Reviewer 1

The authors present a manuscript detailing culture studies of a common algae species under varying CO2 and phosphorus concentrations. Algal blooms can draw down dissolved CO2 to very low levels, and some species have developed mechanisms to compensate for decreased CO2 availability. Some of these mechanisms may be influenced by the presence or absence of bio-available phosphorus, leading to the study design of varying phosphorus levels across test populations of two CO2 levels. While there is probably a compelling study underlying this manuscript, in my opinion there are too many flaws as presented to encourage publication. I feel compelled to point out here that my background is in seawater carbonate chemistry, and my knowledge of cellular biochemical processes is limited. However, based solely on the description of inorganic carbon system measurements I would advise rejecting this manuscript until serious revisions have been done. I will present some major comments below, followed by more minor concerns.

Response: We appreciate these comments and believe the manuscript has been largely improved by responding to all comments raised by the reviewer.

MAJOR COMMENTS -The Materials and Methods section, in particular the sections pertaining to pH and alkalinity measurements, is totally inadequate. Is the pH system an electrode-type system? What pH scale are measurements presented in? How was the pH system calibrated? How were alkalinity titrations performed? No information is presented. These questions are especially critical in the calculation of DIC from pH and alkalinity (P7L129-131), which is very sensitive to relatively minor changes in pH and alkalinity. With no information about the quality of pH and alkalinity measurements, the results of this analysis are impossible to interpret.

Response: We appreciate these comments and apologize for missing these details. The text has been clarified to "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 ^{o}C; Thermo Fisher Scientific Inc., USA). Total alkalinity (TAlk) was determined at 25.0 ^{o}C 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}." at P8L127-135.

-Besides using the barely-described pH system, how did the authors know the CO2 levels of their cultures? Also, adding phosphate to the cultures, at concentrations ranging from 0.5-10 _mol/kg adds a potent buffering agent, as monosodium phosphate has a pKa around 7. How did the authors alter or maintain the pH in the cultures? Were the cultures open to the ambient atmosphere?

Response: We apologize for missing these details. The text has been clarified to "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 variation of culture pH was below ± 0.02 unites 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)." at P8L119-126.

-As previously mentioned, my knowledge of some of the biochemical processes presented here is minimal, and the Introduction did little to help readers like myself. There seem to be connections between plasma membrane redox activity, CAext, rETR, but the manuscript does not explain them. Some terms (i.e. rETR) are

presented with no explanation or definition. Thus the reason for some of the analyses presented was unclear to me. What did measuring the chlorophyll fluorescence inform? The cultures were initiated at the same cell density, but surely the cell density varied between cultures after the treatment period- how was this accounted for?

Response: We apologize for the confusion. The connections between plasma membrane redox activity, CAext, rETR were explained in Discussion. To make readers know them earlier, we have generally explained the connections in Methods. It reads "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." at P9L141-143. rETR has been defined as "relative electron transport" in abstract where it first appeared at P2L11.

In terms of cell density, it did not vary during the two hours of pH treatment as diatom cells usually proliferate at night. This information has been added to the text and it reads "The cell density did not vary during the two hours of pH treatment." at P8L116-117.

-In the Results section the authors present their statistical findings in the form F(1,20)=XX or F(4,20)=XX. I'm assuming these are the results of the two-way ANOVA test mentioned in the 2.8 Statistical Analysis section, but no explanation is given as to what is signified. Are the numbers in parentheses indicating degrees of freedom? What is the threshold for significance?

Response: Yes, the numbers in parentheses indicating degrees of freedom. The threshold for significance is $P < 0.05$, which was stated in section 2.8.

-The Results section is extremely repetitive. Much of the information presented could be more effectively summarized in a table.

Response: The statistical outcomes have been presented in tables as suggested.

MINOR COMMENTS

-The English usage in much of the manuscript could be improved. I will note some points below.

Response: We appreciate the constructive comments very much and revised the manuscript based on all the comments.

-Define rETR in the Abstract (P2L10)

Response: rETR has been defined as “relative electron transport”.

-P2L3 and throughout: define the abbreviations when first used: CO2, DIC, HCO3-, rETR

Response: They have been corrected to “carbon dioxide (CO_2), relative electron transport rate (rETR), bicarbonate (HCO_3^-), dissolved inorganic carbon (DIC)”.

-P2L16 change "is" to "was"

Response: Corrected.

-P3L26 change to "the marine biological"

Response: Corrected.

-P3L31 need a different word than compelling

Response: It has been changed to "key".

-P3L33 and throughout: don’t finish sentences with "etc".

Response: It has been changed to " and so forth".

-P3L34 change "could" to "can".

Response: Corrected.

-P3L36 misselled "dominate"

Response: Corrected

-P3L37 P3L40-41 How is RUBISCO important? What is it, an enzyme?

Response: It has been revised to “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).” at P4L45-49.

-P4L45-48: the carbon concentrating mechanisms are named but not explained. A reader like myself has no way to know what "multiple carbon anhydrase, assumed C4-type pathway" represents

Response: It has been revised to “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 (α, β, γ) and unusual (δ, ζ) 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).” at P5L51-57.

-P4L54 define CCMs (CO2 concentrating mechanisms, right?)

Response: It was defined in the abstract. We have defined it again at line P5L52.

-P4L57: keep consistent units between discussions of CO2 or pCO2. Discuss either CO2 concentration or partial pressure. Reader has no way to compare 5 mol/L CO2 to a pCO2 of 1800 _atm.

Response: It has been revised to “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 CA could be detected even when CO_2 concentration was 27 μmol L^{-1}.” at P5L67-69.

-P5L69: cite the refernces yourself, don’t refer to references therein

Response: It has been revised to “phosphorus acquisition, utilization and storage (Lin *et al.*, 2016; Gao et al., 2018).” at P6L80.

-P5L70: what is the relationship?

Response: It has been revised to “Some studies show the essential role of phosphorus in regulating inorganic carbon acquisition in green algae.” at P6L81-82.

-P5L75-76: remove "the capacity of"

Response: Corrected.

-P5L78-79: all these mechanisms/pathways! How do they interrelate?

Response: It has been revised to "we aimed to test this hypothesis by investigating the variation of CCMs (including active transport of HCO_3^- and carbonic anhydrase activity) and photosynthetic rate under five levels of phosphate and two levels of CO_2 conditions. We also measured redox activity of plasma membrane as it is deemed to be critical to activate carbonic anhydrase (Nimer *et al.*, 1998)." at P6L89-95.

-P5L82: as CO2 is removed by diatom growth, the inorganic carbon equilibria will shift to convert HCO32- to CO2. How do the kinetics of this potentially affect this study?

