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In-vivo single-cell fluorescence and the size-scaling of phytoplankton chlorophyll content

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Running title: *The size-scaling of phytoplankton chlorophyll content*

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Abstract

In unicellular phytoplankton, the size scaling exponent of chlorophyll content per cell decreases with increasing light limitation. Empirical studies have explored this allometry combining data from several species using average values of pigment content and cell size for each species. The resulting allometry, includes thus phylogenetic and size scaling effects. The possibility of measuring single-cell fluorescence with imaging-in-flow cytometry devices allows the study of the size scaling of chlorophyll content at both the inter and intra-specific levels. In this work, the changing allometry of chlorophyll content was estimated for the first time for single phytoplankton populations using data from a series of incubations with monocultures exposed to different light levels. Inter-specifically, our experiments confirm previous modelling and experimental results of increasing size scaling exponents with increasing irradiance. A similar pattern was observed intra-specifically but with a larger variability in size scaling exponents. Our results show that size-based processes and geometrical approaches explain variations in chlorophyll content. We also show that the single cell fluorescence measurements provided by imaging-in-flow devices can be applied to field samples to understand the changes in the size dependence of chlorophyll content in response to environmental variables affecting primary production.

Importance

The chlorophyll concentrations in phytoplankton register physiological adjustments in cellular pigmentation arising mainly from changes in light conditions. The extent of these adjustments is constrained by the size of the phytoplankton cells, even within single populations. Hence, variations in community chlorophyll derived from photoacclimation are also dependent on the phytoplankton size distribution.

Introduction

The growth rate of phytoplankton cells in the oceans is limited by resource availability, such as light and nutrients (1). In order to maximize the efficiency of resource acquisition in a variable environment, cell physiology adjusts through a suite of acclimation processes. Photoacclimation refers to the phenotypic adjustments of the cells in response to variation in irradiance levels and it is typically reflected in a graded reduction in the photosynthetic pigment content with increasing irradiance (2). The mechanistic basis for the pigment content-irradiance relationship is well known (3, 4) and leads to altered cellular pigment composition.

Because all phototrophic plankton contain chlorophyll a (Chl-*a*) as light harvesting pigment, it is arguably the best known and most widely used proxy for autotrophic biomass (5). However, changes in the ratio of Chl-*a* to carbon biomass indicate an adjustment of cellular pigment levels to match the demands for photosynthesis (6) and the concentration of Chl-*a* is thus a biased estimator of phytoplankton biomass expressed in organic carbon terms (1). Instead, the intra-cellular Chl-*a* concentration can give a vision of the physiological status of the cells which can be translated to the community level.

Phytoplankton cells adjust the Chl-*a* to carbon biomass ratio (Chl-*a*:C) in response to an imbalance between the rate of light absorption and the energy demands for photosynthesis and biosynthesis (7). Hence, Chl-*a*:C does not only varies in response to changes in light, but also in growth rate (6). A reduction in growth rate increases Chl-*a*:C, and growth rate explained 39% of Chl-*a*:C variability (8). Nutrient availability also influences Chl-*a*:C (9) since chlorophyll synthesis is a function of the nitrogen status (10). Regarding taxonomy, the average Chl-*a*:C varies for different taxonomical groups of phytoplankton (11).

Light utilization traits, such as pigment content, are thought to be explained in part by cell size (12, 13). When resources are limiting, the cell surface area to volume ratio imposes

fundamental constrains on the rates of resource acquisition (14). The 'package' effect is defined as a reduction in the absorption of pigmented particles within a cell relative to the absorption of the same pigments in solution. The explanation is purely geometrical: as cell volume increases internal optical pathlengths increase, limiting the absorptive efficiency of Chl-a molecules through self-shading, and, as a result, larger cells tend to have relatively lower intracellular concentrations of Chl-a than smaller cells to limit shading effects due to the packaging of pigments (13, 15). This translates into a size dependence of Chl-*a* content that has been reported in laboratory experiments (16) and field studies (17, 18). Additionally, cell size can vary with irradiance and the phase of cellular cycle (19), hence, changes in pigment content can be due to changes in size and not only to photoacclimation processes. It is, then, crucial considering the cellular size when dealing with changes in the elemental composition of phytoplankton.

The size dependence of photoacclimation response results in different allometric exponents in the size-scaling of Chl-*a* content with changing irradiance (15). The photosynthetic response to varying irradiance was described theoretically as a function of cell size (V), considering an optimal light-harvesting strategy (15), and calculating the intracellular pigment concentration required to maximize photosynthesis for a given cell size and a given irradiance. The model predicted that the optimal chlorophyll concentration is proportional to $V^{3/4}$ under light saturation and to $V^{2/3}$ under light limitation. The prediction of different allometric exponents was tested using data from different species cultured in the lab (20), which does not permit exclusion of the variability due to taxonomical composition. The calculation of allometric exponents on a single cell basis would allow testing this prediction in single populations. Hence, the same species evaluated along its whole size range would allow to observe the size-dependence of chlorophyll content without the effect of inter-specific variability.

The cell-level perspective provided by flow cytometry and the capacity to measure large numbers of cells has been used extensively by biological oceanographers to define the distributions and dynamics of marine pico-phytoplankton. Flow cytometry techniques allowed the quantification of optical properties, such as Chl-*a* auto-fluorescence, on individual cells (21). Chl-*a* absorbs energy from light and releases it through several phenomena, being one of them the emission of fluorescence in the red portion of the spectrum, thus, the fluorescence of Chl-*a* reflects the endogenous concentration of this pigment. This is the rationale for the estimation of Chl-*a in situ* by means of fluorometers (22) or for the measurement on discrete samples by means of fluorometric methods (23). Chl-*a* fluorescence intensity quantified by flow cytometry has been shown to scale with cellular Chl-*a* levels in nano-phytoplankton (24), but the relationships reported are not constant for all taxa due to differences in intracellular pigment structure or, even for the same taxa, due to differences in growth conditions. Hence, none of these studies provide a taxon-independent conversion from fluorescence to Chl-*a* that can be applied to natural samples.

