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Responses of the diatom *Asterionellopsis glacialis* to increasing sea water CO_2 concentrations and turbulence

Francesca Gallo[1,*], Kai G. Schulz[2], Eduardo B. Azevedo[1], João Madruga[1], Joana Barcelos e Ramos[1]

[1]Institute for Agricultural and Environmental Research and Technology of the Azores, University of Azores, Rua do Capitão d'Ávila, Pico da Urze 970-0042 Angra do Heroísmo, Açores, Portugal

[2]Centre for Coastal Biogeochemistry, School of Environmental Science and Management, Southern Cross University, PO Box 157, Lismore, NSW 2480, Australia

ABSTRACT: Greenhouse gas emissions, such as carbon dioxide (CO_2), lead to enhanced atmospheric and surface ocean temperatures. At the same time, CO_2 equilibrates between the atmosphere and the surface ocean, resulting in lower seawater pH. The changes in physical and chemical properties of the ocean potentially affect marine primary producers in various ways. A number of researches have addressed the effects of ocean acidification on marine phytoplankton. However, phytoplankton responses to combined effects are still poorly understood. Here, we chose the cosmopolitan chain-forming diatom *Asterionellopsis glacialis* to assess the combined effect of ocean acidification and carbonation (~420 to 2800 µatm) and water motion on its physiological rates. At current CO_2 levels, we observed an increase in growth rates of *A. glacialis* accompanied by a prevalence of longer chains (>6 cells) under enhanced water motion. However, at increasing CO_2 levels (up to ~2800 µatm) and decreasing pH values, enhanced water motion significantly decreased growth rates, chain length and organic matter production of *A. glacialis*. Thus, our study suggests that even though *A. glacialis* benefited from enhanced water motion at present CO_2 concentration, at higher CO_2 levels, the more unstable environment magnified the stress caused by acidification. If in the future the ocean surface layer will be more frequently exposed to storm and wind events, then phytoplankton communities might be more sensitive to lower pH, with potential consequences for community composition and productivity.

KEY WORDS: Phytoplankton · Climate change · Growth rate · Ocean acidification · CO_2 · Water motion

INTRODUCTION

Since the beginning of the Industrial Revolution, human activities such as the burning of fossil fuels, changes in land use and deforestation have led to a considerable increase in carbon dioxide (CO_2) concentrations in the atmosphere (Le Quéré et al. 2015), from pre-industrial levels of approximately 280 µatm, to 400 µatm today. Importantly, present CO_2 levels are above the upper limit of the last 2 million years (Hönisch et al. 2009). This increase translates into a number of global-scale changes, such as modifications in the carbon cycle and the rise of global average temperature, with potential consequences at the ecosystem level (IPCC 2014). The ocean is the largest sink of carbon and heat, mitigating both the effects of anthropogenic CO_2 rise in the atmosphere (Sarmiento & Gruber 2002) and global warming. During the past century, more than one-third of the CO_2 emitted into the atmosphere has been absorbed by the ocean (Sabine et al. 2004), causing unprecedented changes in seawater carbon-

*Corresponding author: effegal@gmail.com

ate chemistry (Pespeni et al. 2013). As a consequence, dissolved inorganic carbon (DIC) and bicarbonate ions (HCO_3^-) have increased in the surface ocean while carbonate ions (CO_3^{2-}) and pH (Wolf-Gladrow et al. 1999) have decreased. In this respect, surface ocean pH has already decreased by 0.1 units since the Industrial Revolution (Rhein et al. 2013) and, in a 'business as usual' CO_2 emission scenario, is projected to drop by an additional 0.4 units by the year 2100 (Raupach et al. 2007, IPCC 2014), shifting the carbonate equilibrium of the seawater towards more acidic conditions, while CO_2 is expected to reach up to 1000 µatm (IPCC 2014).

At the same time, the increase in global average temperature is having a number of effects on the ocean. Heating of the ocean surface acts to enhance stratification of surface waters, shoaling the upper mixed layer (Doney 2006, Rost et al. 2008) and stabilizing the water column. Concomitantly, the amount of total atmospheric water vapor over the global oceans has increased (Trenberth 2005). Enhanced ocean temperatures and water vapor levels in the atmosphere have been related to more intense tropical cyclone winds (Elsner et al. 2008), longer storm lifetimes and greater storm intensities (Emanuel 2005). In fact, there has been an increase of at least 100% in tropical cyclone frequency during the last century in the Atlantic area (Mann & Emanuel 2006, Holland & Webster 2007); moreover, a significant correlation between frequency and duration of hurricanes and increases in sea surface temperatures in the North Atlantic have been reported by Webster et al. (2005). Evidence of global increases in wind speed and wave height in the past 25 yr were also recorded by global satellites (Young et al. 2012, Bertin et al. 2013). Thus, the future surface ocean is expected to be characterized by an overall water column stabilization accompanied by an increase in destabilization events (D'Asaro 2014), such as enhanced wave height and shear turbulence in the very surface layer (Moum & Smyth 2001). These changes in physical properties of the ocean, caused by anthropogenic disturbances, may interact with ocean acidification and have the potential to affect community composition of marine phytoplankton assemblages with potential feedbacks to marine biogeochemical element cycling.

