ULPGC
Universidad de
Las Palmas de
Gran Canaria

Las Palmas de Gran Canaria, June 3, 2024

'Point-by-point response to reviewers' file

https://doi.org/10.5194/bg-2024-1-RC1

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

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

Anonymous Referee #1, 30 Mar 2024

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×A_{663}–0,0989×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 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.

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 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.
- **3.1 Carbonate chemistry parameters**

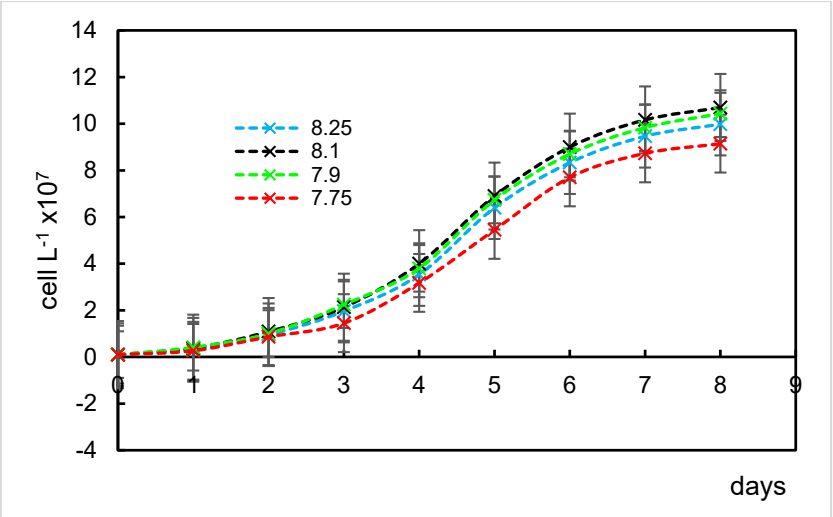
- the fig. 2 is emblematic: in the legend, "diatoms" are mentioned while results cam from Emiliania h.; dark vs light color is referred to what?; these data correspond to which day ?

The title of Figure 2 has been changed as follows:

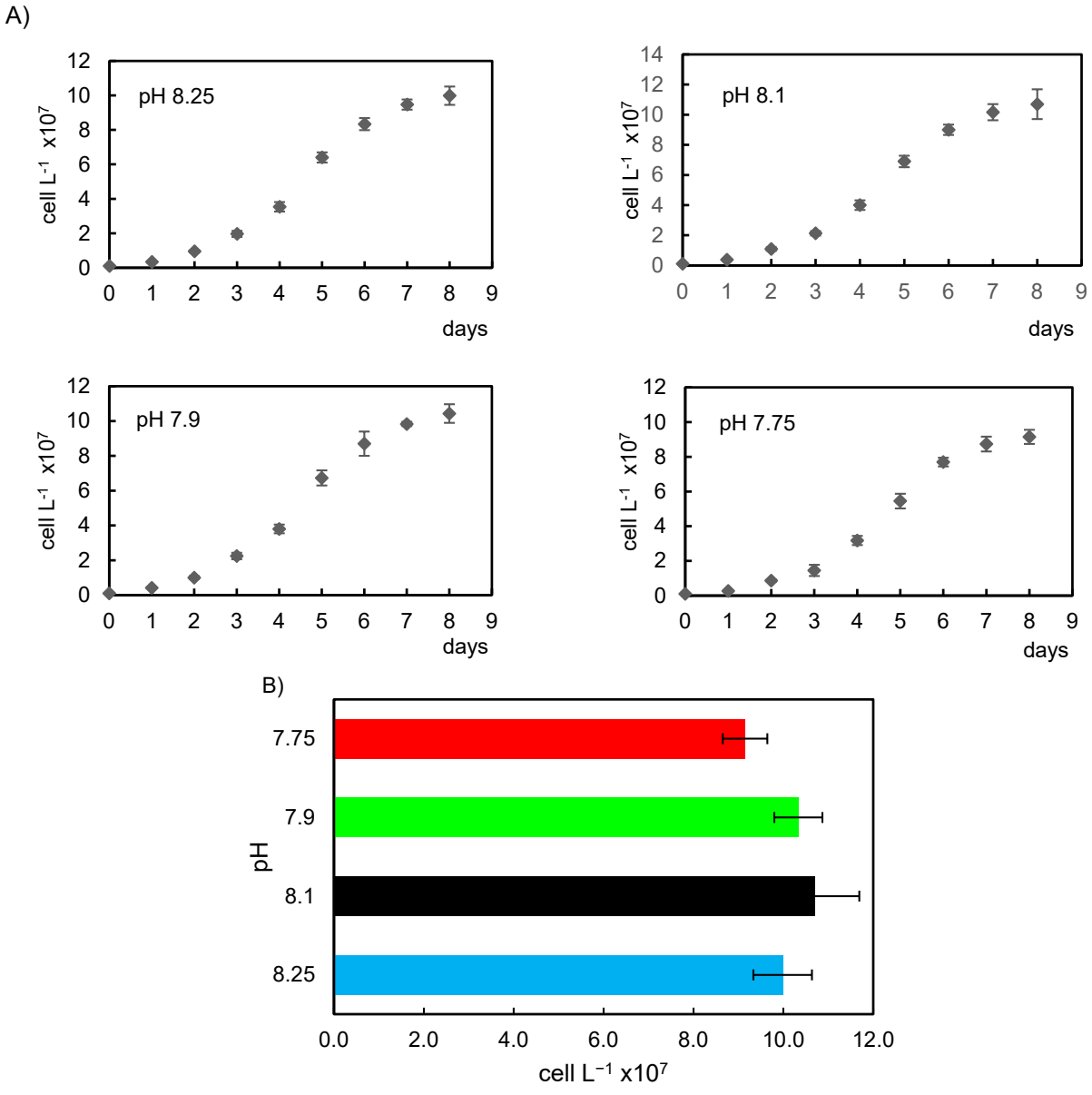
- Figure 2. Total sum of identified intracellular polyphenols expressed as attomole $cell^{-1}$ (light coloured bars) and concentration (nM) of exuded polyphenols (dark coloured bars) by *E. huxleyi* cells grown under reduced pH conditions after eight days of culture.

