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Ocean acidification affects pigment concentration and photoprotection of marine phytoplankton

Paulo Alcaraz-Rocha,[1*] **Júlia Puig-Fàbregas**,[1] **José Luis Garrido**,[2] **Cristina Sobrino**[1*]

[1]Grupo de Oceanografía Biolóxica, Centro de Investigación Mariña (CIM), Universidade de Vigo (UVigo), Vigo, Spain
[2]Grupo de Fotobiología y Toxinología de Fitoplancton, Instituto de Investigaciones Marinas (IIM), Consejo Superior de Investigaciones Científicas (CSIC), Vigo, Spain

Abstract

Ocean acidification produces significant changes on phytoplankton physiology that can affect their growth and primary production. Among them, a downregulation of the enzymatic activity and the production of different cellular metabolites, including chlorophyll *a* (Chl *a*), has been observed in high CO_2 cultures under stable conditions. However, the extent of how phytoplankton metabolism regulation under high CO_2 conditions affects pigment pools and patterns is unknown. This study shows the effect of the atmospheric CO_2 increase on pigment concentration of three important marine primary producers: *Thalassiosira pseudonana*, *Skeletonema costatum*, and *Emiliania huxleyi*. Cultures grown under saturating photosynthetically active radiation were aerated for at least 3 weeks with current concentrations of atmospheric CO_2 (0.04% CO_2 in air) and with CO_2 concentrations expected for future scenarios of climate change (0.1% CO_2 in air) to assess the effect of CO_2 under acclimated metabolism and stable conditions. Moreover, cultures were also subjected to a perturbation (4 h without aeration) to assess responses under non-stable conditions. The results showed that light harvesting and photoprotective pigment concentrations (i.e., Chl *a*, Chl c_2, ββ-carotene, diadinoxanthin, diatoxanthin, fucoxanthin, among others) decreased significantly under high CO_2 and stable conditions, but the response reversed after the perturbation. The de-epoxidation state of xanthophylls, also showed similar patterns, indicating an increase in phytoplankton sensitivity under high CO_2 and stable conditions. The results demonstrate the relevance of CO_2 concentration and acclimation status for phytoplankton light absorption and photoprotective response. They also identify fucoxanthin and Chl c_2 as suitable biomarkers of phytoplankton carbon metabolism under ocean acidification conditions.

Atmosphere–ocean biogeochemical interactions are important in regulating atmospheric chemistry, and hence climate (Bigg et al. 2003). The link between biology and climate primarily functions through the continuous uptake and release of important atmospheric compounds, such as CO_2. Nowadays, the ocean absorbs around 30% of the annual emissions of anthropogenic CO_2 (Gruber et al. 2019). This increase in the oceanic CO_2 concentrations has produced that the global mean surface-ocean pH decreased by ~ 0.11 units from 1770 to 2000 (Jiang et al. 2019), and by ~ 0.018 units in 70% of ocean biomes between 1991 and 2011(Lauvset et al. 2015). Direct evidence, from the period 2015–2019 already experienced a continued increase in CO_2 emissions (WMO 2019), compared to a previous 5-yr assessment period (2011–2015) (WMO 2019). As a consequence, the models predict that ocean acidification may increase by additional 0.3–0.4 units during the 21st century (Caldeira et al. 2005; Orr et al. 2005).

Ocean CO_2 uptake is controlled by different physicochemical and biological mechanisms. The most important biological mechanism is related to phytoplankton carbon fixation through the photosynthetic processes in surface waters, which is responsible for transforming around 50% of atmospheric CO_2 as phytoplankton biomass (Falkowski 2012). CO_2 used for photosynthesis can diffuse through the cell membrane of most phytoplankton species, while both, CO_2 and HCO_3^- can be incorporated through adenosine triphosphate-dependent

*Correspondence: lalcaraz@uvigo.es; sobrinoc@uvigo.es

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mechanisms called CO_2 concentrating mechanisms (CCMs) (Raven 1997). It is expected that future scenarios of high CO_2 will downregulate the activity of CCMs, increasing the energy available for other cellular processes and resulting in increased carbon fixation and growth rates (Raven et al. 2011).

However, studies analyzing the effect of CO_2 on phytoplankton responses show differences among taxonomic groups and within species (Collins et al. 2006; Rost et al. 2008), with increases in photosynthesis and growth in diatoms and diazotrophs but lack of differences in coccolitophores (Kroeker et al. 2013; Dutkiewicz et al. 2015). The lack of a general trend about phytoplankton response to elevated CO_2 seems to be related to differences in their physiological features, including the different strategies for CCMs (Collins et al. 2006).

Besides CO_2, sunlight is one of the major driving forces for phytoplankton photosynthesis, distribution and community structure in the ocean (Lamont and Barlow 2015). To be able to efficiently use solar radiation and to cope with the changes in light quantity and quality in the natural environment, phytoplankton has evolved taxon specific suites of pigments for light absorption in the visible spectral range of 400–700 nm (Jeffrey et al. 2011; Takaichi 2011). The pigment pool is mainly constituted by two functional categories, namely used for light harvesting and for photoprotection. Chlorophylls trap light energy in the blue and red portions of the electromagnetic spectrum, which are used in photosynthesis. A common feature to all phytoplankton is that they contain chlorophyll *a* (Chl *a*), but there are other chlorophylls, such as Chl *b* and Chl *c*, that work as accessory pigments and appear in different phytoplankton groups (Zapata et al. 2004; Jeffrey et al. 2011). Chl *a* concentration and fluorescence are important variables, widely used for lab and satellite estimations of global ocean primary production (Behrenfeld et al. 2005). The second group of pigments, the carotenoids, is also engaged in photoprotection, and some of them (e.g., fucoxanthin or ββ-carotene) can participate in both, light harvesting and photoprotective processes (Takaichi 2011; Mckew et al. 2013).

Changes in photoprotective pigment concentration and composition are usually a fast response (minutes to hours) to environmental conditions (Brunet et al. 2011). At low light intensity, the phytoplankton cell must be able to convert as much available energy as possible into a useful form for the organism. However, under high light conditions, the absorbed light energy may exceed the ability to be used by the photosynthetic system. To avoid damage under such conditions, several adaptive and acclimation mechanisms were evolved. One of the mechanisms that optimize the amount of light necessary for photosynthesis avoiding photodamage is the xanthophyll cycle (Demmig-Adams and Adams 2000, 2006; Lavaud 2007). The xanthophyll cycle involves the enzymatic de-epoxidation of certain xanthophylls containing, at least, an epoxide group, synthesized from ββ-carotene, as a function of absorbed quanta (Lohr and Wilhelm 2001). There are several major groups of xanthophyll cycles which can be defined on the basis of the pigments involved (Kuczynska et al. 2017). The cycle including the one step de-epoxidation of diadinoxanthin into diatoxanthin is characteristic of algae of the red lineage (Jeffrey et al. 2011). Despite light is the major factor affecting pigment concentration and composition, some other factors such as growth phase (Schlüter et al. 2000), iron concentration (Wilhelm et al. 2006), or nutrient status (Henriksen 2005) can also influence pigment composition and concentration within phytoplankton cells. The effects of high CO_2 levels on the whole phytoplankton pigment pool have not been studied yet but decreases in Chl *a* have been observed in high CO_2 acclimated cells (Sobrino et al. 2008, 2014; Rokitta and Rost 2012), although responses are not always straightforward (Trimborn et al. 2017). The elevated CO_2 concentrations also increased the sensitivity of phytoplankton photosynthesis to inhibition by high solar irradiance (Gao et al. 2009, 2012) and UVR (Sobrino et al. 2008, 2014; Wu et al. 2012). This increase in sensitivity under high CO_2 conditions could be related to the decrease in photosystem II D2 protein (PsbD) removal rate (Gao et al. 2018) and also to the downregulation of the CCMs and the photosynthetic machinery, which reduced the intracellular pools of some metabolites, such as Chl *a* or RuBisCo, and the cellular enzymatic activity (Sobrino et al. 2008, 2009). This was observed in previous studies as decreases in esterase activity and the activity of enzymes involved in oxidative stress (Sobrino et al. 2014).

