Phytoplankton calcification as an effective mechanism to alleviate cellular calcium poisoning

Marius N. Müller [1, 2], Joana Barcelos e Ramos [3], Kai G. Schulz [4], Ulf Riebesell [5], Józef Kaźmierczak [6], Francesca Gallo [3], Luke Mackinder [7], Yan Li [8], Pavel N. Nesterenko [8], T. W. Trull [9], Gustaaf M. Hallegraeff [1]

[1] {Institute for Marine and Antarctic Studies (IMAS), University of Tasmania, Private Bag 129, Hobart, TAS 7001, Australia}

[2] {Institute of Oceanography, University of São Paulo, Praça do Oceanográfico 191, 05508-120 São Paulo, SP, Brazil}

[3] {Centre of Climate, Meteorology and Global Change (CMMG), University of Azores, Rua do Capitão d'Ávila, Pico da Urze 970-0042 Angra do Heroísmo, Açores, Portugal}

[4] {Centre for Coastal Biogeochemistry, School of Environmental Science and Management, Southern Cross University, P.O. Box 157, Lismore, NSW 2480, Australia}

[5] {GEOMAR Helmholtz Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany}

[6] {Institute of Paleobiology, Polish Academy of Sciences, Twarda 51/55, 00-818 Warsaw, Poland}

[7] {Department of Plant Biology, Carnegie Institution, 260 Panama Street, Stanford, CA 94305, USA}

[8] {Australian Centre for Research on Separation Science (ACROSS), School of Chemistry, University of Tasmania, Private Bag 75, Hobart TAS 7001, Australia}

[9] {Antarctic Climate and Ecosystems Cooperative Research Centre, University of Tasmania and CSIRO Oceans and Atmosphere Flagship, Hobart, Tasmania 7001, Australia}

Correspondence to: M. N. Müller (mnmuller@usp.br)

Abstract

Marine phytoplankton has developed the remarkable ability to tightly regulate the concentration of free calcium ions in the intracellular cytosol at a level of ~0.1 µmol L^{-1} in the presence of seawater Ca^{2+} concentrations of 10 mmol L^{-1}. The low cytosolic calcium ion concentration is of utmost importance for proper cell signalling function. While the regulatory mechanisms responsible for the tight control of intracellular Ca^{2+} concentration are not completely understood, phytoplankton taxonomic groups appear to have evolved different strategies, which may affect their ability to cope with changes in seawater Ca^{2+} concentrations in their environment on geological time scales. For example, the Cretaceous (145 to 66 Ma ago), an era known for the high abundance of coccolithophores and the production of enormous calcium carbonate deposits, exhibited seawater calcium concentrations up to four times present-day levels. We show that calcifying coccolithophore species (*Emiliania huxleyi*, *Gephyrocapsa oceanica* and *Coccolithus braarudii*) are able to maintain their relative fitness (in terms of growth rate and photosynthesis) at simulated Cretaceous seawater calcium concentrations, whereas these rates are severely reduced under these conditions in some non-calcareous phytoplankton species (*Chaetoceros* sp., *Ceratoneis closterium* and *Heterosigma akashiwo*). Most notably, this also applies to a non-calcifying strain of *E. huxleyi* which displays a calcium-sensitivity similar to the non-calcareous species. We hypothesize that the process of calcification in coccolithophores provides an efficient mechanism to alleviate cellular calcium poisoning and thereby offered a potential key evolutionary advantage, responsible for the proliferation of coccolithophores during times of high seawater calcium concentrations. The exact function of calcification and the reason behind the highly-ornate physical structures of coccoliths remain elusive.

1 Introduction

Calcium is a versatile and crucial ion in biological systems (Case et al., 2007), which is among other functions, essential for cellular signalling, membrane structure and cell division (Sanders et al., 1999). The concentrations of cytosolic free Ca^{2+} in eukaryotes are well regulated and the maintenance of relatively low levels is essential for fast signal transduction. An excessive influx of Ca^{2+} to the cytosol can be lethal as it disturbs intracellular signalling and irreversibly damages the cell (Orrenius et al., 1989; Kader and Lindberg, 2010). Homeostasis of Ca^{2+} in plant cells is predominantly achieved by Ca^{2+}-binding proteins, reducing the effective diffusion coefficient of Ca^{2+} in the cytosol, and ultimately via sequestration by the endoplasmic reticulum, mitochondria and cellular vacuoles (Case et al., 2007). Cytosolic free Ca^{2+} concentrations in marine phytoplankton are about 10^5 times lower than modern seawater concentrations and marine eukaryotes have developed a remarkable capacity to maintain these low cytosolic Ca^{2+} levels (Brownlee et al., 1987; Brownlee et al., 1995). It is, however, unknown if the regulating mechanisms of marine phytoplankton to keep this delicate Ca^{2+} homeostasis differ between species and between functional groups. In freshwater environments, for example, calcium ions play an important role shaping microalgal species composition. Desmid green algae have a narrow tolerance to calcium (Moss, 1972; Tassigny, 1971) and thrive in soft-water lakes, while submersed macrophytes (*Elodea*, *Stratiotes*, *Potamogeton*) and benthic cyanobacteria dominate in hard-water lakes where they can be heavily encrusted with $CaCO_3$ precipitates.

An early hypothesis describes the invention and the process of biomineralization in form of calcium carbonate by marine organisms as a potential Ca^{2+} detoxification mechanism (Simkiss, 1977; Kaźmierczak et al., 1985; Kempe and Degens, 1985). Ocean calcium concentrations have changed remarkably throughout the Phanerozoic eon (past 541 Ma) as documented by fluid inclusions of marine halite (Horita et al., 2002). Over the past 300 Ma, highest seawater Ca^{2+} concentrations are documented for the Cretaceous (145 to 66 Ma ago) (Hönisch et al., 2012), known for massive deposition of biogenic calcareous material produced in the pelagic ocean. Calcifying phytoplankton (coccolithophores) are the dominant planktonic calcifiers in the modern ocean and are responsible for up to half the pelagic production of calcium carbonate (Broecker and Clark, 2009). Coccolithophores form minute calcite plates (coccoliths) inside a specialised cell compartment (coccolith vesicle) from where the coccoliths are subsequently transported to the cell's surface and released via exocytosis. The record of nannofossils and coccoliths has its origin in the Late Triassic (about

225 Ma ago), coinciding with relatively low seawater Ca^{2+} concentrations (Bown et al., 2004). Subsequently, seawater Ca^{2+} concentrations increased, potentially linked to changes in the seafloor spreading rates (Skelton, 2003), and peaked in the Cretaceous at the highest levels since the past 300 Ma (~3 to 4 times the present seawater concentrations of 10 mmol Ca^{2+} L^{-1}). Species diversity and abundance of total nannofossils, including coccolithophores, have increased in concert with high seawater Ca^{2+} concentrations (Fig. 1).

