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Impact of low pH/high pCO_2 on the physiological response and fatty acid content in diatom *Skeletonema pseudocostatum*

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pCO_2/pH perturbation experiments were carried out under two different pCO_2 *levels to evaluate effects of CO_2-driven ocean acidification on semi-continuous cultures of the marine diatom* Skeletonema pseudocostatum *CSA48. Under higher* pCO_2*/lowered pH conditions, our results showed that CO_2-driven acidification had no significant impact on growth rate, chlorophyll-*a*, cellular abundance, gross photosynthesis, dark respiration, particulate organic carbon and particulate organic nitrogen between CO_2-treatments, suggesting that* S. pseudocostatum *is adapted to tolerate changes of ~0.5 units of pH under high* pCO_2 *conditions. However, dissolved organic carbon (DOC) concentration and DOC/POC ratio were significantly higher at high* pCO_2*, indicating that a greater partitioning of organic carbon into the DOC pool was stimulated by high CO_2/low pH conditions. Total fatty acids (FAs) were significantly higher under low* pCO_2 *conditions. The composition of FAs changed from low to high* pCO_2*, with an increase in the concentration of saturated and a reduction of monounsaturated FAs. Polyunsaturated FAs did not show significant differences between pCO_2 treatments. Our results lead to the conclusion that the balance between negative or null effect on* S. pseudocostatum *ecophysiology upon low pH/high pCO_2 conditions constitute an important factor to be considered in order to evaluate the global effect of rising atmospheric CO_2 on primary productivity in coastal ocean. We found a significant decrease in total FAs, however no indications were found for a detrimental effect of ocean acidification on the nutritional quality in terms of essential fatty acids.*

Keywords: Diatom, low pH, high CO_2, fatty acid, DOC, photosynthesis, elemental composition

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INTRODUCTION

Diatoms dominate the phytoplankton community in coastal ecosystems, contributing to ~20% of global primary production (Nelson *et al.*, 1995). Because of their large size and silica ballast, they contribute a major fraction of the downward flux of particulate organic matter into deep ocean (Alldredge & Jackson, 1995) and therefore constitute major players in carbon sequestration from the atmosphere to the deep ocean (Boyd *et al.*, 2010). Chain-forming centric diatoms are the most successful group of eukaryotic primary producers in productive upwelling coastal ecosystems, supporting higher trophic levels. A prerequisite for their high growth rates is an efficient and regulated acquisition of inorganic carbon (Ci) that compensates for the catalytic inefficiency of the enzyme Ribulose-1,5-biphosphate carboxylase/oxygenase (RUBISCO) (Burkhardt *et al.*, 2001).

Ocean acidification (OA) is a consequence of increased inorganic carbon content of the ocean surface water due to rising atmospheric CO_2. The associated drop in the average surface water pH from ~8.2 to 7.8 represents one of the most rapid OA events on earth over the past 300 Myr (Caldeira & Wickett, 2003). It is a controversial issue whether high CO_2/low pH in seawater would significantly promote growth and primary productivity. Responses of diatoms to high pCO_2 and decreased pH are likely to be species-specific, with potential winners, neutral and losers (Gao & Campbell, 2014). Diatoms can downregulate the activity of extracellular carbonic anhydrase (Burkhardt *et al.*, 2001), but they differ in their CO_2 concentrating mechanisms (CCMs) (Hopkinson *et al.*, 2013). Photosynthetic responses to enhanced CO_2 under OA are remarkably diverse, and there is

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a large variability both between and within taxonomic groups. Nevertheless, despite the growing body of literature on the topic, clear trends in the photosynthetic responses of phytoplankton to elevated CO_2 have not emerged, and positive effects, if any, are small (Mackey *et al.*, 2015).

In addition to growth and primary production, the elemental composition of phytoplankton might vary under OA. Increasing CO_2 can lead to increased particulate organic carbon (POC) content relative to N and P quotas, i.e., higher C:N and C:P under elevated CO_2 concentrations (Riebesell *et al.*, 2007; Feng *et al.*, 2008). Nevertheless, systematic increases of particulate organic matter (POM) and C:N ratios have not been observed in response to rising pCO_2 and temperature (Burkhardt *et al.*, 1999; Wohlers-Zöllner *et al.*, 2011). Moreover, the current evidence demostrated that large differences in the elemental composition of marine phytoplankton can arise from nutrient limitation (Geider & LaRoche, 2002), physical factors (Laws & Bannister, 1980; Burkhardt *et al.*, 1999) and interspecific variability among algal species with different C:N:P requirements (Geider & LaRoche, 2002). Elevated pCO_2 and temperature may lead to a greater partitioning of organic carbon into the dissolved organic carbon (DOC) pool (Kim *et al.*, 2011).

