Regulation of inorganic carbon acquisition in a red tide alga (Skeletonema costatum): the importance of phosphorus availability

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Abstract:

*Skeletonema costatum* is a common bloom-forming diatom and encounters eutrophication and severe carbon dioxide (CO$_2$) limitation during red tides. However, little is known regarding the role of phosphorus (P) in modulating inorganic carbon acquisition in *S. costatum*, particularly under CO$_2$ limitation conditions. We cultured *S. costatum* under five phosphate levels (0.05, 0.25, 1, 4, 10 μmol L$^{-1}$) and then treated it with two CO$_2$ conditions (2.8 and 12.6 μmol L$^{-1}$) for two hours. The lower CO$_2$ reduced net photosynthetic rate at lower phosphate levels (< 4 μmol L$^{-1}$) but did not affect it at higher phosphate levels (4 and 10 μmol L$^{-1}$). In contrast, the lower CO$_2$ induced higher dark respiration rate at lower phosphate levels (0.05 and 0.25 μmol L$^{-1}$) and did not affect it at higher phosphate levels (> 1 μmol L$^{-1}$). The lower CO$_2$ did not change relative electron transport rate (rETR) at lower phosphate levels (0.05 and 0.25 μmol L$^{-1}$) and increased it at higher phosphate levels (> 1 μmol L$^{-1}$). Photosynthetic CO$_2$ affinity ($1/K_{0.5}$) increased with phosphate levels. The lower CO$_2$ did not affect photosynthetic CO$_2$ affinity at 0.05 μmol L$^{-1}$ phosphate but enhanced it at the other phosphate levels. Activity of extracellular carbonic anhydrase was dramatically induced by the lower CO$_2$ at phosphate replete conditions (> 0.25 μmol L$^{-1}$) and the same pattern also occurred for redox activity of plasma membrane. Direct bicarbonate (HCO$_3^-$) use was induced when phosphate concentration was more than 1 μmol L$^{-1}$. These findings indicate P enrichment could enhance inorganic carbon acquisition and thus maintain the photosynthesis rate in *S. costatum* grown under CO$_2$ limiting conditions via increasing activity of extracellular carbonic anhydrase and facilitating direct HCO$_3^-$ use. This study sheds light on how bloom-forming algae cope with carbon limitation during the development of red tides.
Keywords: carbonic anhydrase; CO₂ concentrating mechanisms; pH compensation point; photosynthesis; redox activity; respiration
1. Introduction

Diatoms are unicellular photosynthetic microalgae that can be found worldwide in freshwater and oceans. Marine diatoms account for 75% of the primary productivity for coastal and other nutrient-rich zones and approximately 20% of global primary production (Field et al., 1998; Falkowski, 2012), hence playing a vital role in the marine biological carbon pump as well as the biogeochemical cycling of important nutrients, such as nitrogen and silicon (Nelson et al., 1995; Moore et al., 2013; Young & Morel, 2015). Diatoms usually dominate the phytoplankton communities and form large-scale blooms in nutrient–rich zones and upwelling regions (Bruland et al., 2001; Anderson et al., 2008; Barton et al., 2016).

Nutrient enrichment is considered as a key factor that triggers algal blooms albeit the occurrence of diatom blooms may be modulated by other environmental factors, such as temperature, light intensity, salinity, and so forth (Smetacek & Zingone, 2013; Jeong et al., 2015). When inorganic nitrogen and phosphorus are replete, diatoms can out-compete chrysophytes, raphidophytes and dinoflagellates (Berg et al., 1997; Jeong et al., 2015; Barton et al., 2016) and dominate algal blooms due to their quicker nutrient uptake and growth rate.

In normal natural seawater (pH 8.1, salinity 35), HCO$_3^-$ is the majority (~90%) of total dissolved inorganic carbon (DIC, 2.0–2.2 mM). CO$_2$ (1%, 10–15 μM), which is the only direct carbon source that can be assimilated by all photosynthetic organisms, only accounts for 1% of total dissolved inorganic carbon. Diatoms’ ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), catalyzing the primary chemical reaction by which CO$_2$ is transformed into organic carbon, has a relatively low affinity for CO$_2$ and is commonly less than half saturated under current CO$_2$ levels in seawater (Hopkinson & Morel, 2011),
suggesting that CO$_2$ is limiting for marine diatoms’ carbon fixation. To cope with the CO$_2$
limitation in seawater and maintain a high carbon fixation rate under the low CO$_2$ conditions,
diatoms have evolved various inorganic carbon acquisition pathways and CO$_2$ concentrating
mechanisms (CCMs), for instance, active transport of HCO$_3^-$, the passive influx of CO$_2$,
multiple carbonic anhydrase (including both common ($\alpha$, $\beta$, $\gamma$) and unusual ($\delta$, $\zeta$) families that
carries out the fast interconversion of CO$_2$ and HCO$_3^-$), assumed C4-type pathway (using
phosphoenolpyruvate to capture more CO$_2$ in the periplastidal compartment), to increase the
concentration at the location of Rubisco and thus the carbon fixation. (Hopkinson & Morel,
2011; Hopkinson et al., 2016). Skeletonema costatum is a worldwide diatom species that can
be found from equatorial to polar waters. It usually dominates large-scale algal blooms in
eutrophic seawaters (Wang, 2002; Li et al., 2011). When blooms occur, seawater pH increases
and CO$_2$ decreases because the dissolution rate of CO$_2$ from the atmosphere cannot catch up
with its removal rate caused by intensive photosynthesis of algae. For instance, pH level in
the surface waters of the eutrophic Mariager Fjord, Denmark, could be up to 9.75 during algal
blooms (Hansen, 2002). Consequently, S. costatum experiences very severe CO$_2$ limitation
when blooms occur. To deal with it, S. costatum has developed multiple CCMs (Nimer et al.,
1998; Rost et al., 2003). However, contrasting findings were reported. Nimer et al. (1998)
documented that extracellular carbonic anhydrase activity in S. costatum was only induced
when CO$_2$ concentration was less than 5 µmol L$^{-1}$ while Rost et al. (2003) reported that
activity of extracellular carbonic anhydrase could be detected even when CO$_2$ concentration
was 27 µmol L$^{-1}$. Chen and Gao (2004) showed that in S. costatum had little capacity in direct
HCO$_3^-$ utilization. On the other hand, Rost et al. (2003) demonstrated that this species could
take up CO₂ and HCO₃⁻ simultaneously.

