Variations of polyphenols and carbohydrates of *Emiliania huxleyi* grown under simulated ocean acidification conditions

Milagros Rico[1,2], Paula Santiago-Díaz[1,2], Guillermo Samperio-Ramos[3], Melchor González-Dávila[1,2], Juana Magdalena Santana-Casiano[1,2]

5 [1]Departmento de Química, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Spain.
[2]Instituto de Oceanografía y Cambio Global (IOCAG), Universidad de Las Palmas de Gran Canaria, Unidad Asociada ULPGC-CSIC, Las Palmas de Gran Canaria, Spain.
[3]Instituto de Investigaciones Oceanológicas. Universidad Autónoma de Baja California. Carretera Ensenada-Tijuana nº 3917,
10 Fracc. Playitas, Ensenada Baja California. C.P. 22860.

Correspondence to: Milagros Rico (milagros.ricosantos@ulpgc.es)

Abstract. Global environmental changes strongly affect the growth and biochemical composition of microalgae. 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 the adaptive mechanisms of these organisms through changes in phenolic compounds and

15 carbohydrate content and composition under ocean acidification scenarios. The highest algal biomass peaks, 1.07 (± 0.10) and 1.04 (± 0.06) × 10^8 cells L^{-1}, were observed in the microcosms with intermediate CO_2 levels (pH 8.10 and 7.90 respectively). Intra- and extracellular phenolic compounds were identified and quantified by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC). The highest concentrations of total exuded phenolics were found in cultures with lower cell densities, at pH 8.25 (43±3 nM) and 7.75 (18.0±0.9 nM). Accumulation of intracellular phenolic compounds was observed in

20 cells with decreasing pH, reaching the maximum level (9.24±0.19 attomole $cell^{-1}$) at the lowest pH (7.75). The total carbohydrate content inside the cells increased with decreasing pH from 8.25 to 8.10, remaining constant at pH 7.90, and decreasing at lower pH. The presence of antioxidants was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition and ferric-reducing antioxidant power (FRAP) assays. The highest activity in both tests was exhibited by cells grown at pH 7.75.

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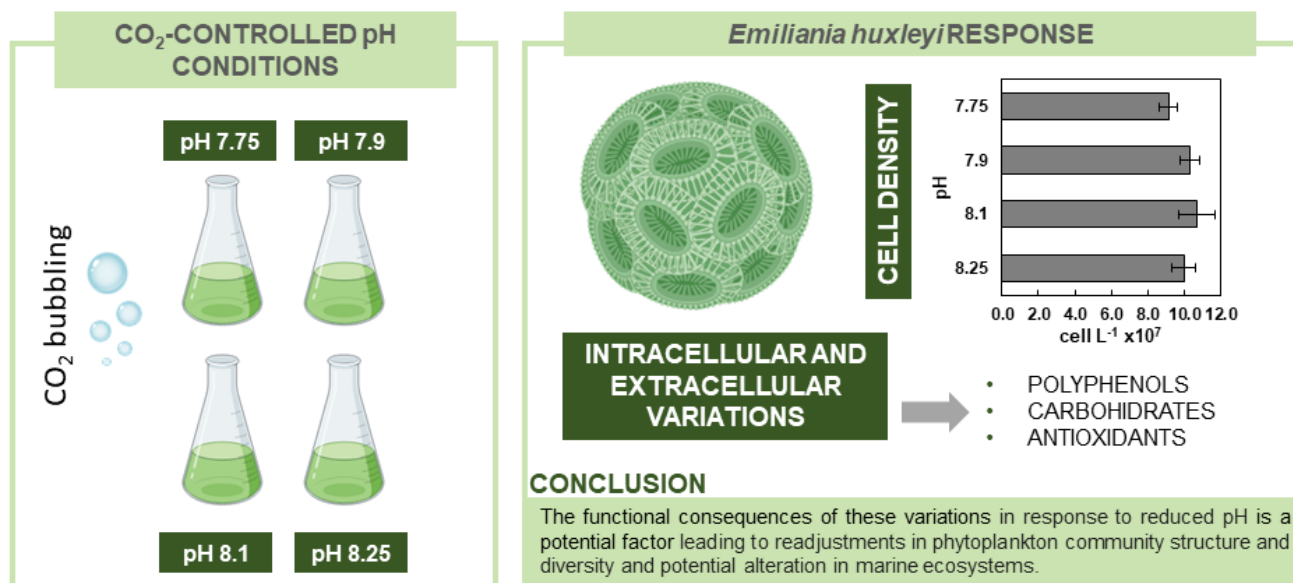
Graphical abstract

