ULPGC
Universidad de
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Las Palmas de Gran Canaria, May 29, 2024

'Point-by-point response to reviewers' file

We really thank and appreciate important comments and suggestions made by Reviewers. Substantial changes have been made in the manuscript in accordance with their suggestions and have been highlighted in blue.

Reviewer's comments followed by our reply (blue color).

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Anonymous Referee #2, 25 Apr 2024

The manuscript by Rico et al., demonstrated the responses of phenolics and carbohydrates of Emiliania huxleyi to ocean acidification conditions. They found that the intracellular phenolic compounds increased under low pH conditions, and these findings have certain implications for the marine food webs and biogeochemical cycles. However, I do have some concerns need to be addressed before it can be accepted for the publication in BG.

This study complements the one previously carried out by our research group and published by Samperio et al. (2017), who cultured the cells studied here (5 replicates) and monitored the changes in several parameters throughout different growth phases (growth, DOC, etc.). Some details of the growing conditions have been included in section 2.2 following suggestions from another reviewer.

- Samperio-Ramos, G., Santana-Casiano, J. M., González-Dávila, M., Ferreira, S., and Coimbra, M. A.: Variability in the organic ligands released by Emiliania huxleyi under simulated ocean acidification conditions, AIMS Environ. Sci., 4(6), https://doi.org/10.3934/environsci.2017.6.788, 2017.

New sections have been included and are listed at the end of this document:

- **2.4 Chlorophyll *a***
- **2.8 Statistical analysis**
- **3.1 Carbonate chemistry parameters**
- **3.3 Chlorophyl a**

Some references have been deleted or replaced by others that are more in line with the amended discussion.

General Comments:

Abstract: The Abstract was not well structed, and is wordy. It should be very concise and highlight the significance of the findings presented in this study. Please rephrase it to be more concise.

The Abstract has been modified 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 improve understanding of its responses to 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 as antioxidants, and 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). However, no significant differences were found between the iron reducing activities and the radical scavenging activities of the compounds present in the exudates. The nature and concentration of the organic compounds present in the culture medium may favour or inhibit the local growth of specific algal species, and influence trace metal bioavailability affecting the biogeochemical cycling of carbon and affecting microbial functional diversity.

Discussion:

1: The discussion could better articulate how the observed changes in polyphenols and carbohydrates could impact broader ecological processes. Specifically, the implications for the marine food web and biogeochemical cycles could be explored in more depth.

Several sentences have been included to highlight the implications for the marine food web in more depth:

- Introduction (lines 46 to 63 discuss this topic): 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 the enhancement of 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 the 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, which leads to a substantial reorganization of the planktonic community, affecting multiple trophic levels from phytoplankton to primary and secondary consumers (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* to increased CO_2, markedly decreasing its growth rate at elevated CO_2 concentrations with bacteria *Idiomarina abyssalis* (*I. 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.

- Section 3.4: These results agree with those reported by Jin et al. (2015), who evidenced that phytoplankton grown under the CO_2 levels predicted for the end of this century showed accumulation of phenolic compounds, increased by 46–212% compared with that obtained at the current CO_2 level. Subsequently, zooplankton fed with phytoplankton grown in acidified seawater showed 28% to 48% higher phenolic content. This transfer of accumulated phenolic compounds to higher trophic levels could have serious consequences for the marine ecosystem and seafood quality. Dupont et al. (2014) have shown that survival of adult boreal shrimp (*Pandalus borealis*) was affected when exposed for 3 weeks at pH 7.5 with an increase in mortality of 63% compared to those grown at pH 8.0 (pH at the sampling site), showing also changes in appearance and taste.

- Section 3.5: Grosse et al. (2020) investigated the effects of seawater acidification on dissolved and particulate amino acids and carbohydrates in arctic and sub-arctic planktonic communities in two large-scale experiments

in a pH range similar to ours here (control mesocosm: pCO_2 185 µatm /pH 8.32; mesocosm pCO_2 between 270 and 1420 µatm/pH 8.18–7.51). The authors concluded that the relative composition of amino acids and carbohydrates did not change as a direct consequence of increased pCO_2, and the observed changes depended mainly on the composition of the phytoplankton community.

