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Influence of CO₂ and nitrogen limitation on the coccolith volume of *Emiliana huxleyi* (Haptophyta)

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Received: 30 March 2012 – Accepted: 10 April 2012 – Published: 25 April 2012

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Published by Copernicus Publications on behalf of the European Geosciences Union.

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Abstract

Coccolithophores, a key phytoplankton group, are one of the best studied organisms with regard to the response to ocean acidification/carbonation. The biogenic production of calcareous coccoliths has made coccolithophores a promising group for paleoceanographic research aiming to reconstruct past environmental conditions. Recently, geochemical and morphological analyses of fossil coccoliths have gained increased interest in regard to changes in seawater carbonate chemistry. The cosmopolitan coccolithophore *Emiliana huxleyi* (Lohm.) Hay and Mohler was cultured over a range of $p\text{CO}_2$ levels in controlled laboratory experiments under nutrient replete and nitrogen limited conditions. Measurements of photosynthetic activity and calcification revealed, as previously published, an increase in organic carbon production and a moderate decrease in calcification from ambient to elevated $p\text{CO}_2$. The enhancement in particulate organic carbon production was accompanied by an increase in cell diameter. Coccolith volume was best correlated with the coccosphere/cell diameter and no significant correlation was found between coccolith volume and particulate inorganic carbon production rate. The conducted experiments revealed that the coccolith volume of *E. huxleyi* is variable with aquatic CO_2 concentration within the tested range but appears to be a primary function of the coccosphere/cell diameter both under nitrogen limited and nutrient replete conditions. Comparing coccolith morphological and geometrical parameters like volume, mass and size to physiological parameters under controlled laboratory conditions is an important step to understand variations in fossil coccolith geometry.

1 Introduction

Coccolithophores, a key functional phytoplankton group, evolved about 225 million years ago and their intracellularly produced coccoliths are subsequently present in the sediment record. Over geological times coccolithophores experienced various

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environmental conditions and are facing nowadays an alteration of the seawater carbonate chemistry due to the anthropogenic release of carbon dioxide. Atmospheric CO₂ is absorbed by the ocean which leads to an increase in dissolved inorganic carbon and a decrease in the ocean's pH, referred to ocean carbonation/acidification. The response of coccolithophores to elevated *p*CO₂ under nutrient replete conditions has been intensively studied in numerous controlled laboratory studies (Riebesell et al., 2000; Langer et al., 2006, 2009; Feng et al., 2008; Barcelos e Ramos et al., 2010; Krug et al., 2011). Especially, the cosmopolitan species *Emiliania huxleyi* is one of the best studied planktonic species in regard to ocean carbonation/acidification. Diverging results on *E. huxleyi* have triggered scientific discussions and a deeper reflection of the conducted experiments (Riebesell et al., 2008; Iglesias-Rodriguez et al., 2008; Shi et al., 2009). However, recent results confirm a rather uniform response of *E. huxleyi* to *p*CO₂ under nutrient replete conditions with differing strain specific sensitivities (Langer et al., 2009; Bach et al., 2011; Findlay et al., 2011; Hoppe et al., 2011).

Studies on *E. huxleyi* under nutrient limited conditions and elevated *p*CO₂ are rare (Sciandra et al., 2003; Leonardos and Geider, 2005; Borchard et al., 2011) whereas nitrogen or phosphate supply in the upper ocean is one of the main factors limiting phytoplankton growth (Davey et al., 2008; Moore et al., 2008). *Emiliania huxleyi* is a poor competitor for nitrate compared to diatoms (Riegmann et al., 1992) but has an extraordinarily high affinity for orthophosphate and is able to utilise organic phosphates (Riegmann et al., 2000), displaying a high competitive ability in phosphate limited areas of the ocean. Nitrogen and phosphorus limitation lead to reduced growth rates and changes in cell diameter of *E. huxleyi*. Phosphate limited conditions cause an increase in cell diameter whereas under nitrogen limitation the cell diameter decreases. Both effects are likely linked to the cellular division cycle (Paasche, 2002; Müller et al., 2008). Inducing high *p*CO₂ levels in nitrogen limited cultures of *E. huxleyi* results in a further decrease in cell diameter/volume (Sciandra et al., 2003).

The intracellularly produced coccoliths vary in volume and mass with coccolithophore species and strain (Young and Ziveri, 2000). Volume and weight estimates of

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coccoliths are widely used in paleoceanographic studies to estimate carbonate fluxes from the surface to the deep ocean (Young and Ziveri, 2000; Beaufort et al., 2007). Recently, changes in seawater carbonate chemistry over the last 40 000 yr have been linked to the distribution of differentially calcified species and morphotypes (Beaufort et al., 2011) but the complexity of environmental factors triggering changes in coccolith mass and size is noted (Beaufort et al., 2011; Herrmann et al., 2012; Poulton et al., 2011). Besides the complex interaction of environmental factors influencing coccolith geometry, Henderiks (2008) indicated for three main genera of coccolithophores (*Reticulofenestra*, *Cyclicargolithus* and *Coccolithus*) that the fossil coccolith size is correlated to the coccosphere/cell diameter. In this study, we used the cosmopolitan coccolithophore species *Emiliana huxleyi* to investigate the effect of changes in the seawater carbonate chemistry on the coccolith volume. Controlled laboratory carbonate chemistry experiments were conducted under nutrient replete and nitrogen limited conditions.

2 Methods

2.1 Cultures

Emiliana huxleyi (Lohm.) Hay and Mohler (morphotype A) was isolated in 2009 in the Raune Fjord (Norway) by K. Lohbeck and cultured in natural seawater under nutrient replete conditions at 20 °C and a light intensity of 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The culture was kept under continuous light to desynchronize the cell division cycle. Desynchronization was checked by cell diameter measurements over 24 h via a Beckman Coulter MultisizerTM 3 (see below), whereby no significant change in cell diameter of the population was detected (data not shown). Culture media were prepared by filtration (0.2 μm pore size) and subsequent autoclaving of Mediterranean sea water (salinity of 38). After autoclaving, seawater was bubbled with ambient sterile air (0.1 μm pore size) to reintroduce inorganic carbon and to equilibrate the carbonate system to ambient

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*p*CO₂ conditions. Precultures of *Emiliana huxleyi* were maintained under dilute batch culture conditions ($<1.5 \times 10^5$ cells ml⁻¹) in exponential growth at ambient *p*CO₂ conditions with macro- and micronutrient addition corresponding to f/20 after Guillard (1975), i.e. nitrate and phosphate concentration of 88.2 and 3.6 μmol l⁻¹, respectively. Precultures of *E. huxleyi* were not acclimated to the applied *p*CO₂ conditions prior to the experiments (see below). However, studies indicate that *E. huxleyi* when exposed to new *p*CO₂ conditions displays after 8 h a similar physiological response compared to cultures acclimated for 10 or more generations (Barcelos e Ramos et al., 2010; Riebesell et al., 2000; Müller et al., 2010). It is therefore assumed that over the course of the conducted experiments (growth of 5 or more generations) cells of *E. huxleyi* were fully acclimated to the experimental *p*CO₂ conditions at the time of sampling.

