ULPGC
Universidad de
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'Point-by-point response to reviewers' file

We really thank and appreciate important comments and suggestions made by Reviewers. Substantial changes have been included in the manuscript in accordance with their suggestions and have been highlighted in blue. The following references have been deleted or replaced by others that are more in line with the amended discussion.

- Antosiewicz, J. M., and Kane, P. M.: Editorial: Intracellular Molecular Processes Affected by pH. Front. Mol. Biosci. Sec. Biophysics, 9, 891533, https://doi: 10.3389/fmolb.2022.891533, 2022.
- Casey, J., Grinstein, S., and Orlowski, J.: Sensors and regulators of intracellular pH, Nat. Rev. Mol. Cell. Biol., 11, 50–61. https://doi.org/10.1038/nrm2820, 2010.
- Salam, U., Ullah, S., Tang, Z.-H., Elateeq, A.A., Khan, Y., Khan, J., Khan, A., and Ali, S.: Plant Metabolomics: An Overview of the Role of Primary and Secondary Metabolites against Different Environmental Stress Factors, Life, 13, 706, https://doi.org/10.3390/life13030706, 2023.
- Santschi, P. H., Hung, C.-C., Schultz, G., Alvarado-Quiroz, N., Guo, L., Pinckney, J., and Walsh, I.: Control of acid polysaccharide production and 234Th and POC export fluxes by marine organisms, Geophys. Res. Lett., 30, 1044, https://doi.org/10.1029/2002GL016046, 2003.
- Shaked, Y., and Lis, H.: Disassembling iron availability to phytoplankton. Front. Microbiol., 3, 123, https://doi.org/10.3389/fmicb.2012.00123, 2012.

The following paragraphs have been deleted:

In section 3.2 Cell growth

- The highest peaks of algae biomass, 1.07 (± 0.10) and 1.04 (± 0.07) × 10^8 cells L^{-1}, were recorded in the microcosms with intermediate CO_2 levels 350 µat and 600 µat (pH 8.10 and 7.90 respectively).

In section 3.4 Phenolic contents of cells and exudates

- The phenolic profile differences found inside and outside the cells could be explained by the different mechanisms to counter pH acidification inside (intrinsic buffers such as ionizable groups on amino acids, phosphates and other molecules; Na^+–H^+ exchangers and bicarbonate transporters, membrane permeability, among others) and the changes of metabolic pathways involved (Barcelos e Ramos et al., 2010; Casey et al., 2010) and outside, limited to membrane permeability and exuded material.

Sections 3.6 Antioxidant activities and Conclusion have been rewritten

Anonymous Referee #1, 30 Mar 2024 (https://doi.org/10.5194/bg-2024-1-RC1)

The manuscript by M. Rico et al presents data from a cultivation of *Emiliania h*. under four pH conditions. results concern the growth, phenolic compounds and total carbohydrate in both cells and medium.

The study might be of interest if the authors add some other variables (pigments, coccoliths,). in its present state, this ms can not be accepted as publication, due to relevant weaknesses.

New sections have been included:

- Section 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) = $0.999 \times A_{663} – 0{,}0989 \times A_{645}$.
- Section 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.

- The ms totally lacks of statistical analysis. It does seem that for most of the data, no significant variation was revealed among the different treatments. if this was true, the results and discussion section has to be thoroughly re-written.

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 reviewer 2.

- 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.

The cell densities (N) were determined from the average of 5 experimental batch cultures at each pCO_2 treatment. The goodness of the fit for each curve was estimated by the coefficient of correlation (r2 > 0.95).

The following new sections have been included in the manuscript. ANOVA studies and correlation matrix are included at the end of this document.

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

- **3.1 Carbonate chemistry parameters**

- the fig. 2 is emblematic: in the legend, "diatoms" are mentioned while results cam from Emiliania h.; dark vs light color is referred to what?; these data correspond to which day ?