1 Introduction

30 Microalgae play a key role in marine ecosystems, forming the basis of the marine food chain as they are responsible for almost half of the total primary production (Usher et al., 2014; Dedman et al., 2023). They constitute a potential feedstock because of their valuable metabolites such as pigments, vitamins, proteins, carbohydrates, and lipids with valued fatty acids. Marine phytoplankton, primarily diatoms and coccolithophorids groups, drive the oceanic carbon cycle by sequestering inorganic carbon from the atmosphere during photosynthesis (Marinov et al., 2010). Coccolithophores are the most abundant calcifying

35 phytoplankton and form gigantic blooms throughout the oceans, especially in mid-latitudes. The coccolithophore *Emiliania huxleyi* (*E. huxleyi*) is the main contributor to calcareous sea sediments, making it particularly sensitive to ocean acidification and playing a crucial ecological role (Arundhathy et al., 2021; Westbroek, 1992). When exposed to elevated CO_2 and low pH, it reduces its growth rate and level of calcification, resulting in thinner coccosphaeres (Kholssi et al., 2023; Mackey et al., 2015; Meier et al., 2014).

40 Global environmental changes, in particular those related to increasing temperature and decreasing pH, profoundly affect ocean ecosystems at many levels, as these are the two main variables controlling all chemical and biological cycles, with a major

impact on the growth and metabolic functions of microalgae (Berge et al., 2010; Dedman et al., 2023; Kholssi et al., 2023; Lu et al., 2013). The absorption of anthropogenic CO_2 into seawater lowers its pH with adverse consequences for marine ecosystems and human societies (Gruber et al., 2023; Jiang et al., 2023; Lida et al., 2021). In fact, pre-industrial seawater pH

45 (8.25) has already dropped to 8.10, and is expected to reach a pH of 7.85 in this century (Jacobson, 2005). For instance, pH homeostasis, which regulates the pH inside and outside the cell, is critical for the growth and metabolism of most microorganisms, including microalgae (Barakat et al., 2021; Guan and Liu, 2020; Lund et al., 2020). Different algal species show different optimal pH ranges for maximum growth (Hoppe et al. 2011; Kholssi et al., 2023). Changes in environmental pH could have consequences on the competitiveness of both sensitive and tolerant microalgae in mixed phytoplankton

50 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). Such changes could also affect the species at a higher trophic level, resulting in a potential shift in biodiversity (Jin and Kirk, 2018; Vasconcelos et al., 2002). 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

55 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

60 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.

Changes in phytoplankton communities due to variation in seawater acidity alter the composition of the organic ligands that

65 these communities release into the surrounding environment (Samperio-Ramos et al., 2017). These ligands are crucial 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 (Harmesa et al., 2022; Santana-Casiano et al., 2014). Iron is an essential micronutrient for phytoplankton involved in fundamental cellular processes, including respiration, photosynthesis, nitrogen uptake, and nitrogen fixation (Raven et al., 1999; Hogle et al., 2014). It controls the

70 productivity, species composition, and trophic structure of microbial communities over large regions of the ocean (González et al., 2019; Hunter and Boyd, 2007). Iron concentrations in ocean waters are very low due to its low solubility and effective removal from the ocean surface by phytoplankton (Liu and Millero, 2002). Complexation with organic compounds is one of the mechanisms for maintaining dissolved iron concentrations above its inorganic solubility, while potentially reducing the concentrations of soluble and bioavailable inorganic species (Hunter and Boyd, 2007; Shaked and Lis, 2012). A decrease in

75 seawater pH from 8.1 to 7.4 will increase Fe(III) solubility by approximately 40%, which could have a large impact on

biogeochemical cycles (Morel and Price, 2003; Millero et al., 2009). Organic matter exuded by marine microorganisms can form Fe(III) complexes that modify Fe(II) oxidation rates and promote the reduction of Fe(III) to Fe(II) in seawater. Under acidifying conditions, some research work has shown that the residence time of the reduced form of essential trace metals increases as their oxidation rate decreases (Pérez-Almeida et al., 2022; Santana-Casiano et al., 2014).

80 Carbohydrates are one of the major components of the dissolved organic carbon (DOC) pool in marine environments, representing 3-50% of dissolved and colloidal organic matter (Giljan et al., 2023; Hassler et al. 2011; Wang et al., 2006). Phenolic compounds are secondary metabolites synthesized as a defense mechanism of organisms exposed to abiotic stresses (salinity, metal toxicity, heat, acidification, cold, extreme light, nutrient deficiency, UV radiation) (López et al. 2015; Parvin et al., 2022). Their antioxidant nature enhances tolerance of organisms to adverse climatic conditions that induce an increase

85 in reactive oxygen species (ROS) (Gauthier et al. 2020; Sachdev et al. 2021; Vázquez et al., 2022). Both types of compounds can influence the iron chemistry and bioavailability (Perez-Almeida et al., 2022; Santana-Casiano et al., 2014; Santschi et al., 2003). Catechin, sinapic acid and gallic acid were found to be weak Fe-binding ligands that increased the persistence of dissolved Fe, regenerating Fe(II) in seawater from 0.05% to 11.92% (González et al. 2019). Hassler et al. (2011) reported that the addition of glucuronic acid to natural planktonic assemblages increased iron bioavailability for eukaryotic phytoplankton.