Other important proxies for food quality in marine food webs are fatty acid (FA) content and composition. Polyunsaturated fatty acids (PUFA) are considered especially important as they represent essential FAs that cannot be synthesized *de novo* by heterotrophic consumers (Müller-Navarra *et al.*, 2004). Phytoplankton production of PUFA is highly dependent on the algal physiology and nutrients, and therefore on the environmental conditions (Klein Breteler *et al.*, 2005; Leu *et al.*, 2013). There is a paucity of information on how OA might impact FA contents. Here, we examine the effects of high CO_2/low pH and excess nutrients on growth and physiological rates, elemental composition, carbon partitioning and nutritional quality of *Skeletonema pseudocostatum* in order to evaluate: (1) how the growth rate, photosynthesis, respiration and carbon partitioning of *Skeletonema pseudocostatum* is affected by a drop of ~0.5 units of pH caused by elevated pCO_2 levels and (2) how physiological rates may affect the fatty acid content under condition of increased CO_2/lowered pH.

MATERIALS AND METHODS

Semi-automatic system for seawater carbonate manipulation

Experiments were conducted using a semi-automatic mesocosm system for seawater carbonate chemistry manipulation at Calfuco Marine Laboratory in south-central Chile (39°78′S, 73°39′W). CO_2-enriched seawater was produced by bubbling seawater with air-CO_2 mixtures, following the method described by Torres *et al.* (2013). The system uses mass flow controllers (MFC) to blend atmospheric air with ultra-pure CO_2 (i.e. research grade) to produce different pCO_2 levels (see Table 1). The seawater was continuously bubbled with either ambient or enriched pCO_2-air. The high level of pCO_2, 1123 µatm, corresponded approximately with projected atmospheric levels between years 2100 and 2150 under the RCP 8.5 scenario (Meinshausen *et al.*, 2011). Air/CO_2 mixtures were produced using a bulk technique, where dry air with pure CO_2 were supplied to seawater using an air mass flow controller (MFC) (Aalborg, model GFC; http://www.aalborg.com) and a CO_2 MFC (Aalborg, Model GFC). Dry and filtered air (through a 1 µm particulate filter) was generated by compressing atmospheric air (117 psi) using an oil-free, 4 piston air compressor (Schulz, model MSV12). Pressure in the air and pure CO_2 were maintained at ~10 psi. Air flow in MFC was set manually to 5 l min^{-1} for treatment and CO_2 flow was set manually to 4.25 ml min^{-1} to produce the high CO_2 treatment. The CO_2 of blended gas was monitored to allow fine regulation of CO_2 through MFC to reach target pCO_2 in seawater. The pCO_2 monitoring system was based on a CO_2 analyser (Qubit System, model S151), primarily for measuring the CO_2 content in the air-CO_2 mixture.

Culture conditions

Skeletonema pseudocostatum (CSA 48, non axenic) was isolated from Yaldad Bay, southern Chile (43.1°S–73.7°W) in March 2009 and obtained from the COPAS Sur-Austral strain collection (http://www.ficolab.cl/), at the Department of Botany, Concepción University. Cells were grown and acclimated in autoclaved (1 l) and filtered (0.1 µm) natural seawater (salinity: 29.8 PSU) within autoclaved glass bottles (1 L) at the same temperature (14.7 ± 0.6°C), light intensity (195 µmol m^{-2} s^{-1}) and at a 16/8 light/dark cycle. Light was measured with a sensor Li-192SA (Li-Cor) and was provided by cold white LED tubes (22 Watts, 6000 K). The time of sampling was kept throughout the acclimatization and experiment. For the acclimatization, seawater was enriched to f/2 medium (Guillard & Ryther, 1962). Cells were maintained in exponential growth phase using a semicontinuous culture. To maintain balanced exponential growth, cultures were diluted with fresh medium every 3–4 days, keeping cell concentrations $<43 \times 10^4$ cell ml^{-1} during the acclimation. During the first (21–25 November) and second round (26–29 November) of the algal acclimation under low pCO_2 levels, mean pH values ranged between 8.106 and 8.230, respectively (Table 1). In cultures under high pCO_2 conditions, mean pH values ranged between 7.676 and 7.654, respectively. Cultures were acclimated to the respective pH/pCO_2 values for 10 generations.

After acclimatization, cells from respective pH/pCO_2 treatments were inoculated in autoclaved polycarbonate carboys filled with 20 l of autoclaved seawater (29 November) at the same temperature and light intensity, and carboys were positioned randomly in the experimental system. Carboys were closed with rubber stoppers pierced with glass capillaries for inlet and outlet of air/CO_2 mixture. Four carboys were used for low pCO_2 and four for high pCO_2 treatments, while two control carboys without cells were followed for monitoring abiotic changes in carbonate system parameters. Under these culture conditions, cells were grown for ~6 generations. Samples for carbonate system parameters were taken on 1 and 3 December (Table 1). The harvesting of samples was carried out on 3 December. Cell concentrations at the time of sampling were ~22×10^4 cell ml^{-1} at low pCO_2 and ~14.5×10^4 cell ml^{-1} at high pCO_2. During the experimental period, the photosynthetic activity and cell density increased, leading to an increase in pH (0.11 unit under low CO_2 and 0.018 unit under high CO_2). The concentration of DIC and

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Table 1. Cell concentration of the marine diatom *S. pseudocostatum* and average (± SE, N = 4) of carbonate system parameters during (A) acclimation and (B) experimental period under two different pCO_2/pH levels; Low pCO_2 (L); High pCO_2 (H).

