Response to Reviewer 2 Comments (RC2):

We thank the reviewer for their constructive comments

RC2-1: Review of the manuscript egusphere-2023-3110: „Lipid remodeling in phytoplankton exposed to multi-environmental drivers in a mesocosm experiment“ by Cantarero et al.

Using samples from the eastern tropical South Pacific off the coast of Callao, Perú, the authors investigated the lipid remodelling of phytoplankton in the mesocosm in response to various environmental stressors

I have a big complaint about the authors constantly talking about TAGs in the discussion in almost all the subsections, even though they have not analysed them. Especially in the Abstract, the authors go too much into the redistribution of IPL to TAG production in response to various stressors. I think this is unacceptable, especially for the Abstract.

I found the manuscript exhausting to read. There are too many unnecessary discussions.

Response RC2-1: Thank you for your valuable comments to improve the state of our manuscript. We agree that the discussion could be more concise and we plan to condense the text to mostly focus on the key findings provided by our own data. We also recognize that since we didn't measure TAGs directly we can only hypothesize as to their relation to the IPL distributions observed in this experiment. We will clarify this and limit speculation in the discussion and abstract.

RC2-2: I suggest that the authors use the common abbreviations for mono- and di-galactosyldiacylglyercols and sulfoquinovosyldiacylglycerol, namely MGDG, DGDG and SQDG.

Response RC2-2: We recognize that other authors typically use the abbreviations mentioned by reviewer 2 for glycolipids common to phytoplankton. However, all IPLs described in this manuscript contain diacylglycerol structures, so in our opinion, the omission of "DG" in the abbreviations of other phospholipids (eg. PG, PC, and PE) may lead to confusion. We plan, also in response to the comment of reviewer 1 as well, to include more structural information of the IPLs reported in our study in the introduction (see Response RC1–2:). We will continue referring to major phytoplankton IPL classes by their headgroups to provide a simple and consistent naming convention.

RC2-3: There is unnecessary over citations in the manuscript. The authors have cited 3 or more articles for one statement, one or two would be enough. Consequently, there are too many references (157).

Response RC2-3: We feel that there is no harm in providing comprehensive citations of relevant and valuable work of our colleagues and predecessors in this field. Furthermore, we are well within the guidelines of the journal regarding citations. We hope that a comprehensive list of references will be helpful for other authors in the future.

RC2-4: The first digit of the three-digit line numbers is not visible. I hope I have estimated the line number correctly in the comments.

Response RC2-4: We regret this and appreciate you bringing it to our attention. We suspect that the format change to PDF may have affected the original Word file. We will take this into consideration for the upcoming submission.

RC2-5: In accordance with the above and the specific comments below, I suggest that the manuscript can be published after Major revision

Response RC2-5: We appreciate the opportunity to address each of the comments.

Abstract

RC2-6: L 45 – cellular P as well

Response RC2-6: Thank you, this will be included.

1 Introduction

RC2-7: L 49-50 – In general, three citations are unnecessary. E.g. Ulloa and Pantoja, 2009 and Thamdrup et al., 2012) would be more than enough. This comment applies to all cases where there are 3 or more citations for a statement.

Response RC2-7: We respectfully disagree with the reviewer. Please refer to the response to comment RC2-3 for further details.

RC2-8: L 72 – The citation Catalanotti et al. (2013) is inadequate and should be removed.

Response RC2-8: We agree and will remove it.

RC2-9: L 79 – All the cell organelles have lipid membrane!

Response RC2-9: We will address this accordingly in the introduction of IPLs.

RC2-10: L 80 – I do not find citations Du and Benning 2016; Morales et al., 2021 appropriate.

Response RC2-10: We agree these references are more pertinent to neutral lipids. Thus, we will remove them and streamline this introductory sentence to focus on relevant literature highlighting specific environmental stressors and their impacts on IPL remodeling.

RC2-11: L 81 – The citations Urzica et al., 2013 and Gordillo et al., 1998 are missing in the References. I suggest to remove it due to the over citations.

Response RC2-11: We agree these citations are not essential and will be removed.

RC2-12: L 82 - Sato et al., 1979 or 1980? Also, the citations Wada and Murata, 1990 and Sinensky, 1974; and Tatsusawa and Takizawa 1996 (first time mentioned) are not appropriate and should be removed.