90 Furthermore, iron bioavailability also increased when three different saccharides were used in cultured and natural eukaryotic phytoplankton populations, suggesting that this is a generalizable phenomenon.

The effect of pH changes on the speciation of metal-organic complexes and on the redox kinetics of metals in the marine environment is not as well characterized as that of inorganic ligands due to the heterogeneous composition of dissolved organic matter and their unknown structure. Therefore, studying the nature of these organic ligands will allow a comprehensive

95 understanding of the consequences of acidification on ocean biogeochemical processes.

This work aimed to determine how marine acidification may affect the composition of cells and exudates from *Emiliania huxleyi*. Therefore, cultures of *E. huxleyi* were grown at four different pH (7.75, 7.90, 8.10, and 8.25) reached by bubbling CO_2 in the culture seawater (Samperio-Ramos et al., 2017). The four experimental scenarios represent interglacial, close to contemporary, and two future ocean acidification conditions based on the Intergovernmental Panel on Climate Change

100 projections (IPCC, 2014). 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. The total carbohydrate content of cells was quantified using the phenol/ sulfuric acid (PSA) assay (Dubois et al., 1956). The presence of antioxidants was assessed by the antioxidant activity of cells and exudates determined by DPPH radical scavenging activity test (RSA) and FRAP assay (Sethi

105 et al., 2020).

Biogeosciences
Discussions

2 Materials and methods

2.1 Chemicals

Methanol (HPLC gradient grade) was purchased from Scharlab (Barcelona, Spain). Formic acid (synthesis grade), $Fe_3Cl \cdot 6H_2O$, and $FeSO_4 \cdot 7H_2O$ were supplied by Panreac (Barcelona, Spain), D-Glucose (Glc), phenol, DPPH and 2,4,6-tri(2-pyridyl)-triazine (TPTZ) by Sigma-Aldrich (St. Louis, MO, USA). 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. Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

2.2 Cultures

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} 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 with an equipment that modulates the CO_2 flow once the desired pH is reached, keeping it constant (±0.02). To maintain suspended cells and homogeneity of CO_2 in solution, the cultures were shaken at 60 rpm with a Teflon-coated magnetic stirrer. All materials were cleaned following a standard protocol (Achterberg et al., 2001) and subsequently autoclaved at 121 °C for 30 min prior to use. Once the seawater reached the desired pCO2/pH value, coccolithophorides were inoculated into the batch cultures. Stock cultures were maintained in the experimental conditions for 48 h before starting each experiment, which allowed acclimation to the CO_2 concentration. Five experimental batch cultures were carried out at each pH treatment. Seawater enriched with exudates was filtered using acid-cleaned and combusted polycarbonate (Nucleopore) syringe-filters (0.45 µm) to prevent cell breakage. A more detailed description is given by Samperio-Ramos et al. (2017).

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 acetone. The samples were sonicated for 2 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 acetone 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 and carbohydrates quantifications and DPPH inhibition and FRAP values determinations.

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.

2.4 Phenolic profile of *E. huxleyi* cells and exudates

Methanol extract of cells (2 mL) prepared as described in section 2.3 was evaporated, the residue was dissolved in methanol 140 (200 µL) and filtered with a syringe filter (0.2 µm). Chromatographic analysis of cells and exudates was made according to Santiago-Díaz et al. (2023), with a Jasco LC-4000 HPLC equipment provided with a PU-4180 quaternary pump, an AS-4150 autosampler, an MD-4015 photodiode array detector, LC-Net ll interface, a Phenomenex C18 column (250 mm x 4.6 mm, 5 µm) and a Phenomenex guard column maintained at 30ºC. The elution was performed with water containing 0.1% formic acid (phase A) and methanol (phase B), with a flow rate of 1 mL min^{-1} and injection volume of 10 µL. The gradient elution method 145 for A was 0 min, 75%; 30 min, 40%; 40 min, 40%; and finally, the column was washed and reconditioned. For quantification, simultaneous monitoring was set at 270 nm (GAL, PCA, CAT, VAN, RU, ECAT, and SYR) and 324 nm (GA, COU, and FA). Samples were analysed by triplicate, and the results were expressed as attomole cell^{-1} (amol cell^{-1}).