We tested two calcifying coccolithophores (*Emiliania huxleyi* and *Gephyrocapsa oceanica*), two diatoms (*Chaetoceros* sp. and *Ceratoneis closterium*) and one raphidophyte (*Heterosigma akashiwo*) to elevated seawater calcium concentrations simulating changes in oceanic Ca^{2+} levels over the past 300 Ma. Representative for a non-calcifying coccolithophore, one non-coccolith carrying (naked) *E. huxleyi* strain was tested. Furthermore, a possible stimulation of coccolith production by increased seawater Ca^{2+} concentration was investigated in two under-calcifying *E. huxleyi* strains. If biogenic calcification represents a viable mechanism to cope with high external Ca^{2+} concentrations, a diverging response in physiological parameters would be expected between calcifiers and non-calcifiers.

2 Materials and Methods

2.1 Culture conditions

Monospecific cultures of the diploid coccolithophores *Gephyrocapsa oceanica* (CS-335/03) and *Emiliania huxleyi* (calcifying CS-370, non-calcifying SO-6.13 and under-calcifying SO-5.25 and SO-8.04), the diatoms *Chaetoceros* sp. (CHsp-TB02) and *Ceratoneis closterium* (CCMMG-3), and the raphidophyte *Heterosigma akashiwo* (CS-169) were grown in sterile artificial seawater (Kester et al., 1967) with macro- and micronutrient additions according to f/2 and f/20 (Guillard, 1975), respectively, or in the case of *G. oceanica* according to GSe/20 (Loeblich and Smith, 1968). The under-calcified populations (strains SO-5.25 and SO-8.04) consist of cells with no or single attached coccoliths. Cells with no coccoliths attached in these populations either lost their coccoliths, lacked the ability to produce coccoliths or did not yet produce coccoliths. *Emiliania huxleyi* strain SO-6.13 was isolated by Suellen Cook in Feb. 2007 from the Southern Ocean (54˚ S, 146˚ E and 65 m depth). Multiple single cell isolates from this water sample resulted in a number of calcified ecotype B/C *E. huxleyi* strains. Strain SO-6.13, however, was naked upon isolation and throughout the conduct of the current study. Much later, in early 2015, strain SO-6.13 switched from a non-calcifying to a calcifying stage and started to produce typical B/C coccoliths.

Calcium concentrations were adjusted by varying additions of $CaCl_2$ with concomitant additions of NaCl, keeping the ionic strength of the artificial seawater constant. *Gephyrocapsa oceanica*, *H. adashiwo* and *E. huxleyi* (CS-370) were obtained from the Australian National Algae Culture Collection. *Ceratoneis closterium* was obtained from the Centre of Climate, Meteorology and Global Change at the University of Azores (CMMG). All other species and strains were obtained from the Algae Culture Collection at the Institute of Marine and Antarctic Studies at the University of Tasmania, Australia.

2.2 Experimental set-up

In the first experiment, cells were acclimated to the experimental conditions (Ca^{2+} range from 1 to 52 mmol L^{-1}) for more than 50 generations and allowed to consume a maximum of 10% (non-calcifiers) or 5% (calcifiers) of dissolved inorganic carbon to avoid major changes in the carbonate chemistry. Cultures were incubated in triplicates at 12°C (16°C for *G. oceanica*), a photon flux density of 100 µmol quanta m^{-2} s^{-1} and a 16:8 h light:dark cycle at the University

of Tasmania. *Ceratoneis closterium* was incubated at 20°C, 250 µmol quanta m^{-2} s^{-1} and a 14:10 h light:dark cycle at the University of Azores. The physiological response of all species (except *C. closterium*) was examined in terms of growth rate, particulate organic and inorganic carbon cell quota and production rate, and maximum quantum yield of the photosystem II (Fv/Fm). Physiology of *C. closterium* was only examined in terms of growth rate. Seawater carbonate chemistry was determined from total alkalinity (A_T) and dissolved inorganic carbon (C_T) samples taken at the start and the end of the experiment.

In the second experiment, two under-calcified *E. huxleyi* strains (SO-5.25 and SO-8.04) were cultured at the University of Tasmania in triplicates for 2 month under dilute semi-continuous batch conditions at the identical conditions as described above with Ca^{2+} concentrations adjusted to 10 or 36 mmol Ca^{2+} L^{-1}. Strain specific growth rate and the number of coccoliths per cell were monitored over time via cell counts and scanning electron microscopy, respectively. Cultures were allowed to grow from ~50 to a maximal cell density of ~80 000 cells mL^{-1} which prevented major changes in the seawater carbonate chemistry.

2.3 Seawater chemistry analysis

Seawater Ca^{2+} concentrations at the start of the experiment were determined via chelation ion chromatography (Meléndez et al., 2013), using an adjusted method to match the different Ca^{2+} concentrations (precision of ±1.4%). Dissolved inorganic carbon and A_T were analyzed as the mean of triplicate measurements with the infrared detection method using an Apollo SciTech DIC-Analyzer (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, USA). Consecutive measurements of the Dickson standard resulted in an average precision of >99.8% for both C_T and A_T. The carbonate system was calculated by equations from Zeebe and Wolf-Gladrow (2001) with dissociation constants for carbonic acid after Roy et al. (1993), modified with sensitivity parameters for [Na^+], [Mg^{2+}] and [Ca^{2+}] (Ben-Yaakov and Goldhaber, 1973). The calcite saturation state (Ω) was calculated with regard to Mg/Ca ratio as described in Tyrrell and Zeebe (2004). Detailed information on the carbonate system parameters can be found in the Supplementary Material.