In the last decades, numerous experiments have addressed potential responses of marine phytoplankton to elevated CO_2 levels (e.g. Gao et al. 2012a). Particularly, the potential influence of enhanced CO_2 concentrations on calcifying phytoplankton (coccolithophores), which are thought to be highly sensitive to ocean acidification, has been investigated intensively (Meyer & Riebesell 2015). Furthermore, a number of studies have assessed the physiological response of the silica-shielded phytoplankton, i.e. diatoms (i.e. Sarthou et al. 2005, Roberts et al. 2007, Sobrino et al. 2008, Tortell et al. 2008, Trimborn et al. 2009, Gao & Campbell 2014, Hennon et al. 2014, King et al. 2015, Wu et al. 2015, Clement et al. 2016), which contribute up to 40% of marine primary production in the ocean and are responsible for a large portion of organic carbon export to the deep ocean (Ducklow et al. 2001, Scott 2005, Hopkinson et al. 2011). Under present-day carbonate chemistry, growth of diatom species can be limited by the availability of inorganic carbon (Gao & Campbell 2014). To circumvent or reduce this limitation, diatoms have developed active carbon concentrating mechanisms (CCMs), which include carbonic anhydrase and putative bicarbonate transporters (Hopkinson et al. 2016). CCMs elevate the CO_2 concentration at the site of Rubisco (the enzyme responsible for the first step of photosynthesis) and account for a significant part of cellular energy expenditure (Raven 1991, Beardall & Giordano 2002, Crawfurd et al. 2011, Hopkinson et al. 2013, Johnson et al. 2013, Gao et al. 2014, Raven et al. 2014, Matsuda et al. 2017). Thus, increasing CO_2 availability may be beneficial for diatoms which can down-regulate the CCM capacity (Giordano et al. 2005, Hopkinson et al. 2011) and save energy to reallocate for growth and carbon fixation (Hein & Sand-Jensen 1997, Wu et al. 2010). On the other hand, the reduction in pH associated with the rise of CO_2 levels might negatively affect cell physiology, namely by increasing energy requirements to counterbalance the external pH decrease (Wu et al. 2010). Specifically, the increase of H^+ concentrations may affect intracellular pH, membrane potential, energy portioning and enzyme activity (Beardall & Raven 2004, Riebesell 2004, Giordano et al. 2005), with consequences for growth rates and photosynthesis. In terms of increased growth and carbon fixation rates, and photosynthetic efficiency, responses of diatoms to varying CO_2 levels range from positive (Chen & Gao 2003, 2004, Wu et al. 2010, McCarthy et al. 2012, Barcelos e Ramos et al. 2014) to absent or negative (Burkhardt et al. 1999, Crawfurd et al. 2011), even under comparable experimental conditions. However, most of these studies focused on the response of diatoms within a narrow range of CO_2 concentrations, but higher levels might be useful to understand their physiological thresholds (Barry et al. 2010).

As outlined above, in the future ocean, phytoplankton will live in a shallower mixed layer, with increased likelihood of destabilization events (D'Asaro et al. 2014) caused by increased frequency and intensity of storms. However, the effects of these destabilizations of the surface ocean on phytoplankton have been poorly investigated (Garrison & Tang 2014). Especially, most of the knowledge concerning the effects of turbulent conditions on phytoplankton have focused on phytoplankton communities (Estrada et al. 1987, Peters & Marrasé 2000, Barton et al. 2014, Zhou et al. 2015), with only a few studies investigating the response of individual species (Berdalet & Estrada 1993, Berdalet et al. 2007, Garrison & Tang 2014). Enhanced turbulence can benefit larger phytoplankton cell size (lower surface to volume ratio) by increasing nutrient flux to the cell surface, since the water motion has the potential to overcome the limits of diffusive transport of nutrients (Gavis 1976, Lazier & Mann 1989, Karp-Boss et al. 1996, Peters et al. 2006, Guasto et al. 2012). Concomitantly, disrupting the diffusive boundary layer that surrounds the cells exposes them to the chemical conditions of bulk seawater. As most field studies have difficulties in separating the effects of turbulence from other variables, such as temperature, light or nutrient concentrations, it is necessary to conduct controlled laboratory experiments. Therefore, in order to better understand the potential response of phytoplankton to ocean turbulence, it is first necessary to assess the response to constant levels of turbulence motions (stationary in time and homogeneous in space by means of orbital shakers or oscillating grid devices) in the laboratory (Guadayol et al. 2009). In our study, we chose the cosmopolitan chain-forming diatom *Asterionellopsis glacialis*, to test the combined effects of constant water motion and CO_2 on the physiological rates of this species in terms of cell growth, organic matter production, cellular elemental quotas and chain formation. Three CO_2 levels were chosen to range from present day to future projections (400, 780 and 1110 µatm) and 1 level at 2800 µatm was added to assess potential physiological thresholds.

