

# Elevated CO<sub>2</sub> and associated seawater chemistry do not benefit a model diatom grown with increased availability of light

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**ABSTRACT:** Elevated CO<sub>2</sub> is leading to a decrease in pH in marine environments (ocean acidification [OA]), altering marine carbonate chemistry. OA can influence the metabolism of many marine organisms; however, no consensus has been reached on its effects on algal photosynthetic carbon fixation and primary production. Here, we found that when the diatom *Phaeodactylum tricornutum* was grown under different pCO<sub>2</sub> levels, it showed different responses to elevated pCO<sub>2</sub> levels under growth-limiting (20 μmol photons m<sup>-2</sup> s<sup>-1</sup>, LL) compared with growth-saturating (200 μmol photons m<sup>-2</sup> s<sup>-1</sup>, HL) light levels. With pCO<sub>2</sub> increased up to 950 μatm, growth rates and primary productivity increased, but in the HL cells, these parameters decreased significantly at higher concentrations up to 5000 μatm, while no difference in growth was observed with pCO<sub>2</sub> for the LL cells. Elevated CO<sub>2</sub> concentrations reduced the size of the intracellular dissolved inorganic carbon (DIC) pool by 81% and 60% under the LL and HL levels, respectively, with the corresponding photosynthetic affinity for DIC decreasing by 48% and 55%. Little photoinhibition was observed across all treatments. These results suggest that the decreased growth rates under higher CO<sub>2</sub> levels in the HL cells were most likely due to acid stress. Low energy demand of growth and energy saving from the down-regulation of the CO<sub>2</sub> concentrating mechanisms (CCM) minimized the effects of acid stress on the growth of the LL cells. These findings imply that OA treatment, except for down-regulating CCM, caused stress on the diatom, reflected in diminished C assimilation and growth rates.

**KEY WORDS:** Ocean acidification · Intracellular DIC · Photosynthesis

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## INTRODUCTION

Driven by human activity, such as fossil fuel combustion and deforestation, the atmospheric partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) has increased by nearly 40% since pre-industrial times (IPCC 2007). As the principal sink, the ocean has absorbed approximately 30% (IPCC 2014) of anthropogenically derived CO<sub>2</sub>, causing ocean acidification (OA), with the ocean pH predicted to decrease by 0.3 to 0.4 units by the end of this century (Sabine et al. 2004, Mikaloff Fletcher et al. 2006, Beardall et al. 2009). In addition to global OA driven by anthropogenic CO<sub>2</sub> release (IGBP 2013),

additional acidification events occur on a regional scale as a consequence of localized upwelling of cold, CO<sub>2</sub>-rich water and of volcanic CO<sub>2</sub> vents such as those in the Mediterranean and Circum-Pacific volcanic arcs, where pH values can be as low as 6.57, approximately equivalent to a pCO<sub>2</sub> of 20753 μatm (Sano & Williams 1996, Hall-Spencer et al. 2008, Dias et al. 2010). Such intensified CO<sub>2</sub> enrichment and altered seawater carbonate chemistry have consequences for primary productivity and growth of various organisms (Häder et al. 2015). Decreases in seawater pH can affect the intracellular acid-base balance of organisms, leading to enhanced energy

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demand; consequently, the membrane electrochemical potential and enzyme activity may be indirectly affected (Madshus 1988, Kramer et al. 2003, Milligan et al. 2009). Cytoplasmic pH is relatively constant under changes of external pH (Smith & Raven 1979), though intracellular pH in a coccolithophore was slightly reduced by the external pH drop associated with OA treatment (Nimer et al. 1994).

However, because of the paucity of knowledge on the physiological effects of OA, it is still controversial whether future carbon sequestration via the function of phytoplankton will increase or decline due to the progressive OA resulting from the accumulation of CO<sub>2</sub> in the atmosphere and its continuous dissolution into oceans. Importantly, while elevated pCO<sub>2</sub> levels can down-regulate CO<sub>2</sub> concentrating mechanisms (CCM) in the many aquatic photosynthetic organisms tested, it is still unclear if lowered energy costs due to down-regulation of CCM activity (Deng et al. 2003, Hopkinson et al. 2011) can compensate for the additional stresses associated with a pH drop, particularly at lower light levels encountered at depths in the oceans (Madshus 1988, Beardall et al. 1998, Kramer et al. 2003, Milligan et al. 2009, Raven et al. 2014). Acclimation to low-photon flux may also lead to a reduced capacity to employ a CCM which down-regulates under sub-saturating irradiance (Beardall & Giordano 2002, Yang & Gao 2012), leading to an increase in cells' reliance on CO<sub>2</sub> diffusion (Fu & Han 2010, Hepburn et al. 2011). Under high irradiance, CCM is expressed under ambient pCO<sub>2</sub> levels but is down-regulated with increasing pCO<sub>2</sub> (Wu et al. 2010, Li et al. 2014). There are documented data showing that elevated pCO<sub>2</sub> stimulated growth of some diatoms grown under low light (Gao et al. 2012, Li & Campbell 2013), but adverse effects on photosynthetic performance and growth (see Gao & Campbell 2014 and literature therein) have also been reported. In addition, whether intracellular inorganic carbon pools in diatoms would increase or decrease under OA conditions has not been experimentally demonstrated.

*Phaeodactylum tricornutum*, widely distributed around the world, is universally known as a model diatom for studies. The completely sequenced genome (<http://genome.jgi-psf.org/Phatr2/Phatr2.home.html>) permits follow-up studies depending on genetic information. Here, we grew the marine diatom *P. tricornutum* under growth-limiting (LL) and growth-saturating (HL) light levels at 4 different pCO<sub>2</sub> levels from 400 to 5000 µatm and showed that OA at the level expected by the end of the century (pH 7.8, 1000 µatm pCO<sub>2</sub>) caused a slight increase in carbon assimilation and growth. However, more intense

acidification at higher levels of pCO<sub>2</sub> partially reduced activity of CCM and internal inorganic carbon pools and had a negative impact on cells' carbon fixation capacity. We also showed that the harmful impacts of OA were enhanced at HL, but much reduced at the LL level, under energy-limited conditions.