Phosphorus (P) is an indispensable element for all living organisms, serving as an integral component of lipids, nucleic acids, ATP and a diverse range of other metabolites. Levels of bioavailable phosphorus are very low in many ocean environments and phosphorus enrichment can commonly increase algal growth and marine primary productivity in the worldwide oceans (Davies & Sleep, 1989; Müller & Mitrovic, 2015; Lin et al., 2016). Due to the essential role of phosphorus, extensive studies have been conducted to investigate the effect of phosphorus on photosynthetic performances (Geider et al., 1998; Liu et al., 2012; Beamud et al., 2016), growth (Jiang et al., 2016; Reed et al., 2016; Mccall et al., 2017), phosphorus acquisition, utilization and storage (Lin et al., 2016; Gao et al., 2018a). Some studies show the essential role of phosphorus in regulating inorganic carbon acquisition in green algae (Beardall et al., 2005; Hu & Zhou, 2010). In terms of S. costatum, studies regarding the inorganic carbon acquisition in S. costatum focus on its response to variation of CO₂ availability. The role of phosphorus in S. costatum’s CCMs remains unknown. Based on the connection between phosphorus and carbon metabolism in diatoms (Brembu et al., 2017), we hypothesize that phosphorus enrichment could enhance inorganic carbon utilization and hence maintain high rates of photosynthesis and growth in S. costatum under CO₂ limitation conditions. In the present study, we aimed to test this hypothesis by investigating the variation of CCMs (including active transport of HCO₃⁻ and carbonic anhydrase activity) and photosynthetic rate under five levels of phosphate and two levels of CO₂ conditions. We also measured redox activity of plasma membrane as it is deemed to be critical to activate carbonic anhydrase (Nimer et al., 1998). Our study would provide helpful insights into how
bloom-forming diatoms overcome CO$_2$ limitation to maintain a quick growth rate during red tides.

2. Materials and Methods

2.1. Culture conditions

*Skeletonema costatum* (Grev.) Cleve from Jinan University, China, was cultured in f/2 artificial seawater with five phosphate levels (0.05, 0.25, 1, 4, 10 μmol L$^{-1}$) by adding different amounts of NaH$_2$PO$_4$·2H$_2$O. The cultures were carried out semi-continuously at 20°C for seven days. The light irradiance was set 200 μmol photons m$^{-2}$ s$^{-1}$, with a light and dark period of 12: 12. The cultures were aerated with ambient air (0.3 L min$^{-1}$) to maintain the pH around 8.2. The cells during exponential phase were collected and rinsed twice with DIC-free seawater that was made according to Xu et al. (2017). Afterwards, cells were resuspended in fresh media with two levels of pH (8.20 and 8.70, respectively corresponding to ambient CO$_2$ (12.6 μmol L$^{-1}$, AC) and low CO$_2$ (2.8 μmol L$^{-1}$, LC) under corresponding phosphate levels for two hours before the following measurements, with a cell density of 1.0 × 10$^6$ mL$^{-1}$. Cell density was determined by direct counting with an improved Neubauer haemocytometer (XB-K-25, Qiu Jing, Shanghai, China). This transfer aimed to investigate the effects of phosphate on DIC acquisition under a CO$_2$ limitation condition. The pH of 8.70 was chosen considering that it is commonly used as a CO$_2$ limitation condition (Nimer et al., 1998; Chen & Gao, 2004) and also occurs during algal bloom (Hansen, 2002). Two hours should be enough to activate CCMs in *S. costatum* (Nimer et al., 1998). The cell density did not vary during the two hours of pH treatment. All experiments were conducted in triplicates.