Figure 2 and its title have been changed as follows:

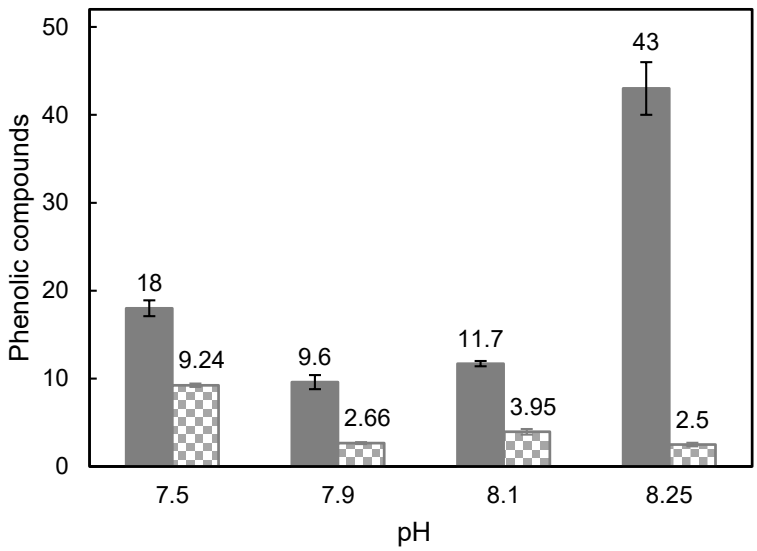
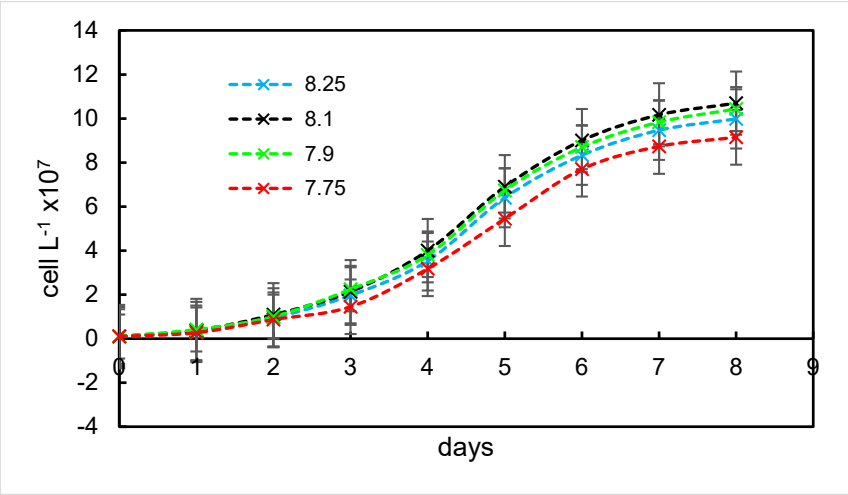


Figure 2 Total sum of the identified intracellular polyphenols expressed as attomol cell^{-1} (non-solid bars) and concentration (nM) of exuded polyphenols (solid bars) by *Emiliania huxleyi* cells grown under reduced pH conditions after eight culture days.

- fig.1: no SD bars

The SD bars overlap in the growth curves and were therefore avoided. Figure 1.B) is related and shows the cell densities with the SDs.



However, Fig. 1 has been changed as presented below.

