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ORIGINAL PAPER

Response of the calcifying coccolithophore *Emiliana huxleyi* to low pH/high pCO₂: from physiology to molecular level

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Abstract The emergence of ocean acidification as a significant threat to calcifying organisms in marine ecosystems creates a pressing need to understand the physiological and molecular mechanisms by which calcification is affected by environmental parameters. We report here, for the first time, changes in gene expression induced by variations in pH/pCO₂ in the widespread and abundant coccolithophore *Emiliana huxleyi*. Batch cultures were subjected to increased partial pressure of CO₂ (pCO₂; i.e. decreased pH), and the changes in expression of four functional gene classes directly or indirectly related to calcification were investigated. Increased pCO₂ did not affect the calcification rate and only carbonic anhydrase transcripts exhibited a significant down-regulation. Our

observation that elevated pCO₂ induces only limited changes in the transcription of several transporters of calcium and bicarbonate gives new significant elements to understand cellular mechanisms underlying the early response of *E. huxleyi* to CO₂-driven ocean acidification.

Introduction

The oceans are the largest active sinks of carbon on Earth, with an estimated 30% of anthropogenic carbon emissions produced since 1800 taken up by oceans (Sabine et al. 2004). This leads to profound changes in the carbonate chemistry of seawater with an increase in pCO₂, dissolved inorganic carbon (DIC) and bicarbonate ions (HCO₃⁻) concentration, and a decrease in the concentration of carbonate ions (CO₃²⁻) and pH. These changes are collectively referred to as ocean acidification, an anthropogenic perturbation that has been identified as a great threat to marine ecosystems (Halpern et al. 2008) and particularly to calcifying organisms (Orr et al. 2005). A decreased availability of carbonate ions could thus affect the ability of calcifying organisms to precipitate CaCO₃. This will directly impact marine ecosystems by weakening CaCO₃ skeletons and it will impact the ocean carbon pump as CaCO₃ is thought to enhance the export of organic carbon in the deep ocean (“carbon ballasting”; Engel et al. 2009). Coccolithophores are the dominant planktonic calcifiers in the present ocean and are estimated to be responsible for about half of all modern precipitation of CaCO₃ (Milliman 1993). Thus it is crucial to understand how these organisms will be affected by ocean acidification in order to effectively predict the response of the ocean to this large-scale perturbation and its future ability to absorb anthropogenic CO₂.

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A large range of coccolithophores responses to elevated $p\text{CO}_2$ have been observed in laboratory cultures (Riebesell et al. 2000; Zondervan et al. 2001; Langer et al. 2006, 2009; Iglesias-Rodriguez et al. 2008; Ridgwell et al. 2009; Shi et al. 2009; Müller et al. 2010). Resolving this diversity in responses requires a better understanding of the cellular and biochemical mechanisms and pathways involved in calcification and how they are affected by changes in $p\text{CO}_2$ and other environmental parameters. The molecular mechanisms involved in coccolithophore biomineralization are still poorly understood despite extensive physiological investigation (reviewed by de Vrind-de Jong and de Vrind 1997; Young et al. 1999; Marsh 2000; González 2000; Paasche 2002; Baeuerlein 2003), and the molecules responsible for the acquisition and intracellular transport of Ca^{2+} , HCO_3^- and CO_3^{2-} , and in the precipitation of CaCO_3 remain to be identified.

However, a whole genome assembly for *E. huxleyi* (strain CCMP1516) has been publicly released by the Joint Genome Institute (available at www.doe.jgi.gov), a growing number of expressed sequence tags (EST) resources for this species are now available (Wahlund et al. 2004; Quinn et al. 2006; von Dassow et al. 2009), and candidate genes likely to be important for biomineralization can now be identified by homology to known eukaryotic proteins involved in the processing of Ca^{2+} and $\text{CO}_2/\text{HCO}_3^-/\text{CO}_3^{2-}$.

In the present study, we chose *E. huxleyi* (Lohmann) Hay and Mohler, the most abundant calcifying phytoplankton on Earth (Westbroek et al. 1993) to investigate the effect of atmospheric CO_2 emission scenarios expected by the end of this century (IPCC 2007) on calcification process and underlying cellular mechanisms. We assessed the growth and calcification rate of a calcifying strain of this species in response to $p\text{CO}_2/\text{pH}$ variations. In parallel, molecular targets were followed for their gene expression using quantitative PCR.

We focused on two classes of proteins tightly involved in cellular pH and/or carbonate chemistry regulation (e.g. carbonic anhydrase and $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger family). We studied two classes of carbonic anhydrase (CA) out of five known (α , β , γ , δ , and ζ) and their role in *E. huxleyi* cells subjected to lower pH. Carbonic anhydrases are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide into bicarbonate and play different roles in physiological processes such as photosynthesis, respiration, pH homeostasis and ion transport.