2.2. Manipulation of seawater carbonate system
The two levels of pH (8.20 and 8.70) were obtained by aerating the ambient air and pure nitrogen (99.999%) till the target value, and were then maintained with a buffer of 50 mM tris (hydroxymethyl) aminomethane-HCl. The cultures were open to the ambient atmosphere and the rise of culture pH was below 0.02 units (corresponding to the rise of CO$_2$ less than 0.7 and 0.2 μmol L$^{-1}$ for pH 8.20 and 8.70 treatments respectively) during the two hours of pH treatment. CO$_2$ level in seawater was calculated via CO2SYS (Pierrot et al., 2006) based on measured pH and TAlk, using the equilibrium constants of K1 and K2 for carbonic acid dissociation (Roy et al., 1993) and the KSO$_4^-$ dissociation constant from Dickson (1990).

2.3. The pH$_{NBS}$ was measured by a pH meter (pH 700, Eutech Instruments, Singapore) that was equipped with an Orion® 8102BN Ross combination electrode (Thermo Electron Co., USA) and calibrated with standard National Bureau of Standards (NBS) buffers (pH = 4.01, 7.00, and 10.01 at 25.0 ºC; Thermo Fisher Scientific Inc., USA). Total alkalinity (TAlk) was determined at 25.0 ºC by Gran acidimetric titration on a 25-ml sample with a TAlk analyzer (AS-ALK1, Apollo SciTech, USA), using the precision pH meter and an Orion® 8102BN Ross electrode for detection. To ensure the accuracy of TAlk, the TAlk analyser was regularly calibrated with certified reference materials from Andrew G. Dickson’s laboratory (Scripps Institute of Oceanography, U.S.A.) at a precision of ± 2 μmol kg$^{-1}$. CO$_2$ level in seawater was calculated via CO2SYS (Pierrot et al., 2006) based on measured pH and TAlk, using the equilibrium constants of K1 and K2 for carbonic acid dissociation (Roy et al., 1993) and the KSO$_4^-$ dissociation constant from Dickson (1990).

Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured with a pulse modulation fluorometer
(PAM-2100, Walz, Germany) to assess electron transport in photosystem II and the possible connection between electron transport and redox activity of the plasma membrane. The measuring light and actinic light were 0.01 and 200 μmol photons m\(^{-2}\) s\(^{-1}\), respectively. The saturating pulse was set 4,000 μmol photons m\(^{-2}\) s\(^{-1}\) (0.8 s). Relative electron transport in photosystem II (rETR, μmol e\(^{-}\) m\(^{-2}\) s\(^{-1}\)) = \(\frac{(F_{M}' - F_t)}{F_{M}'} \times 0.5 \times PFD\) (Gao et al., 2018), where \(F_{M}'\) is the maximal fluorescence levels from algae in the actinic light after application a saturating pulse, \(F_t\) is the fluorescence at an excitation level and PFD is the actinic light density.

2.4. Estimation of photosynthetic oxygen evolution and respiration

The net photosynthetic and respiration rates of \(S.\) costatum were measured using a Clark-type oxygen electrode (YSI Model 5300, USA) that was held in a circulating water bath (Cooling Circulator; Cole Parmer, Chicago, IL, USA) to keep the setting temperature (20°C). Five mL of samples were transferred to the oxygen electrode cuvette and were stirred during measurement. The light intensity and temperature were maintained as the same as that in the growth condition. The illumination was provided by a halogen lamp. The increase of oxygen content in seawater within five minutes was defined as net photosynthetic rate. To measure dark respiration rate, the samples were placed in darkness and the decrease of oxygen content within ten minutes was defined as dark respiration rate given the slower oxygen variation rate for dark respiration. Net photosynthetic rate and dark respiration rate were presented as μmol O\(_2\) (10\(^9\) cells\(^{-1}\)) h\(^{-1}\).

To obtain the curve of net photosynthetic rate versus DIC, seven levels of DIC (0, 0.1, 0.2, 0.5, 1, 2, and 4 mM) were made by adding different amounts of NaHCO\(_3\) to the Tris buffered
DIC-free seawater (pH 8.20). The algal samples were washed twice with DIC-free seawater before transferring to the various DIC solutions. Photosynthetic rates at different DIC levels were measured under saturating irradiance of 400 μmol photons m$^{-2}$ s$^{-1}$ and growth temperature. The algal samples were allowed to equilibrate for 2–3 min at each DIC level during which period a linear change in oxygen concentration was obtained and recorded. The parameter, photosynthetic half saturation constant ($K_{0.5}$, i.e., the DIC concentration required to give half of DIC-saturated maximum rate of photosynthetic $O_2$ evolution), was calculated from the Michaelis-Menten kinetics equation (Caemmerer and Farquhar 1981): $V = V_{max} \times \frac{[S]}{(K_{0.5} + [S])}$, where $V$ is the real-time photosynthetic rate, $V_{max}$ is maximum photosynthetic rate and $[S]$ is the DIC concentration. The value of $1/K_{0.5}$ represents photosynthetic DIC affinity. $K_{0.5}$ for CO$_2$ was calculated via CO2SYS (Pierrot et al., 2006) based on pH and TA, using the equilibrium constants of K1 and K2 for carbonic acid dissociation (Roy et al., 1993) and the KSO$_4^-$ dissociation constant from Dickson (1990).

