Reviewer 1

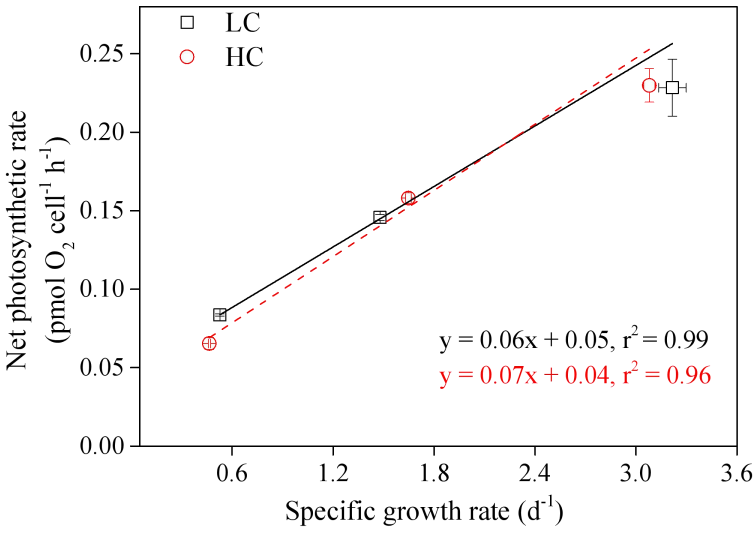
Comments:

I am very happy to see a study of the interaction of increased pCO_2 with temperature and photoperiod on a model diatom. These multifactorial experiments are challenging but necessary. To limit the (infinite) range of possible combinations the authors matched photoperiod to expected seasonal temperature.

I think the data, as presented, is valuable, but under-analyzed. I offer some suggestions for some cross plots of physiological performance at growth condition with achieved growth rate. What is the growth return on O_2 evolution across the conditions?

Response: We thank the reviewer for the useful comments and suggestions that have greatly improved this manuscript.

The following figure is the relationship between net photosynthetic rates (pmol O_2 $cell^{-1}$ h^{-1}) and specific growth rates (d^{-1}) and we will add the figure and the relationships between other physiological parameters and growth in the revised MS.



Abstract: Good

Materials and Methods:

2.3 The authors need to describe whether the growth rates taken every 2 days were averaged within each growth condition, or whether the maximum measured growth rate for a condition is taken. The presented growth rates are fast for a diatom.

Response: We are sorry for the ambiguous description. More details were added in the manuscript. Cultures were diluted every 3 days and cell concentration was counted two and three days after the dilution, and growth rates were calculated based on the concentrations. The data showed in Fig. 1 were averaged growth rates calculated for three times at different dilution days.

Diatoms have diverse cell sizes and thus growth rates of different species vary a lot. For *Skeletonema costatum*, the average growth rate is around 1.0-2.0 d^{-1} under normal (20℃, 100 μmol photons m^{-2} s^{-1}, 12L: 12D) conditions (Balzano et al., 2011; Sakshaug and Andresen, 1986), which accords with the growth rates (1.6 d^{-1}) under autumn condition (15℃, 150μmol photons m^{-2} s^{-1}, 12L: 12D) in this study. In addition, the average growth rate could increase to 2.4 d^{-1} under 12L: 12D light and dark cycle, when temperature rise to 25 ℃ (Zhang et al., 2020). In the present study, for the summer condition, daylength is 16 h, and temperature is 25 ℃, which could lead to higher growth rate.

’ 2.7 Measurements of PSII Proteins’ orå ’2.7 PSII Protein Measurements’å

(current header is technically correct but is archaic usage).

Response: Corrected.

Figures/Results; Figure 1: Amazing growth rate under summer conditions; the fastest I have ever seen I think for a diatom.

Response: Please see our former responses (line 27-35).

Figure 2: Lovely data, congratulations. Very surprising switch of the OA effect in winter. Suggested additional plot: V at growth pCO_2? After all, most of the curve as plotted is above even the OA range of pCO_2. So a hypothetical Vmax may not be as important as the achieved V at growth conditions. Then, I would suggested plotting growth rate vs. V at growth condition (pCO_2 and light).

Response: We appreciate the reviewer for the useful suggestion. DIC concentrations in the culture media are about 2 and 2.1 mmol/L in ambient and OA environment respectively, and in the P-C curve, DIC concentration range from 0 to 4 mmol/L. Photosynthetic rate increased significantly with increasing DIC concentration when DIC is lower than 2 mmol/L, and the rate is relative constant when DIC is higher than 2 mmol/L. In the present study Vmax obtained from the P-C curve showed similar pattern and value with V at growth condition. We will add this information in the results part.

