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RESEARCH

Enhancing *Gephyrocapsa huxleyi* growth with recycled calcium from oyster shells

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Abstract

Coccolitophores, such as *Gephyrocapsa huxleyi*, can have multiple interesting biotechnological applications thanks to their ability to produce complex structures made of $CaCO_3$, called coccolith, and a biomass rich in valuable molecules. Unfortunately, they usually reach low cellular density in standard conditions, hindering actual industrial applications. To enhance growth, $CaCO_3$ may be used instead of standard $CaCl_2$. Here, shell-derived $CaCO_3$ was also tested to valorise oyster shells, which are not recyclable and can cause environmental harm if not disposed of correctly. *G. huxleyi* cultures were grown in f/2 medium prepared using standard artificial seawater, artificial seawater with pure $CaCO_3$ in place of $CaCl_2$, and artificial seawater with the pulverised shells of *Magallana gigas* (Pacific oyster) instead of $CaCl_2$. Cultures were monitored for 14 days, and final dry biomass concentration, photosynthetic pigment and total protein concentrations were evaluated. Moreover, coccolith morphology was checked through scanning electron microscopy. Replacing $CaCl_2$ with pure $CaCO_3$ increased cell density roughly sixfold relative to the control; using pulverised oyster shells yielded the greatest effect, with cell densities about 26 times higher than $CaCl_2$. Cells grown in the medium added with pulverised oyster shells were smaller, exhibited higher pigment content per dry weight and a protein content comparable with the control. The bulk composition of the oyster shell revealed the presence of trace elements, like Mn, Fe and Cu, that could have enhanced the algal growth. Results indicate pulverised oyster shells can be a sustainable and effective calcium source to valorise waste and enhance *G. huxleyi* biomass production.

Keywords Microalgae · *Emiliania huxleyi* · *Gephyrocapsa huxleyi* · *Magallana gigas* · Coccolith · Green economy

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Introduction

Among microalgae, coccolithophores (Prymnesiophyceae) are well-known to generate large oceanic blooms visible even from space, with major implications for the oceanic carbon cycle (Claxton et al. 2022; H. Zhang et al. 2025). Their contribution is mainly linked to their unique ability to produce coccoliths, i.e. scales made of $CaCO_3$ with species-specific shape (Paasche 2001; Vuković and Godrijan 2025; Wheeler et al. 2023). Thanks to their structure, coccoliths could have interesting biotechnological applications, such as for nanomaterial production, for biomaterials production or as enzyme carriers (Al-Mardeai et al. 2024; Jakob et al. 2017; Lo Presti et al. 2021; Lomora et al. 2021; Moreira et al. 2023; Skeffington and Scheffel 2018). In addition to coccoliths production, coccolithophores also produce other interesting and valuable molecules such as carotenoids, including fucoxanthin, polyunsaturated fatty acids and vitamins (Eliason et al. 2024; Lenning et al. 2004; Pond & Harris 1996; Sun et al. 2024). These characteristics make these algae ideal candidates for sustainable applications in multiple sectors, from aquaculture to pharmaceutical production (Araújo et al. 2022; Lo Presti et al. 2021; Vicente et al. 2021; Wang et al. 2021). Moreover, some of them can also produce long-chain ketones called alkenones, with promising applications in the cosmetic industry (Huynh et al. 2019, 2020; McIntosh et al. 2018). Lastly, the biomass of coccolithophores, including the coccoliths, could be used as animal feed, considering that other sources of calcium carbonate are currently studied and used for the preparation of calcium-fortified foods (Aditya et al. 2021; Ahn et al. 2025; Bhagat et al. 2024). For these reasons, and considering the ability of photosynthetic organisms to capture CO_2 through photosynthesis, coccolithophores could have a crucial role in the green economy (Sadvakasova et al. 2023; Sarker and Kaparaju 2024).

Unfortunately, coccolithophores usually reach low cellular density and biomass concentrations when cultivated in standard conditions, making it difficult to harvest all these valuable molecules and hindering interesting applications (Jakob et al. 2018; Rico et al. 2024; Vicente et al. 2021; J. Zhang et al. 2023). Interestingly, by changing only the calcium substrate from $CaCl_2$ to $CaCO_3$, it should be possible to increase cells production in *Gephyrocapsa huxleyi* (Lohmann) P.Reinhardt (formerly known as *Emiliania huxleyi* (Lohmann) W.W.Hay and H.Mohler), a model coccolithophore, due to a stabilization of the carbonate system, an automatic replenishment of calcium ions and a reduced photoinhibition due to the presence of insolubilized $CaCO_3$ particles in the medium (Jakob et al. 2018). However, further investigation is needed to assess how $CaCO_3$ affects microalgal growth and morphology, coccolith structure and production, and metabolite content of the resulting biomass.