- Last paragraph in section 3.6: However, numerous experimental studies show that most coccolithophores cultured at elevated CO_2 reduce their level of calcification with a tendency to produce degraded or aberrant coccoliths that calcify even less and will spread in a future high CO_2 ocean (Langer et al., 2009; Lohbeck et al., 2012; Mackey et al., 2015). The ecological consequences of ocean acidification on the competitiveness of coccolithophores in mixed phytoplankton communities are unclear. The microalgae species *T. Chuii*, *N. gaditana* and *P. tricornutum* showed a higher cell abundance when grown alone under acidification conditions than in multispecies toxicity tests (Bautista-Chamizo et al., 2019). In addition, although oxidative stress in *N. gaditana* was significantly higher in the single-species tests, the ROS levels were higher in *P. tricornutum* and *T. chuii* in the multispecies tests, suggesting that algal interactions had an effect on pH toxicity in these species, but in a species-specific way. However, the effects of competition between these three species were recorded at pH 8, but they were eclipsed by the effect of CO_2 acidification. Several studies evidenced enhanced oxidative stress in cells in acidified seawater, through an increase in ROS that correlated significantly with the accumulation of polyphenols (Bautista-Chamizo et al., 2019; Vázquez et al., 2022). These changes may cause cascading effects in the marine food web, varying the macromolecular composition of consumers (Jia et al., 2024). Ocean acidification will directly affect marine organisms, altering the structure and functions of ecosystems. The accumulation of phenolic compounds leads to functional consequences in primary and secondary producers, with the possibility that fishery industries could be influenced as a result of progressive ocean change (Gattuso et al., 2015; Jin et al., 2015; Trombetta et al., 2019).

The implications of biogeochemical cycles are discussed throughout the manuscript, incorporating some phrases and paragraphs with comparisons with the literature in sections 3.3, 3.4 and 3.5.

- Sections 3.3: Higher level of Chl *a* were also observed in microalgae *Chlorella* sp., *P. tricornutum,* and *C. muelleri* harvested at pH 7.8 and 7.5 than that quantified at pH 8.1, concluding that primary producers that can utilize HCO_3^- as the carbon source benefit from elevated CO_2 concentration (Jia et al., 2024). However, Yu et al. (2022) found no significant changes in cellular and total content of Chl *a* of *E. huxleyi* cells harvested at similar pH than those here (between 7.72-7.76 under elevated CO_2 (1000 µatm) and between 8.03 and 8.07 under atmospheric CO_2). Mackey et al. (2015) concluded that photosynthetic responses to ocean acidification are highly variable throughout species and taxa, with different *E. huxleyi* strains exhibiting opposite responses to elevated CO_2 on maximum photosynthetic rates.

- Sections 3.4: Samperio et al. (2017) reported an increase in dissolved organic carbon (DOC) exudation by 19% and 15% during exponential and stationary phases, respectively, as CO_2 levels increased from 225 µatm (177.06 ± 10.95 fmol C $cell^{-1}$ day^{-1}) to 900 µatm (209.74 ± 50.00 fmol C day^{-1} $cell^{-1}$) in the culture medium. The authors suggested that ocean acidification could significantly enhance the release of phenolic compounds when *E. huxleyi* is grown under low-iron conditions. They detected phenolic compounds only in the stationary phase and their release rate was affected by CO_2 conditions, observing a strong correlation between the concentrations of produced phenolic compounds with exuded DOC, indicating that these compounds constituted a relatively constant fraction of the organic matter excreted by *E. huxleyi*. Extracellular release of phenolic compounds was statistically higher at pH 7.75 (0.41 ± 0.02 fmol C $cell^{-1}$ day^{-1}) than at pH 8.1 (0.36 ± 0.02 fmol C $cell^{-1}day^{-1}$; Tukey contrast: t value=2.495; $p<0.1$). While Samperio et al. (2017) studied the total polyphenol contents through the Arnow spectrophotometric assay, in this study individual polyphenols were identified (Table 2), concluding that the highest concentrations of these polyphenols identified were exuded in the cultures at pH 8.25 (43±3 nM) and 7.75 (18.0±0.9 nM) (Figure 2).

  ….. However, the extracellular phenolic compound's behavior followed the same tendency to that described above for intracellular between pH 8.1 and 7.75, where a high correlation was found between intra- and extracellular phenolic compounds ($p<0.001$). Total exuded phenolic compounds identified here were significantly enhanced in this pH interval ($p<0.05$) to 18.0±0.9 nM at the predicted pH for future scenarios (pH 7.75) over those quantified at pH 8.1 (11.7±0.3). Significant differences were found in PCA and ECAT amounts ($p<0.01$ and 0.05, respectively), which together with RU were detected in all exudates. Wu et al. (2016) studied the interaction between Fe and ten phenols at pH 8.0, finding that Fe(II) was rapidly oxidized under alkali condition (pH 8.0±0.1) even in the presence of gentisic acid, syringic acid, p-coumaric acid, vanillic acid, and ferulic acid, which did not show any protection effect for ferrous iron in these conditions. However, caffeic acid,