We also investigated the homologs of $\text{Cl}^-/\text{bicarbonate}$ exchanger solute carrier family 4 proteins (SLC4), well known for their roles in intracellular pH regulation in animal cell (Romero et al. 2004) and recently described as highly specific to calcifying cells of *E. huxleyi* (von

Dassow et al. 2009). According to von Dassow et al. (2009) study, one of the SLC4 $\text{Cl}^-/\text{bicarbonate}$ transcript (cluster GS05051) was represented by 7/0 reads for calcifying (2 *N*) cells compared to non-calcifying (*N*) cells.

Based on the decrease in calcification (e.g. decrease in PIC) observed in some coccolithophore cultures subjected to $p\text{CO}_2$ increase (Riebesell et al. 2000; Zondervan et al. 2001; Sciandra et al. 2003; Langer et al. 2006, 2009; Feng et al. 2008; Müller et al. 2010; Ridgwell et al. 2009), representative genes of two more protein classes were then investigated. The protein GPA was chosen since it was previously found associated with coccolith polysaccharides and displays Ca^{2+} -binding activity (Corstjens et al. 1998).

We then chose to specifically examine a Ca^{2+} -transporter-related gene. Ca^{2+} ion is not only a regulatory agent in physiological processes but also the primary cation used in biomineralized structures. While Ca^{2+} transporters and specifically the voltage-gated ion channel proteins are described in detail for vertebrates (Dolphin 2009), little is known about such transporters in the protist *E. huxleyi*. However, as in all biomineralization processes, either intracellular or extracellular, the primary event is the entry of Ca^{2+} ions at the cell membrane level. Thus, we hypothesized that those genes might be involved in calcification of *E. huxleyi* as it has been previously shown in the scleractinian coral *Stylophora pistillata* (Zoccola et al. 1999) and in calcification process in general.

In the present study, the hypothetical roles of the genes of interest in calcification in relation to the expression response to $\text{pH}/p\text{CO}_2$ variations and perspectives for the future of coccolithophores in a high CO_2 world are discussed.

Materials and methods

Culture condition and sampling

Diploid (2 *N*) cells of *Emiliania huxleyi* strain RCC1216 (Tasman sea; 42°18'S–169°50'W) were provided by the Alcobank culture collection, Caen, France (<http://www.sb-roscoff.fr/Phyto/RCC>). Many *E. huxleyi* strains lose the capacity to calcify in culture, and cultures often contain a mix of non-calcified and calcified cells complicating interpretations. Haploid and diploid life stages of the studied strain (RCC1217/RCC1216) were first characterized on a flow cytometer. Two distinct groups were identified in cytograms according to their nucleic acid fluorescence and side scatter. The composition of the experimental culture was then confirmed to be mainly diploid. RCC1216 was chosen because a wealth of ESTs is available from this strain and it exhibits high calcification under standard culture conditions. Cultures were

maintained in K/2 (-Si, -Tris) medium prepared from filter-sterilized seawater (Keller et al. 1987) at 17°C under a 14 h light: 10 h dark photoperiod with cool white fluorescent light at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, with a salinity of 38 ‰.

Experimental setup

Two 10-l glass bottles (control and experimental treatments) were filled with sterile culture medium and maintained at 17°C using a thermostated water bath. They were bubbled for 2 h with ambient air (control, ambient pCO_2) or a mixture CO_2 -free air (generated by the use of soda lime) and pure CO_2 stabilized at the desired partial pressure of 760 ppm (experimental treatment, high pCO_2) by a mass flow controller (GFC, Aalborg) coupled with an infrared gas analyzer (LICOR Li-6252), respectively. pH, salinity and total alkalinity (TA) were measured to check the pCO_2 in both treatments. The final pCO_2 values were 440 and 770 ppm in the control and the experimental treatments, respectively. Once the desired pCO_2 was reached, triplicate 2-l Nalgene bottles were filled up with each medium without headspace. An inoculum of 50 cells ml^{-1} (calculated from the stock culture) was added, and the 6 bottles were sealed with Teflon tape to avoid gas exchange between the medium and the atmosphere (Langer et al. 2006). Replicates were transferred to an incubation chamber and kept under the conditions described earlier (see Culture condition and sampling section) during all the experimental period. The cells were harvested at around $50,000 \pm 10,000$ cells ml^{-1} in order to work with low cell densities ensuring well-controlled experimental conditions and the biomass necessary for a reliable analysis. The sampling was performed after an 8-day incubation period at 0900 h (90 min after the beginning of the light period) for all 6 bottles.

Cell density and growth rate

Cell density was checked daily (10.00 a.m.) from day 3, using 500 μl of sample on a flow cytometer (FACSCalibur, BD Biosciences). Coccolithophores were detected by their red autofluorescence in the FL3 channel.

For determination of the growth rate (μ), samples for cell density were taken at the beginning and at the end of experiment. Growth rate (μ) was calculated as: $\mu = (\ln C_1 - \ln C_0) \Delta t^{-1}$ where C_0 and C_1 are the cell concentrations at the beginning (inoculation time) and at the end of experiment (harvesting time), respectively, and Δt is the duration of incubation in days.