Date	Period	Treatment [pCO_2]	Cell ml^{-1}	pH (NBS)	DIC ($\mu mol\ kg^{-1}$)	TA ($\mu mol\ kg^{-1}$)	pCO_2 (μatm)	HCO_3^- ($\mu mol\ kg^{-1}$)	CO_2 ($\mu mol\ kg^{-1}$)
(A)									
21–25.11.2013	1st round	L	28×10^4	8.106 ± 0.07	2129 ± 53	2308 ± 75	495 ± 84	1983 ± 43	19 ± 2.9
26–29.11.2013	2nd round	L	32×10^4	8.230 ± 0.27	2006 ± 77	2253 ± 120	393 ± 209	1819 ± 161	15 ± 8.3
21–25.11.2013	1st round	H	43×10^4	7.676 ± 0.04	2434 ± 82	2462 ± 95	1543 ± 126	2320 ± 79	59 ± 3.9
26–29.11.2013	2nd round	H	40×10^4	7.654 ± 0.03	2249 ± 101	2273 ± 110	1492 ± 63	2143 ± 97	58 ± 2.6
(B)									
29.11.2013	E	L-Control		8.133 ± 0.003	2034 ± 35	2205 ± 56	439 ± 12	1889 ± 36	17 ± 3.5
		H-Control		7.666 ± 0.03	2148 ± 92	2164 ± 18	1394 ± 75	2047 ± 39	53 ± 2.6
1.12.2013	E	L	60×10^3	8.205 ± 0.009	1898 ± 195	2109 ± 213	343 ± 27	1745 ± 177	13 ± 11
		H	39×10^3	7.689 ± 0.021	1740 ± 324	1931 ± 219	1166 ± 351	1658 ± 231	41 ± 13
3.12.2013	E	L	22×10^4	8.249 ± 0.03	1312 ± 165	1584 ± 8.3	212 ± 30	1201 ± 149	8.3 ± 1.2
		H	14×10^4	7.684 ± 0.01	1707 ± 124	1739 ± 126	1050 ± 40	1628 ± 188	41 ± 1.6

DIC, Dissolved inorganic carbon; TA, total alkalinity; HCO_3^-, bicarbonate; CO_2, carbon dioxide.
First round of acclimation was carried out between 21 and 25 November and second round between 26 and 29 November. Experiment started on 29 November and ended on 3 December.

total alkalinity (TA) decreased by 35 and 28% under low CO_2 conditions and 20 and 19% under high CO_2 conditions, respectively.

Chemical analysis

Samples for nutrient analysis (NO_3^-, NO_2^-, PO_4^{3-} and Si $(OH)_4$) were taken every day during the acclimation and experimental periods (Table 2). Samples were filtered (GF/F) and frozen (−20°C) until analysis following Strickland & Parsons (1968). Daily pH samples were collected in 50 ml syringes and immediately transferred to a 25 ml thermostatted cell at 25.0 ± 0.18°C for standardization, and measurements were conducted with a pH electrode with a glass combined double Ag/AgCl junction (Metrohm model 6.0258.600) calibrated with standard National Bureau of Standards (NBS) calibration buffer Metrohm® 4 (Code 6.2307.200), 7 (Code 6.2307.210) and 9 (Code 6.2307.220). The estimated analysis error for this analysis was estimated as <0.01 pH. For dissolved inorganic carbon (DIC) and DOC determination, separate 30 ml subsamples were collected with a sterile syringe and filtered through a Swinex containing a GF/F filter that had been precombusted for 4–5 h at 450°C directly into 40 ml glass 200 Series I-CHEM® vials. For DIC analyses, the septa of vials were exchanged for butyl rubber septa to prevent diffusion of CO_2 (DOE, 1994). Samples for DIC analysis were preserved with 50 µl of a saturated solution of mercuric chloride (DOE, 1994). Immediately after opening the sample bottle, a digital syringe withdrew a small amount of sample (0.5 ml), acidified it with 10% phosphoric acid and subsequently measured the evolved CO_2 with a LICOR 6262 non-dispersive infrared gas analyser. Certified seawater reference materials from A. Dickson were used to ensure the quality of DIC determination by preparing a calibration curve covering the range of DIC from 200–2000 $\mu eq\ l^{-1}$ (Dickson *et al.*, 2003), with a resulting precision averaging ≈ 0.1% (range 0.05–0.5%). Temperature and salinity data were used to calculate the other carbonate system parameters (e.g. pCO_2, HCO_3). Analyses were performed using CO_2SYS software for MS Excel (Pierrot *et al.*, 2006) set with Mehrbach solubility constants (Mehrbach *et al.*, 1973) refitted by Dickson & Millero (1987). The $KHSO_4$ equilibrium constant determined by Dickson (1990) was used for all calculations.