The authors proposed that the decrease in intracellular pools might affect the compounds involved in repair mechanisms and the general defense state of the cells. However, the role of the photoprotective pigments and its likely relation to the increase in sensitivity under high CO_2 conditions has not been studied yet. We hypothesize that the increase in phytoplankton sensitivity to high CO_2 levels observed in previous studies might be related to a lower photoprotective capability, measured in terms of pigment concentration and composition, which contributes to the general defense of phytoplankton cells (Demmig-Adams and Adams 2000, 2006; Lavaud 2007). Furthermore, taking into account that the decrease in intracellular pools, including those related to photoprotection, might be associated to changes in CCM activity and cellular basal metabolism, the response to high CO_2 concentrations was tested in three species with global distribution that were studied under stable and perturbed conditions. The objective of the perturbation was to assess the responses of algae acclimated to elevated CO_2 concentrations which, by any environmental factor, upregulate their CCMs. Eukaryotic algae have developed a finely tuned regulatory system that suppresses expression of CCM-related genes under conditions of replete CO_2 and activates expression of these genes when CO_2 decreases (Wang et al. 2005; Yamano and Fukuzawa 2009). But it is known that CCM activity can also change depending on other environmental factors including

irradiance, temperature and nutrients, among others (Berman-Frank et al. 1998; Beardall and Giordano 2002). Therefore, the experimental design aimed to promote dissimilar effects on CCM activity, providing a wide range of results. The tested species were two diatoms (*Thalassiosira pseudonana* and *Skeletonema costatum*) and one haptophyta (*Emiliania huxleyi*). Approximately 40% of the marine phytoplankton species are diatoms and they are of crucial importance from an ecological and biogeochemical point of view, especially in nutrient-rich systems (Falkowski et al. 2004). The haptophyte group includes the coccolithophores, covered by small regular calcareous plates (coccoliths), which are important in biogeochemical cycles since they are responsible for about half of all modern precipitation of $CaCO_3$ in the ocean (Poulton et al. 2007).

Materials and methods

Culture growth conditions and experimental set up

The experiments were carried out with three phytoplankton strains: two diatoms (*T. pseudonana* [CCAP1085/12] and *S. costatum* [CCAP1077/1C] provided from the Culture Collection of Algae and Protozoa of the Scottish Marine Institute [CCAP]) and a calcifying strain (*E. huxleyi* [CCMP 371] provided from the National Center for Marine Algae and Microbiota). The phytoplankton strains were maintained under exponential growth conditions by following a semi-continuous culture strategy, with dilutions every 2 d, in a temperature controlled growth chamber (18 ± 1°C) and two different CO_2 conditions. The growth media was made from filtered seawater (at 35 PSU salinity) enriched with *f*/2 nutrients, and bubbled according to the CO_2 concentration for each experimental treatment during at least 5 d. Both, cultures and fresh media, were bubbled with regular atmospheric air collected from an open area (low carbon treatment [low CO_2] 40.5 Pa = 0.04% CO_2 in air CO_2) or with a mixture of atmospheric air and CO_2 from a pressurized gas tank (AIRGAS SPAIN, S. A.) to simulate future scenarios of climate change (high carbon treatment [high CO_2] 101.3 Pa = 0.1% CO_2 in air). The flow rate in each flask (100 mL min^{-1}) was controlled by individual flowmeters (Aalborg Inc). Cultures were illuminated with 180 μE m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by cool-white Sylvania fluorescent lamps following a 14 : 10 light: dark photoperiod. PAR was measured in the air with a spherical quantum sensor QSL 2100 (Biospherical Intruments Inc.) placed in a position similar to the center of the flask, facing the lamps. Cultures were acclimated to these experimental conditions for at least 3 weeks. Full acclimation to the experimental conditions was assumed when cells reached maximum photosynthetic efficiency values (i.e., F_v/fm, *see* paragraph below) higher than 0.65 during at least 1 week of semicontinuous growth (*see* Sobrino et al. 2008). After the acclimation period, cultures were diluted to a target concentrations of 1×10^6 cells mL^{-1} for *T. pseudonana* and 0.5×10^6 cells mL^{-1} for *S. costatum* and *E. huxleyi*, and the response of the phytoplankton cells to elevated CO_2 conditions was assessed under two different metabolic status in three independent 2 L cultures: cells acclimated to low CO_2 and high CO_2 under stable conditions (low CO_2 day 1 and high CO_2 day 1), and under perturbed conditions (low CO_2 day 2 and high CO_2 day 2), the latter achieved by stopping the aeration of the cultures for 4 h after the sampling in day 1. A diagram showing the experimental design of this study is shown in the companion paper from Puig-Fàbregas et al. (2021). The temporal decrease in CO_2 availability from the media (perturbation) has been demonstrated to promote significant changes in the expression of a high amount of genes, including those related with the upregulation of the CCMs, in cultures acclimated to high CO_2 conditions as those used for this experimental design (Brueggeman et al. 2012; Puig-Fàbregas et al. 2021). More specifically, results about the upregulation of CCM activity and basal metabolism measured as the response of three specific genes involved in CCM activity (α-carbonic anhydrase: *αca1*), the structure of the cytoskeleton (actin gene [*act*]) and carbon assimilation (RuBisCO: *rbcL*) after the 4-h period without bubbling are shown in Puig-Fàbregas et al. (2021). The perturbation was also expected to change the dynamic equilibrium of the cellular metabolic rates from steady-state to unsteady-state conditions (Halsey and Jones 2015) or, using the terminology from other authors, from balanced to unbalanced growth conditions (Barcelos e Ramos et al. 2010), without damaging the cells. After the perturbation, cultures were diluted again with fresh media, bubbled according to the CO_2 concentration for each experimental treatment, to similar concentrations as day 1 (i.e., 1×10^6 cells mL^{-1} for *T. pseudonana* and 0.5×10^6 cells mL^{-1} for *S. costatum* and *E. huxleyi*) in triplicate and connected again to the aeration to avoid carbon limitation. In order to obtain comparative responses between days 1 and 2, the study shows the results obtained from samples collected, using similar procedures, in the middle of the photoperiod for both days. This sampling time was close to the daily photosynthesis maximum and was aimed to avoid differences due to circadian rhythms on the studied variables (Prézelin and Ley 1980).

