ULPGC
Universidad de
Las Palmas de
Gran Canaria

Las Palmas de Gran Canaria, July 31, 2024

Point-by-point response to reviewers

We really appreciate suggestions made by Reviewers.

Our answers and clarifications are written in blue. Modified texts in the manuscript are highlighted.

Report #1

Checklist for reviewers

1) Scientific significance Does the manuscript represent a substantial contribution to scientific progress within the scope of this journal (substantial new concepts, ideas, methods, or data)?	Excellent **Good** Fair Poor
2) Scientific quality Are the scientific approach and applied methods valid? Are the results discussed in an appropriate and balanced way (consideration of related work, including appropriate references)?	Excellent **Good** Fair Poor
3) Presentation quality Are the scientific results and conclusions presented in a clear, concise, and well structured way (number and quality of figures/tables, appropriate use of English language)?	Excellent **Good** Fair Poor

For final publication, the manuscript should be

accepted as is

accepted subject to **technical corrections**

accepted subject to minor revisions

reconsidered after **major revisions**

rejected

Were a revised manuscript to be sent for another round of reviews:

I would be willing to review the revised manuscript.

I would not be willing to review the revised manuscript.

Suggestions for revision or reasons for rejection

(visible to the public if the article is accepted and published)

The authors have made substantial revisions on the manuscript, however, they are still some questions which need authors to address.

1. I am not satisfied with the current abstract, it need further refinement, some statements should be omitted.
2. The photoperiod was 24 h? Why no dark phase

Point by point response

1. I am not satisfied with the current abstract, it need further refinement, some statements should be omitted.

The abstract has been changed as follows:

Abstract. Cultures of the coccolithophore *Emiliania huxleyi* were grown under four different CO_2-controlled pH conditions (7.75, 7.90, 8.10, and 8.25) to explore variations in intra- and extracellular polyphenols and carbohydrates in response to different ocean acidification scenarios. Acidification did not significantly affect final cell densities and carbohydrate contents. Intra- and extracellular phenolic compounds were identified and quantified by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC), with the highest concentrations of total exuded phenolics at pH 8.25 (43±3 nM) and 7.75 (18.0±0.9 nM). Accumulation of intracellular phenolic compounds was observed in cells with decreasing pH, reaching the maximum level (9.24±0.19 attomole $cell^{-1}$) at the lowest pH (7.75). The phenolic profiles presented significant changes in exuded epicatechin and protocatechuic acid (p<0.05 and 0.01, respectively), and intracellular vanillic acid (p<0.001), which play an essential role in the availability of trace metals. A significant increase in chlorophyll *a* content was observed in cells grown at the most acidic pH (p<0.01), which also showed significantly higher radical inhibition activity (p<0.01). The nature and concentration of these organic compounds present in the culture medium may influence trace metal bioavailability affecting the biogeochemical cycling of carbon and microbial functional diversity.

The photoperiod was 24 h? Why no dark phase

Even that no dark phase was applied in our studies, *E. huxleyi* has often been considered extremely light-tolerant (Jakob et al., 2018; Loebl et al., 2010). Xing et al. (2015) reported that *E. huxleyi* grown under indoor constant light showed higher specific growth rate than those grown under fluctuating outdoor solar radiation. Therefore, we applied a photoperiod of 24 h to provide maximum organic matter production, keeping this condition in all the studies. Moreover, pH, through CO_2 acidification, is the only variable modified in our study focused on the effect of acidification on *E. huxleyi*, so changes in organic matter should be linked only to the effect of this pH change and its consequences (changes in the availability of essential metals such as iron). We used the same strains as well as the cultivation conditions (lighting, seawater, nutrients, temperature, etc.) so the influence of all these factors should be the same in all cultures.

Axenic cultures of *E. huxleyi* (strain RCC1238) were supplied by the Spanish Bank of Algae (BEA) (member of the European Culture Collections Organization (ECCO) located

in Taliarte, SE coast of Gran Canaria), and cultured following its recommendations to increase the productivity. This microalgae Collection is included in the World Data Centre for Microorganisms (WFCC-MIRCEN) and is recognized by the World Intellectual Property Organization (WIPO) as international authority for the deposit of microorganisms (algae) through the Budapest Treaty. It is also included in the European Consortium MIRRI.

