ORIGINAL ARTICLE

Independence of nutrient limitation and carbon dioxide impacts on the Southern Ocean coccolithophore *Emiliania huxleyi*

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Future oceanic conditions induced by anthropogenic greenhouse gas emissions include warming, acidification and reduced nutrient supply due to increased stratification. Some parts of the Southern Ocean are expected to show rapid changes, especially for carbonate mineral saturation. Here we compare the physiological response of the model coccolithophore *Emiliania huxleyi* (strain EHSO 5.14, originating from 50°S, 149°E) with pH/CO_2 gradients (mimicking ocean acidification ranging from 1 to 4 × current $p$$CO_2$ levels) under nutrient-limited (nitrogen and phosphorus) and -replete conditions. Both nutrient limitations decreased per cell photosynthesis (particulate organic carbon (POC) production) and calcification (particulate inorganic carbon (PIC) production) rates for all $p$$CO_2$ levels, with more than 50% reductions under nitrogen limitation. These impacts, however, became indistinguishable from nutrient-replete conditions when normalized to cell volume. Calcification decreased three-fold and linearly with increasing $p$$CO_2$ under all nutrient conditions, and was accompanied by a smaller ~30% nonlinear reduction in POC production, manifested mainly above 3 × current $p$$CO_2$. Our results suggest that normalization to cell volume allows the major impacts of nutrient limitation (changed cell sizes and reduced PIC and POC production rates) to be treated independently of the major impacts of increasing $p$$CO_2$ and, additionally, stresses the importance of including cell volume measurements to the toolbox of standard physiological analysis of coccolithophores in field and laboratory studies.
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Introduction

Anthropogenic carbon dioxide absorbed by the surface ocean rapidly alters the seawater carbonate speciation, resulting in an increase of seawater CO_2 and HCO_3^- concentrations (ocean carbonation) and a decrease of CO_3^{2-} and pH (ocean acidification). This process is accompanied by additional environmental changes such as surface warming, stratification and the decreased supply of nutrients to the surface mixed layer (Sarmiento *et al.*, 1998). These multiple changes in the oceanic environment are acting simultaneously and with different regional and temporal intensities (Boyd and Doney, 2002; Boyd *et al.*, 2008). It has become increasingly important to investigate how multiple drivers affect the physiological response of phytoplankton to predict future eco-physiological changes in phytoplankton

communities (Boyd and Hutchins, 2012; Boyd *et al.*, 2015a; Brennan and Collins, 2015; Riebesell and Gattuso, 2015). In today's ocean, light supply and the availability of macro- and micronutrients in the surface mixed layer represent the main limiting factors for phytoplankton growth (Davey *et al.*, 2008; Moore *et al.*, 2008; Marinov *et al.*, 2010). While it is projected that the underwater light supply for phytoplankton growth will be enhanced due to shallower mixed layers, the nutrient supply from the underlying water column will be decreased (Boyd *et al.*, 2008). Thus, it seems reasonable to assume that in a future ocean nutrient availability will have a pronounced role in limiting phytoplankton growth.

Coccolithophores are the dominant pelagic calcifiers in the ocean and play an important role in the cycling of carbon over both modern and geological time scales due to their ability to photosynthesize and to produce minute calcium carbonate scales (coccoliths) in the sunlit layer of the ocean. The formation and subsequent export and deposition of coccoliths to the sediments reduce alkalinity of the surface ocean, decreasing the efficiency of the

'biological pump' to sequester anthropogenic carbon dioxide. Estimating the distribution and productivity of coccolithophores in comparison with non-calcifying phytoplankton represents an important component for predictive biogeochemical models to assess possible changes in climatic feedback mechanisms. The response of coccolithophores to changing carbonate chemistry has been intensively investigated in nutrient-replete batch laboratory culture experiments and is overall well understood (Bach *et al.*, 2015; Meyer and Riebesell, 2015). The combined effects of changing carbonate chemistry and nutrient availability are less well understood and have been mostly documented for the model species *Emiliania huxleyi* isolated from northern hemisphere waters (Sciandra *et al.*, 2003; Leonardos and Geider, 2005; Borchard *et al.*, 2011; Lefebvre *et al.*, 2012; Müller *et al.*, 2012; Engel *et al.*, 2014). However, coccolithophores from low-latitude oceans and especially the Southern Ocean remain under-studied even though the Southern Ocean is one of the world's regions affected most severely by anthropogenic acidification (Orr *et al.*, 2005; Gille, 2008). *Emiliania huxleyi* is represented in the Southern Ocean by several morphotypes, of which the ecotypes A and B/C are mainly found in the open ocean (Cubillos *et al.*, 2007; Krueger-Hadfield *et al.*, 2014). These ecotypes differ in cell size, coccolith morphology, photosynthetic pigment compositions and physiological response sensitivity to environmental conditions (Cook *et al.*, 2011, 2013). Southern Ocean *E. huxleyi* ecotype A and B/C exhibit lower physiological rates (photosynthesis, calcification and growth rate) compared with its northern hemisphere counterpart and, additionally, a higher sensitivity to changing seawater carbonate speciation (Müller *et al.*, 2015). The Southern Ocean represents a geographically isolated ecosystem for phytoplankton due to the Subantarctic and Polar fronts which reduce the possibility of lateral gene transfer and recombination. It is therefore unknown if mechanistic physiological concepts developed for northern hemisphere ecotypes (e.g. Bach *et al.*, 2015) can be transferred to their Southern Ocean counterparts. Here, we investigated the sensitivity of Southern Ocean *E. huxleyi* ecotype A to future ocean acidification scenarios under nutrient-limited growth using exponentially growing chemostat cultures to which controlled amounts of nitrate and phosphate were supplied. These physicochemical conditions were identical to those applied in our previous study (Müller *et al.*, 2015), facilitating direct comparison of the effects of ocean acidification between nutrient-replete and -limited growing cultures of *E. huxleyi*.

Material and methods

Culture conditions
Emiliania huxleyi originating from the Southern Ocean (strain EHSO 5.14—ecotype A; isolated from 50°S and 149°E by S Cook in February 2007) was grown under axenic conditions as asexual diploids at 14 °C in 0.2 µm filtered natural seawater (collected offshore of Bruny-Island, Tasmania) with a salinity of 35 psu and a continuous photon flux density of 100–115 µmol photons m^{-2} s^{-1}. *Emiliania huxleyi* was cultured under continuous light over more than 6 months prior to experimental use to ensure full entrainment of the cellular division cycle and other intracellular circadian oscillations. The strain was obtained from the Algae Culture Collection at the Institute of Marine and Antarctic Studies at the University of Tasmania, Australia. Macro- and micronutrients were added according to f/20 and f/80, respectively, corresponding to 88 µmol l^{-1} nitrate and 3.6 µmol l^{-1} phosphate (Guillard, 1975), which resulted in improved growth behaviour (Müller *et al.*, 2015).