MATERIALS AND METHODS

Experimental set-up

Monospecific cultures of the cosmopolitan *Asterionellopsis glacialis* (strain CCMMG_1 isolated in 2011 from offshore Terceira Island, Azores) were grown in sterile filtered (0.2 µm) North Atlantic seawater (salinity 35.9) enriched with approximately 4.5 µmol l^{-1} of phosphate, 64 µmol l^{-1} of nitrate and silicate and trace metals and vitamins found in the f/20 medium (Guillard & Ryther 1962). Dilute batch cultures were grown at 20°C under constant light intensity (incident photon flux density of [range] ~170 ± 10 µmol m^{-2} s^{-1}) and a 14:10 h light:dark cycle. *A. glacialis* was tested under repose (control treatment) and constant water motion (enhanced turbulence treatment) conditions and at 4 CO_2 levels (ranging approximately from 420 to 2800 µatm, corresponding to pH_T [total scale] values between ~8.04 and 7.30), resulting in a total of 8 treatments. All cultures were gently rotated vertically (20 times) daily in order to avoid sedimentation. Moreover, cultures grown in the enhanced turbulence treatment were additionally exposed to constant mixing generated by an orbital shaker with 220 rpm speed. Under this condition, cells were kept in suspension at their position while being exposed to constant water motion. Before the start of the experiment, all cultures were acclimated to the experimental conditions for at least 18 generations, ensuring exponential growth throughout the experiment. Particularly, 2 consecutive dilute batch cultures (9 generations for each with a difference in growth rate between the 2 consecutive batch cultures and the experiment always lower than 10%) were maintained at low abundance (average final concentration of <15 000 cells ml^{-1}), to avoid significant changes in seawater carbonate chemistry speciation (DIC consumption <5% as recommended by La Roche et al. 2011). All 8 experimental treatment levels (4 CO_2 levels × 2 water motion regimes) were conducted in triplicate (for more details, see Table 1).

Cell numbers and growth rates

The abundance of *A. glacialis* and the number of cells in each chain were determined from Lugol-fixed samples (2% final concentration) by means of an inverted microscope (Nikon Eclipse TS100, 200× magnification). Cells were harvested during the exponential phase of growth, and cellular growth rates (µ) were determined following Levasseur et al. (1993) as:

$$\mu = \ln(Cf/Ci)/\Delta t \quad (1)$$

where Cf and Ci represent the final and the initial cell concentrations, respectively, and Δt corresponds to the growth period in days.

Table 1. Carbonate chemistry parameters at the beginning and end of the experiment and their averages. Superscripts 'a' and 'b' indicate measured parameters and calculated values, respectively. TA: total alkalinity, pH_T: pH on a total scale, DIC: dissolved inorganic carbon

Treatment	CO_2 treatment	pCO_2 (µatm)[b]	Avg pCO_2 (µatm)[b]	TA (µmol kg^{-1})[a]	pH_T[a]	HCO_3^- (µmol kg^{-1})[b]	CO_3^{2-} (µmol kg^{-1})[b]	CO_2 (µmol kg^{-1})[b]	DIC (µmol kg^{-1})[b]	DIC drawdown (%)[b]
Initial	1	512		2357	7.960	1946	164	16	2127	
	2	1007		2355	7.701	2108	98	32	2238	
	3	1435		2356	7.560	2171	73	46	2290	
	4	3845		2362	7.154	2283	30	123	2467	
Final control	1	330	421	2382	8.123	1823	224	11	2057	3.3
	1	328	420	2375	8.134	1816	224	11	2050	3.6
	1	325	419	2375	8.127	1813	225	10	2048	3.7
	1	308	410	2378	8.146	1797	233	10	2039	4.1
	2	544	775	2376	7.941	1977	160	17	2154	3.8
	2	521	764	2397	7.960	1980	167	17	2164	3.3
	2	545	776	2375	7.940	1976	159	17	2153	3.8
	2	484	746	2372	7.983	1940	172	16	2182	4.9
	3	825	1130	2366	7.781	2075	116	26	2217	3.2
	3	863	1149	2369	7.764	2087	112	28	2227	2.8
	3	789	1112	2367	7.798	2065	120	25	2211	3.5
	3	852	1144	2369	7.769	2084	113	27	2225	2.8
	4	1960	2903	2379	7.428	2235	57	63	2354	3.4
	4	1848	2846	2355	7.448	2206	59	59	2324	4.1
	4	1763	2804	2376	7.471	2219	62	63	2337	3.6
	4	1975	2909	2376	7.424	2231	56	59	2350	4.2
Final enhanced turbulence	1	337	424	2378	8.115	1828	221	11	2059	3.2
	1	347	429	2362	8.102	1827	214	11	2052	3.5
	1	328	420	2372	8.124	1814	223	11	2048	3.7
	2	648	828	2365	7.873	2016	139	21	2176	2.8
	2	717	862	2370	7.836	2045	130	23	2198	1.8
	2	600	804	2369	7.903	1999	148	19	2166	3.2
	3	805	1120	2376	7.792	2077	119	26	2222	3.0
	3	782	1109	2375	7.803	2069	122	25	2216	3.2
	3	800	1118	2379	7.795	2078	120	26	2223	2.9
	4	1718	2782	2384	7.492	2222	64	55	2341	3.9
	4	1736	2790	2373	7.486	2214	63	56	2332	4.3
	4	1711	2778	2374	7.492	2212	64	55	2331	4.3

Carbonate chemistry manipulation, measurements and calculations

The carbonate system manipulation of sterile filtered North Atlantic seawater was done by combined additions of HCl and $NaHCO_3$ (Gattuso et al. 2010), to maintain constant total alkalinity (Schulz et al. 2009). Carbonate chemistry of the media and of the cultures was calculated with the software CO2SYS (Lewis & Wallace 1998), using measured total alkalinity, pH_T, temperature, salinity, phosphate and silicate, and the equilibrium constants determined by Mehrbach et al. (1973) as refitted by Dickson & Millero (1987). The pH_T was measured with an electrode cell (WTW 340i pH meter) and calibrated with a Tris sea water buffer (provided by A. Dickson) according to Dickson et al. (2007). Total alkalinity was measured by potentiometric titration according to Dickson et al. (2003) using a Metrohm 848 Titrino Plus equipped with Metrohm 869 Compact Sample changer. Total alkalinity measurements were corrected with certified reference material (Dickson 2010) at about 20 µmol kg^{-1} accuracy and 2 µmol kg^{-1} precision. Measured (total alkalinity, pH_T, temperature, salinity, phosphate and silicate) and calculated (pCO_2, HCO_3^-, CO_3^{2-}, CO_2 and DIC) parameters are expressed in Table 1 at the beginning and the end of the experiment (time of the harvesting) and as an average of both, which represents each treatment throughout the experiment.