2.5. Measurement of photosynthetic pigment

To determine the photosynthetic pigment (Chl $a$) content, 50 mL of culture were filtered on a Whatman GF/F filter, extracted in 5 mL of 90% acetone for 12 h at 4°C, and centrifuged (3,000 g, 5 min). The optical density of the supernatant was scanned from 200 to 700 nm with a UV-VIS spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The concentration of Chl $a$ was calculated based on the optical density at 630 and 664 nm: Chl $a = 11.47 \times \text{OD}_{664} - 0.40 \times \text{OD}_{630}$, Chl $c = 24.36 \times \text{OD}_{630} - 3.73 \times \text{OD}_{664}$ (Gao et al., 2018b), and was normalized to pg cells$^{-1}$.

2.6. Measurement of extracellular carbonic anhydrase activity
Carbonic anhydrase activity was assessed using the electrometric method (Gao et al., 2009). Cells were harvested by centrifugation at 4, 000 g for five minutes at 20°C, washed once and resuspended in 8 mL Na-barbital buffer (20 mM, pH 8.2). Five mL CO₂-saturated icy distilled water was injected into the cell suspension, and the time required for a pH decrease from 8.2 to 7.2 at 4°C was recorded. Extracellular carbonic anhydrase (CA_{ext}) activity was measured using intact cells. CA activity (E.U.) was calculated using the following formula: E.U. = 10 × (T₀ / T − 1), where T₀ and T represent the time required for the pH change in the absence or presence of the cells, respectively.

2.7. Measurement of redox activity in the plasma membrane

The redox activity of plasma membrane was assayed by monitoring the change in K₃Fe(CN)₆ concentration that accompanied reduction of the ferricyanide to ferrocyanide. The ferricyanide [K₃Fe(CN)₆] cannot penetrate intact cells and has been used as an external electron acceptor (Nimer et al., 1998; Wu & Gao, 2009). Stock solutions of K₃Fe(CN)₆ were freshly prepared before use. Five mL of samples were taken after two hours of incubation with 500 μmol K₃Fe(CN)₆ and centrifuged at 4000 g for 10 min (20°C). The concentration of K₃Fe(CN)₆ in the supernatant was measured spectrophotometrically at 420 nm (Shimadzu UV-1800, Kyoto, Japan). The decrease of K₃Fe(CN)₆ during the two hours of incubation was used to assess the rate of extracellular ferricyanide reduction that was presented as μmol (10⁶ cells)⁻¹ h⁻¹ (Nimer et al., 1998).

2.8. Cell-driving pH drift experiment

To obtain the pH compensation point, the cells were transferred to sealed glass vials containing fresh medium (pH 8.2) with corresponding phosphate levels. The cell
concentration for all treatments was $5.0 \times 10^5$ mL$^{-1}$. The pH drift of the suspension was monitored at 20°C and 200 µmol photons m$^{-2}$ s$^{-1}$ light level. The pH compensation point was obtained when there was no a further increase in pH.

2.9. Statistical analysis

Results were expressed as means of replicates ± standard deviation and data were analyzed using the software SPSS v.21. The data from each treatment conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances could be considered equal (Levene's test, $P > 0.05$). Two-way ANOVAs were conducted to assess the effects of CO$_2$ and phosphate on net photosynthetic rate, dark respiration rate, ratio of net photosynthetic rate to dark respiration rate, rETR, Chl $a$, $K_{0.5}$, $C_{A_{ext}}$, reduction rate of ferricyanide, and pH compensation point. Least Significant Difference (LSD) was conducted for post hoc investigation. Repeated measures ANOVAs were conducted to analyze the effects of DIC on net photosynthetic rate and the effect of incubation time on media pH in a closed system. Bonferroni was conducted for post hoc investigation as it is the best reliable post hoc test for repeated measures ANOVA (Ennos, 2007). The threshold value for determining statistical significance was $P < 0.05$.

3. Results

3.1. Effects of CO$_2$ and phosphate on photosynthetic and respiratory performances

The net photosynthetic rate and dark respiration rate in $S. costatum$ grown at various CO$_2$ and phosphate concentrations were first investigated (Fig. 1). CO$_2$ interacted with phosphate on net photosynthetic rate, with each factor having a main effect (Table 1). *Post hoc* LSD comparison ($P = 0.05$) showed that LC reduced net photosynthetic rate when the phosphate levels was below 4 µmol L$^{-1}$ but did not affect it at the higher phosphate levels. Under AC, net
photosynthetic rate increased with phosphate level and reached the plateau (100.51 ± 9.59 µmol O₂ (10⁹ cells)⁻¹ h⁻¹) at 1 µmol L⁻¹ phosphate. Under LC, net photosynthetic rate also increased with phosphate level but did not hit the peak (101.46 ± 9.19 µmol O₂ (10⁹ cells)⁻¹ h⁻¹) until 4 µmol L⁻¹ phosphate. In terms of dark respiration rate (Fig. 1b), phosphate had a main effect on it and it interacted with CO₂ (Table 1). Specifically, LC increased dark respiration rate at 0.05 and 0.25 µmol L⁻¹ phosphate levels, but did not affect it when phosphate level was above 1 µmol L⁻¹ (LSD, P < 0.05). Regardless of CO₂ level, respiration rate increased with phosphate availability and stopped at 1 µmol L⁻¹.