Experimental chemostat setup
All experiments were conducted in chemostat culture vessels consisted of water-jacketed 2 l cylinders (filled to 1.8 l) connected to a circulating water bath maintained at a constant temperature of 14 °C (light conditions as described above). Chemostat culture vessels were connected to the seawater media supply tanks (10 l cubitainers) by the gas-tight silicon tubing system running through one peristaltic pump (Gilson-Minipuls, Gilson S.A.S., Villiers le Bel, France) per chemostat. Before experimental utilization, the culture vessels and all tubing were cleaned and rinsed completely with a 10% HCl solution. The chemostat vessels were filled for 24 h with 10% HCl and subsequently rinsed with MilliQ-water and sterile seawater. Culture media supplying the chemostat experiments were prepared in 10 l cubitainers (supply tanks), which were pre-washed with 10% HCl and rinsed with deionized water before utilization. The cubitainers were tested for CO_2 permeability by filling them with acidified seawater (pCO_2 of ~ 2100 µatm) leaving no headspace and, subsequently, the total dissolved inorganic carbon (C_T) concentration was monitored over a 1 month period. The C_T concentration in the cubitainer had no significant change over the course of the test period ($C_T = 2085.5 \pm 1.5$ µmol kg^{-1}, $n = 5$).

Seawater was adjusted to the target carbonate chemistry speciation by addition of HCl and NaHCO$_3$. Afterwards, macro- and micronutrients were added according to f/20 and f/80, respectively, except for the limiting nutrient (phosphate or nitrate). When nitrogen was the limiting element, initial nitrate concentrations were set to 5.3 ± 0.9 µmol l^{-1} (1s.d., $n = 4$), resulting in an N:P ratio of ~ 2.8 and when phosphorus was the limiting element, initial phosphate concentrations were set to 0.32 ± 0.6 µmol l^{-1} (1s.d., $n = 4$), resulting in an N:P ratio of ~ 137. Culture medium was transferred from the chemostat supply tanks (cubitainers) to the pre-cleaned chemostat vessels via acid cleaned tubes

passing a 0.2-µm sterile acid cleaned filter. When the chemostat vessels were filled with 1800 ml of culture medium, the supply was stopped, an exponential growing pre-culture of *E. huxleyi* (acclimated for 4–6 generations to target pCO$_2$ levels) was inoculated and the vessel was sealed airtight. *Emiliania huxleyi* (starting density of 90–130 cells ml^{-1}) was allowed to grow exponentially to the maximum population density (growth of 8–9 generations depending on nitrate or phosphate availability) and afterwards the medium inflow (dilution rate) from the supply tanks to the chemostat vessels was restarted. The chemostat vessels were operated at a constant dilution rate ($D = 0.20 \pm 0.01$ day^{-1}), which was periodically checked by weighting the incoming medium. After 7–15 days under limitation (acclimation period), *E. huxleyi* reached equilibrium state conditions (constant cell number with less than 7% variations) and was allowed to grow for another 6–10 days (3–7 generations) under equilibrium before the dilution was stopped and the chemostat culture was sampled. During equilibrium conditions, cell number, coccosphere, cell and coccolith volume were checked daily with a Coulter Multisizer 4 and the chemostat supply tanks were sampled every second day for C_T and total alkalinity (A_T). At the end of the chemostat experiments, samples were taken from the chemostat vessels for C_T, A_T, cell number, coccosphere, cell and coccolith volume, particulate organic carbon (POC) and total particulate carbon (TPC). Cell densities at the termination of the experiment ranged from 29×10^3 to 50×10^3 cells ml^{-1} to avoid major changes in seawater carbonate chemistry and light limitation due to self-shading. Nutrient concentrations (nitrate +nitrate and phosphate) were measured from the chemostat vessel at the end of the experiment.

Experiments were conducted under axenic conditions but bacterial contamination cannot be excluded. Coulter Multisizer 4 analysis of possible bacteria and detritus particles ranging from 0.12 to 2.5 µm^3 indicated a maximum abundance of $\sim 1.8 \times 10^6$ ml^{-1}. This bacterial abundance is comparable to previous *E. huxleyi* chemostat experiments, contributing less than 2% of the total POC (Lefebvre *et al.*, 2012; Müller *et al.*, 2012; Engel *et al.*, 2014), and we therefore assume bacterial activity to be negligible.

Carbonate chemistry
The carbonate system was monitored via C_T and A_T measurements. Dissolved inorganic carbon and A_T were analysed as the mean of triplicate measurements with the infrared detection method using an Apollo SciTech DIC-Analyzer (Apollo SciTech, Newark, DE, USA) (Model AS-C3) and the potentiometric titration method (Dickson *et al.*, 2003), respectively. Data were corrected to Certified Reference Materials (CRM; Scripps Institution of Oceanography, La Jolla, CA, USA). Consecutive measurements of the reference material resulted in

an average precision of $>99.8\%$ for both C_T and A_T. Carbonate system parameters were calculated from temperature, salinity, C_T and A_T (mean values from the start and end of experiments) using CO2SYS (version 2.1 by E Lewis and D W R Wallace), with the stoichiometric equilibrium constants for carbonic acid given in Roy *et al.* (1993).

Cell numbers, coccosphere, cell and coccolith volume
Samples for cell number and coccosphere/cell volume were processed directly after sampling and each measured three times with a Coulter Multisizer 4. Afterwards, the samples were acidified with HCl (0.1 mM) to dissolve all free and attached coccoliths and subsequently measured again to determine the cell and coccolith volume of *E. huxleyi* (Müller *et al.*, 2012). Estimates of average sphere, cell and coccolith volumes were associated with a random error of $<4\%$, which was determined by repeated measurements of identical *E. huxleyi* culture material ($n = 10$).

Elemental analyses
For each experiment, three sub-samples were filtered onto precombusted quartz filters (450 °C for 4 h) and frozen at -20 °C. TPC and POC were measured at the Central Science Laboratory of the University of Tasmania (CSL-UTAS) on separate filters using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser (Thermo Fisher Scientific, Waltham, MA, USA). The filter for POC analysis was treated with fuming HCl (~10 h) to remove all inorganic carbon (Hedges and Stern, 1984). Particulate inorganic carbon (PIC) was calculated from the difference of TPC and POC. Particulate organic nitrogen (PON) was analysed simultaneously with the POC measurements. Cell quota of particulate matter PM (PM = PIC, POC and PON) per cell was calculated as

$$PM/cell = PM_{filter}/(V \times N)$$

where PM$_{filter}$ is the mass (pg) of particulate matter per filter, V is the volume (ml) filtrated and N is the number of cells per ml. Repeated analysis of identical *E. huxleyi* culture material ($n = 5$) resulted in a random error of $<5\%$ for TPC, POC and PON while PIC was associated with an error of $<8\%$ due to error propagation. Cellular particulate matter was additionally calculated on a per cell volume basis as

$$PM/cellV = PM/cell \times cellV^{-1}$$

where cellV is the volume per cell (µm^3 per cell). Production rates of PIC, POC and PON were calculated by multiplying the cell quota (PM/cell or PM/cellV) by the applied chemostat dilution rate, which equals cellular growth rate (μ).

Nutrient analyses
Samples for phosphate and nitrate+nitrite analyses were sterile filtered and stored frozen until analysis.