- 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, Figure 1 has been changed as presented below.



- 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, Chem. Geol. 511, 380-388, https://doi.org/10.1016/j.chemgeo.2018.10.017
    - López, et al. (2015): Phenolic profile of Dunaliella tertiolecta growing under high levels of copper and iron, Environ. Sci. Pollut. Res. 22 (19) 14820-14828, 10.1007/s11356-015-4717-y
    - 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:

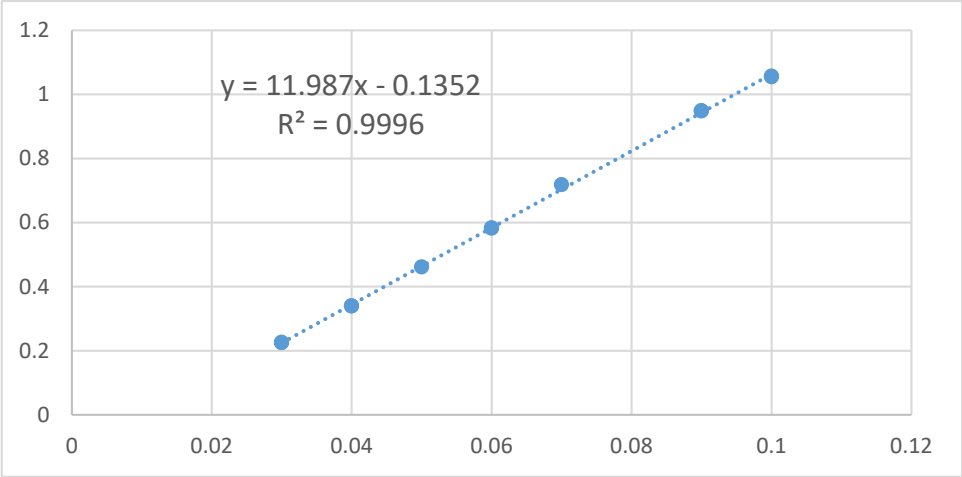
- Introduction (lines 100-102)
  Intra- and extracellular phenolic compounds (gallic acid (GAL), protocatechuic acid (PCA), p-coumaric acid (COU), ferulic acid (FA), catechin (CAT), vanillic acid (VAN), epicatechin (ECAT), syringic acid (SYR), rutin (RU) and gentisic acid (GA)) were identified and quantified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