## MATERIALS AND METHODS

### Organism model and algal culture conditions

A culture of *Phaeodactylum tricornutum* strain CCMA 106 was obtained from the Center for Collections of Marine Bacteria and Phytoplankton (CCMBP) of the State Key Laboratory of Marine Environmental Science (Xiamen University). This strain was originally isolated from the South China Sea. It was grown in artificial seawater with Aquil (Morel et al. 1979) medium enrichment. We diluted the cultures semi-continuously every 24 h and aerated them at 350 ml min<sup>-1</sup> with air containing target CO<sub>2</sub> concentrations of 400, 950, 2250 or 5000 µatm. The pCO<sub>2</sub> levels were obtained by a CO<sub>2</sub> enricher (CE-100B, Wuhan Ruihua Instrument & Equipment, China), and the target pCO<sub>2</sub> levels were checked with a carbon dioxide sensor (GM70, Vaisala, Finland). Elevated pCO<sub>2</sub> levels are the driver for acidification of seawater, and the procedure here follows standard best practice for OA research (Riebesell et al. 2010). Cells at densities between 2.5 × 10<sup>4</sup> and 1.8 × 10<sup>5</sup> ml<sup>-1</sup> were grown under a 12 h light:12 h dark photoperiod at 200 or 20 µmol photons m<sup>-2</sup> s<sup>-1</sup> and 20°C. The compensation point for growth of *P. tricornutum* has been reported to be 0.5 µmol photons m<sup>-2</sup> s<sup>-1</sup> (Geider et al. 1986). Experiments were initiated after the alga had acclimated to each treatment condition for at least 8 generations and only after a constant growth rate had been recorded for 3 further consecutive sampling periods.

### Carbonate system chemistry in cultures

The pH was measured 3 to 5 times prior to dilution for each treatment with a pH meter (Benchtop pH510; OAKTON) calibrated with the National Bureau of Standards buffer solution (Hanna). With the known values of pH, salinity, nutrients, temperature, and pCO<sub>2</sub>, other associated parameters of the carbonate chemistry of the cultures were estimated using the program CO<sub>2</sub>SYS (Lewis & Wallace 1998). The equilibrium constants (K<sub>1</sub> and K<sub>2</sub>) for carbonic

acid (Roy et al. 1993) and boric acid (Dickson 1990) dissociation were used for all calculations.

### Growth rate and chlorophyll measurements

We measured cell numbers with a Coulter Counter (Z-2, Beckman) before and after culture dilution, and growth rate was calculated according to the following equation:  $\mu = (\ln N_1 - \ln N_0) / (t_1 - t_0)$ , where  $N_0$  and  $N_1$  refer to the cell concentration immediately after the dilution and before the next dilution, respectively, and  $t_1 - t_0$  is the time (days, 1 d in this experiment) between the dilutions. For chlorophyll *a* (chl *a*) determination, 150 ml samples under steady state growth conditions were filtered onto a Whatman GF/F filter (25 mm), extracted in 5 ml 100% methanol overnight at 4°C, and then centrifuged at 5000 × *g* for 10 min. The absorbance of the supernatant was scanned from 250 to 800 nm with a scanning spectrophotometer (DU 800, Beckman Coulter). The cellular chl *a* and carotenoid content were calculated according to the equations of Ritchie (2006) and Strickland & Parsons (1968), respectively.

### Photosynthetic carbon fixation

Cells were incubated under the growth light and pCO<sub>2</sub> levels (initial cell concentration was 5000 cell ml<sup>-1</sup>; fresh medium was aerated with target gas before incubation) with 5 μCi (0.185 MBq) NaH<sup>14</sup>CO<sub>3</sub> (ICN Radiochemicals, Irvine, CA, USA) for 12 h (full light period only) or 24 h (light and the following dark period), respectively, and samples were filtered and collected every 12 h. The samples were then exposed to fuming HCl overnight and dried over the following 6 h, as previously reported (Gao et al. 2007). The <sup>14</sup>C assimilated into organic matter was counted using a liquid scintillation counter (Tri-Carb 2800TR, Perkin-Elmer, Waltham, USA). The carbon fixation rate was normalized to the initial cell concentration.

The relationship between photosynthesis and dissolved inorganic carbon (P vs. C curves) for *P. tricornutum* was measured at 20°C with 50 mM HEPES to maintain the pH at 8.0 under a photosynthesis-saturating light level of 400 μmol photons m<sup>-2</sup> s<sup>-1</sup> (Geider et al. 1985, Zou & Gao 2014) at different levels of DIC.

### Determination of intracellular inorganic carbon

The intracellular inorganic carbon pools were measured using the silicone oil centrifugation tech-

nique (Badger et al. 1980, Johnston & Raven 1996, Tortell et al. 2000). Inorganic C-depleted cell suspensions (buffered with 50 mM HEPES) were firstly prepared and placed in the dark. Prior to addition to centrifuge tubes that contained 100 μl of 2.5 N NaOH in 10% methanol (bottom layer) and 200 μl silicone oil (middle layer), 200 μl aliquots of inorganic C-depleted cell suspensions were pre-illuminated for 2 min under 600 μmol photons m<sup>-2</sup> s<sup>-1</sup> (Ratti et al. 2007). Subsequently, as soon as 100 μl of NaH<sup>14</sup>CO<sub>3</sub> stock solution (5 μCi, 0.185 MBq) was injected into the individual tube, the cell suspensions were mixed thoroughly and incubated at the same light intensity for 10 s. Suspensions were then centrifuged at 18 000 × *g* for 40 s to terminate the incubations, separating the cells from the <sup>14</sup>C solution by passage through the middle (oil) layer into the NaOH-methanol solution. We then froze the tubes immediately with liquid nitrogen, followed by removal of the cell pellets and their resuspension into scintillation vials containing 1 ml of 0.5 N NaOH. After mixing thoroughly, 0.5 ml of the resuspended pellet solution was transferred into another fresh vial, thereby obtaining 2 subsamples. One of these was used for estimation of organic C by acidification with 0.5 ml of 2 N HCl and leaving the contents to degas unassimilated <sup>14</sup>HCO<sub>3</sub><sup>-</sup> for at least 12 h. The second sample was left in 0.5 N NaOH and provided a count of inorganic plus organic <sup>14</sup>C. The amount of radioactivity was determined in each sample by liquid scintillation counting. The amount of inorganic C inside the cells is given by the difference between total (inorganic + organic) and acid-stable (organic) <sup>14</sup>C in the cell pellets.