Duplicate samples from each chemostat vessel were analysed colorimetrically (Hansen and Koroleff, 1999).

Fitting procedure, statistical analysis and model application
Chemostat data were compared with results from nutrient-replete conditions by fitting the nutrient-replete data to a first- or second-degree polynomial in a least-square sense using the MATLAB function 'polyfit'. Polynomial prediction intervals of 95% and 99% were calculated with the MATLAB function 'polyconf'. Thus, all chemostat data located outside the 95% or 99% prediction intervals of nutrient-replete conditions can be regarded as significantly or highly significantly different, respectively.

Production rates of POC and PIC were normalized to the nutrient-specific maximum measured rates, which resulted in relative (rel.) production rates ranging between 0 and 1. Relative PIC production was fitted to a recent developed model (Bach *et al.*, 2015). Maximum PIC production rates were encountered at 422, 343 and 366 µatm pCO_2 for EHSO 5.14 under replete, nitrogen- and phosphorus-limited conditions, respectively. The model describes the response of coccolithophore calcification rate as a function of seawater carbonate chemistry, with the sensitivity parameters *a*, *b*, *c* and *d*:

$$\text{Calcification rate} = \frac{a[\text{HCO}_3^-]}{b + [\text{HCO}_3^-]} - e^{-c[\text{CO}_2]} - d[\text{H}^+]$$

Fitting procedure was done as described in Bach *et al.* (2015) using the function 'lsqnonlin' in MATLAB, which solves nonlinear least-squares with optional boundary parameters.

Results

Carbonate chemistry and nutrient availability
The manipulation of the seawater carbonate chemistry in the chemostat supply tanks resulted in a significant change in C_T concentrations (ranging from 2065 to 2304 µmol kg^{-1}, *n* = 8) whereas the A_T remained relatively constant (2299 ± 12 µmol kg^{-1}, *n* = 8). This resulted in a pCO_2 and pH$_{(\text{total scale})}$ range in the chemostat supply tanks from 392 to 1677 µatm and from 8.06 to 7.48, respectively. Dissolved inorganic carbon consumption by coccolithophore growth was in general higher under phosphorus limitation but remained below 5% in all experiments, which resulted in small alterations of carbonate chemistry speciation in the chemostat vessels at the termination of the experiments (Table 1). At nitrogen-induced chemostat equilibrium conditions, nitrate+nitrite concentrations were near or below the detection limit (< 0.2 µmol l^{-1}) and phosphate concentrations were > 1.5 µmol l^{-1}. When phosphorus was the limiting nutrient, no phosphate was

Table 1 Carbonate chemistry in the chemostat vessels at the time of sampling

C_T (µmol kg^{-1})	A_T (µatm)	pCO_2 (µatm)	pH (total scale)	CO_2	HCO_3 (µmol kg^{-1})	CO_3	Ω (calcite)
Nitrogen limitation							
2024	2273	343	8.10	13	1833	178	4.24
2145	2274	699	7.84	27	2013	105	2.51
2212	2276	1113	7.65	43	2097	72	1.71
2261	2300	1362	7.57	52	2147	61	1.47
Phosphorus limitation							
1966	2188	366	8.07	14	1793	159	3.80
2106	2205	812	7.76	31	1987	88	2.10
2139	2197	1100	7.64	42	2029	68	1.62
2264	2287	1514	7.53	58	2150	55	1.32

detectable but nitrate+nitrite concentrations were > 80 µmol l^{-1}.

Cell quota and geometry
Coccolithophore growth rate was determined by the chemostat dilution rate (0.2 day^{-1}) and below the expected maximum growth rate for *E. huxleyi* strain EHSO 5.14 for the applied pCO_2 levels (see Müller *et al.*, 2015). Coccosphere, cell and coccolith volumes as well as cellular quota of POC, PIC and PON, respectively were throughout higher under phosphorus limitation compared with cell quota and coccosphere, cell and coccolith volume under nitrogen limitation (Figure 1 and Table 2). Highest coccosphere and cell volumes were observed under phosphorus limitation and elevated pCO_2 of ~ 1100 µatm whereas lowest volumes were detected under nitrogen limitation and elevated pCO_2 (Table 2). PIC and PON per cell decreased with pCO_2 under nitrogen and phosphorus limitation whereas POC per cell was highest at pCO_2 of around 1100 µatm. No coccolith volume could be determined at the highest tested pCO_2 value under nitrogen limitation because of insufficient coccolith production, which is also indicated by the low PIC content per cell of 0.7 pgC cell^{-1}. Coccolith volume increased with pCO_2 under nitrogen and phosphorus limitation from 0.43 to 0.47 and from 1.40 to 1.45 µm^3, respectively (Table 2). Coccolithophore sphere, cell and coccolith volumes under nutrient limitation were highly significantly different from nutrient-replete conditions (Figures 1a and b). Production rates per cell of POC and PIC were higher under phosphorus compared with nitrogen limitation and indicated similar trends in regard to the applied pCO_2 range (Figure 1). Under nitrogen limitation both POC and PIC production per cell were significantly different from nutrient-replete conditions, whereas under phosphorus limitation this was only indicated for POC production per cell (Figures 1c and d). The cellular PIC:POC ratio linearly decreased from ambient to elevated pCO_2
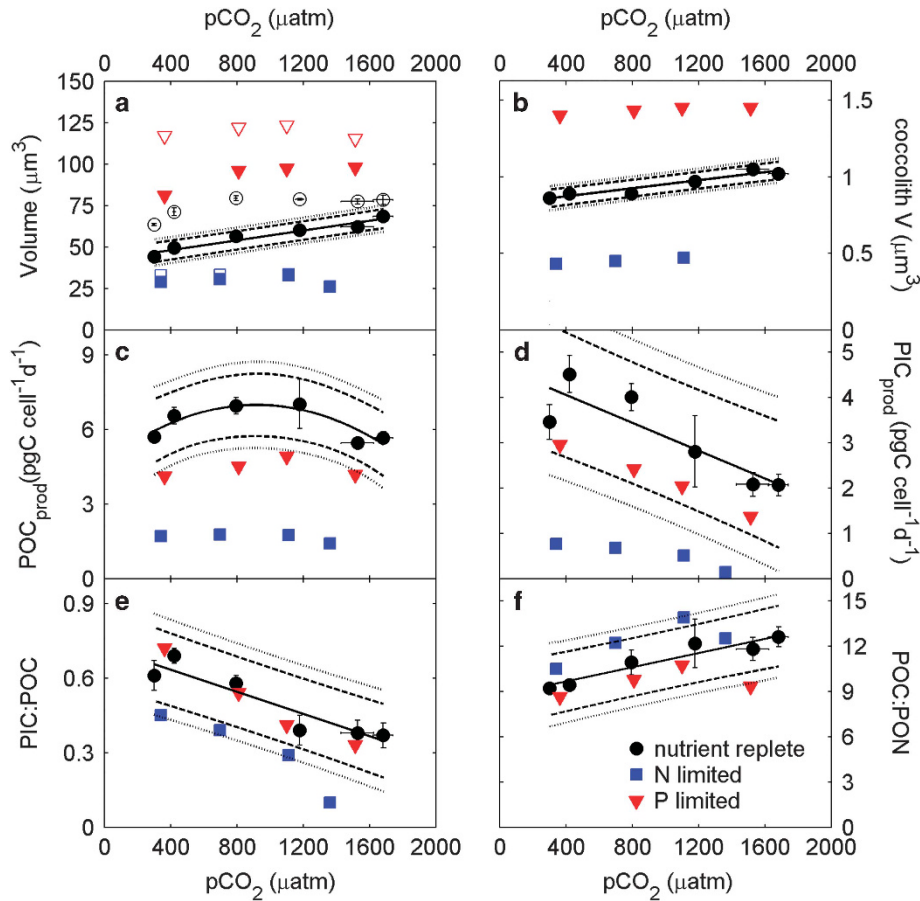
Figure 1 Physiological response of *E. huxleyi* ecotype A, strain SO5.14, to changing carbonate chemistry under nutrient-replete conditions (black circles; Müller *et al.*, 2015), nitrogen (blue squares) and phosphorus (red triangles) limitation. Black lines represent best-fit regressions (first- or second-degree polynomials) through nutrient-replete data with 95% and 99% prediction intervals (dashed and dotted black line, respectively). (**a**) Coccolithophore sphere and cell volume (open and filled markers, respectively), (**b**) coccolith volume, (**c**) particulate organic carbon production, (**d**) particulate inorganic carbon production, (**e**) ratio of particulate inorganic to organic carbon and (**f**) ratio of particulate organic carbon to nitrogen (mol:mol).

