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No detectable effect of CO_2 on elemental stoichiometry of *Emiliania huxleyi* in nutrient-limited, acclimated continuous cultures

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ABSTRACT: Effects of CO_2 concentration on elemental composition of the coccolithophore *Emiliania huxleyi* were studied in phosphorus-limited, continuous cultures that were acclimated to experimental conditions for 30 d prior to the first sampling. We determined phytoplankton and bacterial cell numbers, nutrients, particulate components like organic carbon (POC), inorganic carbon (PIC), nitrogen (PN), organic phosphorus (POP), transparent exopolymer particles (TEP), as well as dissolved organic carbon (DOC) and nitrogen (DON), in addition to carbonate system parameters at CO_2 levels of 180, 380 and 750 µatm. No significant difference between treatments was observed for any of the measured variables during repeated sampling over a 14 d period. We considered several factors that might lead to these results, i.e. light, nutrients, carbon overconsumption and transient versus steady-state growth. We suggest that the absence of a clear CO_2 effect during this study does not necessarily imply the absence of an effect in nature. Instead, the sensitivity of the cell towards environmental stressors such as CO_2 may vary depending on whether growth conditions are transient or sufficiently stable to allow for optimal allocation of energy and resources. We tested this idea on previously published data sets where PIC and POC divided by the corresponding cell abundance of *E. huxleyi* at various pCO_2 levels and growth rates were available.

KEY WORDS: Acidification · Cocolithophores · Element composition · Nutrient limitation · Chemostats

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INTRODUCTION

Large-scale changes in surface ocean chemical equilibrium and in elemental cycling are occurring due to ocean acidification (e.g. Chen & Millero 1979, Brewer et al. 1997, Kleypas et al. 2006). The ocean provides the largest sink for anthropogenic carbon dioxide (CO_2) globally (Sabine et al. 2004). As the inventory of dissolved inorganic carbon (DIC) of the surface ocean increases due to enhanced uptake of CO_2 from the atmosphere, the pH of seawater decreases. CO_2-related changes in carbonate chemistry of the ocean affect marine biota, and thus the cycling of biogenic elements (Wolf-Gladrow et al. 1999, Beardall & Raven 2004). The biological pump, i.e. the sequestration of biologically fixed carbon from surface waters to below the thermocline, sustains a vertical DIC gradient in the ocean that pro-

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motes the uptake of CO_2 from the atmosphere. Because of the vast spatial dimensions of the oceanic system, even small changes in the biological pump could significantly affect atmospheric CO_2 concentration. It has been proposed that calcifying organisms, such as corals, coccolithophores, foraminifera, and pteropods, may particularly suffer from increasing seawater acidification (decreasing pH), because more metabolic energy is required for cellular precipitation of calcium carbonate, which impairs production of mineral shells as well as population growth (Feely et al. 2004).

Many studies of the effect of changing CO_2 on phytoplankton have shown variations in growth, primary production, calcification, the efficiency and regulation of carbon concentration mechanisms, and the production of extracellular organic matter, while others found little or no response (reviewed in Ridgwell et al. 2009, Riebesell & Tortell 2011). Many of these studies use *Emiliania huxleyi* (or a similar coccolithophore) as a model species due to its considerable role in the global carbon cycle and its demonstrated sensitivity to changing pCO_2 (Paasche 2001, Thierstein & Young 2004). Formation of particulate inorganic carbon (PIC) during calcification releases CO_2, whereas particulate organic carbon (POC) and dissolved organic carbon (DOC) formation during photosynthesis uses CO_2. The balance between primary production and calcification is therefore an important ratio to measure whether coccolithophores in the ocean act as a net source or sink of CO_2. The [PIC]:[POC] 'rain' ratio has gathered much attention in studies of the response of coccolithophores to global change, as the ratio of PIC and POC removal from the surface ocean determines CO_2 sequestration on longer time scales. POC concentration in phytoplankton cultures or in the field includes organic carbon in living cells, cell detritus and extracellular particles, such as gel particles. A major class of gel particles is the transparent exopolymer particles (TEP) that form from dissolved polysaccharide precursors by physical coagulation (Engel et al. 2004a).

Acidic polysaccharides originate mainly from phytoplankton exudation, a process that is viewed as a carbon 'overflow' for photosynthesis products under nutrient depletion (e.g. Obernosterer & Herndl 1995, Biddanda & Benner 1997, Søndergaard et al. 2000). As photosynthesis rates increase with CO_2 concentration (Rost et al. 2003), the exudation rate of acidic polysaccharide and therefore TEP production may also rise (Engel 2002). Recent experimental studies indicate that TEP formation increases in response to ocean acidification (Engel 2002, Mari 2008, Engel et al. 2014) and may alter [POC]:[PN] ratios (where PN is particulate nitrogen) during coccolithophore blooms (Engel et al. 2005). Changes in [PIC]:[POC] ratio can be dependent on several factors; the ratio does not automatically reflect changes in the relative amount of precipitated calcium carbonate but can vary solely with carbon quota and TEP concentration.

In contrast to organic matter production, the functionality of calcification in coccolithophores is not well understood. In addition to carbonate chemistry, the calcification rate in *E. huxleyi* is affected by light, salinity, macro- and micronutrient availability, and temperature, and varies with growth rate (Fernández et al. 1996). Indeed, nutrient limitation (particularly phosphorus) has been shown to increase calcification rates relative to photosynthesis, but can also result in formation of malformed or incomplete coccoliths (Paasche 1998, Kaffes et al. 2010). Variations in external growth factors are difficult to control simultaneously under experimental conditions because cell growth itself changes, e.g. nutrient and light availability and therefore growth rate. This challenges the experimental isolation of a single, direct effect of pCO_2 on calcification. Strong co-variations of factors that modify calcification rates must be expected during bloom-like situations, either in the field or during fertilized mesocosm experiments. Even in batch culture experiments, there are problems in continuously tracing nutrient availability and growth rates with time. Different variations in environmental factors thus may partly be responsible for the complex, and in some regard even contrary, picture of the sensitivity of *E. huxleyi* to ocean acidification drawn in previous studies. Increasing pCO_2 was found to either decrease (Riebesell et al. 2000a, Rost et al. 2003, Sciandra et al. 2003a, Zondervan et al. 2002) or increase (Iglesias-Rodriguez et al. 2008a) the concentration of biogenic calcite produced, or to cause no, or a complex, response (Langer et al. 2006, 2009a, De Bodt et al. 2010a). Response to CO_2 of *E. huxleyi* may also involve changes in growth rate (Jones et al. 2013) and may be strain-specific (Raven & Crawfurd 2012). Apart from multiple changes of growth factors occurring in batch cultures, another general challenge of experimental perturbation studies typically is their short duration (days to weeks), which inevitably bears the risk that observed responses may be transient rather than sustained. Recently, it has been suggested that *E. huxleyi* can adapt to high CO_2 and restore calcification rates within a period of 1 yr, relative to cells grown at present day pCO_2 (Lohbeck et al. 2012). Strain selection and adaptation

may further impact calcification response of natural populations.

A sustained exposure of microorganisms to environmental factors like CO_2 or pH, while keeping other growth factors more stable, can be realized in chemostats. Chemostats are continuous culture systems, in which cell abundance is controlled by the nutrient concentration of the inflow media, while growth rate μ (d^{-1}) is in balance with the rate of the flow (d^{-1}) (Novick & Szilard 1950). During steady state, the growth rate equals the flow rate, and cell abundances remain constant over time. Thus, chemostats enable studies of cell physiology and element composition under constant nutrient-limited growth. Few chemostat experiments with phytoplankton at different pCO_2 have been conducted previously (Sciandra et al. 2003a, Leonardos & Geider 2005, Borchard et al. 2011, Borchard & Engel 2012). Interestingly, these studies often showed the largest response to CO_2 during transient phases, i.e. either when transitioning from low to high pCO_2 (Sciandra et al. 2003a, Leonardos & Geider 2005) or during the initial transition from the batch to the dilution phase (Borchard et al. 2011).