Interestingly, $CaCO_3$ is the main component of molluscs shells, such as *Magallana gigas* (Thunberg, 1793), commercially known as Pacific oyster, which are not recyclable due to their resistant composition (Alvarenga et al. 2012; Topić Popović et al. 2023; Vijai Anand et al. 2021). Incorrect disposal of bivalve shells can cause huge environmental harm due to the decomposition of residual organic tissues that can release unpleasant smells, to the excessive land usage for their stockage in landfills and the degradation of near ecosystems (M.-Y. Li et al. 2025; Zhan et al. 2022). Mollusc shell waste is produced by different anthropogenic activities, from bivalve hatcheries to restaurants, so that tonnes of this waste are produced daily (Topić Popović et al. 2023; Zhan et al. 2022). Reuse of mollusc shells is therefore needed. Currently, applications are being tested in different sectors, such as agriculture (Koley et al. 2025; Vijai Anand et al. 2021), cement production (Giosuè et al. 2025), and even as a calcium supplement in feedstock (Laohavisuti et al. 2025; Topić

Popović et al. 2023). Among that, the reuse of shells for microalgae cultivation can also be considered. Indeed, materials usually considered as waste have been used successfully for microalgae cultivation, valorising such resources and producing a valuable biomass with promising applications in biotechnological sectors (Baldisserotto et al. 2021, 2025; Ethiraj et al. 2024; Giovanardi et al. 2013; Lee et al. 2024).

Culture media with extracts from complex matrices, such as soil, or organisms, such as yeast, already exist, and are considered standard preparations for the cultivation of specific organisms (Barsanti and Gualtieri 2022; Spearman et al. 2016). Considering that molluscs shells also contain elements that are essential for microalgae growth, such as Fe, Cu and Zn, they could be considered as an additive in culture media (Fox and Zimba 2018; Kapranov et al. 2025; Maciel et al. 2024; Rajpoot et al. 2025). This research aims to evaluate new ways of reducing waste from the mollusc industry while simultaneously producing valuable microalgal biomass.

Materials and methods

Algal strain and culturing conditions

Gephyrocapsa huxleyi CCAP920/8 (Lohmann) Reinhardt 1972, formerly known as *Emiliania huxley*, was acquired from the Culture Collection of Algae & Protozoa (CCAP; SAMS Limited, SAMS, Dunbeg, Oban, Scotland, United Kingdom) and cultivated in three different media based on the artificial seawater recipe reported in Berges et al. (2001):

1. Standard artificial seawater containing 1.344 g L^{-1} of $CaCl_2 \times 2H_2O$ as calcium source, as control ($CaCl_2$). Final Ca concentration = 366 mg L^{-1}.
2. Modified artificial seawater containing 0.9150 g L^{-1} of pure $CaCO_3$, as calcium source ($CaCO_3$) instead of $CaCl_2 \times 2H_2O$. Final Ca concentration = 366 mg L^{-1}.
3. Modified artificial seawater containing 1.0167 g L^{-1} of pulverised shells of *M. gigas* as calcium source (*M. gigas* shells) instead of $CaCl_2 \times 2H_2O$. Final estimated Ca concentration = 366 mg L^{-1} based on preliminary data collected as described below.

All media were enriched with f/2 nutrients (Guillard & Ryther 1962). Experiments were carried out in a growth chamber (24 ± 1 °C, 30 $\mu mol_{photons}$ m^{-2} s^{-1} PAR, 16:8 h light–dark photoperiod) in 500 mL Erlenmeyer flasks. Cultures were hand-shaken daily.

Oyster shells composition

M. gigas shells were collected from Goro lagoon (Goro, Ferrara, Italy), cleaned manually to remove animal residues, rinsed with Milli-Q water and dried overnight at 50 °C. Then, cleaned shells were pulverised with a Retsch S1 planetary ball mill (Retsch GmbH, Verder Scientific, Haan, Germany).

To determine the bulk composition, 0.5 g of powdered shells was digested in HNO_3 10%, the solution was then filtered with 0.45 µm PVDF syringe filters and analysed using an Agilent 8800 ICP-MS (Agilent Technologies, Santa Clara, CA, USA) for the determination of trace elements. The instrument operating parameters were 1550 W RF power, 8.0 mm sampling depth, 15 L min^{-1} plasma gas. The masses monitored were ^{25} Mg, ^{39} K, ^{49}Ti, ^{51} V, ^{53}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{62}Ni, ^{65}Cu, ^{66}Zn, ^{88}Sr, ^{111}Cd, ^{137}Ba and ^{208}Pb.

Ca and Si were determined using an Optima 3100XL ICP-OES (PerkinElmer, Waltham, MA, USA). The instrument measuring parameters were: 15 L min^{-1} plasma flow, 0.5 L min^{-1} auxiliary flow, 0.65 L min^{-1} nebuliser flow, 1350 W RF power. The analytical lines monitored were 317,933 nm for Ca and 288,158 nm for Si. The analyses were performed in triplicate.

Cell density and dry weight evaluation

Cellular density was monitored for 14 days using Thoma counting chamber. For dry biomass concentration (grams of algal dry weight, DW, per Litre; g_{DW} L^{-1}) at the end of the experiment, aliquots of the different cultures were mixed with 1 N HCl to lower the pH below 5 to dissolve calcium carbonate crystals and coccoliths (Oz et al. 2023). Then, these solutions were filtered through pre-dried and pre-weighted glass fibre filters (Whatman GF/C; 1.2 µm pore size), rinsed with distilled water, dried for 48 h at 60 °C, and weighed to evaluate the biomass yield of the cultures (Popovich et al. 2012).