Temperature, pH, salinity, dissolved inorganic carbon, total alkalinity, and CO_2 analysis

Temperature and pH from each sample were measured with a Crison pH-meter PH 25+ (CRISON Instruments, S.A.) calibrated according to the total hydrogen ion pH scale with a buffer solution of 2-Amino-2-(hydroxymethyl)-1,-3-propanodiol ("Tris base") prepared with artificial marine water (DOE 1994). The salinity was measured with a Thermosalinometer Pioneer 30 (Radiometer Analytical SAS). Samples for dissolved inorganic carbon (DIC) analysis were collected and processed as in Puig-Fàbregas et al. (2021) using a Non-Dispersive Infrared Gas Analyzer Li-7000 (Li-COR Environmental). The system was standardized with freshly made

Na_2CO_3 solutions. The CO_2 and total alkalinity in the water samples were calculated from the pH, salinity, temperature, and DIC measurements performed daily for each sample, using the software csys.m from Zeebe and Wolf-Gladrow (2001).

Cell abundance and growth rates

Cell abundance was measured using a Neubaüer chamber. Growth rates were determined as:

$$\mu = [\ln(X_2) - \ln(X_1)]/\Delta t,$$

where μ = growth rate (d^{-1}), X_1 = cellular density (cells mL^{-1}) at time X_1, X_2 = cellular density (cells mL^{-1}) at time X_2, and Δt refers to the time period between X_1 and X_2 (d).

Maximum photosynthetic efficiency of photosystem II

The maximum photosynthetic efficiency of photosystem II (PSII) (F_v/fm) (Schreiber et al. 1994) was measured daily with a Water-PAM fluorometer (Walz) in order to assess the effect of the CO_2 under stable and perturbed conditions on the acclimation and good physiological status of the cells (*see* Sobrino et al. 2008). Samples collected in duplicate from the three independent replicate cultures, and for each of the experimental CO_2 treatments, were maintained in the dark at room temperature for approximately 20 min before measurements of the F_v/fm values.

Pigment analysis by high-performance liquid chromatography

Five-milliliter samples from each culture were filtered immediately after the sampling under dark conditions on glass-fiber filters (GF/F; Whatman Inc.). The filters were immediately frozen in liquid nitrogen and stored at −80°C until their extraction and analysis by high-performance liquid chromatography (HPLC), following the protocols described in Zapata et al. (2000) (*see* Supporting Information for detailed methods). In the species from this study, the xanthophyll cycle was defined by the presence of diadinoxanthin and diatoxanthin. The de-epoxidation state (DEPS), that has been demonstrated to have a close positive correlation with protective non-photochemical mechanisms that quench singlet-excited chlorophylls and harmlessly dissipate excess excitation energy as heat (i.e., non-photochemical quenching) (e.g., Demmig-Adams 1990), was calculated by normalizing the concentration of the photoprotective pigment (diatoxanthin) to the entire intracellular pool of the two pigments involved in the cycle (diadinoxanthin and diatoxanthin) (Ruban et al. 2004):

$$\text{DEPS} = \text{diatoxanthin}/(\text{diadinoxanthin} + \text{diatoxanthin}).$$

Statistical analysis

The results show the mean and the standard error (SE) of three independent replicates for each of the experimental treatments. Differences between the mean values obtained from the CO_2 treatment (high CO_2 vs. low CO_2) and the metabolic status (stable vs. perturbed), were statistically tested using a using a Student's *t*-test (IBM SPSS 15.0 Statistics Base) and considering 95% as the limit of significance ($p \leq 0.05$ indicates significant differences). The normality and homogeneity of the data were tested with the parametric tests Shapiro–Wilk and Levene, respectively (IBM SPSS 15.0 Statistics Base). A two-way analysis of variance (ANOVA) was also performed on cell density, growth rates, photosynthetic efficiency, DEPS and the cellular pigment content ($p \leq 0.05$ indicates significant differences) to test the interactive effect of CO_2 treatment and metabolic status. The *p* value was standardized by Good (1982) in order to overcome the low number of replicates. Multidimensional scaling (MDS) was also applied to study the simultaneous relationships among phytoplankton pigment responses and CO_2 concentration using the SPSS 15.0 (IBM SPSS 15.0 Statistics Base). The MDS analysis was performed using the cellular pigment content as well as the percentage of variation in cellular pigment content when comparing high CO_2 vs. low CO_2 cells under the different metabolic status. The resulting models were made with a data matrix that included information about the response observed for five pigments (i.e., the five major common pigments: Chl *a*, fucoxanthin, Chl c_2, ββ-carotene, and diadinoxanthin) from the three phytoplankton species under the two metabolic conditions tested in the study and the CO_2 values for each of the different CO_2 treatments. For this MDS analysis the Euclidean distance was used as a dissimilarity measure. The stress value, which is an important fit criterion and represents the average of deviations between the final distance on the map and the initial distances or dissimilarities, was normalized to take values between 0 and 1 (Kruskal 1964).

Results

pH, DIC, and CO_2

The pH in the high CO_2 treatments was significantly lower than in the low CO_2 treatments in the three phytoplankton cultures (Table 1). The average pH value was 8.50 ± 0.13 in low CO_2 treatments and 8.15 ± 0.18 in the high CO_2 treatments (Table 1). The changes in pH resulted in CO_2 values ~ 73% significantly higher in high CO_2 compared to low CO_2 cultures under stable conditions (Table 1). In addition, the DIC concentrations in the high CO_2 treatments were ~ 23% higher than in the low CO_2 treatment for the three species in both sampling days (Table 1). Comparing the three species, average CO_2 in the liquid medium of the high CO_2 and low CO_2 treatments was significantly lower in *T. pseudonana* than in *S. costatum* and *E. huxleyi* cultures (Table 1), as a consequence of

Table 1. Mean (± SE, $n = 3$) of pH, DIC (μmol kg^{-1}), CO_2 (μatm), and TA (μmol kg^{-1}) from *Thalassiosira pseudonana*, *Skeletonema costatum*, and *Emiliania huxleyi* cultures in the low CO_2 (LC) and high CO_2 (HC) treatments under stable conditions (i.e., HC1 and LC1 in day 1) and under perturbed conditions (i.e., LC2 and HC2 in day 2). Significant differences between the HC and LC treatments for each metabolic condition are indicated with an asterisk (*$p < 0.05$).

		pH	DIC	CO_2	TA
Thalassiosira pseudonana	LC1	8.87±0.029	1637±31	50.3±5.4	2493±13.8
	HC1	8.44±0.178*	2283±44*	149.1±31.2*	2875±25.6*
	LC2	8.87±0.040	1877±39	58.7±7.8	2814±14.23
	HC2	8.41±0.035*	2427±47*	275.2±29.5*	2983±23.77
Skeletonema costatum	LC1	8.86±0.040	2272±75	70.5±10.0	3247±43.93
	HC1	8.10±0.100*	3023±42*	753.2±188.5*	3317±109.32
	LC2	8.56±0.050	2331±109	172.8±34.4	2946±72.54
	HC2	7.96±0.010*	2942±53*	975.0±30.9*	3133±51.32
Emiliania huxleyi	LC1	8.14±0.007	1833±9	400.6±8.3	2121±7.89
	HC1	7.80±0.003*	2161±50*	1086.2±21.1*	2321±53.60
	LC2	8.18±0.012	1564±16	312.4±10.6	1842±16.75
	HC2	7.76±0.009*	2248±44*	1222.0±37.8*	2399±45.47*

the differences in growth rates (*see* the explanation below). Among the different species, CO_2 in *E. huxleyi* resembled more closely the bubbled CO_2 values than the two diatom species (Table 1). By contrast to the CO_2 values under stable conditions, average CO_2 in the high CO_2 treatment was ~26% higher under perturbed conditions, which can result from the dilution made after the perturbation or from the biological production of CO_2 derived from changes in growth rate (*see* results in Table 2).