Table 2 *Emiliania huxleyi* cell quota, coccosphere, cell and coccolith volume at the applied pCO_2 levels under nitrogen and phosphorus limitation

pCO_2 (μatm)	POC (pgC cell^{-1})	PIC	PON (pgN cell^{-1})	Sphere V	CellV (μm^3)	Coccolith V
Nitrogen limitation						
343	8.5	3.8	0.69	32.8	28.7	0.43
699	8.8	3.4	0.62	33.3	30.4	0.45
1113	8.8	2.6	0.54	33.6	32.8	0.47
1362	7.0	0.7	0.48	26.3	25.9	—
Phosphorus limitation						
366	20.5	14.7	2.04	116.8	81.0	1.40
812	22.5	12.1	1.98	121.8	95.8	1.43
1100	24.5	10.2	1.96	123.1	97.2	1.45
1514	20.8	6.8	1.91	115.3	97.7	1.45

levels both under nitrogen and phosphorus limitation similar to nutrient-replete conditions. Under nitrogen limitation, however, a significant offset compared with nutrient-replete conditions was observed. POC:PON indicated no significant linear trend with pCO_2 ($r^2 = 0.09$, $P = 0.46$, $n = 8$; Figure 1f)

and was not significantly different from nutrient-replete conditions.

Discussion

Experimental setup
All chemostat experiments were conducted under continuous light exposure to desynchronize the cellular division cycle (cell cycle) of *E. huxleyi*, which results in an independence of sampling time to cellular volume and POC quota (Müller *et al.*, 2008, 2015). Thus, a bias due to different sampling times can be excluded. Alterations of physicochemical culture conditions (light, temperature and nutrient limitation) have the potential to modify the duration of certain cell cycle phases (G1, S, G2 and M) in phytoplankton (Olson *et al.*, 1986; Vaulot *et al.*, 1987; Müller *et al.*, 2008), which can shift the beginning and end of cell cycle phases to different location within the course of a complete cycle. Therefore, it is possible to sample different physiological states related to certain positions in the cell cycle of a synchronized population (light:day cycle)

when sampling at the same time of the day. This potential bias can be avoided when the population's cell cycle is desynchronized and the population is equally distributed in all cell cycle phases.

Chemostat experiments are designed to limit the exponential growth of cells to a desired steady-state level lower than the maximum possible growth rate under the applied physicochemical conditions (e.g. Sciandra *et al.*, 2003; Leonardos and Geider, 2005; Borchard *et al.*, 2011; Lefebvre *et al.*, 2012; Müller *et al.*, 2012; Engel *et al.*, 2014). This design is distinctively different from nutrient-starvation batch experiments (e.g. Vaulot *et al.*, 1987; Parpais *et al.*, 1996; Langer *et al.*, 2012, 2013; Rouco *et al.*, 2013). In these latter experiments, the dynamic transition from the exponential to the stationary growth phase is investigated, in which conditions become unfavourable for cell division until the growth rate approximates zero and cells loose viability. Thus the physiological state of cells in starvation experiments (stationary growth phase) is distinctly different from the physiological state observed in nutrient-replete batch and chemostat experiments (exponential growth phase). This implies that physiological results (e.g. cell quota and production rates) of nutrient-starvation experiments have to be compared with great caution with results from nutrient-replete batch and chemostat experiments. On the other hand, production rates of particulate material from the mid-exponential growth phase at nutrient-replete conditions (e.g. batch cultures) are comparable to exponential growing cultures under nutrient limitation because these rates include accounting for the specific growth (division) rates under each condition. For example, it seems counterintuitive that phosphorus-limited cells of *E. huxleyi* strain EHSO 5.14 have a higher POC quota (20.5 pgC cell^{-1}; Table 2) compared with nutrient-replete conditions at similar conditions (12.6 pgC cell^{-1}; Müller *et al.*, 2015). This increased cell quota under phosphorus limitation, however, is a result of the reduced division rate and concomitant accumulation of organic matter inside the cell. When accounting for the respective growth rates, the results become intuitively understandable with a lower POC production rate under phosphorus limitation compared with nutrient-replete conditions (4.1 compared with 5.7 pgC cell^{-1}day^{-1}, respectively; see also Figure 1).

Physiological response to ocean acidification under nutrient-limited and -replete conditions
Anthropogenic ocean acidification involves multiple changes in the speciation of seawater carbonate chemistry such as the increase of $CO_{2(aq)}$, HCO_3^- and H^+ and, on the other hand, the decrease of CO_3^{2-}. Changes in these parameters have direct influence on coccolithophore physiology as they are associated with cellular functions, such as photosynthesis, calcification and enzyme reactions. An increase of $CO_{2(aq)}$ and HCO_3^- results in enhanced growth, calcification and photosynthesis rates of phytoplankton when keeping pH, respectively, the H^+ concentration, constant (Bach *et al.*, 2011; McMinn *et al.*, 2014). Under ocean acidification scenarios (increase of $CO_{2(aq)}$, HCO_3^- and decrease of pH, CO_3^{2-}), calcification in the coccolithophore *E. huxleyi* is generally reduced due to the higher energetic costs for intracellular H^+ homeostasis and the elevation of the calcium carbonate saturations state ($\Omega_{calcite}$) at the site of calcification (Raven and Crawfurd, 2012). Changing carbonate speciation, however, is one factor that has to be considered when assessing possible changes in coccolithophore physiology in a future ocean. Additional environmental factors are concomitantly changing (e.g. temperature, light and nutrient supply). The importance of multiple driver response has been emphasized (Boyd and Hutchins, 2012) and understanding the single and combined responses will provide a fundamental basis to develop unifying concepts (Riebesell and Gattuso, 2015).