Morphological observations

Cells were observed in bright field using an Axiophot (Zeiss, Oberkochen, Germany) photo-microscope. To evaluate cell dimensions, photographs were taken with a VisiCam Pro 20C digital camera (20 megapixels; VWR International Srl, Milan, Italy) and measurements of cell diameter were carried out through Optika Proview (Optika S.r.l., Ponteranica, Bergamo, Italy, version × 64 4.11).

Scanning electron microscopy (SEM) was used to evaluate differences in the coccolith morphology. Cells from the $CaCl_2$ cultures were collected from the bottom of the flasks through gentle pipetting and deposited on round borosilicate cover glass (12 mm diameter, KnittelGlass, Statlab, McKinney, Texas, U.S.). For the other cultures, since cells didn't deposit, 2 mL of cell suspensions were centrifuged (110 × *g*, 10 min); then, 1.8 mL of supernatants were removed, and the remainders were deposited on the round borosilicate cover glass described above. Samples were then dried at 60 °C for 24 h, gently washed with distilled water to remove salts and dried again at 60 °C for 24 h. Samples were then sputter-coated with platinum and observed through a GeminiSEM 460 (Zeiss, Oberkochen, Germany).

Metabolites quantification

For photosynthetic pigments quantification, biomass was collected through centrifugation (18,000 × *g*, 10 min) and suspended in DMSO (Dimethyl sulfoxide). After 10 min at 55 °C, the supernatant was separated through centrifugation (18,000 × *g*, 10 min) and absorbances were read at 666 nm, 630 nm, 480 nm and 750 nm using a VWR P9 UV/Vis double-beam spectrophotometer (VWR, Avantor Inc., Radnor, Pennsylvania, U.S.). Chlorophyll *a* (Chl *a*) and chlorophyll *c* (Chl *c*) concentrations were quantified as reported by (Ritchie et al. 2021). Carotenoid (Car) concentration was estimated using the formula reported in (Wellburn 1994). Results are expressed both as $\mu g_{PIGMENT}$ $mg_{DW}{}^{-1}$ and as $ng_{PIGMENT}$ 10^{-6} cells.

To evaluate the total protein content, the whole cultures were harvested by centrifugation (500 × *g*, 10 min), and the extraction was carried out following (Baldisserotto et al. 2023). Due to the high volume required for this analysis, only one extract per treatment

was prepared to obtain preliminary result and identify potential differences in protein concentration. Quantification of total proteins in algal extract was performed using a modified Lowry method (Lowry et al. 1951) as described in (Baldisserotto et al. 2023). Each extract was read at least in three analytical replicates. Data are reported both as mg_{PRT} 100 mg_{DW}^{-1} and as μg_{PRT} 10^{-6} cells.

Analyses of the culture media

pH and conductivity of culture media were evaluated using a PH/CO-2500L (VWR, Avantor Inc., Radnor, Pennsylvania, U.S.).

To evaluate nitrate and phosphorous concentrations in culture media, algal biomass was removed through centrifugation ($500 \times g$, 10 min), and supernatant was stored at − 20 °C until analyses. Nitrate ($N\text{-}NO_3^-$) concentration in the media was evaluated spectrophotometrically by reading absorbances at 220 nm and 275 nm following the protocol described in the "Standard Methods Committee of the American Public Health Association" (2022a) using the spectrophotometer described above. Standards for the calibration curve were prepared in artificial seawater (Berges et al. 2001). Phosphate ($P\text{-}PO_4^{3-}$) concentration was evaluated through the molybdenum blue method described in "Standard Methods Committee of the American Public Health Association" (2022b) using a flow-injection autoanalyser (Flowsys, Systea SpA, Company, Roma, Italy).

Statistical analyses

Data were processed with Jamovi 2.6 software (The Jamovi Project, Sydney, Australia). A parametric one-way analysis of variance (ANOVA) was used to verify the presence of significant differences ($p < 0.05$) between treatments, followed by a Tukey test to find where the differences occurred. Data are expressed as means ± standard deviations.

Results

Growth kinetics and cell dimensions

Cultures cultivated in modified media reached higher cellular density compared to the control, as shown in Fig. 1a. In particular, cultures containing the pulverised shells of *M. gigas* as a calcium source had the highest cellular density among all treatments ($2383 \pm 116 \times 10^3$ cells mL^{-1}). This value was about 26-fold the cellular density of the control with $CaCl_2$ ($88 \pm 15 \times 10^3$ cells mL^{-1}). Similarly, the $CaCO_3$ cultures showed an increased final cellular density ($505 \pm 41 \times 10^3$ cells mL^{-1}), although this was still about fourfold lower than the *M. gigas* cultures. Moreover, significant differences were also recorded in cell diameter, with the cells cultivated with the pulverised shells being the smallest (4.06 ± 0.68 µm), followed by the cells cultivated using pure $CaCO_3$ (4.66 ± 0.80 µm), all being significantly smaller than the control (5.28 ± 0.7 µm), as reported in Fig. 1b. The difference in cell dimension can also be observed in the microscopy images in Fig. 2a, b and c, which additionally show the presence of coccolith around cells in all treatments. Overall, culturing with alternative calcium sources increased the final dry biomass concentration compared to the control (8.67 ± 2.31 mg_{DW} L^{-1}), but no significant difference was found between the final dry