- Jakob, I., Weggenmann, F., Posten, C., Cultivation of Emiliania huxleyi for coccolith production, Algal Research, 31, 47-59, https://doi.org/10.1016/j.algal.2018.01.013, 2018.
- Loebl M., Cockshutt A. M., Campbell, D. A., Finkel, Z. V.: Physiological basis for high resistance to photoinhibition under nitrogen depletion in *Emiliania huxleyi*, *Limnology and Oceanography*, 55, 1807-2229, https://doi.org/10.4319/lo.2010.55.5.2150, 2010.
- Xing, T., Gao, K. and Beardall, J.: Response of Growth and Photosynthesis of *Emiliania huxleyi* to Visible and UV Irradiances under Different Light Regimes. Photochem Photobiol, 91: 343-349. https://doi.org/10.1111/php.12403, 2015.

Report #2

Anonymous during peer-review: Yes No

Anonymous in acknowledgements of published article: Yes No

Checklist for reviewers

1) Scientific significance Does the manuscript represent a substantial contribution to scientific progress within the scope of this journal (substantial new concepts, ideas, methods, or data)?	Excellent Good **Fair** Poor
2) Scientific quality Are the scientific approach and applied methods valid? Are the results discussed in an appropriate and balanced way (consideration of related work, including appropriate references)?	Excellent Good Fair **Poor**
3) Presentation quality Are the scientific results and conclusions presented in a clear, concise, and well structured way (number and quality of figures/tables, appropriate use of English language)?	Excellent Good Fair **Poor**

For final publication, the manuscript should be

accepted as is

accepted subject to **technical corrections**

accepted subject to **minor revisions**

reconsidered after **major revisions**

rejected

Were a revised manuscript to be sent for another round of reviews:

I would be willing to review the revised manuscript.

I would not be willing to review the revised manuscript.

Suggestions for revision or reasons for rejection

(visible to the public if the article is accepted and published)

Overview

Technically, I am not inclined to provide support for this published paper in this journal. The focus of this work is on physiological variations of E. hux, which have already been extensively reported. Therefore, this manuscript does not offer anything new except for the testing of four different pH levels. One major concern is the poorly written manuscript with unclear descriptions. For instance, the method section regarding the carbonate system needs to be rewritten, and there is no mention of the CO2 calculation, among other things. In my opinion, it would be more beneficial to include fewer molecular approaches and focus on identifying function genes related to toxicity and polyphenols. Additionally, significant improvements in writing style are necessary before submitting elsewhere.

Down below are fewer suggestions/questions.

Abstract
The response of OA to E. hux has already been extensively documented both physiologically and metabolically. This sentence should be revised to align with the aim of this work, which is to explore variations in polyphenols and carbohydrates. The abstract now appears to be more results-oriented rather than providing an overview. It should be revised to incorporate references to published articles.

Introduction
Reference needed.

Line 43: State as "OA" with a lowering of pH.

The main aim of this study is to investigate ocean acidification (OA). Therefore, a small introduction explaining the definition of OA would be very useful for readers to understand what ocean acidification is.

Lines 40 to 80: This section is too vague and could benefit from reducing and splitting the paragraphs.

Methods
Line 116-120. Rewrite. Too vague.

Do all variations of the conditions have the same temperature and incubation time in the culture incubator? Not clearly written.

Don't you think 200 μmol is too much for E. hux?
Please provide a clearer explanation of how CO2 was introduced into the cultures. Was it done using a single cylinder in different incubators or with multiple cylinders?

Results
How were the values calculated? Did they use the standard protocol and calculation methods, such as CO2SYS software, etc.? A clear description is needed.

The authors discussed other published papers that reported on E. hux at different pH levels, but they examined the concentration of pCO2 injected rather than the pH changes. This raises the question of how much concentration was injected to achieve each pH variation in this study.

Line 435: Is "limited research" completely wrong? Numerous studies have already been reported, not only on E. hux, but also on other phytoplankton, regarding topics such as warming, acidification, etc.

There are many concerns troughout the results and discussion.

Conclusion has to be completely based on the author's own results.

Point by point response:

Studying the nature of the organic ligands in oceanic waters will allow a comprehensive understanding of the consequences of acidification on ocean biogeochemical processes. Catechin, sinapic acid and gallic acid were found to increase the persistence of dissolved Fe, regenerating Fe(II) in seawater from 0.05% to 11.92% (González et al., 2019).

- González, A. G, Cadena-Aizaga, M. I., Sarthou, G., González-Dávila, M., and Santana-Casiano, J. M.: Iron complexation by phenolic ligands in seawater, Chem. Geol., 511, 380–388, https://doi.org/10.1016/j.chemgeo.2018.10.017, 2019.

Introduction
Reference needed.

Line 43: State as "OA" with a lowering of pH.