Figure 4: Without standard curves, be very cautious in interpretation of the immunoblotting data. The example blot result shows near-saturation (non-linear response of signal to target abundance) for many of the bands. So the Y axis dynamic range of the greyscale plots may be considerably compressed relative to the actual change in protein target abundance. Once a band is black, it cannot get any blacker. It

is also very surprising that in winter OA increased RbcL signal.

Response: In this experiment we just calculated the relative value of each protein. The data provide us with a general trend, not the accurate concentration as the reviewer mentioned, among different treatments. And we will add a caveat in the revised MS. Actin (internal control) could correct the experimental error in the process of quantitative sample loading of protein, to ensure the accuracy of the experimental results. The greyscale plots were measured according to density and the area of bands (TanonImage software). Although they are all black, the densities and areas are different in most conditions.

For the increased RbcL signal under OA condition in winter, although some researchers found RubisCO contents decreased at OA (Losh et al., 2013; Endo et al., 2015), RbcL expression of different diatoms (*Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, and *T. weissflogii*) were found slightly increased under OA in nitrogen-replete condition (Hong et al., 2017). And phytoplankton communities showed enhanced Rubisco expression in 800 ppm treatment compared with 350 ppm (Tortell et al., 2000). A coastal isolated *T. pseudonana* and *Emiliania huxleyi* also showed higher RbcL contents in higher CO_2 level, although the offshore isolated *T. pseudonana* showed no significant difference between ambient and high CO_2 conditions (McCarthy et al., 2012). And low temperature could increase the relative amount of RbcL (Devos et al., 1998). We will add further discussion in the revised MS.

References:

82 Balzano S, Sarno D, Kooistra W H C F. Effects of salinity on the growth rate and
83 morphology of ten *Skeletonema* strains. Journal of Plankton Research, 2011, 33:
84 937-945.
85 Devos N, Ingouff M, Loppes R, et al. Rubisco adaptation to low temperatures: a
86 comparative study in psychrophilic and mesophilic unicellular algae. Journal of
87 Phycology, 1998, 34: 655-660.
88 Endo H, Sugie K, Yoshimura T, et al. Effects of CO_2 and iron availability on rbcL
89 gene expression in Bering Sea diatoms. Biogeosciences, 2015, 12: 2247-2259.
90 Hong H, Li D, Lin W, et al. Nitrogen nutritional condition affects the response of
91 energy metabolism in diatoms to elevated carbon dioxide. Marine Ecology Progress
92 Series, 2017, 567: 41-56.
93 Losh J L, Young J N, Morel F M M. Rubisco is a small fraction of total protein in
94 marine phytoplankton. New Phytologist, 2013, 198: 52-58.
95 McCarthy A, Rogers S P, Duffy S J, et al. Elevated carbon dioxide differentially alters
96 the photophysiology of *thalassiosira pseudonana* (bacillariophyceae) and *emiliania*
97 *huxleyi* (haptophyta) 1. Journal of Phycology, 2012, 48: 635-646.
98 Sakshaug E, Andresen K. Effect of light regime upon growth rate and chemical
99 composition of a clone of *Skeletonema costatum* from the Trondheimsfjord,
100 Norway. Journal of Plankton Research, 1986, 8: 619-637.
101 Tortell P D, Rau G H, Morel F M M. Inorganic carbon acquisition in coastal Pacific
102 phytoplankton communities. Limnology and Oceanography, 2000, 45: 1485-1500.
103 Zhang L, Li H, Wu M, Li F, and Xu J.: Effects of seawater acidification on

photosynthetic physiological characteristics of *Skeletonema costatum* at different temperatures, Journal of Jiangsu Ocean University, 2020, 29: 1-7.

Reviewer 2

Comments:

The manuscript titled "Physiological responses of *Skeletonema costatum* to the interactions of seawater acidification and combination of photoperiod and temperature" described a research attempting to explore the impact of high pCO_2 (or ocean acidification, OA) under different seasons (combination of photoperiod and temperature) on the diatom *S. costatum*. The experiments are well conceived, and methods are clearly presented. The most interesting observation is that high pCO_2 (OA) does not uniformly impact *S. costatum* under different seasons: with somewhat negative impact on winter conditions. The authors showed the interesting observation, but the experimental design and data quality can be improved.

Here are some questions and suggestions for the authors.

Response: We thank the reviewer for the recognition of the value of our work and the valuable comments.

On the experimental setup, the authors stated that the cell culture pH did not change over 0.05 units in the 3d of one generation (section 2.3), so one very basic question is: what are the pH values and ranges for the six different conditions (i.e. three seasons and two pCO_2)? Since the manuscript is about how OA impacts *S. costatum* differently during different seasons, the acidification information, which can be

presented easily as pH, is very critical to this whole article, however, this information is missing.