In addition, traditional flow cytometers have a limited capacity to analyze large-sized phytoplankton (>5 µm). There are no measurements of fluorescence on a single cell basis for micro-plankton, a compartment where a significant proportion of the autotrophic activity takes place. The Flow Cytometer And Microscope (FlowCAM) is an automated technique for plankton enumeration that combines flow cytometry and microscopy (25). Although the FlowCAM is not a flow cytometer *per se*, it contains the needed elements to measure the fluorescent response of single cells: a source of light, a fluidics system in which the cells are embedded and a detection sensor. When a suspension of phototrophic cells runs through the fluidics system, the detected fluorescence signals trigger the digital camera to obtain images that allows counting and sizing the cells in the sample. However, the fluorescence signals

114 measured by FlowCAM have not been yet interpreted further than determining cell viability
115 (26).

116 Here, we present a reliable methodology for the estimation of the content of Chl-*a* on
117 single cells that allows the exploration of the changes in the size scaling of Chl-*a* content
118 when cells are exposed to light limitation and that can be applied to field studies. Seven
119 species of marine phytoplankton where grown over a range of irradiances and non-limiting
120 nutrient conditions, and their content in pigments and fluorescence monitored to describe the
121 relationship between the FlowCAM measured fluorescence per cell and the content of Chl-*a*
122 per cell. With those cultured species we explored the changes in the inter-specific and intra-
123 specific size scaling of Chl-*a* content. Finally, the size scaling of Chl-*a* content in field
124 samples was explored and related to the irradiance levels in the water column. Our results
125 showed that the allometry of the content of Chl-*a* changed with light intensity. This
126 physiological responses and the size-dependence of Chl-*a* content can be monitored in field
127 samples thanks to the measurement of Chl-*a* content on a single cell basis with imaging-in-
128 flow devices.

Materials and Methods

Phytoplankton cultures and growth conditions

We performed a series of incubation experiments to induce photoacclimation in cultured phytoplankton, exposing mono-specific suspensions of cells to different light intensities in order to obtain a gradient of intracellular Chl-*a* concentration. Seven species were used: *Isochrysis galbana*, *Emiliania huxleyi*, *Rhodomonas salina*, *Prorocentrum micans*, *Karlodinium veneficum* (*micrum*), *Alexandrium tamarense* and *Protoceratium reticulatum*. The inocula were maintained in exponential growth in F2 media at 15 °C in a culture chamber with photoperiod 12L:12D and low light conditions. With those initial cultures we conducted 12 experiments (Table I).

Each experiment took place in a linear incubator illuminated at one end by a spot light. The incubator was divided into compartments along its axis by means of transverse partitions consisting on a double layer of nylon mesh. The initial culture was divided in 50 mL cellstack culture chambers (Corning), and the chambers placed into the compartments of the incubator. Total irradiance (photosynthetically active radiation, PAR) in each compartment was measured with a LICOR radiometer (Biospherical). The irradiances varied slightly between experiments but in any one experiment light levels ranged between 37 and 1838 µmol photons m^{-2} s^{-1} of PAR. Running water through the incubator maintained the temperature stable at 15.5 ± 0.5 °C. Incubations were kept with constant photoperiod during 6 days in most cases to allow photoacclimation. Samples for analysis were taken always at 9:00AM, two hours after the light was activated. The culture chambers were shaken manually twice a day.

Experiments 1 to 4 were run by duplicate, in parallel incubator lines; samples for microscopy counts, estimation of Chl a concentration and *in vivo* FlowCAM analysis were taken on the last day of each incubation experiment. Experiments 5 to 12 were run in a single

153 incubator line; samples were taken on the first and last days of each incubation experiment,
154 and additionally in the mid-term of experiments 8 and 11 (Table I).

155 Microscopy counts were carried out placing 1 mL of sample in a Sedgwick-Rafter
156 counting cell slide under a NIKON inverted microscope. For the analysis of Chl-*a*
157 concentration, 10mL of each sample were filtered onto Whatman GF/F filters, frozen at -18
158 °C during 24h and extracted in 3 mL of acetone during 24h. Chl-*a* a concentration was
159 determined using a spectrofluorometer (Perkin Elmer LB-50s) with excitation set at 488 nm
160 and emission at 663.5 nm. The signal of the spectrofluorometer was calibrated against Chl-*a*
161 solutions of known concentration. The concentration of extracted Chl-*a* was divided by the
162 number of cells to obtain the Chl-*a* per cell value, and by the total cellular volume to obtain
163 the average intracellular Chl-*a* concentration.

164 *In vivo* FlowCAM analysis provided measurements of cell concentration and of single
165 cell fluorescence and size. Excitation illumination consisted of a blue laser fan of 488 ± 0.04
166 nm, and fluorescence was measured as the emitted light passing a 650 nm long-pass filter
167 reaching a photomultiplier tube (PMT 1). When the fluorescent light reaches the PMT
168 generates a voltage pulse that is described numerically by three parameters: the maximum
169 value reached by the pulse, known as height or peak, the number of consecutive
170 measurements that exceeded the threshold value, called width, and the integrated area under
171 the pulse. The width and the area of the pulse are indicative of particle transit time and hence
172 strongly influenced by the flow speed of sample analysis and the size of the cell being
173 detected (27), while the peak of the signal is expected to reflect the total fluorescence of the
174 cell and therefore it was the parameter chosen to estimate the intra-cellular Chl-*a* content.

