL acclimated cultures was 0.66 (*Synechococcus* sp.) to 0.26 (*A. carterae*) times that of LL acclimated cells (Table 2). The cellular contents of auxiliary chlorophylls and photosynthetic xanthophylls also decreased at the highest irradiances (Table 2). Differences among species were also observed. Chlorophylls  $c_2 + c_1$  were the main auxiliary chlorophylls found in most studied species: a sharp reduction in cellular content with increasing irradiances was observed in *Cryptomonas* sp. and *A. carterae*; this was significantly smaller in *E. huxleyi* (Table 2). Considering photosynthetic xanthophylls, the cellular content of Peridinin in *A. carterae* varied from 1.37 pg cell<sup>-1</sup> in HL to 6.00 pg cell<sup>-1</sup> in LL conditions. The contents of Fucoxanthin in *P. tricornutum* and 19'-HF in *E. huxleyi* for LL conditions were twice those observed in HL conditions. In the case of photoprotective carotenoids, their cellular contents generally increased with increasing irradiances. For instance, Diadinoxanthin cellular concentration in *E. huxleyi* varied from 0.02 pg cell<sup>-1</sup> in LL to 0.09 pg cell<sup>-1</sup> in HL conditions, and Zeaxanthin in *Synechococcus* sp. increased from 0.62 to 1.61 fg cell<sup>-1</sup>. Alloxanthin in *Cryptomonas* sp. was the only photoprotective pigment observed to decrease with increasing irradiances (Table 2), similarly to the results found by Schlüter *et al.* [80] for the cryptophyte *Plagioselmis prolonga*. Similar trends were also observed for pigment-to-TP ratios in the case of *Prochlorococcus* sp., for which no cell counts were available. Dv Chl *a* and Dv Chl *b* decreased with increasing irradiances, while the proportion of Zeaxanthin to TP increased from 23% in LL to 52% in HL conditions (Table 2).

## 2. Intraspecific Variability of Light Absorption Spectra

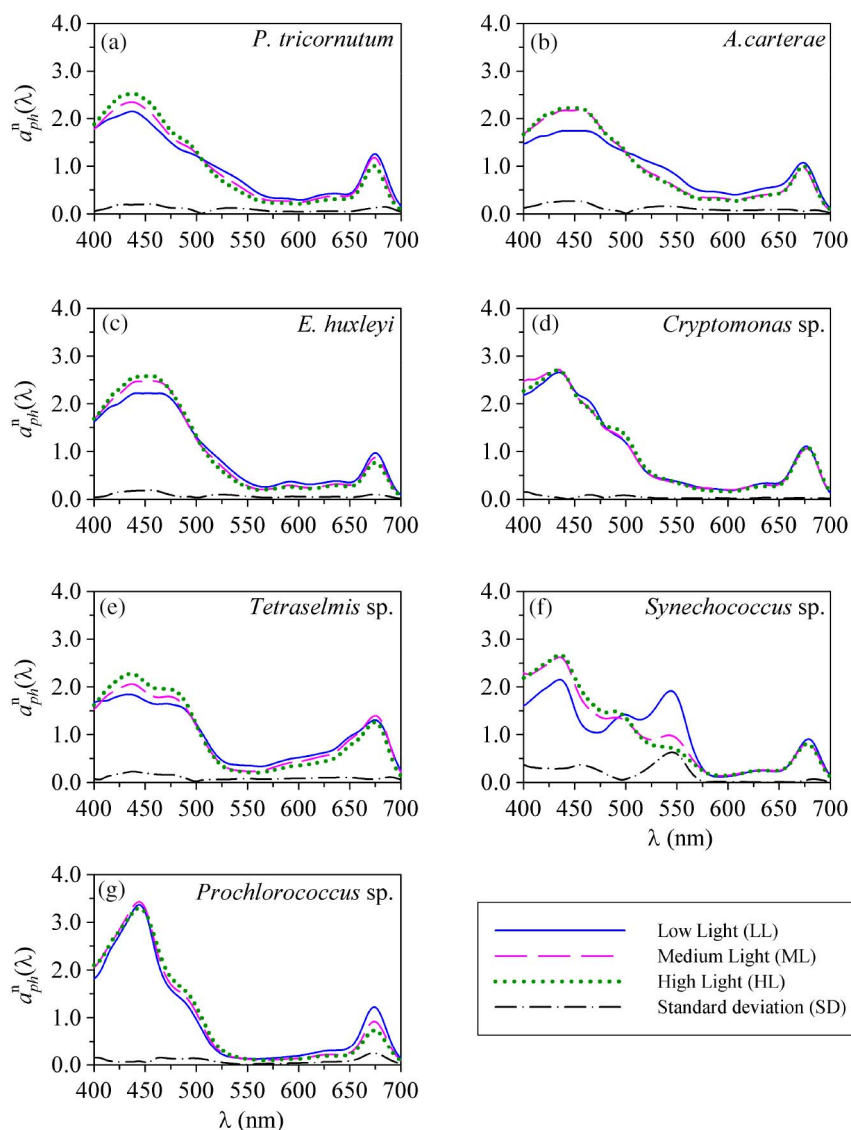
The phytoplankton light absorption spectra, normalized to their mean value between 400 and 700 nm ( $a_{\text{ph}}^n(\lambda)$ ; [17]), of the seven species grown under three different light intensities are shown in Fig. 1. Each spectrum of a given light regime is the average of three replicates from the same culture, then normalized. Spectral coefficient of variation ( $CV(\lambda)$ , that is, the standard deviation to mean ratio) for each group of replicates was generally <15%. Spectral variability occasionally increased up to 27% between 550 and 700 nm. Values up to 35% and 40% were observed at a few wavelengths for *Prochlorococcus* sp. and *Synechococcus* sp., respectively, likely as a consequence of absent or less pronounced features of absorbing pigments other than Chl *a* or DV Chl *a*.

The three irradiance treatments caused changes in the spectral shape of phytoplankton light absorption coefficients. The first striking feature was a flattening of the absorption spectra associated with a change in the irradiance conditions from HL to LL. This was observed for all the studied species except *Cryptomonas* sp. [Fig. 1(d)]. This spectral flattening represented a stronger packaging of pigments within the cells [28,34]. In the case of the experimental conditions (fixed irradiance and excess of nutrients), the observed pigment packaging effect was mainly associated with the increase in the total intracellular pigment contents (Table 2) instead of changes in the average size [28]. Indeed, the one-way ANOVA test ( $p < 0.01$ ) on the diameter of a sphere equivalent to cell volume revealed that the

small changes in cell size observed in the present dataset were significant only for some species or growth conditions (Table 2). A second observed feature is the variability in some spectral bands essentially associated to the absorption bands of carotenoids. The standard deviation spectrum highlighted the wavebands exhibiting maximum variability for each species, and the one-way ANOVA and Kruskal–Wallis tests confirmed, at these bands, significant effects of the irradiance treatments (Fig. 1; Table 3). This was especially striking for *Synechococcus* sp., for which the shape of the light absorption spectrum showed a drastic change (not just spectral flattening) from HL to LL conditions [Fig. 1(f)]. Considering the pigment-absorption band associations proposed by Bidigare *et al.* [31] and Hoepffner and Sathyendranath [32], these significant intraspecific differences in the spectral absorption signatures of the examined species were also related to modifications in intracellular concentrations of those pigments useful for taxonomic identification (Table 3).