Response: It is exactly true that the shift from HCO_3^- to CO_2 will occur as CO_2 is removed by diatom's growth and it usually leads to the increase of OH- and pH in seawater because the dissolution rate of CO_2 from the atmosphere cannot catch up with its removal rate (Gao et al., 1993; Hansen, 2002). However, the pH in the cultures was relatively stable due to the addition of tris (hydroxymethyl) aminomethane-HCl buffer, which resulted in stable CO_2 levels in the cultures. And this is what we aimed to achieve so that the culures could be conducted under two CO_2 levels: one is the aimbient level (12.6 μmol L^{-1}) and the other is CO_2 limiting level (2.8 μmol L^{-1}).

The text has been clarifed to "The cultures were open to the ambient atmosphere and the rise of culture pH was below 0.02 unites (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." at P8L120-123.

Gao K, Aruga Y, Asada K, et al. Enhanced growth of the red alga *Porphyra yezoensis* Ueda in high CO_2 concentrations. Journal of Applied Phycology, 1991, 3(4):

355-362.

Hansen PJ. 2002. Effect of high pH on the growth and survival of marine phytoplankton: implications for species succession. Aquatic Microbial Ecology 28: 279-288.

-P6L105: what does "algae after in light" mean?

Response: It has been clarified to “where F_M’ is the maximal fluorescence levels from algae in the actinic light after application a saturating pulse” at P9L146-147.

-P6L108: change to "photosynthetic and respiration rates"

Response: Corrected.

-P7L114-116: why measure photosynthesis for 5 minutes but respiration for 10?

Response: The oxygen variation rate due to dark respiration is slower than that caused by photosynthesis, so more times are needed for dark respiration measurement. It has been explained “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.” at P9L156-159.

-P7L126: what is "Ci-saturated maximum rate"?

Response: It has been revised to “DIC-saturated maximum rate” at P10L169.

-P8L149: by "samples" do you mean the diatom cells?

Response: Yes, it has been changed to “cells” at P11L194.

-P9L156: what is the exofacial ferricyanide reduction reaction?

Response: Exofacial means extracellular. The text has been clarified to “The redox activity of plasma membrane was assayed by monitoring the change in ferricyanide $K_3Fe(CN)_6$ concentration that accompanied reduction of the ferricyanide to ferrocyanide. The ferricyanide [$K_3Fe(CN)_6$] cannot penetrate intact cells and has been used as an external electron acceptor (Nimer *et al.*, 1998; Wu & Gao, 2009). Stock solutions of $K_3Fe(CN)_6$ were freshly prepared before use. Five mL of samples were taken after two hours of incubation with 500 μmol $K_3Fe(CN)_6$ and centrifuged at 4000 *g* for 10 min (20^{o}C). The concentration of $K_3Fe(CN)_6$ in the supernatant was measured spectrophotometrically at 420 nm (Shimadzu UV-1800, Kyoto, Japan). The decrease of $K_3Fe(CN)_6$ during the two hours of incubation was used to assess the rate of extracellular ferricyanide reduction (Nimer et al., 1998).” at P11L196-206.

-P9L158: "pH drift" connotes instrument drift to me, not pH changes due to cellular Activity

Response: To clarify it, the text has been revised to “Cell-driving pH drift experiment” at P12L207.

-P9L169: change "on differences"

Response: It has been corrected to “to assess the effects of CO_2 and phosphate on net photosynthetic rate” at P12L217-218.

-P9L174: Need more information on the Bonferroni correction.

-P11L200: is the Bonferroni correction the same as the "Post hoc LSD comparison" mentioned here? I don’t think it is? What is this comparison?

Response: The Bonferroni test uses a straight-forward t test but then evaluates that t at $\alpha = 0.05/c$, where c is the number of comparisons. Some of the post-hoc comparisons may not be appropriate for repeated-measure ANOVA while Bonferroni is the best reliable one (Ennos, 2007). The text has been revised to "Bonferroni was conducted for *post hoc* investigation as it is the best reliable *post hoc* test for repeated measures ANOVA (Ennos, 2007)"at P12L223-224.

Ennos A R. Statistical and Data Handling Skills in Biology. Pearson Education, 2007, p.96.

-P11L213: change "access" to "assess"

Response: Corrected.

-P11L216: what does "interplayed" signify?

Response: It has been changed to "interacted".

-P12L230: is the peak the same as the plateau mentioned earlier?

Response: Yes.

-P12L231: what do you mean by "assayed"?

Response: It has been changed to "assessed".

-P13L247: how was the pH compensation point identified?

Response: As mentioned in section 2.7, "the pH compensation point was obtained

when there was no a further increase in pH." at P12L211-212.

-P13L260-261: not sure what "comparative photosynthetic rates" means

Response: It has been corrected to "showed similar photosynthetic rates for the lower and higher CO_2 treatments." at P17L313-314.

-P15L293: does "inorganic carbon" here mean both carbonate and bicarbonate?

Response: No, it means both CO_2 and HCO_3^- because CO_3^{2-} cannot be used for photosynthesis. The text has been revised to " inorganic carbon (CO_2 and HCO_3^-)" P18L338.

-P15L303: change to "increased the redox"

Response: Corrected.

-P15L304: misspelled extracellular

Response: Corrected.

-P16L315: change to "as the CO2"

Response: Corrected.

-P16L317: change to "with a strong"

Response: Corrected.

-P16L319: change to "the red macroalgae"

Response: Corrected.

-P17L340: change to "the potential mechansims"

Response: Corrected.

-P17L342: change to "are hampered"

Response: Corrected.

-P17L348: change to "growth"

Response: Corrected.

Anonymous Referee #2

This manuscript reports results of experiments which aim to investigate the link between P availability and the C uptake by S. costatum diatoms. While apparently interesting interactions were observed, insufficient detail is provided about the methods, and I have reservations about the suitability of the statistical analysis employed.

Response: We appreciated these comments and have improved the manuscript by responding to the comments.

Major Comments

The introduction would benefit from adding hypotheses.

Response: We did state our hypothesis at line and it reads “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 CO_2 limitation conditions." at P6L85-89.

The methods section has a rather low amount of detail for each of the methods presented, with details of instrument manufacturers and models, and references frequently missing. In particular, there is no mention of how cells were counted, and normalizing this is an important aspect of many of the measurements.

Response: We appreciate these comments and have added more details to the Methods. In terms of cell counting, it has been clarified to "Cell density was determined by direct counting with an improved Neubauer haemocytometer (XB-K-25, Qiu Jing, Shanghai, China)." at P7L110-111.

I am also not convinced that 3 replicates of each treatment is sufficient, at least not for the parametric statistical testing that is employed.