Intracellular inorganic C concentrations were calculated by normalizing the amount of carbon to intracellular volume (mannitol-impermeable), which was measured by incubating samples for 30 to 40 s with <sup>3</sup>H<sub>2</sub>O and <sup>14</sup>C-mannitol and centrifugation as described above. These labelled compounds provide an estimation of total biovolume (<sup>3</sup>H<sub>2</sub>O) and extracellular H<sub>2</sub>O space (D-[1-<sup>14</sup>C]-mannitol) (Badger et al. 1980, Ratti et al. 2007).

We carried out the experiment for the determination of intracellular volume and extracellular water under low light and 20°C conditions.

### Photochemical parameters

To estimate the photochemical responses of the cells to changes in pCO<sub>2</sub> and light intensities, the maximum photochemical quantum yield ( $F_v/F_m$ ), effective quantum yield in the light ( $\Phi_{PSII}$ ) and rapid

light curves (RLCs) were measured using a Xenon-Pulse Amplitude Modulated fluorometer (XE-PAM, Walz, Germany).  $F_v$  is the maximum wavelength of the variable fluorescence when all the non-photochemical processes are minimum under dark adaptation state;  $F_m$  is the maximum fluorescence when all the reaction centers of photosynthetic system II are closed and all the non-photochemical processes are minimum under dark adaptation state. RLCs determined the relative electron transport rates (rETR) when cells were exposed to 8 different and increasing light levels (0, 76, 156, 226, 337, 533, 781, 1077 and 1593  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), with a 15 s duration of exposure to each level separated by a 0.8 s saturating pulse (3000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ).

At the middle of the photoperiod, samples were taken and kept in the dark for 15 min prior to determination of  $F_v/F_m$  and RLC.  $\Phi_{\text{PSII}}$  was measured at the actinic light levels close to the growth light intensities 76 (a growth-limiting light level) and 226  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (a growth-saturating light level, Gao et al. 2012). rETR at each light level was calculated as  $\text{rETR} = \Phi_{\text{PSII}} \times 0.5 \times \text{PPFD}$ , where  $\Phi_{\text{PSII}}$  is the effective photochemical quantum yield of PSII, and the factor 0.5 accounts for approximately 50% of all the absorbed energy being allocated to PSII. The rETR data were fitted to light intensity with the equation of Eilers & Peeters (1988):  $y = x/(ax^2 + bx + c)$ , where  $a$ ,  $b$ ,  $c$  are estimated parameters,  $x$  is photon flux density, and  $y$  is the rETR value. Maximal relative electron transport rate ( $\text{rETR}_{\text{max}}$ ) and light saturation point ( $E_k$ ) were then calculated according to the equations:  $\text{rETR}_{\text{max}} = 1/[b + 2(ac)^{1/2}]$ ,  $E_k = c/[b + 2(ac)^{1/2}]$ , and  $\alpha$  (light use efficiency) was determined as  $\alpha = 1/c$ .

### Data analysis

One-way ANOVA and Tukey tests as well as 2-sample  $t$ -tests were used to establish differences and interactions among these treatments. The significance level was set at  $p < 0.05$ .

## RESULTS

### Carbonate system

The pH levels in the different treatments varied from 8.17 ( $\pm 0.01$ ) to 7.19 ( $\pm 0.01$ ), being significantly different ( $p < 0.01$ ) across  $\text{pCO}_2$  levels but not across low- and high-light acclimations under the same  $\text{pCO}_2$  level ( $p > 0.05$ ) (see Table S1 in the Supplement at

[www.int-res.com/articles/suppl/a079p137\\_supp.pdf](http://www.int-res.com/articles/suppl/a079p137_supp.pdf)). With increased  $\text{pCO}_2$  levels, DIC changed from  $1918.91 \pm 45.87$  (SD) to  $2547.50 \pm 16.95 \mu\text{mol kg}^{-1}$ , though the changes in total alkalinity (TA) across all treatments were  $< 6\%$ . During the process of  $\text{pCO}_2$  increasing from 400 to 5000  $\mu\text{atm}$ ,  $\text{HCO}_3^-$  and  $\text{CO}_{2(\text{aq})}$  concentrations increased by 36.67% and 115%, respectively, and  $\text{CO}_3^{2-}$  concentration decreased approximately 7-fold.