Culture growth and physiological state

The highest growth rates were observed in *T. pseudonana* (1.66 d^{-1} ± 0.03) followed by *S. costatum* (0.95 d^{-1} ± 0.02) and *E. huxleyi* cultures (0.80 d^{-1} ± 0.04) (Table 2). Growth increased significantly under high CO_2 and stable conditions in *T. pseudonana* and *E. huxleyi* (Table 2), but the response was not so clear in *S. costatum* (Table 2). By contrast, this trend showing an increase in growth under high CO_2 stable conditions, reversed under perturbed conditions, resulting in significantly lower growth rates in the high CO_2 than in the low CO_2 cultures for the three species (Table 2). The F_v/fm results indicated that the cells in all the cultures were in good photophysiological state independently of the CO_2 or metabolic conditions (Table 2). Nevertheless, significant differences were found in *T. pseudonana* under stable conditions, where F_v/fm was significantly higher in high CO_2 cultures compared to low CO_2 (Table 2) and in *E. huxleyi*, where the high CO_2 cultures showed lower F_v/fm values independently of the metabolic state (Table 2). The two-way ANOVA results showed that the

Table 2. Mean (± SE, $n = 3$) of cell density ($N \times 10^6$ cells mL^{-1}), growth rates (d^{-1}) and maximum photosynthetic efficiency of PSII (F_v/fm) from *Thalassiosira pseudonana*, *Skeletonema costatum*, and *Emiliania huxleyi* cultures in the low CO_2 (LC) and high CO_2 (HC) treatments under stable conditions (i.e., HC1 and LC1 in day 1) and under perturbed conditions (i.e., LC2 and HC2 in day 2). Significant differences between the HC and LC treatments for each metabolic condition are indicated with an asterisk (*$p < 0.05$).

		Cell density	Growth rates	F_v/fm
Thalassiosira pseudonana	LC1	3.58±0.242	1.29±0.07	0.718±0.002
	HC1	4.82±0.142*	1.66±0.03*	0.733±0.003*
	LC2	3.84±0.090	1.53±0.02	0.729±0.007
	HC2	4.24±0.104*	1.42±0.02*	0.728±0.005
Skeletonema costatum	LC1	0.83±0.02	0.95±0.02	0.693±0.003
	HC1	0.90±0.03*	0.95±0.04	0.683±0.011
	LC2	0.44±0.02	0.75±0.04	0.691±0.005
	HC2	0.34±0.01*	0.35±0.04*	0.683±0.004
Emiliania huxleyi	LC1	0.58±0.036	0.42±0.06	0.709±0.002
	HC1	1.03±0.032*	0.56±0.03*	0.701±0.004*
	LC2	0.67±0.027	0.80±0.04	0.719±0.001
	HC2	0.32±0.024*	0.48±0.07*	0.699±0.002*

interaction between CO_2 treatments and metabolic status did not produce significant effects on growth or photosynthetic efficiency of PSII (data not shown).

Pigment composition and concentration

The analysis of the chromatograms for each of the studied species allowed the detection of 16 different pigments (Fig. S1; Table S1).

As expected (Jeffrey et al. 2011), differences between the two diatom species and the coccolithophorid species regarding the number and type of the detected pigments were observed (Fig. S1a–c): *T. pseudonana* and *S. costatum* were similar in terms of pigment composition with Mg-2,4-divinyl pheoporphyrin a_5 monomethyl ester, Chl c_1, Chl c_2, fucoxanthin, diadinoxanthin, Chl *a*, and ββ-carotene as the common pigments (Fig. S1a,b), but differed by the presence of the photoprotective pigment diatoxanthin, which was detected in *T. pseudonana* but not in *S. costatum* (Fig. S1a,b). *E. huxleyi* showed the most complex pigment composition already described (Garrido et al. 2016) (Fig. S1c) with Chl c_3, monovinyl Chl c_3, 19′-butanoyloxyfucoxanthin, 19′-hexanoyloxy-4-ketofucoxanthin, 19′-hexanoyloxyfucoxanthin, Chl c_2 monogalactosyldiacylglyceride ester and βε-carotene in addition to the pigments found in *T. pseudonana* and *S. costatum* (Fig. S1a–c).

The results showed that CO_2 treatments did not produce a significant effect on qualitative pigment composition but significantly changed cellular pigment content. In addition, cellular pigment content in high CO_2 cultures showed a different response pattern, compared to low CO_2 cultures, depending on the metabolic state of the cells. Among the pigments shared by the three species (i.e., Chl *a*, Chl c_2, fucoxanthin, diadinoxanthin, and ββ-carotene), chlorophyll response pattern was very similar in *S. costatum* and *E. huxleyi*, with lower content in the high CO_2 than in the low CO_2 cultures under stable conditions but higher concentration under perturbed conditions (Fig. 1a,b). The average decrease in Chl *a* for both species under high CO_2 and stable conditions was 69% and 11% for *S. costatum* and *E. huxleyi*, respectively, while the increase after the perturbation was 14% and 44% compared to low CO_2 conditions. The same pattern was observed in *S. costatum* and *E. huxleyi* for the photoprotective pigments, although ββ-carotene and diadinoxanthin response was less frequently significant than the response observed for fucoxanthin and both chlorophylls (Chl *a* and Chl c_2) (Figs. 2a–c, 1a,b, respectively). Regarding fucoxanthin, this was also the pigment with the biggest response in *E. huxleyi* when comparing high CO_2 and low CO_2 under stable conditions (43% decrease), and in all the species under perturbed conditions (average increase of 30%) (Fig. 2a). Unlike *T. pseudonana* and *S. costatum*, HPLC analyses also confirmed the presence of fucoxanthin derivatives (fucoxanthin, 19′-hexanoyloxy-4-ketofucoxanthin and 19′-hexanoyloxyfucoxanthin) in *E. huxleyi* (Fig. S1c), which are known to interconvert, depending on the environmental conditions (Garrido et al. 2016). Among the various pigments sharing the fucoxanthin chromophore (i.e., with similar absorption features) that have been characterized in the coccolithophore *E. huxleyi* (Airs and Llewellyn 2006), the most abundant, besides fucoxanthin itself, were 19′-hexanoyloxy-4-ketofucoxanthin and 19′-hexanoyloxyfucoxanthin (Stolte et al. 2000; Zapata et al. 2004). The sum of fucoxanthin pigments content per cell (ΣF) (Fig. 3a) in our study was strongly similar to the response observed for the other photoprotective pigments in *E. huxleyi*, with lower cellular content in the high CO_2 than in the low CO_2 cultures under stable conditions, and vice versa under perturbed conditions (Fig. 3a). A more detailed analysis showing the response of 19′-hexanoyloxyfucoxanthin, the most abundant of these three pigments, also indicated significant differences due to the different CO_2 treatments (Fig. 3b).