Response: We agree that a higher replication would strengthen the study. However, we had to reduce the replication to obtain reliable data as we had 10 treatments for each measurement, indicating 30 samples (10×3) for each measurement.
Three-replicate is fine for parametric statistical tests and it were used in many studies (Riebesell et al., 2007; Gao et al., 2012; Walworth et al., 2016; Hong et al., 2017).

Hong H, Shen R, Zhang F, et al. The complex effects of ocean acidification on the prominent N_2-fixing cyanobacterium *Trichodesmium*. Science, 2017, 356(6337): 527-531.

Riebesell U, Schulz K G, Bellerby R G J, et al. Enhanced biological carbon consumption in a high CO_2 ocean. Nature, 2007, 450(7169): 545-548.

Gao K, Xu J, Gao G, et al. Rising CO_2 and increased light exposure synergistically reduce marine primary productivity. Nature Climate Change, 2012, 2(7): 519-523.

Walworth N G, Fu F X, Webb E A, et al. Mechanisms of increased *Trichodesmium* fitness under iron and phosphorus co-limitation in the present and future ocean. Nature Communications, 2016, 7: 12081.

The results section does not report what the actual values of the measured parameters were, only the results of statistical tests for differences between treatments.

Response: In our previous manuscripts, we were informed by some reviewers that the report of actual values was unnecessary as readers could see them from tables and figures. We anyhow added some actual values to the Results section at P13L226-P16L302.

There is rather limited discussion of the mechanisms behind each of the effects observed.

Response: We agree that we did not discuss the molecular mechanisms for the effects observed and it would be very speculative to do that since our study did not refer to molecular measurements. Instead, we discussed the relevant mechanisms based on our data. For instance, we think P enrichment enhanced rETR and photosynthetic rate via inducing more synthesis of photosynthetic pigment. We also discussed the relationship among extracellular CA activity, redox activity of plasma membrane, and rETR. We think the combined effect of CO_2 limit and P enrichment on extracellular CA activity was implemented by their influence on rETR first and then redox activity of plasma membrane; the increased rETR under the conditions of CO_2 limit and P enrichment could generate excess reducing equivalents which

stimulated redox activity of plasma membrane; redox activity of plasma membrane could induce extracellular CA activity via protonation extrusion of its active centre.

In addition, in the Discussion section, we compared our results to those of similar studies, explained the meaning of our finding, tested our hypothesis by integrating the measured parameters, and finally produced take-home massage "P enrichment could induce activity of extracellular carbonic anhydrase and direct utilization of HCO_3^- in *S. costatum* to help overcome the CO_2 limitation, as well as increasing photosynthetic pigment content and rETR to provide required energy." in the Conclusion section. Honestly, we have no idea of how to improve the discussion of the mechanisms behind each of the effects observed further and hope to hear more specific suggestions.

Minor Comments

Not all of the figures are referred to in the text, or at least not in the correct order (there is no Fig. 3 reference between the first reference for Fig. 2 and that for Fig. 4).

Response: We think all the figures were referred to in the text in order. We did refer to Fig. 3 between Fig. 2 and Fig. 4 at P14L251.

Line 10: Define rETR the first time it is used.

Response: It has been defines as "relative electron transport rate".

Line 43: This should say 'limiting', not 'limited'.

Response: Corrected.

Line 48: Give the name in full the first time it is used, and where it is used at the start of a sentence.

Response: Corrected throughout the text.

Lines 54-60: Please define all these acronyms the first time they are used.

Response: Corrected.

Line 88: I don't think the units given here for irradiance are correct (micromoles per m squared).

Response: It has been corrected to "μmol photons m^{-2} s^{-1}" at P7L103.

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 is was more than 1 μmol L^{-1}. This studyese findings indicates P enrichment could the essential role of P in regulatingenhance 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^- useand CO_2-concentrating mechanisms (CCMs) in *S. costatum* and. This study sheds light on how

bloom-forming algae cope with carbon limitation during the development of red tides.

Keywords: carbonic anhydrase; CO_2 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 ~~compelling~~ 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 ~~etc~~. (Smetacek & Zingone, 2013; Jeong *et al.*, 2015). When inorganic nitrogen and phosphorus are replete, diatoms ~~could~~ can out-compete chrysophytes, raphidophytes and dinoflagellates (Berg *et al.*, 1997; Jeong *et al.*, 2015; Barton *et al.*, 2016) and dom~~a~~inate 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~~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 ~~limited~~ 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 (α, β, γ) and unusual (δ, ζ) 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~~etc~~. (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~~CA~~ could be detected even when ~~*PCO*$_2$~~ CO_2 concentration ~~i~~was ~~1800~~ 27 μmol L^{-1} ~~μatm~~. Chen and Gao (2004) showed

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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.

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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~~and the references therein~~). Some studies show the essential role of ~~relationship between~~ phosphorus ~~availability and~~ 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 CO_2 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 CO_2 limitation conditions. In the present study, we aimed to test this hypothesis by investigat~~ed t~~ing the variation of CCMs (including active transport of HCO_3^- and carbonic anhydrase activity) and photosynthetic rate ~~he inorganic acquisition pathways, photosynthetic~~

~~CO_2 affinity, carbonic anhydrase activity, redox activity of plasma membrane, and photosynthetic rate~~ under five levels of phosphate and two levels of CO_2 conditions ~~to test this hypothesis~~. 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.

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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 $NaH_2PO_4 \cdot 2H_2O$. 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 $\times 10^6$ 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 unites (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).

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 °C; Thermo Fisher Scientific Inc., USA). Total alkalinity (TAlk) was determined at 25.0 °C 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}. ~~All experiments were conducted in triplicates.~~ CO_2 level in seawater was calculated via CO2SYS (Pierrot *et al.*,

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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).

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2.2.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}) = $(F_M' - F_t) / F_M' \times 0.5 \times PFD$ (Gao et al., 2018), where F_M' is the maximal fluorescence levels from algae ~~after~~ in the actinic light after application a saturating pulse, Ft 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 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 8.2). 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 ~~Ci~~DIC-saturated maximum rate of photosynthetic O_2 evolution), was calculated from the Michaelis-Menten kinetics equation (Caemmerer and Farquhar 1981): $V = V_{max} \times [S] / (K_{0.5} + [S])$, 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 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). ~~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

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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 $a = 11.47 \times OD_{664} - 0.40 \times OD_{630}$, Chl $c = 24.36 \times OD_{630} - 3.73 \times OD_{664}$. (Gao et al., 2018b), and was normalized to pg cells^{-1}.

~~2.5.~~2.4. *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 CO_2-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: $E.U. = 10 \times (T_0 / T - 1)$, where T_0 and T represent the time required for the pH change in the absence or presence of the ~~samples~~cells, respectively.