### Growth and chlorophyll responses

Light intensity had a significant effect on the growth of *Phaeodactylum tricornutum*, such that the growth rates of the HL cultured cells were more than twice those at low light ( $p < 0.01$ ). For the HL cultured cells, the peak value of growth rate was recorded at 950  $\mu\text{atm pCO}_2$  (Fig. 1). Compared to the 400  $\mu\text{atm pCO}_2$  treatment, a significant difference ( $p = 0.004$ ) was detected. When  $\text{pCO}_2$  was increased further (to 5000  $\mu\text{atm}$ ), we observed a considerable decline in the growth of the HL grown cells. Additionally, the variation in growth rate with  $\text{pCO}_2$  of the LL cultured cells was relatively minor compared with that of the HL incubated algae. Similarly, compared to the 400  $\mu\text{atm pCO}_2$  treatment, small but statistically significant enhancements of growth were observed for the LL cells grown under the 950  $\mu\text{atm}$  ( $p = 0.045$ ) and 2250  $\mu\text{atm}$  ( $p = 0.018$ ) conditions. However, the

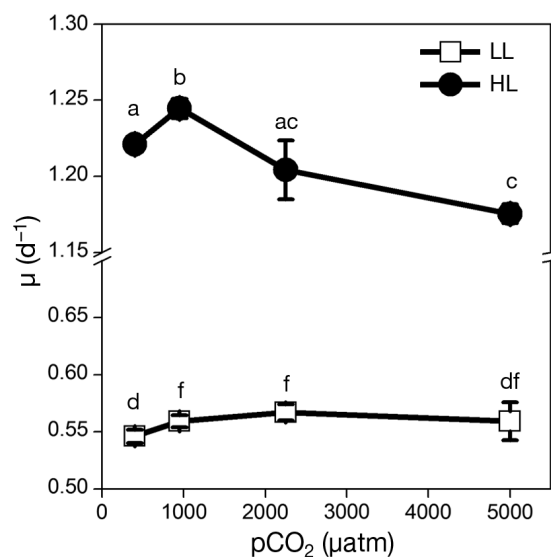


Fig. 1. Growth rates of ( $\mu$ ) *Phaeodactylum tricornutum* under a matrix of  $\text{pCO}_2$  and light. Different letters indicate significant differences among all treatments including different  $\text{pCO}_2$  and light conditions. Data are means  $\pm$  SD,  $n = 3$ . HL: high light, 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; LL: low light, 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$

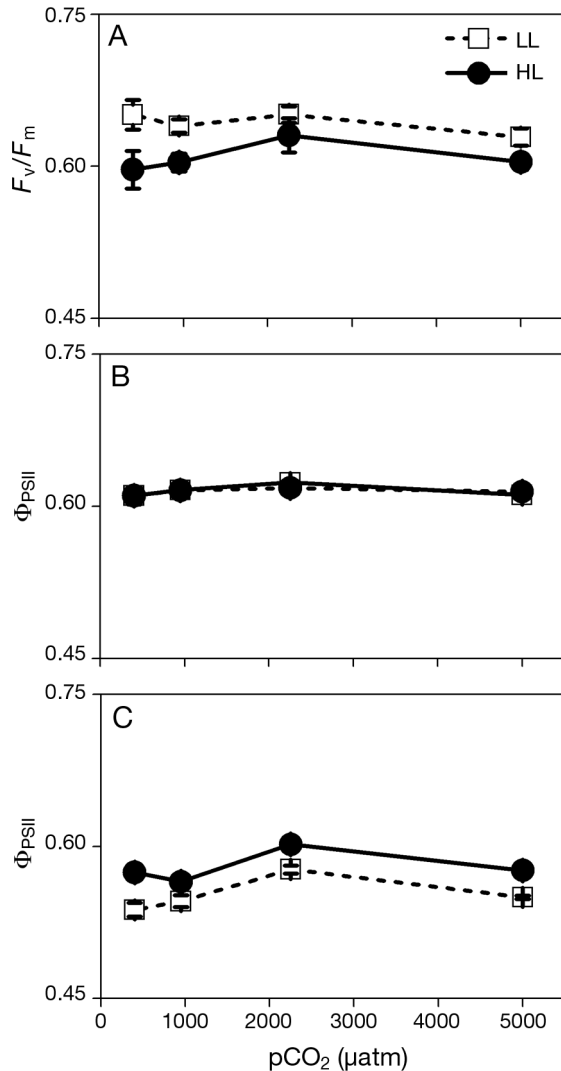


Fig. 2. (A) Maximum photochemical quantum yield ( $F_v/F_m$ ) measured after dark incubation. (B) Effective quantum yield in the light ( $\Phi_{PSII}$ ) assessed under  $76 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , limiting light for growth. (C)  $\Phi_{PSII}$  assessed under  $226 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , saturation light for growth. LL and HL represent cells cultured under 20 and  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for at least 8 generations, respectively. Data are means  $\pm$  SD,  $n = 3$

LL acclimated cells' growth rate showed almost no change when the pCO<sub>2</sub> increased to 5000  $\mu\text{atm}$  (Fig. 1). Overall, the response of growth rate to CO<sub>2</sub> was much less in the LL acclimated cells than in the HL treatments.

Analysing across all the pCO<sub>2</sub> levels, there was a significant difference in chl *a* content between the 2 light intensity treatments ( $p < 0.01$ ; see Table S2 in the Supplement), with marked enhancements, varying from 34.30% to 51.4%, under low-light conditions compared with high light. The chl *a* content was, however, not significantly affected by pCO<sub>2</sub> lev-

els. The carotenoid contents of cells were unaffected by pCO<sub>2</sub> or light levels. The cells grown under high light showed higher ratios of carotenoids to chl *a*, and a significant difference in this ratio was observed between the 2 light levels. For the high light level, compared to the low pCO<sub>2</sub> treatments, this ratio decreased markedly for algae cultured at high pCO<sub>2</sub> levels, including 2250 and 5000  $\mu\text{atm}$  ( $p < 0.001$ ; Table S2).

### Photochemical responses

$F_v/F_m$  and  $\Phi_{PSII}$  at 76 and 226  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  are shown in Fig. 2. The variation of pCO<sub>2</sub> levels had no significant influence on the maximal quantum yield and effective quantum yield.  $E_k$  and  $\text{rETR}_{\text{max}}$  of *P. tricornutum* increased with light and pCO<sub>2</sub> levels (Fig. 3A,B). However, regardless of the different light treatments, the change in the light use efficiency  $\alpha$  with pCO<sub>2</sub> was small at LL and absent in HL cultures (Fig. 3C), although absolute values of  $\alpha$  were 1.20- to 1.34-fold higher in LL cultures than in those of HL cells (Fig. 3D).