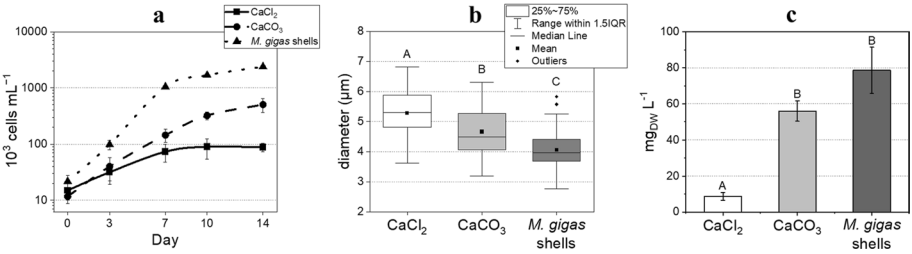

Fig. 1 (**a**) time course variation of cellular density of *Gephyrocapsa huxleyi* cultures cultivated using $CaCl_2 \times 2H_2O$ as calcium source (squares), pure $CaCO_3$ as calcium source (circles) and pulverised shells of *Magallana gigas* as calcium source (triangles); data refer to means ± standard deviations ($n = 3$). (**b**) Boxplot of cell diameter in the different cultures based on 50 measurements; different letters highlight significant differences between groups ($p < 0.05$) found using a one-way ANOVA. (**c**) Final concentration of dry biomass in the different cultures; data refer to means ± standard deviations ($n = 3$); different letters highlight significant differences between groups ($p < 0.05$) found using a one-way ANOVA

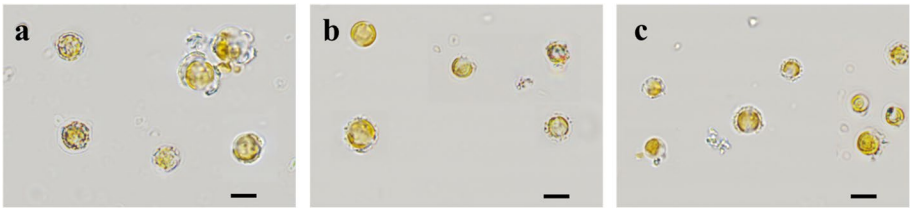

Fig. 2 Light microscopy images of *Gephyrocapsa huxleyi* after 14 days of cultivation with (**a**) $CaCl_2 \times 2H_2O$ as calcium source, (**b**) pure $CaCO_3$ as calcium source and (**c**) pulverised shells of *Magallana gigas* as calcium source. Bars: 5 µm

weight concentration of the cultures cultivated using the oyster shells (78.67 ± 12.86 mg_{DW} L^{-1}) rather than pure $CaCO_3$ (56.00 ± 5.66 mg_{DW} L^{-1}), as shown in Fig. 1c.

SEM observations revealed that coccolith structure seemed to be preserved between all cultures. However, more broken coccoliths were found in the $CaCO_3$ sample, with partial or missing distal elements (Fig. 3b), compared to the control (Fig. 3a) and the culture added with *M. gigas* shells (Fig. 3c). Moreover, more unbound coccolith were observed in the $CaCO_3$ and *M. gigas* samples compared to the control, probably due to the different methodology used.

Elemental composition of the oyster shells

As reported in Table 1, Ca was the most abundant element in the oyster shells (about 345.78 mg g^{-1}), followed by Si (about 1.25 mg g^{-1}) and Sr (about 1.00 mg g^{-1}). Interestingly, the concentration of Mn inside the grams of pulverised shells used for the preparation of the modified medium was higher compared to the concentration in the standard medium, while other elements, such as Sr, Cu and Zn, had a concentration in the same order of magnitude. Considering that the modified medium was prepared by replacing the $CaCl_2 \times 2H_2O$ present in the standard medium with the pulverised shells of *M. gigas,* without other modifications, we cannot exclude the contribution of the pulverised oyster shells

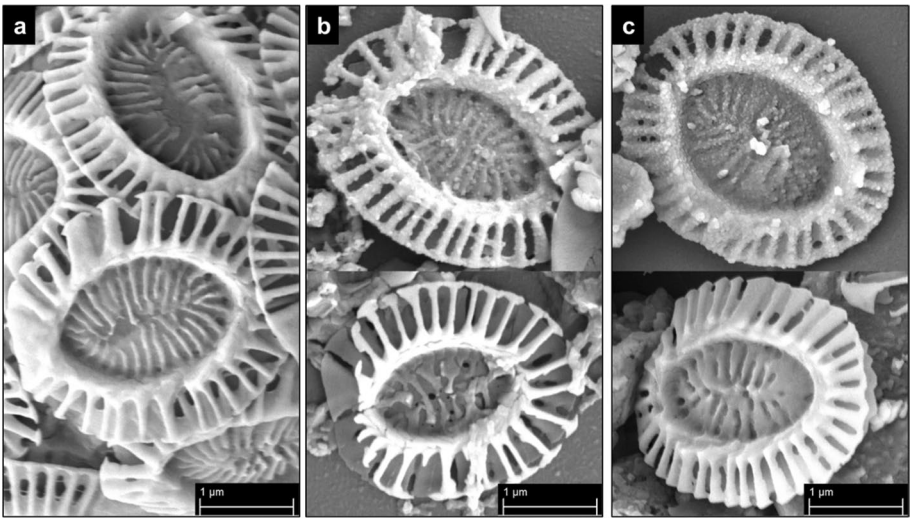

Fig. 3 SEM images of coccoliths of *Gephyrocapsa huxleyi* cultivated using (**a**) $CaCl_2 \times 2H_2O$ as calcium source, (**b**) pure $CaCO_3$ as calcium source, and (**c**) pulverised shells of *Magallana gigas* as calcium source. Bars: 1 µm