~~2.6.~~2.5. *Measurement of redox activity in the plasma membrane*

The redox activity of plasma membrane was assayed by monitoring the change in $K_3Fe(CN)_6$ concentration that accompanied reduction of the ferricyanide to ferrocyanide. The ~~incubating the cells with 500 μmol~~ ferricyanide [$K_3Fe(CN)_6$] ~~that~~ cannot penetrate intact cells and has been used as an external electron acceptor (Nimer *et al.*, 1998; Wu & Gao, 2009). Stock solutions of $K_3Fe(CN)_6$ were freshly prepared before use. Five mL of samples were taken after two hours of incubation with 500 μmol $K_3Fe(CN)_6$ and centrifuged at 4000 g for

10 min (20°C). The concentration of $K_3Fe(CN)_6$ in the supernatant was measured spectrophotometrically at 420 nm (Shimadzu UV-1800, Kyoto, Japan). The decrease of $K_3Fe(CN)_6$ during the two hours of incubation ~~The absorbance of supernatant at 420 nm~~ was ~~measured~~ used ~~immediately~~ to assess the rate of ~~exofacial~~extracellular ferricyanide reduction that was presented as μmol $(10^6$ cells$)^{-1}$ h^{-1}(Nimer et al., 1998).

~~2.7.~~2.6. *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 × 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.

~~2.8.~~2.7. *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 ~~differences~~ net photosynthetic rate, dark respiration rate, ratio of net photosynthetic rate to dark respiration rate, rETR, Chl *a*, $K_{0.5}$, CA_{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 ~~($F_{(4, 20)} = 3.662$, $P = 0.021$, Fig. 1a)~~, with each factor having a main effect (~~$F_{(1, 20)} = 11.286$, $P = 0.003$ for CO_2, $F_{(4, 20)} = 157.925$, $P < 0.001$ for phosphate~~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 O_2 (10^9 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 O_2 (10^9 cells)$^{-1}$ h^{-1}) until 4 μmol L^{-1} phosphate. In terms of dark respiration rate (Fig. 1b), p~~P~~hosphate had a main effect on ~~dark respiration~~ it ~~rate ($F_{(4, 20)} = 169.050$, $P < 0.001$, Fig. 1b),~~ and it interacted with CO_2 (~~$F_{(4, 20)} = 3.226$, $P = 0.034$~~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 CO_2 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 CO_2 and phosphate had a main effect ~~($F_{(1, 20)} = 32.443$, $P < 0.001$ for CO_2, $F_{(4, 20)} = 7.081$, $P = 0.001$ for phosphate)~~, and they ~~interplayed~~ interacted on the ratio of respiration to photosynthesis (~~$F_{(4, 20)}$~~

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246 ~~= 8.299, P < 0.001~~Table 1). LC increased the ratio when phosphate was lower than 4 μmol L^{-1}

247 but did not affect it when phosphate levels were 4 or 10 μmol L^{-1}.

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249 Both CO_2 and phosphate affected rETR ~~($F_{(1, 20)}$ = 28.717, P < 0.001 for CO_2, $F_{(4, 20)}$ =~~

250 ~~127.860, P < 0.001 for phosphate)~~ and they also showed an interactive effect (~~$F_{(4, 20)}$ = 3.296,~~

251 ~~P = 0.031,~~ Fig. 3 & Table 2). For instance, *post hoc* LSD comparison showed that LC did not

252 affect rETR at lower phosphate levels (0.05 and 0.25 μmol L^{-1}) but increased it at higher

253 phosphate levels (1–10 μmol L^{-1}). Regardless of CO_2 treatment, rETR increased with

254 phosphate level (0.05–4 μmol L^{-1}) but the highest phosphate concentration did not result in a

255 further increase in rETR (LSD, P ~~=~~ > 0.05).

256 The content of Chl *a* was measured to investigate the effects of CO_2 and phosphate on

257 photosynthetic pigment in *S. costatum* (Fig. 4). Both CO_2 and phosphate affected the

258 synthesis of Chl *a* ~~($F_{(1, 20)}$ = 32.963, P < 0.001 for CO_2, $F_{(4, 20)}$ = 92.045 P < 0.001 for~~

259 ~~phosphate)~~ and they had an interactive effect (~~$F_{(4, 20)}$ =3.871, P = 0.017~~Table 2). *Post hoc* LSD

260 comparison (P = 0.05) showed that LC did not affect Chl *a* at 0.05 or 0.25 μmol L^{-1} phosphate

261 but stimulated Chl *a* synthesis at higher phosphate levels (1–10 μmol L^{-1}). Irrespective of CO_2

262 treatment, Chl *a* content increased with phosphate level and reached the plateau (0.19 ± 0.01

263 pg $cell^{-1}$ for AC and 0.23 ± 0.01 pg $cell^{-1}$ for LC) at 4 μmol L^{-1} phosphate.

264 To ~~access~~ assess the effects of CO_2 and phosphate on photosynthetic CO_2 affinity in *S.*

265 *costatum*, the net photosynthetic rates of cells exposure to seven levels of DIC were measured

266 (Fig. 5). After curve fitting, the values of $K_{0.5}$ for CO_2 were calculated (Fig. 6). CO_2 and

267 phosphate interplayed on $K_{0.5}$ ~~($F_{(4, 20)}$ = 3.821, P = 0.018)~~ and each had a main effect (~~$F_{(1, 20)}$ =~~

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96.182, $P < 0.001$ for CO_2, $F_{(4, 20)} = 40.497$, $P < 0.001$ for phosphate 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 in $K_{0.5}$ to 2.59 ± 0.29 μmol kg^{-1} seawater compared to the value of 4.00 ± 0.30 μmol kg^{-1} seawater at 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 (Table 3). *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 (0.04–0.10 EU (10^6 cells)$^{-1}$) with phosphate level and stopped increasing at 1 μmol L^{-1} phosphate. Under LC, CA_{ext} activity also increased (0.04–0.35 EU (10^6 cells)$^{-1}$) with phosphate level but reached the peak at 4 μmol L^{-1} phosphate. The redox activity of plasma membrane was also assayed assessed 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 Table 3).

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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 (~~$F_{(10, 100)} = 7604.563, P < 0.001$~~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 (~~$F_{(10, 100)} = 47.469, P < 0.001$~~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_2 treatment did not affect pH compensation point ~~($F_{(1, 20)} = 0.056, P = 0.816$)~~ but phosphate had a main effect~~–~~ (Table 3 ~~$F_{(4, 20)} = 226.196, P < 0.001$~~). 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.

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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*keletonema*~~.~~ *costatum* grown at higher phosphate levels (4 and 10 μmol L^{-1}) also showed ~~comparative~~ similar photosynthetic rates ~~between~~ 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 *S. costatum*.