175 **Field sampling**

176 Field data consisted of 20 samples taken from 12th to 14th April of 2013 in coastal, shelf
177 and oceanic waters of the Cantabrian Sea (Southern Bay of Biscay) (43.51-43.80°N, 5.55-
178 3.73°W, 25-1625 m bathymetry). Sea-water samples were collected at selected depths

179 (ranging from 3 to 100 meters) with Niskin bottles mounted on a rosette sampler system
180 equipped with a CTD (conductivity-temperature-depth) probe (SBE911), and auxiliary sensor
181 for the measurement of underwater PAR (Biospherical/Licor radiometer). Surface PAR on an
182 hourly basis was recorded on a meteorological station sited on land near the sampling area
183 (43.54°N, 5.62°W, at 30 meters above mean sea level).

184 From the in situ PAR profile, the attenuation coefficient (k_d) was estimated with the
185 light extinction equation,

$$I_z = I_0 \times e^{(-z \times k_d)} \qquad \text{Eq. 1}$$

186 where I_z and I_0 represent, respectively, irradiance at a given depth and in the surface, and z is
187 the depth in the water column. To integrate the irradiance regime experienced by the cells of a
188 given sample the field irradiance at the sample depth was estimated taking into account the 24
189 hours PAR regime previous to the time of acquisition of the sample (only light hours),
190 calculated from surface solar radiation and assuming a constant k_d, and weighting the
191 calculated underwater PAR by the elapsed time. Field irradiances ranged from 1 to 1604 µmol
192 photons m^{-2} s^{-1}.

193 Phytoplankton samples were analyzed on board with FlowCAM. They were maintained
194 fresh and in the dark until analysis, which started immediately after collection to minimize
195 pigment degradation. Each sample was split in two: an un-concentrated subsample pre-filtered
196 by 40 µm for analysis with 200× magnification and 50 µm flow chamber (1 mL), and a
197 concentrated subsample pre-filtered by 100 µm (1 liter down to around 20 mL) for analysis
198 with 100× magnification and 100 µm flow chamber (10 mL). The sample concentration was
199 carried out by reverse filtration (28) through a 15 µm mesh to prevent the damage of living
200 cells. A detailed description of the protocol for FlowCAM sample analysis can be consulted
201 elsewhere (29).

202 **Data analysis**

203 For each sample, either cultured or natural, abundance of autotrophic cells was
204 estimated from all cells imaged by the FlowCAM in the fluorescence-triggered mode. The
205 size of the cells was estimated considering only properly focused and uncut single cells. The
206 spherical equivalent diameter (ESD) and geometrical section of each cell were obtained
207 directly from the digital image taken by the FlowCAM, and particle biovolume was calculated
208 as a revolution volume from the ESD of the particles. Fluorescence signals were selected
209 considering only uncut cells captured in the proximities of the laser beam. FlowCAM
210 photographs with more than one particle were rejected.

211 Experiments 1 to 4 were used to explore the variability of fluorescence measurements,
212 in parallel lines of the incubator (experiments 1-4) and in replicate analysis of the same
213 sample (experiment 1). Experiments 5 to 12 were used to obtain a fluorescence to Chl-*a*
214 conversion and to explore the inter- and intra-specific scaling of chlorophyll content. We
215 categorized the day of sample extraction according to the growth phase of the culture, as
216 being in exponential phase or not, by plotting the evolution of carbon biomass. From the
217 initial carbon content (C_0 at time t_0) to the content in the last day of sample extraction (C_t at
218 time t) we estimated the specific growth rate (μ):

$$C_t = C_0 \times e^{\mu(t-t_0)} \qquad \text{Eq. 2}$$

219 and the number of generations (g) experienced by the population from the initial sample.

$$g = log_2(C_t/C_0) \qquad \text{Eq. 3}$$

220 To check whether the photoacclimation has being completed at the end of the incubations, we
221 compared the carbon and chlorophyll cell content between the mid-term and last sample day
222 of experiments 8 and 11 (days 4 and 6).

223 # Results

224 ## Variability of the fluorescence signal

225 Experiments 1 to 4 took place each in two parallel incubator lines, being the treatments
226 analyzed by duplicate (line replicate). Additionally, in experiment 1 samples of the first line
227 were analyzed by duplicate (aliquot replicate). These results were used to account for the
228 variability of the fluorescence signal detected by FlowCAM. Fluorescent beads analyzed each
229 sampling day (between 64 and 768 items) presented average values of 181 ± 14 fluorescence
230 units with the 20x lens and of 41 ± 19 units with the 10x lens. The variability between average
231 values in parallel incubations (Fig. 1A) was ±38 units of fluorescence per cell (95% c.i.),
232 whereas the variability between aliquots of the same incubation line (Fig. 1B) was of ±4 units
233 of fluorescence per cell. The standard deviation within each FlowCAM run was in all cases
234 higher than differences in average values between experimental (line or aliquot) replicates.
235 This indicates that the fluorescence signal was constant enough to compare different
236 experiments or treatments (inter-specific variation) and sensitive enough to distinguish
237 differences among cells of the same population (intra-specific variation).

238 ## Phytoplankton incubations

239 Experiments 5 to 12 were used to obtain a fluorescence to Chl-*a* conversion and to
240 explore the inter- and intra-specific scaling of chlorophyll content. Although the number of
241 generations experienced in each experiment since the beginning to the last day was variable
242 (Table II), the experiments sampled also in the mid-term (8 and 11) twice confirmed that the
243 relative composition of the cells kept constant for most of the light treatments from the the
244 mid-term sample to that at the end of the incubation, indicating that the cells were already
245 acclimated to culture conditions (Fig. 2).