Response: The pH of culture media under different treatments are presented below. And the information will be added in the revised MS.

Treatments	W-LC	W-HC	A-LC	A-HC	S-LC	S-HC
pH	8.10 ± 0.01	7.85 ± 0.01	8.14 ± 0.01	7.89 ± 0.01	8.19 ± 0.02	7.89 ± 0.02

Following the cell culture pH question, the authors measured the photosynthesis (P) vs DIC curve at pH 8.12 (section 2.5) and very likely they did the same with P-I curve. It would be better if the authors measure the responses under lower pH for high pCO_2 treated cells, according to the high pCO_2 (OA) conditions. It should be expected that the pH is lower under OA conditions, and *S. costatum* acclimated to OA conditions may not photosynthesize better under the experimental condition with higher pH (8.12). As a result, the presented P-I and P-DIC curves for *S. costaum* cultured in HC (OA conditions) may not reflect their real physiological status in terms of photosynthesis under OA conditions. Also please note that Tris buffer is known to change pH significantly with different temperature, so it is important to measure or calculate the pH at certain temperature.

Response: Researchers determine the P-C curves of OA treated cells under either ambient pH or culture pH (HC conditions). As the reviewer mentioned, P-C curves determined under culture pH could reflect the real physiological status under OA conditions. However, the percentages of DIC species (CO_2, HCO_3^-, CO_3^{2-}) differ in media with different pH, which is inconvenient to compare K_m of LC and HC cells.

Thus, some studies prefer setting same pH for LC and HC conditions to compare V_{max} and K_m at same pH (Nakajima et al., 2013; Shi et al., 2017). For P-I curves, the measurement time was much shorter than that used for P-C curves, so the pH wouldn't change markedly and Tris buffer was not used for P-I curves in the present study. Cells were resuspended in pre-aerated fresh medium under cultured condition (i.e. pH 8.14 for LC cells, 7.9 for HC cells) to measure P-I curves. Details for P-I measurements were added in the revised MS. We are sorry we missed the effects of Tris buffer on pH at different temperature. For the P-C curves measurements in the present study, Tris was added in the culture medium, and the pH of the medium used for resuspending cells was adjusted at room temperature.

In the results session, the authors only mentioned and cited Fig. 1, while Figure 2, 3, 4 are listed at the end of the manuscript, none of those was referred in the text. In the tables presented, Table 2 and Table 3 do not show any units.

Response: We are sorry for the mistakes, Figure 2, 3, 4 are referred in the text now and units are added in Table 2 and 3 now.

In section 3.5 where the authors present the "PSII protein concentrations", RbcL was included as key PSII proteins. Such claim should be red-flagged, likewise the statement of "RbcL is related to the function of QA". Inclusion of RbcL in PSII proteins is also found in the Abstract and Discussion. The authors should make sure what RbcL really does with creditable citations before writing assumptions or conclusions about RbcL.

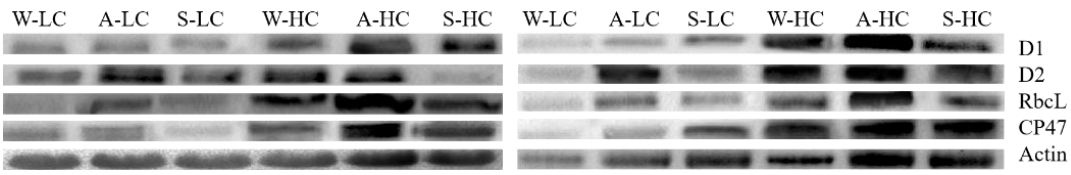
Response: We apologize for the vague statement regarding the RbcL. It's revised in section 3.5, Abstract and Discussion.