2.2 Experimental setup

All experiments were conducted in 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 20 ± 0.6 °C (light conditions as described in Sect. 2.1). Before experimental use, the culture vessels were cleaned and filled completely with a 10 % HCl solution. After incubation for 24 h the HCl was removed and the vessels were rinsed first with MilliQ-water and a second time with sterile seawater (already adjusted to the target carbonate system). The target *p*CO₂ value (see Table 1) was achieved by mixing CO₂ free air with pure carbon dioxide (Air Liquide, France) using mass flow controllers (ANALYT-MTC Model 35823 and Brooks Model 5850 TR) and an air pump (flow rate of 100 ± 10 ml min⁻¹). CO₂ free air was generated by pumping ambient air through an activated carbon filter device to remove organics (Whatmann Carbon Cap) and subsequently passed through soda lime to remove CO₂. This procedure was efficient enough to produce an air stream containing less than 2 ppm CO₂, what was periodically checked using a Licor CO₂ analyser (LI-820) calibrated with a 400 ppm CO₂-air mixture (Deuste Steiniger, Germany). Precision of the Licor CO₂ analyser was about 1.5 %. CO₂ concentration in the target CO₂-air stream was monitored every second by

Licor CO₂ analyzers. The experimental setup created an oscillation around the target CO₂-value of about 14 ± 6 % (1 SD, *n* = 12).

2.2.1 Batch experiments

Batch experiments (B1, B2 and B3) were performed in triplicate for each *p*CO₂ treatment. 1800 ml of culture media (see above but excluding the nutrient additions) was filled into the culture vessels through a 0.2 µm sterile and acid cleaned filter, leaving an atmosphere of 200 ml. The culture media were bubbled with the target *p*CO₂ stream for 4 days. Afterwards, aeration was relocated to the atmosphere of the culture vessel keeping the air over the culture media at target *p*CO₂. The salinity of the growth media was increased to 38.5 due to the aeration with dry CO₂-air and subsequent evaporation of ≈25 ml. Nutrients were added to the growth media according to f/20 (Guillard, 1975) and preculture of *E. huxleyi* was inoculated to a cell density of 1000 cells ml⁻¹. After gently mixing by a magnetic stirrer, samples were taken for dissolved inorganic carbon (DIC), total alkalinity (TA) and bacterial abundance. The exponential growing population of *Emiliania huxleyi* was allowed to grow for 5 to 6 generations under experimental conditions (≈4 days) and subsequently the incubation was terminated for sampling. Samples were taken for DIC, TA, cell number, cell diameter/volume and coccolith volume, particulate organic phosphate (POP), particulate organic carbon (POC), total particulate carbon (TPC), bacterial abundance and scanning electron microscopy (SEM). Samples for cell number were taken before and after the sampling procedure to account for the increase in cell number during the 2 h of sampling.

2.2.2 Chemostat experiments

Culture media for the chemostat experiments (C1, C2 and C3) were prepared in 20 l polycarbonate tanks which were prewashed with HCl and autoclaved before usage. Seawater was bubbled with target *p*CO₂ (as described above) for 1 week to assure equilibrium. Afterwards, sterile filtered nutrients were added to the media supply tanks

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according to f/20, excepting the nitrate concentration which was set to $9.0 \pm 1.4 \mu\text{mol l}^{-1}$ (1 SD, $n = 3$) resulting in a N:P ratio of ≈ 2.5 . Culture media were transferred from the supply tanks to the precleaned culture vessels via acid cleaned tubes passing a $0.2 \mu\text{m}$ sterile acid cleaned filter. Culture vessels were cleaned with HCl, subsequently rinsed with deionised water and culture media (see Sect. 2.2). At the time when culture vessels were filled, the supply of media was stopped and cells of *E. huxleyi* were inoculated. After the cell population reached maximum cell number the dilution of the chemostat was started. The chemostat cultures were operated at a constant dilution rate ($D = 0.49 \pm 0.01 \text{ d}^{-1}$) which was periodically checked by weighing the incoming medium. Cell number, cell diameter/volume and coccolith volume were checked daily with a Beckman Coulter MultisizerTM 3.

After *E. huxleyi* had reached equilibrium state (constant cell density for about 10 days), the dilution was stopped and the culture was sampled. Samples were taken for DIC, TA, cell number, cell diameter/volume and coccolith volume, POP, POC, TPC, nutrient concentration (nitrate+nitrite and phosphate), bacterial abundance and SEM. Samples for cell number were taken before and after the sampling procedure to account for the increase in cell number during the 2 h of sampling. Additionally, DIC and TA were sampled from the media supply tank every second day during the equilibrium state (total sample number of five).

2.3 Carbonate system analysis

The carbonate system was monitored by TA and DIC measurements. DIC samples (25 ml) were taken carefully in duplicate with a disposal single use syringe, avoiding air contact, and filtered through a sterile filter (pore size $0.2 \mu\text{m}$). Samples were sealed air tight, stored at 4°C in the dark and measured within one month after sampling. Duplicate DIC samples were analysed as the mean of triplicate measurements with the infrared detection method by using AIRICA (MARIANDA, Germany) and corrected

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to Dickson seawater standards. Consecutive measurements of the Dickson standard resulted in an average precision of $\pm 0.08\%$ (1 RSD, $n = 15$).