2.4 Physiological parameters

Maximum quantum yield of the photosystem II (Fv/Fm) was measured on dark adapted samples (45 minutes) using a Water-PAM fluorometer (Walz GmbH, Germany). Subsamples for total particulate carbon (TPC) and particulate organic carbon (POC) were filtered onto pre-combusted (7 hours, 450°C) quartz-microfibre filters (pore-size of 0.3 µm) and stored at -24°C. Filters for POC analysis were fumed with saturated HCl for 10 hours to remove all inorganic carbon. TPC and POC were measured on an elemental analyser (Thermo Finnigan EA 1112, Central Science Laboratory of the University of Tasmania). Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. Cell numbers were obtained by means of triplicate measurements with a Multisizer 4 Coulter Counter (Beckman Coulter, USA) or by light microscopy counts. The average cell number was used to calculate the growth rate μ (d^{-1}) as $\mu = (\ln(c_1) - \ln(c_0)) / (t_1 - t_0)$, where c_0 and c_1 are the cell concentrations at the beginning (t_0) and the end of the incubation period (t_1). POC and PIC production rates were calculated from cell quota and species-specific growth rates.

2.5 Scanning electron microscopy

Samples for electron microscopy were filtered gently onto polycarbonate filters, air dried at 60°C and afterwards sputter-coated with gold-palladium. Photographs were taken with a Hitachi SU-70 field emission scanning electron microscope (SEM) at the Central Science Laboratory of the University of Tasmania. During SEM sessions >50 cells were visually evaluated and representative pictures were taken.

3 Results

In the first experiment, at Ca^{2+} concentrations below 2 mmol L^{-1} all species exhibited significantly (*t*-test, $p<0.05$) lower growth, particulate organic carbon (POC) production rates and maximum quantum yield of photosystem II (Fv/Fm) compared to modern seawater concentrations of ~10 mmol Ca^{2+} L^{-1} (Fig. 2). Furthermore, the two calcifying species displayed decreased particulate inorganic carbon (PIC) production rates at Ca^{2+} concentrations below 2 mmol L^{-1} compared to ~10 mmol Ca^{2+} L^{-1} (*t*-test, $p<0.05$). At elevated Ca^{2+} concentrations all non-calcifying species exhibited a severe reduction in growth, POC production and maximum quantum yield (Fig. 2). In the most extreme cases no growth was detected at 42 and 52 mmol Ca^{2+} L^{-1} in *H. akashiwo* and *C. closterium*, respectively. Both tested coccolithophore species, on the other hand, were able to maintain their growth, Fv/Fm, POC and PIC production rates with no substantial change at calcium concentration expected for Cretaceous seawater (25 to 40 mmol Ca^{2+} L^{-1}). A further increase in external Ca^{2+} concentrations up to 52 mmol L^{-1} adversely affected POC and PIC production only in *E. huxleyi*, whereas *G. oceanica* was not impaired. The non-calcifying strain of *E. huxleyi* exhibited a similar response as the diatom and raphidophyte species with reduced physiological rates of up to 84% at Ca^{2+} concentrations of 19 mmol L^{-1} and higher (Fig. 2). To illustrate the diverging physiological response of calcifying coccolithophores and non-calcifying phytoplankton, we normalized growth and POC production rates from the current study and literature data to the species-specific rates exhibited at modern ocean calcium levels (Fig. 3). A linear regression fit (from 9 to 52 mmol Ca^{2+} L^{-1}) through calcifiers and non-calcifiers resulted in a 6.9 times steeper reduction for the latter group in terms of growth rate (Fig. 3A) and a 4.6 times steeper reduction in terms of POC production rates (Fig. 3B).

In the second experiment, the two under-calcified *E. huxleyi* strains (SO-5.25 and SO-8.04) cultured at elevated seawater Ca^{2+} concentrations (36 mmol L^{-1}) displayed no significant change in growth rate (*t*-test, $p>0.05$) compared to strains cultured at modern Ca^{2+} concentrations of 10 mmol L^{-1} (0.67±0.01 and 0.72±0.01 d^{-1} compared to 0.68±0.01 and 0.71±0.01 d^{-1} for the strains SO-5.25 and SO-8.04, respectively). The number of coccoliths per cell, however, increased remarkably from less than two coccoliths per cell at 10 mmol Ca^{2+} L^{-1} to more than 12 coccoliths per cell, forming a complete coccosphere, at 36 mmol Ca^{2+} L^{-1} (Fig. 4).

4 Discussion

The results presented here demonstrate the influence of seawater Ca^{2+} concentrations on marine phytoplankton physiology (in terms of growth and particulate organic carbon production). Whereas previous studies already investigated the effects of elevated seawater Ca^{2+} concentrations on calcifying coccolithophore physiology and coccolith formation (Herfort et al., 2004; Langer et al., 2007; Müller et al. 2011), this study is to our knowledge the first to investigate the Ca^{2+} sensitivity of non-calcifying phytoplankton in the laboratory. Marine phytoplankton presumably operate several mechanisms which contribute to cellular Ca^{2+} regulation such as intra and extra cellular enzymatic binding capacities and/or the influx regulation via selective channels (Gadd, 2010). Over the past decade progress has been made in the discovery of cellular compartments (e.g. endoplasmic reticulum, chloroplast, mitochondria) regulating plant Ca^{2+} homeostasis and signalling (McAinsh & Pittmann, 2009; Webb, 2008; Brownlee and Hetherington, 2011) and on differences in Ca^{2+} channels between eukaryotes and higher plants and mammalian cells (Wheeler and Brownlee, 2008). However, many unknowns remain about phytoplankton intracellular ion regulation and the homeostasis of the major biological active cations like Ca^{2+} and Mg^{2+} and their interaction and possible influence on each other. For example, Ca^{2+} has a higher ion-exchange capacity than Mg^{2+} (Harris, 2010) and when present in high concentrations might interfere with enzymatic reactions where Mg^{2+} acts as a cofactor (Moore et al., 1960; Legong et al., 2001). However, it remains speculative if this is a possible explanation for the observed reduction in growth rate and Fv/Fm of non-calcifying phytoplankton species (Fig. 2).

The non-calcifying strain of *E. huxleyi* showed a comparable response to elevated seawater Ca^{2+} concentrations as the diatom and raphidophyte species (Fig. 3). This indicates that the Ca^{2+} tolerance of calcifying coccolithophores compared to non-calcifying phytoplankton is not a taxon-specific trait but connected to the process of calcification itself and furthermore, suggests that coccolithophore biomineralization acts as an efficient mechanism to cope with high external Ca^{2+} concentrations. Reduced overall fitness triggered by high external Ca^{2+} concentrations is presumably associated to enhanced transmembrane Ca^{2+} influx leading to higher energetic costs for cytosolic Ca^{2+} removal and might ultimately result in a disadvantage in resource competition between phytoplankton species. The chlorophyceae, *Dunaniella* is one of the most tolerant phytoplankton species regarding high external ion concentrations and regularly blooms in highly saline lakes (Oren, 2002, 2005). However, this extremophile species is inhibited in growth by high external Ca^{2+} concentrations and only

forms blooms in hyper saline lakes when the upper water layer becomes sufficiently diluted with regard to its Ca^{2+} concentrations (Baas-Becking, 1931). This emphasises the ecological importance of external Ca^{2+} concentrations for phytoplankton growth dynamics.

