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

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Abstract: 

*S. costatum* is a common bloom-forming diatom and encounters eutrophication and severe CO₂ limitation during red tides. However, little is known regarding the role of phosphorus in modulating inorganic carbon acquisition in *S. costatum*, particularly under CO₂ 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 CO₂ conditions (2.8 and 12.6 μmol L⁻¹) for two hours. The lower CO₂ 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 CO₂ 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 CO₂ did not change 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 CO₂ affinity (*K₀.₅*) decreased with phosphate levels. The lower CO₂ did not affect *K₀.₅* at 0.05 μmol L⁻¹ phosphate but reduced it at the other phosphate levels. Activity of extracellular carbonic anhydrase was dramatically induced by the lower CO₂ at phosphate replete conditions (> 0.25 μmol L⁻¹) and the same pattern also occurred for redox activity of plasma membrane. Direct HCO₃⁻ use was induced when phosphate concentration is more than 1 μmol L⁻¹. This study indicates the essential role of P in regulating inorganic carbon acquisition and CO₂ concentrating mechanisms (CCMs) in *S. costatum* and 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 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 compelling factor that triggers algal blooms albeit the occurrence of diatom blooms may be modulated by other environmental factors, such as temperature, light intensity, salinity etc. (Smetacek & Zingone, 2013; Jeong et al., 2015). When inorganic nitrogen and phosphorus are replete, diatoms could out-compete chrysophytes, raphidophytes and dinoflagellates (Berg et al., 1997; Jeong et al., 2015; Barton et al., 2016) and domainate 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) 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 limited 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, for instance, active transport of HCO$_3^-$, the passive influx of CO$_2$, multiple carbon anhydrase, assumed C4-type pathway, etc. (Hopkinson & Morel, 2011; Hopkinson et al., 2016). *S. 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. 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 $\mu$mol L$^{-1}$ while Rost et al. (2003) reported that activity of extracellular CA could be detected even when $p$CO$_2$ is 1800 $\mu$atm. 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 CO$_2$ and HCO$_3^-$ 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 and the references therein). Some studies show the relationship between phosphorus availability and 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 the capacity of inorganic carbon utilization and hence maintain high rates of photosynthesis and growth in S. costatum under CO₂ limitation conditions. In the present study, we investigated the inorganic acquisition pathways, photosynthetic CO₂ affinity, carbonic anhydrase activity, redox activity of plasma membrane, and photosynthetic rate under five levels of phosphate and two levels of CO₂ conditions to test this hypothesis. Our study would provide helpful insights into how bloom-forming diatoms overcome CO₂ limitation to maintain a quick growth rate during red tides.

2. Materials and Methods

2.1. Culture conditions

S. 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 NaH₂PO₄·2H₂O. The cultures were carried out semi-continuously at 20°C for
seven days. The light irradiance was set 200 μmol 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 (8.2 and 8.7, 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\(^{-1}\). This transfer aimed to investigate the effects of phosphate on DIC acquisition under a CO\(_2\) limitation condition. The pH of 8.7 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). All experiments were conducted in triplicates.

2.2. Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured with a pulse modulation fluorometer (PAM-2100, Walz, Germany). 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 (rETR, μmol e\(^{-}\) m\(^{-2}\) s\(^{-1}\)) = \((F_{M'} - F_t) / F_{M'} \times 0.5 \times PFD\), where \(F_{M'}\) is the maximal fluorescence levels from algae after in light, \(F_t\) is the fluorescence at an excitation level and PFD is the actinic light density.

2.3. Estimation of photosynthetic oxygen evolution and respiration

The net photosynthetic rate and respiration rate 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. Net photosynthetic rate and dark respiration rate were presented as µmol O₂ (10⁹ 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 NaHCO₃ to the Tris buffered DIC-free seawater. 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 (K₀.₅, i.e., the DIC concentration required to give half of Ci-saturated maximum rate of photosynthetic O₂ evolution), was calculated from the Michaelis-Menten kinetics equation (Caemmerer and Farquhar 1981): \[ V = V_{max} \times [S] / (K_{0.5} + [S]) \], where V is the real-time photosynthetic rate, \( V_{max} \) is maximum photosynthetic rate and [S] is the DIC concentration. \( K_{0.5} \) for CO₂ was calculated via CO2SYS (Pierrot et al., 2006), using the equilibrium constants of K1 and K2 for carbonic acid dissociation (Roy et al., 1993) and the KSO₄⁻ dissociation constant from Dickson (1990). Total alkalinity and pH were the...
two input parameters. Seawater pH was measured with a pH meter (pH 700, Eutech Instruments, Singapore) and total alkalinity was measured by titrations.