Carbonate chemistry measurements

The carbonate system of the experiment was monitored by measuring total alkalinity (TA), pH_T , temperature and

salinity in the cultures. Triplicate 25 ml samples were collected for total alkalinity at the beginning, prior to inoculation, and at the end of the experiments (harvesting time). They were immediately filtered onto 0.2- μm filters and analyzed potentiometrically by a custom-made titrator built with a Metrohm pH electrode and a 665 Dosimat titrator. TA was calculated using a Gran function applied to the pH values ranging from 3.5 to 3.0 as described by Dickson et al. (2007). Titrations of an alkalinity standard, provided by A. G. Dickson (batch 80), were within 0.7 $\mu\text{mol kg}^{-1}$ of the nominal value (SD = 2.6 $\mu\text{mol kg}^{-1}$; $N = 8$). According to Brewer and Goldman (1976), 1 μM EDTA added to a phytoplankton culture to maintain Fe in solution contributes about 2 μeq to the alkalinity. In our case, the 125 nM EDTA should contribute about 0.2 μeq to the alkalinity in the medium and can thus be considered as negligible.

pH_T was measured on 20 ml samples using a pH meter (Metrohm, 826 pH mobile) with a glass electrode (Ecotrode, 6.0262.100 Metrohm) calibrated on the total scale using Tris/HCl and 2-aminopyridine/HCl buffer solutions with a salinity of 38 at a temperature of 17°C. pCO_2 , Ω_{calcite} and other parameters of the carbonate system were calculated from given TA and pH using the R package *seacarb* (Lavigne et al. 2008). The carbonate system, at the beginning and at the end of the incubation period (8 days), is described in Table 1.

Particulate inorganic (PIC) and organic (POC) carbon measurements

Triplicate samples ($\sim 150 \mu\text{g C}$ per filter) were filtered onto pre-combusted (4 h, 400°C) glass fiber filters (Whatman GF/F), dried at 60°C overnight and stored in a desiccator pending analysis. For POC measurements, the inorganic carbon was removed from the filters before the analysis by adding 25% HCl (Nieuwenhuize et al. 1994). Cell content for total particulate carbon (TPC) and for particulate organic carbon (POC) (pg cell^{-1}) was subsequently measured on a Thermo Electron Flash EA 1112 Analyzer as described by Nieuwenhuize et al. (1994). Particulate inorganic carbon (PIC) (pg cell^{-1}) was calculated as the difference between TPC and POC. Particulate inorganic carbon production, i.e. calcification rate (PPIC, $\text{pg PIC cell}^{-1} \text{d}^{-1}$) was calculated according to: $\text{PPIC} = \mu \times (\text{cellular inorganic carbon content in pg PIC per cell})$. Particulate organic carbon production (PPOC, $\text{pg POC cell}^{-1} \text{d}^{-1}$) was calculated according to: $\text{PPOC} = \mu \times (\text{cellular organic carbon content in pg POC per cell})$ (Riebesell et al. 2000).

Quantitative reverse transcriptase-polymerase chain reaction (q-RT-PCR)

RNA extraction—Total RNA was isolated from coccolithophores with Trizol reagent (Invitrogen, La Jolla, CA)

Table 1 Parameters of seawater carbonate system at the beginning and at the end of the incubation period

	pCO ₂ (ppm)	DIC (μmol kg ⁻¹)	HCO ₃ ⁻ (μmol kg ⁻¹)	CO ₃ ²⁻ (μmol kg ⁻¹)	TA (μmol kg ⁻¹)	pH _T	Ω _{calcite}
Day 0							
Low pCO ₂	421 ± 10	2219 ± 8	1950 ± 7	258 ± 2	2577 ± 9	8.05 ± 0.01	6.04 ± 0.2
High pCO ₂	765 ± 10	2351 ± 7	2156 ± 6	174 ± 2	2578 ± 7	7.84 ± 0.01	4.07 ± 0.2
Day 8							
Low pCO ₂	399 ± 10	2197 ± 8	1921 ± 11	264 ± 5	2563 ± 6	8.07 ± 0.01	6.18 ± 0.1
High pCO ₂	692 ± 10	2320 ± 8	2116 ± 9	185 ± 2	2565 ± 8	7.85 ± 0.01	4.33 ± 0.05

Values represent the means of three replicates (SD)

according to the suggested protocol. Five hundred milliliter of medium from each bottle was collected by gentle filtration on polycarbonate filter of 1 μm (Whatman) and resuspended in 1 ml of Trizol. Two successive chloroform (≥99%) steps in 200 μl were carried out to precipitate proteins and DNA. RNA was finally precipitated in 500 μl isopropanol (≥99%). The pellets were washed in 75% ethanol and resuspended in RNase-free water. The RNA quality was checked on 1% agarose (w:v) non-denaturing gels and the purity determined using a Nanodrop spectrophotometer (Nanodrop 3300, Thermo scientific). All samples presented ribosomal RNA bands with no sign of degradation. RNA samples were treated with DNase (1U μl⁻¹, Fermentas) and quantified using a RiboGreen RNA Quantification Kit (Molecular Probes). Total RNA concentration was adjusted to a final concentration of 100 ng μl⁻¹ in all samples, and the reverse transcription was carried out using the Affinity Script qPCR cDNA kit (Stratagene). Negative controls (same reagents mix without reverse transcriptase) were prepared simultaneously and run on each plate for each primer pairs to ascertain that no DNA contamination occurred (Ct values were >40 cycles). No template controls were also run in parallel on each plate.