The remarkable tolerance of calcifying coccolithophores to elevated Ca^{2+} concentrations likely results from a tight control on transmembrane Ca^{2+} entry, intracellular transport, and deposition. Seawater Ca^{2+} presumably enters the coccolithophore cell through permeable channels into the peripheral endoplasmatic reticulum. Via the endomembrane transport network it reaches a Golgi-derived organelle, the coccolith vesicle, where it is precipitated as $CaCO_3$ (Mackinder et al., 2010). Precipitation of Ca^{2+} in the form of calcite changes the ion to a biochemically inert state. Large amounts of Ca^{2+} can thereby be sequestered in a finite space and time. For *Emiliania huxleyi* to sustain a typical rate of calcification requires an uptake of $5x10^6$ Ca^{2+} ions s^{-1} (Mackinder et al., 2010). The fact that this massive intracellular Ca^{2+} flux needs to be achieved at a cytosolic concentration of only 100 nmol Ca^{2+} L^{-1} without disturbing the cell's delicate Ca^{2+} homeostasis exemplifies the level of cellular control involved in coccolithophore calcification. It appears reasonable to assume that this tight cellular control of biogenic calcification (which includes $CaCO_3$ precipitation inside the coccolith vesicle and the regulation of cellular Ca^{2+} entrance and distribution) also allows for the observed tolerance to external Ca^{2+} concentrations. The absence of Ca^{2+}-stimulated calcification at levels above modern ocean Ca^{2+} concentrations (Fig. 2F) is in line with previous findings, which indicate saturation of calcification in *E. huxleyi* and *C. braarudii* at ~10 mmol Ca^{2+} L^{-1} (Herfort et al., 2004; Trimborn et al., 2007; Leonardos et al., 2009; Müller et al., 2011). This suggests that in coccolithophores adapted to modern-ocean conditions, factors other than the Ca^{2+} concentration may limit $CaCO_3$ precipitation at higher than ambient Ca^{2+} levels. Potentially limiting factors include dissolved inorganic carbon acquisition and energy supply for the process of calcification (Bolton and Stoll, 2013; Bach et al., 2015).

Emiliania huxleyi is characterized by three distinct different cell forms: (a) the coccolith carrying non-motile diploid form (C-cell), (b) the naked non-motile diploid form (N-cell) and (c) the scaly motile haploid form (S-cell). The latter haploid form possesses organic body scales covering the cell and two flagellates that enable motion (Paasche, 2002). The life cycle of *E. huxleyi* consists of C- and S-cells whereas N-cells are mostly observed in the laboratory after extended culture periods (Paasche, 2002) or under unfavourable culture conditions (Müller et al. 2015). This study investigated only the diploid coccolith carrying (C-cell) and

the naked (N-cell) cell forms of *E. huxleyi*. Our observations and the presence of N- and S-cells in laboratory cultures and natural populations (Paasche, 2002; Frada et al., 2012; Müller et al., 2015) indicate that *E. huxleyi* cells have the ability to control intracellular Ca^{2+} homeostasis at modern Ca^{2+} concentrations without the need of biomineralization.

At modern seawater conditions some *E. huxleyi* strains display an incomplete coccolith cover (coccosphere) with less than 2 coccoliths per cell (Fig. 4A and B) instead of the 10 to 15 that are necessary to form a complete coccosphere (Paasche, 2002). The results of the second experiment indicate that an existent but under-saturated calcification mechanism can be stimulated by increased seawater Ca^{2+} concentrations (Fig. 4C) and, furthermore, might prevent cellular Ca^{2+} poisoning as seen in the non-calcifying *E. huxleyi* strain (Fig. 2 and 3). However, benefits of coccolith formation are expected which evidently outweigh the substantial costs of this energy-consuming process even under modern ocean Ca^{2+} concentrations. Although numerous hypotheses have been proposed concerning the precise function of coccolithophore calcification, including ballasting, protection from viruses, grazers and damaging irradiance, so far none of these is conclusively supported by experimental evidence (Raven and Crawfurd 2012, Barcelos e Ramos et al. 2012).

4.1 Paleoecological implications

Paleoceanographic studies have indicated that the oceanic conditions of the Cretaceous were quite different from those in the modern ocean (e.g. see Zeebe (2001) and Hay (2008)). Besides elevated seawater Ca^{2+} concentrations (Fig. 1), the Cretaceous was marked by a warm greenhouse environment, elevated sea levels, warm shallow shelf seas and altered oceanic circulation. Here we tested whether the biomineralization mechanism in coccolithophores increases their resilience to cellular calcium stress, which indeed is indicated by the physiologically different responses of the three calcifying coccolithophore species (*E. huxleyi*, *G. oceanica* and *C. braarudii*) compared to the non-calcifying species (Fig. 3). Cretaceous seawater Ca^{2+} concentrations may thus have represented a selective advantage for coccolithophores during this period of the geological past. This could explain the proliferation and high productivity of coccolithophores during the Cretaceous compared to non-calcifying phytoplankton. We cannot exclude the possibility of other environmental factors that might have supported the proliferation of coccolithophores or suppressed non-calcifiers in the Cretaceous (e.g. Stanley et al. 2005), but the seawater Ca^{2+} concentrations seems to be a major environmental aspect promoting coccolithophore over non-calcifying phytoplankton growth.

It remains an open question if the onset of calcification in coccolithophores (approx. 225 Ma ago) at relatively low seawater Ca^{2+} concentrations evolved primarily to efficiently regulate cellular Ca^{2+} homeostasis or if calcification had other functions at that time. If calcification in coccolithophores evolved as Ca^{2+} detoxification mechanisms it was presumably an additional instrument to regulate intracellular Ca^{2+} levels because other strategies must have existed in the ancestors of coccolithophores that did not precipitate calcium carbonate. It is reasonable to assume that the rising oceanic Ca^{2+} concentrations represented a selective pressure on phytoplankton populations and may have provided an evolutionary advantage to coccolithophores over non-calcareous phytoplankton during the Jurassic and Cretaceous period (Fig. 1). However, secondary benefits of calcification are likely responsible for its continued operation under modern ocean Ca^{2+} concentrations. Interestingly, *E. huxleyi* and *G. oceanica*, the dominant coccolithophores in the modern ocean, are two of the few coccolithophore species that have a non-calcifying haploid life stage whereas the haploid life-stage of the majority of coccolithophores is calcified (Billard and Inouye, 2004). This let us suggest that these two species in the modern ocean don't rely on cellular Ca^{2+} detoxification by biomineralization.