For POC and particulate organic nitrogen (PON) analysis, a subsample (1 l) was filtered through combusted (4–5 h at 450°C) GF/F filters to concentrate particles. Filters were dried at ~60°C for 24 h and held in a desiccator until analysed. DOC samples were bubbled with CO_2-free nitrogen for 7 min to ensure complete removal of DIC. DOC and POC measurements were conducted by the G.G. Stable Isotope Hatch, Laboratories at the University of Ottawa, Canada, with an analytical precision of 2%. All DOC and POC samples were run on an total inorganic carbon-total organic carbon (TIC-TOC) analyser (OI Analytical Analyzer, Model 1030). Data were normalized using internal standards.

Determination of fatty acids (FAs) was conducted from water samples onto filtered through MFS GF/F filters to concentrate particles. Saturated (SAFA) and unsaturated fatty acids (MUFA: monounsaturated and PUFA: polyunsaturated) were measured on separate filters dried at 50°C for 24 h and held in a desiccator until analysed. The fatty acid concentrations were measured after extraction and methylation

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Table 2. Inorganic nutrient concentrations (±SE, N = 4) and nutrient ratios (±SE, N = 4) during (A) acclimation and (B) experimental period and under two different CO_2 concentrations; Low pCO_2 (L); High pCO_2 (H).

Date	Period	Treatment [pCO_2]	NO_3^- [µM]	PO_4^{-3} [µM]	NO_2^- [µM]	$Si(OH)_4$ [µM]	N:P	Si:N
(A)								
21–25.11.2013	1st round	L	315 ± 37	12 ± 0.8	1.6 ± 0.5	88 ± 29	26 ± 2.5	0.28 ± 0.09
26–29.11.2013	2nd round	L	244 ± 13	10 ± 2.3	2.2 ± 1.4	97 ± 53	25 ± 7	0.39 ± 0.2
21–25.11.2013	1st round	H	345 ± 25	12 ± 1.2	1.5 ± 0.8	99 ± 32	29 ± 3.5	0.28 ± 0.08
26–29.11.2013	2nd round	H	268 ± 47	12 ± 3.5	1.7 ± 0.8	105 ± 30	22 ± 3.7	0.38 ± 0.06
(B)								
1.12.2013	E	L	282 ± 10	11.09 ± 0.5	1.01 ± 0.5	84.1 ± 6	25.5 ± 0.7	0.31 ± 0.02
		H	280 ± 19	11.79 ± 0.2	0.9 ± 0.7	85.73 ± 3	23.8 ± 1.3	0.33 ± 0.02
3.12.2013	E	L	196 ± 25	10.5 ± 0.8	3.7 ± 2.2	48.4 ± 12	16.5 ± 3.7	0.27 ± 0.07
		H	197 ± 45	11.43 ± 0.8	0.85 ± 0.7	65.24 ± 12	17.4 ± 4.7	0.33 ± 0.1

First round of acclimation was carried out between 21 and 25 November and second round between 26 and 29 November. Experiment started in 29 November and ended on 3 December.

(Kattner & Fricke, 1986) with a gas chromatograph Perkin Elmer Sigma 300 equipped with a programmable temperature vaporizer-injector, a fused Omegawax 53 capillary column, and a flame ionization detector.

Biological measurements

Concentrations of *S. pseudocostatum* cells were determined from samples preserved with acid Lugol's. Cell counting was performed using a Neubauer hemocytometer and optical microscope (OLYMPUS CX31). Specific growth rates were calculated using an exponential curve fitted for each replicate of the treatments. The slope of the exponential curve was considered as the growth rate for each pCO_2 treatment. Cell size was measured using an epifluorescence microscope (OLYMPUS IX51), choosing at random 40 individual cells for each replicate of each pCO_2 treatment. For biovolume calculation we used a cylinder geometric model according to Sun & Liu (2003). For determination of total chl-*a*, samples were filtered onto GF/F filters and stored at −20°C. Chl-*a* was extracted in acetone 95% and measured with a fluorometer (Trilogy Model 7200–040, Turner Designs, Sunnyvale, CA, USA) before and after acidification (Lorenzen, 1966). Chl-*a*, POC, PON and DOC concentrations were normalized per cell (pg $cell^{-1}$), assuming that changes in Chl-*a*, N and C from other sources (e.g. lysis) were not significant in our cultures. Gross photosynthesis (GP) and dark respiration (DR) rates were estimated from changes observed in dissolved oxygen concentrations after incubating *in vitro* in light and dark bottles (Strickland, 1960). Water from three 20 l carboys was transferred to 125 ml borosilicate (i.e. gravimetrically calibrated) using a silicone tube; three time-zero bottles, three light bottles and three dark bottles per replicate were used. The light and dark bottles were incubated at the same temperature and light regime as the 20 l polycarbonate carboy cultures for 6 h; the dissolved oxygen from time-zero bottles was measured at the beginning of the experiment. Dissolved oxygen was measured using a fibre optical oxygen transmitter (Optical Oxygen meter FIBOX, PreSens®). The average of coefficient of variation for replicates was 0.8%. Net photosynthesis (NP) was calculated as the difference in the dissolved oxygen concentration between 'light' incubated samples and 'time zero' samples. Dark respiration (DR) was calculated as the difference between 'dark' incubated samples and 'time zero' samples. Dark respiration rates are expressed as a negative O_2 flux. Gross photosynthesis (GP) was calculated as the difference between NP and DR (Gaarder & Gran, 1927). GP and DR per cell were expressed in fmol $cell^{-1}$ h^{-1}.