The introduction has been modified to address this and the following suggestion as follows (lines 40-42):

The absorption of anthropogenic CO_2 into seawater alters the natural chemical balance of the CO_2-carbonate system resulting in a decrease of the chemical bases in seawater, increasing protons (H^+) and lowering its pH in a process termed "ocean acidification" with adverse consequences for marine ecosystems and human societies (Bates et al., 2014; Jiang et al., 2023; Lida et al., 2021). In fact, pre-industrial seawater pH (8.25) has already dropped to 8.10, and is expected to reach a pH of 7.85 in this century (Jacobson, 2005).

The reference Gruber et al. (2023) has been deleted and replaced with Bates et al. (2014), from which the definition of OA has been taken.

Gruber, N., Bakker, D. C. E., DeVries, T., Gregor, L., Hauck, J., Landschützer, P., McKinley G. A.: Trends and variability in the ocean carbon sink, Nat. Rev. Earth Environ., 4, 119–134, https://doi.org/10.1038/s43017-022-00381-x, 2023.

Bates, N. R., Astor, Y. M., Church, M. J., Currie, K., Dore, J. E., González-Dávila, M., Lorenzoni, L., Muller-Karger, F., Olafsson, J., & Santana-Casiano, J. M. (2014). A Time-Series View of Changing Surface Ocean Chemistry Due to Ocean Uptake of Anthropogenic CO_2 and Ocean Acidification, Oceanography, 27(1), 126–141, http://www.jstor.org/stable/24862128, 2014.

The main aim of this study is to investigate ocean acidification (OA). Therefore, a small introduction explaining the definition of OA would be very useful for readers to understand what ocean acidification is.

The definition has been included as follows (lines 40-42):

The absorption of anthropogenic CO_2 into seawater alters the natural chemical equilibrium of CO_2-carbonate system resulting in a decrease of the chemical bases in seawater, increasing protons (H^+) and lowering its pH in a process termed "ocean

acidification" with adverse consequences for marine ecosystems and human societies (Bates et al., 2014; Jiang et al., 2023; Lida et al., 2021). In fact, pre-industrial seawater pH (8.25) has already dropped to 8.10, and is expected to reach a pH of 7.85 in this century (Jacobson, 2005).

Lines 40 to 80: This section is too vague and could benefit from reducing and splitting the paragraphs.

Several sentences were included in this section according to the suggestion of previous reviewers. However, the introduction has been changed as follows:

Global environmental changes, in particular those related to increasing temperature and decreasing pH, profoundly affect ocean ecosystems at many levels~~, as these are the two main variables controlling all chemical and biological cycles, with a major impact on the growth and metabolic functions of microalgae~~ (Berge et al., 2010; Dedman et al., 2023; Kholssi et al., 2023; Lu et al., 2013). The absorption of anthropogenic CO_2 into seawater alters the natural chemical equilibrium of CO_2-carbonate system resulting in a decrease of the chemical bases, increasing protons concentration (H^+) and lowering its pH in a process termed "ocean acidification~~" lowers its pH~~ with adverse consequences for marine ecosystems and human societies (Bates et al., 2014; ~~Gruber et al., 2023;~~ Jiang et al., 2023; Lida et al., 2021). In fact, pre-industrial seawater pH (8.25) has already dropped to 8.10, and is expected to reach a pH of 7.85 in this century (Jacobson, 2005).

~~For instance, pH homeostasis, which regulates the pH inside and outside the cell, is critical for the growth and metabolism of most microorganisms, including microalgae (; Guan and Liu, 2020; Lund et al., 2020).~~ Different algal species show different optimal pH ranges for maximum growth (Hoppe et al., 2011; Kholssi et al., 2023). Changes in environmental pH could have consequences on the competitiveness of both sensitive and tolerant microalgae in mixed phytoplankton communities, modifying their structure, composition, and distribution, which are crucial in mitigating global environmental change by fixing and transporting carbon from the upper to the deep ocean in the major global carbon sink (Eltanahy and Torky, 2021; Kholssi et al., 2023, Marinov et al., 2010). Vasconcelos et al. (2002) found that exudates from *Phaeodactylumn tricornutum* (*P. tricornutum*) diatoms caused a toxic effect on *E. huxleyi*, while those from *Enteromorpha* spp. caused an increase in final cell yield, concluding that specific exudates produced by the bloom of one algal species may favour or inhibit the local growth of other species. Such changes could also affect species at a higher trophic level, resulting in a potential shift in biodiversity (Jin and Kirk, 2018). Spisla et al. (2021) reported that extreme CO_2 events modify the composition of particulate organic matter in the ocean, which leads to a substantial reorganization of the planktonic community, affecting multiple trophic levels from phytoplankton to primary and secondary consumers (Nelson et al., 2020; Trombetta et al., 2019;). ~~Nelson et al. (2020) found modifications of planktonic and benthic communities in response to reduced seawater pH (from pH 8.1 to 7.8 and 7.4), concluding that a re-arrangement of the biofilm microbial communities occurred through a potential shift from autotrophic to heterotrophic dominated biofilms. In addition, microbial biofilms obtained under reduced pH altered settlement rates in invertebrate larvae of *Galeolaria hystrix*.~~ Barcelos e Ramos et al. (2022) showed that coexistence with