2.5 Determination of carbohydrate content

The total carbohydrates contents in *E. huxleyi* cells were determined following the phenol sulfuric acid method (Dubois et al., 150 1956) with some modifications. The cells extract (1.5 mL) was concentrated, and the residue was dissolved in 0.8 mL of methanol. The reaction was carried out by mixing 80 µL of sample or standard with 150 µL of phenol (5%) and 1 mL of concentrated sulfuric acid. The resulting solution was heated at 100ºC for 5 min, and the absorbance was recorded at 490 nm in a UV-VIS spectrophotometer. The results were expressed as femtomole of glucose equivalent per cell (fmol Glc eq cell^{-1}) calculated from the calibration curve of 0.14 to 2.78 mM glucose, $y=0.0032x + 0.0863$; $R^2=0.9971$.

155 2.6 Antioxidant activities of algae and exudates

The antioxidant properties of the cell extracts were evaluated according to Sethi et al. (2020) with modifications. The ability to reduce complex TPTZ-Fe(III) to TPTZ-Fe(II) by donating an electron was measured as follows: The FRAP reagent was prepared with acetate buffer solution 0.3 M (pH 3.6), 10 mM of TPTZ in HCl (40 mM), and 2.5 mL of FeCl$_3$·6H$_2$O solution (20 mM) (in the ratio of 10:1:1). Methanol extracts (50 µL) were mixed with 1 mL of freshly prepared and pre-warmed FRAP 160 reagent (37ºC) for 13 min. The mixture was introduced in an ice bath, and the absorbance was measured at 593 nm. Results were expressed as fmol of reduced Fe(III) per cell and calculated from a calibration curve constructed with FeSO$_4$·7H$_2$O concentrations ranging from 0.15 to 2.20 mM (regression line equation $y = 0.3942x + 0.1497$, $R^2 =0.996$). The estimation was carried out in triplicate, and the results were averaged.

The capacity of cell extracts and exuded compounds of inhibiting DPPH radical was determined by mixing 0.8 mL of DPPH 165 solution (0.053 mM) with 30 µL of cell extracts, and with 50 µL for each sample of exuded compounds. The absorbance was recorded after 20 min at 515 nm using a UV-visible spectrophotometer (Shimadzu Pharmaspec 1800). The total amount of

inhibited DPPH radical per cell was calculated from a calibration curve of 0.03 to 0.1 mM DPPH, y = 11.987x - 0.1352; R^2=0.9996.

3 Results and discussion

3.1 Cell growth

The acidification conditions did not significantly affect the final cell densities, which increased during 8 days from an initial value of 10^6 cells L^{-1} to 9.98 ($\pm$ 0.53) $\times$ 10^7 (pH 8.25), 1.07 ($\pm$ 0.13) $\times$ 10^8 (pH 8.10), 1.04 ($\pm$ 0.07) $\times$ 10^8 (pH 7.90) and 9.15 ($\pm$ 0.41) $\times$ 10^7 (pH 7.75) (Fig. 1). The highest peaks of algae biomass, 1.07 ($\pm$ 0.10) and 1.04 ($\pm$ 0.07) $\times$ 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) (Samperio-Ramos et al., 2017). After 8 days, growth rates remained fairly constant in all experimental acidification conditions, indicating that the stationary phase had been reached (Fig.1). Heidenreich et al. (2019) also concluded that growth rates of both haploid and diploid *E. huxleyi* cells were unaffected by ocean acidification (culture medium adjusted to pCO_2 400 vs. 1000 µatm). Fukuda et al. (2014) reported similar results for *E. huxleyi* harvested in CO_2-enriched seawater, whose growth was unaffected. However, the authors observed different responses depending on the acidification method, i.e. *E. huxleyi* showed severe growth damage in experiments carried out by acidification by HCl, which also changes the seawater alkalinity that does not happen when bubbling CO_2 is used, concluding that coccolithophore *E. huxleyi* has the ability to respond positively to CO_2 acidification. In contrast to these results, Vázquez et al. (2022) found that CO_2 enrichment aeration up to lower pH than those here (1200 µatm, pH 7.62) induced growth rates of the coccolithophore more negatively affected concerning the control (400 µatm, pH 8.10) than low pH reached without CO_2 enrichment.