2.4. 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: 

\[ \text{Chl a} = 11.47 \times \text{OD}_{664} - 0.40 \times \text{OD}_{630} \]

2.5. 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: 

\[ \text{E.U.} = 10 \times \left( T_{0} / T - 1 \right) \]

where \( T_{0} \) and \( T \) represent the time required for the pH change in the absence or presence of the samples, respectively.

2.6. Measurement of redox activity in the plasma membrane

The redox activity of plasma membrane was assayed by incubating the cells with 500 μmol ferricyanide [K₃Fe(CN)₆] that 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 and centrifuged at 4000 g for 10 min (20°C). The absorbance of supernatant at 420 nm was measured immediately to assess the rate of exofacial ferricyanide reduction (Nimer et al., 1998).

2.7. 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 × 10⁵ 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.

2.8. 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 CO₂ and phosphate on differences net photosynthetic rate, dark respiration rate, ratio of net photosynthetic rate to dark respiration rate, rETR, Chl a, K₀.₅, CAₑₓₙₙ, 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. The threshold value for determining statistical significance was P < 0.05.
3. Results

3.1. Effects of CO₂ and phosphate on photosynthetic and respiratory performances

The net photosynthetic rate and dark respiration rate in *S. costatum* grown at various CO₂ and phosphate concentrations were first investigated (Fig. 1). CO₂ interacted with phosphate on net photosynthetic rate ($F_{(4, 20)} = 3.662, P = 0.021$, Fig. 1a), with each factor having a main effect ($F_{(1, 20)} = 11.286, P = 0.003$ for CO₂, $F_{(4, 20)} = 157.925, P < 0.001$ for phosphate). 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 at 1 µmol L⁻¹ phosphate. Under LC, net photosynthetic rate also increased with phosphate level but did not hit the peak until 4 µmol L⁻¹ phosphate. Phosphate had a main effect on dark respiration rate ($F_{(4, 20)} = 169.050, P < 0.001$, Fig. 1b), and it interacted with CO₂ ($F_{(4, 20)} = 3.226, P = 0.034$). 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 ($F_{(1, 20)} = 32.443, P < 0.001$ for CO₂, $F_{(4, 20)} = 7.081, P = 0.001$ for phosphate), and they interplayed on the ratio of respiration to photosynthesis ($F_{(4, 20)} = 8.299, P < 0.001$). 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 CO$_2$ and phosphate affected rETR ($F_{(1, 20)} = 28.717, P < 0.001$ for CO$_2$, $F_{(4, 20)} = 127.860, P < 0.001$ for phosphate) and they also showed an interactive effect ($F_{(4, 20)} = 3.296, P = 0.031$, Fig. 3). 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 CO$_2$ 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 CO$_2$ and phosphate on photosynthetic pigment in S. costatum (Fig. 4). Both CO$_2$ and phosphate affected the synthesis of Chl $a$ ($F_{(1, 20)} = 32.963, P < 0.001$ for CO$_2$, $F_{(4, 20)} = 92.045 P < 0.001$ for phosphate) and they had an interactive effect ($F_{(4, 20)} = 3.871, P = 0.017$). 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 CO$_2$ treatment, Chl $a$ content increased with phosphate level and reached the plateau at 4 μmol L$^{-1}$ phosphate.

To access the effects of CO$_2$ and phosphate on photosynthetic CO$_2$ 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 CO$_2$ were calculated (Fig. 6). CO$_2$ and phosphate interplayed on $K_{0.5}$ ($F_{(4, 20)} = 3.821, P = 0.018$) and each had a main effect ($F_{(1, 20)} = 96.182, P < 0.001$ for CO$_2$, $F_{(4, 20)} = 40.497, P < 0.001$ for phosphate). 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 in $K_{0.5}$ compared to 0.05 µmol L$^{-1}$ phosphate. The pattern with phosphate under LC was the same as the AC.