In order to evaluate how changes in irradiance growth conditions influenced the classification of a given species through the entire absorption spectrum, we applied a HCA on the fourth derivative of the absorption spectra of the seven species used in Experiment 1. Recent studies [15,18,19,24,81] stressed the use of hyperspectral measurements and the potential of spectral derivative analysis for retrieving information on the phytoplankton community structure in the natural environment. Among the various methods used for pursuing this aim, the classification of algal assemblages using derivative spectra of light absorption through HCA worked successfully [15,19,81].

The dendrogram resulting from HCA yielded well-identified clusters, each comprising the three absorption spectra (LL, ML, and HL) from a single species (Fig. 2). The cophenetic correlation coefficient of 0.89 indicated highly reliable results of the cluster analysis. This suggests that even when different growth conditions provoke significant changes in cellular pigment concentrations and thus in the light absorption features as reported previously, the spectral absorption signature of a given phytoplankton species is still recognizable from that of other species. However, the similarity level at which the spectra of a species were identified as a cluster varied depending on the considered species. Somehow expected from unequally spaced growth irradiances, the distance between the spectra of the cultures acclimated to ML and HL conditions was shorter than that between the ML-acclimated and LL-acclimated spectra, except for *E. huxleyi* (Fig. 2). The similarity between LL-acclimated spectra and those for cultures acclimated to HL and ML conditions was, however, high for *P. tricornutum*, *A. carterae*, and *Cryptomonas* sp. (0.81–0.90). This suggested low intraspecific variability in the light absorption spectra for these species and examined growth conditions. The level of spectral similarity was instead lower than 0.68 for LL-acclimated spectra of *Tetraselmis* sp., *Synechococcus* sp., and *Prochlorococcus* sp. with respect to ML- and HL-acclimated cultures. This highlighted notable intraspecific differences, likely caused by the synergistic effect of limited light and excess of nutrients that enhanced pigment production [35,79] and provoked more drastic changes in the absorption spectral features.



F1:1 **Fig. 1.** *In vivo* light absorption spectra normalized to the mean between 400 and 700 nm [ $a_{ph}^n(\lambda)$ ] for seven species grown at three irradiances.  
 F1:2 Each spectrum is the average of three replicates, then mean normalized. The standard deviation among the normalized spectra representing the three  
 F1:3 growth irradiances is also shown.

**Table 3. Wavebands ( $\lambda$ ; nm) of Standard Deviation Maxima Calculated between Mean-Normalized Absorption Spectra of Each Species Grown under Three Light Regimes (Fig. 1)<sup>a</sup>**

T3:1	Species	$\lambda_1$	$\lambda_2$	$\lambda_3$	$\lambda_4$	$\lambda_5$	$\lambda_6$
T3:2	<i>P. tricornutum</i>	427 <sup>b</sup>	456 <sup>c</sup>	485 <sup>c</sup>	534 <sup>c</sup> (MP)	626 <sup>b</sup>	683 <sup>b</sup>
T3:3	<i>A. carterae</i>	455 <sup>c</sup>	538 <sup>c</sup> (MP)	654 <sup>c</sup>	685 <sup>c</sup>		
T3:4	<i>E. huxleyi</i>	456 <sup>c</sup>	492 <sup>b</sup>	523 <sup>c</sup> (MP)	594 <sup>c</sup>	675 <sup>c</sup>	
T3:5	<i>Cryptomonas</i> sp.	465 <sup>c</sup>	498 <sup>c</sup> (MP)	640 <sup>c</sup>			
T3:6	<i>Tetraselmis</i> sp.	438 <sup>c</sup>	470 <sup>b</sup>	643 <sup>c</sup> (MP)	689 <sup>c</sup>		
T3:7	<i>Synechococcus</i> sp.	455 <sup>c</sup> (MP)	545 <sup>c</sup>				
T3:8	<i>Prochlorococcus</i> sp.	465 <sup>c</sup>	496 <sup>b</sup>	676 <sup>c</sup> (MP)			

<sup>a</sup>One-way ANOVA and Kruskal-Wallis tests:

<sup>b</sup>significant,  $p < 0.05$ ;

<sup>c</sup>highly significant,  $p < 0.01$ . MP, band associated to the corresponding marker pigment.

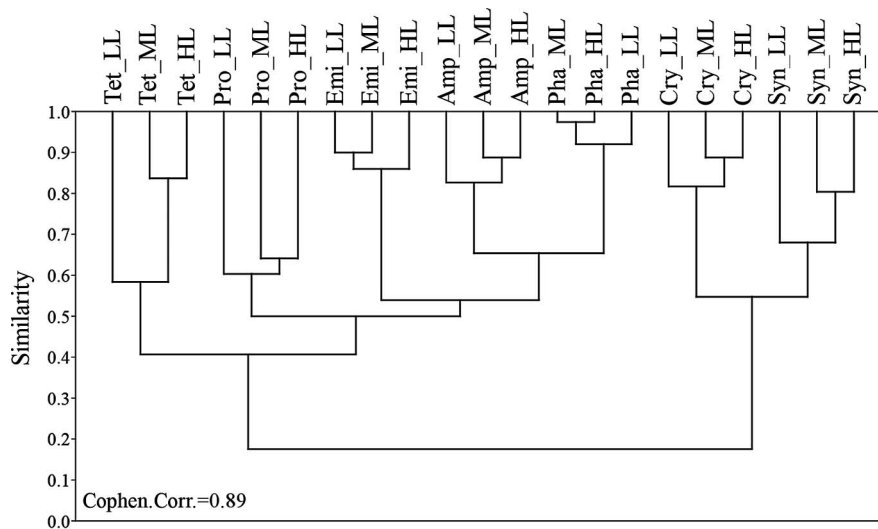
466 **3. Interspecific Variability of Light Absorption Spectra**

467 The next step of Experiment 1 was to quantify the differences  
 468 between the shapes of the light absorption spectra among the

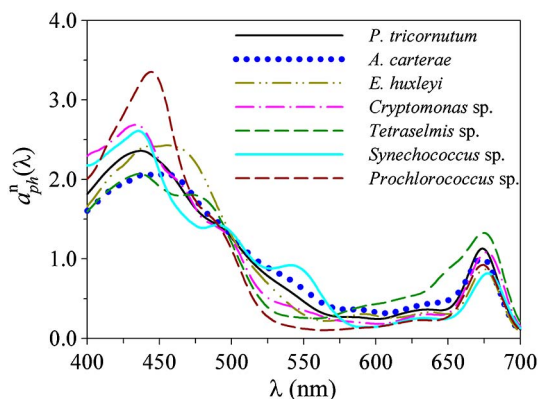
seven studied species (i.e., interspecific differences). For this  
 purpose, a cluster analysis was applied to the fourth derivative  
 of absorption spectra of each light growth condition (LL, ML,

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**Fig. 2.** Results of the hierarchical cluster analysis performed on the fourth-derivative of light absorption spectra (400–700 nm) of seven algal species for three different light growth conditions (LL, ML, and HL): *P. tricornutum* (Pha), *A. carterae* (Amp), *E. huxleyi* (Emi), *Cryptomonas* sp. (Cry), *Tetraselmis* sp. (Tet), *Synechococcus* sp. (Syn), *Prochlorococcus* sp. (Pro). The cophenetic correlation coefficient of the cluster analysis (Cophen. Corr.) is reported.