The ratio of respiration to photosynthesis ranged from 0.23 to 0.40 (Fig. 2). Both CO₂ and phosphate had a main effect, and they interacted on the ratio of respiration to photosynthesis (Table 1). LC increased the ratio when phosphate was lower than 4 µmol L⁻¹ but did not affect it when phosphate levels were 4 or 10 µmol L⁻¹.

Both CO₂ and phosphate affected rETR and they also showed an interactive effect (Fig. 3 & Table 2). For instance, post hoc LSD comparison showed that LC did not affect rETR at lower phosphate levels (0.05 and 0.25 µmol L⁻¹) but increased it at higher phosphate levels (1–10 µmol L⁻¹). Regardless of CO₂ treatment, rETR increased with phosphate level (0.05–4 µmol L⁻¹) but the highest phosphate concentration did not result in a further increase in rETR (LSD, P > 0.05).

The content of Chl a was measured to investigate the effects of CO₂ and phosphate on photosynthetic pigment in S. costatum (Fig. 4). Both CO₂ and phosphate affected the synthesis of Chl a and they had an interactive effect (Table 2). Post hoc LSD comparison (P = 0.05) showed that LC did not affect Chl a at 0.05 or 0.25 µmol L⁻¹ phosphate but stimulated
Chl a synthesis at higher phosphate levels (1–10 µmol L⁻¹). Irrespective of CO₂ treatment, Chl a content increased with phosphate level and reached the plateau (0.19 ± 0.01 pg cell⁻¹ for AC and 0.23 ± 0.01 pg cell⁻¹ for LC) at 4 µmol L⁻¹ phosphate.

To assess the effects of CO₂ and phosphate on photosynthetic CO₂ affinity in S. costatum, the net photosynthetic rates of cells exposure to seven levels of DIC were measured (Fig. 5). After curve fitting, the values of $K_{0.5}$ for CO₂ were calculated (Fig. 6). CO₂ and phosphate interplayed on $K_{0.5}$ and each had a main effect (Table 2). LC did not affect $K_{0.5}$ at the lowest phosphate level but reduced it at the other phosphate levels. Under AC, higher phosphate levels (0.25–4 µmol L⁻¹) reduced $K_{0.5}$ and the highest phosphate level led to a further decrease to 2.59 ± 0.29 µmol kg⁻¹ seawater compared to the value of 4.00 ± 0.30 µmol kg⁻¹ seawater at 0.05 µmol L⁻¹ phosphate. The pattern with phosphate under LC was the same as the AC.

### 3.3. The effects of CO₂ and phosphate on inorganic carbon acquisition

To investigate the potential mechanisms that cells overcame CO₂ limitation during algal blooms, the activity of CAext, a CCM related enzyme, was estimated under various CO₂ and phosphate conditions (Fig. 7a). Both CO₂ and phosphate had a main effect and they interacted on CAext activity (Table 3). Post hoc LSD comparison ($P = 0.05$) showed that LC induced more CAext activity under all phosphate conditions except for 0.05 µmol L⁻¹ levels, compared to AC. Under AC, CAext activity increased (0.04–0.10 EU (10⁶ cells)⁻¹) with phosphate level and stopped increasing at 1 µmol L⁻¹ phosphate. Under LC, CAext activity also increased (0.04–0.35 EU (10⁶ cells)⁻¹) with phosphate level but reached the peak at 4 µmol L⁻¹ phosphate. The redox activity of plasma membrane was also assessed to investigate the factors that modulate CAext activity (Fig. 7b). The pattern of redox activity of plasma
membrane under various CO\textsubscript{2} and phosphate conditions was the same as that of CA\textsubscript{ext} activity. That is, CO\textsubscript{2} and phosphate had an interactive effect on redox activity of plasma membrane, each having a main effect (Table 3).

To test cells’ tolerance to high pH and obtain pH compensation points in \textit{S. costatum} grown under various CO\textsubscript{2} and phosphate levels, changes of media pH in a closed system were monitored (Fig. 8). The media pH under all phosphate conditions increased with incubation time (Table 4). Specifically speaking, there was a steep increase in pH during the first three hours, afterwards the increase became slower and it reached a plateau in six hours (Bonferroni, \( P < 0.05 \)). Phosphate had an interactive effect with incubation time (Table 4). For instance, there was no significant difference in media pH among phosphate levels during first two hours of incubation but then divergence occurred and they stopped at different points. Two-way ANOVA analysis showed that CO\textsubscript{2} treatment did not affect pH compensation point but phosphate had a main effect (Table 3). Under each CO\textsubscript{2} treatment, pH compensation point increased with phosphate level, with lowest of 9.03 ± 0.03 at 0.05 µmol L\textsuperscript{-1} and highest of 9.36 ± 0.04 at 10 µmol L\textsuperscript{-1} phosphate.