other microorganisms modifies the response of *E. huxleyi* subjected to high CO_2 concentration, markedly decreasing its growth rate and cellular organic carbon and increasing its organic carbon in the presence of ~~at elevated CO_2 concentrations with~~ the bacteria *Idiomarina abyssalis* and *Brachybacterium* sp. ~~Moreover, elevated CO_2 concentrations increased organic carbon and decreased inorganic carbon content of *E. huxleyi* cells in the presence of *I. abyssalis*, but not *Brachybacterium* sp.~~

Changes in phytoplankton communities due to variation in seawater acidity alter the composition of the organic ligands that these communities released into the surrounding environment (Samperio-Ramos et al., 2017). These ligands are crucial ~~in the formation of metal complexes~~ for acquiring micronutrients, sequestering toxic metals, and establishing electrochemical gradients that result in changes in speciation, bioavailability, and toxicity of trace metals (Harmesa et al., 2022; Santana-Casiano et al., 2014). Iron is an essential micronutrient for phytoplankton involved in fundamental cellular processes~~, including respiration, photosynthesis, nitrogen uptake, and nitrogen fixation~~ (Raven et al., 1999; Hogle et al., 2014), controlling productivity, species composition and trophic structure of microbial communities over large regions of the ocean (González et al., 2019; Hunter and Boyd, 2007). ~~Iron concentrations in ocean waters are very low due to its low solubility and effective removal from the ocean surface by phytoplankton (Liu and Millero, 2002).~~ Complexation with organic compounds is one of the mechanisms for maintaining dissolved iron concentrations above its inorganic solubility~~, while potentially reducing the concentrations of soluble and bioavailable inorganic species~~ (Hunter and Boyd, 2007; Shaked et al., 2020). A decrease in seawater pH from 8.1 to 7.4 will increase Fe(III) solubility by approximately 40%, which could have a large impact on biogeochemical cycles (Morel and Price, 2003; Millero et al., 2009).~~Organic matter exuded by marine microorganisms can form Fe(III) complexes that modify Fe(II) oxidation rates and promote the reduction of Fe(III) to Fe(II) in seawater.~~ In addition, some research work has shown that the residence time of the reduced form of essential trace metals increases as their oxidation rate decreases under acidifying conditions (Pérez-Almeida et al., 2022; Santana-Casiano et al., 2014).

Methods

Line 116-120. Rewrite. Too vague.

The highlighted information has been included (line 110):

Axenic cultures of *E. huxleyi* (strain RCC1238) were supplied by the Spanish Bank of Algae (BEA) in f/2 medium. *E. huxleyi* coccolithophore was cultured for 8 days in an incubator clean chamber (Friocell FC111) at a constant temperature of 25 ºC with an initial cell density of 10^6 cells L^{-1}, under complete photoperiod (24 h) with light intensity of 200 µmol photons m^{-2} and under different pCO_2-controlled seawater pH conditions (7.75, 7.90, 8.10, and 8.25), measured on the free hydrogen ion scale $pH_F = -\log[H+]$ with a Ross Combination glass body electrode calibrated daily with TRIS buffer solutions. A gaseous mixture of CO_2-free air and pure CO_2 was bubbled in the culture medium to CO_2 levels of 900 µatm (pH 7.75), 600 µatm (pH 7.90), 350 µatm (pH 8.10), and 225 µatm (pH 8.25). Two gas cylinders were used for each incubator. To ensure quasi-constant seawater carbonate chemistry, a solenoid valve connected to both gas cylinders (pure

CO_2-free air cylinder and pure CO_2 cylinder) and a pH controller modulates the CO_2 flow rate once the desired pH is reached, keeping it constant (±0.02) (Samperio-Ramos et al., 2017). The culture medium was sterile filtered (0.1 µm) North Atlantic seawater (S = 36.48) obtained at the ESTOC site (29º10' N, 15º30' W).

Do all variations of the conditions have the same temperature and incubation time in the culture incubator? Not clearly written.