**Fig. 3.** *In vivo* light absorption spectra normalized to the mean between 400 and 700 nm [ $a_{ph}^0(\lambda)$ ] computed as the average of the absorption spectra measured under LL, ML, and HL growth conditions (i.e., AS spectrum), then mean normalized.

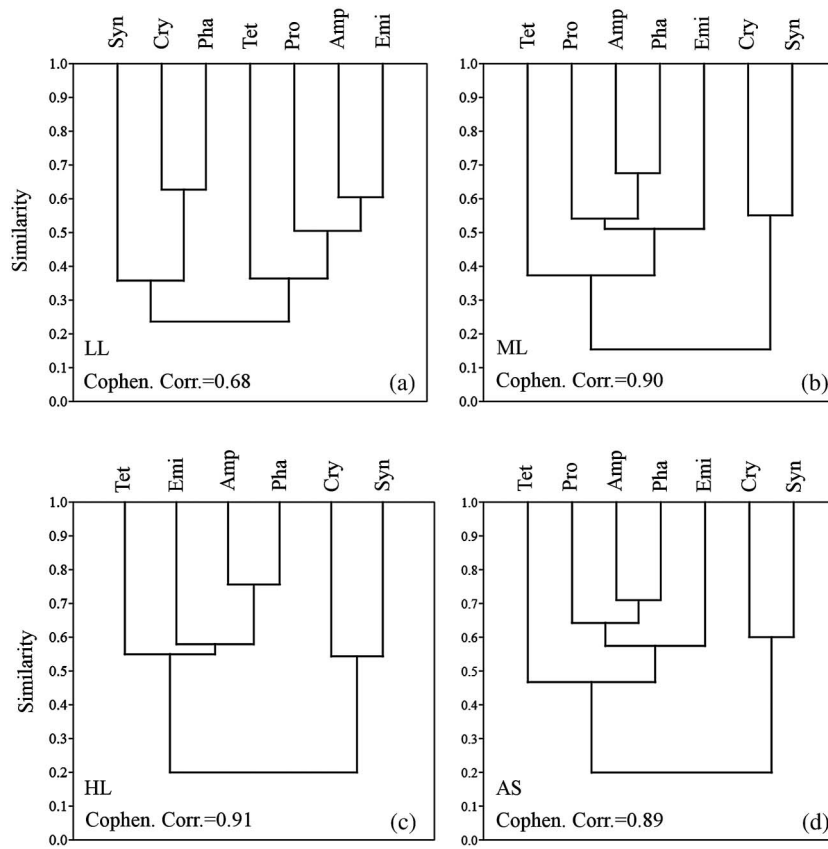
and HL). In addition, we computed the average of the three absorption spectra obtained in the three different light conditions (Fig. 3) and applied a cluster analysis to the fourth derivative of the average spectra (hereafter AS).

The classifications of ML, HL, and AS spectra were similar with high cophenetic correlation coefficients (0.89–0.91). The results of this application evidenced that the absorption spectra of the examined species could be split into two major clusters [Figs. 4(b)–4(d)]. The first one was composed by the spectra of the cryptophyte *Cryptomonas* sp. and the cyanobacterium *Synechococcus* sp., which were characterized by a similarity ranging from 0.54 to 0.60. The second group included all the other species [Figs. 4(b)–4(d)]. Note that *Prochlorococcus* sp. is not displayed in Fig. 4(c) because of the insufficient growth rate observed at 300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Within this cluster,

the absorption spectrum of the prasinophyte *Tetraselmis* sp. was the most different (similarity level between 0.39 and 0.55). The most similar spectra, indicating small interspecific differences as also recently observed by Xi et al. [81], were those of the diatom *P. tricornutum* and the dinoflagellate *A. carterae* (similarity level >0.69). The classification of absorption spectra obtained for the species grown in LL conditions (cophenetic correlation coefficient of 0.68) evidenced instead a high similarity between the spectra of the diatom *P. tricornutum* and the cryptophyte *Cryptomonas* sp. (similarity level of 0.63), and between the dinoflagellate *A. carterae* and the coccolithophore *E. huxleyi* within the other cluster [Fig. 4(a)].

The clusters given by this analysis could actually be explained by similarities and differences in pigment composition that characterized the examined species grown under fixed irradiance and nutrient-enriched conditions. *Cryptomonas* sp. and *Synechococcus* sp. were the only two species containing phycobilins such as phycoerythrin, a pigment with outstanding spectral signatures [82]. *P. tricornutum*, *A. carterae*, and *E. huxleyi* had the same accessory chlorophylls (chlorophyll *c*, Table 2) and photosynthetic xanthophylls (Fuco, Perid, and 19'-HF) with very similar spectral absorption signatures [30,31]. *Tetraselmis* sp. and *Prochlorococcus* sp. contained chlorophyll *b* and divinyl-chlorophyll *b*, respectively, two pigments with very similar light absorption features, and photoprotective pigments with optical properties close to those present in other cluster members. Another result of the cluster application to be emphasized is the low similarity observed between the two zeaxanthin-containing species *Prochlorococcus* sp. and *Synechococcus* sp. (Figs. 3, 4). Given the similar cell size of these species (nominally 0.6 and 1  $\mu\text{m}$  for *Prochlorococcus* sp. and *Synechococcus* sp., respectively), previous size-based absorption approaches detected these two species as a single group [17,18,83]. The low similarity here observed is probably related to the absorption bump at around 550 nm in *Synechococcus* sp.,

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**Fig. 4.** Results of the HCA performed on the fourth derivative of light absorption spectra (400–700 nm) of seven algal species: (a) LL growth condition ( $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ); (b) ML growth conditions ( $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ); (c) HL growth conditions ( $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ); (d) absorption spectra representing the average of modifications induced by three different light growth conditions (AS spectra). In panel (c) *Prochlorococcus* sp. is not included because of the insufficient growth rate observed at  $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . In each panel, the cophenetic correlation coefficient of cluster analysis (Cophen. Corr.) is reported. Abbreviation of species name: *P. tricornutum* (Pha), *A. carterae* (Amp), *E. huxleyi* (Emi), *Cryptomonas* sp. (Cry), *Tetraselmis* sp. (Tet), *Synechococcus* sp. (Syn), *Prochlorococcus* sp. (Pro).