4. Discussion

4.1. Photosynthetic performances under various CO\textsubscript{2} and phosphate conditions

The lower CO\textsubscript{2} availability reduced the net photosynthetic rate of \textit{S. costatum} grown at the lower phosphate levels in the present study. However, Nimer \textit{et al.} (1998) demonstrated that the increase in pH (8.3–9.5) did not reduce photosynthetic CO\textsubscript{2} fixation of \textit{S. costatum} and Chen and Gao (2004) reported that a higher pH (8.7) even stimulated the photosynthetic rate of \textit{S. costatum} compared to the control (pH 8.2). The divergence between our and the
previous studies may be due to different nutrient supply. Both Nimer et al. (1998) and Chen and Gao (2004) used f/2 media to grow algae. The phosphate concentration in f/2 media is \(\sim 36 \, \mu\text{mol} \, \text{L}^{-1}\), which is replete for physiological activities in \textit{S. costatum}. \textit{Skeletonema costatum} grown at higher phosphate levels (4 and 10 \(\mu\text{mol} \, \text{L}^{-1}\)) also showed similar photosynthetic rates for the lower and higher CO\(_2\) treatments. Our finding combined with the previous studies indicates phosphorus plays an important role in dealing with low CO\(_2\) availability for photosynthesis in \textit{S. costatum}.

Different from net photosynthetic rate, LC did not affect rETR at lower phosphate levels (0.05 and 0.25 \(\mu\text{mol} \, \text{L}^{-1}\)) and stimulated it at higher phosphate levels (1–10 \(\mu\text{mol} \, \text{L}^{-1}\)). This interactive effect of CO\(_2\) and phosphate may be due to their effects on Chl \textit{a}. LC induced more synthesis of Chl \textit{a} at higher phosphate levels (1–10 \(\mu\text{mol} \, \text{L}^{-1}\)). This induction of LC on photosynthetic pigment is also reported in green algae (Gao \textit{et al.}, 2016). More energy is required under LC to address the more severe CO\(_2\) limitation and thus more Chl \textit{a} are synthesized to capture more light energy, particularly when phosphate was replete. Although P is not an integral component for chlorophyll, it plays an important role in cell energetics through high-energy phosphate bonds, i.e. ATP, which could support chlorophyll synthesis. The stimulating effect of P enrichment on photosynthetic pigment is also found in green alga \textit{Dunaliella tertiolecta} (Geider \textit{et al.}, 1998) and brown alga \textit{Sargassum muticum} (Xu \textit{et al.}, 2017). The increased photosynthetic pigment in \textit{S. costatum} could partially explain the increased rETR and photosynthetic rate under the higher P conditions.

4.6. \textit{Ratio of respiration to photosynthesis}

The ratio of respiration to photosynthesis in algae indicates carbon balance in cells and
carbon flux in marine ecosystems as well (Zou & Gao, 2013). LC increased this ratio in *S. costatum* grown at the lower P conditions but did not affect it under the higher P conditions, indicating that P enrichment can offset the carbon loss caused by carbon limitation. To cope with CO\(_2\) limitation, cells might have to obtain energy from dark respiration under lower P conditions as it seems infeasible to acquire energy from the low rETR, which led to the increased dark respiration. However, LC induced higher rETR under P replete conditions and energy used for inorganic carbon (CO\(_2\) and HCO\(_3^-\)) acquisition could be from the increased rETR. Therefore, additional dark respiration was not triggered, avoiding carbon loss. Most studies regarding the effect of CO\(_2\) on ratio of respiration to photosynthesis focus on higher plants (Gifford, 1995; Ziska & Bunce, 1998; Cheng *et al.*, 2010; Smith & Dukes, 2013), little is known on phytoplankton. Our study suggests that CO\(_2\) limitation may lead to carbon loss in phytoplankton but P enrichment could alter this trend, regulating carbon balance in phytoplankton and thus their capacity in carbon sequestration.

4.3. Inorganic carbon acquisition under CO\(_2\) limitation and phosphate enrichment

Decreased CO\(_2\) can usually induce higher inorganic carbon affinity in algae (Raven *et al.*, 2012; Wu *et al.*, 2012; Raven *et al.*, 2017; Xu *et al.*, 2017). In the present study, the lower CO\(_2\) did increase inorganic carbon affinity when P level was higher than 0.25 µmol L\(^{-1}\) but did not affect it when P was 0.05 µmol L\(^{-1}\), indicating the important role of P in regulating cells’ CCMs in response to environmental CO\(_2\) changes. LC induced larger CA activity when P was above 0.25 µmol L\(^{-1}\) but did not increase it at 0.05 µmol L\(^{-1}\) of P, which could explain the interactive effect of P and CO\(_2\) on inorganic carbon affinity as CA can accelerate the equilibrium between HCO\(_3^-\) and CO\(_2\) and increase inorganic carbon affinity. Regardless of
CO₂, P enrichment alone increased CA activity and inorganic carbon affinity. P enrichment may stimulate the synthesis of CA by supplying required ATP. In addition, P enrichment increased the redox activity of plasma membrane in this study. It has been proposed that redox activity of plasma membrane could induce extracellular CA activity via protonation extrusion of its active center (Nimer et al., 1998). Our result that the pattern of CA is exactly same as that of redox activity of plasma membrane shows a compelling correlation between CA and redox activity of plasma membrane. The stimulating effect of P on redox activity of plasma membrane may be due to its effect on rETR. The increased rETR could generate excess reducing equivalents, particularly under CO₂ limiting conditions. These excess reducing equivalents would be transported from the chloroplast into the cytosol (Heber, 1974), supporting the redox chain in the plasma membrane (Rubinstein & Luster, 1993; Nimer et al., 1999) and triggering CA activity.