GAL and PCA protected 69%, 64% and 33% of the initial concentration of Fe(II), respectively, due to the chelating capacity of the catechol and galloyl groups with Fe(II). They reported the formation of relatively stable phenolic-Fe complexes under alkaline conditions helping to weaken iron precipitation. Therefore, the high level of polyphenols with chelating ability exuded at pH 8.25 in this study could be due to the redox chemistry of inorganic Fe, intimately linked to the pH (Pérez-Almeida et al., 2019), specifically the low Fe concentration under these conditions. When the pH increases, the oxidation rate constants of Fe(II) also increases and the solubility of inorganic Fe(III) decreases. Polyphenols modify Fe(II) oxidation rates by promoting the reduction of Fe(III) to Fe(II) in seawater. The effect of GAL on Fe oxidation and reduction was studied by Pérez-Almeida et al. (2022), concluding that it reduces Fe(III) to Fe(II) in seawater, with a more pronounced effect as pH decreases, allowing Fe(II) to be present for longer periods and improving its bioavailability. The authors found that 69.3% of the initial Fe(II) was oxidized after 10 min at pH 8.0 in the absence of GAL, while only 37.5% was oxidized in its presence at a concentration of 100 nM after 30 min. The reduction of Fe(III) to Fe(II) by GAL was faster as pH decreased. The same results were observed for catechin and sinapic acid, which also favoured the regeneration of Fe(II) in seawater, increasing the amount of regenerated Fe(II) as pH decreased, concluding that acidification may contribute to an increase in the level of reduced iron in the environment (Santana-Casiano et al., 2014). This could be the reason for the decrease of GAL and the remaining phenolic compounds in the extracellular medium until pH 7.9 is reached, where acidification does not seem to produce extreme adverse effects in *E. huxleyi*, as observed in other species (Bautista-Chamizo et al., 2019), and the increase of their presence inside the cell as the pH decreases. Changes in the bioavailability of Fe and other trace metals linked to pH variation could affect the control of phytoplankton growth and have a major influence on the biogeochemical cycling of carbon and other bioactive elements in the ocean (Shaked et al., 2020; Tagliabue et al., 2017).

- Sections 3.5: …. These results agree partially with those reported by Jia et al. (2024), who found that lowering seawater pH promoted protein synthesis in microalgae *Chlorella* sp., *P. tricornutum*, and *C. muelleri* grown at pH 7.8 and 7.5, compared to those harvested at pH 8.1, concluding that acidification improves the efficiency of carbon assimilation and provides more carbon skeletons for amino acids and protein synthesis.

2: The manuscript could benefit from a more detailed comparison with existing literature. Specifically, how do these results align or contrast with the findings of similar studies in terms of the magnitude and direction of changes in polyphenol and carbohydrate content?

We have included the following sentences where results of exposure to acidification of other microalgae species are discussed.

- Sections 2.4 and 3.3 with Chl *a* analysis and comparison with other species has also been included.

2.4 Chlorophyll a

Chlorophyll *a* (Chl *a*) was determined according to Branisa et al. (2014) with modifications. Frozen cells were suspended in 5 mL of acetone:hexane (3:2) and sonicated for 3 min in an ice water bath. Homogenates were centrifuged at 7500 rpm for 5 minutes and the absorbance (A) of supernatants was measured at 645 and 663 nm. Chl *a* was expressed as femtogram cell^{-1} and quantified spectrophotometrically according to equation: Chl *a* (mg/100 mL) = $0.999\times A_{663}–0{,}0989\times A_{645}$.

3.3 Chlorophyl *a*

After 8 culture days, the concentration of Chl *a* per cell decreases with decreasing pH from 56.6±2.8 fmol cell^{-1} (pH 8.25) to 26.8±1.4 fmol 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 fmol cell^{-1}) with a significant increase observed between pH 8.1 (45.1±3 fmol cell^{-1}) and 7.75 (p<0.01). These results agree with those reported by Vázquez et al. (2022), who observed significantly increased cellular concentration of Chl *a* in the high pCO_2 treatment of *E. huxleyi* cells (1200 µatm, pH 7.62) with respect to control (400 µatm, pH 8.10). Crawford et al. (2011) also studied the effect of elevated CO_2 on cultures of the diatom *Thalassiosira pseudonana* CCMP1335 in a pH range between 7.8 and 8.1 (760 µatm and 380 µatm, respectively), concluding that chlorophyll content increased in cells grown at pH 7.8 compared to those grown at pH 8.1, but photosynthetic efficiency remained constant in both experiments. Higher level of Chl *a* were also observed in microalgae *Chlorella* sp., *P. tricornutum,* and *C. muelleri* harvested at pH

7.8 and 7.5 than that quantified at pH 8.1, concluding that primary producers that can utilize HCO_3^- as the carbon source benefit from elevated CO_2 concentration (Jia et al., 2024). However, Yu et al. (2022) found no significant changes in cellular and total content of Chl *a* of *E. huxleyi* cells harvested at similar pH than those here (between 7.72-7.76 under elevated CO_2 (1000 µatm) and between 8.03 and 8.07 under atmospheric CO_2). Mackey et al. (2015) concluded that photosynthetic responses to ocean acidification are highly variable throughout species and taxa, with different *E. huxleyi* strains exhibiting opposite responses to elevated CO_2 on maximum photosynthetic rates.