246 The average fluorescence per unit volume and the average intracellular Chl-*a*
247 concentration in the last day of incubation varied as a function of growth irradiance (Fig. 3) in
248 all experiments. The pigment concentration obtained through Chl-*a* extraction decreased as
249 expected with increased irradiance and the same pattern was captured by the FlowCAM-
250 measured fluorescence: fluorescence intensity decreased with irradiance in parallel to intra-
251 cellular pigment content. The only exception was experiment 11, which showed higher
252 fluorescence at intermediate growth irradiances, but maintaining the general decreasing
253 pattern for higher values.

254 **Fluorescence to Chl-a conversion**

255 To explore the relationship between the fluorescence measured by the FlowCAM and
256 the Chl-*a* content, we analyzed the relationship between the average (peak) fluorescence per
257 cell (arbitrary units $cell^{-1}$) and the average intracellular pigment content per cell (pg Chl-*a*
258 $cell^{-1}$) in the last day of the incubation experiments (Fig. 4). Since the fluorescence signals
259 measured with different combinations of FlowCAM magnification lenses / flow chamber
260 dimensions (FC) were not comparable due to optical differences, the relationship was
261 explored for each of the applied combinations (200x/FC50 and 100x/FC100).

262 A priori, a linear relation would be expected between fluorescence and the absorption
263 cross section of single cells (30), which implies a nonlinear relationship with the Chl-*a*
264 content given the package effect. The package effect entails that larger cells can expose
265 relatively less pigments for light harvesting than smaller cells due to constraints imposed by
266 the relationship between cell surface area and volume (3, 4, 31). The best fit (lowest Akaike
267 score) between fluorescence and Chl-*a* per cell was given by a curvilinear model. We
268 performed a linear regression over the log-log transformed data to adjust an exponential curve
269 to the experimental data obtained with the 200×/FC50 ($F = 75.46 \times \text{Chl-}a^{0.4}$, $R^2 = 0.85$, p-

value<0.001, Fig. 4A) and the 100×/FC100 ($F = 40.63 \times \text{Chl-}a^{0.28}$, $R^2 = 0.91$, p-value<0.001, Fig. 4B).

Inter-specific scaling of chlorophyll content

For each irradiance level, we combined the data for the different species and explored the relationship between Chl-*a* content and cell biovolume. Figure 5 shows both the analytical Chl-*a* estimates (upper panels) and the estimates derived from FlowCAM's fluorescence using the fluorescence to Chl-*a* conversions described above (lower panels), as a function of body size for the different irradiance treatments. A reduced major axis regression on the log-transformed data was used to estimate the allometric size-scaling exponent at each irradiance level (Fig. 5). The size-scaling exponents were estimated additionally (dashed lines) only with experiments that kept growing in exponential phase as indicated in Table II. The size-scaling exponents of Chl-*a* content increased with irradiance from 0.65 to 0.83 (Fig. 5A-E) which translates in the decrease of the size scaling exponent with increasing light limitation (Fig. 6A). Similarly, the pattern of decrease of the size scaling exponent in light limited conditions was captured by the FlowCAM-measured fluorescence (Fig. 6B). Considering this Chl-*a* predicted from fluorescence, the exponents of the size dependence of pigment content at each irradiance level were slightly higher, varying between 0.77 and 0.90 (Fig. 5F-J).

Intra-specific scaling of chlorophyll content

The measurement of fluorescence on a single-cell basis allowed us to explore the size dependence of Chl-*a* content within single populations. To analyze the size dependence of Chl-*a* content as a function of irradiance within each treatment, the fluorescence of each single cell was converted to Chl-*a* content and plotted against cellular biovolume in a log-log scale. As an example, the five irradiance treatments in experiment 9 (*K. micrum*) appear in

293 Figure 7 (increasing irradiance from panel A to E). The distributions of biovolume and Chl-*a*
294 per cell were log-normal, as indicated by the bar plots in the x and y axis. The dispersion of
295 the data and the presence of outliers are more evident at high irradiance levels. The size
296 scaling exponent was obtained using robust linear regression, an alternative to least squares
297 regression less sensitive to outliers (32), and tested for statistical significance.

298 Considering a single species, the size scaling exponent tended to decrease in light
299 limited conditions, similarly to what happens at the inter-specific level. Figure 8 shows the
300 variation of the size-scaling exponents that resulted significant (p-value<0.05) as a function of
301 irradiance for each of the eight experiments. To visualize the trend, we adjusted linear and
302 logarithmic models to the data and plotted the 95% confidence interval of the model with the
303 lowest Akaike score. All experiments except 5 and 10 showed a pattern of increase of the
304 size-scaling exponent at increased growth irradiance levels. Experiment 5 did not show this
305 pattern, probably due to the small size of *I. galbana*, very close to the detection limit of the
306 FlowCAM.

307 The intra-specific scaling exponents were not limited to 1, as found at the inter-specific
308 scale, but fluctuated between 0.35 and 3.04 with variability within each experiment ranging
309 between 0.1 and 1.52 units. Despite the reduced biovolume range within a single species, the
310 relationship with body size was significant in most cases (in 41 of 42 light treatments, p-
311 value<0.05) and body size explained between 3 and 28% of the variability in the Chl-*a*
312 content.