### Carbon fixation

The net carbon fixation rates at the growth light levels over 12 h or 24 h are shown in Fig. 4. The carbon fixation rate for the HL acclimated cells was 2.1- to 3.9-fold that of the LL acclimated cells. Across all the light and incubation period treatments, carbon fixation varied in the same way with different pCO<sub>2</sub> treatments. As shown in Fig. 4, the same trend of an initial slight increase to 950  $\mu\text{atm}$  then a decrease in fixation rates at the highest pCO<sub>2</sub> was observed. However, except for the HL and 5000  $\mu\text{atm}$  acclimated cells, no significant difference of 24 h carbon fixation rates with pCO<sub>2</sub> were found due to a large variance in the data (Fig. 4B). As shown in Fig. 4A, considerable changes in 12 h carbon fixation rate were observed for both light intensity treatments, but with a similar trend of a slight increase from 400 to 950  $\mu\text{atm}$  and then a decrease at higher CO<sub>2</sub> levels.

### Affinity of cells for dissolved inorganic carbon

The conventional measure of affinity of cells for inorganic carbon is  $K_{0.5}$ ; however, this parameter was strongly affected by the significant decrease in maximal rates of carbon fixation and truncation of P vs.

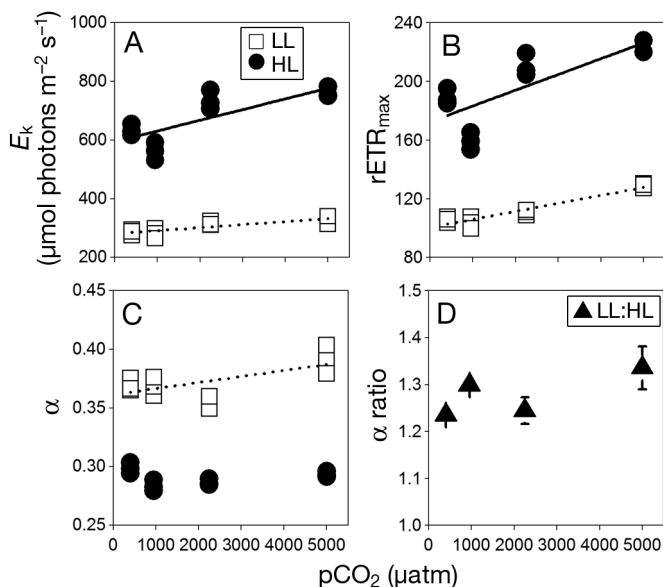


Fig. 3. Fluorescence parameters calculated from the rapid light curve relative to  $p\text{CO}_2$  levels under different light intensities. (A) Light saturation point ( $E_k$ ),  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; low light (LL,  $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ );  $R^2 = 0.65$  ( $p < 0.01$ ); high light (HL,  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ),  $R^2 = 0.58$  ( $p < 0.01$ ). (B) Maximal relative electron transport rate ( $r\text{ETR}_{\text{max}}$ ); LL:  $R^2 = 0.90$  ( $p < 0.01$ ); HL:  $R^2 = 0.55$  ( $p < 0.01$ ). (C) Light use efficiency ( $\alpha$ ); LL:  $R^2 = 0.36$  ( $p < 0.05$ ); HL: showed very low  $R^2$ . (D) The ratio of  $\alpha$  for LL acclimated cells to that of HL acclimated cells showed very low  $R^2$ . The solid line and short dotted line represent the best fit for the HL and LL cultured cells, respectively. LL and HL represent cells cultured under 20 and  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for at least 8 generations, respectively. Data are means  $\pm$  SD,  $n = 3$ . One data point, for  $\alpha$  at  $2250 \mu\text{atm}$  at low light, was an evident outlier and was deleted from the analysis

DIC curves in cells shown in Fig. S1 (in the Supplement), and so is not used here (data shown in Table S3 in the Supplement), though for the cells grown under HL,  $K_{0.5}$  values increased significantly from 400 to  $950 \mu\text{atm}$  then leveled off at higher  $\text{CO}_2$  levels. Values of the slopes of carbon fixation vs. DIC (conductance) were higher in the cells grown under HL than in cells from LL conditions, but in both HL and LL cells, values were approximately stable until  $p\text{CO}_2$  for growth exceeded  $2250 \mu\text{atm}$  (Fig. 5A). However, in comparison to the  $400 \mu\text{atm}$ , the conductance declined considerably at  $5000 \mu\text{atm}$ , by 48% and 55% for the LL and HL treatments, respectively.

Maximal values of  $V_{\text{max}}$  were recorded at 950 and  $2250 \mu\text{atm}$  for the LL and HL cultures, respectively (Fig. 5B).  $V_{\text{max}}$  of cells grown under  $5000 \mu\text{atm}$  was significantly lower for both light treatments, compared to the maximum values. When rates of C fixation at the growth  $\text{CO}_2$  concentrations are calculated from the data shown in Fig. S1, a peak in C fixation