- Section 3.4 has been completely changed: …In contrast, no significant changes were observed by Jia et al. (2024) in the three microalgae species *Chlorella* sp., *P. tricornutum,* and *C. muelleri* harvested under elevated pCO_2 (pH 8.1, 7.8, and 7.5), and
- …. Previous studies on diatoms *P. tricornutum* grown under copper stress showed a significant correlation ($r=0.9999$; $p<0.05$) between accumulated phenolic compounds and malondialdehyde (MDA), commonly produced by an increase in ROS (Rico et al., 2024). ROS production was also correlated with acidification stress conditions in single and multispecies toxicity tests performed by Bautista-Chamizo et al. (2019) using *T. Chuii*, *N. gaditana* and *P. tricornutum* microalgae, where *P. tricornutum* and *N. gaditana* exhibited a significant increase in the percentage of intracellular ROS when exposed to pH 7.4 and pH 6.0, which was more pronounced for *N. gaditana* cells at pH 6.0. Consistent with the loss of cell membrane integrity observed after 48 h at pH 6.0, a 10% and 56% of non-viable cells were found for *P. tricornutum* and *N. gaditana*, respectively, while viable cells remained close to the control for *T. chuii* cells.
- Section 3.5: During the exponential phase, the contribution of dissolved carbohydrates to excreted DOC was higher (18–37%) than during the stationary phase (14–23%), significantly increased as time elapsed from the exponential to the stationary phase (Samperio et al., 2017). However, acidification of the culture medium with CO_2 did not affect the levels of carbohydrates exuded per cell in any of the three growth phases, as these levels did not change significantly in any of them as the pH dropped to pH 7.75. The amount of total intracellular carbohydrates also remained constant between pH 8.25 and pH 7.75. The coccolithophore *E. huxleyi* have shown diverse metabolic responses to ocean acidification and to combinations of ocean acidification with other environmental factors with significant differences between strains (Gafar et al., 2019; Langer et al., 2009; Mackey et al., 2015; Tong et al. 2017;).
- ….. These results partially agree with those reported by Jia et al. (2024), who found that lowering seawater pH up to 7.8 and 7.5 promoted protein synthesis in microalgae *Chlorella* sp., *P. tricornutum*, and *C. muelleri* compared to those harvested at pH 8.1, concluding that acidification improves the efficiency of carbon assimilation and provides more carbon skeletons for amino acids and protein synthesis.
- Grosse et al. (2020) investigated the effects of seawater acidification on dissolved and particulate amino acids and carbohydrates in arctic and sub-arctic planktonic communities in two large-scale experiments in a pH range similar to ours here (control mesocosm: pCO_2 185 µatm /pH 8.32; mesocosm pCO_2 between 270 and 1420 µatm/pH 8.18–7.51). The authors concluded that the relative composition of amino acids and carbohydrates did not change as a direct consequence of increased pCO_2, and the observed changes depended mainly on the composition of the phytoplankton community.

Regarding the magnitudes, section 3.3 cites the article by Lopez et al. (2015) in which the concentration of polyphenols is in the same range (between 9.4 and 8.4 nM). The contents in the cells are in the same range than here (attomole):

- These results partially agree with those reported by López et al. (2015) for *Dunaliella tertiolecta* growing under stress conditions induced by high levels of copper, where the concentration of phenolic compounds declined from 9.4±0.6 nM in seawater cultures without Cu addition to 8.4±0.4 and 8.6±0.4 nM in the copper enriched seawater, and increased 1.4 times concerning the control into the cells grown under the highest Cu level.

3: Broader Impacts: The results are discussed primarily in the context of *Emiliania huxleyi*. Expanding the discussion to consider potential impacts on other phytoplankton species, could provide a more holistic view of ocean acidification impacts.