In this study, nutrient-limited chemostat experiments were conducted with the dominant Southern Ocean *E. huxleyi* ecotype A that was previously investigated in laboratory batch experiment under nutrient-replete conditions (Müller *et al.*, 2015). The same experimental conditions (temperature and light irradiance) and comparable pCO$_2$ gradients (336–1551 µatm) were applied and cellular growth was limited by the supply rate of either nitrogen or phosphorus.

Nitrogen limitation induced clear reduction in POC$_{prod}$, PIC$_{prod}$ per cell and cellular volume in *E. huxleyi* compared with non-limited conditions (Figure 1). Nitrogen is crucial for protein synthesis and the accumulation of cellular biomass. Reduced nitrogen availability presumably results in physiological adjustments towards minimal cellular biomass and reserves requirements for cell division, which is accompanied by reduced average cell volumes (Figure 1a). PIC:POC ratios were generally lower under nitrogen limitation in comparison with nutrient-replete conditions, which is in good agreement with previous chemostat studies indicating a reduction of PIC:POC under nitrogen limitation (Müller *et al.*, 2012). This suggests that under nitrogen limitation physiological preference is given towards maintaining photosynthetic activity compared with calcification. Some studies report increased PIC:POC ratios under nitrogen starvation (Paasche, 2002; Müller *et al.*, 2008). These observations were retrieved from batch experiments in which *E. huxleyi* entered the stationary growth phase due to nitrogen starvation. This is, however, not comparable to the limited exponential growth achieved in chemostat experiments (see the section above) and the increased PIC:POC ratio presumably results from continuous cellular organic resource consumption under nitrogen starvation.

Phosphorus-limited growth of *E. huxleyi* induced a reduction in POC_{prod} and PIC_{prod} per cell compared with nutrient-replete growth (Figure 2). However, only the reduction in POC_{prod} per cell was determined to be significantly different compared with nutrient-replete conditions. This is presumably related to the higher associated error of PIC data resulting in broader prediction intervals. Interestingly, the PIC:POC ratio under phosphorus limitation followed a similar trend as observed under nutrient-replete conditions, which indicates that both POC_{prod} and PIC_{prod} per cell are equally affected by reduced phosphorus supply. Highest coccosphere and cell volumes were detected in phosphorus-limited cells of morphotype A (Figure 1a). This confirms the observations described in the literature of *E. huxleyi* 'overproducing' coccoliths under phosphorus limitation and covering the cell in multiple layers of coccoliths (see the review of Paasche, 2002). Phosphorus is highly required for nucleic acid and phospholipid membrane synthesis (Geider and LaRoche, 2002) and the presence of sufficient phosphorus reserves are crucial to ensure completion of cell division and to prevent corruption of nuclear material (Antia *et al.*, 1990). The 'overproduction' of coccoliths is a result of an induced elongation of the G1 phase of the cellular division cycle by phosphorus limitation (Müller *et al.*, 2008). Calcification and photosynthesis take place in the G1 phase and an increased accumulation of POC and PIC occurs in the cell before sufficient amounts of phosphorus are concentrated to initiate functional cell division. This leads to increased cell and coccosphere volumes while PIC and POC production rates per cell are lower compared with nutrient-replete conditions (Figures 1c and d).

One of the most striking differences between phosphorus and nitrogen limitation is the difference in coccolith volume (Figure 1b). Highest coccolith and cell volumes were observed under phosphorus limitation, in line with previous studies, indicating an elevated calcium content of coccoliths produced under reduced phosphorus availability (Paasche, 1998). An overall correlation was observed between cell and coccolith volume regardless of nutrient conditions: $V_{coccolith} = 0.015$ $V_{cell} + 0.076$ ($r^2 = 0.95$, $P < 0.001$, $n = 13$), confirming previous observations of the relationship between coccolithophore cell diameter and coccolith volume (Müller *et al.*, 2012; Aloisi, 2015).
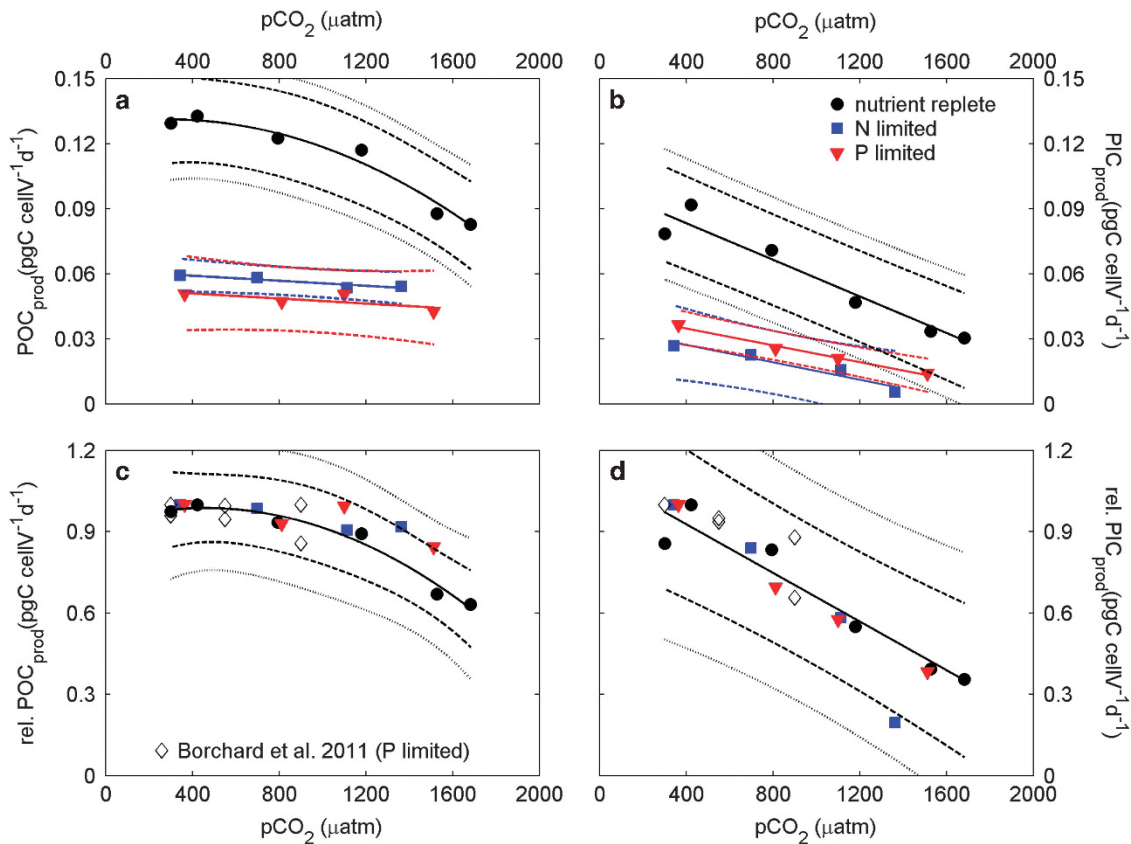

Figure 2 POC (**a**) and PIC (**b**) production rates per cell volume of *E. huxleyi* at nutrient-limited and -replete conditions and relative POC (**c**) and PIC (**d**) production rates per cell volume (normalized to the nutrient-specific maximum measured rate) with additional data from phosphorus limitation experiments (conducted at the same temperature of 14 °C; Borchard *et al.*, 2011), indicating the relative change induced by pCO_2. Solid lines represent best-fit regressions (first- or second-degree polynomials) through nutrient replete, nitrogen and phosphorus limited data (black, blue and red, respectively) with 95% and 99% prediction intervals (dashed and dotted lines, respectively).