4.4. Direct HCO₃⁻ utilization due to phosphate enrichment

A pH compensation point over 9.2 has been considered a sign of direct HCO₃⁻ use for algae (Axelsson & Uusitalo, 1988) as the CO₂ concentration is nearly zero at pH above 9.2. This criterion has been justified based on the experiments for both micro and macro-algae. For instance, the marine diatom Phaeodactylum tricornutum, with a strong capacity for direct HCO₃⁻ utilization, has a higher pH compensation point of 10.3 (Chen et al., 2006). In contrast, the red macroalgae, Lomentaria articulata and Phycodrys rubens that cannot utilize HCO₃⁻ directly and photosynthesis only depends on CO₂ diffusion, have pH compensation points of less than 9.2 (Maberly, 1990). In terms of S. costatum, it has been reported to have a pH compensation point of 9.12, indicating a very weak capacity in direct HCO₃⁻ utilization (Chen
& Gao, 2004). Our study demonstrates that the pH compensation point of *S. costatum* varies with the availability of P. It is lower than 9.2 under P limiting conditions but higher than 9.2 under P replete conditions, suggesting that the capacity of direct HCO$_3^-$ utilization is regulated by P availability. Contrary to CO$_2$ passive diffusion, the direct use of HCO$_3^-$ depends on positive transport that requires energy (Hopkinson & Morel, 2011). P enrichment increased rETR in the present study and the ATP produced during the process of electron transport could be used to support HCO$_3^-$ positive transport. In addition, the increased respiration at higher P levels can also generate ATP to help HCO$_3^-$ positive transport. Our study indicates that P enrichment could trigger HCO$_3^-$ direct utilization and hence increase inorganic acquisition capacity of *S. costatum* to cope with CO$_2$ limitation.

4.5. CCMs and red tides

With the development of red tides, the pH in seawater could be very high along with extremely low CO$_2$ availability due to intensive photosynthesis (Hansen, 2002; Hinga, 2002). For instance, pH level in the surface waters of the eutrophic Mariager Fjord, Denmark, is often above 9 during dinoflagellate blooms (Hansen, 2002). Diatoms are the causative species for red tides and *S. costatum* could outcompete other bloom algae (dinoflagellates *Prorocentrum minimum* and *Alexandrium tamarense*) under nutrient replete conditions (Hu et al., 2011). However, the potential mechanisms are poorly understood. Our study demonstrates *S. costatum* has multiple CCMs to cope with CO$_2$ limitation and the operation of CCMs is regulated by P availability. The CCMs of *S. costatum* are hampered under P limiting conditions and only function when P is replete. Therefore, P enrichment would be critical for *S. costatum* to overcome carbon limitation during algal bloom and to dominate red tides.
The present study investigated the role of P in regulating inorganic carbon acquisition and CO₂ concentrating mechanisms in diatoms for the first time. The intensive photosynthesis and quick growth during algal blooms usually result in noticeable increase of pH and decrease of CO₂. Our study demonstrates that P enrichment could induce activity of extracellular carbonic anhydrase and direct utilization of HCO₃⁻ in *S. costatum* to help overcome the CO₂ limitation, as well as increasing photosynthetic pigment content and rETR to provide required energy. This study provides important insight into the connection of phosphorus and carbon acquisition in diatoms and the mechanisms that *S. costatum* dominates algal blooms.

**Author contribution**

JX and GG designed the experiments, and GG, JY, JF and XZ carried them out. GG prepared the manuscript with contributions from all co-authors.

**Acknowledgements**

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Table 1 Two-way analysis of variance for the effects of CO$_2$ and phosphate on net photosynthetic rate, dark respiration rate and ratio of respiration to photosynthesis of *S. costatum*. CO$_2$*phosphate* means the interactive effect of CO$_2$ and phosphate, df means degree of freedom, F means the value of F statistic, and Sig. means p-value.

<table>
<thead>
<tr>
<th>Source</th>
<th>Net photosynthetic rate</th>
<th>Dark respiration rate</th>
<th>Ratio of respiration to photosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>Sig.</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>1</td>
<td>11.286</td>
<td>0.003</td>
</tr>
<tr>
<td>Phosphate</td>
<td>4</td>
<td>157.925</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CO$_2$*phosphate</td>
<td>4</td>
<td>3.662</td>
<td>0.021</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 2 Two-way analysis of variance for the effects of CO$_2$ and phosphate on relative electron transport rate (rETR), Chl $a$, and CO$_2$ level required to give half of DIC-saturated maximum rate of photosynthetic O$_2$ evolution ($K_{0.5}$) of *S. costatum*. CO$_2$*phosphate* means the interactive effect of CO$_2$ and phosphate, df means degree of freedom, F means the value of F statistic, and Sig. means p-value.