In addition to all the comparisons and references already included in the manuscript, the following sentences have been added

- Section 3.2: Bautista-Chamizo et al. (2019) exposed microalgae *Tetraselmis chuii* (*T. Chuii*), *Nannochloropsis gaditana* (*N. gaditana)* and *P. tricornutum* to pH 6.0, 7.4 and pH 8.0 as a control, observing growth inhibition of 12%, 61% and 66% at pH 6.0, respectively, in single toxicity tests, with *T. chuii* being the most resistant species to CO_2 enrichment. At pH 7.4 only *P. tricornutum* showed a significant decrease in cell abundance (16%), while no differences were found for *T. chuii* and *N. gaditana*, demonstrating that sensitivity to acidification depends on the microalgae species. However, the growth rate of all three microalgae *Chlorella* sp., *P. tricornutum* and *Chaetocetos muelleri (C. muelleri)* harvested at pH levels (8.1, 7.8, and 7.5) was significantly enhanced (Jia et al., 2024). The differences observed in the growth behavior of microalgae, mainly of *P. tricornutun* diatoms, in these two last studies could be due to the culture conditions, e.g. in the first study, Bautista-Chamizo et al. (2019) used seawater collected in the Bay of Cádiz (Spain) (with initial pH 8.0, salinity of 34, temperature at 23 °C and initial cell density 3×10^4 cells mL^{-1}), and in the second study Jai et al. (2024) used artificial seawater prepared by mixing sea salt (Haizhixun, China) with purified water (salinity of 30 ± 1‰, temperature maintained at 18 ± 0.5 °C, initial pH at 8.1, no data on initial cell density).

- Section 3.3 focuses on chlorophyll analysis and the results are compared with those obtained in other acidification studies with *E. huxleyi* and other microalgae species:

- Crawford et al. (2011) also studied the effect of elevated CO_2 on cultures of the diatom *Thalassiosira pseudonana* CCMP1335 in a pH range between 7.8 and 8.1 (760 µatm and 380 µatm, respectively), concluding that chlorophyll content increased in cells grown at pH 7.8 compared to those grown at pH 8.1, but photosynthetic efficiency remained constant in both experiments. Higher level of Chl *a* were also observed in microalgae *Chlorella* sp., *P. tricornutum,* and *C. muelleri* harvested at pH 7.8 and 7.5 than that quantified at pH 8.1, concluding that primary producers that can utilize HCO_3^- as the carbon source benefits from elevated CO_2 concentration (Jia et al., 2024). However, Yu et al. (2022) found no significant changes in cellular and total content of Chl *a* of *E. huxleyi* cells harvested at similar pH than those here (between 7.72-7.76 under elevated CO_2 (1000 µatm) and between 8.03 and 8.07 under atmospheric CO_2). Mackey et al. (2015) concluded that photosynthetic responses to ocean acidification are highly variable throughout species and taxa, with different *E. huxleyi* strains exhibiting opposite responses to elevated CO_2 on maximum photosynthetic rates.

- Section 3.4:

- In contrast, no significant changes were observed by Jia et al. (2024) in the three microalgae species *Chlorella* sp., *P. tricornutum,* and *C. muelleri harvested under elevated pCO_2* (pH 8.1, 7.8, and 7.5).

- … Previous studies on diatoms *P. tricornutum* grown under copper stress showed a significant correlation ($r=0.9999$; $p<0.05$) between accumulated phenolic compounds and malondialdehyde (MDA), commonly produced by an increase in ROS (Rico et al., 2024). ROS production was also correlated with acidification stress conditions in single and multispecies toxicity tests performed by Bautista-Chamizo et al. (2019) using *T. Chuii*, *N. gaditana* and *P. tricornutum* microalgae, where *P. tricornutum* and *N. gaditana* exhibited a significant increase in the percentage of intracellular ROS when exposed to pH 7.4 and pH 6.0, which was more pronounced for *N. gaditana* cells at pH 6.0. Consistent with the loss of cell membrane integrity observed after 48 h at pH 6.0, a 10% and 56% of non-viable cells were found for *P. tricornutum* and *N. gaditana*, respectively, while viable cells remained close to the control for *T. chuii* cells.

- Section 3.5:

- These results agree partially with those reported by Jia et al. (2024), who found that lowering seawater pH up to 7.8 and 7.5 promoted protein synthesis in microalgae *Chlorella* sp., *P. tricornutum*, and *C. muelleri* compared to those harvested at pH 8.1, concluding that acidification improves the efficiency of carbon assimilation and provides more carbon skeletons for amino acids and protein synthesis).

- Grosse et al. (2020) investigated the effects of seawater acidification on dissolved and particulate amino acids and carbohydrates in arctic and sub-arctic planktonic communities in two large-scale experiments in a pH range similar to ours here (control mesocosm: pCO_2 185 µatm /pH 8.32; mesocosm pCO_2 between 270 and 1420 µatm/pH 8.18–7.51). The authors concluded that the relative composition of amino acids and carbohydrates did not change as a direct consequence of increased pCO_2, and the observed changes depended mainly on the composition of the phytoplankton community. Different results were observed in diatoms.