Discrepancies in experimental results may be due to different environmental factors, culture, light conditions, etc. (Tong et al. 2017; Gafar et al. 2019). Langer et al. (2009) observed different responses to acidification among four strains of *E. huxleyi* in all parameters tested, and no strain showed a previously described response pattern for this species, i.e. growth rate exhibited a marked increase in one strain and slight changes in the remaining strains, decreasing in two of them and increasing in the other.
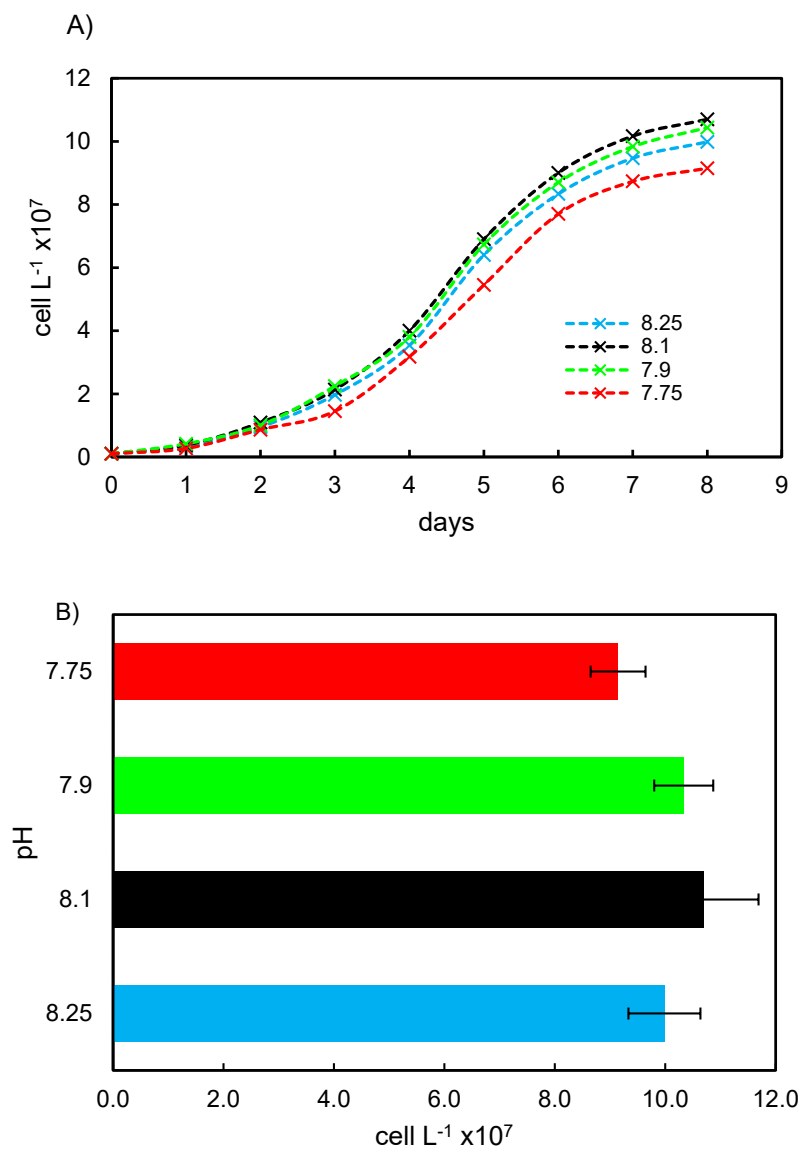
A)

B)

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

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3.2 Phenolic contents of cells and exudates

The phenolic profile of cell extracts and seawater samples enriched with *E. huxleyi* exudates are summarized in Table 1. GAL, PA, ECAT, and RU were detected in both cells and exudates, whereas VAN and COU were only detected in cells. The other

polyphenols were below the limit of quantification (LOQ). At all pH conditions, PA, ECAT, and RU were detected in the
200   seawater enriched with exudates and VAN in cells.

Table 1. Phenolic compounds in cells and exudates of *E. huxleyi* under different pH conditions.

Phenolic compound[a]	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	0.13±0.03	-	-	-	0.25±0.07	-	-	45±0
PCA	-	74±9	0.16±0.01	34±2	-	26.3±0.2	-	298±22
ECAT	1.2±0.1	34±1	-	40±5	-	70±2	-	77±6
VAN	6.44±0.06	-	2.5±0.1	-	2.30±0.05	-	2.5±0.2	-
COU	-	-	-	-	1.4±0.2	-	-	-
RU	1.47±0	12.1±0.4		20±2		13.2±0.3		11.2±0.8
Sum	9.24±0.19	120.1±10.4	2.66±0.11	94±9	3.95±0.32	109.5±2.5	2.5±0.2	431.2±28.8
Levels[b] (nM)	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.