Cellular element quotas and production rates

At the end of the experiment, samples for cellular particulate organic carbon (POC), nitrogen (PON)

and phosphorus (POP) were gently filtered (200 mbar) onto pre-combusted GF/F filters (6 h, 450°C) and stored at −20°C until analyses. POC and PON filters were then dried at 60°C for 4 h, packed in tin boats and analyzed following Sharp (1974) using an elemental analyzer (Thermo Flash EA) coupled to an isotope ratio mass spectrometer (Thermo Delta V Plus) via a Thermo Conflo V manifold. POP filters were oxidized to dissolved inorganic phosphorus with potassium peroxydisulfate and measured colorimetrically by means of a spectrophotometer (Cary 50) following Hansen & Koroleff (1999). POC, PON and POP production rates were calculated by multiplying cellular quotas with the corresponding growth rates (μ).

Dissolved inorganic phosphate and silicate

Samples for the determination of dissolved inorganic phosphate and silicate concentrations were taken at the beginning and at the end of the experiment. Samples were filtered through 0.2 μm polyethersulfone syringe filters and stored at −20°C until analysis. Concentrations of dissolved inorganic silicate and phosphate were determined spectrophotometrically (Cary 50 Probe, Varian) following Hansen & Koroleff (1999) and used in the calculation of the carbonate chemistry.

Statistical analysis and growth rate fitting procedures

Statistical significance was assessed by means of 1-way analysis of variance (ANOVA) with the program SigmaPlot 11.5, and values of $p < 0.01$ by Tukey test were considered to be significant.

RESULTS

The diatom *Asterionellopsis glacialis* was grown at increasing CO_2 concentrations under relatively stable and enhanced turbulence conditions. The carbonate chemistry data are presented in Table 1. For simplicity and clarity, the CO_2 treatments are represented in the graphs by the average of the initial and the final values of calculated pCO_2 as values representative of each treatment throughout the experiment.

Growth rate (μ)

Under stable conditions (control treatment), growth rates of *A. glacialis* peaked at a CO_2 level of ~780 μatm. Particularly, we observed a significant ($p < 0.001$) 26% increase from ~420 to ~780 μatm, followed by a 40 and 22% decrease between ~780 and ~1110 μatm and ~1110 and ~2800 μatm, respectively. When exposed to constant water motion (enhanced turbulence treatment), *A. glacialis* appeared to shift the optimum growth rate towards lower CO_2 concentrations. Growth rates decreased by 25% from CO_2 levels of ~420 to ~780 μatm, followed by a further decrease of 48 and 21% from ~780 to 1110 μatm and from ~1110 to ~2800 μatm, respectively. When comparing the control with enhanced turbulence treatments, we observed that at present-day CO_2 concentrations, growth rate was 29% lower ($p < 0.01$) under control conditions. At enhanced CO_2 levels, however, cell division rates of *A. glacialis* were higher under control (19, 41 and 44% at CO_2 concentrations of 780, 1100 and 2800 μatm, respectively) than under enhanced turbulence conditions (Fig. 1).

Cell quotas and organic matter production

In the control treatment, cellular element quotas for POC, PON and POP were not significantly ($p > 0.01$) affected by increasing CO_2 levels from ~420 to ~780 μatm (Fig. 2). However, when CO_2 rose from approximately 780 to ~1110 μatm, cellular element quotas were significantly increased ($p < 0.01$). This increase of cellular element quotas was not sustained and decreased to the initial values from ~1110

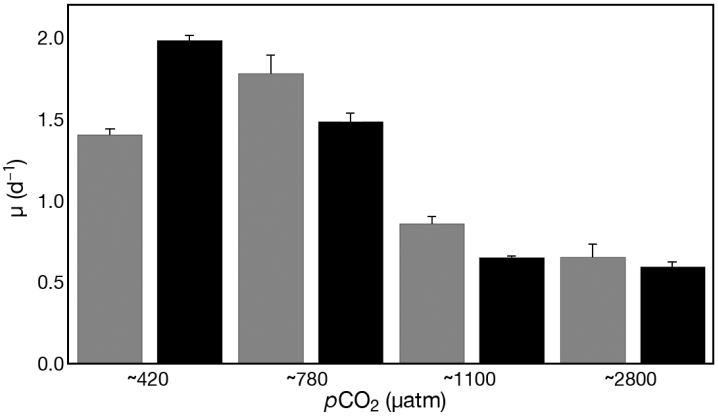

Fig. 1. Growth rates (μ) of *Asterionellopsis glacialis* at increasing CO_2 levels (pCO_2) for the control (grey bars) and enhanced turbulence (black bars) treatments with standard deviation