Statistical analysis

In order to evaluate algal responses to experimental conditions, Student's t-test was used for each chemical and biological parameter. The Shapiro–Wilk statistic (Shapiro & Wilk, 1965) was used to check the data for normality distribution and a Levene test checked the homoscedasticity.

RESULTS

Carbonate system

During the experimental period (day 3.12.2013), the carbonate system parameters under simulated CO_2-driven ocean acidification showed significant differences in the pCO_2 concentration ($t = -11.52$, df = 6, $P < 0.0001$) and pH values ($t = 26.05$, df = 6, $P < 0.0001$) between both CO_2 treatments (Table 1). Significant differences were also found in HCO_3^- ($t = -2.88$, df = 6, $P = 0.028$) and CO_2 ($t = -11.44$, df = 6, $P < 0.0001$). There were no significant differences in DIC concentration ($t = -2.41$, df = 6, $P = 0.05$) between CO_2 treatments, although it was close to the minimal acepted probability. As expected, no significant differences were found in the total alkalinity (TA) ($t = -1.22$, df = 6, $P = 0.26$).

Biological parameters

The impact of high pCO_2 on *S. pseudocostatum* physiology was assessed by comparing 12 parameters between low and high pCO_2 treatments (Figure 1). Although cell volume was higher at low pCO_2 level (442 ± 103 µm^3) compared with high pCO_2 level (361 ± 42 µm^3), there were no significant differences between pCO_2 treatments ($t = 1.38$, df = 6, $P > 0.05$). There were also no significant differences in growth rates ($t = 0.53$, df = 6, $P > 0.05$), cell-normalized Chl-*a* ($t = 2.28$, df = 6, $P > 0.05$), cellular abundance ($t = 2.16$, df = 4, $P > 0.05$), gross photosynthesis and respiration rates ($t = 2.08$, df = 4, $P > 0.05$; $t = -0.74$, df = 1.1, $P > 0.05$, respectively), POC ($t = -0.08$, df = 5, $P > 0.05$), and

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PON ($t = -0.75$, df = 5, $P > 0.05$) C:N ratio ($t = 1.57$, df = 5, $P > 0.05$), R:P ratio ($t = -1.72$, df = 1.1, $P > 0.05$). In contrast, DOC/POC ratio and DOC per cell significantly increased at high pCO_2 levels ($t = -3.91$, df = 2.4, $P < 0.05$; $t = -2.51$, df = 6, $P < 0.05$, respectively). In percentage terms, DOC/POC and DOC per cell increased by 40.4 and 48.4% at high pCO_2, respectively.

The high pCO_2 treatment exhibited significant differences in FA concentration and composition. Total FA concentration was significantly decreased ($t = 5.69$, df = 6, $P < 0.05$) under high pCO_2 ($0.208 \pm 0.04\ \mu g\ l^{-1}$) compared with low pCO_2 ($0.07 \pm 0.01\ \mu g\ l^{-1}$) (Figure 2A). The relative amount of SAFAs was significantly higher ($t = -4.53$, df = 6, $P < 0.05$) and the amount of MUFAs lower ($t = 16.1$, df = 6, $P < 0.05$) at high pCO_2 compared with low pCO_2 treatment. In contrast, polyunsaturated fatty acids (PUFA) did not show significant differences between low pCO_2 ($t = -2.2$, df = 6, $P > 0.05$) (Figure 2B). This is exemplified by some essential fatty acids such as docosahexaenoic acid (DHA, 22:6) and arachidonic acid (ARA, 20:4), which showed similar concentrations under low ($0.0008\ \mu g\ l^{-1}$ and $0.001\ \mu g\ l^{-1}$, respectively) and high ($0.0002\ \mu g\ l^{-1}$ and $0.002\ \mu g\ l^{-1}$, respectively) pCO_2 treatments.

DISCUSSION

In the coastal domain, surface waters are commonly exposed to levels in partial pCO_2 higher than expected at equilibrium with the atmosphere (Hofmann *et al.*, 2011; Yu *et al.*, 2011), which is mostly associated with biological processes such as daily time cycles of photosynthesis and respiration (Shamberger *et al.*, 2011) and oceanographic processes such as riverine discharges and coastal upwelling events (Cao *et al.*, 2011). In consequence, diatoms inhabiting coastal areas may be capable of tolerating larger ranges of pH and pCO_2. However, this high variability also may mean that planktonic organisms inhabiting coastal regions are already operating at the limits of their physiological tolerances. Thus, future OA may drive the physiology of these marine organisms up to the edge in their tolerance range.