In the experiments described below, we use nutrient-limited, continuous cultures of *Emiliania huxleyi* to disentangle potential effects of pCO_2 from those that are caused by variation in growth rate, as well as by other environmental control factors that impact phytoplankton cell numbers, elemental composition (e.g. PIC:POC), and DOC and TEP concentrations. It has been suggested that calcification rates in coccolithophores are affected by the calcite saturation state (Ω-Ca), and strongly decline at a value of Ω-Ca < 2 (Gehlen et al. 2007). Due to high cell abundance, growth and calcification in coccolithophore cultures may reduce Ω-Ca of the seawater medium to values <2 (de Bodt et al. 2008), or even cause undersaturation during low dilution rates in chemostats (Borchard et al. 2011). Ocean acidification will likely cause a decline in $CaCO_3$ saturation state; however, Ω-Ca values in surface waters will be >2 in most parts of the future ocean. During this study, we therefore followed the suggestions of LaRoche et al. (2010) to circumvent strong declines in Ω-Ca by increasing alkalinity in the initial medium reservoir. Cultures were acclimated to the different pCO_2 values for about 1 mo when steady state growth was reached and cell abundances were comparable between different chemostats; this minimized transient responses. Thus, our experiment aimed at studying sustained responses in element composition of *E. huxleyi* to realistic changes in pCO_2 and Ω-Ca, while reducing variations in environmental co-factors.

MATERIALS AND METHODS

To separate potential effects of pCO_2 from other environmental factors, we conducted a chemostat experiment with constant cell growth rate, temperature and nutrient supply, while CO_2 aeration was varied to achieve partial pressures of 180, 380 and 750 μatm.

We used the calcifying strain of *Emiliania huxleyi* (PML B92/11A) because it had responded to changes in pCO_2 during previous batch experiments (Riebesell et al. 2000a, Zondervan et al. 2002). As mentioned above, cells were acclimated over a period of 1 mo prior to the first sampling to avoid short-term stress effects on cell composition. Sampling was repeated 5 times during steady-state growth over a period of 14 d to assure the reproducibility of results. We chose a low flow rate of media (0.2 d^{-1}) because *E. huxleyi* often occurs in nutrient-limited areas (Tyrrell & Taylor 1996, Lessard et al. 2005) where low growth rates prevail. In addition to the particulate elements POC, PIC, PN and POP, we sampled for DOC and TEP to ascertain the role of gel particle formation in POC production.

Experimental set-up. A total of 5 chemostats (60 cm height × 15 cm diameter) with culture volumes of 9.2 l each were used during this study. The chemostat and its associated components are described in detail in Borchard et al. (2011). Desired CO_2 concentrations of 180, 380 (duplicate chemostats) and 750 μatm (duplicate chemostats) were achieved by continuous aeration with CO_2 gas. CO_2 parameters were measured as also described in Borchard et al. (2011). Experiments were conducted at 14°C under P-limitation (N:P of 30:1) and at 190 μE m^{-2} s^{-1}; 16:8 light: dark. Although light intensity and light/dark cycle can be adjusted individually for each chemostat (TL-D DeluxPro, Philips; QSL 100) in our system, the same light conditions were provided for each chemostat culture during this study. Light was measured with a light sensor (Biospherical Instruments) in the air above the seawater inside the chemostats.

Preparation of *E. huxleyi* culture and addition to chemostats followed the description in Borchard et al. (2011). Culture medium for the experiment was prepared from 0.2 μm filtered natural North Sea seawater with a salinity of 33, pH of 8.20 and total alkalinity (TA) of 2250 μmol kg^{-1}, and enriched with macronutrients to yield final concentrations of 43 μM NO_3^- and 1.5 μM PO_4^{3-} to favor phosphorus limiting conditions, as P limitation can initiate blooms of *E. huxleyi* in the field (Aksnes & Egge 1991, Aksnes et al. 1994). Draw-down of carbonates in a coccolitho-

phore culture reduces alkalinity of seawater, and can strongly affect seawater pH or even cause carbonate undersaturation that could impact calcification in *E. huxleyi* (Borchard et al. 2011). To minimize effects of calcification by *E. huxleyi* on carbonate chemistry in the incubators, TA in the reservoir tank was increased by addition of bicarbonate to 2460 µmol kg^{-1} (LaRoche et al. 2010).

Target temperature and pCO_2 were established in incubators within 2 d prior to cell addition. *E. huxleyi* cells were inoculated into each chemostat to a final density of 5000 cells ml^{-1}, and grown in batch mode for 5 d. Afterwards, flow was started and then continued for 38 d at a dilution rate (D) of 0.2 d^{-1}. Gentle mixing of the culture at 50 rpm was applied to keep cells in suspension. Steady state in a chemostat is reached when biomass (or cell abundance) remains constant over time, as the dilution rate equals the growth rate (µ), and was established on Day 28 of this experiment (Fig. 1).

Distinct pCO_2 concentrations in the chemostat cultures were achieved using a CO_2 regulation system consisting of commercially available mass flow controllers (Type 1179 Mass Flo Controller; MKS Instruments) and a pressure vessel. Target CO_2 concentrations are reached by 2-step-down regulation of pure CO_2 (CO_2 4.5; ≥99.995 vol %; Air Liquide) to the target CO_2 level using CO_2-free air (domnick hunter CO_2 scrubber CO2RP280; accuracy <1 µatm). CO_2 concentrations in the air/CO_2 mixture of the inflow were determined before and after the experiment using an infrared CO_2 analyzer (LI-COR, 6252). Previous evaluation of the system showed high precision and stability of air flows with variations of <2% (Borchard et al. 2011). For continuous evaluation of the system, pH and temperature inside each chemostat were recorded hourly over the whole duration of the experiment.

All pipes and tubes were acid rinsed (10% HCl) and sterilized by autoclaving (121°C for 30 min). All non-autoclavable parts, e.g. incubator and reservoir vessels, were cleaned with phosphate-free detergent, soaked in 10% HCl for 2 h and then thoroughly rinsed with deionized water.

Total alkalinity (TA). TA in 25 ml samples was measured by titrating with 0.05 M HCl until the buffering capacity of the water samples was consumed and all bases of interest were protonated to zero level species. Analysis was accomplished with an automatic titrator (TitroLine® alpha *plus*, SI Analytics) equipped with a sample changer (TW *alpha plus*, SI Analytics) and a piston burette (Titronic®110 *plus*, SI Analytics). The concentration of TA (µmol kg^{-1} seawater) was calculated from linear regression of the absolute numbers of protons in solution and the total volume (sample plus HCl) in the range of pH 3 to 4.

pH and temperature. pH and temperature values were recorded every hour on a data logger throughout the entire experiment using WTW pH 340i handheld meters and WTW standard DIN/NBS buffers (PL 4, PL 7 and PL 9) recalibrated using Tris based reference material provided by A. Dickson (pers. comm.). Accuracy of measurement was better than ±0.005 pH and ±0.1°C for temperature.

