



## Phytoplankton calcification as an effective mechanism to alleviate cellular calcium poisoning

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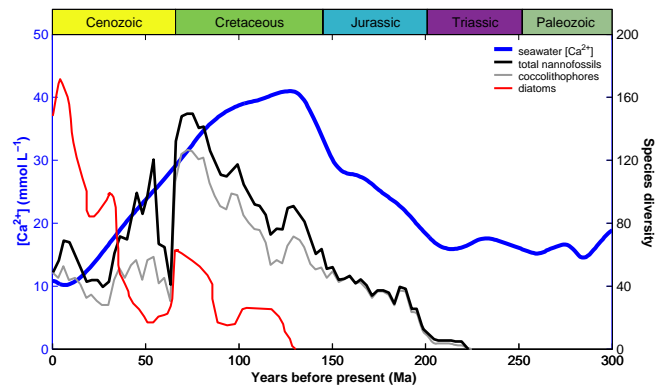
**Abstract.** Marine phytoplankton have developed the remarkable ability to tightly regulate the concentration of free calcium ions in the intracellular cytosol at a level of  $\sim 0.1 \mu\text{mol L}^{-1}$  in the presence of seawater  $\text{Ca}^{2+}$  concentrations of  $10 \text{ mmol L}^{-1}$ . 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentrations in their environment on geological timescales. For example, the Cretaceous (145 to 66 Ma), an era known for the high abundance of coccolithophores and the production of enormous calcium carbonate deposits, exhibited seawater calcium concentrations up to 4 times present-day levels. We show that calcifying coccolithophore species (*Emiliana 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  $\text{Ca}^{2+}$  in eukaryotes are well regulated and the maintenance of relatively low levels is essential for fast signal transduction. An excessive influx of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  in plant cells is predominantly achieved by  $\text{Ca}^{2+}$ -binding proteins, reducing the effective diffusion coefficient of  $\text{Ca}^{2+}$  in the cytosol, and ultimately via sequestration by the endoplasmic reticulum, mitochondria and cellular vacuoles (Case et al., 2007). Cytosolic free  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  levels (Brownlee et al., 1987, 1995). It is, however, unknown whether the regulating mechanisms of marine phytoplankton to keep this delicate  $\text{Ca}^{2+}$  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  $\text{CaCO}_3$  precipitates.

An early hypothesis describes the invention and the process of biomineralization in the form of calcium carbonate by marine organisms as a potential  $\text{Ca}^{2+}$  detoxification mechanism (Simkiss, 1977; Kaźmierczak et al., 1985; Kempe and Degens, 1985). Ocean calcium concentrations have changed remarkably throughout the Phanerozoic eon (past 541 Myr) as documented by fluid inclusions of marine halite (Horita et al., 2002). Over the past 300 Myr, highest seawater  $\text{Ca}^{2+}$  concentrations are documented for the Cretaceous (145 to 66 Ma; Hönlisch 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 specialized 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), coinciding with relatively low seawater  $\text{Ca}^{2+}$  concentrations (Bown et al., 2004). Subsequently, seawater  $\text{Ca}^{2+}$  concentrations increased, potentially linked to changes in the seafloor spreading rates (Skelton, 2003),



**Figure 1.** Seawater  $\text{Ca}^{2+}$  concentration and fossil phytoplankton diversity over the past 300 Myr. Model-reconstructed seawater  $\text{Ca}^{2+}$  concentration (blue line; data retrieved from Hönlisch 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).

and peaked in the Cretaceous at the highest levels since the past 300 Myr ( $\sim 3$  to  $4$  times the present seawater concentrations of  $10 \text{ mmol Ca}^{2+} \text{ L}^{-1}$ ). Species diversity and abundance of total nannofossils, including coccolithophores, have increased in concert with high seawater  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  levels over the past 300 Myr. 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  $\text{Ca}^{2+}$  concentration was investigated in two under-calcifying *E. huxleyi* strains. If biogenic calcification represents a viable mechanism to cope with high external  $\text{Ca}^{2+}$  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. *Emiliana huxleyi* strain SO-6.13 was isolated by Suellen Cook in February 2007 from the Southern Ocean (54° S, 146° E; 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  $\text{CaCl}_2$  with concomitant additions of NaCl, keeping the ionic strength of the artificial seawater constant. *Gephyrocapsa oceanica*, *H. akashiwo* 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 setup