Nutrient-limited POC and PIC production rates per cell of *E. huxleyi* followed similar patterns over the applied pCO$_2$ gradient compared with nutrient-replete conditions (Figure 1). This similarity amplified when accounting for the differences in cellular volume among the nutrient treatments. Calculating the POC and PIC production rates per cell specific volumes resulted in identical rates of nitrogen- and phosphorus-limited treatments while the offset from the nutrient-replete treatments decreased over the applied pCO$_2$ range (Figures 2a and b), suggesting that the degree of nutrient limitation was reduced with increasing pCO$_2$ levels. This was indeed the case in our experiments because growth rates under nitrogen and phosphorus limitation were fixed to 0.20 day^{-1} while maximum growth rates under nutrient-replete conditions decreased from 0.45 to 0.21 day^{-1} with increasing pCO$_2$ levels (Müller *et al.*, 2015). The pCO$_2$ induced changes in the relative POC and PIC production per cell volume (normalized to the nutrient-specific maximum measured rates at 422, 343 and 366 uatm pCO$_2$ for replete, nitrate and phosphorus limited conditions, respectively) indicated no difference and relative rates under nutrient limitation were not significantly different from nutrient-replete conditions (Figures 2c and d). This suggests the absence of combined effects of pCO$_2$ and macronutrient limitation on the POC and PIC production rates per cell volume of *E. huxleyi* within the applied pCO$_2$ range.

It remains to be demonstrated if this independence of macronutrient and pCO$_2$ impacts on production rates can be transferred to micronutrient limitation (e.g. iron or zinc). Zinc limitation triggers similar physiological changes compared with phosphorus limitation, with an increase in cell volume and elevated numbers of coccoliths per cell (Schulz *et al.*, 2004). It has been speculated that zinc and phosphorus limitation have similar effects on the cellular division cycle (impeding transition from the G1 to the S phase), which possibly could explain the similarity in observed physiological response (Müller *et al.*, 2008). Iron limitation, on the other hand, introduces a reduction in cell volume (similar to nitrogen limitation), which is presumably associated with physiological adjustments to meet cellular iron requirement and/or impairment of the photosynthetic machinery and concomitant reduction of cellular organic material build-up (Muggli and Harrison, 1996; Schulz *et al.*, 2004). Future studies focusing on the effects of micronutrient limitation and the cellular division cycle of *E. huxleyi* might reveal promising results helping to understand general response mechanisms. The absence of a difference in POC and PIC production rates between nitrogen and phosphorus limitation, after accounting for changes in cell volume, stresses the importance of including this trait to the toolbox of standard physiological measurements of coccolithophores in field and laboratory studies. This will then allow assessments of potential responses to multiple drivers in the future ocean to proceed in an additive way, for example, by first estimating cell volume changes from changing nutrient availability and then adding the effects of increasing pCO$_2$ using cell volume normalized experimental results.

Recently, a unifying concept has been developed to understand the mechanistic response of northern hemisphere coccolithophores to ocean acidification (Bach *et al.*, 2015), as a balancing of positive response to increased bicarbonate availability, negative impacts of decreasing pH and a minimum requirement for molecular CO$_2$. Here, we applied this model for Southern Ocean *E. huxleyi* ecotype A and included the chemostat data of this study together with the data available for strain EHSO 5.14 and 5.30 under nutrient-replete conditions (Müller *et al.*, 2015). The significant correlation of all measured and modelled rates for calcification (Figure 3a) and the absence of a significant difference between nutrient-replete and -limited conditions (Figure 3b) indicates that (1) this concept can be applied to both nutrient-replete and macronutrient-limited exponentially growing cells of *E. huxleyi* and (2) the Southern Ocean *E. huxleyi* ecotype A exhibits similar physiological regulating mechanisms for calcification in response to changing seawater carbonate chemistry compared with northern hemisphere strains, which is intriguing regarding their geographical distance. However, a high phenotypic variability has been reported for *E. huxleyi* (Blanco-Ameijeiras *et al.*, 2016) and it remains to be shown whether this concept can be transferred to other ecotypes. The calculated sensitivity parameters for Southern Ocean *E. huxleyi* ecotype A (Figure 3) do differ from the parameters reported for the northern hemisphere ecotype A (Bach *et al.*, 2015). This is not surprising because the model does not account for other environmental parameters such as temperature, light and salinity, which can influence the response sensitivity of *E. huxleyi* to changing seawater carbonate chemistry (Sett *et al.*, 2014). However, it is intriguing that this relatively simple model can satisfactorily describe the response of cellular calcification to seawater carbonate chemistry, thus motivating further experimentation and model development including additional environmental parameters.

The complex interplay of globally and regionally changing environmental drivers on phytoplankton physiology has received recent attention (Boyd *et al.*, 2015a) and sophisticated experiments have been conducted to give detailed explanations and predictions of regional species-specific future performance (Boyd *et al.*, 2015b). Most physiological rates of phytoplankton species follow optimum (e.g. temperature) or saturation (e.g. nutrient availability) curve behaviour in regard to changing environmental conditions. The response to ocean acidification, on the other hand, is presumably a combination of substrate limitation (CO$_2$ and HCO$_3^-$) and H$^+$-inhibition, which results in an optimum curve like behaviour (Bach *et al.*, 2015; Müller *et al.*, 2015).
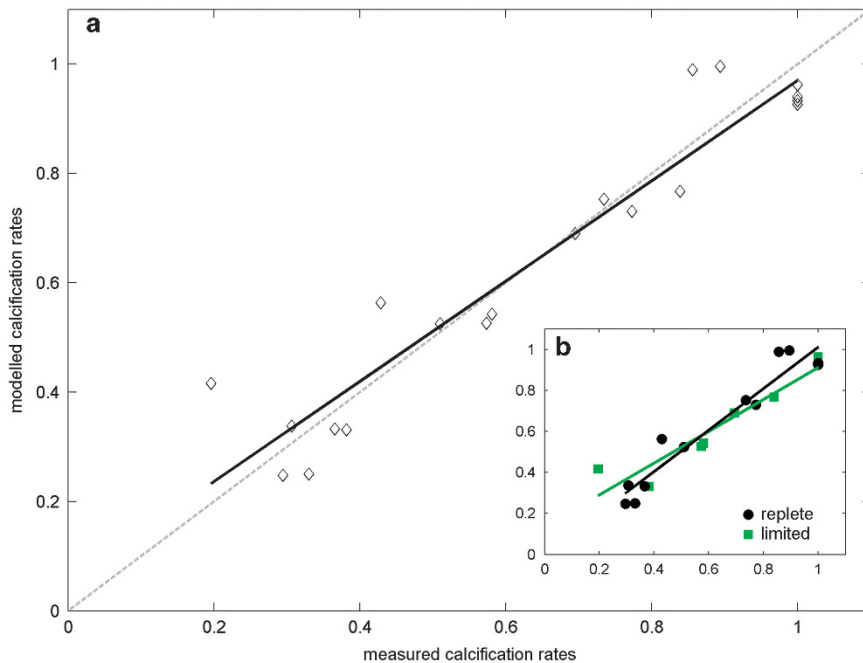
Figure 3 Measured calcification rates (relative to the nutrient-specific maximum measured calcification rate) of *E. huxleyi* SO5.14 and 5.30 compared with modelled calcification rates calculated according to Bach *et al.* (2015) using Equation (1) with the estimated sensitivity parameters ($a = 1.205$, $b = 2.06 \times 10^{-5}$ mol kg^{-1}, $c = 5.82 \times 10^{6}$ kg mol^{-1} and $d = 3.46 \times 10^{7}$ kg mol^{-1}) and the corresponding carbonate chemistry data (Table 1 and Müller *et al.*, 2015). (**a**) All available data of Southern Ocean *E. huxleyi* ecotype A under nutrient-replete and -limited conditions. The grey dashed line denotes the perfect fit (1:1 ratio). Linear regression analysis (black line) resulted in $y = 0.9168x + 0.0530$ ($r^2 = 0.91$, $P < 0.001$, $n = 20$). (**b**) Individual regressions of nutrient-replete (black markers and line) and nutrient-limited (green markers and line) conditions. Statistical analysis comparing both regression slopes indicated no difference (t-value $= 2.09$, $P = 0.053$).