5 Concluding remarks

The concept of biocalcification as a Ca^{2+} detoxification mechanism in marine organisms has been proposed earlier (Simkiss, 1977; Kaźmierczak et al., 1985) and, based on the results of this study, is supported for coccolithophores. The occurrence of calcified cyanobacteria in the geological record during the Phanerozoic also appears to be connected to elevated seawater Ca^{2+} concentrations (Arp et al., 2001), suggesting similarities in the benefits of calcification in fossil cyanobacteria and coccolithophores. It remains speculative to extend the "Ca^{2+}-detoxification concept" to other marine calcifying groups or to the onset of biocalcification in the Precambrian/Cambrian transition (Kempe and Kaźmierczak, 1994; Brennan et al., 2004). However, in view of the substantial variability in seawater Ca^{2+} concentration during Earth's history and the observed Ca^{2+} sensitivity of dominant marine phytoplankton species, the ocean's Ca^{2+} ion concentration should be considered a potential factor influencing the evolution of marine life on Earth.

Acknowledgements

We thank D. Davis for laboratory assistance and A. McMinn for providing a PAM fluorometer. We are grateful for the constructive comments of one anonymous reviewer, T. Tyrrell and J. Young. Additional comments from a research group meeting (composed of L. Munns, M. Duret, C. Daniels, K. Mayers, A. Poulton and R. Sheward) further increased the quality of the manuscript. The work was funded by the Australian Research Council (DP 1093801 to G. M. Hallegraeff and T. W. Trull) and the "Conselho Nacional de Desenvolvimento Científico e Tecnológico Brasil (CNPq, Processo: 405585/2013-6)". K. G. Schulz is the recipient of an Australian Research Council Future Fellowship (FT120100384).

References

Arp, G., Reimer, A., and Reitner, J.: Photosynthesis-Induced Biofilm Calcification and Calcium Concentrations in Phanerozoic Oceans, Science, 292, 1701-1704, 2001.

Baas-Becking, L. G. M.: Salt effects on swarmers of Dunaliella virdis Teod., J. Gen. Physiol., 14, 765-779, 1931.

Bach, L. T., Riebesell, U., Gutowska, M. A., Federwisch, L., and Schulz, K. G.: A unifying concept of coccolithophore sensitivity to changing carbonate chemistry embedded in an ecological framework. Prog. Oceanogr., 135, 125-138, 2015.

Barcelos e Ramos, J., Schulz, K. G., Febiri, S., and Riebesell, U.: Photoacclimation to abrupt changes in light intensity by *Phaeodactylum tricornutum* and *Emiliania huxleyi*: the role of calcification, Mar. Ecol. Prog. Ser., 452, 11-26, 2012.

Ben-Yaakov, S. and Goldhaber, M. B.: The influence of sea water composition on the apparent constants of the carbonate system, Deep Sea Res., 20, 87–99, 1973.

Billard, C. and Inouye, I.: What is new in coccolithophore biology, in: Coccolithophores – From Molecular Processes to Global Impact, Springer, New York, USA, 481-508, 2004.

Bolton, C. T. and Stoll, H. M.: Late Miocene threshold response of marine algae to carbon dioxide limitation, Nature, 500, 558-562, 2013.

Bown, P. R., Lees, J. A., and Young, J. R.: Calcareous nannoplankton evolution and diversity through time, in: Coccolithophores – From Molecular Processes to Global Impact, Springer, New York, USA, 481-508, 2004.

Brennan, S. T., Lowenstein, T. K., and Horita, J.: Seawater chemistry and the advent of biocalcification, Geology, 32, 473-476, 2004.

Broecker, W. and Clark, E.: Ratio of coccolith CaCO3 to foraminifera CaCO3 in the late Holocene deeper-sea sediments, Paleoceanogr., 24, PA3205, 2009.

Brownlee, C., Wood, J. W., and Briton, D.: Cytoplasmic free calcium in single cells of centric diatoms. The use of Fura-2, Protoplasma, 140, 118-122, 1987.

Brownlee, C., Davies, M., Nimer, N., Dong, L. F., and Merrett, M.: Calcification, photosynthesis and intracellular regulation in *Emiliania huxleyi*, Bulletin de l'Institute océanographique de Monaco, 14, 19–35, 1995.

Brownlee, C. and Hetherington, A.: Introduction to a Virtual Special Issue on calcium signalling in plants, New Phytologist 192, 786–789, 2011.

Case, R. M., Eisner, D., Gurney, A., Jones, O., Muallem, S., and Verkhratsky, A.: Evolution of calcium homeostasis: From birth of the first cell to an omnipresent signalling system, Cell Calcium, 42, 345-350, 2007.

Dickson, A. G., Afghan, J. D., and Anderson, G. C.: Reference materials for oceanic CO_2 analysis: a method for the certification of total alkalinity. Mar. Chem., 80, 185−197, 2003.

Frada, M. J., Bidle, K. D., Probert, I., and de Vargas, C.: In situ survey of life cycle phases of the coccolithophore *Emiliania huxleyi* (Haptophyta), Environmental Microbiol., 14, 1558-1569, 2012.

Gadd, G. M.: Metals, minerals and microbes: geomicrobiology and bioremediation, Microbiol., 156, 609-643, 2010.

Guillard, R.: Culture of phytoplankton for feeding marine invertebrates, in: Culture of Marine Invertebrate Animals, Springer, New York, USA, 29-60, 1975.

Harris, D. C.: Quantitative Chemical Analysis, W.H. Freeman, 8th Edition, 2010.

Hay, W. W.: Evolving ideas about the Cretaceous climate and ocean circulation. Cret. Res. 29, 725-753, 2008.

Herfort, L., Loste, E., Meldrun, F., and Thake, B.: Structural and physiological effects of calcium and magnesium in *Emiliania huxleyi* (Hay and Mohler), J. Struct. Biol., 148, 307–314, 2004.