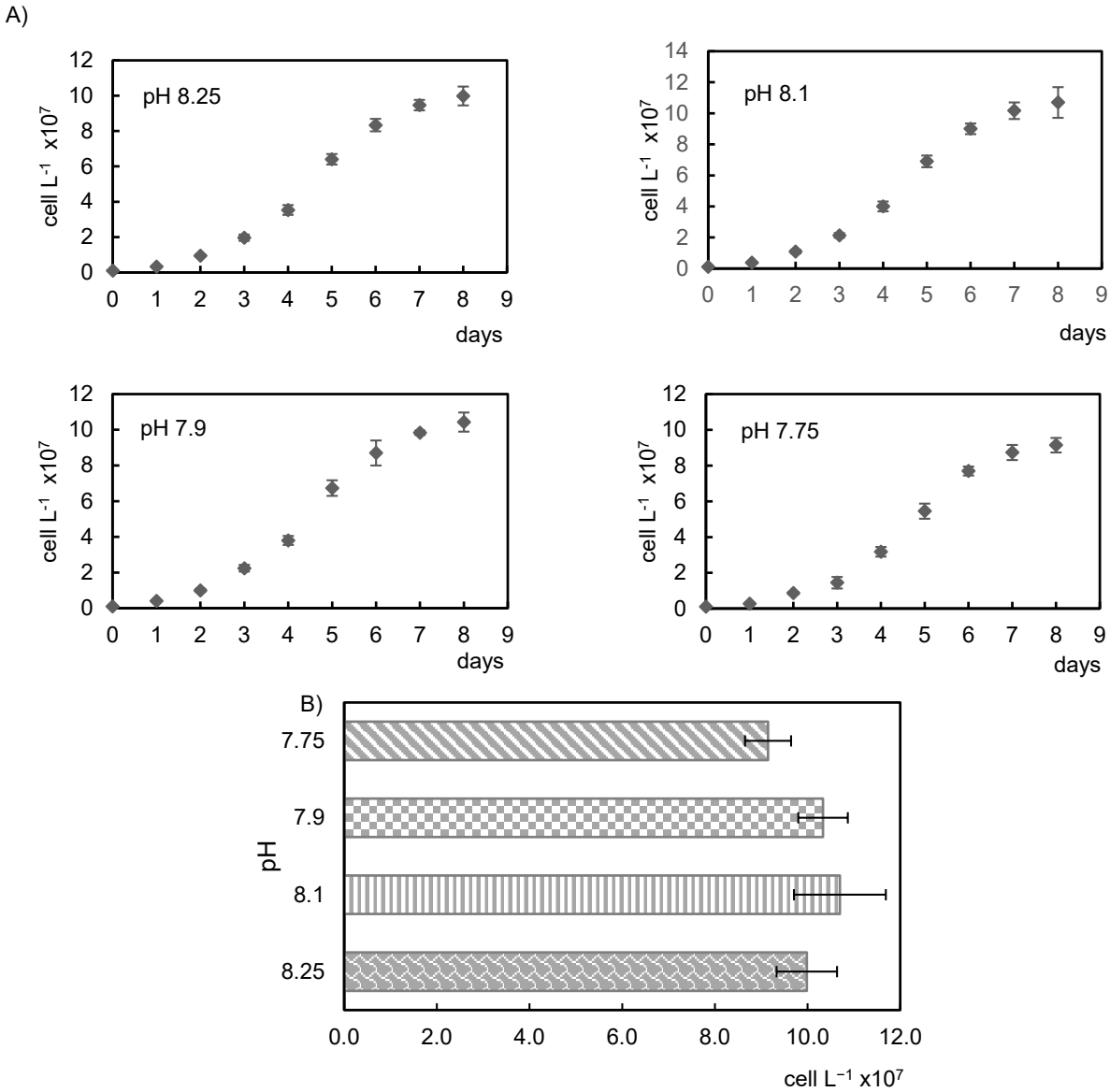


Figure 1 Growth curves (A) and cell densities (B) of coccolithophore *Emiliania huxleyi* cultivated under four different pH conditions.

- material and methods: no mention on light, photoperiod, temperature...: these factors are probably the most relevant to shape the microalgal physiology.

- The following sentences with the requested information have been included in the text:

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 with an initial cell density of 10^6 cells L^{-1} at a constant temperature of 25 ºC, 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. For this purpose, a gaseous mixture of CO_2-free air and pure CO_2 (up to CO_2 levels 900, 600, 350, and 225 µatm, respectively) was bubbled in the culture medium (sterile filtered (0.1 µm) North Atlantic seawater (S = 36.48) obtained at the ESTOC site (29º10' N, 15º30' W) with an equipment that modulates the CO_2 flow once the desired pH is reached, keeping it constant (±0.02). Cells were frozen and stored at -80ºC.