Unlike *S. costatum* and *E. huxleyi*, *T. pseudonana* cellular pigment content did not follow the observed pattern under stable conditions. Nevertheless, they responded similarly than in the other tested species under perturbed conditions (Figs. 1a,b, 2a–c). The results showed significantly higher Chl *a*, fucoxanthin and ββ-carotene content and lack of differences in Chl c_2 and diadinoxanthin under high CO_2 compared to low CO_2 under stable conditions. However, despite the disagreement between these responses and the general pattern observed in *S. costatum* and *E. huxleyi*, the analysis of the DEPS results supported the later, showing a decrease in the response under high CO_2 stable conditions but an increase under perturbed conditions (Fig. 4). Since diatoxanthin was not detected in *S. costatum* in our study, the results did not allow to calculate the DEPS for this species. The two-way ANOVA showed significant effects about the interaction between CO_2 treatments and the metabolic conditions regarding cellular pigment content for Chl *a*, Chl c_2, fucoxanthin, diadinoxanthin, and ββ-carotene as well as for DEPS (data not shown).

In order to explore the pigment response patterns in relation to the effect of the elevated CO_2 concentration in the two different metabolic states tested for the three studied species, a MDS analysis was used to identify the pigmentary signature which best explained the photophysiological responses to CO_2 (Fig. 5a,b). The analysis was performed using the cellular pigment content as well as the percentage of variation in cellular pigment content (Fig. 5a,b) and the CO_2 values for each of the different treatments (Table 1, column 3). The resulting model explained 96.73% and 97.25% of the variation, respectively, and included five pigments (i.e., the major common pigments in the three tested phytoplankton species): Chl *a*, fucoxanthin, Chl c_2, ββ-carotene, and diadinoxanthin. The analysis of the cellular pigment content showed a clear cluster of pigments formed by Chl c_2, ββ-carotene, and diadinoxanthin, and separated from Chl *a* and fucoxanthin, which also appeared quite different between them (Fig. 5a). However, in the case of the percentage of variation in cellular pigment content depending on the experimental treatments,

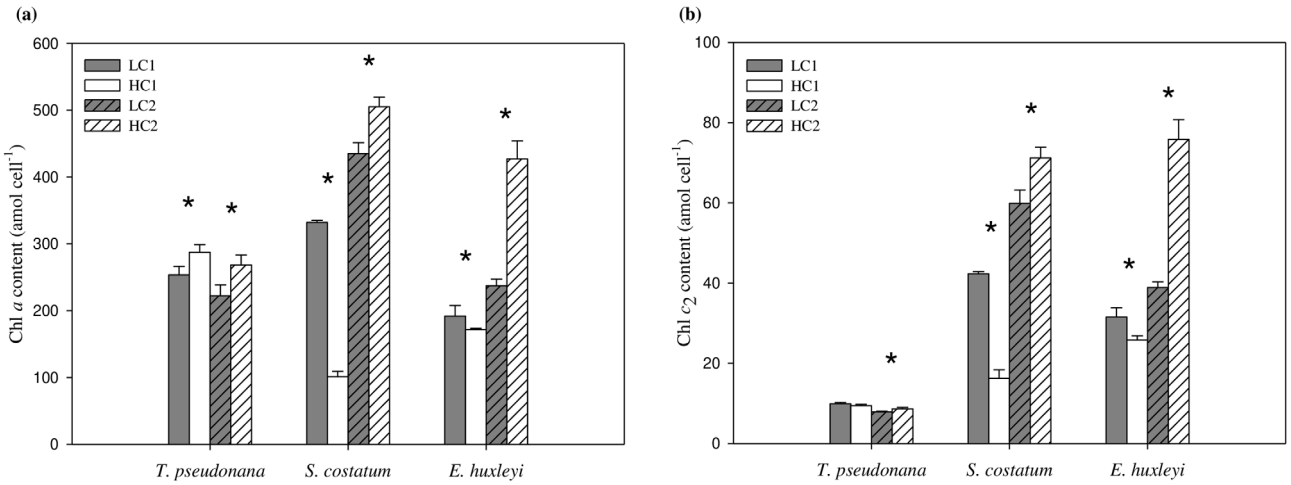

Fig. 1. Chlorophyll cellular content (amol cell^{-1}) detected using Zapata et al. (2000) protocol for *Thalassiosira pseudonana*, *Skeletonema costatum*, and *Emiliania huxleyi* cultures in this study: (**a**) chlorophyll *a* (Chl *a*) cellular content and (**b**) chlorophyll c_2 (Chl c_2) cellular content in the low CO_2 (LC) and high CO_2 (HC) treatments under stable conditions (i.e., HC1 and LC1 in day 1) and under perturbed conditions (i.e., LC2 and HC2 in day 2). Error bars indicate the SE. Significant differences between the HC and LC treatments for each metabolic condition are indicated with an asterisk (*$p < 0.05$).

fucoxanthin and Chl c_2 were the pigments that appear isolated and separated from the main cluster, formed in this case by diadinoxanthin, Chl *a*, and ββ-carotene (Fig. 5b). Excluding or including *T. pseudonana* under stable conditions from the analysis did not change the main results but increased the stress value from 0.006 to 0.011 when comparing the cellular pigment content and from 0.006 to 0.008 when using the percentage of variation, respectively (data not shown). In all the cases, these stress values are considered as excellent (Kruskal 1964).

Discussion and conclusions

Phytoplankton photoacclimation encloses a set of processes that cover a wide range of physiological responses, involving changes in cellular components and structures over different time scales (seconds to days). These processes are aimed to optimize several cell activities, such as growth, respiration, and division, when dealing to changing irradiances in the environment (Brunet et al. 2011). However, despite light is the main environmental factor directly controlling photoacclimation processes in the phytoplankton cell, other factors such as nutrients (Henriksen 2005) or growth phase (Schlüter et al. 2000) can also impact organism physiology altering pigment composition and structure in the cell. In this study, CO_2 manipulation experiments were designed to assess potential changes in phytoplankton physiology resulting from environmental CO_2 variability. The results of these experiments indicated that phytoplankton photophysiology responds to increased CO_2 concentrations expected for future scenarios of climate change. Moreover, the results show that the response is not unidirectional and can change depending on the metabolic status of the cells.