Transcript levels were derived from the accumulation of SYBR green fluorescence measured with a Light Cycler 480 (Roche). The PCR conditions were as follows: 1× SYBR green mix (Roche, Cat. nb: 04707516001), 500 nM primers and 1 μl (100 ng) of cDNA in a total volume of 20 μl. Each sample was run in triplicate (mean ± SD < 0.2). The dissociation curves showed a single amplification product and no primer dimer. For each primer pairs, the amplification efficiency (*E*) was determined on a 5 points 10-time dilution series of 100 ng cDNA extracted from the two tested conditions (control and experimental pCO₂) to check for primer specificity. The reaction efficiencies had values between 80 and 100% with a corresponding amplification factor between 1.8 and 2.0, respectively, for all primer combinations. This value allows for a transformation of the observed changes in cycle threshold (C_T).

RNA transcription levels were determined by the method of direct comparison of C_T values between target genes and a reference gene. Several genes from *E. huxleyi* strain CCMP1516 (JGI, USA) commonly used as house-keeping genes (HKG) (e.g. *actin* (JGI, ID 226687), *β-tubulin* (JGI, ID 451245) and *RPLP0* (JGI, ID 456254)) were tested for their expression stability in experimental samples using the program geNorm (Vandesompele et al. 2002). While none of them was stable enough to normalize the data, calmodulin (JGI, ID 442625) was identified as the most stable gene and used further to normalize the data by the ΔΔCt method (Livak and Schmittgen 2001). Data were then transformed into linear form by: 2^{-DDCT} where -DDCT = (Ct_{Target} - Ct_{HKG})_{Tx} - (Ct_{Target} - Ct_{HKG})_{T0}. Data were analyzed using one-way analyses of variance (ANOVA). Since all the steps from RNA extraction to RT qPCR efficiency have been checked for accuracy, high standards deviations (SD) reported in Fig. 3 were mainly attributed to biological variability in experimental batch cultures.

Genes of interest and primer design

The sequences of 4 of the genes investigated here (*α*- and *γ*-CA, Ca²⁺-channel and *gpa*) were obtained from *E. huxleyi* strain CCMP1516 genome portal (<http://shake.jgi-psf.org/Emihu1/Emihu1.home.html>). The transcripts that encode Cl⁻/HCO₃⁻ exchanger homologs (SLC4 family) were annotated from the Sanger reads of *E. huxleyi* (strains RCC1216/RCC1217) cDNA libraries (von Dassow et al. 2009). Up to 7 homologs have been investigated (GS00443, GS02476, GS12371, GS03121, GS05051, GS09941, GS05509) but only 6 are presented in this study (GS00443 was weakly represented and not significantly detected by qPCR).

In order to characterize the coding sequences (partial or complete) chosen as part of this study, the amino acid (aa) sequences (*α*- and *γ*-CA, Ca²⁺-channel and GPA) or nucleotide sequences (Cl⁻/HCO₃⁻ exchanger homologs) were blasted to UniProt/Swiss-Prot databases (Consortium

U 2009) and NCBI/CDD (Conserved Domains database) (Marchler-Bauer et al. 2009). The characteristics of the given sequences are detailed in Tables 2, 3.

qPCR primer sequences were designed using the Primer3 software to have a G + C content ranging from 50 to 60% and C's > G's 3 identical dNTPs in a row at the 3' ends to avoid self complementarities of the primer sequence. Primers were chosen to generate equivalent amplicon lengths (see Table 4). The melting temperature of the primers was set at 58°C. The qPCR products were sequenced (MWG, Germany) and all matched the anticipated product. For PCR products obtained with primers designed from *E. huxleyi* strain CCMP1516 (e.g. α - and γ _CA, Ca²⁺-channel and GPA), sequences from both strains (CCMP1516 and RCC1216) were aligned and showed 100% identity.

Results and discussion

While previous molecular studies on *E. huxleyi* dealt with identification of genes that are associated with the

calcification mechanism (Quinn et al. 2006; Wahlund et al. 2004; Nguyen et al. 2005; von Dassow et al. 2009), our experiment is the first to investigate gene expression in response to CO₂-driven ocean acidification. Our approach provides new elements on the molecular and physiological role of genes of interest in calcification and helps understand the diverse response of coccolithophores to projected ocean acidification.