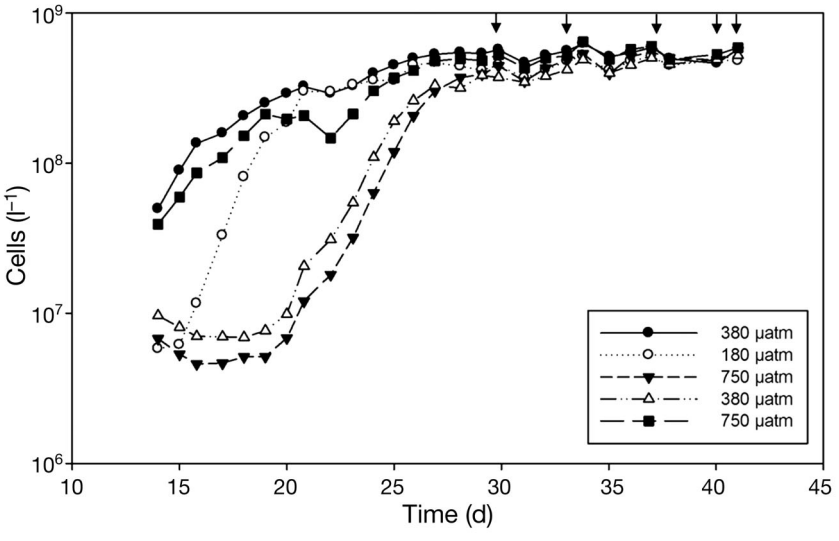

Fig. 1. Number of *Emiliania huxleyi* cells (l^{-1}) over time during continuous flow in the chemostats as well as sampling times during the steady-state phases (arrows). Duplicate chemostats were run at 380 µatm and 750 µatm CO_2, while a single chemostat was run at 180 µatm CO_2

***p*CO_2, and calcite saturation state (Ω-Ca).** The full carbonate system speciation and Ω-Ca in chemostat cultures and medium reservoirs were calculated from pH and TA values at each sampling time with the CO2sys program (Lewis & Wallace 1998), using the carbonic acid dissociation constants of Mehrbach et al. (1973) refitted by Dickson & Millero (1987), and the HSO_4^- dissociation constant of Dickson et al. (1990) on the US National Bureau of Standards scale. The saturation state (Ω-Ca = $[Ca^{2+}][CO_3^{2-}]/K_{sp}$) of calcite was computed using the calcite solubility (K_{sp}) of Mucci (1983).

***E. huxleyi* abundance.** *E. huxleyi* cell numbers in all chemostats were measured daily, but samples for all

other parameters were regularly taken only after steady state was reached, i.e. on Days 30, 34, 38, 42, and 44 of the experiment (Fig. 1). Samples were always taken at the same time of the day (3 h after lights on). Cell abundance and volume were determined in triplicate using a Beckman Coulter Multisizer III. Particles with an equivalent spherical diameter in a range of 3.27 µm to 7.96 µm were identified as *E. huxleyi*, after microscopic inspection. Steady state was assumed when variations in cell abundance were <10% for 3 consecutive days (Leonardos & Geider 2005).

Chlorophyll *a*. Concentrations of chlorophyll *a* (chl *a*) were determined by ion-pairing reverse-phase high-pressure liquid chromatography (HPLC) after extraction into acetone (Mantoura & Llewellyn 1983, Bidigare et al. 1985, Sun et al. 1991). Briefly, duplicate 20 ml samples from each chemostat were filtered onto combusted GF/F filters; samples were frozen until analysis. To extract chl *a*, the filters were sonicated in HPLC-grade acetone; 2 successive 5 ml extracts were combined and filtered through a 0.2 µm Zetapor membrane. Samples were covered with aluminum foil to protect them from light during handling and analysis. Chl *a* was separated from other chloropigments by HPLC on a 5 µm Adsorbosphere C-18 column as described by Sun et al. (1991). Chl *a* was detected by fluorescence (excitation λ= 440 nm, emission λ= 660 nm), and was quantified using an authentic standard (Turner).

Bacterial cell counts. Samples (4.5 ml) were preserved with glutardialdehyde (GDA, AppliChem, 1.1% v/v final concentration), and stored at −20°C for 3 mo until flow cytometry analysis using a Facs Calibur flow cytometer (Becton Dickinson) following the method of Gasol & Del Giorgio (2000). Briefly, nucleic acids in the bacteria were stained with SybrGreen I (Invitrogen). The stock solution of SybrGreen I (10000×) was diluted 1:40 with dimethyl sulfoxide (DMSO, Sigma Aldrich), followed by a 1:40 dilution with the sample (final dilution 10^{-3}, final concentration 6.25×). Shortly before analysis, thawed samples were incubated for 30 min in the dark. As an internal standard, yellow-green fluorescent latex beads (0.94 µm diameter, Polyscience) were used for the volume normalization of counted events. Bacterial cell numbers were calculated using Cell Quest Pro software.

POC, PIC, PN, and POP. Particulate C and N contents were run in duplicate using a Carlo Erba EA-1112 CNS analyzer. For this analyzer, the level of uncertainty is ±2% for C and ±5% for N analysis. Inorganic carbon was removed using 10% HCl, and PIC was calculated as the difference between TPC (total particulate carbon) and POC. For POP, 30 to 80 ml of sample was filtered gently (<200 mm Hg) onto combusted GF/F filters. POP was determined colorimetrically after persulfate oxidation of POP with a precision of ±0.02 µmol l^{-1} (Koroleff & Grasshof 1983).

Nutrients. Samples were filtered through 0.2 µm syringe filters and frozen at −20°C until analysis. Measurements of NO_3^-, NO_2^-, NH_4^+ and PO_4^{3-} were made spectrophotometrically after Grasshof et al. (1999) using an Evolution 3 autoanalyzer (Alliance Instruments). Detection limits were 0.3 µmol l^{-1} for N and 0.01 µmol l^{-1} for P.

Transparent exopolymer particles (TEP). TEP were detected by staining with Alcian Blue, a cationic copper phthalocyanine dye that complexes carboxyl ($-COO^-$) and half-ester sulfate (OSO_3^-) reactive groups of acidic polysaccharides. The amount of Alcian Blue adsorption per sample volume is a measure of TEP concentration, and was determined colorimetrically according to Passow & Alldredge (1995) from 50 to 100 ml samples filtered onto 0.4 µm Nuclepore filters. All filters were prepared in duplicate. Microscopic analysis of TEP size and abundance was performed after Engel (2009). Semi-permanent slides (CytoClear) were prepared by filtration (<200 mbar) of 5 to 10 ml samples onto 0.4 µm Nuclepore filters (25 mm) and the TEP stained with 1 ml Alcian Blue. All filters were prepared in duplicate and stored frozen at −20°C until analysis. TEP slides were examined using a compound light microscope and a digital AxioCam HRc camera (Zeiss) with 200 to 400× magnification. Microscopic inspection revealed only stained coccospheres and no 'free' TEP present, so further microscopic analysis of TEP slides was discontinued.

Dissolved organic carbon (DOC). Samples for DOC (20 ml) were collected in combusted glass ampoules after filtration through combusted GF/F filters. Samples were acidified with 100 µl of 85% phosphoric acid, heat sealed immediately, and stored at 4°C in the dark until analysis. DOC samples were analyzed using the high-temperature combustion method (TOC-VCSH, Shimadzu) (Qian & Mopper 1996). A multi-point calibration curve was prepared each day of measurement using a potassium hydrogen phthalate standard prepared in MilliQ water. Additionally, 2 reference seawater standards (from Dennis Hansell, RSMAS, University of Miami) were used to determine the instrument blank. Each sample was measured in quadruplicate.

Total dissolved nitrogen (TDN). TDN was determined simultaneously with DOC using the TNM-1

detector on the Shimadzu analyzer. TDN is combusted and converted to NOx, which chemiluminesces when mixed with ozone and can be detected using a photomultiplier (Dickson et al. 2007). DON was calculated from TDN by subtraction of NO_3^-, NO_2^-, and NH_4^+ concentrations.

Data analysis. Differences in data as revealed by statistical tests (*t*-test, ANOVA Kruskal-Wallis One Way Analysis of Variance on Ranks, Kolmogorov-Smirnov test) were accepted as significant for $p < 0.05$. Average values for total concentrations are given by their arithmetic mean ±1 standard deviation (SD), averages for ratios by their geometric mean. Calculations, statistical tests and illustration of the data were performed with the software packages Microsoft Office Excel 2010 and Sigma Plot 12.0 (Systat).