Table 1 Concentration of elements in (1) *Magallana gigas* shells, (2) standard artificial seawater (Berges et al. 2001) enriched with f/2 nutrients, (3) estimated concentrations in the modified medium with the pulverised shells of *M. gigas* as the calcium source. For the modified medium, values represent the sum of the concentrations in the standard artificial seawater and those contributed by the added oyster powder (concentration used = 1.0167 g L^{-1}). Data refer to mean ± standard deviation ($n = 3$)

	(1) Magallana gigas *shells*		(2) *Standard medium*		(3) *Modified medium enriched with* M. gigas *shells (estimated)*	
Ca	345.78 ± 17.30	mg g^{-1}	366.30	mg L^{-1}	351.55	mg L^{-1}
Si	1.23 ± 0.25	mg g^{-1}	/	mg L^{-1}	1.25	mg L^{-1}
Mg	238.25 ± 25.56	µg g^{-1}	1144.53	mg L^{-1}	1144.74	mg L^{-1}
K	367.79 ± 47.56	µg g^{-1}	342.31	mg L^{-1}	342.67	mg L^{-1}
Ti	0.66 ± 0.06	µg g^{-1}	/	µg L^{-1}	0.67	µg L^{-1}
V	0.39 ± 0.13	µg g^{-1}	/	µg L^{-1}	0.40	µg L^{-1}
Cr	0.11 ± 0.02	µg g^{-1}	/	µg L^{-1}	0.12	µg L^{-1}
Mn	227.57 ± 116.20	µg g^{-1}	50.00	µg L^{-1}	281.37	µg L^{-1}
Fe	97.59 ± 0.21	µg g^{-1}	653.39	µg L^{-1}	752.61	µg L^{-1}
Co	0.71 ± 0.41	µg g^{-1}	2.48	µg L^{-1}	3.19	µg L^{-1}
Ni	1.46 ± 0.26	µg g^{-1}	/	µg L^{-1}	1.49	µg L^{-1}
Cu	1.64 ± 0.17	µg g^{-1}	2.50	µg L^{-1}	4.17	µg L^{-1}
Zn	3.61 ± 0.67	µg g^{-1}	5.00	µg L^{-1}	8.67	µg L^{-1}
Sr	1000.17 ± 20.51	µg g^{-1}	7167.32	µg L^{-1}	8184.19	µg L^{-1}
Cd	0.49 ± 0.20	µg g^{-1}	/	µg L^{-1}	0.50	µg L^{-1}
Ba	4.38 ± 0.92	µg g^{-1}	/	µg L^{-1}	4.46	µg L^{-1}
Pb	0.45 ± 0.05	µg g^{-1}	/	µg L^{-1}	0.45	µg L^{-1}

to the final concentration of these elements. Moreover, some elements, such as Si, Ni and Ba, were abundant in the oyster shells and are not present in the standard medium.

Culture medium physicochemical parameters

In Table 2 the values of conductivity, pH, and the initial and final $N\text{-}NO_3^-$ concentrations are reported. Conductivity values were similar between the different culture media. On the other hand, initial pH values were different between the different cultures. Interestingly, the final pH values of the commercial $CaCl_2$ and $CaCO_3$ cultures were similar to the initial values, while the final pH of the cultures added with $CaCO_3$ from *M. gigas* powder was significantly higher compared to the initial one. Overall, the addition of the pulverised oyster shells did not change the concentration of nitrate and phosphorous in the medium. By the end of cultivation, nitrate was abundant in all the cultures, with similar concentrations in the controls and in the $CaCO_3$ cultures and a slightly but significantly lower concentration in the cultures added with *M. gigas* powder (Table 2). Similarly, cultures cultivated using the pulverised oyster shells consumed more phosphorous compared to the other cultures, but the cultures cultivated using pure $CaCO_3$ consumed less of this element also compared to the control, as shown in Table 2.

Metabolites production

Interestingly, the concentrations of photosynthetic pigments per million of cells didn't differ significantly between the different cultures (Fig. 4a), but significant differences were recorded when considering the pigment concentrations over dry weight (Fig. 4b). In fact, the cultures cultivated with the pulverised shells of *M. gigas* had over two times the pigment concentration of the other cultures in terms of $\mu g_{PIGMENT}\ mg_{DW}^{-1}$. No significant difference was recorded between the $CaCO_3$ cultures and the $CaCl_2$ control.