Carbonate chemistry was monitored continuously in the experimental media and determined from pH, total alkalinity (TA), and total dissolved inorganic carbon (DIC). 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 DIC were determined using a VINDTA 3C system (González-Dávila et al., 2011). TA was determined by potentiometric titration with hydrochloric acid until the endpoint of carbonic acid was reached. DIC was analyzed by coulometric procedure after phosphoric acid addition. 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 DIC, 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).

- material and methods: it is not clear: seawater or medium?

- Experimental cultures were grown in sterile filtered (0.1 µm) North Atlantic seawater (S = 36.48) obtained at the ESTOC site (29º10' N, 15º30' W).
- The following sentences have been included in the text:

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 with an initial cell density of 10^6 cells L^{-1} at a constant temperature of 25 ºC, 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. For this purpose, a gaseous mixture of CO_2-free air and pure CO_2 (up to CO_2 levels 900, 600, 350, and 225 µatm, respectively) was bubbled in the culture medium (sterile filtered (0.1 µm) North Atlantic seawater (S = 36.48) obtained at the ESTOC site (29º10' N, 15º30' W) with an equipment that modulates the CO_2 flow once the desired pH is reached, keeping it constant (±0.02).

- which was the target of pH manipulation?

- The main aim of this work is stated in the first sentence of the last paragraph of the introduction:

This work aimed to determine how marine acidification may affect the composition of cells and exudates from *Emiliania huxleyi*.

- 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.

Changes in pH generate stress conditions, either because at high pH drastically decrease the availability of trace metals such as Fe(II), a restrictive element for primary productivity (Wu et al., 2016), or because ROS are increased at acid pH (Bautista-Chamizo et al., 2019; Vázquez et al., 2022). The characterization of compounds exuded into the environment under stress conditions has allowed the development of important new lines of research in iron chemistry, for example, in different acidification scenarios. These compounds are crucial ligands in the formation of metal complexes to acquire micronutrients, sequester toxic metals, and to establish electrochemical gradients resulting in changes in the speciation, the bioavailability, and the toxicity of trace metals. Our research team has tested several compounds identified in the exudates of microalgae to study the effects on copper and iron chemistry in seawater:

- Pérez-Almeida et al., 2022. Ocean Acidification Effect on the Iron-Gallic Acid Redox Interaction in Seawater. Front. Mar. Sci., Sec. Marine Biogeochemistry, 9. https://doi.org/10.3389/fmars.2022.837363
- Arnone et al. (2024). Distribution of copper-binding ligands in Fram Strait and influences from the Greenland Shelf (GEOTRACES GN05): Science of The Total Environment, 909, 168162, https://doi.org/10.1016/j.scitotenv.2023.168162
- González et al., 2018. Iron complexation by phenolic ligands in seawater. Chemical Geology. 511, 380-388, 2018.https://doi.org/10.1016/j.chemgeo.2018.10.017
- A. López, et al., 2015. Phenolic profile of Dunaliella tertiolecta growing under high levels of copper and ironEnvironmental Science Pollution Res. 22 (19) 14820-14828, 2015. 10.1007/s11356-015-4717-y
- J. M. Santana-Casiano et al. 2014, Characterization of phenolic exudates from *Phaeodactylum tricornutum* and their effects on the chemistry of Fe(II)-Fe(III). Mar. Chem., 158, 10-16. https://doi.org/10.1016/j.marchem.2013.11.001

- material and methods: 48h (line 124)? does it mean that the experiment started after 48 h of cultivation of E.h. under the different conditions? in the fig.1 the day 0 corresponded to this time? which was the cell concentration at this time?

- These two days correspond to the log phase and are included in the 8 days of culture monitoring.
- We distinguish the three stages in the growth curves of *E. huxleyi*: initial or log phase (these two days, until 2nd day = the first 48h mentioned above), exponential (EP, from 3rd to 5th day) and steady (SP, from 6th to 8th day) phases.