RESULTS

Chemostat nutrient and carbonate chemistry

Nitrate and phosphate were below detection levels in all samples. Nitrite concentration was 0.15 to 0.16 µmol l^{-1} and did not differ significantly between CO_2 treatments.

Throughout the steady-state period, average pH was 8.46 ± 0.08 (180 µatm), 8.24 ± 0.06 (380 µatm) and 8.02 ± 0.07 (750 µatm) (Fig. 2a). TA was not significantly different between treatments, yielding 2400 ± 206 (180 µatm), 2473 ± 226 (280 µatm), and 2450 ± 255 (760 µatm) µmol l^{-1}. Calculations of pCO_2(aq) from pH and TA yielded values of 190 ± 53 (180), 377 ± 28 (380), and 647 ± 69 (750) µatm (Fig. 2b). Both pCO_2(aq) and pH differed significantly between the treatments during the steady-state period as well as during the entire experiment (ANOVA, $p < 0.01$). Calcite saturation (Ω-Ca) was 6.36 ± 0.42 (180 µatm), 4.27 ± 0.49 (380 µatm), and 2.83 ± 0.57 (750 µatm) on average and hence >2 in all samples (Fig. 2c).

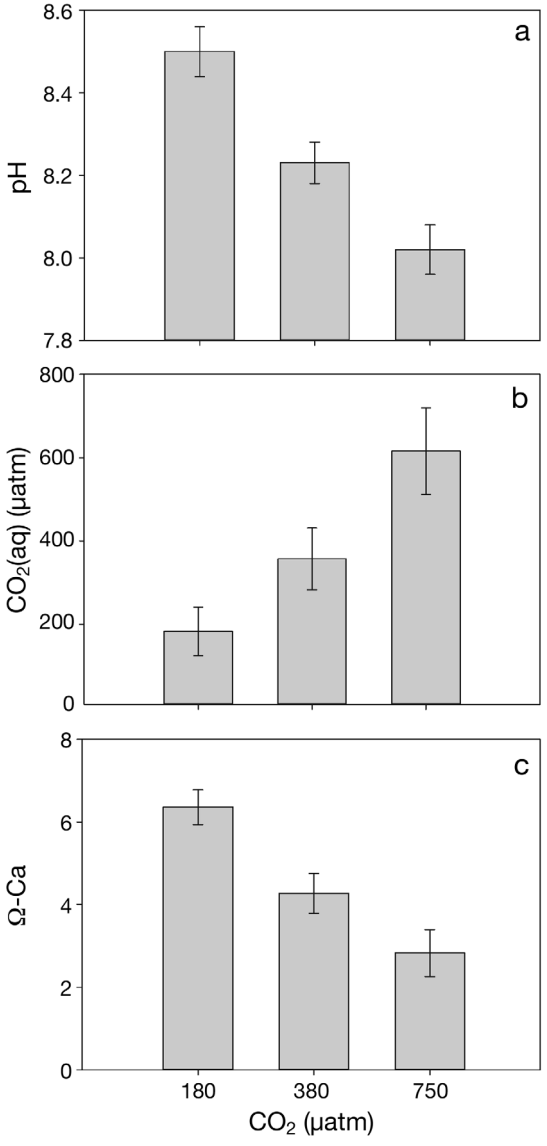

Fig. 2. Variations of (a) pH, (b) CO_2(aq), and (c) calcite saturation state (Ω-Ca) strongly differed during the steady state phase of the *Emiliania huxleyi* chemostat experiment adjusted to 180, 380 and 750 µatm CO_2. Error bars: ±1 SD, n = 5 for 180 µatm and n = 10 for 380 and 750 µatm

Cell abundance and state-steady growth

Emiliania huxleyi was grown inside the chemostat chambers in batch mode for 5 d. Flow through the chemostat was started on Day 6 and run for ca. 3 wk at a rate of $D = 0.2$ d^{-1} until steady state was achieved on Day 28; sampling began on Day 30 (Fig. 1). As expected, cell concentrations initially decreased when chemostat flow began, but after 1 week increased again towards steady state. After Day 28, temporal variation of cell abundance within each chemostat was <10%, defining steady-state cell growth. Cell abundance at the 3 CO_2 concentrations was not significantly different between treatments: 4.8 ± 0.85 × 10^8 l^{-1} (180 µatm), 4.8 ± 1.0 × 10^8 l^{-1} (380 µatm) and 5.3 × 10^8 l^{-1} ± 9.16 × 10^7 (750 µatm) (Fig. 3a). No significant difference between treatments was observed in the chl *a* content of *E. huxleyi* cells (Fig. 3b). Numbers of heterotrophic bacteria varied between 2 × 10^9 l^{-1} and 3 × 10^9 l^{-1}, also with no CO_2 effect. The number of bacteria during this experiment was rela-

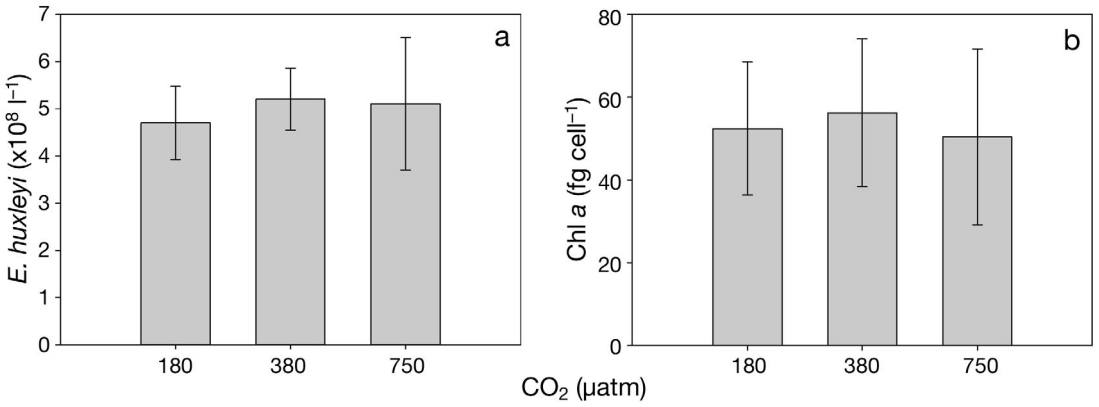

Fig. 3. No significant differences were observed in (a) the abundance of *Emiliania huxleyi* cells or (b) the chlorophyll *a* (chl *a*) content of cells during the steady-state phase of the chemostat experiments adjusted to 180, 380 and 750 µatm CO_2. Error bars: ±1 SD, n = 5 for 180 µatm and n = 10 for 380 and 750 µatm

tively high and can be attributed to the long duration of the experiment and to the large volume of water required for continuous culture. However, estimating a bacterial cell quota of 30 fg C, 5 fg N and 1.4 fg P (upper level for natural marine bacteria given in Fagerbakke et al. 1996), bacteria contributed at most 1 to 2% POC, 3 to 4% PN and 7 to 10% POP. Because the impact of bacteria on element stoichiometry during this study was therefore presumably small, and element quotas of bacteria are highly variable, both within and between species, bacterial contribution to POC, PN and POP will not be resolved in this study.