In the first experiment, cells were acclimated to the experimental conditions ( $\text{Ca}^{2+}$  range from 1 to 52  $\text{mmol L}^{-1}$ ) 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  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and a 16 : 8 h light : dark cycle at the University of Tasmania. *Ceratoneis closterium* was incubated at 20 °C, 250  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  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 months under dilute semi-continuous batch conditions at the identical conditions as described above with  $\text{Ca}^{2+}$  concentrations adjusted to 10 or 36  $\text{mmol Ca}^{2+} \text{L}^{-1}$ . 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  $\sim 50$  to a maximal cell density of  $\sim 80\,000$  cells  $\text{mL}^{-1}$ , which prevented major changes in the seawater carbonate chemistry.

## 2.3 Seawater chemistry analysis

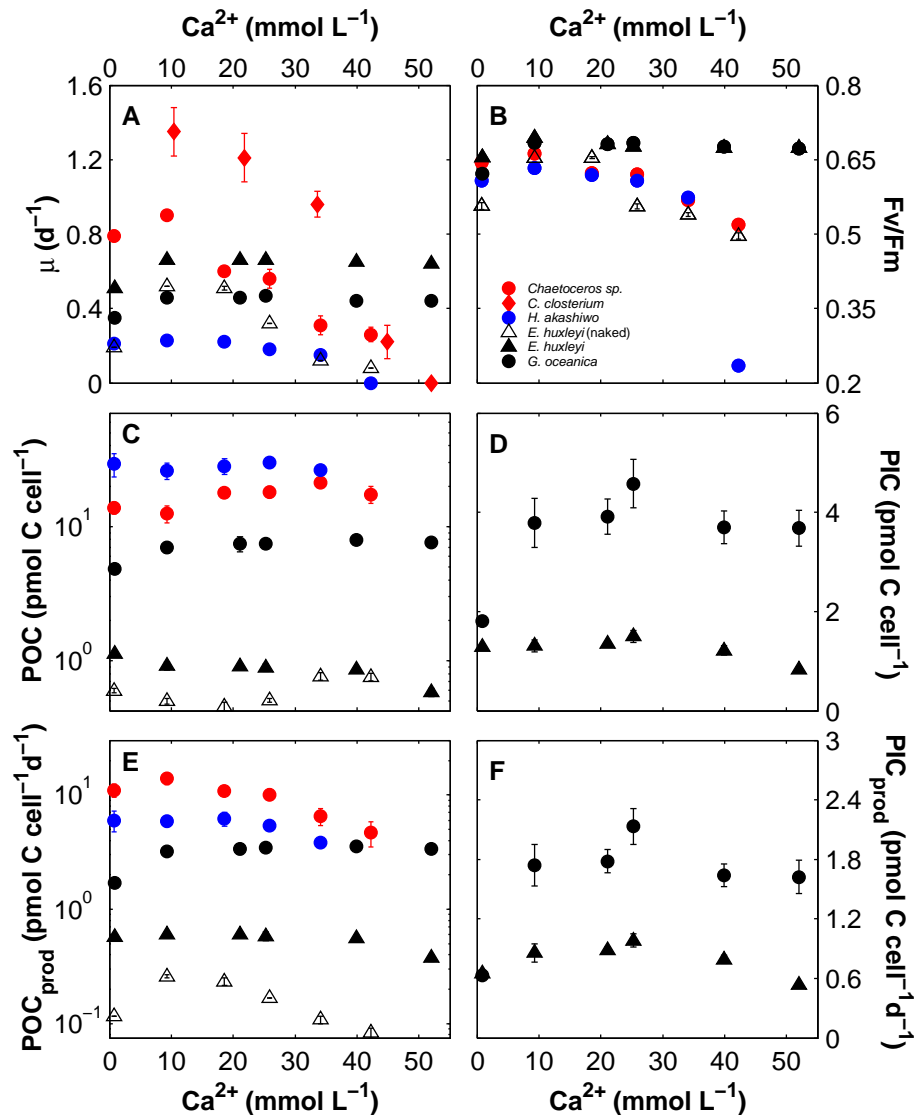
Seawater  $\text{Ca}^{2+}$  concentrations at the start of the experiment were determined via chelation ion chromatography (Meléndez et al., 2013), using an adjusted method to match the different  $\text{Ca}^{2+}$  concentrations (precision of  $\pm 1.4\%$ ). Dissolved inorganic carbon and  $A_T$  were analysed as the mean of triplicate measurements with the infrared detection method using an Apollo SciTech DIC analyser (model AS-C3) and the potentiometric titration method (Dickson et al., 2003), respectively. Data were corrected to certified reference materials (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 using equations from Zeebe and Wolf-Gladrow (2001) with dissociation constants for carbonic acid after Roy et al. (1993), modified with sensitivity parameters for  $[\text{Na}^+]$ ,  $[\text{Mg}^{2+}]$  and  $[\text{Ca}^{2+}]$  (Ben-Yaakov and Goldhaber, 1973). The calcite saturation state ( $\Omega$ ) was calculated with regard to the Mg / Ca ratio as described in Tyrrell and Zeebe (2004). Detailed information on the carbonate system parameters can be found in the Supplement.