Physiological and biochemical response to decreasing pH

The experimental setup was designed following recommendations of best practices (Riebesell et al. 2010), and batch cultures were used as many other previous studies (Riebesell et al. 2000; Zondervan et al. 2001, 2002; Langer et al. 2006, 2009; Iglesias-Rodriguez et al. 2008), in order to ensure that data comparison between studies is possible. The manipulation of the carbonate system was achieved by bubbling the culture medium with CO₂ and/or air before the inoculation, and the experiment was

Table 2 Genes targeted in *E. huxleyi* strain RCC1216 and related characteristics

Name	Suggested protein	EMBL [acc. nb.]	Prot ID (JGI)	Gene scaffold (JGI)	KOG class	KOG ID	UniProt [acc. nb.]	Best hit	E-value
α -CA	α -carbonic anhydrase	na	456048	scaffold_166	(1)	KOG0382	B6BNC3	Carbonic anhydrase [<i>Campylobacteriales bacterium</i> GD.1]	1.00E ⁻²⁷
γ -CA	γ -carbonic anhydrase	na	432493	scaffold_5	(2)	KOG0382	Q0ZB85	Gamma carbonic anhydrase [<i>Emiliania huxleyi</i>]	1.00E ⁻¹³⁰
CAC	Ca ²⁺ ion channel	na	na	scaffold_11	(3)	KOG2301	C1FH96	Voltage-gated ion channel superfamily [<i>Micromonas</i> sp. RCC299]	1.00E ⁻¹³⁸
<i>gpa</i>	calcium-binding protein	FP217524	na	scaffold_1	(3)	KOG2643	Q0MYW8	Putative calcium-binding protein [<i>Emiliania huxleyi</i>]	–
<i>GS00443</i>	Cl ⁻ /HCO ₃ ⁻ exchangers	FP221416	na	nomap	(3)	KOG1172	B5Y5V6	Predicted protein [<i>Phaeodactylum tricorutum</i> CCAP 1055/1]	3.00E ⁻⁴⁵
<i>GS02476</i>	Cl ⁻ /HCO ₃ ⁻ exchangers	FP180858	na	nomap	(3)	KOG1172	Q7T1P6	Anion exchanger 1 [<i>Raja erinacea</i>]	2.00E ⁻³²
<i>GS12371</i>	Cl ⁻ /HCO ₃ ⁻ exchangers	FP187041	na	scaffold_18	(3)	KOG1172	B3RRA7	Putative uncharacterized protein [<i>Trichoplax adhaerens</i>]	7.00E ⁻⁰⁹
<i>GS03121</i>	Cl ⁻ /HCO ₃ ⁻ exchangers	FP180021	na	scaffold_51	(3)	KOG1172	Q4WXW0	Anion exchange family protein [<i>Aspergillus fumigatus</i> strain CEA10]	1.00E ⁻³⁸
<i>GS05051</i>	Cl ⁻ /HCO ₃ ⁻ exchangers	FP185544	na	scaffold_21	(3)	KOG1172	C1E0U4	Anion exchanger family [<i>Micromonas</i> sp. RCC299]	8.00E ⁻¹⁸
<i>GS09941</i>	Cl ⁻ /HCO ₃ ⁻ exchangers	FP163914	450694	scaffold_31	(3)	KOG1172	B7FQY4	Predicted protein [<i>Phaeodactylum tricorutum</i> CCAP 1055/1]	1.00E ⁻⁰⁸
<i>GS05509</i>	Cl ⁻ /HCO ₃ ⁻ exchangers	FP183003	196760	scaffold_4	(3)	KOG1172	B7FQY4	Predicted protein [<i>Phaeodactylum tricorutum</i> CCAP 1055/1]	2.00E ⁻³⁶

EMBL accession numbers have been provided for the clusters annotated as part of von Dassow et al. (2009) (see also von Dassow et al. 2009 Additional file 2). KOG (NCBI eukaryote orthologous group) class [(1) general function, (2) cytoskeleton and (3) inorganic ion transport and metabolism] is also mentioned

na Not available

Table 3 Genes targeted in *E. huxleyi* strain RCC1216. Identified conserved domains and *E*-values are mentioned