All experiments have the same incubation time (8 days) and temperature (25ºC). This has been clarified in section 2.2 as follows:

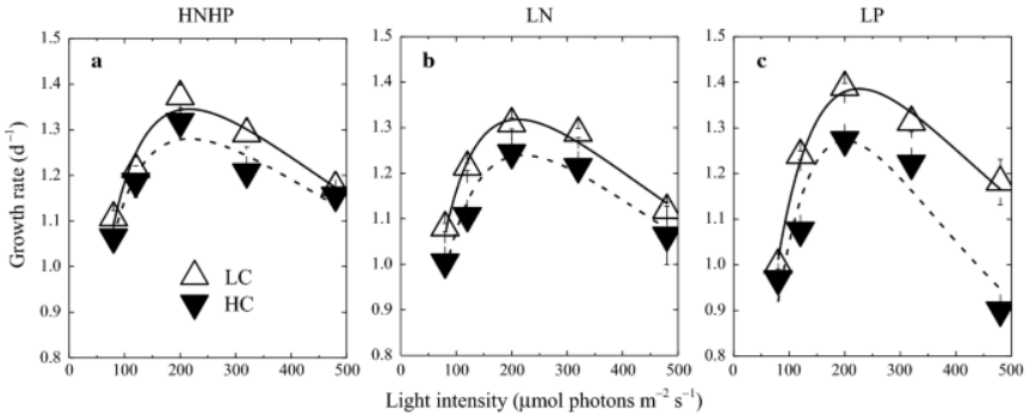
- Axenic cultures of *E. huxleyi* (strain RCC1238) were supplied by the Spanish Bank of Algae (BEA) in f/2 medium. *E. huxleyi* coccolithophore was cultured for 8 days in an incubator clean chamber (Friocell FC111) at a constant temperature of 25 ºC with an initial cell density of 10^6 cells L^{-1},…

Furthermore, in the same section (2.2 culture), it is stated (lines 132-133):

- Gas equilibrium in the media of each treatment was reached after a maximum of 24 h, as observed by the pH evolution. TA and DIC were measured at the beginning and end of the experiment on days 0 and 8 using a VINDTA 3C system (González-Dávila et al.…

Don't you think 200 µmol is too much for E. hux?

Coccolithophore growth rates usually increase with increased light intensity, level off at saturated light intensity and decline at inhibiting high light intensity (Zhang et al., 2019). Growth rates of *E. huxleyi* increased with elevated light intensity up to 200 µmol photons m^{-2} s^{-1} and significantly declined thereafter (all $P < 0.001$) (The following figure has been extracted from Zhang et al., 2019).



Growth rate of *Emiliania huxleyi* as a function of light intensities at low pCO_2 (LC, hollow) and high pCO_2 levels (HC, solid) under **a** high dissolved inorganic nitrogen (DIN) and phosphate (DIP) concentrations (HNHP), **b** low DIN and high DIP concentrations (LN), and **c** high DIN and low DIP concentrations (LP). The lines in each panel were fitted using the model provided by Eilers and Peeters (1988). The values represent the mean ± standard deviation for four replicates

In addition, axenic cultures of *E. huxleyi* (strain RCC1238) were supplied by the Spanish Bank of Algae (BEA) and cultured following its recommendations. This microalgae Collection is member of the European Culture Collections Organization (ECCO) located in Taliarte, SE coast of Gran Canaria. It is included in the World Data Centre for Microorganisms (WFCC-MIRCEN) and is recognized by the World Intellectual Property Organization (WIPO) as international authority for the deposit of microorganisms (algae) through the Budapest Treaty. It is also included in the European Consortium MIRRI.

- Zhang, Y., Fu, F., Hutchins, D.A . *et al.* Combined effects of CO_2 level, light intensity, and nutrient availability on the coccolithophore *Emiliania huxleyi*. *Hydrobiologia* **842**, 127–141 (2019). https://doi.org/10.1007/s10750-019-04031-0

Please provide a clearer explanation of how CO2 was introduced into the cultures. Was it done using a single cylinder in different incubators or with multiple cylinders?

Two cylinders were used to ensure quasi-constant seawater carbonate chemistry. When the seawater pH values reached the target value, a solenoid valve, connected to both gas cylinders (pure CO_2-free air cylinder and pure CO_2 cylinder) and a pH controller, modulated the CO_2 flux, maintaining the set pH (±0.02).