- Section 3.6:

However, numerous experimental studies show that most coccolithophores cultured at elevated CO_2 reduce their level of calcification with a tendency to produce degraded or aberrant coccoliths that calcify even less and will spread in a future high CO_2 ocean (Langer et al., 2009; Lohbeck et al., 2012; Mackey et al., 2015). The ecological

consequences of ocean acidification on the competitiveness of coccolithophores in mixed phytoplankton communities are unclear. The microalgae species *T. Chuii*, *N. gaditana* and *P. tricornutum* showed a higher cell abundance when grown alone under acidification conditions than in multispecies toxicity tests (Bautista-Chamizo et al., 2019). In addition, although oxidative stress in *N. gaditana* was significantly higher in the single-species tests, the ROS levels were higher in *P. tricornutum* and *T. chuii* in the multispecies tests, suggesting that algal interactions had an effect on pH toxicity in these species, but in a species-specific way. However, the effects of competition between these three species were recorded at pH 8, but they were eclipsed by the effect of CO_2 acidification. Several studies evidenced enhanced oxidative stress in cells in acidified seawater, through an increase in ROS that correlated significantly with the accumulation of polyphenols (Bautista-Chamizo et al., 2019; Vázquez et al., 2022). These changes may cause cascading effects in the marine food web, varying the macromolecular composition of consumers (Jia et al., 2024).

Specific comments:

1: Line 37-39: Some controversial findings regarding the calcification of coccolithophore to ocean acidification should be mentioned.

We agree with the reviewer that a study of the controversial findings regarding the calcification would be interesting, but that part is beyond the scope of our research, which focuses more on the change in both polyphenols and carbohydrates with pH variation. However, as indicated in the previous paragraph we have included same references to this effect.

2: Line 82-84: There were at least two studies examined the responses of phenolic compounds in marine primary producers to ocean acidification (Arnold et al., 2012, PLOS ONE; Jin et al., 2015, Nature Communications, 6:8714), where are more relevant to the present study should be acknowledged here.

The authors agree and therefore cite the article twice in the manuscript, and completed the information as follows:

- Line 214:

- The contents of phenolic compounds inside the cells increased with the decline of pH up to pH 7.75, reaching the maximum level (9.24±0.19 amol $cell^{-1}$). These results agree with those reported by Jin et al. (2015), who evidenced that phytoplankton grown under the CO_2 levels predicted for the end of this century showed accumulation of phenolic compounds, increased by 46–212% compared with that obtained at the current CO_2 level. Subsequently, zooplankton fed with phytoplankton grown in acidified seawater showed 28% to 48% higher phenolic content. This transfer of accumulated phenolic compounds to higher trophic levels could have serious consequences for the marine ecosystem and seafood quality. Dupont et al. (2014) have shown that survival of adult boreal shrimp (*Pandalus borealis*) was affected when exposed for 3 weeks at pH 7.5 with an increase in mortality of 63% compared to those grown at pH 8.0 (at the sampling site), showing also changes in appearance and taste.

- Section 3.6, in the last paragraph:

  Several studies evidenced enhanced oxidative stress in cells in acidified seawater, through an increase in ROS that correlated significantly with the accumulation of polyphenols (Bautista-Chamizo et al., 2019; Vázquez et al., 2022). Ocean acidification will directly affect marine organisms, altering the structure and functions of ecosystems. These changes may cause cascading effects in the marine food web, varying the macromolecular composition of consumers (Jia et al., 2024). The accumulation of phenolic compounds leads to functional consequences in primary and secondary producers, with the possibility that fishery industries could be influenced as a result of progressive ocean change (Gattuso et al., 2015; Jin et al., 2015; Trombetta et al., 2019).

By other hand, Arnold et al. (2012) study is focused on marine plants. The authors consider it important to mention this effect seen in higher plants, but not to focus our discussion on them, but rather on microalgae.

Line 284: However, Arnold et al. (2012) reported a loss of phenolics in the seagrasses *Cymodocea nodosa, Ruppia maritima,* and *Potamogeton perfoliatus* grown in acidified seawater, where the pH decreased up to 7.3, and the CO_2 level increased ten-fold.

3: Line 115-125: Have you monitored the carbonate chemistry parameters over the 8 days experimental duration? These parameters are important for a typic OA research. Please clarify.