Hönisch, B., Ridgwell, A., Schmidt, D. N., Thomas, E., Gibbs, S. J., Sluijs, A., Zeebe, R., Kump, L., Martindale, R. C., Greene, S. E., Kiessling, W., Ries, J., Zachos, J. C., Royer, D. L., Barker, S., Marchitto, Jr T. M., Moyer, R., Pelejero, C., Ziveri, P., Foster, G. L., and Williams, B., The Geological Record of Ocean Acidification, Science, 335, 1058-1063, 2012.

Horita, J., Zimmermann, H., and Holland, H. D.: Chemical evolution of seawater during the Phanerozoic: Implications from the record of marine evaporites, Geochim. Cosmochim. Acta, 66, 3733-3756, 2002.

Kader, M. A. and Lindberg, S.: Cytosolic calcium and pH signaling in plants under salinity stress, Plant Signal. Behav., 5, 233-238, 2010.

Kaźmierczak, J., Ittekott, V., and Degens, E.T.: Biocalcification through time: environmental challenge and cellular response, Palaeontol. Zeitschr., 59, 15-33, 1985.

Kempe, S. and Degens, E. T.: An early soda ocean, Chem. Geol., 53, 95-108, 1985.

Kempe, S. and Kaźmierczak, J.: The role of alkalinity in the evolution of ocean chemistry, organization of living systems and biocalcification processes, in: Past and Present Biomineralization Processes: Considerations about the Carbonate Cycle, Bulletin de l'Institute océanographique de Monaco no. spec., Monaco, 13, 61-117, 1994.

Kester, D. R., Duedall, I. W., Conners, D. N., and Pytkowicz, R. M.: Preparation of artificial seawater, Limnol. Oceanogr. 12, 176–179, 1967.

Kooistra, W. H. C. F., Gersonde, R., Medlin, L. K., and Mann, D. G.: The Origin and Evolution of the Diatoms: Their Adaptation to a Planktonic Existence, in: Evolution of Primary Producers in the Sea, Elsevier, Amsterdam, Netherlands, 207-249, 2007.

Langer, G., Gussone, N., Nehrke, G., Riebesell, U., Eisenhauer, A., and Thoms, S.: Calcium isotope fractionation during coccolith formation in *Emiliania huxleyi*: Independence of growth and calcification rate, Geochem. Geophys. Geosyst., 8, Q05007, doi:10.1029/2006GC001422, 2007.

Legong, L., Tutone, A. F., Drummond, R. S. M., Gardner, R. C., and Luan, S.: A novel family of magnesium transport genes in Arabidopsis, Plant Cell, 13, 2761–2775, 2001.

Leonardos, N., Read, B., Thake, B., and Young, J. R.: No mechanistic dependence of photosynthesis on calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyta), J. Phycol., 45, 1046-1051, 2009.

Loeblich, A. R. 3rd and Smith, V. E.: Chloroplast pigments of the marine dinoflagellate *Gyrodinium resplendens*, Lipids, 3, 5–13, 1968.

Mackinder, L., Wheeler, G., Schroeder, D., Riebesell, U., and Brownlee, C.: Molecular mechanisms underlying calcification in coccolithophores, Geomicrobiol. J., 27, 585–595, 2010.

McAinsh, M. R. and Pittman, J. K.: Shaping the calcium signature, New Phytologist 181, 275–294, 2009.

Meléndez, M., Nesterenko, E. P., Nesterenko, P. N., and Corredor, J. E.: Direct chromatographic separation and quantification of calcium and magnesium in seawater and sediment porewaters, Limnol. Oceanogr. Met. 11, 466-474, 2013.

Moore, D. P., Overstreet, R., and Jacobson, L.: Uptake of magnesium and its interaction with calcium in excised barley roots. Plant Physiol., 36, 290–295, 1960.

Moss, B.: The influence of environmental factors on the distribution of freshwater alga: An experimental study. I. Introduction and the influence of calcium concentration, J. Ecol. 60, 917-932, 1972.

Müller, M. N., Kisakürek, B., Buhl, D., Gutperlet, R., Kolevica, A., Riebesell, U., Stoll, H. M., and Eisenhauer, A.: Response of the coccolithophores *Emiliania huxleyi* and *Coccolithus braarudii* to changing seawater Mg^{2+} and Ca^{2+} concentrations: Mg/Ca, Sr/Ca ratios and $\delta^{44/40}Ca$, $\delta^{26/24}Mg$ of coccolith calcite, Geochim. Cosmochim. Acta, 75, 2088–2102, 2011.

Müller, M. N., Trull, T. W., and Hallegraeff, G. M.: Differing responses of three Southern Ocean *Emiliania huxleyi* ecotypes to changing seawater carbonate chemistry. Mar. Ecol. Prog. Ser., 531, 81-90, 2015.

Oren, A.: Halophilic Microorganisms and their Environment, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2002.

Oren, A.: A hundred years of Dunaliella research: 1905-2005, Saline Systems, 1:2, doi:10.1186/1746-1448-1-2, 2005.

Orrenius, S., McConkey, D. J., Bellomo, G., and Nicotera, P.: Role of Ca^{2+} in toxic cell killing, Trends Pharmacol. Sci., 10, 281-285, 1989.

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

Raven, J. A. and Crawfurd, K.: Environmental controls on coccolithophore calcification, Mar. Ecol. Prog. Ser., 470, 137-166, 2012.

Roy, R., Roy, L., Vogel, K., Porter-Moore, C., Pearson, T., Good, C., Millero, F., and Campbell, D.: The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and temperatures 0 to 45 ˚C, Mar. Chem., 44, 249–267, 1993.

Sanders, D., Brownlee, C., and Harper, J. F.: Communicating with Calcium, The Plant Cell, 11, 691-706, 1999.

Simkiss, K.: Biomineralization and detoxification, Calc. Tiss. Res., 24, 199-200, 1977.

Skelton, P.: The Cretaceous World, Academic Press, Cambridge, UK, 2003.

Stanley, S. M., Ries, J. B., and Hardie, L. A.: Seawater chemistry, coccolithophore population growth, and the origin of Cretaceous chalk, Geology, 33, 593-596, 2005.

Tassigny, M.: Action du calcium sur la croissance de desmidies axeniques, Mitt. int. Verein. theor. angew. Limnol. 19, 292-313, 1971.

Trimborn, S., Langer, G., and Rost, B.: Effect of varying calcium concentrations and light intensities on calcification and photosynthesis in *Emiliania huxleyi*, Limnol. Oceanogr., 52, 2285–2293, 2007.

Tyrrell, T. and Zeebe, R. E.: History of carbonate ion concentration over the last 100 million years, Geochim. Cosmochim. Acta, 68, 3521–3530, 2004.