The highest concentrations of total exuded polyphenols were found in cultures at pH 8.25 (43±3 nM) and 7.75 (18.0±0.9 nM),
205   where cell densities were lower (9.98×10^7 and 9.15×10^7 respectively). Under these pH conditions, each cell exuded a higher
amount of phenolic compounds than those exuded under pH 7.90 and 8.10, where a greater number of cells (1.04×10^8 and
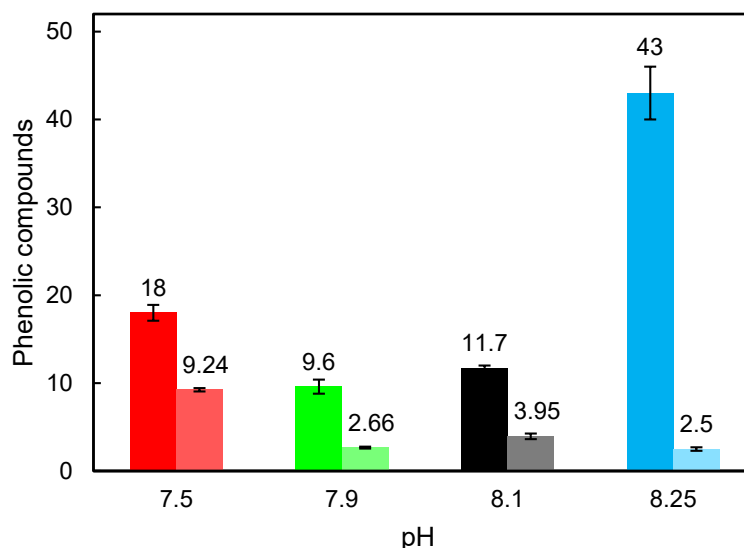1.07×10^8 respectively) exuded less polyphenols (Fig. 2).

Figure 2. Total sum of the identified intracellular polyphenols expressed as attomole cell^{-1} (in light color) and concentration
210 (nM) of exuded polyphenols (in dark color) by diatoms grown under reduced pH conditions.

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
215 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. Polyphenol accumulation has
also been observed in terrestrial plants (Bai et al., 2019; Kaur et al., 2022; Lwalaba et al., 2020). However, Arnold et al. (2012)
reported a loss of phenolics in the seagrasses *Cymodocea nodosa, Ruppia maritima*, and *Potamogeton perfoliatus* grown in
220 acidified seawater, where the pH decreased up to 7.3, and the CO_2 level increased ten-fold.
Vázquez et al. (2022) showed that CO_2 enrichment aeration (1200 µatm, pH 7.62) induced metabolic stress and accumulation
of reactive oxygen species (ROS) in the *E. huxleyi* strain used in their experiments. This could explain the accumulation of
phenolic compounds at pH 7.75 (9.24±0.19 amol cell^{-1}), considered to play a significant role as a defense mechanism due to
their antioxidant and ROS scavenging properties (Salam et al., 2023).
225 No correlation was found between phenolic compounds inside and outside the cell. The extracellular phenolic compound's
behavior was opposite to that described above for intracellular. The exuded compounds decreased from 43 ± 3 nM at pH 8.25
to 9.6±0.8 nM at pH 7.90 (Table 1). These results partially agree with those reported by López et al. (2015) for *Dunaliella
tertiolecta* growing under 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

230   concerning the control into the cells grown under the highest Cu level. However, at the lowest pH of 7.75, the level of phenolic
        compounds was enhanced to 18.0±0.9 nM, higher than that at pH 7.90 (9.6 ± 0.8 nM) but lower than the initial value at pH
        8.25 (43 ±3). At this pH, the intracellular content experienced the maximum increase with respect to the initial one (3.7 times
        higher). This increase may be explained by their potential to scavenge harmful ROS, whose enhanced production has been
        linked to the pH decrease by Vázquez et al. (2022). The phenolic profile differences found inside and outside the cells could

235   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.

        At pH 7.90, an exception was found in this study. The content of intracellular phenolic compounds decreased compared to that

240   found at pH 8.10, but increases with respect to the initial pH 8.25 (1.06 times higher). The decreases in pH in this study were
        0.15, 0.35 and 0.50 units with respect to the initial pH 8.25 (pH 8.1, 7.90 and 7.75, respectively). Suffrian et al. (2011) reported
        measurements of intracellular pH in *E: huxleyi* showing the presence of mechanisms that counteract cell acidification despite
        elevated membrane proton permeability and that an external pH shift of 1 was required to acidify the cell by 0.56 pH units.
        The impact of these changes does not necessarily have to be proportional to the pH change, taking into account the wide range

245   of biological processes dependent on the acid-base properties of the molecules involved, and the different physiological state
        of cells grown under different conditions, as well as the impact of these changes on the structure, and functions of organelles,
        as well as on the overall organization of the cell (Antosiewicz and Kane, 2022).

        Polyphenols modify Fe(II) oxidation rates by promoting the reduction of Fe(III) to Fe(II) in seawater. The effect of the
        polyphenol gallic acid on Fe oxidation and reduction was studied by Pérez-Almeida et al. (2022), concluding that gallic acid

250   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 gallic acid, while only 37.5% was oxidized with 100 nM gallic acid and after 30 min. The reduction of
        Fe(III) to Fe(II) by gallic acid 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

255   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 gallic acid and the remaining phenolic compounds in the extracellular medium,
        and the increase of their presence inside the cell.