Table 1. Average concentrations and specific variations in particulate organic matter (POM) — inorganic carbon (PIC), organic carbon (POC), nitrogen (PON) and phosphorus (POP) — at different levels of CO_2, determined at 5 different time-points over a period of 14 d in phosphorus-limited chemostats. Duplicate chemostats were used for 380 and 750 µatm treatments

POM	180 µatm		380 µatm		750 µatm	
(µmol l^{-1})	(avg.; n = 5)	SD (%)	(avg.; n = 10)	SD (%)	(avg.; n = 10)	SD (%)
POC	399	14	401	17	418	19
PIC	645	18	715	33	700	16
PON	24.2	11	24.4	8	24.6	9
POP	1.59	40	1.36	37	1.49	32

Biogeochemical composition

Concentrations of particulate elements are shown in Table 1. Particulate C, N and P in chemostats at the 3 levels of CO_2 were normalized to cell abundance and compared for the steady state period of observation. Average cell quotas ranged from 1.3 to 1.5 pmol PIC $cell^{-1}$ (Fig. 4a), 0.79 to 0.82 pmol POC $cell^{-1}$ (Fig. 4b), 0.048 to 0.053 pmol PN $cell^{-1}$ (Fig. 4c) and 2.8 to 3.2 fmol POP $cell^{-1}$ (Fig. 4d). No significant differences between CO_2 treatments were observed for any of the cell quotas. The spread in elemental concentrations and cell quotas for each CO_2 treatment represents the variability over time during the steady state growth period sampled. Variability of cell quotas within each treatment differed between elements. Lowest within-treatment variability was observed for the cell quota of POC, followed by PIC, PN and POP.

Concentrations of DOC and DON in the initial culture medium averaged 183 ± 0.1 µmol C l^{-1} and 20 ± 4.8 µmol N l^{-1}, respectively. Concentrations were higher inside the chemostat by about 7 µmol DOC l^{-1} and 2 µmol DON l^{-1}, indicating net biological production. No significant influence of the CO_2 treatment was observed on concentrations of DOC (Fig. 5a) or DON (Fig. 5b). Instead, both components of dissolved organic matter were remarkably stable over time and very similar in all chemostats. For dissolved organic components, variability over time was generally lower than for particulate components (Table 1), i.e. 2.9 to 7.5% for DOC and 2.6 to 3.3% for DON.

Concentration ratios determined during the study are depicted in Fig. 6a-d. As was the case for individual components, no significant influence of CO_2 treatment was determined for any of the following ratios: [PIC]:[POC], [POC]:[PN], [PN]:[POP] or [DOC]:[DON]. Average ratios of [PIC]:[POC] ranged between 1.6 and 1.8, indicating fully calcified cells in all treatments. Average ratios of [POC]:[PN] varied between 16.6 and 17.2, and were clearly above the Redfield ratio of 6.6 throughout the study. Average

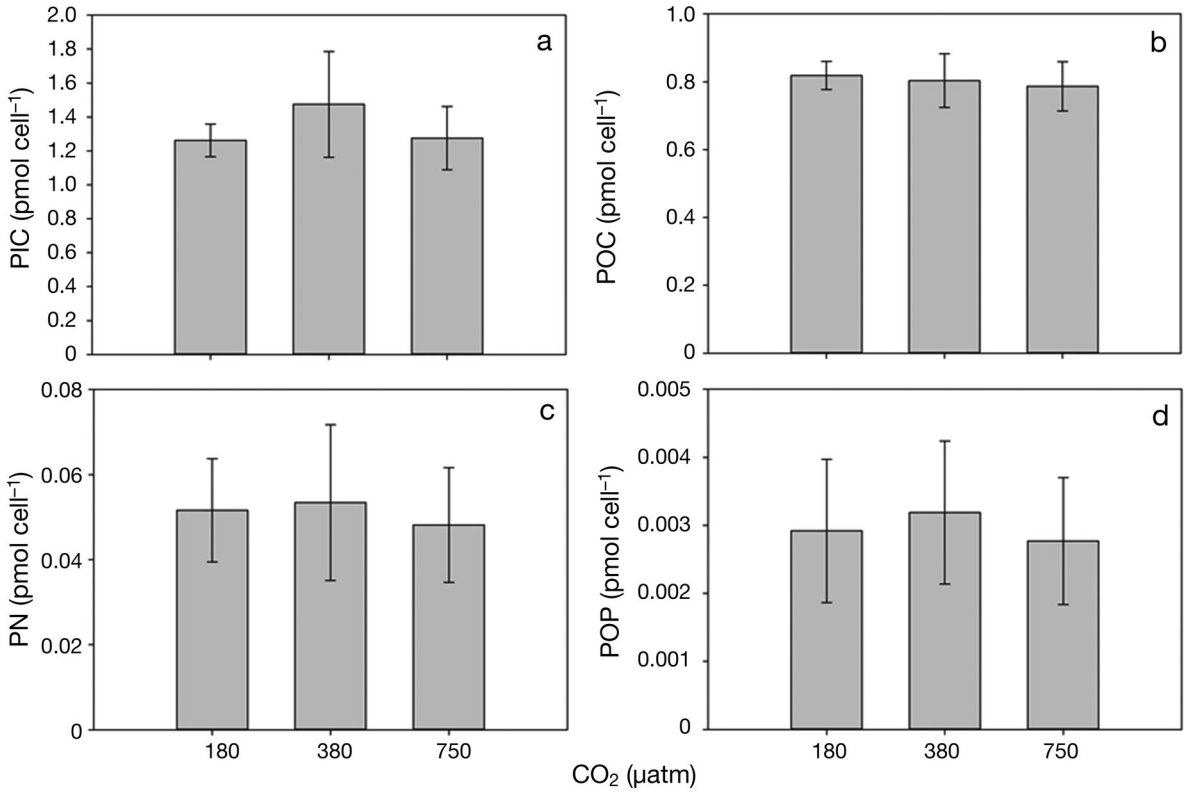

Fig. 4. Variations of particulate (a) inorganic carbon (PIC), (b) organic carbon (POC), (c) nitrogen (PN) and (d) organic phosphorus (POP) were normalized to *Emiliania huxleyi* cell abundances and did not differ significantly in chemostats adjusted to 180, 380 and 750 µatm CO_2. Error bars: ±1 SD, n = 5 for 180 µatm and n = 10 for 380 and 750 µatm

[PN]:[POP] ratios were 17.3 to 19.7 and higher than the Redfield ratio of 16 (*t*-test, $p = 0.06$ for sampling Days 30 to 44; $p < 0.001$ for samplings Days 34 to 44), reflecting the relative P shortage applied in the nutrient media (Fig. 6c). Ratios for [DOC]:[DON] ranged between 8.4 and 8.6 (Fig. 6d) and were thus clearly lower than [POC]:[PN] ratios, albeit still above the Redfield reference value.

TEP

No individual gel particles were observed by microscopic examination throughout the steady-state phase in any of the chemostats. It has been shown that coccoliths of *E. huxleyi* include an acidic polysaccharide that adsorbs Alcian Blue (de Jong et al. 1976, Fichtinger-Schepman et al. 1979). This poly-

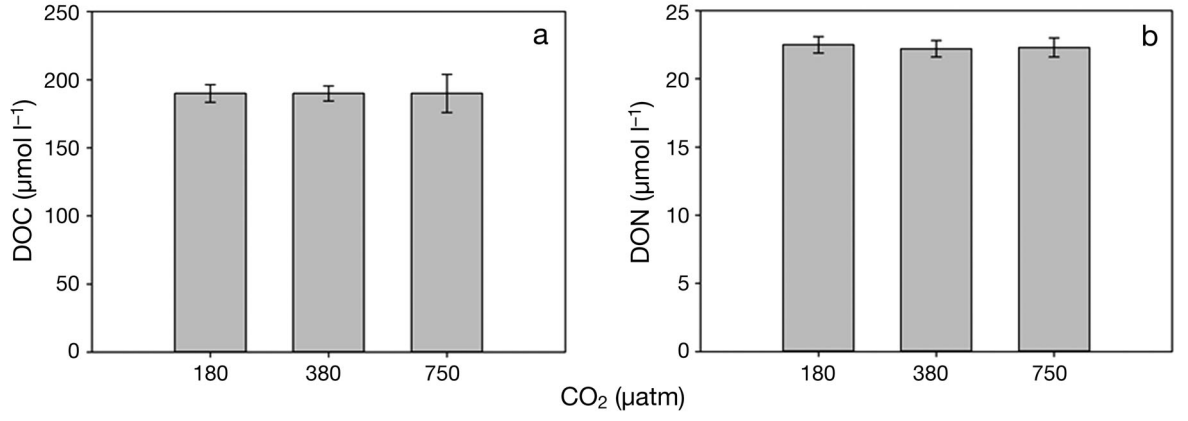

Fig. 5. Concentrations of (a) dissolved organic carbon (DOC) and (b) dissolved organic nitrogen (DON) during the steady-state phase of the *Emiliania huxleyi* chemostat experiment were very similar between CO_2 treatments. Error bars: ±1 SD, n = 5 for 180 µatm and n = 10 for 380 and 750 µatm.