Depending on the species- and strain-specific optima and half saturation constants to specific environmental parameters, the collated results of laboratory experiments comparing ambient to single point predicted future oceanic conditions may lead to a diverse range of species-specific physiological responses (positive, negative or no-effects). The recorded collection of changing environmental parameters, due to anthropogenic climate change, is accumulating with conducted research and environmental monitoring, which is leading to an increased complexity of multiple driver permutation. While it is of the utmost importance to increase our capability to predict future ecosystem functions by experimentally testing the relevant parameters, we emphasize that understanding the principal physiological response to single environmental parameters and their interplay will help to develop unifying physiological concepts.

Conclusions

Southern Ocean *E. huxleyi* ecotype A exhibits an identical relative physiological response (in terms of photosynthesis and calcification) to ocean acidification under nutrient-replete and nutrient-limited conditions (nitrogen and phosphorus), indicating independent effects of macronutrient limitation and ocean acidification. Our results agree well with a recently developed unifying concept (Bach *et al.*, 2015) and provide evidence of our increasing capacity to understand the principal physiological response of coccolithophores to multiple changing environmental conditions. This is useful for the design of multi-driver experiments, the assessment of future Southern Ocean ecological impacts and the interpretation of coccolithophore fossil records.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

References

Aloisi G. (2015). Co-variation of metabolic rates and cell-size in coccolithophores. *Biogeosciences* **10**: 4665–4692.

Antia AN, Carpenter EJ, Chang J. (1990). Species specific phytoplankton growth rates via diel DNA synthesis cycles. III. Accuracy of growth rate measurement in the

dinoflagellate *Prorocentrum minimum*. *Mar Ecol Prog Ser* **63**: 273–279.

Bach LT, Riebesell U, Schulz KG. (2011). Distinguishing between the effects of ocean acidification and ocean carbonation in the coccolithophore *Emiliania huxleyi*. *Limnol Oceanogr* **56**: 2040–2050.

Bach LT, Riebesell U, Gutowska MA, Federwisch L, Schulz KG. (2015). A unifying concept of coccolithophore sensitivity to changing carbonate chemistry embedded in an ecological framework. *Prog Oceanogr* **135**: 125–138.

Blanco-Ameijeiras S, Lebrato M, Stoll HM, Iglesias-Rodriguez D, Müller MN, Méndez-Vicente A *et al.* (2016). Phenotypic variability in the coccolithophore *Emiliania huxleyi*. *PLoS ONE* **11**: e0157697.

Borchard C, Borges AV, Händel N, Engel A. (2011). Biogeochemical response of *Emiliania huxleyi* (PML B92/11) to elevated CO_2 and temperature under phosphorous limitation: a chemostat study. *J Exp Mar Biol Ecol* **410**: 61–71.

Boyd PW, Doney SC. (2002). Modelling regional responses by marine pelagic ecosystems to global climate change. *Geophys Res Lett* **29**: 53-1–53-4.

Boyd PW, Doney SC, Strzepek R, Dusenberry J, Lindsay K, Fung I. (2008). Climate-mediated changes to mixed-layer properties in the Southern Ocean: assessing the phytoplankton response. *Biogeosciences* **5**: 847–864.

Boyd PW, Hutchins DA. (2012). Understanding the response of ocean biota to a complex matrix of cumulative anthropogenic change. *Mar Ecol Prog Ser* **470**: 125–135.

Boyd PW, Lennartz ST, Glover DM, Doney SC. (2015a). Biological ramification of climate-change-mediated oceanic multi-stressors. *Nat Clim Change* **5**: 71–79.

Boyd PW, Dillingham PW, McGraw CM, Armstrong EA, Cornwall CE, Feng Y-Y *et al.* (2015b). Physiological responses of a Southern Ocean diatom to complex future ocean conditions. *Nat Clim Change* **6**: 207–213.

Brennan G, Collins S. (2015). Growth response of a green alga to multiple environmental drivers. *Nat Clim Change* **5**: 892–897.

Cook SS, Jones RC, Vaillancourt RE, Hallegraeff GM. (2013). Genetic differentiation among Australian and Southern Ocean populations of the ubiquitous coccolithophore *Emiliania huxleyi* (Haptophyta). *Phycologia* **52**: 368–374.

Cook SS, Whittock L, Wright SW, Hallegraeff GM. (2011). Photosynthetic pigment and genetic differences between two Southern Ocean morphotypes of *Emiliania huxleyi* (Haptophyta). *J Phycol* **47**: 615–626.

Cubillos JC, Wright SW, Nash G, de Salas MF, Griffiths B, Tilbrook B *et al.* (2007). Calcification morphotypes of the coccolithophorid *Emiliania huxleyi* in the Southern Ocean: changes in 2001 to 2006 compared to historical data. *Mar Ecol Prog Ser* **348**: 47–54.

Davey M, Tarran GA, Mills MM, Ridame C, Geider RJ, LaRoche J. (2008). Nutrient limitation of picophytoplankton photosynthesis and growth in the tropical North Atlantic. *Limnol Oceanogr* **53**: 1722–1733.

Dickson AG, Afghan JD, Anderson GC. (2003). Reference materials for oceanic CO_2 analysis: a method for the certification of total alkalinity. *Mar Chem* **80**: 185–197.