Response RC2-12: Thank you, this citation has been corrected to Sato et al., 1980. We agree that Wada and Murata 1990 as well as Tatsusawa and Takizawa 1996 could be removed. We would like to include the earlier work of Sinensky (1974) which laid the foundation for the concept of homeoviscous membrane adaptation.

RC2-13: L 84-85 - The citation Gombos et al., 2002; Pineau et al., 2004; Simionato et al., 2013; Gašparović et al., are not appropriate and should be removed.

Response RC2-13: Thank you, we agree that Gombos et al., 2002, Pineau et al., 2004, and Simionato et al., 2013 are misplaced here. We disagree about Gašparović et al. as this study is relevant to the potential impacts of light availability on IPL distribution.

RC2-14: L 91 - community level and in time series, s, and the associated

Response RC2-14: This will be corrected.

2 Methods

RC2-15: L 123-124 - It is not clear on which day the ODL water was added. Days 5 and 10?

Response RC2-15: We will clarify this aspect in the revised version of the manuscript. Days 5 and 10 are when the ODZ waters were collected from stations 1 and 3, respectively. 20 m^3 of ODZ water from station 3 was added to mesocosms M2, M3, M6, and M7 on day 11, and 20 m^3 of ODZ water from station 1 was added to mesocosms M1, M4, M5, M8 on day 12.

RC2-16: L 176 – I did not find the protocol for lipid extraction in the paper by Wormer et al. (2013). Therefore, this citation is inadequate.

Response RC2-16: Thank you for flagging this mistake. We will include the proper citation shown below. Wörmer L., Lipp J. S., Hinrichs K.-U. "Comprehensive analysis of microbial lipids in environmental samples through HPLC-MS protocols" in *Hydrocarbon and lipid microbiology protocols: Petroleum, hydrocarbon and lipid analysis*. eds. McGenity T. J., Timmis K. N., Nogales B. (Berlin, Heidelberg: Springer Protocols Handbooks. Springer), 289–317, 2015

3 Results

RC2-17: L 285, 314, 325, 330, 347, 354, 369, 373, 377, 379 – It would be easier for me to follow the text and Figure 2 if the mesocosms were listed in the order shown in Figure 2. E.g. to say in line 325: ... mesocosms 7, 5 and 8.

Response RC2-17: We will make this adjustment for improved clarity.

RC2-18: L 301-302- Fig. 2: The concentrations of the nitrogen species ranged from less than 1 umol/L. The Si concentration in Table 1 is also high (> 17 umol/L), whereas it is not shown in Fig. 2. Is this a question of dilution?

Response RC2-18: The values reported in Table 1 represent the source ODZ waters before their addition to the mesocosms (~20 m^3), whereas Fig. 2 shows the values in the mesocosm throughout the experiment. We will ensure that this is clearly explained in the revised version of the manuscript.

RC2-19: L 417 – I am not familiar with Card and Random Forest. Perhaps it would be good to include in the Supplements an introduction to understanding Figures 4-7.

Response RC2-19: We agree that a more extensive explanation of these statistical analyses would be beneficial for readers, which we will provide in the revised version of the manuscript. We plan to add details on the final predictor selection process and the conservative cutoffs that are meant to highlight that only the most significant predictors are included in our final CART and RF figures (Figures 4-7).

Regression tree (CART) splitting criteria are determined by evaluating the sum of squared deviations in all possible splits and selecting those that result in the greatest reduction of residual error. In order to prevent overfitting in the CART analysis, a pruning procedure is run to remove nodes that contribute little to the model accuracy based on a cost complexity measure. This procedure allows us to simplify the CART results to focus our interpretations on the most significant predictors of IPL headgroups only. In the Random Forest model, following the averaged cross validated accuracy estimates, we implemented a cutoff of 5% reduction in RMSE to eliminate variables that do not significantly reduce the error of the model prediction. This cutoff allows us to focus our interpretation of only variables that contribute significantly to the out of bag predictor performance.