In our study the increase in CO_2 of the media and the acclimation to the high CO_2 concentrations expected for future scenarios of global change was achieved by continuously aerating the cultures with 0.1% CO_2 in air during at least 14 generations. This experimental design allowed detecting the expected CO_2 concentrations in *E. huxleyi* culture media but the diatom cultures did show lower values than predicted. Nevertheless, DIC and CO_2 in high CO_2 and low CO_2 cultures were still significantly higher under the former conditions and CO_2 concentrations were in line with realistic conditions where certain diatom species, such as *T. pseudonana* or *S. costatum*, are able to form intense blooms due to their capacity to overcome low CO_2 concentrations at high population densities (Dai et al. 2008). Low CO_2 concentrations are observed in cultures and during phytoplankton blooms in natural waters due to the high phytoplankton biomass and photosynthetic activity (e.g., Berman-Frank et al. 1998; Dai et al. 2008). *T. pseudonana* and *S. costatum* under high CO_2 conditions produced that the CO_2 incorporation in the water (culture media) was slower than the biological CO_2 incorporation, due to the high cellular densities resulting from the high growth rates. This dynamic change in the chemical conditions did not allow for the full stabilization of *T. pseudonana* physiology expected under the stable conditions treatment. As a consequence, *T. pseudonana* results under stable conditions did not always follow the general trend observed in *E. huxleyi* and *S. costatum*. In addition, in the experimental design of this study, the change in the basal metabolism was achieved by temporarily disrupting the continuous flow of CO_2 in the cultures acclimated to the high CO_2 concentrations. It has been demonstrated that this perturbation promotes the upregulation of the CCMs in cultures acclimated to high CO_2 conditions as those used for this experimental design

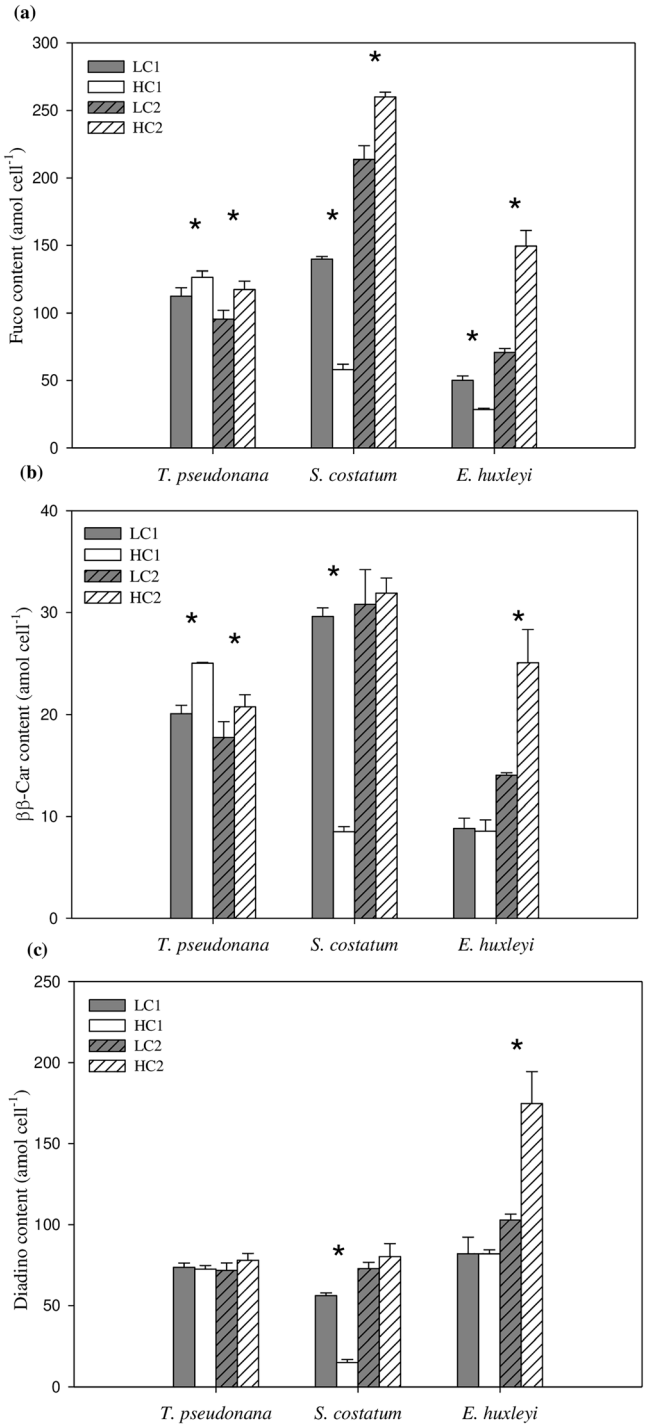

Fig. 2. Carotenoids cellular content (amol cell^{-1}) obtained with Zapata et al. (2000) protocol: (**a**) fucoxanthin (Fuco) cellular content, (**b**) ββ-carotene (ββ-Car) cellular content, (**c**) diadinoxanthin (Diadino) cellular content, from *Thalassiosira pseudonana*, *Skeletonema costatum*, and *Emiliania huxleyi* cultures in the low CO_2 (LC) and high CO_2 (HC) treatments under stable conditions (i.e., HC1 and LC1 in day 1) and under perturbed conditions (i.e., LC2 and HC2 in day 2). Error bars indicate the SE. Significant differences between the HC and LC treatments for each metabolic condition are indicated with an asterisk (*$p < 0.05$).

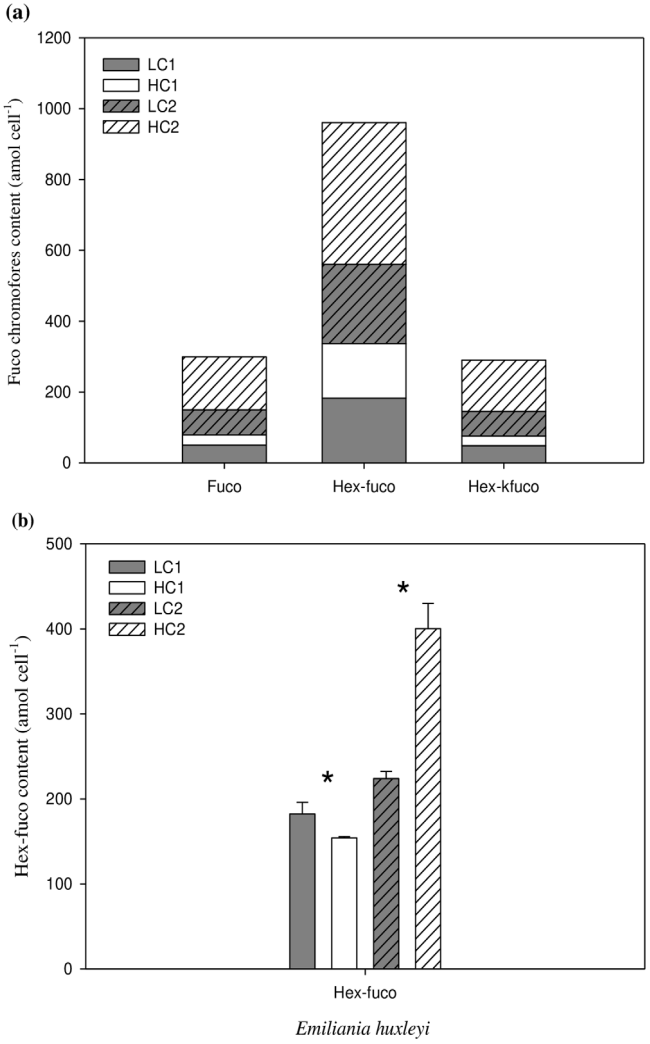

Fig. 3. (**a**) Sum of fucoxanthin pigments content per cell (ΣF) (amol cell^{-1}) detected using Zapata et al. (2000) protocol: fucoxanthin (Fuco), 19′-hexanoyloxyfucoxanthin (Hex-fuco) and 19′-hexanoyloxy-4-ketofucoxanthin (Hex-kfuco) for *Emiliania huxleyi* cultures grown in the low CO_2 (LC) and high CO_2 (HC) treatments under stable conditions (i.e., HC1 and LC1 in day 1) and under perturbed conditions (i.e., LC2 and HC2 in day 2). (**b**) 19′-Hexanoyloxyfucoxanthin cellular content (amol cell^{-1}) detected using Zapata et al. (2000) protocol for *Emiliania huxleyi* cultures grown in the low CO_2 (LC) and high CO_2 (HC) treatments under stable conditions (i.e., HC1 and LC1 in day 1) and under perturbed conditions (i.e., LC2 and HC2 in day 2). Error bars indicate the SE. Significant differences between the HC and LC treatments are marked with an asterisk (*$p < 0.05$).