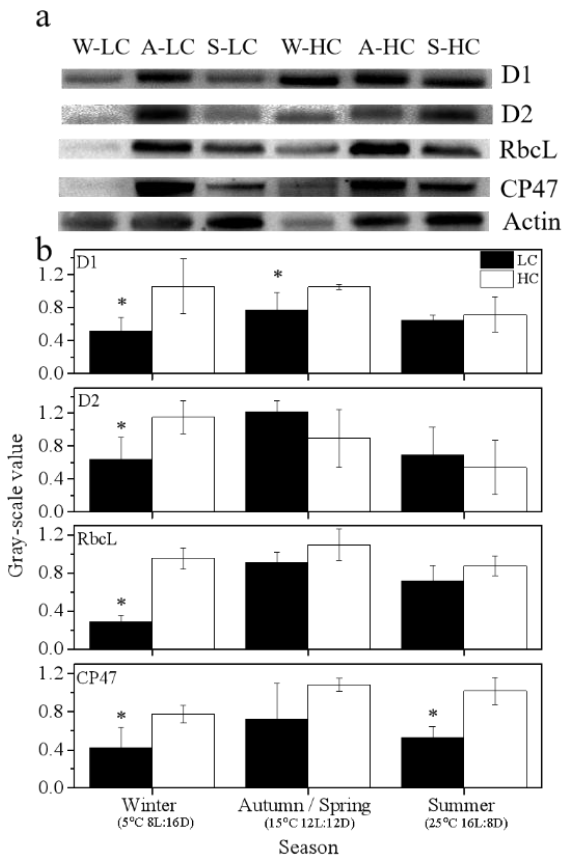
The description of methodology "Values of Actin were divided by other densitometric scanning values of protein to calculate Gray-scale values" should be modified to indicate the supposing meaning of normalizing density to Actin.

Response: Corrected. It has been revised as "Actin was used as internal control in order to correct the experimental error in the process of quantitative sample loading of protein, to ensure the accuracy of the experimental results."

With the data presented in Figure 4, panel (a) and panel (b) do not seem to agree with each other. The western blot data does not look like a representative of the statistical data. For example, in W-HC (winter high pCO_2) condition, the D1 density is much higher than Actin (Fig 4a), so such value is greater than 2 if analyzed using ImageJ, however, the data presented in statistics showed a value very tightly close to 0.9 (Fig 4b). It would be nice if all raw data (immunoblots) are presented to support the statistics in Fig 4b.

Response: We thank the reviewer for pointing this out. We checked the data and revised the mistake. In Fig. 4 panel (a), the D1 value is 1.55 according to TanonImage software. The immunoblots data and revised Fig. 4 are presented as following:





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186 Other minor concerns:

187 Section 2.7, “1000 and 34.5 are constants”, what are the units? Or at least provide the

188 unit of “where C represents total chlorophyll concentration”.

189 **Response:** The unit of chlorophyll concentration is μg / ml. It is revised in the

190 manuscript.

191 The use of letter “C” is ambiguous in the text. C was also used as carbon in line 219:

192 “C fixation”.

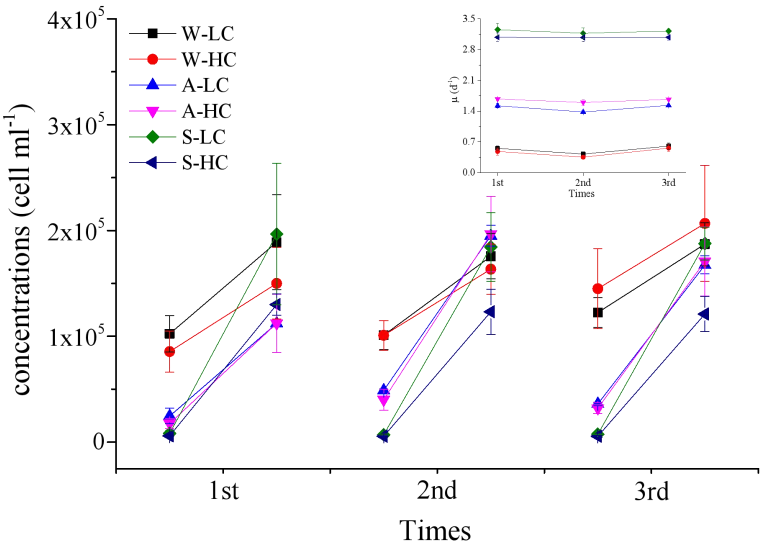
193 **Response:** “C fixation” is revised as “carbon fixation”.

194 For the measurement of specific growth rate, more details on how data were collected

195 would be helpful. It would be better to have the raw data, cell concentration vs time

196 (days), presented.

Response: More details will be added in the revised MS. Cells concentration was diluted every 3 days and cell concentration was counted two and three days after the dilution, such growth rate data in Fig. 1 were calculated for three times at different dilution days. The following figure presents cell concentrations and specific growth rate at three different times.



Line 229, “initial slop” should be “initial slope”.

Response: Corrected.

References

Shi Q, Xiahou W, Wu H. Photosynthetic responses of the marine diatom *Thalassiosira pseudonana* to CO_2-induced seawater acidification. Hydrobiologia, 2017, 788: 361-369.

Nakajima K, Tanaka A, Matsuda Y. SLC4 family transporters in a marine diatom directly pump bicarbonate from seawater. Proceedings of the National Academy of Sciences, 2013, 110: 1767-1772.