Webb, A. A. R.: The chloroplast as a regulator of Ca^{2+} signalling. New Phytologist, 179, 568–570, 2008.

Wheeler, G. L. and Brownlee, C.: Ca^{2+} signalling in plants and green algae--changing channels, Trends Plant Sci., 13(9), 506-14, 2008.

Zeebe, R. and Wolf-Gladrow, D.: CO2 in Seawater: Equilibrium, Kinetics, Isotopes, Elsevier Science B.V., Amsterdam, Netherlands, 2001.

Zeebe, R. E.: Seawater pH and isotopic paleotemperatures of Cretaceous oceans, Palaeogeogr., Palaeoclimatol., Palaeoecol., 170, 49-57, 2001.

Figures

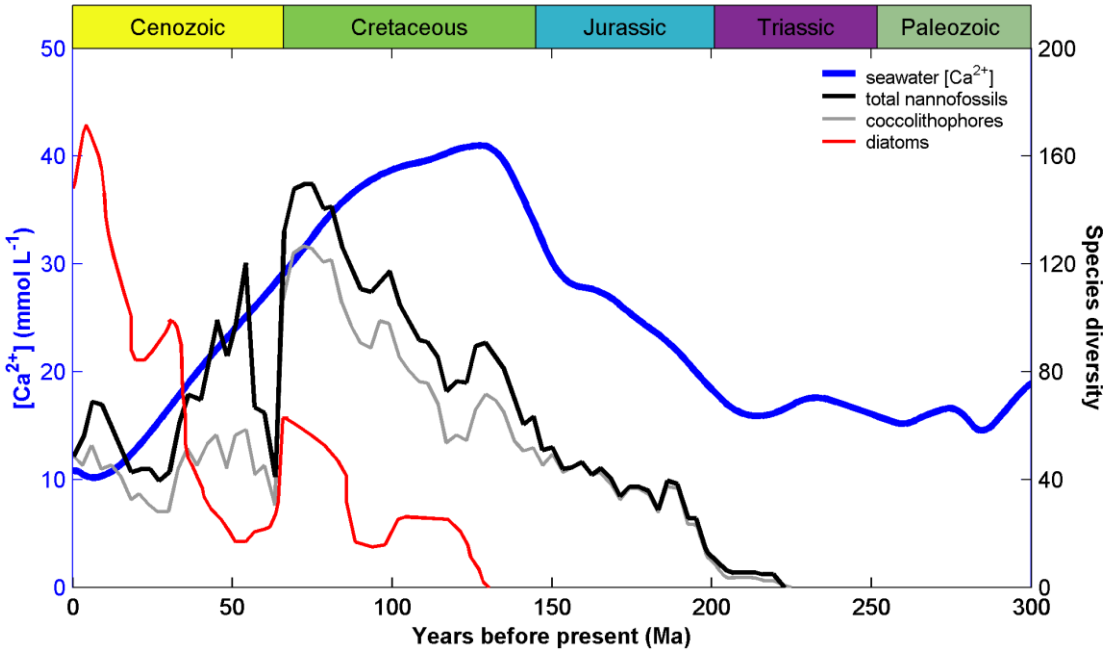

Figure 1: Seawater Ca^{2+} concentration and fossil phytoplankton diversity over the past 300 Ma. Model-reconstructed seawater Ca^{2+} concentration (blue line; data retrieved from Hönisch et al. (2012)), fossil species diversity of diatoms (red line; data retrieved from Kooistra et al. (2007)), total nannofossils and coccolithophores (black and grey line, respectively; data retrieved from Bown et al. (2004)).