Samples for TA (100 ml) were sterile filtered (0.2 μm poresize) and stored dark at 4 °C prior to analysis (within 5 days after sampling). TA was measured in duplicate by the potentiometric titration method after Dickson (1981) and corrected to Dickson seawater standards. Consecutive measurements of the Dickson standard resulted in an average precision of $\pm 3.6 \mu\text{mol kg}^{-1}$ (1 SD, $n = 14$). The carbonate system was calculated by using the program CO2sys (version 1.05 by E. Lewis and D. W. R. Wallace) with dissociation constants for carbonic acid after Roy et al. (1993).

2.4 Cell number, cell diameter/volume and coccolith volume

All samples were counted three times with a Beckman Coulter Multisizer™ 3. The mean cell number was used to calculate the growth rate μ (d^{-1}) during the batch culture experiments as

$$\mu = \frac{(\ln c_1 - \ln c_0)}{t_1 - t_0} \quad (1)$$

where c_0 and c_1 are the cell concentrations at the beginning (t_0) and end of the incubation period (t_1), expressed in days. The growth rate in the chemostat experiments equals the dilution rate (D) under equilibrium conditions and therefore $\mu = D$.

Afterwards, samples were acidified with 0.1 mmol l^{-1} HCl to dissolve all free and attached coccoliths and subsequently measured again. These measurements revealed the diameter/volume of *E. huxleyi* without coccosphere (Fig. 1a, grey line) and additionally, were used as background measurements to determine the mean volume of the free coccoliths. Subtracting the acidified-sample-spectrum from the non-acidified-sample-spectrum resulted in a spectrum to determine the mean free coccolith volume (Fig. 1b).

The “Coulter Counter Principle” is based on changes in the resistance across a sensing zone. Changes in the resistance are recorded as voltage or current pulses. The

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number of pulses can be transferred to the number of particles measured whereas the amplitude is transferred to the volume of the particle. The measured volume can be translated to the particle's diameter if the particle is of spherical appearance. This accounts for the cell and the coccosphere of *E. huxleyi* but does not apply for the coccoliths. In this regard, coccolith measurements are expressed in volume (μm^3) and cell/coccosphere measurements are expressed in diameter (μm). The MultisizerTM 3 was calibrated with $5.1\ \mu\text{m}$ latex beads (Coulter[®] CC L5) and resulted in a precision of $\pm 0.03\ \mu\text{m}$ (1 SD, $n = 20$).

2.5 Production rates of particulate inorganic and organic carbon, particulate organic phosphate and total particulate nitrogen

For each experiment, 4 sub-samples were filtered onto precombusted GF/F filters (450°C for 7 h) and frozen at -20°C . TPC and POC were measured on separate filters using an "Euro EA Elemental Analyser" (Ehrhardt and Koeve, 1999), where the filter for POC analysis was treated with HCl to remove all inorganic carbon. Particulate inorganic carbon (PIC) was calculated from the difference of TPC and POC. Total particulate nitrogen (TPN) was analysed simultaneously with the TPC measurements. POP was measured by wet oxidation in acid persulfate (Koroleff, 1999). Production rates of PIC, POC, POP and TPN were calculated by multiplying the cell quota with the growth rate (μ).

2.6 Nutrient measurements

Samples for nutrient concentrations (nitrite+nitrate and phosphate) were sterile filtered and stored at -20°C until analyses. The concentrations of nitrate and nitrite were measured with a precision of $\pm 0.1\ \mu\text{mol l}^{-1}$ using a Technicon Auto-analyser (Malara and Sciandra, 1991). Phosphate analyses were performed photometrically (precision of $\pm 0.03\ \mu\text{mol l}^{-1}$) according to Hansen and Koroleff (1999). Nutrients were sampled under equilibrium conditions during the chemostat experiments. Additionally,

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nitrate+nitrate concentrations were determined in the media reservoir tanks supplying the chemostat culture vessel.

2.7 Bacterial abundance

Water samples for bacteria abundance were taken at the start and end of the batch experiments as well as under equilibrium condition in the chemostat experiments to estimate particulate organic carbon production by bacteria. Total bacterial abundance was determined by direct counts. Water samples were preserved with 2 % (wt/vol) formaldehyde and stained with 4'6-diamino-2-phenylindole (DAPI, final concentration $0.25 \mu\text{g ml}^{-1}$) and filtered onto black $0.2 \mu\text{m}$ polycarbonate filters (Porter and Feig, 1980). Between 500 and 600 bacteria were counted with an Axiophot-Zeiss epifluorescence microscope at $\times 1000$ magnification. Organic carbon due to bacterial biomass was calculated from bacterial abundance under the assumption of a carbon content of 30 fg cell^{-1} (average for coastal samples according to Fukuda et al., 1998).

2.8 Scanning electron microscopy

Samples for SEM were filtered onto cellulose acetate filter ($0.45 \mu\text{m}$ poresize) and afterwards dried at 60°C pending analyses. Sputter coated (Gold-Palladium) filter portions were observed on a Hitachi S-3000N SEM.

3 Results

Manipulation of the seawater carbonate system by changing DIC concentrations and keeping total alkalinity constant resulted in a $p\text{CO}_2$ range from 280 to $1080 \mu\text{atm}$ and 210 to $1180 \mu\text{atm}$ in the batch and chemostat experiments, respectively (Table 1). DIC consumption by biological activity in the batch and chemostat experiments was less than 5 % and 4 %, respectively.

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A sufficient macro- and micronutrient concentration at the end of the batch experiments (nutrient replete) was assured by growing *E. huxleyi* to low cell densities and therefore keeping depletion of nutrients at a minimum (Table 3). This resulted at the termination of the batch experiments in an approximate concentration of $\text{NO}_3^- + \text{NO}_2^-$ and PO_4^{3-} of >73.0 and $>3.0 \mu\text{mol l}^{-1}$, respectively (Table 3). During equilibrium conditions of the chemostat experiments $\text{NO}_3^- + \text{NO}_2^-$ concentrations were near or below the detection limit ($<0.2 \mu\text{mol l}^{-1}$) whereas PO_4^{3-} concentrations were above $1 \mu\text{mol l}^{-1}$ (Table 3).

Production rates of POC and TPN were decreased under equilibrium conditions in the chemostat experiments (C1–C3, nitrogen limited) by over 50 % compared to nutrient replete conditions (B1–B3, Table 2) and were accompanied by a reduction in cellular POC and TPN content (Table 3). On the other hand, cellular POP quota increased under nitrogen limitation whereas the POP_{prod} decreased (Tables 2 and 3). Bacterial POC was less than 2 % of the total POC during all experiments and can be therefore neglected.