522 which may be due to the absorption of phycoerythrin, a pig- 544  
 523 ment missing in *Prochlorococcus* sp. Although phycoerythrin 545  
 524 abundance may have been drastically enhanced by the 546  
 525 experimental high nutrient concentrations [79], this outcome 547  
 526 suggests the possibility of using their specific pigment absorp- 548  
 527 tion signatures to distinguish their presence when they co-occur 549  
 528 in the algal community. 550

529 **B. Assessing the Contribution of a Given Species** 544  
 530 **from Assemblages with Mixed Taxonomic** 545  
 531 **Composition** 546

532 In the following sections, the results obtained from Experiment 2 547  
 533 are presented. Discussion focuses on the feasibility to extract the 548  
 534 absorption spectrum of a given species from the bulk absorption 549  
 535 properties of an assemblage with mixed taxonomic composition 550  
 536 and to quantify its contribution within it. Analysis is conducted 551  
 537 with the spectral light absorption reference of a given species 552  
 538 coming both from similar and different light growth conditions 553  
 539 to that of mixed assemblages. 554

540 **1. Taxonomic Structure and Bio-optical Characteristics of** 544  
 541 **Simulated Algal Assemblages** 545

542 Taxonomic structure and bio-optical characteristics of algal 546  
 543 assemblages composed by varying proportions (in terms of TChl 547

544 *a*) of *P. tricornutum*, *A. carterae*, *E. huxleyi*, *Synechococcus* sp., 545  
 546 and *Prochlorococcus* sp. (Experiment 2) are here presented and 546  
 547 compared to those of natural assemblages in literature. It is ac- 547  
 548 knowledged that the use of only one species to represent a taxo- 548  
 549 nomic group cannot fully cover the intragroup variability and/ 549  
 550 or the intergroup similarities of light absorption spectral fea- 550  
 551 tures that can be found in natural environments. The reduced 551  
 552 taxonomical complexity of mixed algal assemblages helped 552  
 553 minimize any change in cellular pigment content, cell number, 553  
 554 and thus optical properties during the execution of the experi- 554  
 555 ment. In addition, as a consequence of controlled and nutrient- 555  
 556 enriched conditions of growth, simulated algal mixtures were 556  
 557 characterized by total chlorophyll *a* concentrations higher than 557  
 558 those of natural assemblages [30]. In terms of varying contri- 558  
 559 butions of each species with respect to total chlorophyll *a*, taxo- 559  
 560 nomic and bio-optical characteristics of simulated algal 560  
 561 assemblages were however consistent with those observed in 561  
 562 natural conditions. 562

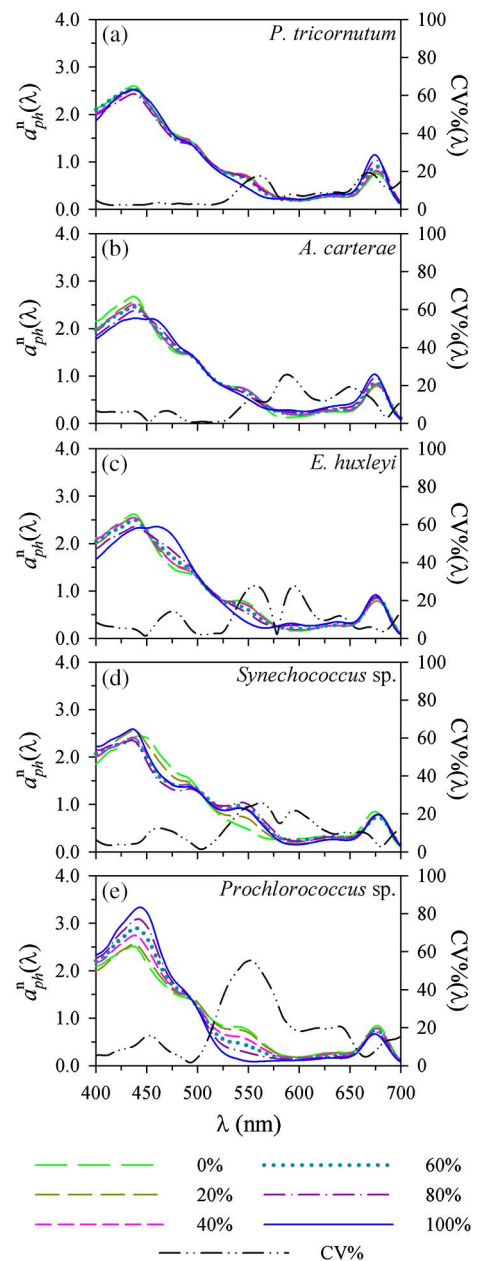
562 The contribution of each phytoplankton size class in the 562  
 563 simulated mixed algal assemblages, calculated according to 563  
 564 Uitz *et al.* [83], ranged from contributions <13% up to more 564  
 565 than 77%, a range of variation consistent with that of natural 565  
 566 phytoplankton communities observed at the global scale [30]. 566

567 The ratios of various groups of pigments (total chlorophylls *c*;  
 568 photosynthetic and photoprotective carotenoids) with respect to  
 569 TChl *a* also varied with trends and within ranges similar (0–0.38,  
 570 0–0.90, and 0.16–1.29, respectively) to those observed in open  
 571 ocean algal populations [30,57,84]. Only the ratios between pho-  
 572 tosynthetic carotenoids to total chlorophyll *a* increased with TChl  
 573 *a*, while no specific trends were observed in natural populations  
 574 [30,57,84]. Chlorophyll-specific phytoplankton light absorption  
 575 4 coefficients at 438 and 675 nm [ $a_{ph}^*(\lambda)$ ] of the simulated mixed  
 576 assemblages varied in the ranges 0.025–0.20 and 0.011–  
 577 0.057 m<sup>2</sup> mg TChl *a*<sup>-1</sup>, respectively, and decreased as a function  
 578 of TChl *a* according to a power law ( $r^2 = 0.75$  for  $a_{ph}^*(438)$  and  
 579  $r^2 = 0.57$  for  $a_{ph}^*(675)$ ); [84]]. The observed coefficients were  
 580 consistent with those observed for various open ocean waters  
 581 [30,57,84–86], except the ultra-oligotrophic surface waters of  
 582 the South Pacific Ocean [87]. However,  $a_{ph}^*(675)$  values up  
 583 to 0.057 m<sup>2</sup> mg TChl *a*<sup>-1</sup> instead of 0.038 m<sup>2</sup> mg TChl *a*<sup>-1</sup>  
 584 [84,86] were observed in simulated mixed assemblages, which  
 585 suggested a weaker pigment packaging effect of TChl *a* within  
 586 algal cells than that found in natural assemblages.