The present study showed that high pCO_2 had no significant impact on cell volume, growth rate, abundance, chl-*a*, C/N ratio, and photosynthesis rates in the diatom

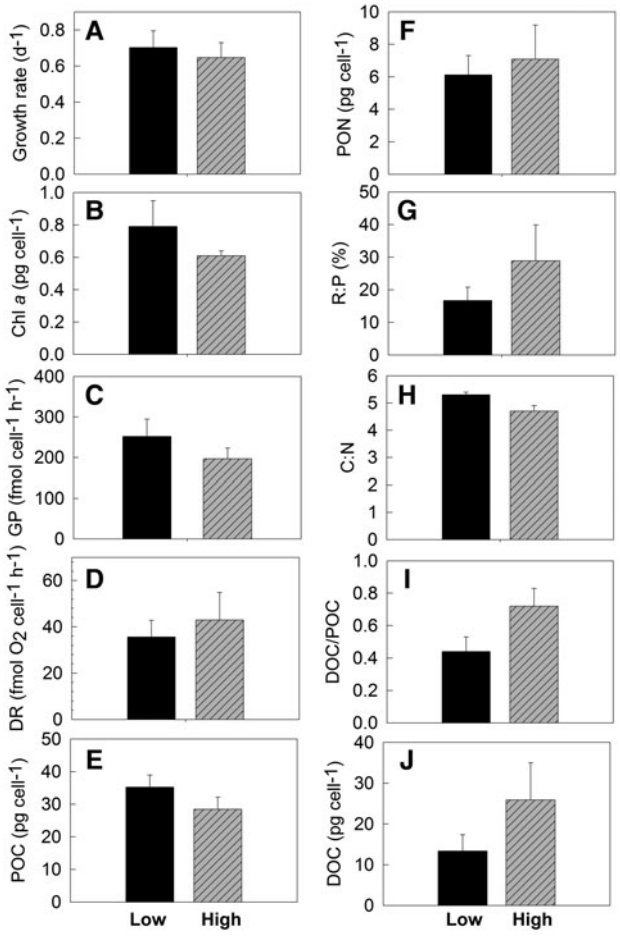

Fig. 1. Physiological parameters: growth rate, gross photosynthesis (GP), dark respiration (DR), cellular concentration of Chl-*a*, particulate organic carbon (POC) and particulate organic nitrogen (PON), respiration losses in % (R:P ratio), C/N ratio, carbon partitioning (DOC/POC ratio) and DOC concentration measured during semi-continuous cultures of *S. pseudocostatum* grown at exponential growth phase under two different CO_2 concentrations; Low pCO_2: 212 ± 30 µatm; High pCO_2: 1050 ± 40 µatm. Error bars indicate standard errors.

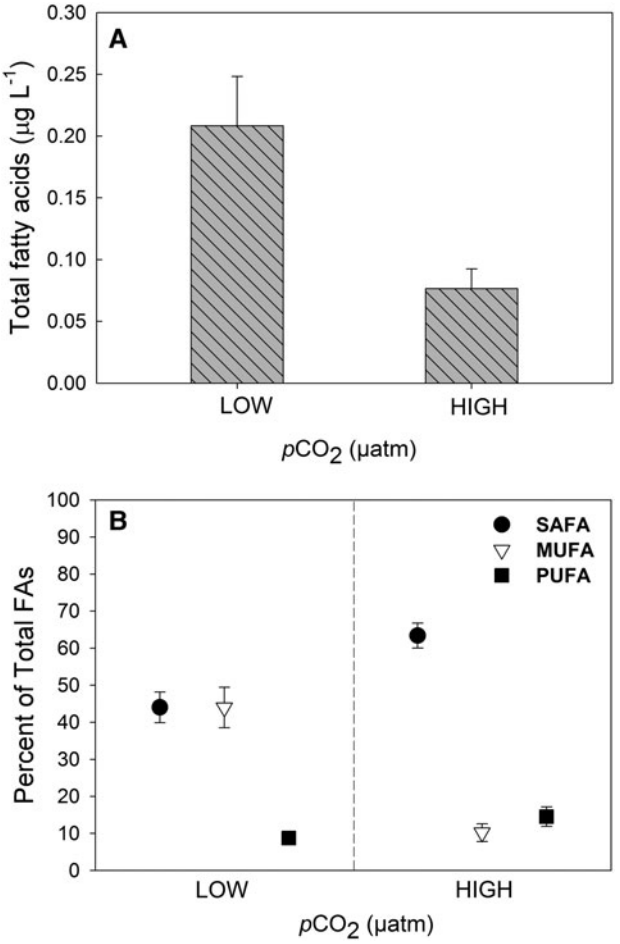

Fig. 2. Fatty acid concentration and composition of *Skeletonema pseudocostatum* cultured at different pCO_2 treatments. (A) Total fatty acid and (B) percentage of saturated (SAFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids relative to total fatty acids during the exponential growth phase cultured at low pCO_2: 212 µatm (N = 3) and high pCO_2: 1050 µatm (N = 3) treatments. Error bars indicate standard errors.