- material and methods: please explain why a first extraction in acetone and then in methanol. what is the role of acetone?

This is a mistake. The described procedure was used for chlorophyll measurement with a mixture of acetone and hexane as described in section 2.4. For the extraction of polyphenols, and for the DPPH and FRAP assays, methanol was used following the usual protocol in our laboratory (López et al., 2015; Santiago-Díaz et al., 2023) and that used by Vicente et al. (2021), and for the extraction of carbohydrates, 5 mL of acidified water (pH = 2) was used, 1.5 mL was freeze-dried and the residue was dissolved as described in section 2.3.

- López et al.: Phenolic profile of *Dunaliella tertiolecta* growing under high levels of copper and iron, Environ. Sci. Pollut. Res. 22, 14820–14828. https://doi.org/10.1007/s11356-015-4717-y, 2015.
- Santiago-Díaz et al.: Copper toxicity leads to accumulation of free amino acids and polyphenols in Phaeodactylum tricornutum diatoms, Environ. Sci. Pollut. Res., 30, 51261–51270, https://doi.org/10.1007/s11356-023-25939-0, 2023.
- Vicente et al. Production and bioaccessibility of *Emiliania huxleyi* biomass and bioactivity of its aqueous and ethanolic extracts, J. Appl. Phycol. 33, 3719–3729, https://doi.org/10.1007/s10811-021-02551-8, 2021

- quantification of phenols: did the authors used some pure standards?

We used the standards described in the following sections:

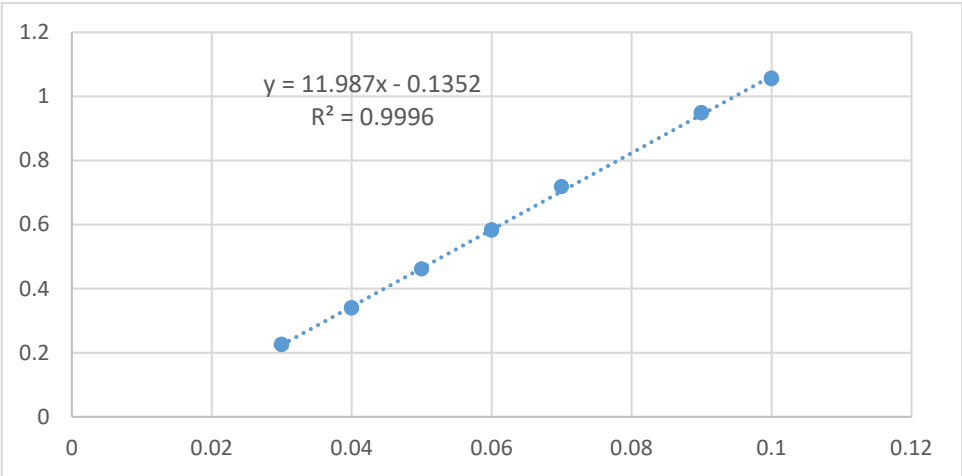
- Introduction (lines 100-102)
  Intra- and extracellular phenolic compounds (gallic acid (GAL), protocatechuic acid (PCA), p-coumaric acid (COU), ferulic acid (FA), catechin (CAT), vanillic acid (VAN), epicatechin (ECAT), syringic acid (SYR), rutin (RU) and gentisic acid (GA)) were identified and quantified by RP-HPLC.

- 2.1 Chemicals (lines 110-111)
  Polyphenol standards were supplied as follows: GAL, PCA, COU, FA, CAT, VAN, ECAT, and SYR by Sigma–Aldrich Chemie (Steinheim, Germany); RU and GA by Merck (Darmstadt, Germany

- The description of the chromatographic analysis can be found in section 2.5, where the standards were cited (lines 166-167): For quantification, simultaneous monitoring was set at 270 nm (GAL, PCA, CAT, VAN, RU, ECAT, and SYR) and 324 nm (GA, COU, and FA).