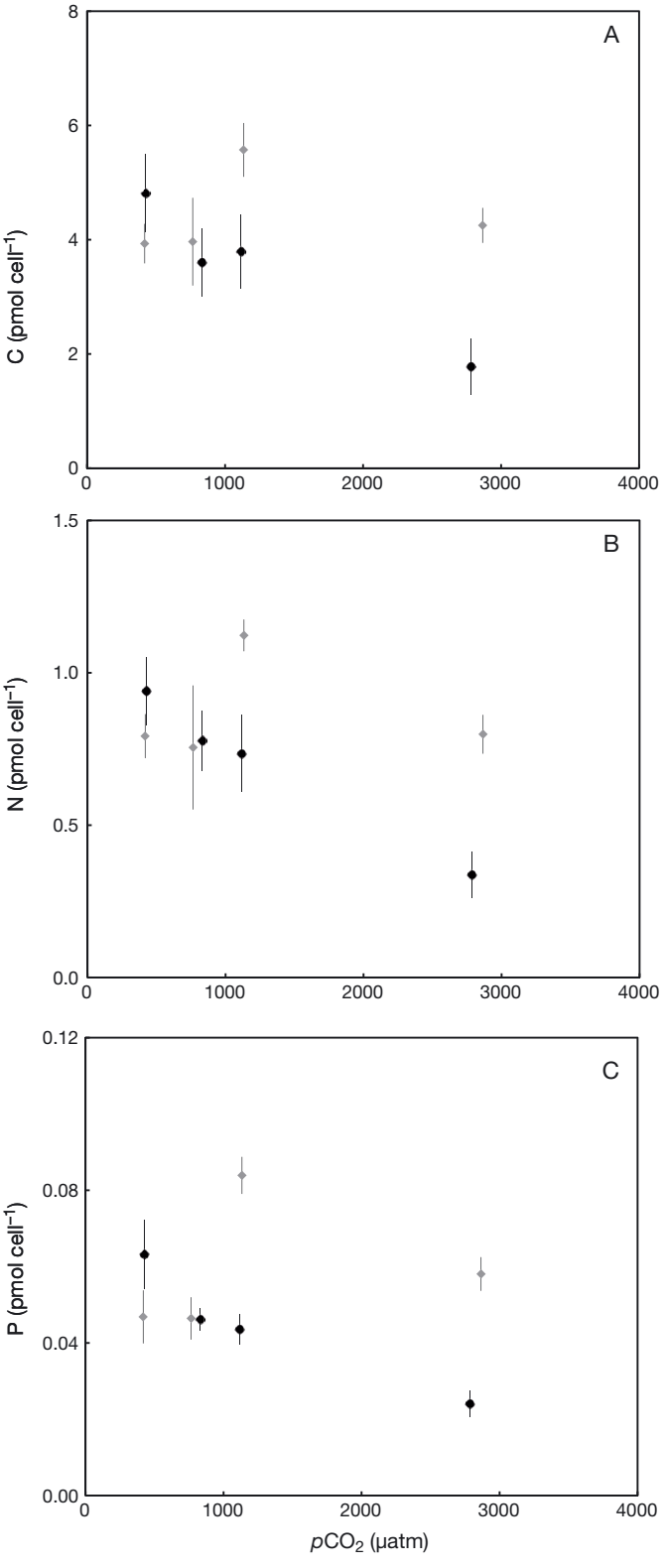

Fig. 2. Cellular element quotas of *Asterionellopsis glacialis* at increasing CO_2 levels (pCO_2) under control (grey diamonds) and enhanced turbulence treatments (black diamonds), expressed as means and standard deviation: (A) carbon, (B) nitrogen and (C) phosphorus

to ~2850 µatm. Cells in the enhanced turbulence treatment decreased their cellular element quotas linearly with increasing CO_2 levels. Comparing the control with the enhanced turbulence treatment, we observed that at CO_2 levels higher than ~780 µatm, cellular element quotas were significantly reduced by turbulence (reduction of 32, 35 and 48% of POC, PON and POP quotas, respectively, at CO_2 levels of ~1110 µatm and a reduction of ~58% POC, PON and POP quotas at CO_2 levels of ~2800 µatm, $p < 0.01$; Fig. 2).

Carbon, nitrogen and phosphorus production rates of the particulate organic matter followed the trends of growth rates (Fig. 3). In the control treatment, carbon and nitrogen production rates peaked at a CO_2 concentration of ~780 µatm and phosphorus production rates at ~1110 µatm. When *A. glacialis* was exposed to constant water motion (enhanced turbulence treatment), the organic matter production rates were highest at present-day CO_2 levels (~420 µatm). Thus, within similar CO_2 concentrations, the constant water motion appeared beneficial only at present-day CO_2, while at higher CO_2 levels, organic matter production rates were negatively affected (Fig. 3).

Particulate organic matter ratios (C:N, N:P, C:P) were not significantly affected by varying CO_2 levels nor by turbulence ($p > 0.01$; Fig. 4).

Relative number of cells per chain

The relative number of cells chain^{-1} was strongly influenced by both increasing CO_2 levels and enhanced turbulence (Fig. 5). Under control conditions, the relative abundance of colonies with more than 6 cells increased from 7% at ~420 µatm to 60% at ~2850 µatm, at the expense of chains composed of 1 to 3 cells, which decreased linearly from 67% in the same CO_2 interval to 15%; meanwhile no change in the abundance of chains comprising 4 to 6 cells was observed. However, the opposite trend was observed upon exposure to enhanced turbulence. In fact, the relative abundance of short chains (<3 cells) increased significantly ($p < 0.01$) with increasing CO_2 concentrations from 2% at ~420 µatm to 98% at ~2850 µatm; furthermore, chains with >6 cells were only observed at CO_2 levels of ~420 and ~780 µatm, decreasing from 83 to 22%. The observed trends in colony size were not related to cell size, since no remarkable difference in cell size (frustule size) was found between treatments (data not shown).