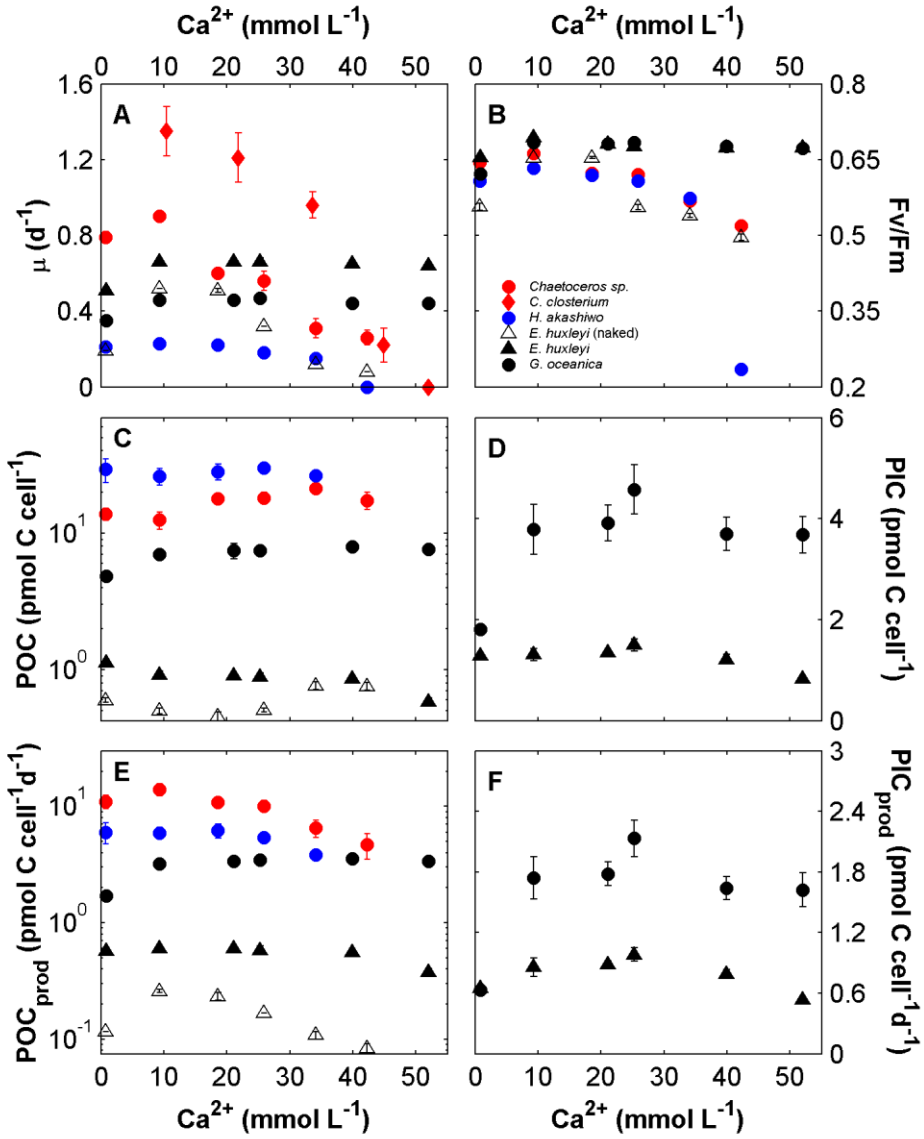

Figure 2: Phytoplankton physiological responses to seawater Ca^{2+} concentration. Displayed are laboratory cultured strains of diatoms (red markers), raphidophyte (blue markers), coccolithophores (black markers) and a non-calcifying coccolithophore (black-open marker): (A), Species-specific growth rate; (B), maximum quantum yield of photosynthesis (Fv/Fm); (C), Cellular POC and (D), PIC quotas; (E), Cellular POC and (F), PIC production rates as a function of seawater Ca^{2+} concentration. Error bars denote ± 1sd (n=3). Note that the physiological response of *Ceratoneis closterium* was only determined via growth rate measurements. POC quota of *H. akashiwo* could not be determined at a Ca^{2+} concentration of 42 mmol L^{-1} due to lack of growth.

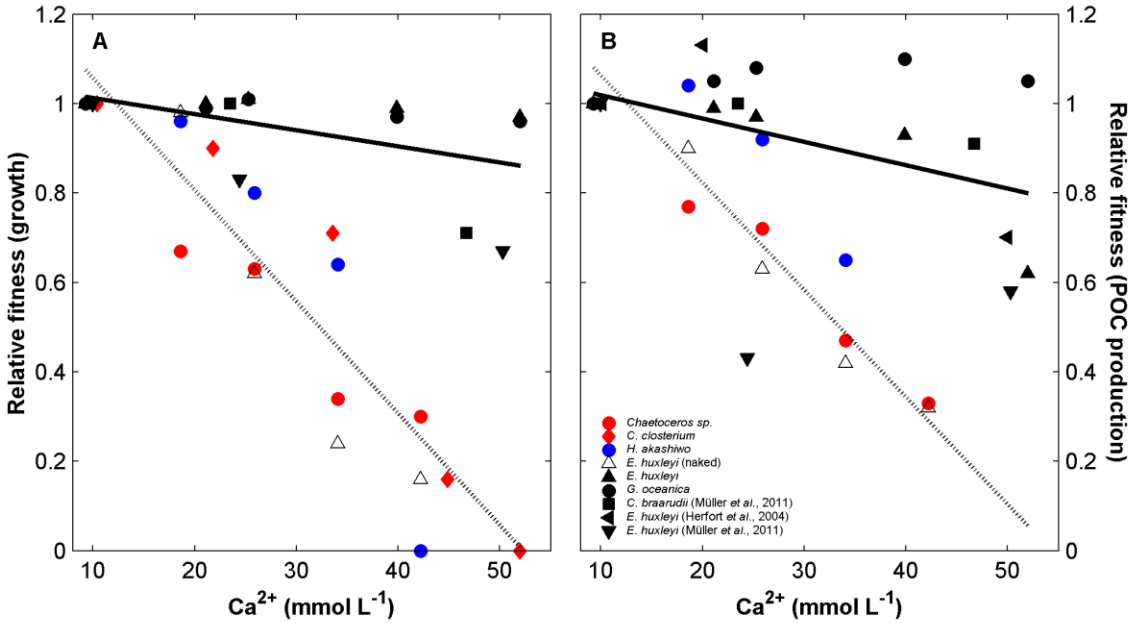

Figure 3: Relative physiological response of phytoplankton species to seawater Ca^{2+} concentration. Relative fitness expressed in terms of (A), growth rate and (B), POC production of all tested species normalised to ambient seawater Ca^{2+} concentration of ~10 mmol L^{-1}, and supplemented with coccolithophore literature data from Müller et al. (2011) and Herfort et al. (2004), to illustrate the effect of calcium poisoning on calcifiers and non-calcifiers. Solid lines indicate regressions through calcifiers: (A) y=-0.0036x+1.0483 (r^2=0.278, p=0.035, n=16) and (B) y=-0.0052x + 1.0704 (r^2=0.184, p=0.067, n=19). Dotted lines indicate regressions through non-calcifiers: (A) y=-0.025x+1.307 (r^2=0.858, p<0.0001, n=20) and (B) y = -0.024 x + 1.303 (r^2=0.826, p<0.0001, n=15).

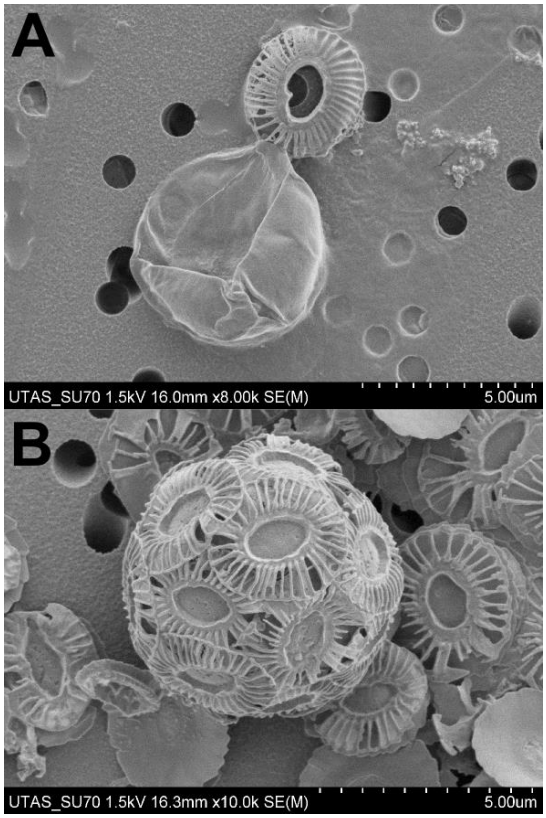

Figure 4: Representative SEM photographs of the under-calcified *E. huxleyi* strain SO-8.04 cultured at modern seawater Ca^{2+} concentration of 10 mmol L-1, showing no or only single attached coccoliths (A). When cultured for two month at elevated Ca^{2+} concentration of 36 mmol Ca^{2+} kg^{-1}, *E. huxleyi* strain SO-8.04 produced a sufficient number of coccoliths to cover the whole cell (B).