Preliminary results on protein concentrations showed different trends between the content over one million cells and the content over dry biomass. Considering the concentration of total proteins over one million cells, the cultures cultivated in the modified media showed a similar protein content, but lower compared to the control (Fig. 5a). However,

Table 2 Conductivity (mS cm^{-1}), initial and final pH values, initial and final $N\text{-}NO_3^-$ concentration (mg L^{-1}), initial and final $P\text{-}PO_4^{3-}$ concentration (mg L^{-1}) in the different media of *Gephyrocapsa huxleyi* cultures. For conductivity, data refers to a single measurement ($n=1$). For all the other parameters, data refers to means ± standard deviations ($n=3$). Different letters highlight a significant difference ($p<0.05$) in the subset (row) found using a one-way ANOVA, while * indicates a significant difference between the final and the initial values

Parameter			*$CaCl_2$*	*$CaCO_3$*	M. gigas *shells*
Conductivity		mS cm^{-1}	41.42	40.17	40.37
pH	Initial		7.92 ± 0.01^{a}	8.15 ± 0.01^{b}	8.26 ± 0.01^{c}
	Final		7.92 ± 0.07^{a}	8.16 ± 0.04^{b}	$8.64 \pm 0.03^{c*}$
$N\text{-}NO_3^-$	Initial	mg L^{-1}	10.16 ± 0.73^{a}	10.86 ± 1.06^{a}	10.93 ± 0.94^{a}
	Final	mg L^{-1}	$9.38 \pm 0.02^{a*}$	$9.42 \pm 0.10^{a*}$	$7.59 \pm 0.15^{b*}$
$P\text{-}PO_4^{3-}$	Initial	mg L^{-1}	1.38 ± 0.04^{a}	1.41 ± 0.08^{a}	1.43 ± 0.04^{a}
	Final	mg L^{-1}	$0.74 \pm 0.01^{a*}$	$0.83 \pm 0.02^{b*}$	$0.53 \pm 0.04^{c*}$

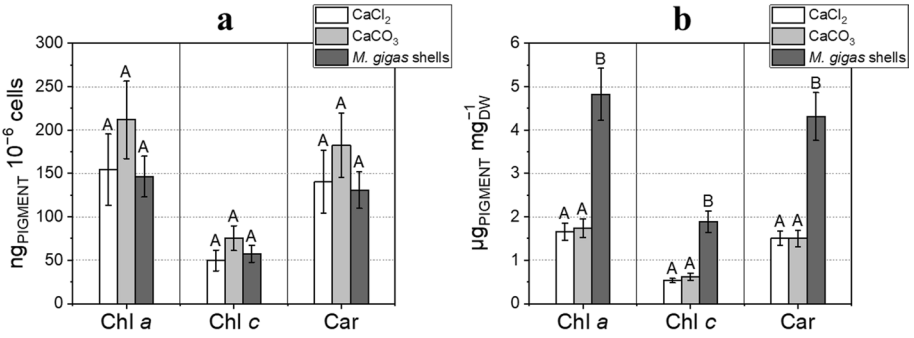

Fig. 4 Concentration of photosynthetic pigments in *Gephyrocapsa huxleyi* after 14 days of cultivation with $CaCl_2 \times 2H_2O$ as calcium source, pure $CaCO_3$ as calcium source or pulverised shells of *Magallana gigas* as calcium source, expressed as (**a**) $ng_{PIGMENT}$ 10^{-6} cells and (**b**) $\mu g_{PIGMENT}$ mg_{DW}^{-1}. Data refer to means ± standard deviations ($n = 3$). Different letters highlight significant differences ($p < 0.05$) found using a one-way ANOVA

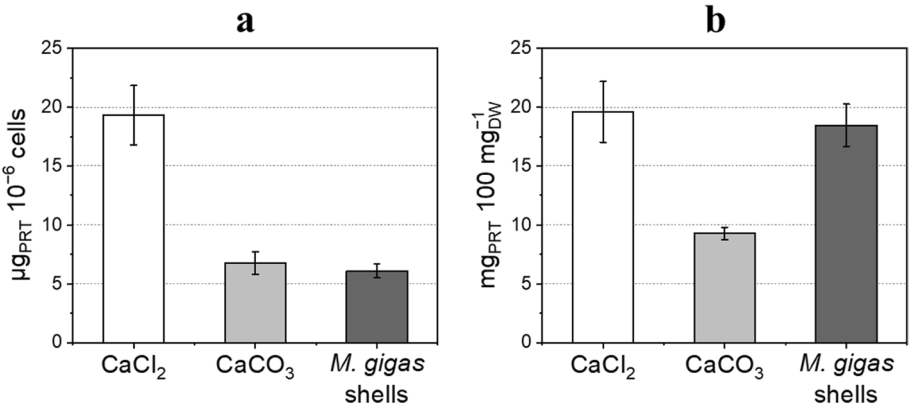

Fig. 5 Trends in the concentration of total proteins in *Gephyrocapsa huxleyi* after 14 days of cultivation with $CaCl_2 \times 2H_2O$ as calcium source, pure $CaCO_3$ as calcium source or pulverised shells of *Magallana gigas* as calcium source, expressed as (**a**) µg of proteins in 10^{-6} cells and (**b**) mg of proteins in 100 mg of dry biomass. Analysis was performed on a single biological replica per treatment; therefore, no statistical analysis was performed. Data refer to means ± standard deviations ($n = 3$)

considering the total protein concentration over 100 mg of dry biomass, the cultures cultivated using the pulverised shells of *M. gigas* showed a similar protein content compared to the control, while the cultures cultivated using pure $CaCO_3$ had a lower total protein concentration compared to the other cultures (Fig. 5b).