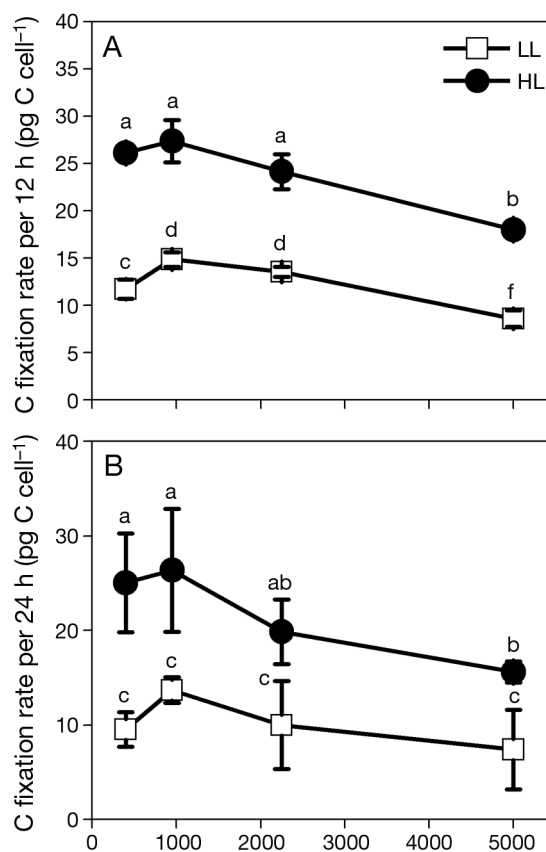


Fig. 4. Carbon fixation per cell during (A) daytime (12 h) and (B) on a daily basis (24 h) in *Phaeodactylum tricornutum* cells grown under different levels of  $p\text{CO}_2$  and light (LL:  $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; HL:  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Different letters above the symbols present significant differences among the treatments. Data are the means  $\pm$  SD,  $n = 3$  (triplicate independent cultures)

rate is found at  $2250 \mu\text{atm}$  with rates decreasing at higher  $\text{CO}_2$  concentration (Fig. S3).

The silicone oil centrifugation data showed that the intracellular volume was about  $60 \mu\text{m}^3$  with no significant difference among the treatments. However, as shown in Fig. 6A, cells cultured at  $5000 \mu\text{atm}$   $p\text{CO}_2$  had much lower (60.1 to 80.6%) intracellular inorganic carbon concentration than those grown at  $400 \mu\text{atm}$   $p\text{CO}_2$ . In the cells grown under  $5000 \mu\text{atm}$   $p\text{CO}_2$ , the internal pool was 603 and  $1639 \mu\text{mol l}^{-1}$  under the LL and HL conditions respectively, representing about a 5.3-fold drop ( $2603.5 \pm 282.8 \mu\text{mol l}^{-1}$ ) in the intracellular DIC pool for the LL cultures and a 2.5-fold decline ( $2473.2 \pm 248.1 \mu\text{mol l}^{-1}$ ) in the cells grown under HL compared to the cells acclimated to  $400 \mu\text{atm}$  (Fig. 5A). The percentage decline of internal DIC concentration was also considerably ( $p = 0.032$ ,  $t$ -test) impacted by different light treatments.

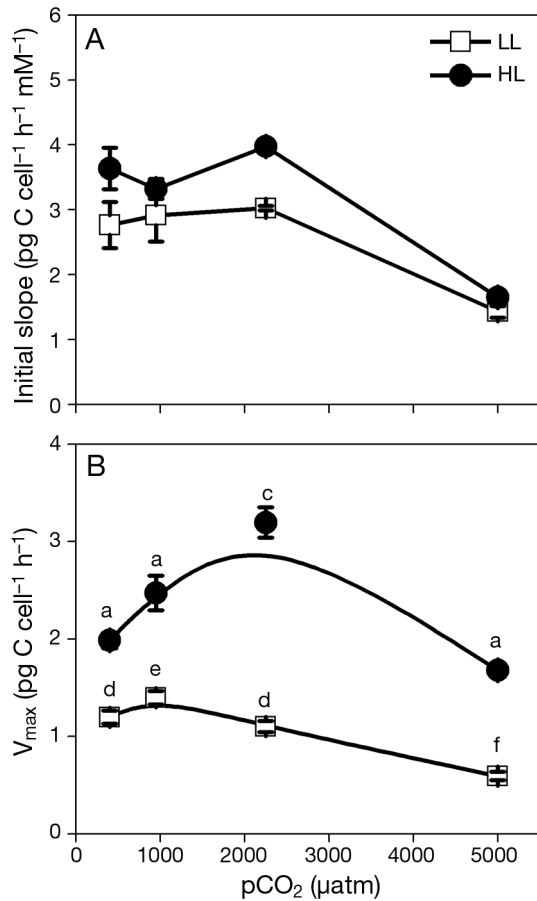


Fig. 5. Parameters derived from Fig. S1 are shown under a matrix of pCO<sub>2</sub> and light for *Phaeodactylum tricornutum*. (A) Initial slope (see Fig. S1) of photosynthesis vs. dissolved inorganic carbon (DIC, pg C cell<sup>-1</sup> h<sup>-1</sup> mM<sup>-1</sup>); (B) DIC-saturated and light-saturated rates of photosynthesis, V<sub>max</sub>, which was calculated according to the Michaelis-Menten function. Data are the means ± SD, n = 3. Different letters above the symbols indicate significant differences among the treatments, each of which was represented by triplicate cultures grown under low light (LL, 20 μmol photons m<sup>-2</sup> s<sup>-1</sup>) and high light (HL, 200 μmol photons m<sup>-2</sup> s<sup>-1</sup>) conditions

Since it is the increased availability of CO<sub>2</sub> at the active site of Rubisco (the pyrenoid, in species that have them; Beardall 1991, Beardall & Giordano 2002, Hopkinson et al. 2011) rather than overall cellular accumulation of DIC that determines CCM activity (but acknowledging that it is impossible to determine [DIC] and pH in specific cell organelles), we used a value for mean internal pH of 7.59 for microalgae cells (Burns & Beardall 1987, Raven 1990) and thus assessed the accumulation factors ([CO<sub>2</sub>]<sub>inside</sub>: [CO<sub>2</sub>]<sub>outside</sub>) based on the calculated average internal CO<sub>2</sub> levels. These factors were estimated to be 14.7 and 11.5 for cells grown at 400 μatm under HL and LL conditions, respectively (Fig. 6B). At 5000 μatm