Engel A, Novoa CC, Wurst M, Endres S, Tang T, Schartau M *et al.* (2014). No detectable effect of CO_2 on elemental stoichiometry of *Emiliania huxleyi* in nutrient-limited, acclimated continuous cultures. *Mar Ecol Prog Ser* **507**: 15–30.

Geider R, LaRoche J. (2002). Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur J Phycol* **37**: 1–17.

Gille ST. (2008). Decadal-scale temperature trends in the Southern Hemisphere ocean. *J Clim* **21**: 4749–4765.

Guillard R. (1975). Culture of phytoplankton for feeding marine invertebrates. In: Smith W, Chanley MH (eds). *Culture of Marine Invertebrate Animals*. Springer: New York, USA, pp 29–60.

Hansen HP, Koroleff F (1999). Determination of nutrients. In: Grasshoff K, Kremling K, Erhardt M (eds). *Methods of Seawater Analysis*, 3rd edn. Wiley-VCH: Weinheim, Germany, pp 159–226.

Hedges JI, Stern JH. (1984). Carbon and nitrogen determinations of carbonate-containing solids. *Limnol Oceanogr* **29**: 657–663.

Krueger-Hadfield SA, Balestreri C, Schroeder J, Highfield A, Helaouët P, Allum J *et al.* (2014). Genotyping an *Emiliania huxleyi* (Prymnesiophyceae) bloom event in the North Sea reveals evidence of asexual reproduction. *Biogeosciences* **11**: 5215–5234.

Langer G, Oetjen K, Brenneis T. (2012). Calcification of *Calcidiscus leptoporus* under nitrogen and phosphorus limitation. *J Exp Mar Biol Ecol* **413**: 131–137.

Langer G, Oetjen K, Brenneis T. (2013). Coccolithophores do not increase particulate carbon production under nutrient limitation: a case study using *Emiliania huxleyi* (PML B92/11). *J Exp Mar Biol Ecol* **443**: 155–161.

Lefebvre SC, Benner I, Stillman JH, Parker AE, Drake MK, Rossignol PE *et al.* (2012). Nitrogen source and pCO$_2$ synergistically affect carbon allocation, growth and morphology of the coccolithophore *Emiliania huxleyi*: potential implications of ocean acidification for the carbon cycle. *Glob Change Biol* **18**: 493–503.

Leonardos N, Geider RJ. (2005). Elevated atmospheric carbon dioxide increases organic carbon fixation by *Emiliania huxleyi* (Haptophyta), under nutrient-limited high-light conditions. *J Phycol* **41**: 1196–1203.

Marinov I, Doney SC, Lima ID. (2010). Response of ocean phytoplankton community structure to climate change over the 21st century: partitioning the effects of nutrients, temperature and light. *Biogeosciences* **7**: 3941–3959.

McMinn A, Müller MN, Martin A, Ryan KG. (2014). The response of Antarctic sea ice algae to changes in pH and CO_2. *PLoS ONE* **9**: e86984.

Meyer J, Riebesell U. (2015). Reviews and synthesis: responses of coccolithophores to ocean acidification: a meta-analysis. *Biogeosciences* **12**: 1671–1682.

Moore CM, Mills MM, Langlois R, Milne A, Achterberg EP, LaRoche J *et al.* (2008). Relative influence of nitrogen and phosphorus availability on phytoplankton physiology and productivity in the oligotrophic subtropical North Atlantic Ocean. *Limnol Oceanogr* **53**: 291–305.

Muggli DL, Harrison PJ. (1996). Effects of nitrogen source on the physiology and metal nutrition of *Emiliania huxleyi* grown under different iron and light conditions. *Mar Ecol Prog Ser* **130**: 255–267.

Müller MN, Antia AN, LaRoche J. (2008). Influence of cell cycle phase on calcification in the coccolithophore *Emiliania huxleyi*. *Limnol Oceanogr* **53**: 506–512.

Müller MN, Beaufort L, Bernard O, Pedrotti ML, Talec A, Sciandra A. (2012). Influence of CO_2 and nitrogen limitation on the coccolith volume of *Emiliania huxleyi* (Haptophyta). *Biogeosciences* **9**: 4155–4167.

Müller MN, Trull TW, Hallegraeff GM. (2015). Differing responses of three Southern Ocean *Emiliania huxleyi* ecotypes to changing seawater carbonate chemistry. *Mar Ecol Prog Ser* **531**: 81–90.

Olson RJ, Vaulot D, Chisholm SW. (1986). Effects of environmental stresses on the cell cycle of two marine phytoplankton species. *Plant Physiol* **80**: 918–925.

Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, Feely RA *et al.* (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**: 681–686.

Paasche E. (1998). Roles of nitrogen and phosphorus in coccolith formation in *Emiliania huxleyi* (Prymnesiophyceae). *Europ J Phycol* **33**: 33–42.

Paasche E. (2002). A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. *Phycologia* **40**: 503–529.

Parpais J, Marie D, Partensky F, Morin P, Vaulot D. (1996). Effect of phosphorus starvation on the cell cycle of the photosynthetic prokaryote *Prochlorococcus* spp. *Mar Ecol Prog Ser* **132**: 265–274.

Raven JA, Crawfurd K. (2012). Environmental controls on coccolithophore calcification. *Mar Ecol Prog Ser* **470**: 137–166.

Riebesell U, Gattuso J-P. (2015). Lessons learned from ocean acidification research. *Nature Clim Change* **5**: 12–14.

Rouco M, Branson O, Lebrato M, Iglesias-Rodriguez MD. (2013). The effect of nitrate and phosphate availability on *Emiliania huxleyi* (NZEH) physiology under different CO_2 scenarios. *Front Microbiol* **4**: 155.

Roy RN, Roy LN, Vogel KM, Porter-Moore C, Pearson T, Good CE *et al.* (1993). The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and temperatures 0 to 45 ˚C. *Mar Chem* **44**: 249–267.

Sarmiento JL, Hughes TMC, Stouffer RJ, Manabe S. (1998). Simulated response of the ocean carbon cycle to anthropogenic climate warming. *Nature* **393**: 245–249.

Schulz KG, Zondervan I, Gerringa LJA, Timmermans KR, Veldhuis MJW, Riebesell U. (2004). Effect of trace metal availability on coccolithophorid calcification. *Nature* **430**: 673–676.

Sciandra A, Harlay J, Lefevre D, Lemee R, Rimmelin P, Denis M *et al.* (2003). Response of coccolithophorid *Emiliania huxleyi* to elevated partial pressure of CO_2 under nitrogen limitation. *Mar Ecol Prog Ser* **261**: 111–122.

Sett S, Bach LT, Schulz KG, Koch-Klavsen S, Lebrato M, Riebesell U. (2014). Temperature modulates coccolithophorid sensitivity of growth, photosynthesis and calcification to increasing seawater $p CO_2$. *PLoS ONE* **9**: e88308.

Vaulot D, Olson RJ, Merkel S, Chisholm SW. (1987). Cell-cycle response to nutrient starvation in two phytoplankton species, *Thalassiosira weissflogii* and *Hymenomonas carterae*. *Mar Biol* **95**: 625–630.