Name	Conserved domains	<i>E</i> -values
α -CA	cd03124, alpha_CA_prokaryotic_like, Carbonic anhydrase alpha, prokaryotic-like subfamily	2.00E ⁻³⁷
γ -CA	cd04645, LbH_gamma_CA_like, Gamma carbonic anhydrase-like	9.00E ⁻⁴⁵
CAC	cd00051, EFh, EF-hand, calcium-binding motif	5.00E ⁻¹³
<i>gpa</i>	cd00051, EFh, EF-hand, calcium-binding motif	5.00E ⁻¹³
<i>GS00443</i>	pfam00955, HCO ₃ cotransp, HCO ₃ ⁻ transporter family	6.00E ⁻¹¹
<i>GS02476</i>	pfam00955, HCO ₃ cotransp, HCO ₃ ⁻ transporter family	7.00E ⁻³⁷
<i>GS12371</i>	pfam00955, HCO ₃ cotransp, HCO ₃ ⁻ transporter family	3.00E ⁻¹⁰
	TIGR00834, ae, anion exchange protein	1.00E ⁻¹⁰
<i>GS03121</i>	pfam00955, HCO ₃ cotransp, HCO ₃ ⁻ transporter family	1.00E ⁻³⁹
<i>GS05051</i>	pfam00955, HCO ₃ cotransp, HCO ₃ ⁻ transporter family	9.00E ⁻¹⁹
<i>GS09941</i>	TIGR00834, ae, anion exchange protein	5.00E ⁻⁰⁵
<i>GS05509</i>	No hit	–

Table 4 Genes targeted in *E. huxleyi* strain RCC1216. Amplified product length, primer pair sequences are mentioned

Name	Amplicon size (bp)	Primers sequence (5'–3')
<i>CaM</i>	151	ATCGACTTCCCGAGTTCT CGAGGTTGGTCATGATGTG
α -CA	134	AGAGCAGAGCCCTATCAACA TCGTCTCGAAGAGCTGGAA
γ -CA	150	GCAAGAGTAGCATCGGAGAC CAACCACCGCAAAGTTGT
CAC	114	GACATCTACGAGCCGAACTC CATCCACTTGAGGAGCATCT
<i>gpa</i>	70	GTTACGCGTGCTCTCCGAG AGGCCTTCTCCAGCATCAT
<i>GS00443</i>	111	GCTCAAGTATTGGCACGTCT TTGAACTTTGGGTCCTGTG
<i>GS02476</i>	158	CATCACCTCGCTACCA AGGCGGACTTCTTGACG
<i>GS12371</i>	126	CAAGAAGGACTACGACACCTG GCCATCAGCATCACGAA
<i>GS03121</i>	137	GATGCGGAACGATCTCAA GGCGCAATACTCGTGAAG
<i>GS05051</i>	134	AAGGGGAAGAAGCCCATC AGAGGCAGGCGAAGAAGAG
<i>GS09941</i>	101	GAGGAGAGAACAGCCCTTGT AACTGAGCAACCGTGTGTG
<i>GS05509</i>	141	TCGTGTCTGGCGTCTTTC CCAGCGCAACCATCTCT

consequently performed in a closed system avoiding gas exchanges with the atmosphere. As in the natural environment, this method involves changes in pCO₂, DIC and pH, while TA remains constant (Gattuso and Lavigne 2009). The stress caused to the cultures by the air bubbling and consequent variability of the response

to tested parameters are eliminated, and the shift in carbonate parameters due to cell activity is negligible. Consequently, any change during the experiment can exclusively be attributed to physiological changes in response to the CO₂ perturbation (Fiorini 2010).

In the past few years, parameters such as growth rate and organic and inorganic carbon production have been widely investigated in calcifiers in order to predict the impact of ocean acidification (Buitenhuis et al. 1999; Clark and Flynn, 2000; Riebesell et al. 2000; Rost et al. 2002; Sciandra et al. 2003; Iglesias-Rodriguez et al. 2008; Shi et al. 2009; Barcelos e Ramos et al. 2010; Müller et al. 2010; Langer et al. 2009). In this study, the response regarding those parameters is in agreement with the diverse responses already described for *E. huxleyi* strains in the literature. We found a minor effect of elevated pCO₂ on the physiology of *E. huxleyi* RCC1216. Cell density was not significantly changed at elevated pCO₂ (Student *t* test $P < 0.1$) (Fig. 1), and growth rate remained unchanged with $\mu = 0.79 \pm 0.02$ and 0.76 ± 0.02 for cultures subjected to control and elevated pCO₂, respectively (Student *t* test $P < 0.1$).

Likewise, no significant change in production of particulate organic (PPOC) and inorganic (PPIC) carbon (Fig. 2a) (Student *t* test $P < 0.1$) and PIC/POC ratio (Fig. 2b) (Student *t* test $P < 0.4$) was observed in cultures subjected to low or high pCO₂. A recent work by Langer et al. (2009), dealing with the response of *E. huxleyi* strain RCC1216 to changing seawater carbonate chemistry, showed both a decrease in PIC cellular content and production in cultures subjected to a pCO₂ of 729 μ atm. The reasons for the discrepancy might relate to differences in culture conditions. Whereas cultures were pre-adapted to experimental conditions for 12 generations by Langer et al. (2009), we only subjected our cultures to an 8-day treatment without acclimation period.