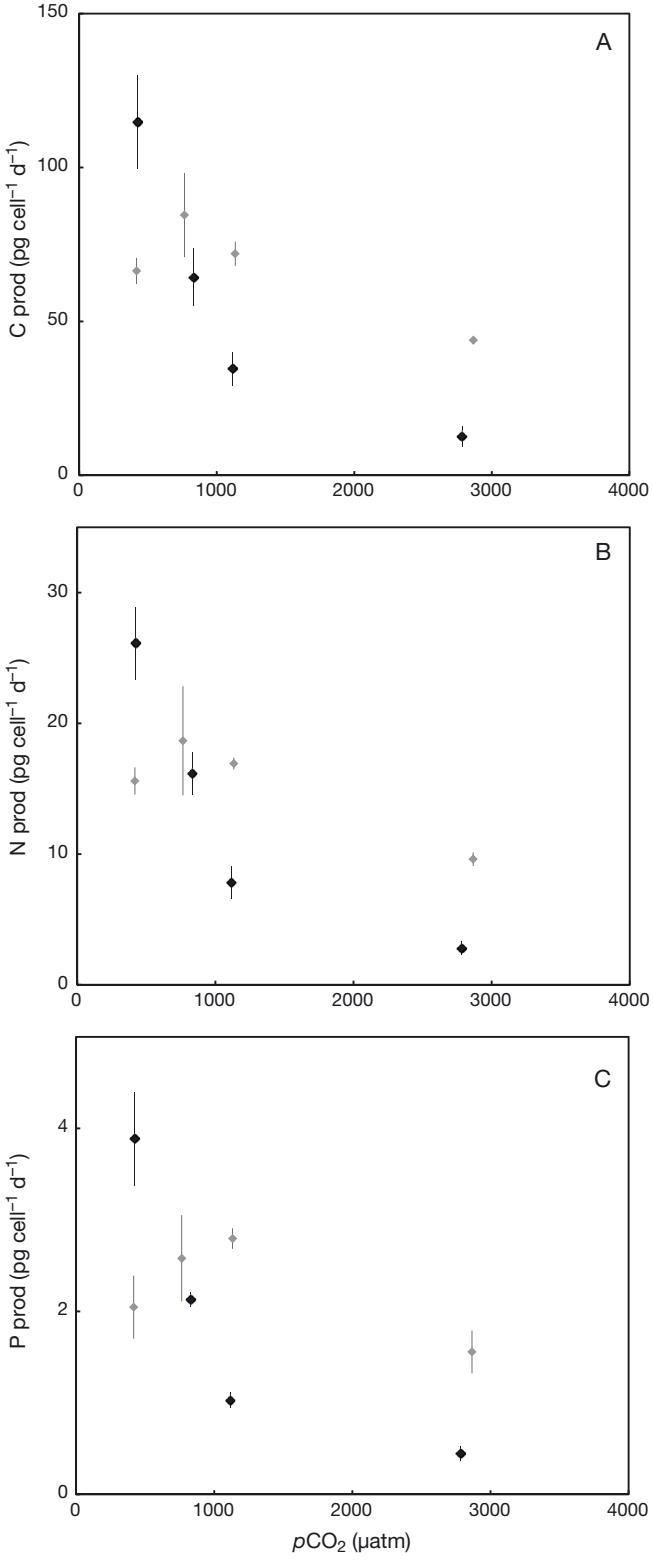

Fig. 3. Organic matter production rates of *Asterionellopsis glacialis* at increasing CO_2 levels (pCO_2) under control (grey diamonds) and enhanced turbulence treatments (black diamonds), expressed as means and standard deviation: (A) carbon, (B) nitrogen and (C) phosphorus