To clarify this, section 2.2 has been modified as follows (lines 113-119):

A gaseous mixture of CO_2-free air and pure CO_2 was bubbled into the culture medium to CO_2 levels of 900 µatm (pH 7.75), 600 µatm (pH 7.90), 350 µatm (pH 8.10), and 225 µatm (pH 8.25). To ensure quasi-constant seawater carbonate chemistry a solenoid valve connected to a pH controller and both gas cylinders (pure CO_2-free air cylinder and pure CO_2 cylinder) modulates the CO_2 flow rate once the desired pH is reached, keeping it constant (±0.02) (Samperio-Ramos et al., 2017). The culture medium was sterile filtered (0.1 µm) North Atlantic seawater (S = 36.48) obtained at the ESTOC site (29º10' N, 15º30' W))

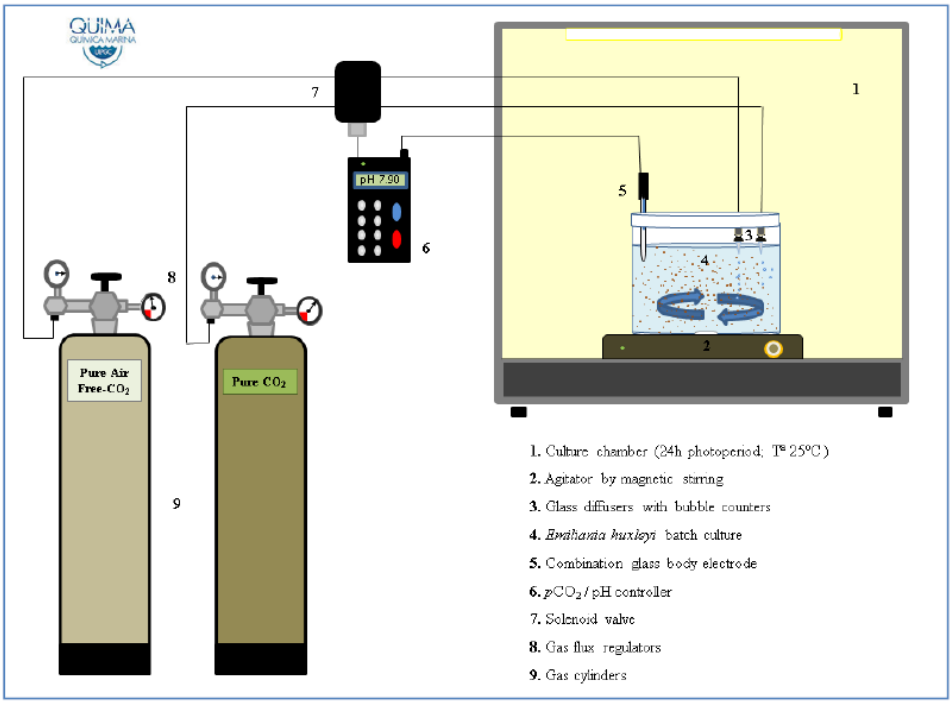


Figure 1. CO_2/pH perturbation experiment set-up, indicating the components.

(Figure extracted from Samperio et al. (2017))

All protocols and methods are described in detail in Samperio et al. (2017) and González Dávila et al. (2011), cited in the manuscript.

The authors discussed other published papers that reported on E. hux at different pH levels, but they examined the concentration of pCO_2 injected rather than the pH changes. This raises the question of how much concentration was injected to achieve each pH variation in this study.

We simulated in our study past, nowadays and future CO_2 concentrations. Injections of CO_2 do not change alkalinity (TA) but increases partial pressure of CO_2 (pCO_2) and total dissolved inorganic carbon (DIC) and decreases pH (e.g., Bates et al., 2014). Therefore, using the initial measured TA, the pH is controlled by the CO_2 injected. Moreover, the pH was continuously monitored during the experiment. The pair TA and DIC was also measured at the end of the study to confirm the concentrations and values were maintained.

Table 1. Carbonate chemistry parameters in experimental media for each pH treatment at day 0 and day 8: total alkalinity (TA), total dissolved inorganic carbon concentration (DIC) and estimated pCO_2 (µatm).

pH-Treatments	TA (µmol kg^{-1})		DIC (µmol kg^{-1})		pCO_2 (µatm)	
	Day 0	Day 8	Day 0	Day 8	Day 0	Day 8
8.25	2376±12	2335±25	1905±26	1869±61	225±1	221±4
8.10	2380±15	2329±28	2012±28	1971±44	353±2	349±5
7.90	2390±17	2347±40	2129±47	2085±36	616±12	599±8
7.75	2401±14	2365±26	2215±16	2178±39	914±18	925±27

Means and standard deviations were calculated from sampling (n = 3).

Results
How were the values calculated? Did they use the standard protocol and calculation methods, such as CO2SYS software, etc.? A clear description is needed.

The measurements and calculation of the carbon dioxide system for this study was previously described by Samperio et al. (2017). The Seawater Carbonate package (Seacarb version 3.0), developed for R Studio software (R Development Core Team), was employed to calculate the values of pCO_2, using the experimental results of pH, dissolved inorganic carbon and total alkalinity, and considering the carbonic acid dissociation constants of Millero et al. (2006).