Discussion

Coccolithophores have great potential for their use in different biotechnological industries (Huynh et al. 2019; Jakob et al. 2017; Lomora et al. 2021; Moreira et al. 2023; Skeffington and Scheffel 2018). Their low biomass production, however, represents a major problem

for their actual implementation in the industry (Occhipinti et al. 2024; Rozenberg et al. 2024). As a solution to enhance growth in coccolithophores, pure $CaCO_3$ has been proposed (Jakob et al. 2018). Our results confirm that pure $CaCO_3$ can be a promising way to increase biomass production, since the dry weight of $CaCO_3$ cultures was sevenfold the dry weight of the control, but the cultures added with oyster shells reached even higher cellular densities, about 26-fold the control with $CaCl_2$ and about fourfold the cellular density of the $CaCO_3$ cultures. As hypothesised by Jakob and colleagues (2018), $CaCO_3$ may enhance growth through a stabilisation of a carbonate system, since high values of carbonate concentration were recorded in their experiment; furthermore, carbonate saturation is one of the conditions required for coccolithophore blooms to happen in natural environments (Tyrrell and Merico 2004). This might explain why cultures with $CaCO_3$ reached higher cellular densities compared to the control; however, it doesn't explain why the cultures grown in the presence of the pulverised shells reached even higher cellular densities. One reason could be the composition of the shell of *M. gigas*, since analyses revealed the presence of multiple elements found in metalloenzymes essential for microalgae growth, such as Fe, Cu, Zn and Mn (Camacho-Rodríguez et al. 2021; Fox and Zimba 2018; Maciel et al. 2024). For example, Mn plays a crucial role in the oxygen-evolving complex, and high concentrations of this element can lead to enhanced photosynthetic productivity, increased oxidative stress resistance and quicker biomass production (Ciurli et al. 2021; Smythers et al. 2023). Similarly, Zn is present in carbonic anhydrase as a cofactor, a fundamental enzyme for both photosynthetic activity and coccolith formation (Shire and Kustka 2022; H. Zhang et al. 2021), while Fe is present in key enzymes of important metabolic pathways, including photosynthesis (S. Li et al. 2024; Probert and Houdan 2004; Schulz et al. 2004). *G. huxleyi* is known to thrive even at low concentrations of Fe and Zn, but higher concentrations of these elements are known to support a higher growth rate (Schulz et al. 2004). However, high concentrations of these elements, along with Cu, can also adversely affect microalgal growth, but these concentrations are usually higher compared to those in the oyster shells (Debelius et al. 2009; Levy et al. 2008; Nowicka 2022; Santomauro et al. 2016). Conversely, other elements also found in the oyster shell, such as Cr and Pb, don't have an active role in the metabolism and can have toxic effects even at low concentrations (Debelius et al. 2009; Nowicka 2022; Purushanahalli Shivagangaiah et al. 2021). *G. huxleyi* has been reported to thrive at high concentrations of these elements with limited impacts on growth and coccolith morphology (Faucher et al. 2017), but it has also been reported to concentrate some of these elements in the biomass, in particular Pb, at concentrations about twofold the external one (Vasconcelos et al. 2002). Presence of these elements inside the resulting biomass could hinder further applications since they can be toxic for other organisms; therefore, it needs to be monitored (Parmar & Patel 2025). Lastly, other elements, such as Sr and Ba, can follow the same pathways as calcium and be included in coccoliths, with no known impact on their morphology (Hermoso et al. 2017; Langer et al. 2009; Meyer et al. 2020). Overall, the addition of pulverised oyster shells to the culture medium had a positive effect on biomass production, suggesting that the beneficial effects of some elements, such as Mn and Fe, could have balanced the presence of some potentially toxic metals, such as Pb and Cr.

Interestingly, the cells grown both in the presence of pure $CaCO_3$ and the pulverised oyster shells were significantly smaller. Microalgae can modulate their volume in response to different abiotic factors, such as culture density, nutrient availability, light exploitation, presence of toxins (Finkel et al. 2016; Lerf et al. 2024; Liu et al. 2023; Machado and Soares 2014). As a matter of fact, a higher concentration of heavy metals, such as Cu, is usually linked to an increase in cellular volume (Bucková et al. 2023; Levy et al. 2008; Machado

and Soares 2014). Considering also that a mild reduction in cell size was also recorded in the $CaCO_3$ cultures, where the concentration of metals was the same as the control cultures, other causes of cell shrinkage need to be found. A strong link between cell size and nitrogen limitation is known to exist in *G. huxleyi* (Müller et al. 2012; Riegman et al. 2000; Sciandra et al. 2003). In our experiment, however, nitrogen was still abundant after 14 days in both cultures, suggesting that other or multiple phenomena could be occurring at the same time. For example, an increase in CO_2 concentration can lead to strain-specific variation in cell volume, with some algae reducing their sizes and others increasing it (Jin et al. 2013; Lim et al. 2022). Increased inorganic carbon availability when using $CaCO_3$ has already been reported by Jakob and colleagues (2018), so variation in the C:N ratio and imbalances in nutrient concentration may have led to the decrease in cell size (Geider and Roche 2002; Hu and Zhou 2010; S. Li et al. 2020). It is also important to consider that cell size can affect sinking rate, with larger cells sinking faster than smaller ones (Malerba et al. 2018; Miettinen et al. 2024). Considering the presence of fast-sinking particulate matter in the modified media, reduced cell size, and thus slower sinking rate, could also be an adaptation to avoid the shading from the insolubilized particles.