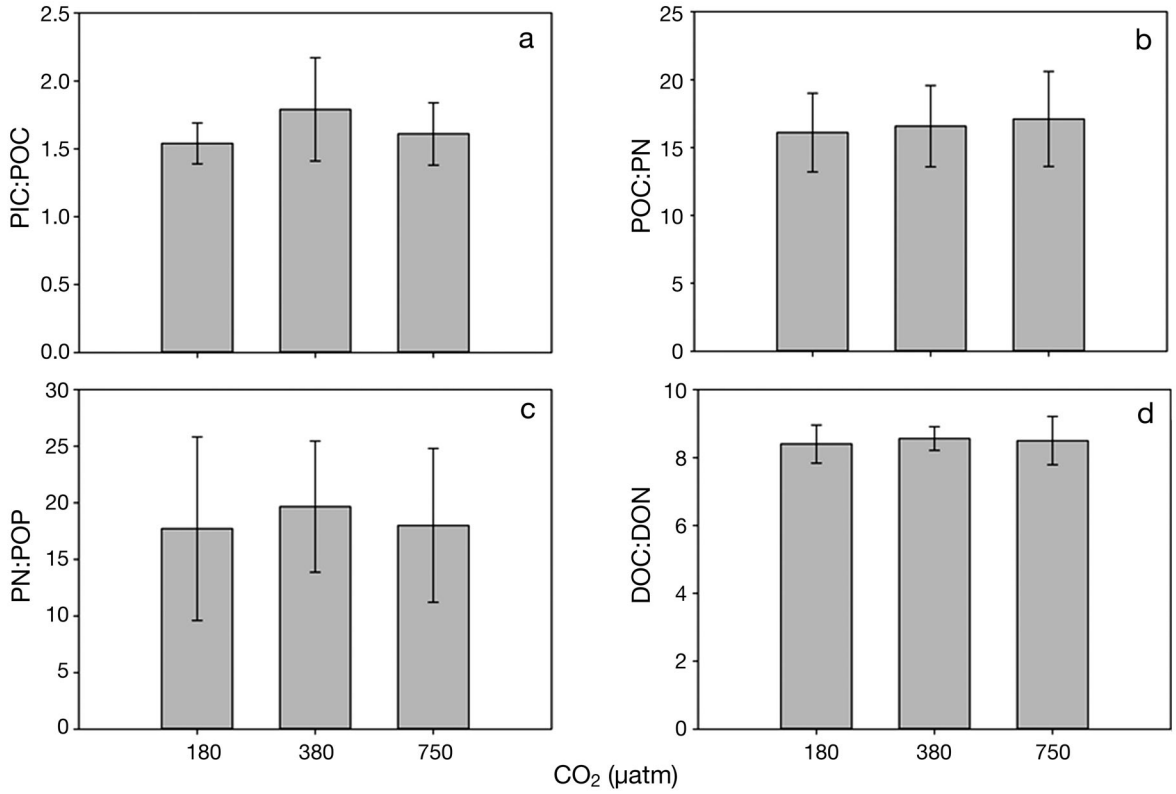

Fig. 6. Element ratios determined during steady state growth of *Emiliania huxleyi* did not differ significantly between CO_2 treatments for (a) PIC:POC, (b) POC:PN, (c) PN:POP, or (d) DOC:DON. Error bars: ±1 SD, n = 5 for 180 µatm and n = 10 for 380 and 750 µatm

saccharide is assumed to play a role in the biomineralisation process and probably also in the agglutination of coccoliths in the coccosphere (Van Emburg et al. 1986). Engel et al. (2004b) showed that the amount of Alcian Blue dye adsorption (AB_{ad}) in exponentially growing, nutrient-replete *E. huxleyi* cultures without TEP was directly related to cell abundance, equivalent to 2.55 ± 0.4 pg Xanthan equivalents (Xeq. $cell^{-1}$ During this study, a significant linear relationship between AB_{ad} and cell abundance was also observed ($p < 0.01$; data not shown), yielding $\Delta[AB_{ad}]$: Δ[cell] of 3.6 ± 0.02 pg Xeq. $cell^{-1}$. This slightly higher value may be attributable to the high degree of calcification determined during this study, but is still low compared to previous mesocosms or culture studies that showed production of TEP by *E. huxleyi* (Engel et al. 2004b, Harlay et al. 2009, Borchard & Engel 2012), and in accordance with the observed absence of TEP during this study.

DISCUSSION

During this study, the growth rate of *Emiliania huxleyi* was controlled by the flow rate in our chemostats, which was maintained at steady state (0.2 d^{-1}). The relatively long period (30 d) that cells grew in the chemostat under constant conditions before sampling allowed cells to acclimate to their environment, specifically to the low supply of phosphate. Element cell quotas and stoichiometry under these growing conditions were insensitive to different pCO_2 levels, indicating that PIC, POC, PN and POP were primarily constrained by the nutrient and light regime (equal in all treatments). It has been suggested that environmental co-factors such as light and nutrient availability may alter the response of *E. huxleyi* to CO_2 changes. We will therefore discuss whether these cofactors may have attenuated cell response to CO_2 during this study.

Light as a cofactor

The amount of energy, i.e. light, available for metabolic processes may play a role in whether *E. huxleyi* is sensitive to changes in pCO_2 (Zondervan 2007, Raven & Crawfurd 2012). Zondervan et al. (2002) observed no response of calcification to CO_2 when cells were grown at low light (<100 µE m^{-2} s^{-1}). Rokitta &

Rost (2012a) also observed a stronger decrease in PIC per cell for *E. huxleyi* at high compared to low light. During our study, light was not limiting, as the available 190 µE m^{-2} s^{-1} is close to the light saturation level of 200 µE m^{-2} s^{-1} reported for *E. huxleyi* by Nielsen (1997). Zondervan et al. (2002) observed highest, i.e. light-saturated, growth rates at 150 µE m^{-2} s^{-1} at 15°C — a temperature similar to that used during this study (14°C). This suggests that irradiance applied during this study was not limiting cell growth, and not low enough to account for the observed absence of a CO_2 effect on element stoichiometry.

Nutrients as cofactors

Another factor potentially modulating CO_2 responses is nutrient availability. During our chemostat study, the culture medium was nutrient-depleted. Phosphate limitation has been shown to increase the number of coccolith platelets per cell, resulting in higher [PIC]:[POC] (Paasche 1998, Riegman et al. 2000). Cell quotas determined during this study were >1 pmol PIC $cell^{-1}$ and thus relatively high compared to cells growing in nutrient-replete conditions, i.e. 0.25 to 0.87 pmol PIC $cell^{-1}$ (e.g. Riebesell et al. 2000a, Lohbeck et al. 2012, Iglesias-Rodriguez et al. 2008a), or in nitrogen-limited cultures (~0.16 pmol IC $cell^{-1}$) (Müller et al. 2012). Moreover, [PIC]:[POC] was always above 1, reflecting a high degree of calcification. These findings are in accordance with earlier findings showing that [PIC]:[POC] can rise from below 1 up to 2.2 under phosphate limitation (Paasche 2001). Using a similar experimental set-up, Borchard et al. (2011) also observed no effect of CO_2 aeration on C and N quotas of *E. huxleyi* under phosphate-limited growth at 0.3 and 0.1 d^{-1}. An attenuated effect of pCO_2 on element ratios of *E. huxleyi* was observed for cells grown under high [N]:[P] by Matthiessen et al. (2012). Thus, phosphorus limitation may be a factor in the suppression of CO_2 effects on element stoichiometry. This may be highly relevant for estimating future changes in the ocean's biogeochemical cycling due to CO_2 responses of *E. huxleyi*, as this species is known to become abundant and form blooms in low-nutrient waters, especially when [N]:[P] is high (Tyrrell & Merico 2004).