In regard to increasing $p\text{CO}_2$, production rates of POC, TPC and POP and cell quota displayed a positive trend under replete nutrients and nitrogen limitation (Tables 2 and 3). PIC_{prod} rate was highest at nutrient replete and intermediate $p\text{CO}_2$ condition ($440 \mu\text{atm}$) and decreased towards elevated $p\text{CO}_2$ ($1080 \mu\text{atm}$) from 21.3 ± 3.2 to $12.1 \pm 2.9 \text{ pgC cell}^{-1} \text{ d}^{-1}$. A similar response was observed in the growth rate within the batch experiments. PIC:POC ratio decreased with increasing $p\text{CO}_2$ levels in the batch and chemostat experiments (Table 2). POC:POP and TPN:POP ratios displayed both an increasing trend with $p\text{CO}_2$ under replete and nitrogen limited conditions whereas POC:TPN ratio was not significantly different under the tested $p\text{CO}_2$ levels (Table 2). Under nitrogen limitation a lower PIC_{prod} was observed when elevating the $p\text{CO}_2$ from 544 to $1180 \mu\text{atm}$ (4.0 to $3.7 \text{ pgC cell}^{-1} \text{ d}^{-1}$). This decreasing trend was confirmed by applying the alkalinity anomaly technique (Sciandra et al., 2003), which resulted in an

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estimated calcification rate of 2.5, 2.4 and 2.2 pgC cell⁻¹ d⁻¹ from low, over intermediate, to elevated pCO₂, respectively.

Coccosphere and cell diameter increased with pCO₂ (Fig. 2) ranging from 3.98 (nitrogen limited) to 5.72 μm (nitrogen replete) and from 3.68 to 4.92 μm, respectively (Table 4). In general, single coccolith volume was reduced under nitrogen limitation compared to nutrient replete conditions (Fig. 3). Coccolith volume increased within the applied pCO₂ range in the batch and chemostat experiments with highest volume under nutrient replete and elevated pCO₂ (Table 4). Best correlation was found between the coccosphere diameter and coccolith volume (Fig. 4, $r^2 = 0.88$, $p < 0.001$, $n = 12$).

4 Discussion

4.1 Cellular rates and ratios

During chemostat equilibrium conditions a seawater medium NO₃⁻ + NO₂⁻ concentration was below or near the detection limit (Table 3). The NO₃⁻ + NO₂⁻ concentration is not an indicator of the level of cellular nitrogen limitation because NO₃⁻ + NO₂⁻ concentration will stay at a very low level even at significant cellular growth rates and will only increase if the applied dilution rate approaches the maximum growth rate. However, a reduction in cellular TPN quota of about 50% was measured during chemostat equilibrium conditions compared to batch culture conditions indicating cellular nitrogen limitation induced by the applied low media NO₃⁻ + NO₂⁻ inflow (Table 3). Additionally, cellular ratios (POC:TPN, POC:POP and TPN:POP) were similar to previously reported values from chemostat studies using nitrogen limitation with a similar low N:P media inflow (Leonardos and Geider, 2005). Interestingly, TPN:POP and POC:POP ratios decreased under nitrogen limited compared to nutrient replete conditions while POC:TPN ratios remained unchanged (Table 2) which suggests that the phosphorus metabolism was partly decoupled from the C and N dynamics.

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The response in photosynthesis and calcification of *E. huxleyi* to changing carbonate chemistry has been studied intensively in laboratory experiments over the last decades. A summary of the responses is given by Ridgwell et al. (2009) and Hoppe et al. (2011). Most laboratory experiments were performed under nutrient replete conditions (Riebesell et al., 2000; Langer et al., 2009; Feng et al., 2009; Hoppe et al., 2011). In the tested $p\text{CO}_2$ range from 282 to 1077 μatm , POC_{prod} increased similar to previous findings under replete nutrient conditions (Riebesell et al., 2000; Zondervan et al., 2002; Barcelos e Ramos et al., 2010). Increasing POC_{prod} was accompanied by increasing trends in TPN_{prod} and POP_{prod} (Table 2), whereas the TPN:POC ratio displayed no change with $p\text{CO}_2$ as reported by Müller et al. (2010). Cellular ratios of POC:POP and TPN:POP increased with $p\text{CO}_2$ which led to a change of the canonical stoichiometry (C:N:P) of *E. huxleyi* under nutrient replete and increased $p\text{CO}_2$ conditions (Table 2).

The response in calcification rate and PIC:POC ratio of *E. huxleyi* has commonly been described to be negatively affected by increasing $p\text{CO}_2$ with species and strain specific sensitivities (Hoppe et al., 2011; Langer et al., 2009; Feng et al., 2008; Barcelos e Ramos et al., 2010; Findlay et al., 2011). The current results fit well into the overall picture of the physiological response of *E. huxleyi* to elevated $p\text{CO}_2$ levels under nutrient replete conditions.

A few laboratory studies dealt with the combined effect of rising $p\text{CO}_2$ and nutrient limitation (Sciandra et al., 2003; Leonardos and Geider, 2005). In contrast to Sciandra et al. (2003) a positive trend in POC_{prod} was observed as reported by Leonardos and Geider (2005) for a non-calcifying strain of *E. huxleyi*. Differences in the experimental set up of Sciandra et al. (2003) and this study might be an explanation for the diverging response observed in POC_{prod} . In the current study, the biomass during chemostat equilibrium condition was of about 10 times lower and was not bubbled with a gas mixture which might interfere with phytoplankton growth and performance (Shi et al., 2009). Additionally, high light intensities (as used here and in Leonardos and Geider, 2005) are known to amplify the positive effect of $p\text{CO}_2$ on POC_{prod} (Zondervan et al., 2002).

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4.2 Cell diameters and coccolith volumes

Coccosphere and cell diameter were reduced by $>0.5\ \mu\text{m}$ ($>10\%$) under nitrogen limited compared to nutrient replete conditions (Table 4). Similar nitrogen depletion effects on coccosphere/cell diameter of *E. huxleyi* were previously observed and reported in the literature (Paasche, 2002; Sciandra et al., 2003) and are presumably related to lengthening and/or compression of certain phases in the cellular division cycle (Müller et al., 2008).