313 Field sampling

314 The size scaling of Chl-*a* content was reproduced in the natural samples analyzed. The
315 fluorescence values in the two subsamples analyzed for each sample were converted to Chl-*a*
316 content and combined to estimate a size scaling exponent for each sample. The exponents of

317 the size dependence were estimated by robust linear regression and test for significance (p-
318 value<0.05). Figure 9A shows an example of a natural sample, with cells imaged with the
319 200×/FC50 (light dots) and with the 100×/F100 (dark dots), and the linear fit. Among all
320 samples, the size scaling exponent ranged from 0.5 to 1 (Fig. 9B) and body size explained
321 between 27 to 59% of the variability in the predicted Chl-*a* content. In natural samples, the
322 influence of other environmental variables, such as temperature and nutrient concentrations,
323 and the physiological (ontogenetic) status of the cells (exponential growth, stationary or
324 senescence phases) resulted in a poor correlation between the size-scaling exponent and field
325 irradiance.

326 **Discussion**

327 The existence of a relationship between the fluorescence measured by flow cytometers
328 and the Chl-*a* per cell has been described before (24) but, for the best of our knowledge, this
329 is the first time that this relationship is explored using FlowCAM and extended to
330 microphytoplankton. The intracellular concentration of Chl-*a* has been estimated for the
331 whole phytoplankton community or for specific compartments (33, 34), but we have shown
332 that the simultaneous acquisition of estimates of cell size, cell abundance and fluorescence per
333 cell by means of FlowCAM offers an integrated methodology to estimate intracellular
334 concentration of Chl-*a* of nano- and micro-phytoplankton on an individual, single cell basis,
335 allowing the characterization of the size dependence of Chl-*a* content in natural samples.

336 Although useful as a proxy for Chl-*a* content, there are limitations when estimating Chl-
337 *a* from fluorescence, given the presence of other energy dissipating processes and also the
338 variability of the fluorescence quantum yield itself. There are diverse processes competing
339 with fluorescence to de-excite the molecule of Chl-*a*, such as internal conversion, resonance
340 energy transfer, quenching and bleaching (35, 36), that may change the energy exchange rate,
341 making the fluorescence signal no longer proportional to the amount of pigment. Through
342 photochemical quenching, the liberation rate of electrons from photosystems increases due to
343 enzymatic activity, and through non-photochemical quenching the heat dissipation is
344 increased. Whereas photochemical quenching can be relevant in solar-stimulated Chl-*a*
345 fluorescence, it is negligible in the laser-stimulated fluorescence, provided the flash is of high
346 intensity and short length (37). It is not possible to inhibit heat dissipation totally but it can be
347 minimized if the measurements are taken on dark adapted, non-stressed cells (38).

348 On the other hand, the quantum yield for chlorophyll fluorescence is not constant across
349 species, neither across physiological conditions. There are variations in the emission of
350 fluorescence of Chl-*a* under constant light conditions, which can indicate a vital cycle in Chl-

351 *a* synthesis (39). When performing a calibration, this problem is common to all fluorimetric
352 methods for chlorophyll estimation, and reference values already account for variations in
353 quantum yield. In our experiments, spectrofluorimetry-determined Chl-a and FlowCAM-
354 determined fluorescence suffered the same bias and hence, we considered both approaches
355 comparable. A different scenario occurs when the fluorescence measurement is applied to
356 new samples without reference values. Since different environmental conditions, such as
357 nutrient availability or temperature, may lead to widely different quantum yield of
358 chlorophyll, it is a matter of debate common to all fluorometric methods that estimate
359 chlorophyll from *in situ* fluorescence to what extent a calibration made with a limited set of
360 samples can be extrapolated to other samples acquired for a different range of environmental
361 conditions.

362 Beyond overall limitations, we consider that the extrapolation to natural samples of the
363 fluorescence to Chl-*a* conversion we have obtained is reasonably because our relationship has
364 been estimated combining different species and different growth conditions. Previous
365 correlations between fluorescence and Chl-*a* in single cells (24) were species specific, which
366 prevented its use it in a routine basis for the estimation of Chl-*a* content for the whole
367 phytoplankton community. We found a curvilinear correlation between fluorescence and Chl-
368 *a* content that resulted taxon independent and, if not independent of growth status, at least it
369 encompassed the variability in pigment content due to growth rate.

370 **Inter-specific size dependence of chlorophyll content**

371 A taxon independent conversion allowed us to estimate the Chl-*a* content in different
372 cultures covering an ample size range, and hence, enabling the analysis of the size scaling of
373 chlorophyll content. Larger cells tend to have lower cellular pigment concentrations than
374 smaller cells under similar environmental conditions (40), to counteract the package effect

375 associated with increasing cell size (15). This means that Chl-*a* content per cell scales with
376 size with exponents lower than one, which is supported by geometrical considerations (3, 4).
377 This theoretical predictions were supported by empirical data (16, 20) and consistent with our
378 results based on fluorescence-based Chl-*a* estimates.

379 As growth irradiance decreased, the exponent of the size-scaling of Chl-*a* content
380 decreased, as described by previous empirical works (20). All cells incremented their pigment
381 content but, relative to body size, the increment of pigment was larger in small cells than in
382 larger cells. Theoretical models have predicted an upper limit for the exponent of ¾ under
383 saturating irradiance (15), but our exponents surpass this predicted value.

384 We are aware, however, that the results derived from the inter-specific analyses need to
385 be taken with caution for two reasons. The first one is that in order to get cells of different
386 sizes it was necessary to combine different species. Changes in light harvesting characteristics
387 that are correlated with cell size can alter the size scaling of chlorophyll content. For example,
388 a systematic shift in cell shape can reduce the package effect and mitigate the potential
389 reduction in the size-scaling exponent of cellular pigment concentration and growth rates
390 (15). The second caveat is that the growth status of the combined cultures must be the same,
391 e.g. exponential growth. When estimating the exponent of the size-scaling of Chl-a content
392 only with cultures in exponential growth, the number of data are reduced and the exponents
393 differed from previous described (Fig. 6A). So the relevance of the size scaling exponents
394 obtained combining all cultures could be obscured by the fact that not all populations where
395 in the same growth phase.