RC2-20: My biggest problem is understanding where in Figures 4-7 there is confirmation of what some authors say. I would like an answer to this:

- How can I see that Oxygen concentration was important in predicting MGDSs in Fig. 4E and F?
- How can I see that Oxygen concentration was important in predicting DGDSs in Fig. 5A and B?
- How can I see that temperatue was important in predicting PE predictions in Figs. 6E-H?
- How can I see that Various forms of biologically available nitrogen were important in predicting MGDS in Fig. 4E?
- How can I see that NH4 was important in predicting BLs in Fig. 5E?
- How can I see that NH4 was important in predicting PEs in Fig. 6E-H?
- How can I see that PO4 was important in predicting SQDGs in Fig. 4A?

- How can I see that PO4 was important in predicting MGDGs in Fig. 4E?
- How can I see that light availability was important in predicting SQDGs in Fig. 4A?
- How can I see that light availability was important in predicting MGDGs in Fig. 4E?
- How can I see that light availability was important in predicting PEs in Fig. 6E-H?
- How can I see that light availability was important in predicting PCs in Fig. 7A?

Response RC2-20: Thank you for making us aware that these important points are not clearly conveyed in the current version of the manuscript. We will expand on the interpretation of these statistical techniques in the methods section as well as in the results and discussion as to aid the reader. Only significant predictors remain in the final CART and RF figures. The pruning and RMSE cutoff procedures described above are intended to remove predictors with little impact on the model performance.

4 Discussion

RC2-21: L 563 – only Fig. 3

Response RC2-21: This typo will be corrected.

RC2-22: L 563-565 – I do not understand this sentence. First the authors state that there is more unsaturated IPL at lower pH values (i.e. in the subsurface), then they state that this is most clearly observed in surface waters???

Response RC2-22: We agree that this point is unclear and we will clarify the relationship between pH and IPL class proportions and unsaturated moieties. This sentenced will be changed to:

The negative correlation between unsaturated IPLs and pH_T (namely amongst glycolipid moieties) is most apparent in surface waters where the variability in pH_T is greatest (± 0.2).

RC2-23: L 573-574 – pCO2 is not concentration but the partial pressure of CO2.

Response RC2-23: This will be corrected.

RC2-24: L 579 - Hu and Gao et al., 2004 ???

Response RC2-24: Thank you for bringing this to our attention. We will include the proper reference below: Hu, H. and Gao, K.: Optimization of growth and fatty acid composition of a unicellular marine picoplankton, Nannochloropsis sp., with enriched carbon sources, Biotechnol. Lett., 25(5), 421–425, doi:10.1023/A:1022489108980, 2003.

RC2-25: L 581-618 – The authors have devoted 4 paragraphs to the discussion of the TAG synthesis. I agree that it's fine to assume that TAG accumulates under unfavourable conditions,

but considering that they did not analyse TAG, I think such a long discussion on TAG is an exaggeration.

Response RC2-25: We agree with the reviewer and appreciate this comment. We will condense the discussion around TAGs in the revised version of the manuscript and reduce speculation.

RC2-26: L 608-609 – I suggest that the authors consider why dinoflagellates are more dominant in the surface layer. Dinoflagellates are possibly mixotrophic, some are also heterotrophic, while diatoms are probably limited by Si availability.

Response RC2-26: Whereas we briefly address this point in section 4.3.1, we plan to expand on it in this particular section of the manuscript as suggested. Namely, we will discuss that mixotrophic dinoflagellates scavenging N from the PON pool likely grants them a considerable advantage in N-depleted surface waters. Their presence in the KOSMOS 2017 experiment, particularly in the final phases, has been reported by Bach et al. (2020) and Min et al. (2023).

We will include the recent publication in our references:

Min, M.A., Needham, D.M., Sudek, S., Truelove, N.K., Pitz, K.J., Chavez, G.M., Poirier, C., Gardeler, B., von der Esch, E., Ludwig, A., Riebesell, U., Worden, A.Z., Chavez, F.P., 2023. Ecological divergence of a mesocosm in an eastern boundary upwelling system assessed with multi-marker environmental DNA metabarcoding. Biogeosciences 20, 1277–1298. https://doi.org/10.5194/bg-20-1277-2023

RC2-27: L 631 – How is 125-220 µmol/L just 28% more than ~15-75 µmol/L?

Response RC2-27: This appears to be a typo that we plan to correct, as it should refer to O_2 concentration in the surface vs subsurface.