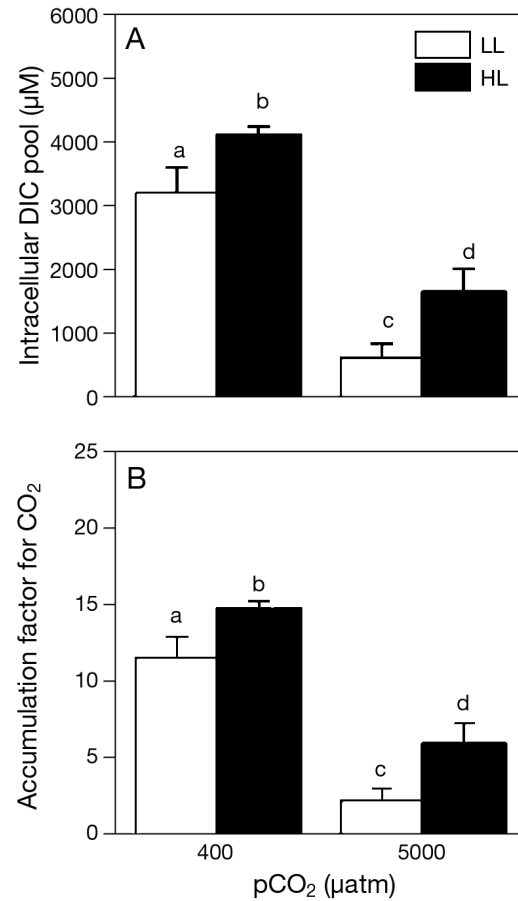


Fig. 6. Intracellular DIC pool and accumulation factor for CO<sub>2</sub> measured through silicone oil centrifugation experiments. (A) intracellular dissolved inorganic carbon (DIC) pools of *Phaeodactylum tricornutum* grown under 400 and 5000 μatm pCO<sub>2</sub> as well as the low light (LL, 20 μmol photons m<sup>-2</sup> s<sup>-1</sup>) and high light (HL, 200 μmol photons m<sup>-2</sup> s<sup>-1</sup>) levels; (B) accumulation factor for CO<sub>2</sub> for different treatments. Different letters above the columns indicate significant differences among the treatments, each of which was represented by triplicate cultures. Error bars = 1 SD

pCO<sub>2</sub>, they dropped to 5.9 at HL and to 2.2 (Fig. 6B) at LL. In short, therefore, the cells grown under LL and high CO<sub>2</sub> had the smallest intracellular DIC pools: about 5.8-fold smaller than that in the cells grown under the HL and low CO<sub>2</sub> levels.

## DISCUSSION

### Acid stress under high CO<sub>2</sub>?

Under the high light level for growth, elevated pCO<sub>2</sub> at the concentration expected by the end of the century caused a small, but statistically significant, rise in growth rates; pCO<sub>2</sub> at the higher concentra-

tions, representing what might be found in upwellings and volcanic seeps, caused a decline in growth. However, the decline in growth rate at higher  $p\text{CO}_2$  levels was not the case at low light, where growth was almost independent of  $p\text{CO}_2$ . The reasons contributing to this phenomenon, observed under HL conditions, could be twofold: (1) photoinhibition and (2) acid stress.

In relation to photoinhibition, cells grown under both HL and LL conditions showed no effect on maximal quantum yield of PSII with increased  $p\text{CO}_2$  and exhibited increases in parameters such as  $E_k$  and  $r\text{ETR}_{\text{max}}$  at higher  $\text{CO}_2$  levels. Similar responses to elevated  $p\text{CO}_2$  have also been shown for the cyanobacterium *Cylindrospermopsis* (Pierangelini et al. 2014), indicating that higher electron transport rates were achieved when higher  $p\text{CO}_2$  was applied to the cells, and that photoinhibition, in terms of  $r\text{ETR}$  at least, was not occurring under any of the treatments, though photorespiratory  $\text{O}_2$  consumption has been reported to be enhanced by ~26% in the same species grown under 1000  $\mu\text{atm } p\text{CO}_2$  (Gao et al. 2012). This lack of photoinhibition was also demonstrated by the absence of significant non-photochemical quenching (NPQ) for the cells under all incubation conditions in this experiment (data not shown). Thus, photoinhibition is out of the question as a driver of the significantly decreased growth rates at high  $p\text{CO}_2$ , and acid stress might therefore be the cause. The effects of  $p\text{CO}_2$  were stronger at saturating light intensity than under limiting light, except for the light harvesting efficiency ( $\alpha$ ), which was enhanced at low light but decreased with increased photosynthetic affinity for  $\text{CO}_2$  (CCM activity) (Fig. S2). Meanwhile, these results demonstrate that OA did not stress the photochemical reactions of PSII, even under intense OA induced at 5000  $\mu\text{atm } p\text{CO}_2$ .

#### Acid stress vs. downregulation of the $\text{CO}_2$ concentrating mechanisms

It is possible that the impact of acidification overwhelmed any performance-enhancing effects of increased  $p\text{CO}_2$  and energy saving from down-regulation of the CCM, a feature previously suggested for a coccolithorporid (Fukuda et al. 2014). When pH *in milieu* declines due to increased  $p\text{CO}_2$ , cells usually acclimate to maintain the normal operation of redox systems on the plasma membrane (Suffrian et al. 2011) and drive  $\text{H}^+$  efflux to maintain a constant intracellular pH environment via proton pumps and/or operation of the  $\text{H}^+$ -translocating ATPase

(Madhus 1988), a process that is energy demanding (Smith & Raven 1979, Suffrian et al. 2011). Extra  $\text{H}^+$ -efflux by  $\text{H}^+$ -ATPase leads to a more acidic local environment surrounding the cell, which could have impacts on the redox systems of the plasma membrane, associated with nutrient (iron, nitrate, etc.) uptake, through the influence on the activities of transmembrane and apoplastic-side plasma membrane enzymes (Jones & Morel 1988, Moog & Brüggemann 1994). While down-regulation of the CCM in diatoms can save energy involved in active inorganic carbon (Ci) acquisition (Hopkinson et al. 2011, Hennon et al. 2014), direct measurements of intracellular  $p\text{CO}_2$  levels in these cells under OA have not previously been carried out. In the current work, we have demonstrated that the inorganic carbon pools within the diatom cells declined with elevated  $p\text{CO}_2$  within the range expected from climate change and natural high  $\text{CO}_2$  systems in the oceans.