587 The light absorption spectra of simulated mixed assemblages  
 588 that will be used, in the following sections, to assess the capability  
 589 of discrimination of a given species from bulk light absorption  
 590 spectral properties are shown in Fig. 5. Each spectrum is the average  
 591 of three replicates from the same mixed culture, then mean-  
 592 normalized. Analysis of coefficients of variation [CV( $\lambda$ )] between  
 593 replicates (calculated as the ratio of the standard deviation to the  
 594 average spectrum) showed spectral variability varying between  
 595 1% and 20%, except on a few occasions. Instead, when observing  
 596 CV( $\lambda$ ) values resulting from a variety of mixed assemblages, re-  
 597 gions of maximum spectral variability, that is, the wavebands of *in*  
 598 *vivo* absorption of auxiliary pigments (marker pigments included;  
 599 [30–32]) were evidenced (Fig. 5). High CV values (up to 55%)  
 600 were generally observed around 550 nm (Fig. 5), a source of  
 601 variability that could be mainly ascribed to the varying propor-  
 602 tions of phycoerythrin in *Synechococcus* sp., Fucoxanthin in  
 603 *P. tricornutum*, Peridinin in *A. carterae*, and 19'-HF in *E. huxleyi*.  
 604 High variability (up to 27%) was also observed at 590 and  
 605 640 nm, as a result of the variable occurrence of chlorophylls *c*,  
 606 and within the range 400–500 nm (up to 16%), probably as a  
 607 consequence of the different spectral contributions of the various  
 608 photoprotective pigments.

## 609 2. Discrimination of a Given Species from Assemblages 610 Adapted to the Same Light Regime

611 Previous studies have demonstrated the potential of the spectral  
 612 similarity analysis [21] and use of SI (Eq. 1) for detecting and  
 613 quantifying a given phytoplankton species from light absorption  
 614 spectra, even in natural mixed assemblages [22]. SI values, as  
 615 derived from pairwise comparison between a reference spectrum  
 616 of a given species and that of an assemblage with unknown taxo-  
 617 nomic structure, were observed to vary accordingly with the frac-  
 618 tion of a species [21–23,25] or cell abundance [24]. SI was thus  
 619 promoted as a possible quantitative indicator of the presence of  
 620 given phytoplankton groups within assemblages [24]. Hence, in  
 621 order to investigate the possibility to detect the spectral signature  
 622 of multiple species and quantify their abundances within mixed  
 623 assemblages, we applied here the spectral similarity analysis on  
 624 the fourth derivative of absorption spectra of the algal assemb-



625 **Fig. 5.** *In vivo* light absorption spectra normalized to the mean  
 626 between 400 and 700 nm [ $a_{ph}^*(\lambda)$ ] of 26 mixed assemblages obtained  
 627 using five cultured species together with the spectral coefficient of vari-  
 628 ations (CV, in %). Each spectrum is the average of three replicates,  
 629 then mean-normalized. Assemblages were obtained varying the con-  
 630 tribution to TChl *a* of a species at a time from 0 to 100% (20% steps):  
 631 (a) *P. tricornutum*, (b) *A. carterae*, (c) *E. huxleyi*, (d) *Synechococcus* sp.,  
 632 (e) *Prochlorococcus* sp.

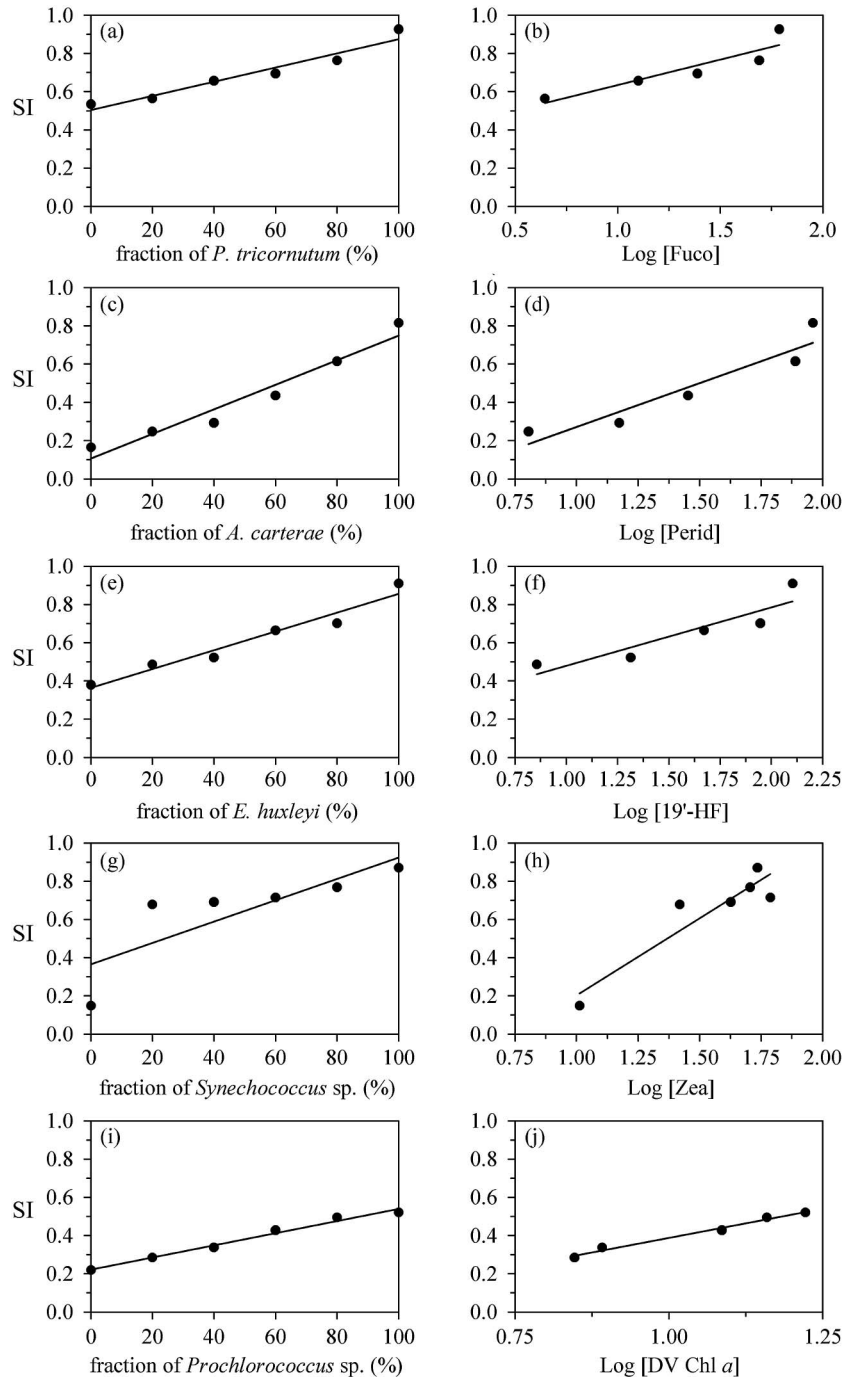
625 lages simulated during Experiment 2. In this context, and differ-  
 626 ently from other algorithms (e.g., [18]), spectral similarity  
 627 analysis can be applied regardless of any prior model training.