396 It is crucial to compare organisms with similar pigment composition under similar
397 growth conditions when calculating and comparing the size-scaling exponent of cellular
398 pigment concentration. The estimation of the allometric exponents on single populations

399 (thanks to the single cell approach) avoids the interspecific variability, but also covers a size
400 range where all cells are in the same growth regime.

401 **Intra-specific size dependence of chlorophyll content**

402 At the intra-specific scale, the variability of fluorescence values, and hence Chl-*a*
403 content, was higher than that for size. Size only explained a comparatively small part of the
404 variability of intracellular Chl-*a* content because of the narrow biovolume range covered, but
405 also due to phenotypic (and to some extent also genotypic) variability among the individuals
406 of the population and differences in the phase of the cellular cycle and physiological status.
407 Nevertheless, the same pattern of decrease of the size scaling exponent of Chl-*a* content per
408 cell in light limited conditions was found when focusing on a single species, which means
409 that, for a generalized increment in pigment content in light limited conditions, the smaller
410 cells had a larger margin for pigment accumulation.

411 This is the first time this fact is observed in single populations and it means that not
412 only smaller species maintain higher photosynthetic rates under light limitation (41) but also
413 smaller cells within the same population. The optical absorption cross section of pigments and
414 the intra-cellular concentration of components that capture photon energy are physiological
415 traits that underlie growth/production traits (42). Consequently, different cell sizes limited in
416 the range of their plasticity for pigment content results in intra-specific differences in the
417 integrated growth response to irradiance. Thus, the variation in the size scaling of Chl-*a*
418 content also predicts shifts in the size scaling of growth and photosynthesis with the light
419 regime. All phytoplankton require light for growth and the variable light regimes experienced
420 by the cells and imposed by water column mixing may explain the size-structure of the
421 phytoplankton community (43, 44). As a result, the differences in pigment content as a
422 function of cell size lead to different physiological strategies and niche partitioning.

423 We have found that the size-scaling of Chl-*a* content per cell of a single population has
424 exponents higher than one for some species, which means that larger cells have higher Chl-*a*
425 content per unit volume than smaller cells. This result can be counter-intuitive. It may be
426 explained, however, if the difference in cell size and pigment content within the population is
427 due, to great extent, to the phase of the cellular cycle experienced by the cells. In unicellular
428 phytoplankton, within a given population, small cells are generated from larger cells, so, cell
429 division may be important reducing the package effect for young daughter cells (39). A large
430 cell with a given Chl-*a* concentration that enters the division phase of the cellular cycle will
431 give place to two smaller cells with half the Chl-*a* content, which is lower than the potential
432 concentration that their new surface-to-volume ratio may allow. In this case, the concentration
433 of Chl-*a* will be independent of cell size and the pigment content per cell will scale with size
434 with an exponent around one. On the other extreme, a large cell can increase its pigment
435 content above the limits imposed by its surface-to-volume ratio in order to divide this pigment
436 between the two daughter cells, as it happens in coccolithophorids (45).

437 For the smallest species, other biovolume constrains can be considered. Very small cells
438 might be prevented from increasing their scalable components (photosynthetic units) due to
439 the necessity of maintaining a constant quota of non-scalable essential components, such as
440 the gene pool, within a very small cell biovolume (46). In this case, only those cells above a
441 critical size will be able to increase their intracellular quota of molecules involved in
442 metabolic processes.

443 Another source of uncertainty is the one related with the variability in the cellular
444 content of other pigments. Phytoplankton cells might be able to respond to changing light
445 levels through the production of other pigments, such as accessory light-harvesting pigments
446 or photo-protective pigments (47). The single-cell fluorescence method accounted for the
447 specific fluorescent signature of a given pigment, which permitted to distinguish the patterns

448 found for different pigments (see Supplementary material for an analysis of phycoerythrin
449 content in *R. salina* experiments). The pattern in the size dependence of the content in a
450 photo-protective pigment is expected to follow the opposite pattern of a light-harvesting
451 pigment, increasing the intracellular concentration and decreasing the size scaling exponent at
452 light saturated conditions.

453 ***In situ* size dependence of chlorophyll content**

454 Our approach, the analysis of single cells, is the unique choice to apply the same
455 methodology to field samples and explore the size dependence of Chl-*a* content *in situ* under
456 different environmental conditions. It also opens the door to the estimation of size-scaling per
457 functional or taxonomic group. In this work, the natural samples yielded exponents lower than
458 1, in agreement with the results of the inter-specific size-scaling. However, a question that
459 remained unsolved is whether the upper limit of the exponent is more likely to be 1 (17) or ¾
460 (15). To the best of our knowledge, we reported for the first time the size scaling of Chl-*a*
461 content in single cells, and our results showed maximum exponents lower than 1 but higher
462 than ¾, both in natural samples and cultures. An isometric scaling between cell volume and
463 intracellular Chl-*a* has been reported on the basis of size-fractionation of Chl-*a* in natural
464 samples by (17), who argued that the discrepancies between the exponents obtained in natural
465 and in laboratory conditions could be due to the different growth conditions experienced by
466 cultured cells and the small number of species used in laboratory studies.