In regard to $p\text{CO}_2$, cell diameter increased by about $0.08\ \mu\text{m}$ or $0.05\ \mu\text{m}$ per $100\ \mu\text{atm}$ $p\text{CO}_2$ under nutrient replete or nitrogen limited conditions, respectively, assuming a linear correlation with $p\text{CO}_2$. However, the observed increase in coccosphere/cell diameter with $p\text{CO}_2$ can only be applied within the tested range of $p\text{CO}_2$ as recent results indicate a steady decrease in coccosphere diameter of *E. huxleyi* when $p\text{CO}_2$ is exceeding values $>1500\ \mu\text{atm}$ (Bach et al., 2011). Changes in the average cell diameter of *E. huxleyi* will have direct implications on metabolic rates, nutrient diffusion/uptake, grazing and sinking rates and broader ecological processes (Engel et al., 2008; Finkel et al., 2010).

In the present study, measured coccolith volumes (via Beckman Coulter Multisizer™ 3) ranged from 0.76 to $3.43\ \mu\text{m}^3$ which is comparable to volume estimates derived from coccolith length measurements via scanning electron microscopy (0.3 to $3.6\ \mu\text{m}^3$, Young and Ziveri, 2000). Converting the measured coccolith volumes to coccolith masses using the density of pure calcite ($2.71\ \text{pg}\ \mu\text{m}^{-3}$) results in coccolith masses from 2.0 to $2.4\ \text{pg}\ \text{CaCO}_3$ and 5.7 to $9.3\ \text{pg}\ \text{CaCO}_3$ per coccolith under nitrogen limited (C1–C3, Table 4) and nutrient replete conditions (B1–B3, Table 4), respectively. Poulton et al. (2011) determined via SEM morphometrics an average coccolith mass of $1.9 \pm 0.7\ \text{pg}\ \text{CaCO}_3$ for coccoliths of *E. huxleyi* morphotype A in surface waters from the Patagonian Shelf. These values are comparable to the current measurements under nitrogen limited conditions and coccolith mass estimates from past laboratory studies under nutrient replete conditions (Paasche, 1998, 1999). Interestingly, coccolith mass

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estimates under nutrient replete conditions of the present study (B1–B3) are exceeding values reported for *E. huxleyi* morphotype A (summarised in Paasche, 2002). However, the latter mentioned experiments were conducted with relatively high cell densities which is likely to cause a high consumption of the available DIC (>20 %) and therefore exposing the growing cells to new carbonate system values (e.g. pH, aquatic CO₂ and CO₃ ion concentration). Unfortunately, past studies on coccolithophores, which were not related to the field of ocean acidification, are almost entirely lacking a description of the carbonate system and are thus difficult to compare to the current results.

Applying Eq. (2), developed by Young and Ziveri (2000), to estimate the coccolith distal shield length (DSL) from coccolith volume (*V*) with the species specific constant $k_s = 0.02$ (as given for normal calcified coccoliths of *E. huxleyi* morphotype A) results in an average coccolith DSL ranging from 4.7 to 5.6 μm under nutrient replete conditions (B1–B3).

$$DSL(\mu m) = \sqrt[3]{\frac{V(\mu m^3)}{k_s}} \quad (2)$$

Corresponding to the estimates for coccolith masses under nutrient replete conditions, the calculated DSLs are more than 1 μm longer than average DSL of ≈3.5 μm derived from oceanic samples of *E. huxleyi* coccoliths morphotype A (Young and Ziveri, 2000; Poulton et al., 2011). Visual inspection of coccoliths from the batch culture experiments via scanning electron microscopy confirmed the presence of coccoliths with DSL >4.5 μm (Fig. 5). Unfortunately, inappropriate filter material and storage problems hindered an adequate coccolith analysis via SEM of the conducted experiments, thus Fig. 5 does not stringently represent average coccoliths from the batch culture experiments. However, previous observations of coccoliths with a DSL >4.5 μm from an *E. huxleyi* morphotype A (Cubillos et al., 2007) and the present SEM picture let us assume that the calculated DSLs (and consequently the coccolith volume measurements) from the current experiments are valid and comparable to previous applied methods

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measuring the geometrics of coccoliths. In this regard, an interlaboratory comparison of the different methods to estimate coccolith volumes and mass (birefringence, DSL measurements and resistive method) is urgently needed to validate and confirm results on coccolith geometrics as previously mentioned by Poulton et al. (2011). Controlled laboratory experiments will provide a suitable basis for a methodological comparison because sufficient sample material can be produced and experimental parameters are regulated and monitored.

A positive trend in coccolith volume was observed with increasing $p\text{CO}_2$ under nutrient replete and nitrogen limited conditions (Fig. 3). Thus, highest coccolith volume (and hypothetical coccolith mass) was found at high $p\text{CO}_2$ (low pH) and nutrient replete conditions. A similar phenomenon was observed in nutrient rich and low pH Chilean upwelling waters by Beaufort et al. (2011) who measured coccolith masses of *E. huxleyi* morphotype E with values $>8 \text{ pg CaCO}_3$. High coccolith masses at elevated $p\text{CO}_2$ and low pH seems to be counterintuitive considering the predominantly reported decrease in calcification rate of coccolithophores with $p\text{CO}_2$ (Riebesell et al., 2000; Langer et al., 2009; Hoppe et al., 2011; Ridgwell et al., 2009). However, coccolith mass or volume (one point in time) are not comparable to the rate of calcification or PIC_{prod} (change over time). A comparable observation has been reported for phosphate limited cells of *E. huxleyi* which produce coccoliths with a higher calcite content than under nutrient replete conditions (Paasche, 2002). Even though the calcite content per coccolith, the PIC cell quota and the cell volume increases with phosphate limitation, the calcification rate or PIC_{prod} decreases (Riegmann et al., 2000; Müller et al., 2008).

Interestingly, estimated numbers of free/detached coccoliths during our experiments suggest a lower production of coccoliths per cell with elevated $p\text{CO}_2$ which would be an explanation for the decrease in PIC_{prod} with a concomitant increase in coccolith volume. However, only the free/detached coccoliths in the culture media were measured and not the total produced coccoliths (free/detached + attached). Although samples were equally treated before analyses, the observed trend might be biased by the physical

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treatment (rough or gentle mixing) of the sample and a change in coccolith density cannot be excluded.