628 The index of spectral similarity, SI, was first computed  
 629 between the spectra measured for each simulated mixed assem-  
 630 blage where the contribution of a given species varied from 0%  
 631 to 20% of TChl *a*, and the reference spectrum of the corre-  
 632 sponding species. The absorption spectrum of a given species

633 cultured at a light intensity of  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  and  
 634 obtained from Experiment 1 was chosen as the reference spec-  
 635 trum, as it represented the same experimental light conditions as  
 636 those of the mixed assemblages. Hence, this comparison allowed  
 637 investigating the discrimination among species regardless the in-  
 638 fluence of light-induced spectral modifications. The resulting SI  
 639 was then regressed against (1) the relative abundance (in term of  
 640 TChl *a*) of the considered species within the mixed assemblage

and (2) the log10 concentration of the corresponding marker  
 641 pigment (Fig. 6). Marker pigments were chosen as indicative  
 642 of the abundance of a given species within the assemblage fol-  
 643 lowing Jeffrey and Veski [45].

The resulting SI values were related to the fraction of a given  
 645 species within the assemblages ( $r^2 > 0.68$ , Table 4, Fig. 6 left  
 646 column) and to the concentration of the corresponding marker  
 647 pigment ( $r^2 > 0.83$ , Table 4, Fig. 6 right column). These  
 648



F6:1 **Fig. 6.** Relationships between SI values, computed from the comparison between the fourth-derivative spectra of each assemblage and the spec-  
 F6:2 trum of the species grown at  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and the relative fraction to TChl *a* (left column) or the logarithm of MP concentrations  
 F6:3 (right column) of a species within the mixed assemblages: (a) *P. tricorntutum*, (b) *A. carterae*, (c) *E. huxleyi*, (d) *Synechococcus* sp., (e) *Prochlorococcus* sp.  
 F6:4 Statistics of linear regressions are reported in Table 4.

**Table 4. Parameters of Linear Regressions Displayed in Fig. 6:  $n$  = Number of Observations;  $b$  = Regression Slope;  $a$  =  $y$ -intercept;  $r^2$  = Determination Coefficient<sup>a</sup>**

T4:1	Equation	Species	Reference Spectrum	$n$	$b$ ( $^b10^2$ )	$a$	$r^2$	SI range
T4:2	$SI = b * \%(species) + a$	<i>P. tricornutum</i>	ML	6	0.4	0.50	0.94 <sup>c</sup>	0.53–0.92
T4:3		<i>A. carterae</i>	ML	6	0.6	0.11	0.95 <sup>c</sup>	0.16–0.81
T4:4		<i>E. huxleyi</i>	ML	6	0.5	0.36	0.95 <sup>c</sup>	0.38–0.91
T4:5		<i>Synechococcus</i> sp.	ML	6	0.6	0.37	0.68 <sup>b</sup>	0.15–0.87
T4:6		<i>Prochlorococcus</i> sp.	HL	6	0.3	0.22	0.98 <sup>c</sup>	0.22–0.52
T4:7	Equation	Marker Pigment	Reference Spectrum	$n$	$b$	$a$	$r^2$	SI Range
T4:8	$SI = b * \text{Log}[\text{MP}] + a$	Fuco	ML	5	0.27	0.37	0.84 <sup>b</sup>	0.56–0.92
T4:9		Perid	ML	5	0.46	-0.19	0.89 <sup>b</sup>	0.25–0.81
T4:10		19'-HF	ML	5	0.31	0.17	0.83 <sup>b</sup>	0.49–0.91
T4:11		Zea	ML	6	0.81	-0.60	0.86 <sup>c</sup>	0.15–0.87
T4:12		DV Chl $a$	HL	5	0.61	-0.22	0.98 <sup>c</sup>	0.28–0.52

<sup>a</sup>Student's  $t$ -test:<sup>b</sup> $p < 0.05$ ; ns, not significant.<sup>c</sup> $p < 0.01$ ;

649 results clearly indicated that the spectral signature of a given  
650 species substantially influences the bulk absorption spectrum  
651 of the assemblage. More importantly, results evidenced that  
652 the contribution of each species to the assemblage structure  
653 could be quantified using its absorption properties, also when  
654 the relative abundances of all contributing species were similar  
655 (i.e., 20% of TChl  $a$ ).

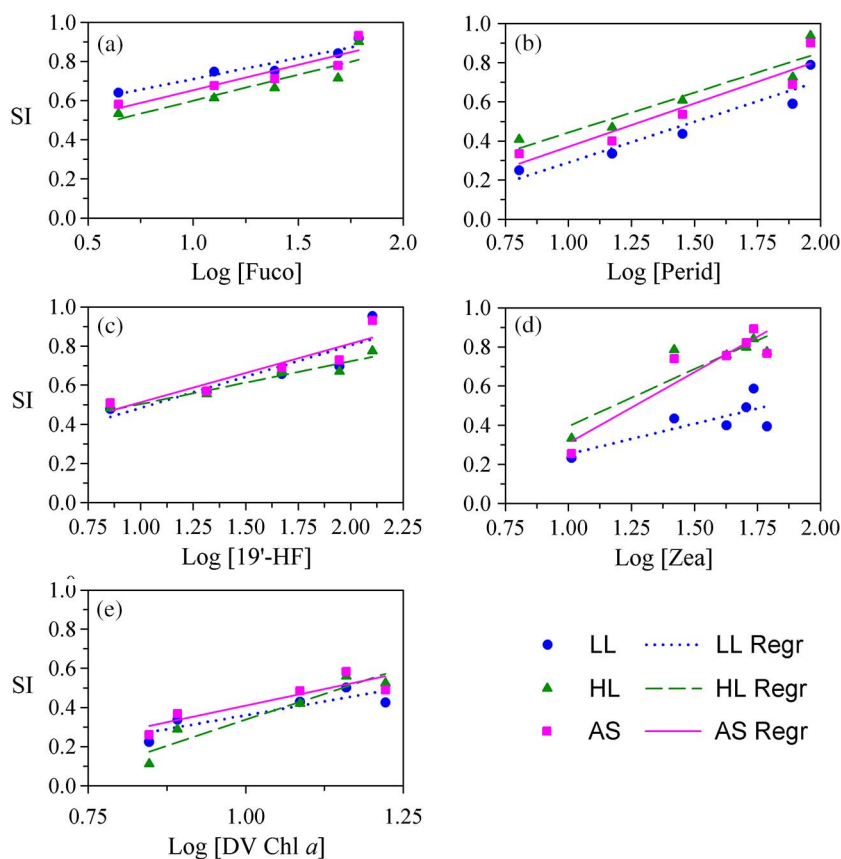
656 However, the analysis of the variation ranges of the SI values  
657 and regression parameters (Table 4) suggested that the overall  
658 capability of discriminating a phytoplankton species using the  
659 bulk absorption spectrum of the assemblage was more or less  
660 robust depending on the considered species. For a null fraction  
661 (0%) of a given species (Fig. 6 left column), the SI values ap-  
662 peared to be always different from zero and were even high in  
663 the case of *P. tricornutum* and *E. huxleyi* (0.53 and 0.38, re-  
664 spectively; Table 4). They were, however, low for *A. carterae*  
665 (0.16), *Synechococcus* sp. (0.15), and *Prochlorococcus* sp.  
666 (0.22). This suggested that all the various reference spectra  
667 we studied shared some level of similarity in terms of shape.  
668 In addition, the similarity between the reference spectrum  
669 and the spectrum measured for an assemblage of 100% of a  
670 given species never reached 1, although they were cultured  
671 under the same controlled growth conditions. This may be be-  
672 cause it is impossible to reproduce exactly the same absorption  
673 spectrum of a given species and for given growth conditions  
674 twice, as a consequence of multiple biological responses that  
675 organisms may have with respect to the same environmental  
676 factors. The impact of methodological errors cannot, however,  
677 be excluded. SI values were close to 1 in the case of *P. tricorn-*  
678 *nutum* and *E. huxleyi* (0.92 and 0.91, respectively; Table 4),  
679 slightly lower for *Synechococcus* sp. (0.87) and *A. carterae*  
680 (0.81), and surprisingly low in the case of *Prochlorococcus* sp.  
681 (0.52). In particular, the case of *Prochlorococcus* sp. could be  
682 related to a low signal-to-noise ratio in those parts of the spec-  
683 trum where there is no absorbing pigment [e.g., 550–650 nm  
684 for *Prochlorococcus* sp.; Fig. 5(e)], which could possibly affect  
685 the sensitivity of the fourth derivative method [18]. A compar-  
686 ison among replicates of spectra for those assemblages with  
687 100% of a given species further strengthened the possible oc-  
688 currence of methodological errors, as SI values no higher than