RC2-28: L 682-683 – Did the authors analysed the influence of temperature on each individual IPL separately?

Response RC2-28: The influence of temperature was analyzed through the change in unsaturations for the entire set of IPLs. For example, supplemental figure 2 investigates the relationship between the number of unsaturations (weighted average) in all IPL moieties combined, whereas Figure 3 investigates linear relationships for each individual IPL separately. Neither analysis provided evidence for a consistent correlation between the degree of unsaturation and temperature.

RC2-29: 4.2.5 Light Availability - Considering that the mesocosms had low light conditions and not high light conditions, I think it is unnecessary to discuss other works about high light conditions.

Response RC2-29: We agree that this section can be significantly reduced and more focused on the impacts of moderate to low light conditions. We do cite works that suggest the levels observed in this mesocosm experiment are high enough to result in significant production of neutral lipids (TAGs) though, and we plan to reduce this discussion given that we do not provide TAG measurements.

RC2-30: L 740 - 741 – The authors should read more recent works on the role of SQDG, e.g. DOI: 10.1042/BCJ20170047 ; DOI: 10.1074/jbc.RA118.004304

Response RC2-30: Thank you for these recommendations. We will review and include them in the revised discussion of SQDG's function in the photosynthetic apparatus.

Gabruk, M., Mysliwa-Kurdziel, B., and Kruk, J.. MGDG, PG and SQDG regulate the activity of light-dependent protochlorophyllide oxidoreductase. Biochem. J. 474, 1307–1320. doi: 10.1042/BCJ20170047, 2017.

Nakajima, Y., Umena, Y., Nagao, R., Endo, K., Kobayashi, K., Akita, F., Suga, M., Wada, H., Noguchi, T., and Shen, J.-R. J. Biol. Chem., 293, 14786–14797, 2018.

RC2-31: L 766-767 – I assume it should be written: ... LOW light availability may amplify the effects of other environmental stressors…

Response RC2-31: This will be corrected.

RC2-32: L 783 - 4.3.1 Relative adaptability of Phytoplankton Classes to environmental change: Most of the text in this subsection, including Fig. 8, should be moved to the Results section.

Response RC2-32: We use the RF model in this subsection to highlight the most predictive lipid moieties and lipid classes for each phytoplankton class, and to suggest what their most likely dominant biological sources are. This subsection and Figure 8 are meant to provide an overview of the IPL remodeling strategies potentially available to the major phytoplankton classes of this experiment and to hypothesize as to how these remodeling strategies may play a role in their distribution in this environment. We prefer to leave this section in the discussion as it provides an overview relating the interplay of IPL distributions, phytoplankton distributions, and changing environmental conditions.

Figures

RC2-33: Fig. 2c – The legend of this figure lacks an explanation for the black line (chl a).

Response RC2-33: This will be corrected.

RC2-34: I suggest to change Chl-a y-axe from 0-10.

Response RC2-34: Unfortunately, there are several high Chl-a observations that would be removed with this y axis limit. We prefer to keep the axis limits as they are to prevent the omission of data.

RC2-35: Fig. 2d – it would be better to organise the IPLs according to their cellular origin. I would suggest the following order: MGDG, DGDG, SQDG, PG, PC, PE, BL and others.

Response RC2-35: We will modify this accordingly to improve clarity.

References

RC2-36: In some references the name of the journal is written with the full name, in others with an abbreviation.

Response RC2-36: We will correct these inconsistencies in the citations export.

RC2-37: The reference Jiang and Jónasdóttir should be separated from the reference Hutchins and Jiang

RC2-38: Reference Guckert, J. B., and Cooksey, K.E.: to correct: …high Ph-induced... DOI: 10.1111/j.0022-3646.1990.00072.x

RC2-39: Reference Schubotz, F., Xie, S., Lipp, J. S., Hinrichs, K. and Wakeham, S. G.: is poorly written.

RC2-40: Also, The reference "Schubotz, F., Xie, S., Lipp, J. S., Hinrichs, K. and Wakeham, S. G" should be separated from the reference Shulz and Riebessel

RC2-41: Reference Cantarero should be improved: ... Front. Mar. Sci. 7:540643. doi: 10.3389/fmars.2020.540643, 2020...

Response RC2-37-41: These will be corrected.