The coccolith volume was found to correlate best with the coccosphere/cell diameter (Fig. 4). Similar indications are given by Henderiks (2008) who reported a relationship of the coccosphere diameter with the coccolith size (distal shield length) for several fossil coccolithophore species. However, it remains to be tested how changes in coccosphere/cell diameter induced by other environmental parameters (e.g. temperature, irradiance and nutrient availability) will influence coccolith volume or size. The complexity in coccolith size variability of natural observations as a result of various environmental parameters has been indicated (Poulton et al., 2011; Herrmann et al., 2012).

In contrast to our study, De Bodt et al. (2010) (nutrient replete conditions) and Scianra et al. (2003) (nitrogen limited conditions) reported a decrease in coccosphere diameter/volume of *E. huxleyi* with increasing $p\text{CO}_2$ levels. These diverging observations are presumably an effect of the unaltered (De Bodt et al., 2010) and decreased (Scianra et al., 2003) POC_{prod} which in combination with a diminished PIC_{prod} leads to a reduction in coccosphere diameter. Additionally, the latter experiments were performed under lower light intensities ($150\text{--}170\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$) than compared to Leonardos and Geider (2005) and this study ($300\text{--}500\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$). Increasing light intensities are reported to amplify the effect of elevated POC_{prod} with $p\text{CO}_2$ under nutrient replete conditions (Zondervan et al., 2002). Another point might be the optical measuring principle (HIAC) to determine the cell diameter/volume used in Scianra et al. (2003) compared to the resistance method (Coulter). The coccosphere highly scatters and reflects light of different wavelengths which might interfere with measurements based on attenuation and absorption of light. For example, a thinning of the coccosphere layer surrounding the cell might result in a lower light scatter and therefore in an optical-measured decrease in volume even if the coccosphere diameter/volume is unaltered due to an concomitant increase in the cell volume.

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This study was conducted with a desynchronised population of *E. huxleyi* induced by using continuous light. It should be mentioned that measuring cell volumes under a light:dark cycle (synchronised division) has to be conducted with great care. Sampling has to be well timed because changes in cell volume occur within less than an hour depending in which phase of the cell cycle the population is located. However, future experiments using synchronised cultures under altered $p\text{CO}_2$ conditions and following the cellular cycle may resolve the underlying mechanisms leading to changes in cellular volume and growth rate.

5 Conclusions

It is demonstrated that the average coccolith volume of *Emiliana huxleyi* varies with changes in the seawater carbonate chemistry. However, coccolith volume was found to be primarily a function of the coccosphere/cell diameter both under nutrient replete and nitrogen limited conditions and indications are given that *E. huxleyi* produces more voluminous and a lower number of coccoliths with increased $p\text{CO}_2$ values resulting in a reduction of the particulate inorganic carbon production. It remains to be tested if the increase in coccolith volume with coccosphere/cell diameter might be a cascading effect of the higher particulate organic carbon cell quota induced by elevated aquatic CO_2 concentrations.

Acknowledgements. We thank Nathalie Leblond for CN measurements. We thank Kai Lohbeck for providing us *Emiliana huxleyi* to conduct this study and Scarlett Sett for POP measurements. This work was funded by the REMECCA project (Fondation TOTAL), by the BMBF project “Bioacid” (BMBF, FKZ 03F0608A) and by the Australian Research Council (ARC) Discovery Project (DP) 1093801. We thank G. Hallegraeff for proof reading the manuscript and further comments. Additionally, we are grateful for the comments made by three anonymous reviewers to an earlier version of this manuscript.

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Table 2. Physiological parameters and cellular ratios of the batch (1 SD, $n = 3$) and chemostat experiments. Significance was tested for the batch culture experiments using a one-way ANOVA ($p < 0.05$).

Exp. code	μ (d ⁻¹)	PIC _{prod} (pgC cell ⁻¹ d ⁻¹)	POC _{prod} (pgC cell ⁻¹ d ⁻¹)	TPN _{prod} (pgN cell ⁻¹ d ⁻¹)	POP _{prod} (pgP cell ⁻¹ d ⁻¹)	PIC:POC (mol:mol)	POC:TPN (mol:mol)	POC:POP (mol:mol)	TPN:POP (mol:mol)
Batch experiments									
B1	1.02 ± 0.07	15.7 ± 1.8	11.3 ± 1.0	1.36 ± 0.12	0.20 ± 0.02	1.41 ± 0.27	9.68 ± 0.70	142 ± 5	14.8 ± 0.9
B2	1.32 ± 0.09	21.3 ± 3.2	16.3 ± 2.6	2.67 ± 0.93	0.27 ± 0.04	1.31 ± 0.11	7.55 ± 1.83	159 ± 11	21.9 ± 5.7
B3	0.94 ± 0.12	12.1 ± 2.9	25.6 ± 2.8	3.16 ± 0.11	0.29 ± 0.02	0.47 ± 0.08	9.45 ± 1.13	231 ± 41	24.4 ± 1.6
p	<0.01	0.02	<0.001	0.02	0.02	<0.01	0.17	<0.01	0.03
F	12.86	8.87	29.99	8.81	7.92	25.78	2.41	10.98	6.29
Chemostat experiments									
C1	0.49 ± 0.01	–	4.5	–	0.16	–	–	73.4	–
C2	0.49 ± 0.01	4.0	5.4	0.54	0.17	0.75	11.7	79.6	6.8
C3	0.50 ± 0.01	3.7	7.6	0.82	0.19	0.48	10.8	104	9.6

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Table 3. Overview of cell density, cell quota of particulate nitrogen and phosphor and culture media nutrient concentrations ($\text{NO}_3^- + \text{NO}_2^-$ and PO_4^{3-}) at the end of the batch and chemostat experiments. $\text{NO}_3^- + \text{NO}_2^-$ and PO_4^{3-} concentrations at the start of the batch experiments (B1–B3) were set to $\approx 88.2 \mu\text{mol l}^{-1}$ and $\approx 3.6 \mu\text{mol l}^{-1}$, respectively. $\text{NO}_3^- + \text{NO}_2^-$ and PO_4^{3-} concentrations of the reservoir tanks during the chemostat experiments (C1–C3) were set to $9.0 \pm 1.4 \mu\text{mol l}^{-1}$ and $\approx 3.6 \mu\text{mol l}^{-1}$, respectively.