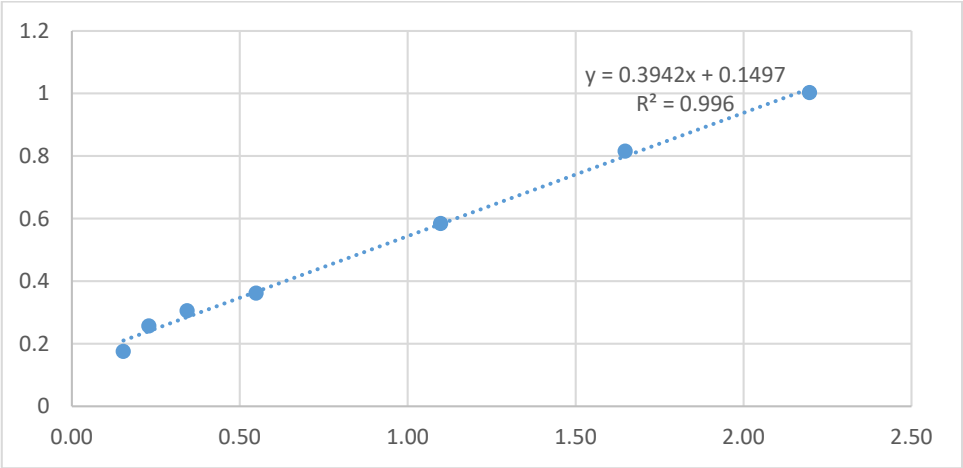
- calibration curve: please explain why there is a "b" factor (y = ax + b; for carbohydrates, frap) also especially when it is negative (dpph)?

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:



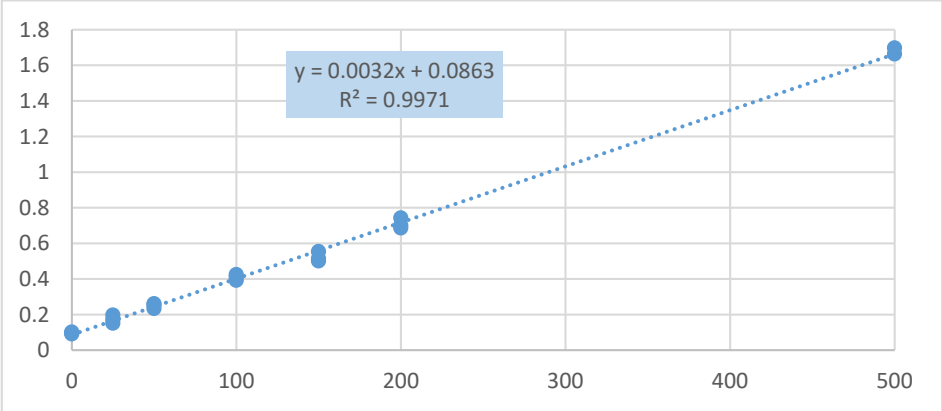
The absorbances of the algal samples correspond to the concentration of not inhibited DPPH. Therefore, subtracting the concentrations obtained through the calibration curve from the initial DPPH concentration allows us to calculate the amount of inhibited DPPH.

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 06*x*+0.1887. https://doi.org/10.1016/j.apjtm.2017.07.024.
- *Alam et al. 2014: y*=5.4901*x*+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 and placed at the end of section 3.2:

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. (Tong et al. 2017; Gafar et al. 2019). 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.

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

New sections and paragraphs included and/or changed in the manuscript:

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.

2.3 Cells and exudates extract preparation prior to HPLC, carbohydrates, and antioxidant activities quantifications

Cells cultured for 8 days in 700 mL of seawater were freeze-dried and suspended in 5 mL of methanol. The samples were sonicated for 10 min, stirred for 5 min, and centrifuged at 7000 rpm for 15 min. The supernatant was separated, and the residue was extracted with 15 mL of methanol for 30 min and centrifuged again. All supernatants were collected and evaporated to dryness, and the resulting residue was dissolved in 5 mL of methanol. These samples were used for phenolic compounds identification, DPPH inhibition and FRAP values determinations. For carbohydrates, freeze-dried cells were suspended in 5 mL of acidified water (pH=2), the samples were sonicated for 10 min, stirred for 5 min, and centrifuged at 7000 rpm for 15 min and the supernatant was separated.

Seawater samples (700 mL) enriched with exudates were previously subjected to solid phase extraction (SPE) at a flow rate of 2 mL min^{-1} using Macherey-Nagel Chromabond Easy cartridges (500 mg). The retained analytes were eluted with 12 mL of methanol, which was subsequently evaporated on a rotary evaporator. The residue was dissolved in 300 µl of methanol and filtered through a 0.22 µm filter to be injected into the HPLC equipment. These samples were used to quantify phenolic compounds and DPPH inhibition values.

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 DIC values ranged from 1905±26 to 2215±16 at a TA mean value of 2386±16 µmol kg^{-1} (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 research TA and DIC 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 (DIC) and estimated pCO_2 (µatm).

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

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