In chemostat studies where cultures were limited by nitrogen rather than phosphorus, a different pattern is seen. Sciandra et al. (2003a) grew *E. huxleyi* in continuous culture at light intensities (170 µE m^{-2} s^{-1}) similar to our experiments, but under N-limitation and at a higher growth rate (0.5 d^{-1}). They observed an immediate response of cells to changes in pCO_2 and a decrease in net calcification and photosynthesis rates, yielding, however, no effect of pCO_2 on [PIC]:[POC]. Also during N-limited growth in chemostats, Müller et al. (2012) observed a decreasing trend in [PIC]:[POC], and an increasing trend in [POC]:[POP] and [TPN]:[POP] with pCO_2, whereas [POC]:[PN] was unaffected by CO_2. Thus, nitrogen limitation may not suppress CO_2 sensitivities of *E. huxleyi*.

Transient versus steady state growth

Rokitta & Rost (2012a) emphasized that in previous studies of *E. huxleyi* grown at present CO_2 levels (or higher), the TPC content remained stable despite a change in PIC and POC quotas. A decrease in PIC was associated with an increase in POC or vice versa. They concluded that stable TPC production, in spite of different pCO_2 levels, suggests that overall CO_2 uptake does not change, but that the carbon is allocated between POC and PIC differently under ocean acidification. In our study, PIC and POC cell quotas were rather constant over time and similar between pCO_2 treatments. This suggests that *E. huxleyi* is in principle capable of acclimating to different CO_2 concentrations, so that no differences in its cellular elemental content could be resolved during this experiment. Steady state (balanced) growth, i.e. growth at a defined rate, over an extended period is a prerequisite to investigate the physiological response of cells to a limiting factor. On the one hand, the chemostat system is therefore more advantageous than the batch approach, where growth rates and environmental conditions, like nutrient supply, are constantly changing (Jannasch 1974, Langer et al. 2013). On the other hand, balanced growth conditions are hardly met in a natural environment. In nature, cell growth is a transient process and the cell's physiology is dynamic, constantly pursuing optimum conditions for growth. A CO_2 effect may therefore be related to the cell's physiological ability to respond to a changing microenvironment.

One important process in this respect is the partitioning of metabolic products under nutrient-limiting conditions. For *E. huxleyi*, it has been shown that an increase in POC production under nutrient limiting conditions was associated with the production of extracellular particulate organic carbon, such as TEP (de Bodt et al. 2008, Borchard & Engel 2012). An increase in TEP concentration was also part of the

explanation for increasing POC concentrations during mesocosm studies with and without pCO_2 alterations (Engel et al. 2002, Engel et al. 2004b). Other studies have revealed that TEP formation increases with increasing ocean acidification (Engel 2002, Mari 2008, Borchard & Engel 2012). It is known that CO_2 can alleviate rate limitation of primary production in phytoplankton, such as *E. huxleyi,* that are inefficient in carbon acquisition (Rost et al. 2003). Higher pCO_2 in exponentially growing cells can therefore support higher primary production that, under nutrient limitation, is exuded in the form of carbohydrates, eventually forming TEP.

Production of TEP is viewed as a carbon 'overflow' for photosynthesis products under nutrient depletion. Schartau et al. (2007) showed that in a model study the production of organic carbon in excess of nitrogen uptake and Redfield stoichiometry, frequently referred to as 'carbon overconsumption' (Toggweiler 1993), can be split into 2 types. The first type explains a small amount of excess carbon uptake during exponential growth. This excess carbon is well allocated within the cell and contributes to changes in cell quota while the release of carbohydrates remains small. The second type is a transient phenomenon that occurs when nutrients limit cell growth. In this case, the carbon assimilation is too extreme and cannot be allocated within the cell to promote growth. The cells are in a state of severe acclimation, and excessive carbon is released as carbohydrates. This transient period of growth limitation is largely determined by the timescale of possible physiological acclimation. Obviously, it is this transient growth phase where we must expect the largest sensitivity in TEP formation to variations in pCO_2. Our chemostat experiment does not cover a transient growth period, which could explain the observed negligible TEP concentrations.

Comparison with previous studies

The absence of a clear CO_2 signal on element composition or TEP production during our study does not necessarily imply the absence of a CO_2 effect on *E. huxleyi* in general, but demands a more careful consideration of (short-term) variations in growth history. For example, comparable low growth rates can be achieved under steady state conditions, or they can occur during the course of a bloom experiment, e.g. when approaching nutrient depletion. Both situations differ with respect to the cell's physiological acclimation state and likely exhibit different sensitivities to environmental variations such as in pCO_2.

Previous studies of CO_2 effects on growth and calcification of *E. huxleyi* differed in their experimental design and covered diverse growing conditions, including transient growth phases. Since we would not expect transient growth periods to happen during experiments with sufficient nutrient supply and high growth rate, we anticipated larger differences in elemental content between independent studies at lower rather than at high growth rates. Likewise, studies that included periods of physiological acclimation, i.e. due to nutrient limitation, should have shown larger differences in elemental content between different pCO_2 conditions than those studies where high growth rates were maintained by nutrient saturation.

To compare our results of growth under steady-state conditions with previous findings, we compiled published data of PIC and POC divided by the corresponding cell abundance of *E. huxleyi* at various pCO_2 levels and growth rates. The collected data originate from 20 independent studies, of which 17 datasets were retrieved from the EPOCA database; www.epoca-project.eu/index.php/data/data-sets.html (Fig. 7a-d). The pH levels in these studies cover a wide range, from 7.09 ($pCO_2 \approx 5869$ µatm) to 9.12 ($pCO_2 \approx 28$ µatm). Observed growth rates varied between 0.01 and 1.67 d^{-1} (Fig. 7a). The dataset was split into an upper and lower range of relative growth rates (RGR). The separation is defined at RGR = 0.8 d^{-1}, which approximates the middle of the variational growth range and splits data into 2 subsets of comparable size (n = 178 for RGR > 0.8 d^{-1} and n = 220 for RGR < 0.8 d^{-1}). In addition, we differently labeled data subsets according to their pH variational range. Mean pH of all measurements was $\overline{pH} = 8.04$, with a standard deviation of $\sigma = 0.25$. The upper range considered data with pH > 8.29 = $\overline{pH} + \sigma$, the middle range included data that fulfilled pH < $\overline{pH} \pm \sigma <$ pH, and data with pH below 7.79 ($\overline{pH} - \sigma < 7.79$) (Fig. 7a). The purpose of splitting data into smaller subsets was to visually accentuate how variability in POC and PIC cell quotas and the corresponding [PIC]:[POC] depended on RGR and pH (Fig. 7b-d).