## 2.4 Physiological parameters

Maximum quantum yield of the photosystem II (Fv/Fm) was measured on dark-adapted samples (45 min) using a WATER-PAM fluorometer (Walz GmbH, Germany). Sub-samples for total particulate carbon (TPC) and particulate organic carbon (POC) were filtered onto pre-combusted (7 h, 450 °C) quartz-microfibre filters (pore-size of 0.3  $\mu\text{m}$ ) and stored at  $-24$  °C. Filters for POC analysis were fumed with saturated HCl for 10 h 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  $\mu$  ( $\text{d}^{-1}$ ) 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



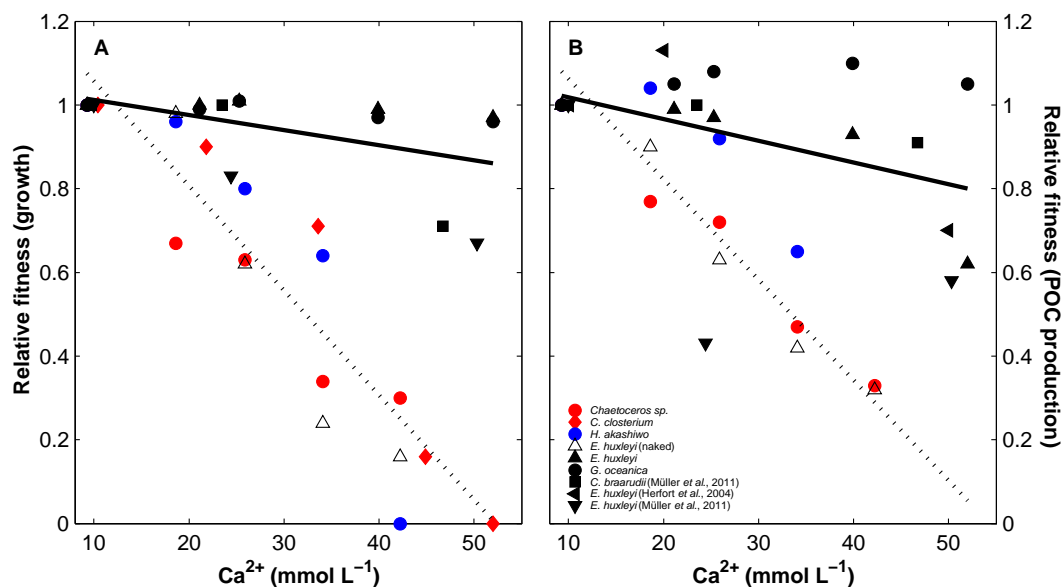
**Figure 2.** Phytoplankton physiological responses to seawater  $\text{Ca}^{2+}$  concentration. Displayed are laboratory-cultured strains of diatoms (red markers), raphidophytes (blue markers), coccolithophores (black markers) and a non-calcifying coccolithophore (black open marker): (a) species-specific 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  $\text{Ca}^{2+}$  concentration. Error bars denote  $\pm 1$  SD ( $n = 3$ ). 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  $\text{Ca}^{2+}$  concentration of 42  $\text{mmol L}^{-1}$  due to lack of growth.