All these parameters were monitored, discussed and reported previously by Samperio et al. (2017). We have included the following paragraph in section 2.2 and a new section called 3.1 Carbonate chemistry parameters

- Section 2.2:
- Carbonate chemistry was monitored continuously in the experimental media and determined from pH, total alkalinity (TA), and total dissolved inorganic carbon (TC). pH in the treatments was measured on the free hydrogen ion scale ($pH = -\log [H^+]$), by immersing Orion Ross combination glass electrodes in the experimental media. The electrodes were calibrated daily using TRIS buffer solutions. The equilibration of the gas in the media of each treatment was achieved after a maximum of 24 h, observed by the evolution of pH. TA and TC were measured at the beginning and end of the experiment on days 0 and 8. TA was determined by potentiometric titration with hydrochloric acid until the endpoint of carbonic acid was reached utilizing a VINDTA 3C system (Mintrop et al., 2000). TC was analyzed by coulometric procedure after phosphoric acid addition (González-Dávila et al. 2011). Certified Reference Material (provided by A. Dickson at Scripps Institution of Oceanography) was employed to assess the performance of the titration system, yielding an accuracy of 1.5 and 1.0 µmol kg^{-1} for TA and TC, respectively. 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, considering the carbonic acid dissociation constants. A more detailed description is given by Samperio-Ramos et al. (2017).

- **3.1 Carbonate chemistry parameters**
- Preliminary tests indicated good stability of carbonate chemistry parameters in media, obtained by the CO_2 regulation system (Table 1). After CO_2-equilibration, initial TC values ranged from 1905 ± 26 to 2215 ± 16 at a TA mean value of 2386 ± 16 µmol kg^{-1} (one-way ANOVA; F=1.729, p=0.2382 among treatments), corresponding to a CO_2 range of 225 ± 1 and 914 ± 13 µatm. Although carbonate chemistry in CO_2-manipulated experiments can be strongly affected by biological activity (Howes et al. 2017; Miller and Kelley 2021), during our experiments TA and TC remained fairly stable (t-tests; p>0.05) within treatments, over the 8-day experimental period.

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 (TC)) and estimated pCO_2 (µatm). Means and standard deviations were calculated from sampling (n = 3).

pH-Treatments	TA (µmol kg^{-1})		TC (µmol kg^{-1})		*p*CO_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

In addition, the following paragraphs have been included in the sections 3.4 and 3.5

- Samperio et al. (2017) reported an increase in dissolved organic carbon exudation by 19% and 15% during exponential and stationary phases, respectively, as CO_2 levels increased from 225 µatm (177.06 ± 10.95 fmol C $cell^{-1}$ day^{-1}) to 900 µatm (209.74 ± 50.00 fmol C day^{-1} $cell^{-1}$) in the culture medium. The authors suggested that ocean acidification could significantly enhance the release of phenolic compounds when *E. huxleyi is* grown

under low-iron conditions. They detected phenolic compounds only in the stationary phase and their release rate was affected by CO_2 conditions, observing a strong correlation between the concentrations of produced phenolic compounds with exuded dissolved organic carbon, indicating that these compounds constituted a relatively constant fraction of the organic matter excreted by *E. huxleyi*. Extracellular release of phenolic compounds was statistically higher at pCO_2 900 µatm (0.41 ± 0.02 fmol C cell^{-1} day^{-1}) than at pCO_2 350µatm (0.36 ± 0.02 fmol C cell^{-1}day^{-1}; Tukey contrast: t value = 2.495; $p < 0.1$). While Samperio et al. (2017) studied the total polyphenol contents through the Arnow spectrophotometric assay, in our study we have identified some polyphenols (Table 1), concluding that the highest concentrations of these polyphenols identified were exuded in the cultures at pH 8.25 (43±3 nM) and 7.75 (18.0±0.9 nM). The high level of these exuded polyphenols at pH 8.25 could be due to the redox chemistry of inorganic Fe, intimately linked to the pH (Pérez-Almeida et al., 2019). When the pH increases the solubility of inorganic Fe(III) decreases and the oxidation rate constants of Fe(II) increases. Wu et al. (2016) studied the interaction between Fe and ten phenols at pH = 8.0, finding that only caffeic acid, gallic acid, and protocatechuic acid protected 69%, 64% and 33% of the initial iron (II), respectively, due to the chelating capacity of the catechol and galloyl groups with Fe(II).

- Samperio et al. (2017) reported higher contributions of dissolved carbohydrates to excreted DOC during the exponential phase (18–37%) than during the stationary phase (14–23%), significantly increased as time elapsed from the exponential to the stationary phase. However, acidification of the culture medium with CO_2 did not affect the levels of carbohydrates exuded per cell in any of the three growth phases, as these levels did not change significantly in any of them as the pH dropped to pH 7.75. The amount of total intracellular carbohydrates also remained constant between pH 8.25 and pH 7.75. The coccolithophore *E. huxleyi* have shown diverse metabolic responses to ocean acidification and to combinations of ocean acidification with other environmental factors with significant differences between strains (Langer et al., 2009; Tong et al. 2017; Gafar et al. 2019).