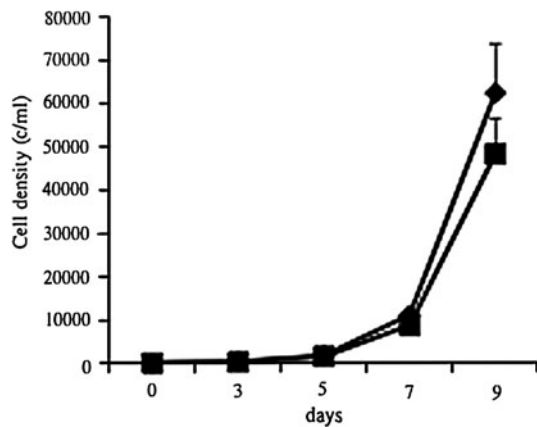


Fig. 1 Cell density (number of cells. ml⁻¹) in *E. huxleyi* cultures subjected to present (diamond) and 2100 (square) predicted pCO₂ condition on an 8-day incubation period

Molecular responses to decreasing pH

In this study, we have used gene expression profiling to explore some molecular mechanisms that may underlie tolerance to future ocean acidification conditions. The genes investigated were related to the Ca²⁺ metabolism or speciation and DIC transport (see Table 2). The expression of up to 11 genes was followed and mainly showed no significant response to elevated pCO₂/lower pH (Fig. 3).

Although all of the genes of interest were previously described as potentially associated with the biomineralization process in a wide range of organisms, from pelagic coccolithophores (Wahlund et al. 2004; Dyhrman et al. 2006; Soto et al. 2006; Quinn et al. 2006; Richier et al. 2009) to benthic invertebrates (Zoccola et al. 1999; Moya et al. 2008), this is the first time that genes related to the SLC4 family have been investigated for their role in the carbonate chemistry of marine calcifiers and their response to environmental threats (e.g. ocean acidification). The family of SLC4 anion exchanger (AE) proteins includes the Na⁺-independent Cl⁻/HCO₃⁻ exchanger that is critical for the regulation of several physiological processes including intracellular pH (pH_i) and the HCO₃⁻/CO₃²⁻ balance in eukaryotic cells (Alper et al. 2001; Alper 2009; Romero et al. 2004). In *E. huxleyi*, an inhibitor-based study has

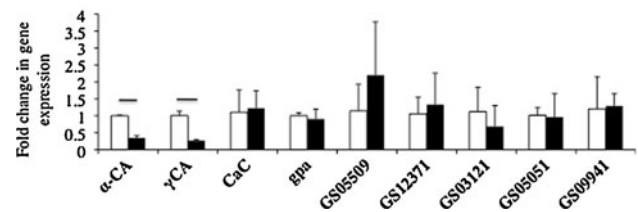


Fig. 3 Fold change in gene expression with increasing pCO₂ from present (white bars) to predicted 2100 pCO₂ value (black bars). Bars indicate a significant difference between pCO₂ treatments (ANOVA one-way, $P < 0.05$). Data are presented as means \pm standard deviations for three independent cultures

indicated the involvement of the Cl⁻/HCO₃⁻ exchangers in DIC uptake (Herfort et al. 2002), and von Dassow et al. (2009) recently suggested that SLC4 homolog might function to maintain optimal balance of pH and carbonate/bicarbonate in the coccolith deposition vesicle for calcification. However, SLC4-like homolog ability to transport either HCO₃⁻ or CO₃²⁻ in *E. huxleyi* is still unknown (Mackinder et al. 2010). In the present study, no significant variation in the Cl⁻/HCO₃⁻ exchanger homolog's gene expression was observed under tested conditions (Fig. 3; ANOVA one-way, $P < 0.5$). Those results suggest either undetectable or no effect of the tested pH/pCO₂ perturbation on targeted genes. In fact, the low variations in the carbonate system (i.e. ΔHCO₃⁻/CO₃²⁻) highlighted in our experiment (see Table 1) could explain the unchanged Cl⁻/HCO₃⁻ exchanger gene expression. In sea urchin larvae subjected to similar pCO₂ condition, unchanged mRNA transcript levels of the Cl⁻/HCO₃⁻ exchanger were also reported (Todgham and Hofmann 2009).

Looking further into genes related to DIC transport proteins, the expression of α- and γ-CA genes was investigated as part of this study. Information on the molecular characterization of CA is scarce in phytoplankton, and especially in coccolithophores. The involvement of these two CAs in biomineralization has yet to be discussed but given the role of CA in acid/base compensation, it is probable that one or more of them may be regulated by the acid–base imbalance that could have resulted from the decrease in pH. Despite up to 12 CA transcripts recently identified in *E. huxleyi* by von Dassow et al. (2009), little