(Brueggeman et al. 2012; Puig-Fàbregas et al. 2021), which would be able to change the dynamic equilibrium of the cellular metabolic rates (Barcelos e Ramos et al. 2010; Halsey and Jones 2015). The change in the dynamic equilibrium of the metabolic rates can happen by any environmental factor, such as a different metabolic status, from stable to perturbed conditions, as used in the present study. Nevertheless, it is important to notice that the perturbation used to upregulate the

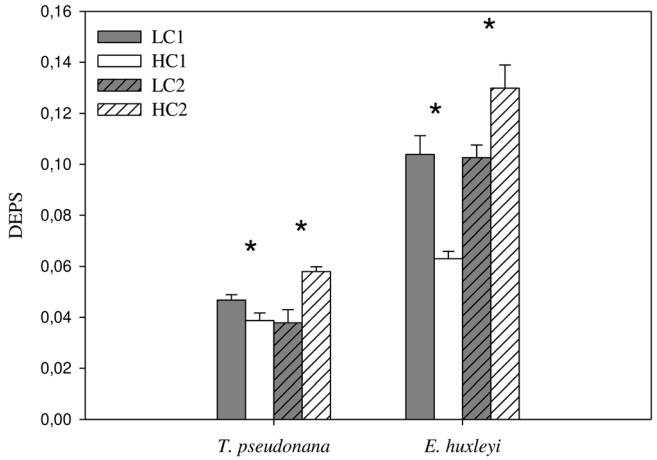

Fig. 4. De-epoxidation state (DEPS = diatoxanthin/[diatoxanthin + diadinoxanthin]) values obtained in *Thalassiosira pseudonana* and *Emiliania huxleyi* cultures grown in the low CO_2 (LC) and high CO_2 (HC) treatments under stable conditions (i.e., HC1 and LC1 in day 1) and under perturbed conditions (i.e., LC2 and HC2 in day 2). Error bars indicate the SE. Significant differences between the HC and LC treatments are indicated with an asterisk (*$p < 0.05$).

basal metabolism in this study was not related to a damaging effect. The lack of damage in the studied phytoplankton cultures is demonstrated by the high F_v/fm values observed in the cells for all the experimental treatments, as corresponds to cultures grown under optimum and saturating nutrient, light, and temperature growth conditions. In fact, values were within the highest observed range as corresponds to analysis made with a Water PAM fluorometer (Walz) that induces multiple photochemical turnovers of individual PSII reaction centres instead a single turnover.

The results from the present study clearly showed that elevated CO_2 levels can affect phytoplankton biomass and cellular pigment content. Both variables are interlinked and connected through the carbon metabolism of the cells despite the former is usually related to processes involved in the dark phase of photosynthesis while the later acts during the light phase. However, the responses observed for the growth rates and the cellular pigment content described opposite patterns showing, as a general trend, an increase in growth rates and a decrease in pigment content in high CO_2 compared to low CO_2 under stable conditions, and a decrease in growth rates and an increase in pigment content in high CO_2 compared to low CO_2 under perturbed conditions. The interaction between experimental treatments was also more significant for the cellular pigment content than for the growth culture variables, which indicates the high sensitivity of pigments to carbon metabolism. The results from the growth rates agree with previously published studies that indicate that diatoms are likely to be benefitted by increasing the availability of dissolved CO_2 as a substrate for photosynthesis (Rost et al. 2008). They also agree with other studies reporting increases in CO_2-induced growth rates in both natural and cultured diatom populations

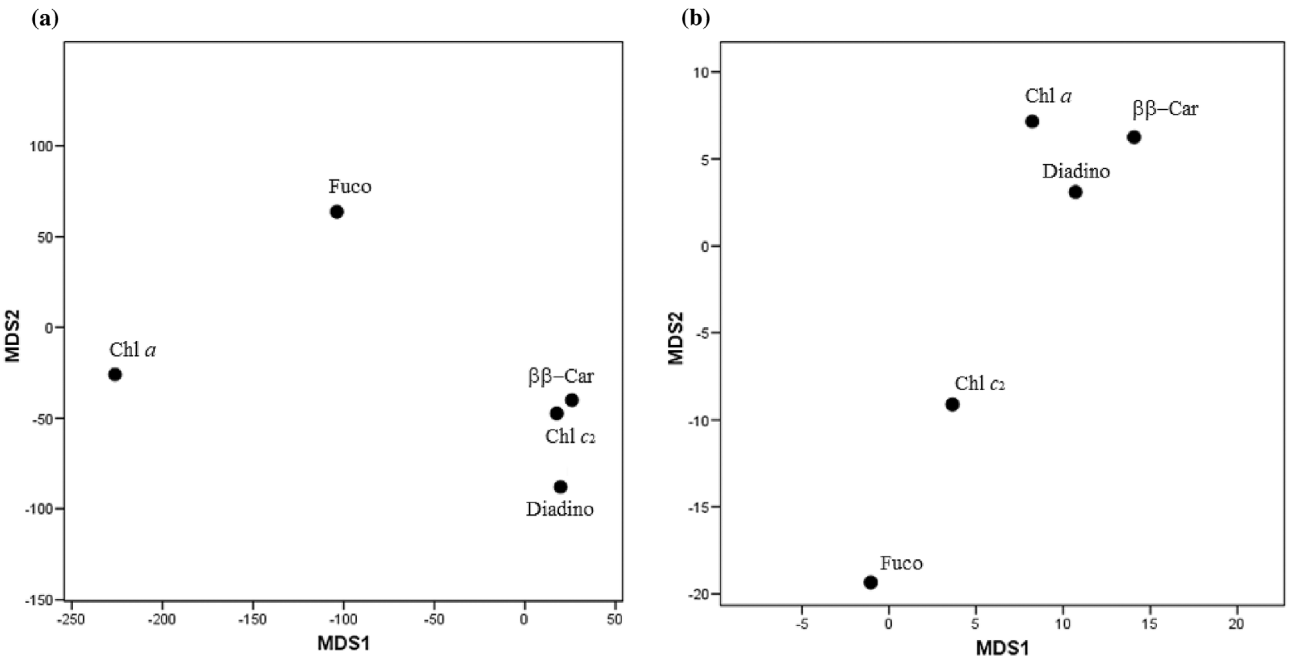

Fig. 5. Two-dimensional scatter plot obtained from multidimensional scaling (MDS) analysis to represent: (**a**) the cellular pigment content when comparing high CO_2 (HC) vs. low CO_2 (LC) cells under the different metabolic conditions in a single plane (stress = 0.0082 [0.82%, excellent adjustment]; explained variance = 0.9673 [96.73%]), and (**b**) the percentage of variation in cellular pigment content when comparing HC vs. LC cells under the different metabolic status in a single plane (stress = 0.0117 [1.17%, excellent adjustment]; explained variance = 0.9725 [97.25%]). Abbreviations as in Figs. 1, 2.