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 *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 alga *Dunaliella tertiolecta* (Geider *et al.*, 1998) and brown alga *Sargassum muticum* (Xu et al., 2017). The increased photosynthetic pigment in *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 *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 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 the redox activity of plasma membrane in this study. It has been proposed that redox activity of plasma membrane could induce eextracellular 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 CO_2 limited ing 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 HCO_3^- utilization due to phosphate enrichment

A pH compensation point over 9.2 has been considered a sign of direct HCO_3^- use for algae (Axelsson & Uusitalo, 1988) as the CO_2 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 HCO_3^- 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 HCO_3^- directly and photosynthesis only depends on CO_2 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 HCO_3^- 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 casautive 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* ~~is~~ 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.

5. Conclusions

The present study investigated the role of P in regulating inorganic carbon acquisition and

400 CO_2 concentrating mechanisms in diatoms for the first time. The intensive photosynthesis and
401 quick growth during algal blooms usually result in noticeable increase of pH and decrease of
402 CO_2. Our study demonstrates that P enrichment could induce activity of extracellular carbonic
403 anhydrase and direct utilization of HCO_3^- in *S. costatum* to help overcome the CO_2 limitation,
404 as well as increasing photosynthetic pigment content and rETR to provide required energy.
405 This study provides important insight into the connection of phosphorus and carbon
406 acquisition in diatoms and the mechanisms that ***S. costatum*** dominates algal blooms.

407 **Author contribution**

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

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575 Table 1 Two-way analysis of variance for the effects of CO_2 and phosphate on net
576 photosynthetic rate, dark respiration rate and ratio of respiration to photosynthesis of *S.*
577 *costatum*. CO_2*phosphate means the interactive effect of CO_2 and phosphate, df means degree
578 of freedom, F means the value of F statistic, and Sig. means p-value.
579

Source	Net photosynthetic rate			Dark respiration rate			Ratio of respiration to photosynthesis		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
CO_2	1	11.286	0.003	1	1.262	0.275	1	32.443	<0.001
Phosphate	4	157.925	<0.001	4	169.050	<0.001	4	7.081	0.001
CO_2*phosphate	4	3.662	0.021	4	3.226	0.034	4	8.299	<0.001
Error	20			20			20		

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.

Source	rETR			Chl *a*			$K_{0.5}$		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
CO_2	1	28.717	<0.001	1	32.963	<0.001	1	96.182	<0.001
Phosphate	4	127.860	<0.001	4	92.045	<0.001	4	40.497	<0.001
CO_2*phosphate	4	3.296	0.031	4	3.871	0.017	4	3.821	0.018
Error	20			20			20		

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.

Source	CA_{ext} activity			redox activity of plasma membrane			pH compensation point		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
CO_2	1	569.585	<0.001	1	937.963	<0.001	1	0.056	0.816
Phosphate	4	176.392	<0.001	4	276.362	<0.001	4	226.196	<0.001
CO_2*phosphate	4	87.380	<0.001	4	137.050	<0.001	4	0.040	0.997
Error	20			20			20		

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.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	40.766	10	4.077	8737.941	<0.001
Time*CO_2	0.003	10	<0.001	0.569	0.838
Time*phosphate	0.886	40	0.022	47.496	<0.001
Time*CO_2*phosphate	0.002	40	<0.001	0.112	1.000
Error	0.093	200	<0.001		

596 **Figure legends**

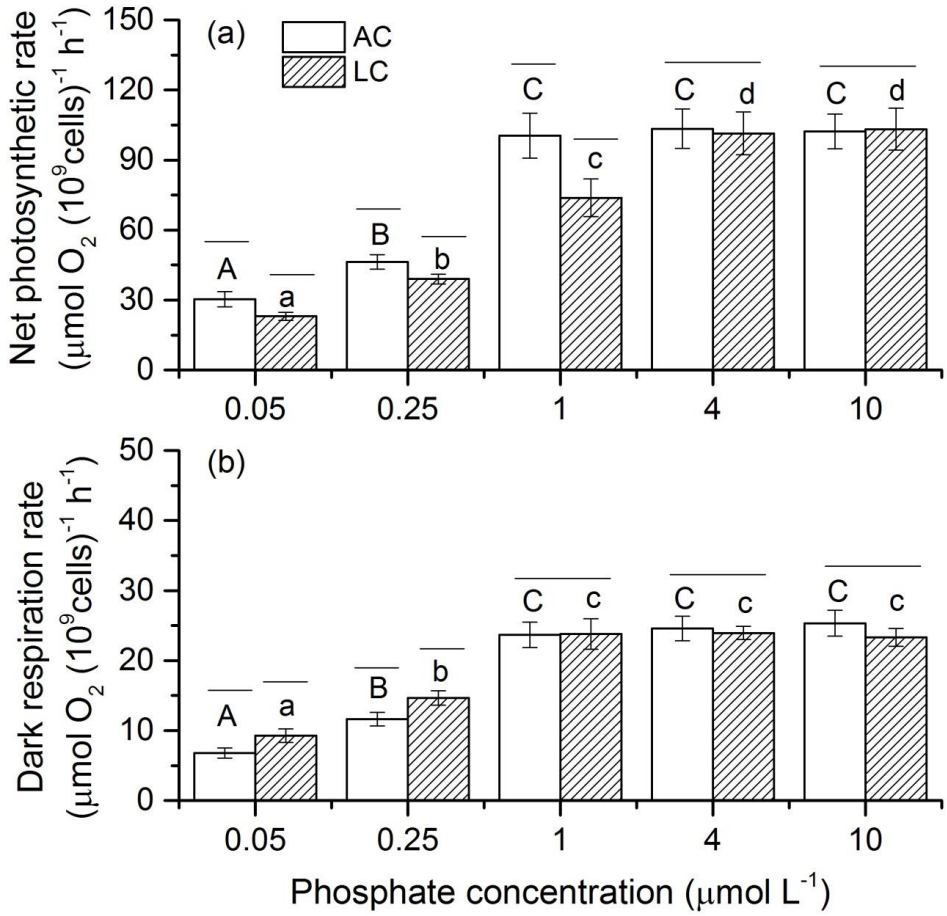
597 **Fig. 1.** Net photosynthetic rate (a) and dark respiration rate (b) in *S. costatum* grown at
598 various phosphate concentrations after ambient (AC) and low CO_2 (LC) treatments. The error
599 bars indicate the standard deviations (n = 3). Different letters represent the significant
600 difference ($P < 0.05$) among phosphate concentrations (capital for AC, lower case for LC).
601 Horizontal lines represent significant difference ($P < 0.05$) between CO_2 treatments.
602 **Fig. 2.** Ratio of respiration rate to net photosynthetic rate in *S. costatum* grown at various
603 phosphate concentrations after ambient (AC) and low CO_2 (LC) treatments. The error bars
604 indicate the standard deviations (n = 3). Different letters represent the significant difference (P
605 < 0.05) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines
606 represent significant difference ($P < 0.05$) between CO_2 treatments.
607 **Fig. 3.** Relative electron transport rate (rETR) in *S. costatum* grown at various phosphate
608 concentrations after ambient (AC) and low CO_2 (LC) treatments. The error bars indicate the
609 standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$)
610 among phosphate concentrations (Capital for AC lower case for LC). Horizontal lines
611 represent significant difference ($P < 0.05$) between CO_2 treatments.
612 **Fig. 4.** Photosynthetic Chl *a* content in *S. costatum* grown at various phosphate concentrations
613 after ambient (AC) and low CO_2 (LC) treatments. The error bars indicate the standard
614 deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among
615 phosphate concentrations (capital for AC, lower case for LC). Horizontal lines represent
616 significant difference ($P < 0.05$) between CO_2 treatments.
617 **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 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.