3.3. The effects of CO$_2$ and phosphate on inorganic carbon acquisition

To investigate the potential mechanisms that cells overcame CO$_2$ limitation during algal blooms, the activity of CA$_{ext}$, a CCM related enzyme, was estimated under various CO$_2$ and phosphate conditions (Fig. 7a). Both CO$_2$ ($F_{(1,20)} = 569.585, P < 0.001$) and phosphate ($F_{(4,20)} = 176.392, P < 0.001$) had a main effect and they interacted ($F_{(4,20)} = 87.380, P < 0.001$) on CA$_{ext}$ activity. Post hoc LSD comparison ($P = 0.05$) showed that LC induced more CA$_{ext}$ activity under all phosphate conditions except for 0.05 µmol L$^{-1}$ levels, compared to AC. Under AC, CA$_{ext}$ activity increased with phosphate level and stopped increasing at 1 µmol L$^{-1}$ phosphate. Under LC, CA$_{ext}$ activity also increased with phosphate level but reached the peak at 4 µmol L$^{-1}$ phosphate. The redox activity of plasma membrane was also assayed to investigate the factors that modulate CA$_{ext}$ activity (Fig. 7b). The pattern of redox activity of plasma membrane under various CO$_2$ and phosphate conditions was the same as that of CA$_{ext}$ activity. That is, CO$_2$ and phosphate had an interactive effect ($F_{(4,20)} = 137.050, P < 0.001$) on redox activity of plasma membrane, each having a main effect ($F_{(1,20)} = 937.963, P < 0.001$ for CO$_2$; $F_{(4,20)} = 276.362, P < 0.001$ for phosphate).

To test cells’ tolerance to high pH and obtain pH compensation points in *S. costatum* grown under various CO$_2$ 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 ($F_{(10,100)} = 7604.563, P < 0.001$). 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

\[ F(10, 100) = 47.469, P < 0.001 \]

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$_2$ treatment did not affect pH compensation point ($F(1, 20) = 0.056, P = 0.816$) but phosphate had a main effect ($F(4, 20) = 226.196, P < 0.001$). Under each CO$_2$ treatment, pH compensation point increased with phosphate level, with lowest of 9.03 ± 0.03 at 0.05 µmol L$^{-1}$ and highest of 9.36 ± 0.04 at 10 µmol L$^{-1}$ phosphate.

4. Discussion

4.1. Photosynthetic performances under various CO$_2$ and phosphate conditions

The lower CO$_2$ availability reduced the net photosynthetic rate of *S. costatum* grown at the lower phosphate levels in the present study. However, Nimer et al. (1998) demonstrated that the increase in pH (8.3–9.5) did not reduce photosynthetic CO$_2$ fixation of *S. costatum* and Chen and Gao (2004) reported that a higher pH (8.7) even stimulated the photosynthetic rate of *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 ~36 µmol L$^{-1}$, which is replete for physiological activities in *S. costatum*. *S. costatum* grown at higher phosphate levels (4 and 10 µmol L$^{-1}$) also showed comparative photosynthetic rates between 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 *S. costatum*. 

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Different from net photosynthetic rate, LC did not affect rETR at lower phosphate levels (0.05 and 0.25 μmol L\(^{-1}\)) and stimulated it at higher phosphate levels (1–10 μmol L\(^{-1}\)). This interactive effect of CO\(_2\) and phosphate may be due to their effects on Chl \(a\). LC induced more synthesis of Chl \(a\) at higher phosphate levels (1–10 μmol 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 \(a\) 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 algae \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.

4.2. 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 \textit{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 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 $\mu$mol L$^{-1}$ but did not affect it when P was 0.05 $\mu$mol 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 $\mu$mol L$^{-1}$ but did not increase it at 0.05 $\mu$mol 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 CO$_2$, 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 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 limited 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 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 strong capacity for direct HCO₃⁻ utilization, has a higher pH compensation point of 10.3 (Chen et al., 2006). In contrast, 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 HCO₃⁻ utilization is regulated by P availability. Contrary to CO₂ passive diffusion, the direct use of HCO₃⁻ 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 HCO₃⁻ positive transport. In addition, the increased respiration at higher P
levels can also generate ATP to help HCO₃⁻ positive transport. Our study indicates that P enrichment could trigger HCO₃⁻ direct utilization and hence increase inorganic acquisition capacity of *S. costatum* to cope with CO₂ 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 CO₂ 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, potential mechanisms are poorly understood. Our study demonstrates *S. costatum* has multiple CCMs to cope with CO₂ limitation and the operation of CCMs is regulated by P availability. The CCMs of *S. costatum* is 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.

### 5. Conclusions

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.

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**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.** $CA_{ex}$ 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
Fig. 4
Fig. 5
Fig. 6

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Fig. 7
Fig. 8