4: Line 170-175: Should it be possible to calculate the specific growth rates for the exponential phase of each growth curve and then compare them under different pH conditions?

No significant differences were found between the specific growth rates of five replicates:

pH	Specific Growth Rate (day^-1)
7.75	0.564±0.009
7.9	0.582±0.014
8.1	0.587±0.014
8.25	0.575±0.011
ANOVA (p=0.336)	

5: Line 210: diatoms?

This has been corrected

10: Line 255: Typo of the citation “Santana_Casiano et al., 2014”

This has been corrected to Santana-Casiano et al., 2014

A Pearson’s correlation test was performed to determine the degree of relationship between pairs of variables and a one-way ANOVA to determine statistically significant differences between measurements. Both studies were conducted using the Jamovi program (2022) and p-values of <0.05 were considered statistically significant.

Correlation Matrix between pH 8.25 – 7.75

		Intracellular TCH	Extracellular TCH	Intra TPC	Extra TPC	RSA cells	RSA exudates	FRAP cells
Intracellular TCH	R de Pearson	—						
	valor p	—						
Extracellular TCH	R de Pearson	-0.257	—					
	valor p	0.731	—					
Intra TPC	R de Pearson	-0.615	0.416	—				
	valor p	0.948	0.153	—				
Extra TPC	R de Pearson	0.396	-0.810	-0.073	—			
	valor p	0.166	0.993	0.568	—			
RSA cells	R de Pearson	-0.280	0.728 *	0.829 **	-0.240	—		
	valor p	0.749	0.020	0.005	0.716	—		
RSA exuded	R de Pearson	0.213	0.456	-0.567	-0.730	-0.249	—	
	valor p	0.306	0.128	0.929	0.980	0.724	—	
FRAP	R de Pearson	-0.543	0.129	0.718 *	0.133	0.527	-0.565	—
	valor p	0.918	0.380	0.022	0.376	0.090	0.928	—

* $p < .05$, ** $p < .01$, *** $p < .001$

TCH: Total carbohydrates
TFC Total phenolic content
RSA: Radical Scavenging Activity
FRAP: Ferric Reducing Power Assay

Correlation Matrix between pH 8.1 and 7.75

		Intracellular TCH	Extracellular TCH	Intra TPC	Extra TPC	RSA cells	RSA exuded	FRAP
Intracellular TCH	R of Pearson	—						
	p value	—						
Extracellular TCH	R of Pearson	0.463	—					
	p value	0.177	—					
Intra TPC	R of Pearson	-0.567	0.307	—				
	p value	0.879	0.277	—				
Extra TPC	R of Pearson	-0.662	0.104	0.965 ***	—			
	p value	0.924	0.423	< .001	—			
RSA cells	R of Pearson	-0.083	0.803 *	0.810 *	0.665	—		
	p value	0.562	0.027	0.025	0.075	—		
RSA exuded	R of Pearson	0.736 *	-0.043	-0.941	-0.976	-0.627	—	
	p value	0.048	0.532	0.997	1.000	0.909	—	
FRAP	R of Pearson	-0.625	0.201	0.778 *	0.786 *	0.588	-0.709	—
	p value	0.908	0.351	0.034	0.032	0.110	0.943	—

Nota. * $p < .05$, ** $p < .01$, *** $p < .001$

TCH: Total carbohydrates
TPC: Total phenolic content
RSA: Radical Scavenging Activity
FRAP: Ferric Reducing Power Assay

One way factor ANOVA

	F	gl1	gl2	*P*	*p*
RSA cells	290.89	3	2.07	0.003	< .01
RSA exudates	30.38	3	1.90	0.037	< .05
FRAP cells	1.26	3	2.12	0.464	
Total exuded phenolics (nM)	64.69	3	1.88	0.019	< .05
Exuded phenolics per cell	14529.66	3	1.67	< .001	
Protocatechuic acid	256.02	3	2.00	0.004	< .01
Epicatechin	40.88	3	1.94	0.026	< .05
Rutin	7.25	3	1.70	0.152	
Intracellular-phenolics	173.30	3	2.18	0.004	< .01
Intra-Vanillic acid	1308.13	3	2.08	< .001	

RSA: Radical Scavenging Activity
FRAP: Ferric Reducing Power Assay
TCH: Total carbohydrates
TPC: Total phenolic content