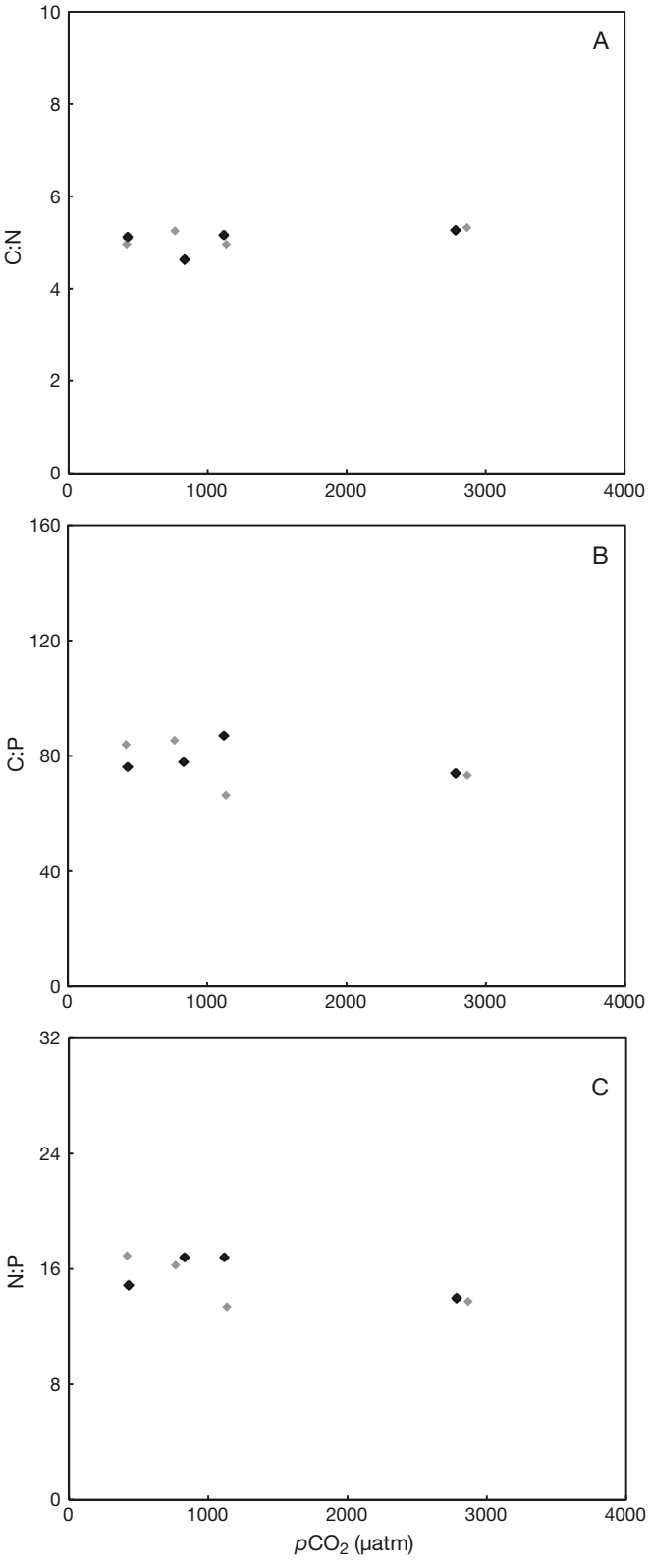

Fig. 4. Particulate organic matter ratios of *Asterionellopsis glacialis* at increasing CO_2 levels (pCO_2) under control (grey diamonds) and enhanced turbulence treatments (black diamonds): (A) C:N, (B) C:P and (C) N:P

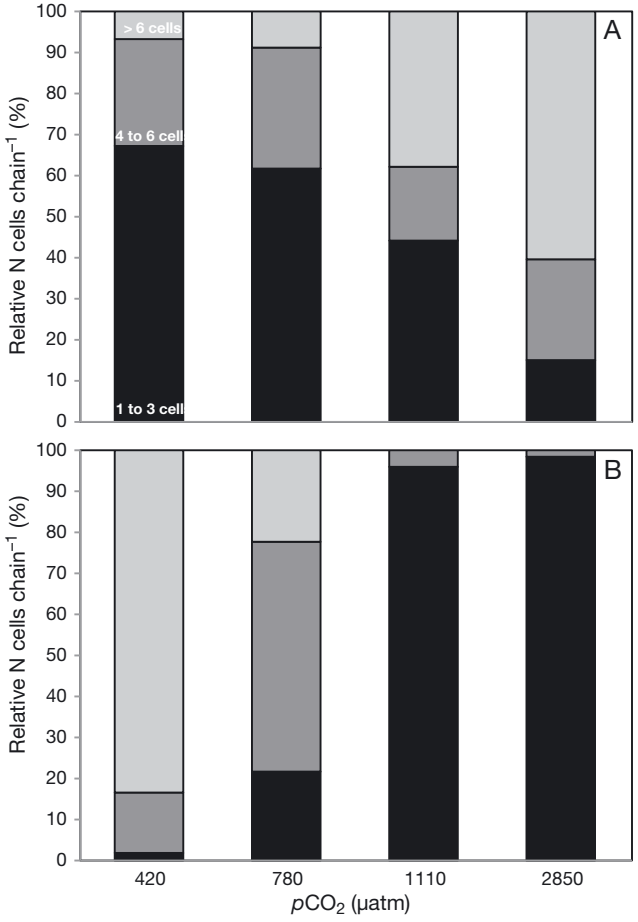

Fig. 5. Relative number of cells per chain of *Asterionellopsis glacialis* at increasing CO_2 levels (pCO_2) under (A) stable and (B) turbulent environmental conditions

DISCUSSION

Influence of CO_2 and turbulence on growth rates

At present-day CO_2 concentrations, phytoplankton can be limited by the availability of DIC (Raven et al. 2014), meaning that diffusive CO_2 supply (Miller et al. 1991, Rotatore et al. 1995, Li & Canvin 1998, Burkhardt et al. 2001) and active uptake of DIC are not sufficient to support maximum photosynthetic rates (Riebesell et al. 1993, Morel et al. 2002). In the enhanced turbulence treatment, the effects of constant water motion near the cells might disrupt or eliminate the diffusive boundary layer, increasing dissolved inorganic nutrients and carbon concentrations at the cell surface during daytime. As a consequence, the diffusion and the uptake of CO_2 and HCO_3^- are likely enhanced when compared to cells grown under more stable conditions. Cells exposed to constant water motion could therefore down-regulate the CCMs, saving energy, which potentially could be reallocated to growth or other energy-demanding processes. Here, it seems that *Asterionellopsis glacialis* used the excess energy to enhance C, N and P production rates and growth, reaching values significantly higher than under more stable conditions.