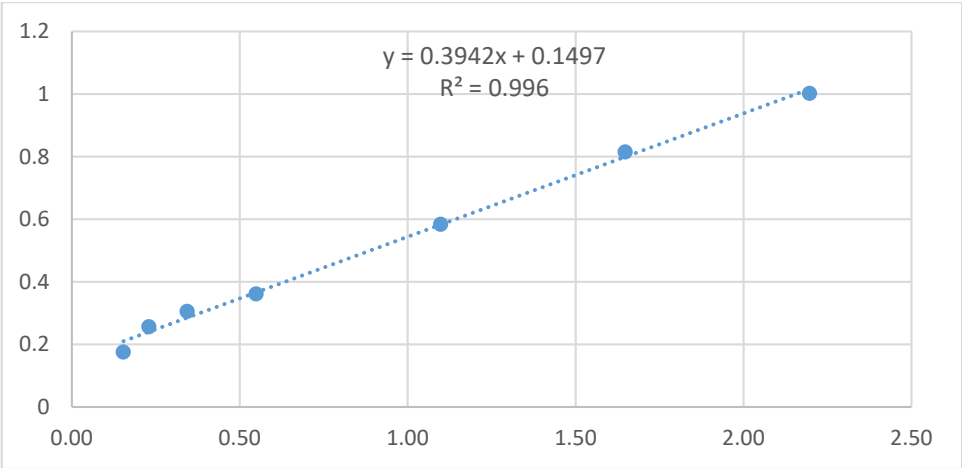
- calibration curve: please explain why there is a "b" factor (y = ax + b; for carbohydrates, frap) also especially when it is negative (dpph)?

DPPH assay: We prepare a calibration curve of DPPH in methanol at different concentrations. The absorbance corresponding to each concentration is measured and the following calibration curve is constructed:



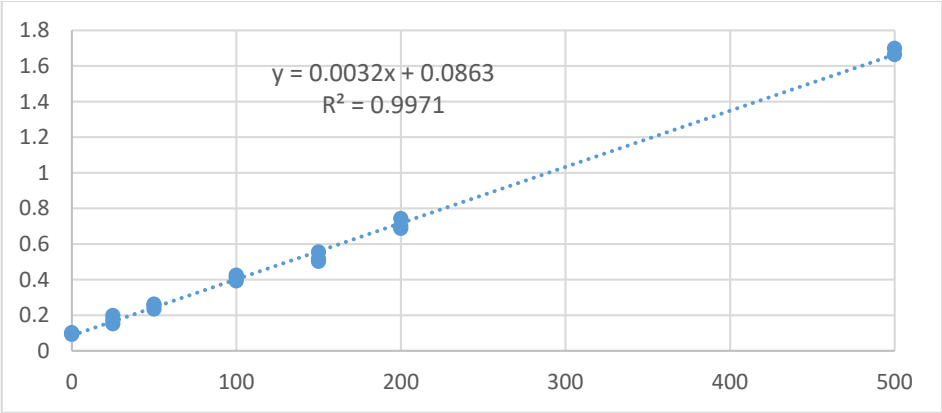
The absorbances of the algal samples correspond to the concentration of not inhibited DPPH. Therefore, subtracting the concentrations obtained through the calibration curve from the initial DPPH concentration allows us to calculate the amount of inhibited DPPH.

Calibration curve for FRAP determination:



This method has been performed according to the literature, where this factor is always present, probably due to parallel reactions or impurities in the reagents, which are present in all standards and samples, and therefore do not affect the final result. Regardless of whether the reducing power is measured as reduced Fe(III) from a calibration curve prepared with Fe(II) or whether it is measured with a standard such as Trolox, this factor b is present.

Glucose calibration curve:



The following articles corroborates this:

- *Noreen et al., 2017:* y=0.000 06x+0.1887. https://doi.org/10.1016/j.apjtm.2017.07.024.
- *Alam et al. 2014:* y=5.4901x+0.2547. https://doi.org/10.1155/2014/296063.
- *Mukherjee et al.,* 2019. https://link.springer.com/article/10.1007/s12649-017-0053-4

- line 165: cell extracts? how they have been done?