467 The variability found on the intra-specific size-scaling of Chl-*a* content appeared also in
468 natural samples, since different populations of the phytoplankton community present different
469 physiologies and phases of their respective life cycles. The variation of the size scaling
470 exponent as a function of irradiance was obscured in the field sampling, probably because the
471 measured Chl-*a* content depends also on many other factors (e.g. the nutritional status of the
472 cells) (6). Also, we have cultured only spherical or elliptical cells, but, in natural

communities, cells have developed strategies to escape from geometrical constrains on pigment content, such as the non-spherical shapes of diatoms and the presence of vacuoles (48).

The analysis of natural samples covers a wide range of environmental scenarios, from light or nutrient limitation to saturating conditions, which can help to understand how resource acquisition affect the productivity of natural communities. The size dependence of Chl-*a* content relates directly with the size scaling of photosynthetic rates. This can be determinant in primary productivity models since phytoplankton production can be modelled on a more realistic way improving current estimates derived from global primary production models. State of the art net primary production (NPP) models use carbon biomass instead of Chl-*a* concentration, since variability in intracellular Chl-*a* content from light acclimation and nutrient stress confounds the relationship between Chl-*a* and phytoplankton biomass (49, 50). *In situ* datasets as those obtained with FlowCAM, including cell size and Chl-*a* content, can be useful in the validation of these models.

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624 **Table I. Incubation conditions.** Description of growth conditions of the incubation
625 experiments carried out to obtain a gradient of Chl-*a* content per cell in seven different
626 phytoplankton species.

Experiment	Species	Number of light treatments	Photo-period	Total days	Days of measurements	Lens / flow chamber	Number of points for the calibration
1	*Isochrisis*	5	12/12	4	4	20x/FC50	-
2	*Isochrisis*	5	12/12	6	6	20x/FC50	-
3	*Isochrisis*	5	12/12	1	1	20x/FC50	-
4	*Prorocentrum*	2	12/12	1	1	20x/FC50	-
5	*Isochrisis*	5	12/12	6	1 and 6	20x/FC50	5
6	*Emiliana*	5	12/12	5	1 and 5	20x/FC50	5
7	*Rhodomonas*	5	12/12	6	1 and 6	20x/FC50	5
8	*Rhodomonas*	6	14/10	6	1, 4 and 6	20x/FC50	12
9	*Karlodinium*	5	12/12	6	1 and 6	20x/FC50	5
10	*Alexandrium*	5	12/12	6	1 and 6	10x/FC100	5
11	*Alexandrium*	6	14/10	6	1, 4 and 6	10x/FC100	12
12	*Protoceratium*	5	12/12	6	1 and 6	10x/FC100	5

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Table II. Incubation results. Cell-size, cellular composition (carbon and analytical Chl-a), fluorescence peak, growth rate (μ) and number of generations experienced (g) during the six experiments (Exp. 5 to 12, Table I) gathered to estimate the conversion from fluorescence to intra-cellular Chl-*a*, and the allometry of Chl-*a* content. Note that for experiments 8 and 11, the composition and growth parameters have been estimated not only for the last day of the incubation but also for the mid-term sampling (i.e. day 4). The asterisks in μ values indicate those cultures that kept growing in exponential phase.

Exp	Light [μmol photons m^{-2} s^{-1}]	day	ESD [μm]	C [pg $cell^{-1}$]	Chl-a [pg $cell^{-1}$]	Fluorescence peak ± sd	μ [d^{-1}]	g
5	115	6	4.0	5.6	0.28	45 + 20	0.29*	2.5
5	230	6	4.4	7.1	0.18	41 + 19	0.45*	3.9
5	460	6	4.2	6.4	0.14	37 + 18	0.57*	5.0
5	920	6	4.5	7.3	0.15	30 + 17	0.54*	4.7
5	1839	6	4.7	8.4	0.10	27 + 14	0.57*	5.0
6	115	5	4.5	7.7	0.98	50 + 22	-0.50	-2.3
6	230	5	5.1	10.6	0.86	46 + 28	-0.45	-1.9
6	460	5	5.1	10.4	0.55	56 + 27	-0.43	-1.3
6	920	5	5.0	10.1	0.21	55 + 25	0.00	0.7
6	1839	5	4.4	7.1	0.20	40 + 21	0.37*	1.8
7	115	6	7.3	26.1	1.93	109 + 20	0.38*	3.6
7	230	6	7.8	30.5	2.05	108 + 20	0.66*	5.4
7	460	6	8.2	35.3	1.74	98 + 22	0.74*	6.3
7	920	6	7.8	31.0	1.35	85 + 23	0.91*	6.6
7	1839	6	7.8	30.9	0.47	87 + 25	0.35*	5.9
8	55	4	8.1	34.1	2.40	109 + 29	0.03	0.2
8	92	4	8.6	40.1	2.23	100 + 26	0.15	0.8
8	230	4	8.5	38.0	2.27	83 + 25	0.23	1.3
8	382	4	9.0	44.0	2.20	100 + 27	0.24	1.4
8	828	4	8.7	40.3	1.50	83 + 24	0.39*	2.2
8	1563	4	8.6	39.1	1.46	80 + 26	0.18	1.0
8	55	6	8.4	37.4	3.42	105 + 26	-0.06	0.0
8	92	6	8.4	36.9	3.36	105 + 27	0.01	0.8
8	230	6	8.4	37.2	2.53	105 + 25	0.06	1.4
8	382	6	8.6	39.8	2.03	87 + 26	0.43*	2.7
8	828	6	8.4	36.5	1.86	88 + 25	0.26*	2.9
8	1563	6	9.8	55.3	1.56	99 + 27	0.25*	1.8
9	115	6	10.1	60.5	4.42	168 + 25	0.46*	1.1
9	230	6	10.4	65.7	3.34	154 + 28	0.48*	2.5
9	460	6	10.3	64.9	2.48	133 + 27	0.51*	3.3
9	920	6	10.9	73.4	1.78	115 + 27	0.66*	3.5
9	1839	6	10.2	62.8	1.56	106 + 28	0.61*	3.6
10	115	6	31.2	2018.8	30.57	101 + 39	-0.16	-1.1
10	230	6	35.3	2889.5	31.34	98 + 36	-0.20	-1.1
10	460	6	33.9	2498.7	21.64	104 + 32	-0.18	-0.8
10	920	6	35.2	2836.9	15.51	102 + 29	-0.29	-1.3
10	1839	6	37.1	3286.7	9.89	94 + 27	-0.19	-1.1
11	37	4	23.7	888.2	10.99	73 + 24	-0.19	-1.1
11	69	4	23.4	867.3	7.96	76 + 24	-0.12	-0.7
11	152	4	22.3	754.8	7.42	71 + 26	-0.08	-0.5
11	276	4	21.4	677.8	4.55	65 + 25	0.05	0.3
11	598	4	21.9	720.3	3.66	60 + 26	0.07	0.4
11	1563	4	21.5	684.3	2.53	49 + 23	0.10	0.6
11	37	6	23.5	884.6	8.07	60 + 24	0.04	-0.9
11	69	6	23.4	864.8	7.46	70 + 22	-0.10	-1.0
11	152	6	22.3	756.3	6.33	72 + 24	0.02	-0.4
11	276	6	21.2	654.6	4.94	62 + 26	-0.06	0.1
11	598	6	21.1	642.6	3.71	55 + 25	-0.16	-0.2
11	1563	6	20.8	616.6	2.47	51 + 25	-0.15	0.1
12	115	6	28.7	1621.7	239.82	136 + 31	-0.87	-5.6
12	230	6	30.2	1849.0	53.34	126 + 26	-0.20	-3.7
12	460	6	32.0	2162.3	50.71	119 + 29	0.04	0.4
12	920	6	32.3	2225.8	48.86	109 + 27	-0.19	-0.5
12	1839	6	32.4	2231.7	40.73	111 + 26	-0.10	-1.7

