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Interactive comment on "Regulation of inorganic carbon acquisition in a red tide alga (*Skeletonema costatum*): the importance of phosphorus availability" *by* Guang Gao et al.

Guang Gao et al.

jrxia@gzhu.edu.cn

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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 com-

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pelled 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 pHNBS was measured by a pH meter (pH 700, Eutech Instruments, Singapore) that was equipped with an Orion® 8102BN Ross combination electrode (Thermo Electron Co., USA) and calibrated with standard National Bureau of Standards (NBS) buffers (pH = 4.01, 7.00, and 10.01 at 25.0 oC; Thermo Fisher Scientific Inc., USA). Total alkalinity (TAlk) was determined at 25.0 oC by Gran acidimetric titration on a 25-ml sample with a TAlk analyzer (AS-ALK1, Apollo SciTech, USA), using the precision pH meter and an Orion® 8102BN Ross electrode for detection. To ensure the accuracy of TAlk, the TAlk analyser was regularly calibrated with certified reference materials from Andrew G. Dickson's laboratory (Scripps Institute of Oceanography, U.S.A.) at a precision of $\pm$ 2 μmol kg-1." at P8L127-135.

-Besides using the barely-described pH system, how did the authors know the CO2

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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 $\pm$ 0.02 unites during the two hours of pH treatment. CO2 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 KSO4- 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 (CO2), relative electron transport rate (rETR), bicarbonate (HCO3-), dissolved inorganic carbon (DIC)”.

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-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 CO2 is transformed into organic carbon, has a relatively low affinity for CO2 and is commonly less than half saturated under current CO2 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 CO2 concentrating mechanisms (CCMs), for instance, active

transport of HCO3-, the passive influx of CO2, multiple carbonic anhydrase (including both common (α, β, γ) and unusual (δ, ζ) families that carries out the fast interconversion of CO2 and HCO3-), assumed C4-type pathway (using phosphoenolpyruvate to capture more CO2 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 CO2 concentration was less than 5 μmol L-1 while Rost et al. (2003) reported that activity of extracellular CA could be detected even when CO2 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?

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Response: It has been revised to “we aimed to test this hypothesis by investigating the variation of CCMs (including active transport of HCO3- and carbonic anhydrase activity) and photosynthetic rate under five levels of phosphate and two levels of CO2 conditions. We also measured redox activity of plasma membrane as it is deemed to be critical to activate carbonic anhydrase (Nimer et al., 1998).” 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 HCO3- to CO2 will occur as CO2 is removed by diatom’s growth and it usually leads to the increase of OH- and pH in seawater because the dissolution rate of CO2 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 CO2 levels in the cultures. And this is what we aimed to achieve so that the culures could be conducted under two CO2 levels: one is the aimbient level (12.6 μmol L-1) and the other is CO2 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 CO2 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 CO2 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 FM’ 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 K3Fe(CN)6 concentration that accompanied reduction of the ferricyanide to ferrocyanide. The ferricyanide [K3Fe(CN)6] cannot penetrate intact cells and has been used as an external electron acceptor (Nimer et al., 1998; Wu & Gao, 2009). Stock solutions of K3Fe(CN)6 were freshly prepared before use. Five mL of samples were taken after two hours of incubation with 500 μmol K3Fe(CN)6 and centrifuged at 4000 g for 10 min (20oC). The concentration of K3Fe(CN)6 in the supernatant was measured spectrophotometrically at 420 nm (Shimadzu UV-1800, Kyoto, Japan). The decrease of K3Fe(CN)6 during the two hours of incubation was used to assess the rate of extracellular ferricyanide reduction (Nimer et al., 1998).” at P11L196-206.

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-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 CO2 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 α = 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 CO2 treatments.” at P17L313-314.

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

Response: No, it means both CO2 and HCO3- because CO32- cannot be used for photosynthesis. The text has been revised to “ inorganic carbon (CO2 and HCO3-)” 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.

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-P17L340: change to "the potential mechansims"

Response: Corrected.

-P17L342: change to "are hampered"

Response: Corrected.

-P17L348: change to "growth"

Response: Corrected.

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