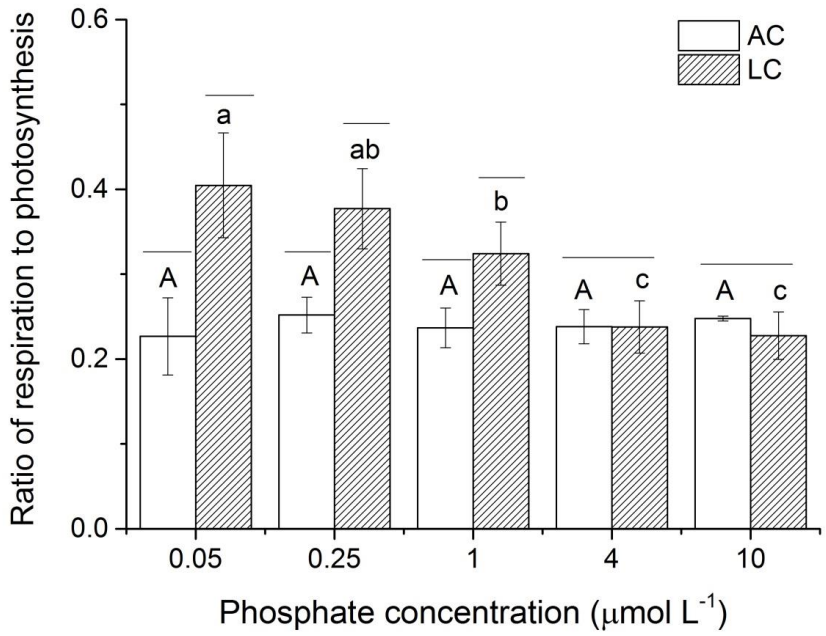
Fig. 7. CA_{ext} activity (a) and reduction rate of ferricyanide (b) in *S. costatum* grown at various phosphate concentrations after ambient (AC) and low CO_2 (LC) treatments. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) 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).


(a)
AC
LC
Net photosynthetic rate
(μmol O2 (10^9cells)^-1 h^-1)
150
120
90
60
30
0
A
a
B
b
C
c
C
d
C
d
0.05
0.25
1
4
10
(b)
Dark respiration rate
(μmol O2 (10^9cells)^-1 h^-1)
50
40
30
20
10
0
A
a
B
b
C
c
C
c
C
c
0.05
0.25
1
4
10
Phosphate concentration (μmol L^-1)

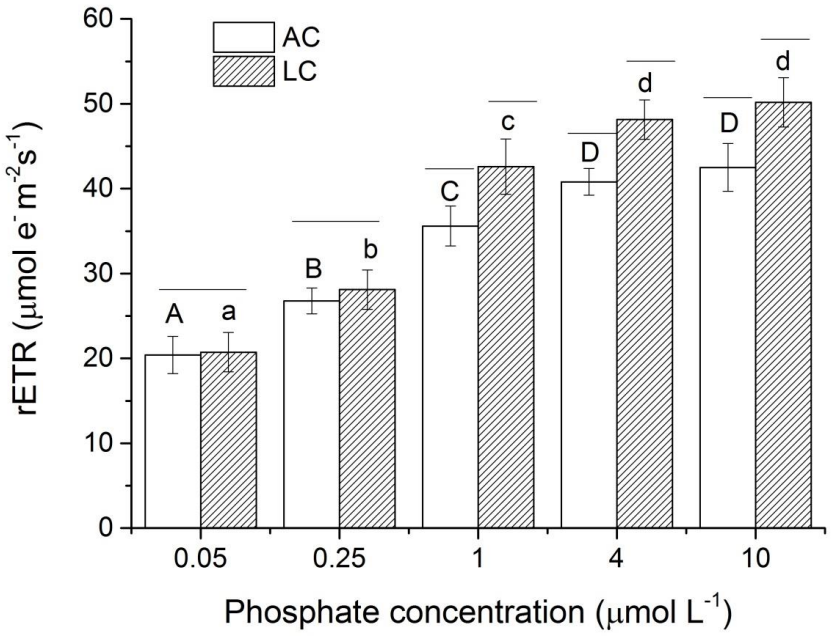
633

634 **Fig. 1**


Ratio of respiration to photosynthesis
0.6
0.4
0.2
0.0
AC
LC
a
ab
b
A
A
A
A
c
A
c
0.05
0.25
1
4
10
Phosphate concentration (μmol L-1)

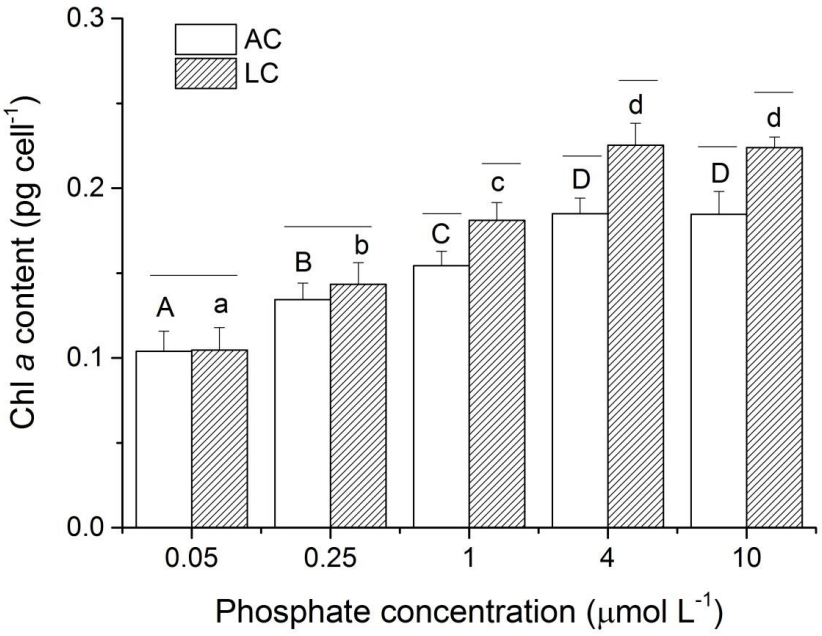
635

636 **Fig. 2**


AC
LC
rETR (μmol e⁻ m⁻² s⁻¹)
60
50
40
30
20
10
0
A
a
B
b
C
c
D
d
D
d
0.05
0.25
1
4
10
Phosphate concentration (μmol L⁻¹)