Exp. code	$p\text{CO}_2$ (μatm)	Cell No. ($\text{cells ml}^{-1} \times 10^4$)	TPN (pgN cell^{-1})	POP (pgP cell^{-1})	$\text{NO}_3^- + \text{NO}_2^-$ ($\mu\text{mol l}^{-1}$)	PO_4^{3-} ($\mu\text{mol l}^{-1}$)
Batch experiments						
B1	282 ± 21	5.1 ± 1.5	1.34 ± 0.21	0.20 ± 0.03	$83.4 \pm 0.7^*$	$3.28 \pm 0.04^*$
B2	442 ± 15	5.4 ± 3.2	2.00 ± 0.60	0.20 ± 0.02	$81.4 \pm 1.5^*$	$3.26 \pm 0.18^*$
B3	1077 ± 165	6.1 ± 2.6	3.40 ± 0.53	0.31 ± 0.06	$73.9 \pm 4.7^*$	$3.01 \pm 0.21^*$
Chemostat experiments						
C1	235	6.8 ± 0.6	–	0.33	0.1	1.08
C2	544	6.1 ± 0.6	1.10	0.35	0.1	1.07
C3	1180	5.8 ± 0.4	1.64	0.38	0.2	1.78

* Values were estimated by subtracting the nutrient consumption (determined from cell densities and cell quota of TPN and POP) from the nutrient concentration at the beginning of the batch experiments.

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Table 4. Coccosphere/cell diameters and coccolith volume of the batch (1 SD, $n=3$) and chemostat (1 SD, $n=10$) experiments.

Exp. code	Coccosphere diameter (μm)	Cell diameter (μm)	Vol. liths (μm^3)
Batch experiments			
B1	5.10 ± 0.04	4.18 ± 0.15	2.09 ± 0.26
B2	5.31 ± 0.09	4.37 ± 0.05	2.71 ± 0.34
B3	5.72 ± 0.11	4.92 ± 0.21	3.43 ± 0.63
p	<0.001	<0.01	0.027
F	39.4	19.0	6.9
Chemostat experiments			
C1	3.98 ± 0.03	3.68 ± 0.03	0.76 ± 0.05
C2	4.27 ± 0.05	3.86 ± 0.03	0.80 ± 0.01
C3	4.45 ± 0.09	4.10 ± 0.09	0.89 ± 0.01

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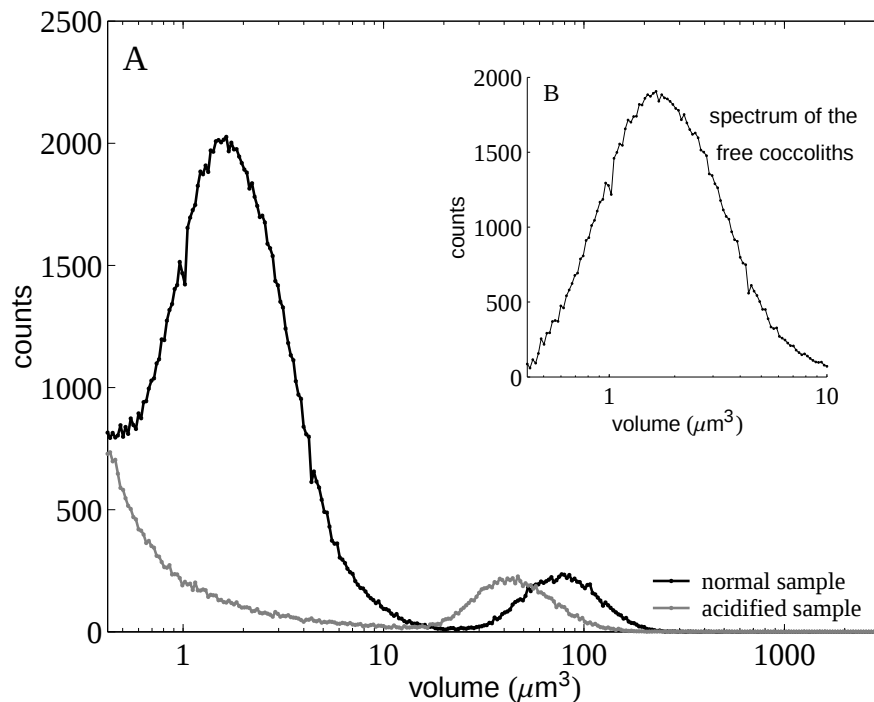


Fig. 1. MultisizerTM 3 volume spectra. **(A)** Spectra of the *E. huxleyi* population (black line) and after treatment with HCl (grey line). **(B)** Volume spectrum of the free coccoliths after subtracting the acidified sample from the normal sample.

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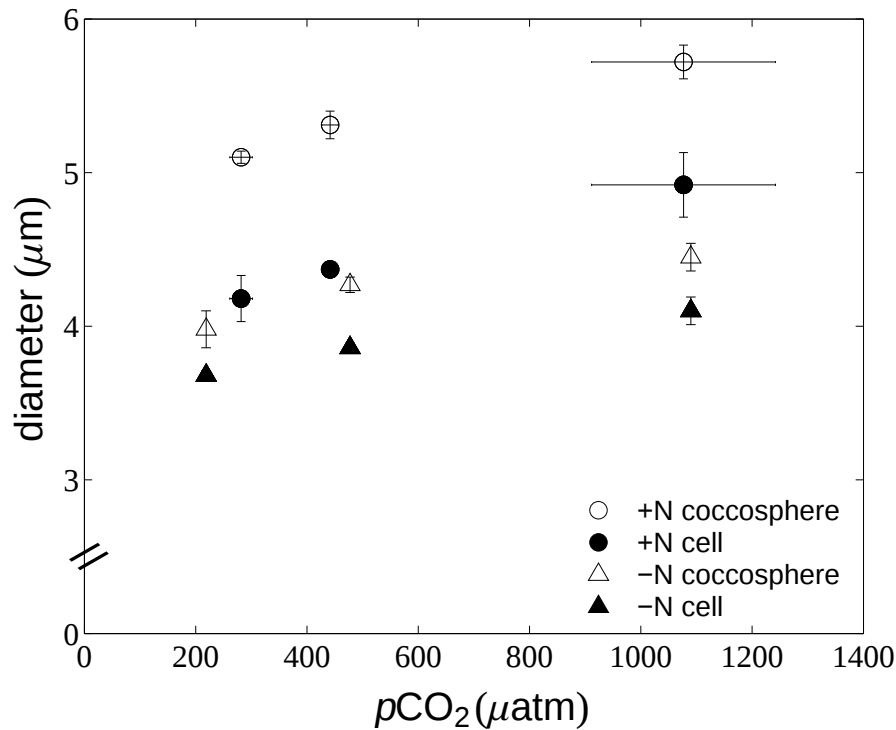


Fig. 2. Coccosphere (open symbols) and cell (filled symbols) diameter under nutrient replete (+N, circles) and nitrogen limitation (-N, triangles) in regard to $p\text{CO}_2$ levels.