Morphological changes, in particular reduction in cell size, are also linked to changes in metabolite production and concentration inside the organism (Finkel et al. 2016; Liu et al. 2023; Yan et al. 2021). Even if data regarding protein concentrations are only preliminary and further investigation is needed, interesting trends were observed. Indeed, the decreased protein content per cell in the cultures added with pure or recycled $CaCO_3$ could be linked to an increased C:N ratio in the media, supporting this hypothesis (Gao et al. 2018; S. Li et al. 2020). Interestingly, there seems to be no difference in the concentration of protein per 100 mg of biomass between the control and the cells cultivated using the shells of *M. gigas* as a calcium source. The concentration of protein is one of the key factors when considering microalgae for animal feeding, since low values can lead to an unbalanced diet (Madacussengua et al. 2025; Madeira et al. 2017). This preliminary result may open the possibility of further research in the use of this alga as animal feedstock, and further analyses are needed also to check for variations in single amino acid abundance (Martins et al. 2021; Urrutia-Mazzuca et al. 2025).

Similarly to protein concentration, also pigments showed a different trend between the concentration per cell and the concentration per dry biomass. Interestingly, the photosynthetic pigments concentration per cell was similar among all treatments. However, due to the morphological differences, the biomass obtained using the pulverised shells of *M. gigas* resulted to be enriched in all the photosynthetic pigments. Among microalgal pigments, carotenoids are well-regarded also for their multiple biotechnological applications in the pharmaceutical, cosmetic and nutraceutical sectors (Razzak 2024; Sirohi et al. 2022). Moreover, carotenoids are also fundamental in animal diets, in particular when speaking of poultry, since they are required for the development of the pigmentation on both the animal and the yolk (Coudert et al. 2020; Madacussengua et al. 2025). In particular, fucoxanthin, the most abundant carotenoid in *G. huxleyi*, is currently being studied for its beneficial effects on human health (Khaw et al. 2022; Kim et al. 2012; J. Zhang et al. 2023). Moreover, it also showed valuable effects when added to animal feed, increasing the percentage of important fatty acids, such as docosahexaenoic acid, in quail egg yolks (Guo et al. 2025). The increased content of total carotenoids found in the biomass of *G. huxleyi* cultivated using the oyster shell opens to the potential exploitation of this alga in animal nutrition with potential positive effects.

Environmental parameters are known to affect coccolith ultrastructure, with major malformations recorded at pH lower than 7.5 (Bach et al. 2012), and shrinkage recorded

when changing temperature and salinity (Saruwatari et al. 2016). Another important factor influencing coccolith production is Ca^{2+} availability, with malformations found at values around 1 mmol L^{-1}, and no calcification happening with concentrations of Ca^{2+} around 0.1 mmol L^{-1} (Trimborn et al. 2007). Even though in our trials, pH, salinity and temperature were optimal for coccolith production, we could not gather any information about the actual Ca^{2+} concentration. Since commonly used methods for Ca^{2+} quantification in water require acidification or use delicate equipment, they cannot be easily applied when particulate matter or insolubilized $CaCO_3$ is present, as they would lead to overestimation or machine breakage (He et al. 2020; Jakob et al. 2018; Stoodley et al. 2014). However, both light microscopy and SEM showed the presence of coccolith when culturing with alternative calcium sources, suggesting that the conditions required for calcification were still present. Moreover, SEM imaging suggested that coccolith in the $CaCO_3$ cultures seemed more susceptible to damage or partially broken, whereas coccolith in the *M. gigas* cultures were fully formed and intact. These observations suggest that using pulverised shells of *M. gigas* as calcium source, rather than pure $CaCO_3$ or $CaCl_2$, does not affect coccolith morphology and could be an implementation in the culture media also for coccoliths production. Considering that coccoliths are mainly composed of $CaCO_3$, their presence in the biomass of *G. huxleyi* cultivated using *M. gigas* shells suggests that it could be used as supplement in calcium-fortified feed, similar to how other calcium sources are already used (Aditya et al. 2021; Ahn et al. 2025; Laohavisuti et al. 2025).

Conclusions

Mollusc shells are not recyclable and need to be disposed of in landfills, with potential negative impact on the environment. Our findings suggest that using mollusc shells for the cultivation of coccolithophores could be a promising and sustainable way to valorise this waste while also producing a valuable biomass. Indeed, both cells and dry biomass productions were increased when using the pulverised oyster shells for the cultivation of *G. huxleyi*. Notably, the biomass resulted to be enriched in carotenoids, while still being able to produce coccoliths. These results open to the possibility of further investigation about biotechnological applications of *G. huxleyi*. In this regard, future research should focus on changes in biomass composition in terms of both valuable molecules and heavy metal concentration, to evaluate safe uses of biomass of *G. huxleyi* cultivated using recycled oyster shells in remunerative fields, such as in the preparation of calcium and carotenoid fortified feed for animals, in particular for poultry.

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Data availability The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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