- this aspect has been clarified above.

- LINE 173: The highest peak? but NO SIGNIFICANT!

- This sentence has been deleted.

- lines 186-189: re-write!

These lines have been rewritten as follows:

> Discrepancies found in the literature regarding acidification effects and responses of *E. huxleyi* coccolithophores may be due not only to different environmental factors and culture conditions, etc. (Gafar et al., 2019; Tong et al., 2017). Langer et al. (2009) observed substantial differences in sensitivity to acidification in four different *E. huxleyi* strains with different responses in all parameters tested.

- table 1: unit of cell and exuded? seems to be different, also from the "levels" (nM).

The title of Table 1 has been changed as follows and a mistake was corrected in the amount of PCA exuded at pH 7.75. The total concentration was correct.

Table 1. Amounts of intracellular and exuded phenolic compounds by cells of *E. huxleyi* grown under different pH conditions.

Phenolic compound	pH 7.75 (pCO_2 900µat)		pH 7.90 (pCO_2 600µat)		pH 8.10 (pCO_2 350µat)		pH 8.25 (pCO_2 225µat)	
	Cell	Exuded	Cell	Exuded	Cell	Exuded	Cell	Exuded
GAL[a]	0.13±0.03	-	-	-	0.25±0.07	-	-	45±0
PCA[a]	-	151±12	0.16±0.01	34±2	-	26.3±0.2	-	298±22
ECAT[a]	1.2±0.1	34±1	-	40±5	-	70±2	-	77±6
VAN[a]	6.44±0.06	-	2.5±0.1	-	2.30±0.05	-	2.5±0.2	-
COU[a]	-	-	-	-	1.4±0.2	-	-	-
RU[a]	1.47±0	12.1±0.4		20±2		13.2±0.3		11.2±0.8
Sum[a]	9.24±0.19	197.1±12.3	2.66±0.11	94±9	3.95±0.32	109.5±2.5	2.5±0.2	431.2±28.8
Concentration (nM)[b]		18.0±0.9		9.6±0.8		11.7±0.3		43±3

[a]Results are expressed as attomole $cell^{-1}$ (means ± standard deviation of three measurements).

[b]Results are expressed as nanomole L^{-1} (means ± standard deviation of three measurements).

Abbreviations: GAL: gallic acid; PCA: protocatechuic acid; ECAT: epicatechin; VAN: vanillic acid; COU: p-coumaric acid; RU: rutin.

All quantities are expressed as attomole $cell^{-1}$, specified with the superscripts[a], except for the nanomolar concentration, specified with the superscripts[b] and footnotes in the table.

- I suggest also to the authors to give a look on 10.1080/07388551.2021.1874284, that might help for the discussion.

The aim of the study suggested by the reviewer is to provide an overview of current knowledge on phenolic compounds in microalgae. The article focuses on factors that influence the variation in total polyphenol and flavonoid content: microalgal biodiversity, chemodiversity between groups, different analytical methodologies, physiological state based on how the cells are maintained or cultured, e.g. light or other factors. The study cites two of our previously reported manuscripts (references 19 and 23):

- 19. Rico et al.: Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress. Limnol Oceanogr. 2013; 58: 144–152.
- 23. López et al.: Phenolic profile of *Dunaliella tertiolecta* growing under high levels of copper and iron. Environ Sci Pollut Res Int. 2015; 22: 14820–14828.

However, pH is the only variable modified in our study focused on the effect of acidification on *E. huxleyi* through CO_2 acidification, 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.

- legend: three measurements: three replicates? (different cultures? or technical replicates?)

- Five experimental batch cultures were carried out at each pH treatment. Three replicates refer to different cultures.

- lack of significativity tests in all the studies

The version 2.3 of jamovi program (2022) has been used for statistical analyses (retrieved from https://www.jamovi.org).

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

Anonymous Referee #2, 25 Apr 2024 (https://doi.org/10.5194/bg-2024-1-RC2)

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

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***

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 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})		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

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