0.98  $\pm$  0.003 (*E. huxleyi*) were observed. All regression slopes  
of linear models computed both with the relative abundance to  
TChl  $a$  (Fig. 6 left column) and MP concentrations (Fig. 6  
right column) were significant, but high up to 0.81 only in  
the case of the cyanobacterium *Synechococcus* sp. [Table 4;  
Figs. 6(g)–6(h)]. The lower regression slopes especially for  
*P. tricornutum*, *A. carterae*, and *E. huxleyi* (Table 4) may be  
a consequence of the co-occurrence of similar pigment com-  
positions and shared spectral shapes. In these cases, the level of  
similarity can lower performances in properly quantifying  
the presence of these algal groups from the bulk absorption  
spectrum of the assemblage.

### 3. Discrimination of a Given Species from Assemblages Adapted to Different Light Regimes

In this section, we evaluate the effects of light-induced spectral changes in the absorption coefficients for the quantification of a given species in assemblages with mixed taxonomic structure. Similar to the analyses presented in Section 3.B.2, we calculated the SI by pairwise comparison between each absorption spectrum of a simulated mixed assemblage (Fig. 5) and the spectrum of each given species when acclimated to different light growth conditions from the mixed assemblage as a reference, thus LL and HL (ML for *Prochlorococcus* sp.) spectra coming from Experiment 1 (Fig. 1). References obtained by averaging absorption spectra measured under the three light conditions (AS spectra, Fig. 3) of each given species were also used. The resulting SI was then regressed against the log10 concentration of the corresponding marker pigment within the mixed assemblage (Fig. 7).

The analysis of the variation ranges of SI values and regression parameters (Fig. 7, Table 5) revealed that the contribution of a species was detected within the absorption spectrum of a mixed assemblage, even when the reference spectra representing different light growth conditions were used. However, different behaviors were observed among species and according to the reference used. In the cases of *P. tricornutum*, *A. carterae*, and *E. huxleyi*, all SI values were significantly linearly correlated ( $r^2 > 0.79$ ; Table 5) to the logarithm of concentrations of Fuco, Perid, and 19'-HF, respectively. Nevertheless, SI values



**Fig. 7.** As Fig. 6 (right column), for SI values obtained using reference spectra of species acclimated to LL, HL (ML for *Prochlorococcus* sp.) conditions and the AS spectra: (a) *P. tricornutum*, (b) *A. carterae*, (c) *E. huxleyi*, (d) *Synechococcus* sp., (e) *Prochlorococcus* sp. Statistics of linear regressions are reported in Table 5.

**Table 5. Parameters of Linear Regressions Displayed in Fig. 7: *n* = Number of Observations; *b* = Regression Slope; *a* = *y*-intercept; *r*<sup>2</sup> = Determination Coefficient<sup>a</sup>**

T5:1	Equation	Marker Pigment	Reference Spectrum	<i>n</i>	<i>b</i>	<i>a</i>	<i>r</i> <sup>2</sup>	SI range
T5:2	SI = <i>b</i> * Log[MP] + <i>a</i>	Fuco	LL	5	0.22	0.49	0.91 <sup>b</sup>	0.64–0.92
T5:3			HL	5	0.26	0.33	0.79 <sup>b</sup>	0.53–0.90
T5:4			AS	5	0.26	0.39	0.85 <sup>b</sup>	0.58–0.93
T5:5		Perid	LL	5	0.42	-0.13	0.90 <sup>b</sup>	0.25–0.79
T5:6			HL	5	0.41	0.03	0.88 <sup>b</sup>	0.41–0.94
T5:7			AS	5	0.44	-0.07	0.89 <sup>b</sup>	0.34–0.90
T5:8		19'-HF	LL	5	0.32	0.16	0.81 <sup>b</sup>	0.48–0.95
T5:9			HL	5	0.22	0.28	0.94 <sup>c</sup>	0.48–0.77
T5:10			AS	5	0.30	0.21	0.86 <sup>b</sup>	0.51–0.93
T5:11		Zea	LL	6	0.31	-0.06	0.60 ns	0.23–0.59
T5:12			HL	6	0.59	-0.19	0.82 <sup>b</sup>	0.33–0.84
T5:13			AS	6	0.72	-0.41	0.86 <sup>c</sup>	0.26–0.89
T5:14		DV Chl <i>a</i>	LL	5	0.57	-0.21	0.77 ns	0.22–0.50
T5:15			ML	5	1.06	-0.72	0.90 <sup>b</sup>	0.11–0.56
T5:16			AS	5	0.68	-0.27	0.80 <sup>b</sup>	0.26–0.58

<sup>a</sup>Student's *t*-test:

<sup>b</sup>*p* < 0.05, ns not significant.

<sup>c</sup>*p* < 0.01;

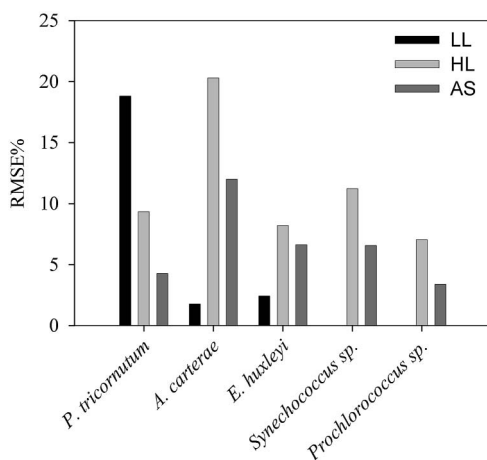
728 in *P. tricornutum* [Fig. 7(a)] were generally higher when the  
 729 spectrum measured under the LL conditions was used as a  
 730 reference instead of the HL or AS spectra (Table 5). The exact  
 731 opposite situation occurred in *A. carterae*, for which the SI

values were maximum when the HL spectrum was used as  
 the reference [Fig. 7(b), Table 5]. In the case of the two  
 cyanobacteria *Synechococcus* sp. and *Prochlorococcus* sp., no sig-  
 nificant relationships were found between SI and MPs when

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absorption spectra of mixed assemblages were compared with the reference spectrum of the low irradiance condition [Table 5; Figs. 7(d)–7(e)]. This was probably a consequence of the high intraspecific spectral variability observed for these two species at the given experimental conditions.