This information has been included in section 2.2 (lines 138-141) and the bibliography cited in the references section.

Line 435: Is "limited research" completely wrong? Numerous studies have already been reported, not only on E. hux, but also on other phytoplankton, regarding topics such as warming, acidification, etc.
This sentence has been deleted following the last recommendation here.

There are many concerns troughout the results and discussion.
Conclusion has to be completely based on the author's own results.
The conclusion has been modified as follows to focus on our results following this recommendation:

Acidification between the current pH of the oceans (8.1) and the future scenario of pH 7.75 leads to an increase in polyphenol production in *E. huxleyi* cells and their free radical inhibitory activity. More importantly, the change in the polyphenol profile between cells and exudates and between pH conditions should be closely related not only to their antioxidant activity under stress conditions, but also to the chemistry of iron and other trace metals and their bioavailability under different pH conditions. Intra- and extracellular carbohydrate levels did not show modifications with decreasing pH. These changes in metabolites with different capacity to inhibit radicals and complex metals, whose accumulation is associated with enhanced oxidative stress, are potential factors leading to readjustments in phytoplankton community structure and diversity and possible alteration in marine ecosystems.

Report #3

Anonymous during peer-review: Yes No

Anonymous in acknowledgements of published article: Yes No

Checklist for reviewers

1) Scientific significance Does the manuscript represent a substantial contribution to scientific progress within the scope of this journal (substantial new concepts, ideas, methods, or data)?	Excellent **Good** Fair Poor
2) Scientific quality Are the scientific approach and applied methods valid? Are the results discussed in an appropriate and balanced way (consideration of related work, including appropriate references)?	Excellent **Good** Fair Poor
3) Presentation quality Are the scientific results and conclusions presented in a clear, concise, and well structured way (number and quality of figures/tables, appropriate use of English language)?	Excellent **Good** Fair Poor

For final publication, the manuscript should be

accepted as is

accepted subject to **technical corrections**

accepted subject to minor revisions

reconsidered after **major revisions**

rejected

Were a revised manuscript to be sent for another round of reviews:

I would be willing to review the revised manuscript.

I would not be willing to review the revised manuscript.

Suggestions for revision or reasons for rejection

(visible to the public if the article is accepted and published)

Comments on "Variations of polyphenols and carbohydrates of Emiliania huxleyi grown under simulated ocean acidification conditions."

This study reports the effect of ocean acidification on phenols and carbohydrates contents of Emiliania huxleyi. The idea is nice and the experimental setup is good. The data shown here are collected and analyzed clearly, and provide useful information for the biogeochemical cycling of carbon in future ocean acidification. I only have a few minor comments:

(1) Can you explain the relationships between carbohydrates and phenols in the introduction section of this manuscript?
(2) Lines 216-218: "In contrast to these results, ……". This sentence is too long,

please rewrite it.
(3) Lines 237-240: The unit of Chl a is “fmol cell–1” here. Please also show it in “pg cell–1” in a bracket, such as 56.6±2.8 fmol cell–1 (**±** pg cell–1).
(4) For figure 2, the first point in the x-axis should be “7.75” rather than “7.5”. Please change it.
(5) Lines 292-296: “ROS production was also correlated with ……..for N. gaditana cells at pH 6.0”. It is so difficult to understand this sentence. Please rewrite it.
(6) In the introduction and discussion sections, such as in lines 70-79 and 317-337, the authors talk about the contents of Fe. It is better to measure the Fe concentration in the seawater at the beginning and end of the incubations in future study.
(7) Lines 415-416, there are logistic problems about this sentence “Engel (2015) reported that …… in their study”. Please rewrite it.

Point by point response:

(1) Can you explain the relationships between carbohydrates and phenols in the introduction section of this manuscript?

The authors are not sure which relationship the reviewer is referring to. These compounds were selected because they may influence the chemistry of iron and its bioavailability, as indicated in the introduction (lines 72-83) and in lines 318-338.

The antioxidant activities of complex carbohydrates have been attributed mainly to phenolic and protein components, rather than to carbohydrate molecules. Covalent and non-covalent interactions between carbohydrates and phenols are possible. Polysaccharides with covalently bound phenolic compounds acquire metal reducing properties, ability to inhibit oxidative enzymes and enhanced metal chelating properties due to the contribution of the electronegative character of the polyhydroxylated phenolic aromatic ring. Polysaccharides can also interact with phenolic compounds by means of hydrophobic effects, hydrogen bonding and Van der Waals interactions. Multiple binding sites along the polysaccharide backbone results in the formation of highly stable carbohydrate/phenolic complexes. (Fernandes et al., 2023)
The antioxidant activity of polysaccharides is highly dependent on several factors (solubility, molecular weight, occurrence of positive or negatively charged groups among others), being the presence of linked phenolic compounds the major contribution, which allows them to show RSA and metal reducing ability that polysaccharides devoid of phenolic and proteins groups do not exhibit (Chen et al. 2024; Fernandes and Coimbra, 2023). The antioxidant properties increase with the degree of polysaccharides substitution with phenolic compounds.