636 **Figure legends**

637

638 **Figure 1.** Variability (mean ± sd) of the fluorescence signal within (**A**) parallel lines of the
639 incubator during experiments 1 to 4 and (**B**) duplicate sample analysis during
640 experiment 1. Dashed lines indicate the 95% confidence interval.

641 **Figure 2.** Relative composition of the cells of (**A**) *Rhodomonas sp.* (exp. 8) and (**B**)
642 *Alexandrium tamarense* (exp. 11) in terms of the Chl-a to carbon ratio (mg g^{-1}) as a
643 function of incubation time in the different light treatments (graded from light to dark
644 grey corresponding from high to low irradiance levels).

645 **Figure 3.** Incubations overview. Fluorescence per biovolume unit (black dots) and
646 intracellular Chl-*a* concentration (white dots) as a function of growth irradiance
647 intensity (µmol photons m^{-2} s^{-1}) in the last day of each experiment.

648 **Figure 4.** Fluorescence to Chl-*a* conversion. Fluorescence peak per cell measured by the
649 FlowCAM as a function of analytic Chl-*a* per cell, for the experiments analyzed with
650 the (**A**) 200×-FC50 and (**B**) 100×-FC100 lens-flow chamber combinations. Solid
651 line, equation and R^2 indicate the fitted conversion from fluorescence peak to Chl-*a*.

652 **Figure 5.** Inter-specific size scaling of Chl-*a* per cell. Size scaling obtained combining the
653 results from experiments 5 to 12 for each of the increasing (**A** to **E**) irradiance
654 treatments for analytical Chl-*a* (each experiment provided a unique Chl-*a* value,
655 hence vertical error bars are not available) and for the increasing (**F** to **J**) irradiance
656 treatments for fluorescence-based Chl-*a* (standard deviation shown in vertical bars).
657 Solid lines include all experiments and dashed line in the upper panels only cultures
658 in exponential growth (Table II).

659 **Figure 6.** Size-scaling exponent of Chl-*a* per cell as a function of growth irradiance, (**A**) for
660 analytical Chl-*a* and (**B**) for fluorescence-based Chl-*a*. Size scaling exponent was

calculated from reduced major axis regression with standard deviation shown in vertical bars. Solid lines include all experiments and dashed line only cultures in exponential growth.

Figure 7. Intra-specific size scaling of fluorescence-based Chl-*a* per cell. Population size scaling in the five increasing (**A** to **E**) irradiance treatments of experiment 9 (*K. micrum*). The size scaling exponent was obtained through robust linear regression, and the slope, R^2 of the relationship and the number of cells counted are shown in each panel. The asterisk in each panel indicates that the relationship was significant (p-value<0.05). Bar plots attached to the x and y axis show the distribution of cell biovolume and predicted Chl-*a* respectively.

Figure 8. Change of the intra-specific size scaling with light. Intra-specific size scaling exponent for fluorescence-based Chl-*a* per cell as a function of growth irradiance for experiments 5 to 12 (Table II). The size scaling exponent was calculated from robust linear regression with standard deviation show in vertical bars. Solid lines indicate the confidence interval of the fitting with the lowest Akaike score, and the asterisk in each panel indicates that the relationship was significant (p-value<0.05).

Figure 9. Application to field sampling. (**A**) Example of the size scaling of fluorescence-based Chl-*a* per cell in a natural sample analyzed with the 200x (light dots) and the 100x magnification (dark dots). The size scaling exponent was obtained through robust linear regression, and the slope and R^2 of the fitting appear within the panel. (**B**) Size scaling exponent of fluorescence-based Chl-*a* per cell in a set of natural samples as a function of 24 hours field irradiance history.