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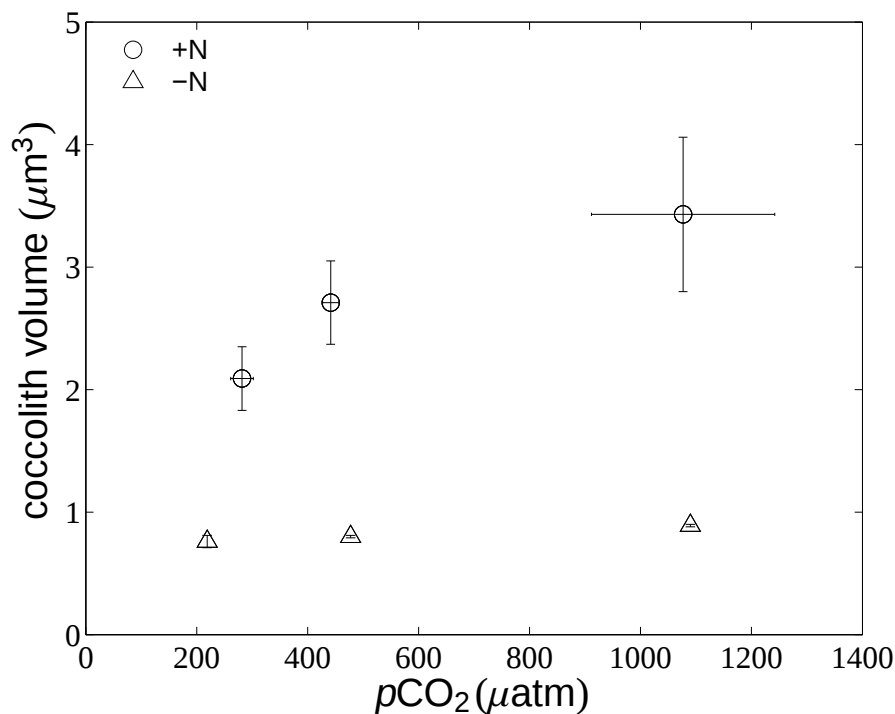


Fig. 3. Coccolith volumes under nutrient replete (+N, circles) and nitrogen limitation (-N, triangles) in regard to the applied $p\text{CO}_2$ levels.

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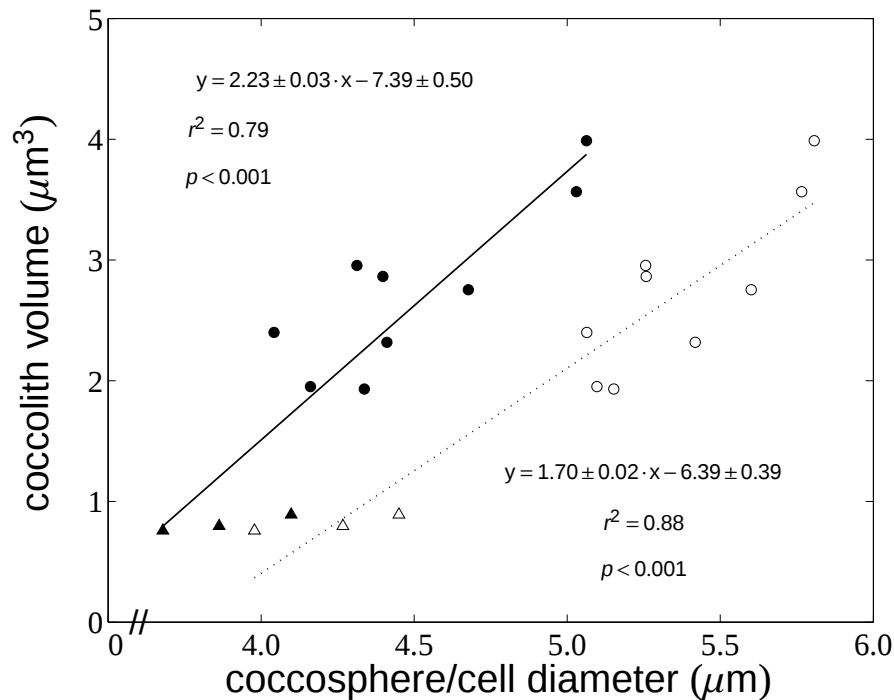


Fig. 4. Coccolith volume as a function of the cell (filled symbols) and coccosphere (open symbols) diameter. Circles and triangles indicate the batch and chemostat experiments, respectively.

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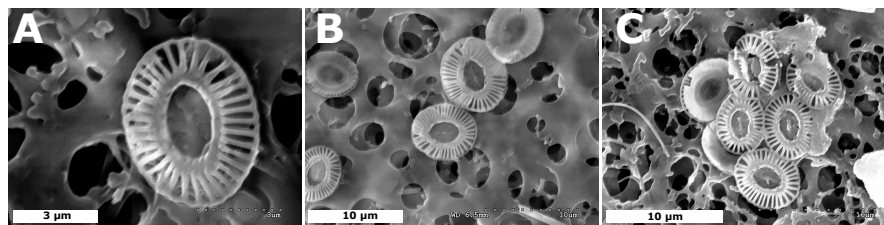


Fig. 5. SEM pictures of *E. huxleyi* cultures from the batch experiments under nutrient replete conditions. Displayed are coccoliths produced by *E. huxleyi* exposed to a $p\text{CO}_2$ of (A) $282 \pm 21 \mu\text{atm}$, (B) $442 \pm 15 \mu\text{atm}$ and (C) $1077 \pm 165 \mu\text{atm}$.

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