At CO_2 concentrations of ~800 µatm, *A. glacialis* exposed to stable conditions showed a peak in growth rate likely due to a compromise between increasing carbon availability and less favorable pH conditions. Stimulation of growth rate (Kim et al. 2006, Wu et al. 2010, Gao et al. 2012a, McCarthy et al. 2012, Yang & Gao 2012, Barcelos e Ramos et al. 2014) and/or photosynthesis (Sun et al. 2011, Yang & Gao 2012, Gao et al. 2014) have been reported for some species of diatoms grown at similar CO_2 concentrations, although some species might be more sensitive as reported for *Chaetoceros muelleri* (Ihnken et al. 2011). Furthermore, it has been suggested that doubling of ambient CO_2 concentrations could reduce the energy spent with CCM operation in several species of diatoms by up to 20%, decreasing the total energy used for carbon fixation by 3 to 6% (Hopkinson et al. 2011). However, in the enhanced turbulence treatment, growth rates continuously decreased from ambient to increased CO_2 conditions, due to a shift of optimum growth towards lower CO_2 concentrations. This might be a consequence of higher energy demand for maintaining intracellular pH than energy savings with CCM operation at a disrupted boundary layer (Berdalet & Estrada 2005). Hence, the concomitantly mixed environment might alleviate potential inorganic carbon limitation at low CO_2 concentrations while at the same time exposing the cells to unfavorably low pH conditions earlier on.

At CO_2 concentrations higher than 1000 µatm (as expected for the year 2100), cell division rates of *A. glacialis* significantly decreased in the control and enhanced turbulence treatments, though more noticeably in the latter. This is most likely related to the decrease in sea water pH associated with the rise in CO_2 levels. In coccolithophores, intracellular pH regulation is mediated by voltage-gated H^+ channels (Hv channels) placed in the plasma membrane which dispose of excess protons (Taylor et al. 2012). At extracellular pH above 8.2, the H^+ efflux across the Hv channels occurs passively, making the process energetically favorable. However, at lower pH concentrations, the membrane potential changes and the H^+ efflux becomes an energy-demanding process. In order to guarantee cellular homeostasis, cells are forced to invest energy with the operation of the Hv

channels to cope with the external pH decrease. Indeed, acidified environments can compromise the diffusive boundary layer (Flynn et al. 2012) and the intracellular enzyme and protein structure and activities (Beardall et al. 2009, Berge et al. 2010, Lu et al. 2011). Particularly, the activity of extracellular carbonic anhydrase is inhibited by low pH levels (Aizawa & Miyachi 1986, Sultemeyer 1998, Bozzo & Colman 2000, Gao et al. 2012b, Hopkinson et al. 2013).

Influence of enhanced CO_2 and turbulence on chain length and cell physiology

Under control conditions, we observed a linear increase in chain length with rising CO_2 concentrations. Shorter chains (1 to 3 cells chain^{-1}) have thinner boundary layers which decrease limitation of inorganic carbon near the cells (Barcelos e Ramos et al. 2014). Thus, cells in these chains could save energy by down-regulating the CCM. In agreement, Tchernov & Lipschultz (2008) found that in larger colonies of *Trichodesmium* spp., the diffusion of CO_2 into the cells is limited. When exposed to constant water motion, the presence of longer chains of *A. glacialis* decreased as CO_2 concentrations were enhanced. Similar behavior has been observed in the diatoms *Chaetoceros* spp. and *Pseudo-nitzschia* spp., which showed longer chains in turbulent environments (Arin et al. 2002).

Increasing CO_2 levels triggered opposing trends in the control and enhanced turbulence treatments. The increase in chain length with enhanced CO_2, observed under stable conditions, might elevate the pH in the interior of the colonies and protect the cells from the acidified environment as seen previously (Barcelos e Ramos et al. 2014). However, in the case of *Skeletonema costatum*, the optimum chain length was associated with favorable CO_2 growth conditions and high growth rates (Takabayashi et al. 2006). In contrast, in the enhanced turbulence treatment at increased CO_2 levels, we observed a reduction in the number of cells per chain associated with decreased growth rates, cellular elemental quotas and organic matter production. The reason for this may be related to a disrupted and reduced boundary layer which directly exposes the cells to lower pH levels. Therefore, cells must reallocate energy to regulate intracellular pH and cellular processes such as nutrient uptake or production of organic matter, and extracellular polysaccharides which bind adjacent cells are compromised.

Particulate organic ratios

The bonds between cells of *A. glacialis* are made of mucilage polysaccharide pads associated with high C:N and C:P ratios (Beardall et al. 2009, Barcelos e Ramos et al. 2014). However, in agreement with previous studies conducted on *A. glacialis* (Barcelos e Ramos et al. 2014), *T. pseudonana* (McCarthy et al. 2012) and *E. huxleyi* (Borchard et al. 2011, McCarthy et al. 2012), no changes were observed in the C:N and C:P ratios across the experimental CO_2 range.

CONCLUSIONS

This study showed that constant water motion can impact the CO_2 response of *A. glacialis*. At CO_2 concentrations comparable to present day, *A. glacialis* benefited from constant water motion. This might be related to a reduction in the diffusive boundary layer and the consequent increase in inorganic carbon and nutrient availability near the cell. As a consequence, this could allow for down-regulating CCM operation, and the energy saved could have been invested in other energy-demanding processes. Under enhanced CO_2 levels, the costs of intracellular pH regulation outweighed the benefits of increased CO_2 concentrations. Thus, under enhanced turbulence and CO_2 concentrations, cells probably had to increase the energy investment in cellular homeostasis while growth and organic matter production rates were reduced. Consequently, even though *A. glacialis* benefited from constant water motion at present-day CO_2, under future CO_2 scenarios, it might be negatively affected, with potential consequences for the phytoplankton community composition.

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