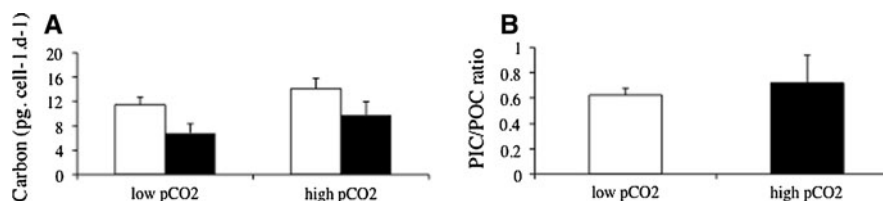


Fig. 2 Production of particulate inorganic carbon (PPIC) and organic carbon (PPOC) per cell and per day (carbon (pg. cell⁻¹ d⁻¹)) (a) and PIC/POC ratio (b). Black and white bars represent PIC and POC,

respectively. Data are presented as means \pm standard deviations for three independent cultures

information is available about the localization and role of these genes in coccolithophores. A first attempt to characterize CA isoforms in *E. huxleyi* was performed by Soto et al. (2006) who speculated on a location for γ -EhCA2 protein in the coccolith vesicle with a 25-fold up-regulation in γ -EhCA2 transcripts under calcifying versus non-calcifying condition. The role of γ -CA isoform in calcification was also supported by previous studies with up-regulated transcripts in cultures where calcification was stimulated by phosphate-depletion (Quinn et al. 2006) and significantly higher during the light period in calcifying cells (RCC1216 strain) compared to non-calcifying ones (RCC1217) (Richier et al. 2009).

In the present study, the CA sequences were searched against databases for their conserved domains (see Table 2). A conserved domain homolog to an “alpha_CA_prokaryotic like” carbonic anhydrase was detected in α -CA. In this sub-family, the enzyme has been reported to be part of the organic matrix layer in shells. Other members of this family may be involved in maintaining pH balance, in facilitating transport of CO₂ or H₂CO₃, or in sensing carbon dioxide levels in the environment. We thus deliberately chose here to analyze γ -CA isoform, for the reasons outlined earlier, and α -CA isoform for its widespread distribution in several kingdoms of life (vertebrates, invertebrates, bacteria, and some chlorophytes) and its role in biomineralization of benthic organisms (Moya et al. 2008). We showed that α - and γ -CA genes were down-regulated when exposed to decreasing pH resulting in a fold change of 2.3 and 3.8, respectively (ANOVA one-way, $P < 0.05$) (Fig. 3). A previous study on *E. huxleyi* intracellular CA activity showed no clear trend with increasing pCO₂ (from 36 ppmv up to 1,800 ppmv) (Rost et al. 2003). However, the measurements in that study did not discriminate between CA isoform classes and it might be that the regulation of CA genes is class specific.

Additionally, it has been previously suggested that the CA enzymes and SLC4 anion exchangers may interact (Vince and Reithmeier, 2000; Sterling et al. 2001, 2002; Morgan et al. 2007). In mammalian cell lines, the cytoplasmic carboxy terminal of AE1 has a carbonic anhydrase II (CAII) binding site that upon inhibition reduces AE1-mediated Cl⁻/HCO₃⁻ exchange by 50–60% (Sterling et al. 2001). Carbonic anhydrase IV (CAIV) interaction sites have also been identified on the extracellular surface of AE1 isoform. According to the authors, CAII and IV would increase HCO₃⁻ transport by altering localized HCO₃⁻ levels enhancing the HCO₃⁻ concentration gradient (McMurtrie et al. 2003). A similar function may occur in coccolithophores with CA interacting with the Cl⁻/HCO₃⁻ exchanger facilitating the conversion of HCO₃⁻ into CO₂ at the cytosolic face of the plasma membrane decreasing the local concentration of HCO₃⁻ at the cytosolic transport site

(Mackinder et al. 2010). In our study, we could speculate that increasing pCO₂ inhibits both α - and γ -CA genes transcription and consequently the activity of their relative proteins. Thus, no interactions with SLC4 homologs would occur, which is reflected by unchanged Cl⁻/HCO₃⁻ exchanger transcript level under experimental condition. In the same way, the unchanged Ca²⁺-channel (CAC) and *gpa* transcript level, in response to tested conditions, would suggest no reduced capacity of the protein to transport or bind Ca²⁺ to the sites of calcification and supports the unchanged calcification rate observed in the tested cultures. However, the regulation of gene of interest related proteins was not investigated as part of this study. Simultaneous analyses of both transcripts and corresponding proteins are required to conclude on any proteins regulation and function.

In conclusion, all the results shown by our study constitute new elements in molecular exploration of genes involved in *E. huxleyi* early response to an acidifying world. No major physiological changes were observed in the chosen strain in response to ocean acidification and only CA isoforms, among the tested genes, appeared significantly regulated under the experimental condition. However, no significant variation in expression of most of the genes might either suggest (1) no major effect of the near future pCO₂ condition in the ocean on the tested strain or (2) no direct role of the targeted genes in early response to high pCO₂/low pH. An exhaustive investigation into *E. huxleyi* transcriptome would be required to identify all the genes/cellular mechanisms involved in response to pCO₂/pH variation.

Nonetheless, the fact high pCO₂-treatment did not induce major molecular and physiological changes in this calcified phytoplankton suggests that it may have the capacity to adapt to future ocean acidification.

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