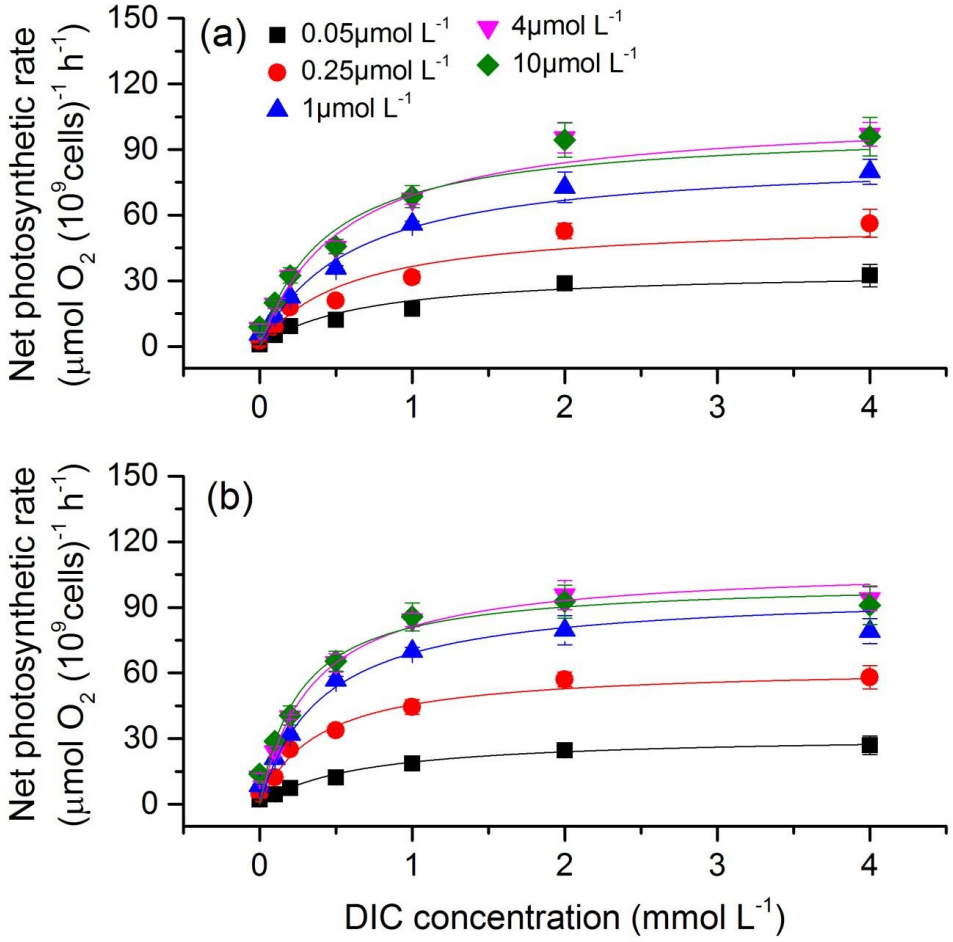
637

638 **Fig. 3**


AC
LC
Chl a content (pg cell-1)
0.0
0.1
0.2
0.3
A
a
B
b
C
c
D
d
D
d
0.05
0.25
1
4
10
Phosphate concentration (μmol L-1)

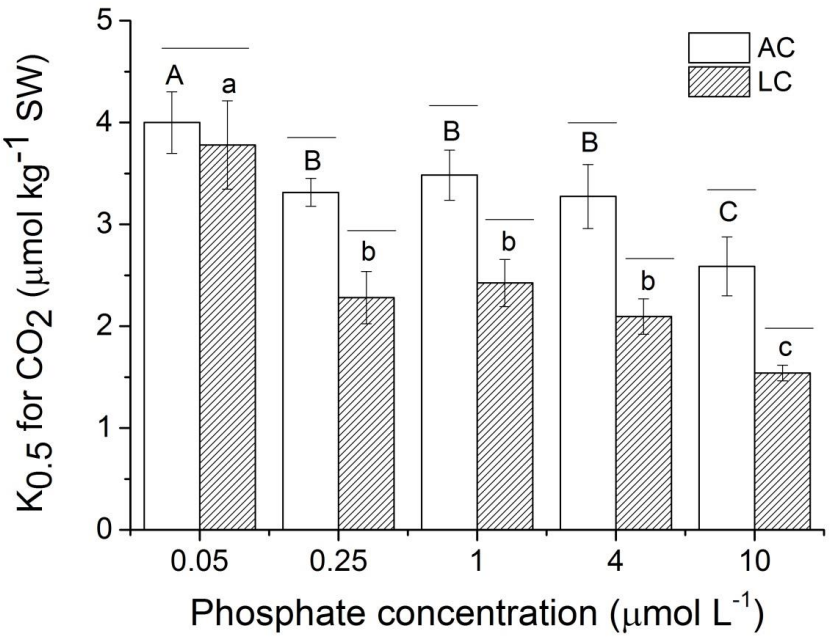
639

640 **Fig. 4**


(a)
0.05μmol L-1
0.25μmol L-1
1μmol L-1
4μmol L-1
10μmol L-1
Net photosynthetic rate (μmol O2 (10^9cells)^-1 h^-1)
150
120
90
60
30
0
0
1
2
3
4
(b)
Net photosynthetic rate (μmol O2 (10^9cells)^-1 h^-1)
DIC concentration (mmol L-1)