As reflected in the initial slope of photosynthesis-dissolved inorganic carbon curves, which correlates with photosynthetic affinity for DIC (Beardall 1991, Johnston et al. 1992), CCM activity of the cells grown under HL and LL was significantly down-regulated with increasing  $p\text{CO}_2$ . The intracellular dissolved inorganic carbon pool, assessed with the silicone oil technique, distinctly decreased under the elevated  $\text{CO}_2$  level, regardless of HL and LL conditions (there was a significant difference between the decline for the 2 light intensities,  $p = 0.032$ ). The percentages of CCM down-regulation for the HL and LL cells were 60% and 81%, respectively, confirming that the down-regulation of the CCM under the high  $\text{CO}_2$  conditions (400  $\mu\text{atm}$  vs. 5000  $\mu\text{atm}$ ) was more pronounced under the LL treatment.

The mismatch between changes in the photosynthetic affinity of cells for DIC and differences in intracellular DIC pools could be explained by the localized DIC hypothesis (Raven 1997), whereby cells could accumulate  $\text{CO}_2$  around the site of Rubisco activity without the necessity for a high overall intracellular DIC (Matsuda et al. 2011, Hopkinson 2014). Therefore, the relatively lower intracellular DIC pool for the cells grown under 5000  $\mu\text{atm } p\text{CO}_2$  may not reflect the true activity of steady state CCM for *P. tricornutum*. Associated with the combined effects of both increased harm from the pH decline and benefits from increased DIC availability at elevated  $p\text{CO}_2$ , we expected that there may be a point beyond which the CCM is not further down-regulated, though the point cannot be demonstrated well from our data. On the basis of the changes in initial slope of P vs. DIC curves within our experiments, we



expect that the point for this balance could be above 5000  $\mu\text{atm}$  and vary under different light treatments. Additionally, the balance point may be quite different for different DIC utilization mechanisms that are used by other algae (Aizawa & Miyachi 1986, Shiraiwa 2003, Tsuji et al. 2009).

In addition, although no impact on light harvesting efficiency ( $\alpha$ ) from increased CO<sub>2</sub> was observed for the HL cultured cells, that for the LL grown cells was positively related to the increasing pCO<sub>2</sub> (Fig. 3C). These observations may be due to the energetic limitation on the capacity of the cells grown under LL to utilize HCO<sub>3</sub><sup>-</sup> by active transport and to develop CCM, thus enhancing their reliance on CO<sub>2</sub> diffusion (Hepburn et al. 2011, Gao & Campbell 2014). However, relatively highly expressed CCM was employed by the cells grown under HL. Furthermore, the growth rate and carbon fixation rate as well as relative electron transport were much higher for the HL cultured cells, which thus needed more DIC to achieve their growth rates compared to the ones grown under LL. As a result, the energy saved from the down-regulation of CCM activity may be used by the LL cultured cells to better deal with the pH decline as the pCO<sub>2</sub> increased in comparison with the HL cells (Raven et al. 2000, Li & Campbell 2013, Wu et al. 2010). This is supported by Fig. S2, which shows that  $\alpha$  was negatively correlated with the initial slope of P vs. DIC curves at LL, but not in HL cultures, i.e. lower CCM activity was associated with greater efficiency of light harvesting. The major energy expenditure on the CCM is in the transport of DIC into the chloroplast (1 ATP per HCO<sub>3</sub><sup>-</sup> molecule; Raven et al. 2000, Hopkinson et al. 2011) with 0.5 ATP for HCO<sub>3</sub><sup>-</sup> transported across the external membrane of the cell. A 50% reduction in CCM capacity in cells grown at 5000  $\mu\text{atm}$  CO<sub>2</sub> would thus save 0.75 ATP per CO<sub>2</sub> delivered, with the suppression of the photosynthetic carbon oxidation cycle by elevated CO<sub>2</sub> at the active site providing additional energy savings. Energy costs of pH regulation are complex, depending on a range of factors, including nutrient source and mechanism of pH homeostasis (Smith & Raven 1979). The influence of increased environmental H<sup>+</sup> concentration on the cell is not only related to pumping out the extra H<sup>+</sup> from the cells but also involves other complex cellular activities, so the energy costs associated with the decreased external pH cannot be calculated easily. Growth rate and carbon fixation rate may be more reliable metrics for demonstrating the pH effects.

Therefore, the decreased growth rates under higher pCO<sub>2</sub> levels in the HL cells were most likely attrib-

table to acid stress. Compared to the large requirement for DIC acquisition by the HL cultured alga, the CO<sub>2</sub>-diffusion-dependent cells grown under LL with little CCM activity must have benefited more from the increased CO<sub>2(aq)</sub>. Our data demonstrate that seawater acidification, whether global due to anthropogenic forcings or regional due to coastal upwelling or areas of CO<sub>2</sub> seeps, will influence the physiology of diatoms, as exemplified by *P. tricornutum*. The down-regulation of CCM operation, leading to energy savings, partially overcame the negative impact of exacerbated acidity resulting from very high pCO<sub>2</sub>. When we consider the impacts of regional acidification, the light available at different depths of water is likely to play an important role in regulating the physiological performance of phytoplankton, and interactions between CO<sub>2</sub> and light levels will be critical in this situation. Clearly, the responses shown here for *P. tricornutum* need to be investigated in a wider range of ecologically relevant species before any broad conclusions can be reached.

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