(Kroeker et al. 2013; Dutkiewicz et al. 2015). In addition, the fact that the response reversed depending on the basal metabolic activity of the cells help to understand the lack of an unique response when analyzing the effect of CO_2 on phytoplankton, and more specially on cocolithophorids, that have been usually showing a more variable response (Kroeker et al. 2013). Moreover, they are in agreement with a previous hypothesis that proposes that the increase in growth rates and the decrease in cell metabolites under high CO_2 in acclimated phytoplankton cultures results from a cascade effect related to the downregulation of the CCMs (Sobrino et al. 2014). Briefly, the theoretical model proposes that under high CO_2 conditions, similar to those used in this study, aquatic CO_2 is able to saturate the active center of RuBisCO without the need for active transporters, leading to energy savings and optimization of photosynthetic related processes so that there is more energy available for other metabolic pathways, and finally resulting in higher growth rates. This adjustment of cell physiology includes a decrease in the incorporation and/or synthesis of new metabolic components, thus reducing intracellular pools and the enzymatic activity, and increasing metabolic efficiency (Sobrino et al. 2008, 2009, 2014). However, some contrasting responses have also been observed (García-Gómez et al. 2014) and further information about the molecular mechanisms and the consequences of downregulation for cell metabolism is still needed (Hopkinson et al. 2010; Raven et al. 2014). Regarding pigment pools in phytoplankton, Sobrino et al. (2008) results show that high CO_2 acclimated cultures of *T. pseudonana* experienced decreases in cellular Chl *a* with concomitant increases in growth and photosynthetic rates than low CO_2 cultures grown under stable growth conditions. However, the present study demonstrates that the high CO_2 effect applies to practically the whole set of pigments, independently of differences in pigment composition from species belonging to different taxonomical groups and independently of the functional role of these pigments in the cell. The decreased pigment content under high CO_2 conditions has been described for plants and is termed pigment economy (Gao et al. 2016). Moreover, it also demonstrates that this trend can reverse when the metabolism is activated due to the displacement of the basal metabolism from its steady-state. In this study the activation was induced through a perturbation that triggered the upregulation of the CCMs (Brueggeman et al. 2012; Puig-Fàbregas et al. 2021), which in consequence increased the metabolic demand and a higher synthesis of cellular pigments under high CO_2 compared to low CO_2 conditions. The response in high CO_2 cultures with downregulated CCMs and stable growth conditions resembles high light conditions, as observed in other studies (Rokitta and Rost 2012) but it simulates dark conditions when the perturbation creates the unbalance between the energy consuming processes (CCM upregulation) and the energy generating processes.

The results also demonstrate that the CO_2 concentration can modify the photoprotective capability of the phytoplankton through changes in the photoprotective pigment content and in the DEPS of the xanthophylls. This was demonstrated in our study through the analysis of DEPS in *T. pseudonana* and *E. huxleyi*, but not in *S. costatum* since diatoxanthin was not detected in this species. This result is not unusual, since diatoxanthin concentration in this species is usually low, or even zero, also after exposure to different light conditions (Lacour et al. 2020).

The species used in this study possess a xanthophyll cycle where the specific xanthophyll diadinoxanthin is being converted to diatoxanthin by an enzymatic single de-epoxidation step to dissipate excess light energy (Brunet et al. 2011). High CO_2 significantly decreased the photoprotective capability of the cells under stable conditions but increased the photoprotective response when the metabolism was upregulated due to the external perturbation. The two-way ANOVA results highlighted that the cellular pigment content and the photoprotective capability were further stimulated by the interaction of both, CO_2 increase and metabolism perturbation. These results are in line with the responses observed in Sobrino et al. (2008, 2009) that showed that at high CO_2 values under stable conditions cells were more sensitive to UVR exposures. Similar conclusions were also obtained in Gao et al. (2012), working with natural marine communities exposed to solar radiation. The reason for this increase in sensitivity was not demonstrated in those studies but was explained as the result from changes in the balance between photosynthetic damage and repair rates under UVR exposures (Sobrino et al. 2005, 2008), which in the last instance are related to the protoprotective and photorepair capability of the cells. The present results reinforce and demonstrate the important role of the effect of elevated CO_2 on pigment-related photoprotection.

One of the main objectives that arose from these results was to find a suitable molecule that may act as physiological indicator of the basal metabolic activity of phytoplankton for predicting responses under simulated ocean acidification scenarios. Taking into account the role of CCMs activity for triggering the downregulation or upregulation of the photosynthetic metabolism under high CO_2 conditions it seems plausible to consider these molecules as one of the most interesting molecules for this purpose. Nevertheless our results indicate that pigment content or the DEPS state, besides being more easily quantified than CCM activity, may be also useful.

In this sense, the MDS analysis, based on a distance or dissimilarity matrix obtained from the results of the three species tested, was a great tool to visualize the most outstanding characteristics of the data. The analysis evidenced that fucoxanthin and Chl c_2 were the pigments with the greatest relevance to fulfill the objective, since they are the pigments that show the highest sensitivity to CO_2 and that appear in a concentration high enough to be easily detected. Low cellular pigment contents, such as those quantified for diadinoxanthin, result in values close to the detection limit, calculated in this study

as the concentration of component providing a signal-to-noise ratio (S/N) of three (typically 0.237 pm per injection for diadinoxanthin), increasing the replicate variability and decreasing the probability of resulting in significant responses. The decrease in both, fucoxanthin and Chl c_2, could be useful indicating a downregulation of the photosynthetic machinery under high CO_2 stable conditions, while the increase in fucoxanthin stands out for upregulated conditions. Changes in Chl *a*, a usual variable for satellite estimations of ocean primary production (Behrenfeld et al. 2005), were also frequently significant and therefore should be analyzed with caution when predicting responses in simulated future scenarios. Its concentration can be assessed very easily through fluorometry or spectrophotometry, so it can be used as a fast check for the upregulation or downregulation state under high CO_2 conditions. However, the results from *T. pseudonana* indicated that its cellular content can change very rapidly depending on growth rate and therefore decreasing its value as pigment biomarker for ocean acidification scenarios.

In conclusion, the results from this study demonstrate that the pigment content and the photoprotective capability related to xanthophylls de-epoxidation and other photoprotective pigments in phytoplankton can change depending on CO_2 concentration. However, the response depends on the basal metabolic activity of the cells. Therefore, under stable environmental conditions that promote steady-state growth, phytoplankton cells decrease pigment content and photoprotective capability, making phytoplankton more sensitive to environmental stressors. On the contrary, if an external perturbation activates the basal metabolic state, pigment content, and photoprotection capability can be higher in cells under high CO_2 than in cells under low CO_2 conditions. The results show two contrasting endpoint metabolic states obtained under controlled conditions in the laboratory and did not aim to explain the responses observed in nature, which may be similar or somewhere in between, depending on the interaction with other environmental drivers. However, they support previous studies that describe the important role of the physiological regulation of the photosynthetic system under high CO_2 conditions to understand the responses of phytoplankton in future scenarios of global change.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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Conflict of Interest

None declared.

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