3.3 Total carbohydrate content of cells

        Total carbohydrates in cells and exudates of *E. huxleyi* are shown in Table 2. No correlation was found between intra- and

260   extracellular contents. The amount of total intracellular carbohydrates remained almost constant between pH 8.25 and pH 7.9,
        and decreased slightly at pH 7.75. Similar results were observed for exuded carbohydrates, which did not undergo any change
        as the pH dropped to pH 7.75. Our results agree with those of Araujo and Tabano (2005), who reported that CO_2 addition to

the culture seawater lowered the carbohydrates content of marine diatom *Chaetoceros* cf. *wighamii.* Thornton (2009) reported that the planktonic diatom *Chaetoceros muelleri* grown at pH 6.8, 7.4, 7.9, and 8.2 showed a decreased proportion of total

265   carbohydrate within the cells and increased levels of dissolved exuded carbohydrates into the surrounding medium with the decrease in pH. Thornton used a different method to maintain pH, a biological buffer (25 mM HEPES (Sigma-Aldrich, St. Louis, Mo, USA)) and daily titration with the addition of a small amount of HCl, which changes the seawater alkalinity. Fukuda et al. (2014) observed increased production and storage of polysaccharides by the coccolithophore *E. huxleyi* stimulated by acidification with CO_2 enrichment. Jones et al. (2013) compared the response of the coccolithophore species *E.*

270   *huxleyi* cultured at two pH conditions reached by bubbling CO_2 (pH 7.94 and 7.47 at the time of the harvesting), when as little as 5% of dissolved inorganic carbon (DIC) was consumed, indicating low chemical shift throughout the experiments. The higher CO_2 level evidenced cellular responses to stress such as decreased growth rates, but proteins associated with many key metabolic processes remained unaltered, thus maintaining many biological functions. Diverse metabolic responses of the coccolithophore *E. huxleyi* to ocean acidification and to combinations of ocean acidification with other environmental factors

275   have been described (Tong et al. 2017; Gafar et al. 2019). Xie et al. (2021) studied the effects of high and low DIC concentration (from 900 to 4,930 µmolkg[-1]) and reduced pH value (from 8.04 to 7.70) on physiological rhythms, element contents and macromolecules of the coccolithophore *Emiliania huxleyi*, concluding that its response is highly dependent on the DIC. Compared to high pH conditions, low pH and DIC concentration led to increases in particulate organic carbon (POC) and particulate organic nitrogen (PON) contents with less impact on protein and carbohydrate contents; however, high DIC

280   and low pH reduced POC, PON, protein, and carbohydrate contents. 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

285   composition of the phytoplankton community.

Table 2. Intracellular and extracellular carbohydrates of *Emiliania huxleyi* harvested under different pH conditions

pH	Intracellular carbohydrates[a]	Extracellular carbohydrates[b]
8.25 (pCO$_2$ 225µat)	27.0 ± 2.2	59.24 ± 3.97
8.10 (pCO$_2$ 350µat)	30.4 ± 3.2	60.99 ± 6.57
7.90 (pCO$_2$ 600µat)	30.3 ± 3.7	63.19 ± 9.81
7.75 (pCO$_2$ 900µat)	23.8 ± 1.9	62.60 ± 5.23

[a]The results are expressed as femtomole glucose equivalent cell[-1]

[b]Extracellular released carbohydrates (femtomole of glucose equivalent cell[-1]).

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The results are expressed as means ± standard deviation of three measurements.

Similar effects to those observed here have been found in the response of macroalgae to elevating ocean acidification. Gao et
290 al. (2017) reported that pCO_2 does not affect the carbohydrate content of algae *Ulva rigida* investigated under pH 7.95 and
7.55. Barakat et al. (2021) studied the effect of the acid stress on the green alga *Ulva fasciata* subjected to four levels of pCO_2,
280, 550, 750, and 1050 µatm (pH values 7.2, 7.6, 7.86, 8.1 (control)), and found similar production of carbohydrates at the
three lowest pH (46.13, 46.96 and 46.04% of dry weight respectively), while the control (pH 8.1) showed 42.37 % of dry
weight.