The compiled PIC and POC cell quotas with RGR >0.8 d^{-1} were normally distributed with median ≈ mean = 0.9 ± 0.2 pmol $cell^{-1}$ for POC and median ≈ mean = 0.7 ± 0.2 pmol $cell^{-1}$ for PIC. Likewise, the corresponding distribution of [PIC]:[POC] values was normal (median ≈ mean = 0.8 ± 0.2 pmol $cell^{-1}$). Cell quotas of POC and PIC with RGR < 0.8 d^{-1} revealed much greater variability, emphasizing

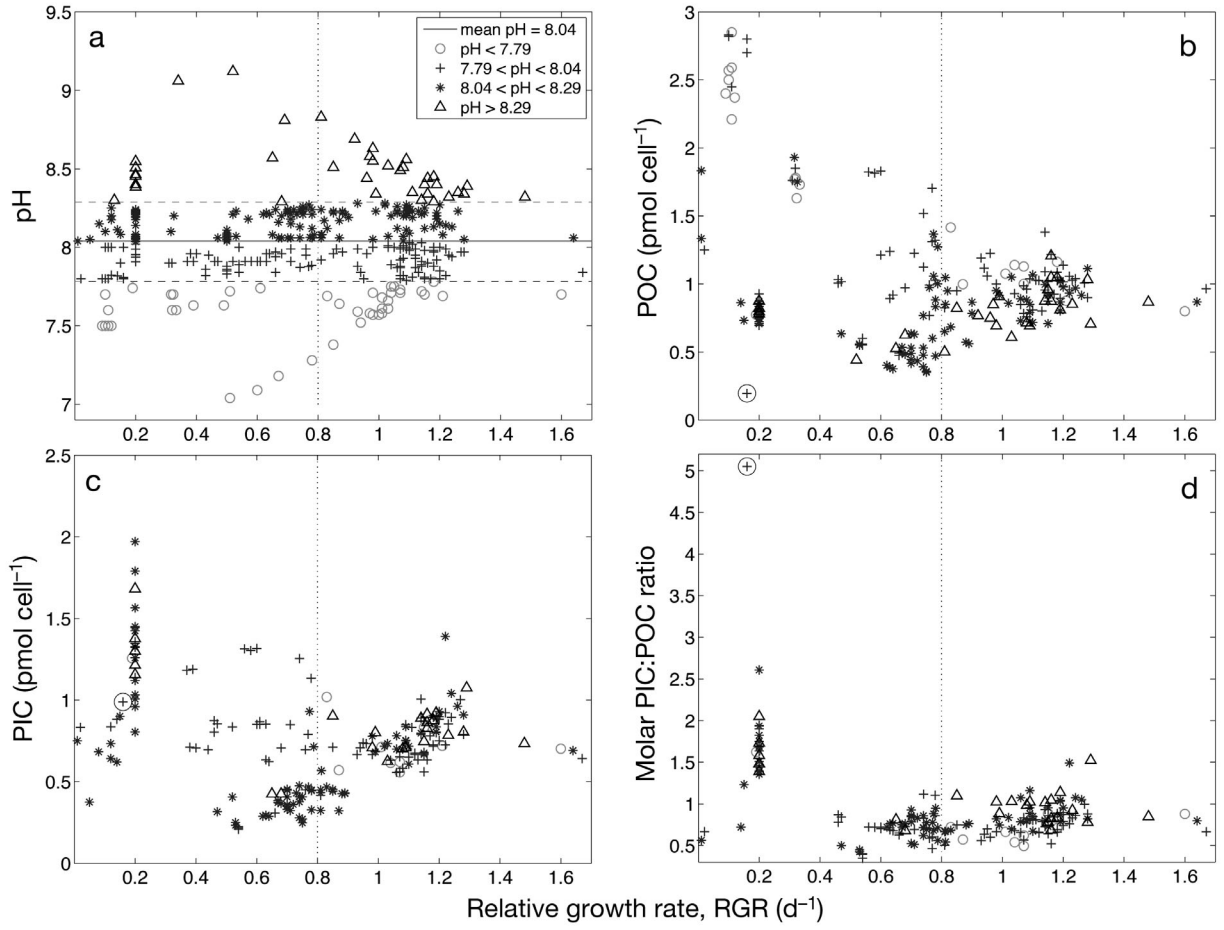

Fig. 7. Compiled dataset for *Emiliania huxleyi* split according to the 4 pH ranges applied during these studies; plotted against relative growth rate (RGR) are (a) pH, (b) POC normalized to cell abundance, (c) PIC normalized to cell abundance, and (d) [PIC]:[POC] molar ratios. Solid and dashed lines indicate mean pH and mean pH ± standard deviation (μ ± σ pH), respectively. Vertical dotted lines mark the separation of data into subsets of RGR < 0.8 d^{-1} < RGR, explanation in text. Data included from: Biermann & Engel (2012), Borchard et al. (2012), Barcelos e Ramos et al. (2010), Richier et al. (2011), Koch (2010), Iglesias-Rodriguez et al. (2008b), Langer et al. (2009b), Lefebvre et al. (2011), Riebesell et al. (2000a,b), Sciandra et al. (2003b), Fiorini et al. (2010), Bach et al. (2011), de Bodt et al. (2010b), Fiorini et al. (2011), Hoppe et al. (2011), Müller et al. (2010), Rokitta & Rost (2012b), Lohbeck et al. (2012), and the present study. One data point was encircled as an outlier due to its very low C-quota

physiological variations with different impacts on element stoichiometry. At low RGR (<0.8 d^{-1}), the subset of POC and PIC measurements deviated from their means, with more data exhibiting elevated values. For example, the data subset that corresponded with the lower pH range (pH < 7.79) included up to threefold higher values than the mean value observed at RGR > 0.8 d^{-1}. For RGR < 0.4 d^{-1} and pH < 7.79 in particular, POC quotas were significantly higher at high pCO_2 levels, with values between 1.6 and 2.9 pmol $cell^{-1}$, which is well above the median value of 0.92 pmol $cell^{-1}$ (Fig. 7b). Under low pCO_2 conditions (pH > 8.26), we found no POC above 1.2 pmol $cell^{-1}$, and all values for RGR < 0.8 d^{-1} were below the respective reference median of RGR >0.8 d^{-1}. The medium pH ranges (7.79 < pH < 8.04 and 8.04 < pH < 8.29) included POC measurements that ranged from 0.2 up to 1.9 pmol $cell^{-1}$. PIC data showed similar behavior compared to POC, but with one exception: elevated PIC values between 1.1 and 1.7 pmol $cell^{-1}$, i.e. higher than the reference average of 0.7 pmol $cell^{-1}$, were also observed under low pCO_2 conditions (pH > 8.26) at low RGR. These values yielded elevated [PIC]:[POC] between 1.4 and 2.0 (Fig. 7d), and encompassed the range observed during this study. For *E. huxleyi*, elevated [PIC]:[POC] at low RGR can be explained by (1) an increasing number of calcite platelets per cell when cell growth ceases while calcification continues, and (2) a harvest of detached coccoliths after they have been shed by growth-limited cells of growth-limited cultures dur-

ing sampling. At higher RGR, cell quotas of PIC and POC revealed similar trends, yielding no clear difference of [PIC]:[POC] between pH settings.

Overall, the variability seen in these subsets makes the extraction of a generic CO_2 response signal difficult, if not impossible. To approach this, further details about differences between experimental setups are required to untangle variations in growth condition. Experimental settings of the compiled dataset shown in Fig. 7 include variations in pCO_2, temperature, light, species strain, nutrient stoichiometry, and growth rate as well as data from chemostats and batch cultures. To deduce a clear response signal in PIC or POC quotas due to pCO_2 or pH change remains difficult, particularly for the medium pH range (7.79 < pH < 8.29) that may be representative of future ocean acidification (Frölicher & Joos 2010). Here, element quotas are seemingly highly sensitive to a variety of environmental conditions and vary with RGR. Data obtained during our chemostat study fall well within the range of the previous observations, but no direct effect of pCO_2 on elemental stoichiometry of *E. huxleyi* was observed while RGR was constant. With respect to climate change, variations in the spatial and temporal distribution of *E. huxleyi* will occur in association with changes in temperature, nutrient availability, grazing pressure, and ocean acidification. A better understanding of the cell's physiological constraints to optimize resources, i.e. element uptake and incorporation, and to acclimate to a heterogeneous and continuously changing environment, might be necessary before we can truly estimate the future response of elemental composition of *E. huxleyi* to environmental stressors such as ocean acidification.

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