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S. pseudocostatum (Figure 1). Although some studies have indicated that elevated pCO_2 is expected to have a stimulative effect on growth rates (Kim *et al.*, 2006; King *et al.*, 2011; Low-Décarie *et al.*, 2011) and primary productivities (Riebesell *et al.*, 2007), many other studies have shown that elevated pCO_2 concentration did not affect the growth rates in diatom monocultures, including *S. costatum* (Chen & Gao, 2003, 2004), *Thalassiosira pseudonana* (Crawfurd *et al.*, 2011; King *et al.*, 2015), and *Chaetoceros brevis* (Boelen *et al.*, 2011), or in diatom-dominated natural phytoplankton assemblages (Tortell *et al.*, 2000). Furthermore, Berge *et al.* (2010) have also shown that low pH/high pCO_2 conditions do not affect the growth rate and production rates of eight species of phytoplankton representing diatoms, dinoflagellates, cryptophytes and haptophytes. Berge *et al.* (2010) also showed that 49 strains of a total 33 species of phytoplankton exhibited similar growth rates at pH ~7.8 compared with more alkaline levels of pH (8.1–8.2), suggesting that marine phytoplankton are adapted to tolerate the modelled average pH drop due to ocean acidification by the year 2100. Our findings are consistent with these findings, suggesting that *S. pseudocostatum* tolerates well changes of 0.5 units of pH due to manipulated pCO_2 levels.

Diatoms have the capacity for simultaneous transport of CO_2 and HCO_3^- during photosynthesis and increase their affinities of both transport systems in response to diminishing supply of carbon substrate (Burkhardt *et al.*, 2001). However, the proportion at which CO_2 and HCO_3^- are taken up and the extent to which Ci uptake is affected by changes in CO_2 supply vary among phytoplankton species (Nimer *et al.*, 1998; Elzenga *et al.*, 2000; Burkhardt *et al.*, 2001; Rost *et al.*, 2003). For example, *Phaeodactylum tricornutum* takes up CO_2 preferentially over HCO_3^- from seawater whereas *Thalassiosira weissflogii* takes up HCO_3^- preferentially to CO_2 under depletion of CO_2; thus for *Phaeodactylum tricornutum* one would expect a pronounced response in photosynthetic C fixation under enhanced CO_2. However, *P. tricornutum* showed increased photosynthetic electron transport rates, but no change or very modest increases in growth (5–13%; Wu *et al.*, 2010; Li *et al.*, 2014) or carbon fixation (Burkhardt *et al.*, 2001) under high pCO_2. In our experiment (3 December), significant differences in HCO_3^- concentrations were observed between pCO_2 treatments (Table 1), suggesting that both free CO_2 and HCO_3^- were probably an important inorganic carbon source for *Skeletonema pseudocostatum* cells.

Rising pCO_2 might affect primary producers in terms of saving energy required for active inorganic carbon acquisition, whereas low pH could potentially increase metabolic demand to maintain celular homeostasis relative to the increased acidity (Gao *et al.*, 2012). Nevertheless, our results did not show significant differences in the respiration rates between low and high pCO_2 levels (Figure 1), which suggest no significant changes in the balance between production and consumption (see R:P ratio) between low and high pCO_2 levels.

Furthermore, our results also showed that there were no significant differences in the elemental composition (C:N ratio) between the pCO_2 treatments (Figure 1), indicating that increasing CO_2 would not increase the POC:PON ratio. In contrast, DOC per cell and the DOC/POC ratio were significantly higher at high pCO_2, suggesting that extracellular carbon release relative to particulate carbon production increased under elevated pCO_2. The extracellular release of photosynthesis products is especially common during nutrient-depleted growth conditions (Kim *et al.*, 2011; Borchard & Engel, 2012), since phytoplankton exude DOC to the environment to reduce the energy costs associated with storing surplus compounds (Wood & Van Valen, 1990). However, it has also been reported to occur independent of nutrient availability (Hessen *et al.*, 2004; Hessen & Anderson, 2008) and under continuous CO_2 enrichment (Song *et al.*, 2013). In our experiment cells were under nutrient-replete conditions (Table 2) and continuous CO_2 enrichment, so the DOC increase under high pCO_2 conditions seems not to be nutrient dependent. The higher DOC release triggered by elevated CO_2 is consistent with other studies carried out in natural phytoplankton assemblages (Riebesell *et al.*, 2007; Kim *et al.*, 2011; Engel *et al.*, 2013) and monocultures (Engel *et al.*, 2004; Borchard & Engel, 2012). Most experimental studies have suggested that greater assimilation of carbon into organic matter at high CO_2 levels may increase the extracellular organic matter release from phytoplankton. However, our study showed that gross photosynthesis, POC concentration and C:N ratio were not significantly different between pCO_2 treatments, which rules out this process. Enrichment of CO_2 and increased acidity have also been found to stimulate photorespiration in diatoms *T. pseudonana* and *P. tricornutum* (Gao *et al.*, 2012), a process by which oxygen is consumed and CO_2 released under light conditions, as well as a process by which glycolate is lost to the outside medium as an excreted product. It has been suggested that photorespiration is important for maintaining electron flow to prevent photoinhibition under stress conditions (i.e. high CO_2 levels) (Heber *et al.*, 1996), as well as under drought stress (Wingler *et al.*, 1999). In addition, the formation of photorespiratory metabolites, such as glycine, serine and glycolate has also been measured under salt stress in C3 plants (Downton, 1977; Di Martino *et al.*, 1999). Therefore, photorespiration may play a protective role under stress conditions and consequently contribute partially to the release of DOC triggered under high pCO_2 levels. We also recognize that our experiment was not axenic and consequently the increase of DOC in the bottles could also be produced by lysis and transformation of POC to DOC by bacteria or chemical hydrolysis (Carlson, 2002). The greater partitioning of organic carbon into the DOC pool under high CO_2/low pH conditions may have implications for long-term C storage in aquatic ecosystems, as DOC components can be both important precursors in the creation of large particle aggregates and also in the formation of recalcitrant DOC (Engel 2002) via heterotrophic metabolism in the upper layers of aquatic ecosystems (Jiao *et al.*, 2010). Important questions arise regarding the increase of DOC production under acidification condition scenarios, e.g. (i) How the increase of DOC will affect the C cycling through bacteria; (ii) the formation of transparent exopolymer particles and consequently, the export of organic matter to the deep ocean (Passow, 2002); and (iii) nutrient competition between bacteria and phytoplankton.