<table>
<thead>
<tr>
<th>Source</th>
<th>rETR</th>
<th>Chl $a$</th>
<th>$K_{0.5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>Sig.</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>1</td>
<td>28.717</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phosphate</td>
<td>4</td>
<td>127.860</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CO$_2$*phosphate</td>
<td>4</td>
<td>3.296</td>
<td>0.031</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 3 Two-way analysis of variance for the effects of CO$_2$ and phosphate on CA$_{ext}$ activity, redox activity of plasma membrane and pH compensation point of *S. costatum*. CO$_2$*phosphate* means the interactive effect of CO$_2$ and phosphate, df means degree of freedom, F means the value of F statistic, and Sig. means p-value.

<table>
<thead>
<tr>
<th>Source</th>
<th>CA$_{ext}$ activity</th>
<th>Redox activity of plasma membrane</th>
<th>pH compensation point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>Sig.</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>1</td>
<td>569.585</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phosphate</td>
<td>4</td>
<td>176.392</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CO$_2$*phosphate</td>
<td>4</td>
<td>87.380</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 4 Repeated measures analysis of variance for the effects of CO$_2$ and phosphate on pH change during 10 hours of incubation. Time*CO$_2$ means the interactive effect of incubation time and CO$_2$, Time*phosphate means the interactive effect of incubation time and phosphate, Time*CO$_2$*phosphate means the interactive effect of incubation time, CO$_2$ and phosphate, df means degree of freedom, F means the value of F statistic, and Sig. means p-value.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>40.766</td>
<td>10</td>
<td>4.077</td>
<td>8737.941</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time*CO$_2$</td>
<td>0.003</td>
<td>10</td>
<td>&lt;0.001</td>
<td>0.569</td>
<td>0.838</td>
</tr>
<tr>
<td>Time*phosphate</td>
<td>0.886</td>
<td>40</td>
<td>0.022</td>
<td>47.496</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time*CO$_2$*phosphate</td>
<td>0.002</td>
<td>40</td>
<td>&lt;0.001</td>
<td>0.112</td>
<td>1.000</td>
</tr>
<tr>
<td>Error</td>
<td>0.093</td>
<td>200</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

**Fig. 1.** Net photosynthetic rate (a) and dark respiration rate (b) in *S. costatum* grown at various phosphate concentrations after ambient (AC) and low CO$_2$ (LC) treatments. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines represent significant difference ($P < 0.05$) between CO$_2$ treatments.

**Fig. 2.** Ratio of respiration rate to net photosynthetic rate in *S. costatum* grown at various phosphate concentrations after ambient (AC) and low CO$_2$ (LC) treatments. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines represent significant difference ($P < 0.05$) between CO$_2$ treatments.

**Fig. 3.** Relative electron transport rate (rETR) in *S. costatum* grown at various phosphate concentrations after ambient (AC) and low CO$_2$ (LC) treatments. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among phosphate concentrations (capital for AC lower case for LC). Horizontal lines represent significant difference ($P < 0.05$) between CO$_2$ treatments.

**Fig. 4.** Photosynthetic Chl $a$ content in *S. costatum* grown at various phosphate concentrations after ambient (AC) and low CO$_2$ (LC) treatments. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines represent significant difference ($P < 0.05$) between CO$_2$ treatments.

**Fig. 5.** Net photosynthetic rate as a function of DIC for *S. costatum* grown at various
phosphate concentrations after ambient (a) and low CO$_2$ (b) treatments. The error bars
indicate the standard deviations (n = 3).

**Fig. 6.** Half saturation constant ($K_{0.5}$) for CO$_2$ in *S. costatum* grown at various phosphate
concentrations after ambient (AC) and low CO$_2$ (LC) treatments. The error bars indicate the
standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$)
among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines
represent significant difference ($P < 0.05$) between CO$_2$ treatments.

**Fig. 7.** CA$_{ext}$ activity (a) and reduction rate of ferricyanide (b) in *S. costatum* grown at various
phosphate concentrations after ambient (AC) and low CO$_2$ (LC) treatments. The error bars
indicate the standard deviations (n = 3). Different letters represent the significant difference ($P$
$< 0.05$) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines
represent significant difference ($P < 0.05$) between CO$_2$ treatments.

**Fig. 8.** Changes of pH in a closed system caused by photosynthesis of *S. costatum* grown at
various phosphate concentrations after ambient (AC) and low CO$_2$ (LC) treatments. The error
bars indicate the standard deviations (n = 3).
Fig. 1
Fig. 2
Fig. 3

The graph shows the relationship between rETR (µmol e⁻•m⁻²•s⁻¹) and phosphate concentration (µmol L⁻¹) for AC and LC treatments. The bars are labeled with letters indicating statistical significance: A, B, C, D, and lowercase letters a, b, c, d. The graph indicates that rETR increases with increasing phosphate concentration, with AC and LC treatments showing different trends at various concentrations.
Fig. 4

Chl a content (pg cell⁻¹)

Phosphate concentration (µmol L⁻¹)
Fig. 5
Fig. 6
Fig. 7
Fig. 8