The results of this experiment have implications in the context of operational application of algorithms used for the optical discrimination of phytoplankton groups. Frequently, in order to discriminate phytoplankton groups from spectra of assemblages with unknown taxonomic structure, absorption spectra of cultured or monospecific algal communities are used as a reference [17,23–25,75]. Evidently, this is made by assuming that similar growth conditions, and thus a similar level of photoacclimation, exist between the reference and the studied absorption spectrum. This can be a source of uncertainty affecting the performances of the retrievals. The next step was, therefore, to attempt to predict the concentration of the five marker pigments (and assess the errors) by applying the linear models shown in Table 5 to a range of SI values. The SI ranges, falling within the ranges observed from linear models (Table 5) and including SI values corresponding to increments of 0.05, were 0.65–0.90 for *P. tricornutum*, 0.45–0.75 for *A. carterae*, 0.55–0.75 for *E. huxleyi*, 0.40–0.80 for *Synechococcus* sp., and 0.30–0.50 for *Prochlorococcus* sp. Then we evaluated the predictive skills of the models by comparing the predicted MPs to the measured MPs in the different cultures. Because the five species used to obtain mixed algal assemblages were cultured at a light intensity of 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , the MPs concentration obtained from linear models in Table 4 (i.e., comparison with ML-acclimated reference spectrum) were used as the measured MPs concentrations. RMSE% values (Eq. 2) were calculated for each statistically significant relationship of Table 5. RMSE% values varied from about 2% to 21% (Fig. 8). The HL-regression model generally produced RMSE% values higher than those resulting from the LL- and AS-regression models, except for *P. tricornutum*. MPs concentrations predicted from



**Fig. 8.** RMSE% computed between the logarithm of MP concentrations estimated by LL, HL (ML for *Prochlorococcus* sp.), and AS regressions (Fig. 7) and those obtained from regressions in Fig. 6. MP concentrations were retrieved for a range of SI values representative of each species (see text). RMSE% values were calculated only for statistically significant regressions of Fig. 7 (see also Table 5).

AS-regression models were generally the lowest and ranged from 3% to 12% (Fig. 8). These results evidenced that the error in quantifying the abundance of different marker pigments representative of different taxonomic groups was generally low and slightly affected by changes in light growth conditions. In particular, these investigations showed that the average spectrum of three light conditions (AS spectrum) could actually reduce the error in quantifying the abundance of a given species within assemblages characterized by a mixed taxonomic composition.

#### 4. CONCLUSIONS

Following the recommendations of the international community of phytoplankton functional type algorithm developers [12,43,44], two experiments on marine algal cultures representing different taxonomic groups were dedicated to investigate the extent to which the plasticity and/or similarity of spectral light absorption coefficients may affect the accuracy in optically detecting phytoplankton taxonomic composition. In particular, the datasets of pigments and light absorption spectra provided by the two presented experiments were exploited to specifically assess (i) what is the effect of light-driven spectral modifications in the accuracy of phytoplankton taxonomic composition retrievals by light absorption coefficients, and (ii) how many phytoplankton groups can be discriminated from the bulk spectral light absorption properties of marine algal communities characterized by mixed taxonomic composition. The presented experiments were not intended for any algorithm development and/or validation.

Results of the two experiments showed encouraging directions to follow for improving current spectral absorption-based algorithms and/or exploring new approaches for the retrieval of multiple phytoplankton groups. In particular,

- The spectral signature of a given species substantially influences the bulk phytoplankton light absorption spectrum of the assemblage. Spectral signatures of five taxonomically different groups can be extracted and used for quantifying their relative contributions in terms of TChl *a* and marker pigment concentrations.

- Intraspecific plasticity of phytoplankton light absorption spectra due to changes in light conditions does not significantly affect optical classification and discrimination of five phytoplankton groups from assemblages with mixed taxonomic composition (RMSE < 21%).

- The use of a reference spectrum coming from the average of various light regimes actually reduces the error in quantifying the abundance of a given species from bulk light absorption properties of mixed assemblages (RMSE < 12%).

- The cyanobacteria, *Synechococcus* sp. and *Prochlorococcus* sp., can be discriminated as two separated groups within the same assemblage.

The analysis of the experiments also highlighted some limitations that might be taken in account when new algorithm development is planned and/or retrieval accuracy of the current approaches has to be evaluated. In particular,

- All light absorption spectra of the examined algal groups share some level of similarity in term of shape, which limits the accuracy of retrievals.

• The high spectral similarity observed between diatoms and dinoflagellates further reduces their discrimination capability when co-occurring within the same assemblage.

• Contributions <20% of a given group to TChl *a* within a mixed assemblage are hard to detect.

• Detection of the full dominance (i.e., 100%) of a given group using phytoplankton light absorption spectra is also affected by errors, which vary according to the group.

The analyses here presented are only the first step to understand the limits and to untangle the effects of growth light (photoacclimation/adaptation) in the detection of phytoplankton groups from bulk light absorption properties of assemblages with mixed taxonomic composition such those characterizing most oceanic environments. We acknowledge that there are some limitations to working with cultures and differences from natural populations (in terms of proportions among groups, total chlorophyll concentration of assemblages, and nutrient/light availability), but cultures represent the best way to individually assess the role of environmental factors acting in natural systems and the detection limits for a given algal group. A comparison of pigment distribution and bio-optical properties between simulated and natural algal assemblages suggested, however, that considerations resulting from these experiments could be extended also to open ocean waters and thus be relevant for improving methods of detection of phytoplankton from *in situ* and remote sensing platforms and for ecological and biogeochemical studies (e.g., primary production modeling [88]). It appears clear, however, that other aspects should be studied in depth in order to better simulate environmental conditions such as the analysis of the synergic effects of nutrient depletion and light limitation in modifying the spectral absorption coefficients and/or adding complexity to simulated taxonomic structures in terms of number of species and taxa. It is also envisaged to perform such experiments and analyses for spectral light backscattering coefficients in order to provide dedicated phytoplankton functional type algorithms [12,13] with similar information and to complement and/or enhance light absorption discrimination capabilities. Finally, since a hyperspectral resolution of ocean color sensors is planned for scheduled satellite missions [89], further efforts should be directed also to the investigation of the minimal spectral resolution required for achieving a comprehensive taxonomic knowledge of the phytoplankton community structure, in addition to specific groups [90], and at the same time make use of the technological and measurement maturity of hyperspectral sensors.

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