The effects of lowered pH and increased pCO_2 was also evaluated on nutritional quality of *S. pseudocostatum*. Total FAs were significatively different between pCO_2-treatments, being 63.26% higher under low pCO_2 compared with high pCO_2 treatment (Figure 2A). These results agree with other studies that showed a significant decline in total FAs of the

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centric diatom *T. pseudonana* under elevated CO_2 (750 μatm) compared with present-day CO_2 (380 μatm) (Rossoll *et al.*, 2012). These authors found that the relative amount of SAFAs was significantly higher at high CO_2 and a ~20% decline in the relative amount of PUFAs. Our findings showed that the relative amount of SAFAs was significantly higher (44 to 63%) and MUFAs significantly lower (44 to 10%) at high pCO_2 compared with the low pCO_2 treatment. In contrast, lowered pH and elevated CO_2 did not affect the contribution of PUFAs to total fatty acids significantly (Figure 2B). The important increase of saturated and decrease of monounsaturated FA contents and total FAs under acidification may affect the transfer of lipids to higher trophic levels. However, the nutritional quality in terms of essential FAs remains unchanged. Most lipids consist mainly of hydrocarbon chains with varying numbers of double bonds. SAFA have hydrocarbon chains with single bonds while polyunsaturated FAs contain more than one double bond and include many compounds essential for higher trophic levels, such as for copepod egg production, hatching and maturity (Jonasdottir *et al.*, 2005; Klein Breteler *et al.*, 2005). Our findings are consistent with other studies showing no detrimental effects of high pCO_2 on the nutritional quality in terms of essential fatty acids (Leu *et al.*, 2013). Many other responses can be expected in the total FAs and components depending on phytoplankton functional group and species. For example, declining PUFA content at elevated pCO_2 was reported for the Antarctic prasinophyte *Pyramimonas gelidicola* (Wynn-Edwards *et al.*, 2014), the sea-ice diatom *N. lecointei* (Torstensson *et al.*, 2013) and the diatom *Cylindrotheca fusiformis* (Bermúdez *et al.*, 2015). No detectable differences attributable to pCO_2 treatment in the fatty acids component has been observed for the centric diatoms *T. pseudonana* and *T. weissflogii*, the green algae *Dunaliella salina*, the euryhaline microalgae *Chlorella autotrophica* (King *et al.*, 2015) and the dinoflagellate *Gymnodinium* sp. (Wynn-Edwards *et al.*, 2014). In contrast, high CO_2 increased the accumulation of total lipids and polyunsaturated fatty acids in the chlorophytes *Scenedesmus obliquus* and *Chlorella pyrenoidosa* (Tang *et al.*, 2011). The cellular processes involved in FA synthesis under changing pH and pCO_2 levels are not fully understood. Because pH might act as a regulation signal for the formation of cell membranes by controlling the production of its synthesizing enzymes (Young *et al.*, 2010), it has been proposed that a higher saturation degree at high CO_2 levels (increase of SAFA) may be a mechanism to control the internal cell-pH because a membrane built of short chain FA is less fluid and permeable to CO_2 (Rossoll *et al.*, 2012).

Our findings suggest that growth, gross photosynthesis and C:N ratio were not necessarily connected to CO_2-driven changes in composition and content of FAs in *S. pseudocostatum*. In agreement, other studies showed that CO_2-driven changes in the growth rate of the centric diatom *Thalassiosira weissflogii* were not reflected by significant changes in the elemental composition and fatty acid composition, which indicate bidirectional responses to changes in CO_2 (King *et al.*, 2015). Since ocean acidification has the potential to alter phytoplankton biochemistry, our results highlight the importance for understanding the cellular processes involved in FA synthesis under rising CO_2/decreasing pH, which will finally determine the carbon transfer efficiency to higher trophic levels in a changing ocean.

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