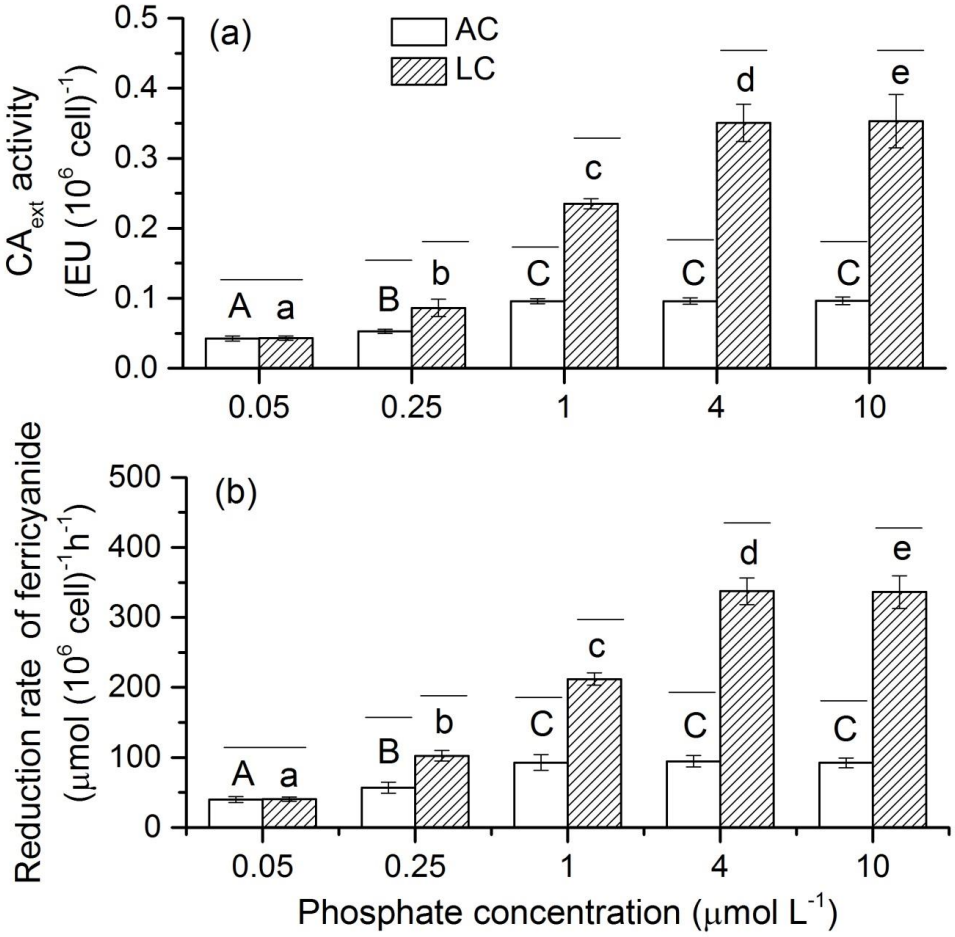
641

642 **Fig. 5**


AC
LC
A
a
B
b
B
b
B
b
C
c
0
1
2
3
4
5
0.05
0.25
1
4
10
$K_{0.5}$ for CO_2 (μmol kg^{-1} SW)
Phosphate concentration (μmol L^{-1})

643

644 **Fig. 6**


(a)
AC
LC
CA_{ext} activity
(EU (10^6 cell)$^{-1}$)
0.5
0.4
0.3
0.2
0.1
0.0
A a
B b
C c
C d
C e
0.05
0.25
1
4
10
(b)
Reduction rate of ferricyanide
(μmol (10^6 cell)$^{-1}$h^{-1})
500
400
300
200
100
0
A a
B b
C c
C d
C e
0.05
0.25
1
4
10
Phosphate concentration (μmol L^{-1})

645

646 **Fig. 7**

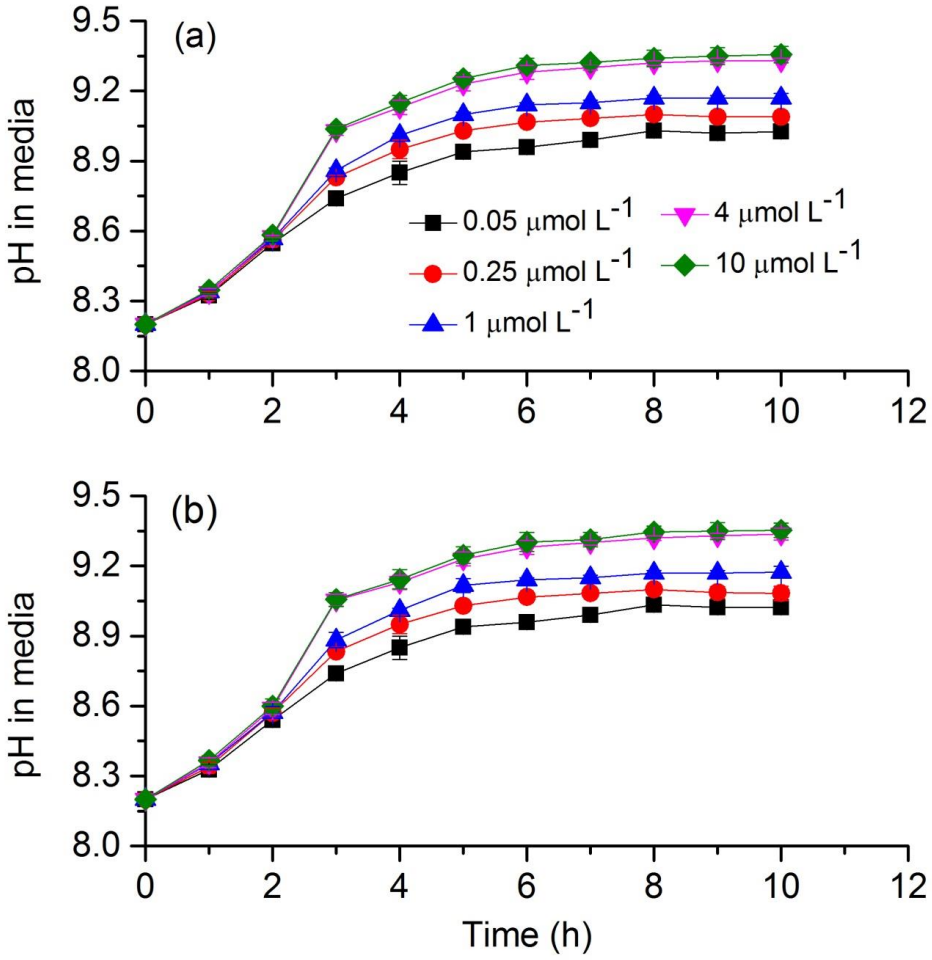

(a)
(b)
pH in media
Time (h)
0.05 μmol L-1
0.25 μmol L-1
1 μmol L-1
4 μmol L-1
10 μmol L-1
9.5
9.2
8.9
8.6
8.3
8.0
0
2
4
6
8
10
12

Fig. 8