The following sentence and the cited reference have been included in the introduction (lines 83-86) and in the reference section respectively:

The metal reducing and chelating properties of polysaccharides are highly dependent on several factors, with the presence of covalently and non-covalently bound phenolic and protein components being the main contributing factor, allowing them to exhibit radical scavenging activity (RSA) and metal reduction capacity that they would not exhibit if devoid of these components (Fernandes and Coimbra, 2023).

- Fernandes P. A. R., Coimbra, M. A.: The antioxidant activity of polysaccharides: A structure-function relationship overview, Carbohyd. Polym., 314, 120965, https://doi.org/10.1016/j.carbpol.2023.120965, 2023.

(2) Lines 216-218: "In contrast to these results, ……". This sentence is too long, please rewrite it.

This sentence (lines 215-218) has been changed as follows:

In contrast to these results, Vázquez et al. (2022) found that acidification with CO_2 (1200 µatm, pH 7.62) induced lower coccolithophore growth rates than acidification reached without CO_2 enrichment compared to the control (400 µatm, pH 8.10). In addition, elevated CO_2 affected cell viability and promoted the ROS accumulation, effects not observed under low pH without CO_2 additions.

(3) Lines 237-240: The unit of Chl a is fmol cell^{-1}" here. Please also show it in "pg cell^{-1}" in a bracket, such as 56.6±2.8 fmol cell^{-1} (**±** pg cell^{-1}).

There is an error in the amounts indicated in the manuscript that has been corrected. In the experimental section, the units are correctly stated: "Chl *a* was expressed as femtogram cell^{-1} and quantified spectrophotometrically according to the equation: Chl *a* (mg/100 mL) = $0.999\times A_{663}-0.0989\times A_{645}$". From the equation applied for quantification, the quantity is obtained directly in mass units. The quantities given in section 3.3 are calculated correctly, the error affects the units. The paragraph has been changed as follows

After 8 culture days, the concentration of Chl *a* per cell decreases with decreasing pH from 56.6±2.8 fg cell^{-1} (pH 8.25) to 26.8±1.4 fg cell^{-1} (pH 7.9). However, cells grown in the most acidic conditions (pH 7.75) show the highest amount of Chl *a* (67.3±2.0 fg cell^{-1}) with a significant increase observed between pH 8.1 (45.1±3.0 fg cell^{-1}) and 7.75 (p<0.01).

(4) For figure 2, the first point in the x-axis should be "7.75" rather than "7.5". Please change it.

This has been corrected.

(5) Lines 292-296: "ROS production was also correlated with ……..for N. gaditana cells at pH 6.0". It is so difficult to understand this sentence. Please rewrite it.

The sentence (lines 292-298) has been changed as follows:

Bautista-Chamizo et al. (2019) reported an increase in ROS production by decreasing the pH of the culture medium in single- and multispecies toxicity assays conducted with microalgae *T. Chuii*, *N. gaditana* and *P. tricornutum*. The species *P. tricornutum* and *N.*

gaditana exposed to pH 7.4 and pH 6.0 exhibited a significant increase in the percentage of intracellular ROS, which was more pronounced for *N. gaditana* cells at pH 6.0.

(6) In the introduction and discussion sections, such as in lines 70-79 and 317-337, the authors talk about the contents of Fe. It is better to measure the Fe concentration in the seawater at the beginning and end of the incubations in future study.

We agree with the reviewer that Fe concentration should be measured at the beginning and at the end of the incubations. In this study, iron was added to seawater from a stock solution (1 mM) of ferric chloride (Sigma) obtaining an initial concentration of 2.5 nM to avoid iron deficiency.

- Wei Jin, C., You, G. Y., & Zheng, S. J.: The iron deficiency-induced phenolics secretion plays multiple important roles in plant iron acquisition underground. Plant Signaling & Behavior, 3(1), 60–61. https://doi.org/10.4161/psb.3.1.4902, 2008.

(7) Lines 415-416, there are logistic problems about this sentence "Engel (2015) reported that …… in their study". Please rewrite it.

The sentence has been rewritten as follows (lines 414-416):

Borchard and Engel (2015) found no significant differences between growth rates and primary production (composed of dissolved and particulate organic carbon) of *E. huxleyi* grown at current and high CO_2 concentrations due to its ability to acclimate.