295 **3.4 Antioxidant activities**

Table 3 shows the antioxidant activities of extracts of cells *E. huxleyi* grown in seawater enriched with several levels of CO_2
and those of the compounds exuded by these cells. Cells with the highest phenolic content (pH 7.75) gave the most increased
scavenging activity (8.1±0.1 fmol cell[-1]) and iron-reducing capacity (10.2±0.5 fmol cell[-1]), indicating that these cells grown in
the most acidified media produce relevant amounts of antioxidants. At higher pH, the ability to reduce iron remains almost
300 constant. At the same time, the free RSA increases as pH decreases, following the same tendency as the content of analysed
polyphenols. However, the exudates do not exhibit the same tendency. As the pH decreases, the total content of exuded
polyphenols also decreases and the exuded compounds' RSA increases up to pH 7.90, stating that polyphenols are not
responsible for this activity. By lowering from pH 7.90 to pH 7.75, polyphenols increase and DPPH inhibition decreases.
Significant correlation was found between FRAP and total intracellular phenolic content (r=0.928, *p*<0.05) suggesting that the
305 reducing power in cells is associated with these compounds, which reduce Fe(III) to Fe(II). This could also corroborate that
the polyphenols exuded at lower pH decrease as their reducing power is no longer necessary because under these conditions
the persistence of essential metals such as Fe(II) increases, and their concentration and reducing power inside the cell increases
to compensate for oxidative stress. The FRAP test is based on the transfer of an electron, whereas the DPPH test includes the
transfer of both a hydrogen atom and an electron. These different chemical reactions mechanisms and their kinetics could
310 explain the differences between FRAP and DPPH results and may be the reason for the lack of correlation between the phenolic
content and RSA.

Table 3. Antioxidant activities of exudates and extracts of *E. huxleyi* cells grown in seawater enriched with CO_2.

pH	FRAP[a]	RSA	
		Cells[b]	Exudates[c]
8.25 (pCO₂ 225µat)	9.5±0.6	3.4±0.2	26±1
8.10 (pCO₂ 350µat)	9.3±0.3	2.3±0.3	37±2
7.90 (pCO₂ 600µat)	9.3±0.7	6.0±0.2	40±2

7.75 (pCO$_2$ 900µat)	10.2±0.5	8.1±0.1	26.1±0.4

[a]Results are expressed as fmol of reduced Fe(III) per cell.

[b]The results are expressed as fmol of inhibited DPPH per cell

[c]The results are expressed as nmol of inhibited DPPH L^{-1}

All results are expressed as means ± standard deviation of three measurements

Abbreviations: FRAP: ferric-reducing antioxidant power; RSA: radical scavenging activity

315 The results found in the literature on the effect of acid stress on the biochemical composition of microorganisms and their exudates are apparently contradictory. However, a large number of factors (initial cell density, grown phase, culture media composition, light photoperiod and light intensity applied during culture, pH, among others) strongly influence the toxicity of pollutants in algal bioassay stimulating and/or inhibiting the production of different metabolites through the regulation of metabolic pathways (Barcelos e Ramos et al., 2010; Santiago-Díaz et al., 2023; Singh and Shrivastava, 2016; Tandon et al.,

320 2017; Zhang et al., 2019). In addition, the antioxidant activity of cell extracts and exudates depends on their complex mixture of compounds and the interactions between them, as well as the assay mechanism/kinetic (Šamec et al., 2021; Sethi et al., 2020). We have detected different carbohydrate contents that showed significant negative correlation with the FRAP values, which may indicate that these compounds are not responsible for this reducing activity. In addition, we only identified a small sample of phenolics.

325 On the other hand, the pH range for optimal growth varies depending on the microalgae species, and even the sensitivity of *E. huxleyi* to acidification appears to be strain-specific (Langer et al., 2009). Borchard and Engel (2015) reported that *E. huxleyi* ability to acclimate to different CO$_2$ concentrations during the stationary phase of growth was responsible for the absence of a CO$_2$ effect found on primary production and exudation in their study.

The responses of marine organisms to ocean acidification are very complex and appear to depend on many factors (Grosse et

330 al., 2020). Changes in seawater pH influence the protonation of biological molecules and could modify their charge and negatively affect metabolic processes. Several mechanisms have been described to maintain the pH inside cells, such as changing the lipid composition of the cytoplasmic membrane or increasing the production of cyclopropane fatty acids to reduce proton permeability (Lund et al., 2020). Ocean acidification will directly affect marine organisms, altering the structure and functions of marine ecosystems. The accumulation of phenolic compounds leads to functional consequences in primary and

335 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).

Conclusion

Global environmental change influences the growth and metabolic functions of microalgae affecting their communities' structure and compositions depending on the sensitivity of different taxonomic groups, with implications for higher trophic

340   levels and biodiversity loss. Limited research has been focused on the ecological consequences of joint action of seawater pH decrease, warming, and changes in salinity, among others. In this study, the phenolic profiles of *E. huxleyi* cells grown under four marine acidification scenarios, as well as their exudates, have been determined for the first time. Different phenolic profiles of cells and exudates were found. Intra- and extracellular carbohydrate levels also showed slight modifications with a pH decrease. These changes in metabolites increased the intracellular reducing ability (FRAP) and the capacity to inhibit

345   radicals, whose accumulation is associated with enhanced oxidative stress. The functional consequences of these variations observed here in response to reduced pH are a potential factor leading to readjustments in phytoplankton community structure and diversity and possible alteration in marine ecosystems.

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