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  $\text{Ca}^{2+}$  concentrations below 2  $\text{mmol L}^{-1}$ , all species exhibited significantly ( $t$  test,  $p < 0.05$ ) lower growth, particulate organic carbon (POC) production rates and maximum quantum yield of photo-

system II (Fv/Fm) compared to modern seawater concentrations of  $\sim 10 \text{ mmol Ca}^{2+} \text{ L}^{-1}$  (Fig. 2). Furthermore, the two calcifying species displayed decreased particulate inorganic carbon (PIC) production rates at  $\text{Ca}^{2+}$  concentrations below 2  $\text{mmol L}^{-1}$  compared to  $\sim 10 \text{ mmol Ca}^{2+} \text{ L}^{-1}$  ( $t$  test,  $p < 0.05$ ). At elevated  $\text{Ca}^{2+}$  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  $\text{mmol Ca}^{2+} \text{ L}^{-1}$  in *H. akashiwo* and *C. closterium*, respectively. Both tested coccolithophore species, on the other



**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 normalized to ambient seawater  $Ca^{2+}$  concentration of  $\sim 10 \text{ mmol L}^{-1}$ , 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)  $y = -0.0036 \times +1.0483$  ( $r^2 = 0.278$ ,  $p = 0.035$ ,  $n = 16$ ) and (b)  $y = -0.0052 \times +1.0704$  ( $r^2 = 0.184$ ,  $p = 0.067$ ,  $n = 19$ ). Dotted lines indicate regressions through non-calcifiers: (a)  $y = -0.025 \times +1.307$  ( $r^2 = 0.858$ ,  $p < 0.0001$ ,  $n = 20$ ) and (b)  $y = -0.024 \times +1.303$  ( $r^2 = 0.826$ ,  $p < 0.0001$ ,  $n = 15$ ).

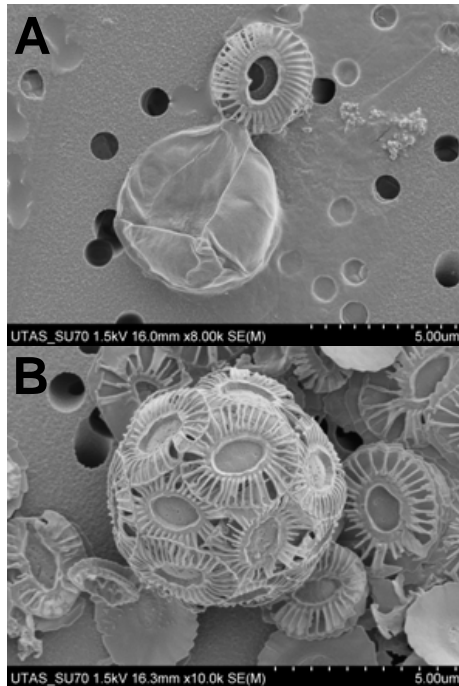
hand, were able to maintain their growth, Fv/Fm, POC and PIC production rates with no substantial change at calcium concentration expected for Cretaceous seawater (25 to 40  $mmol Ca^{2+} L^{-1}$ ). A further increase in external  $Ca^{2+}$  concentrations up to 52  $mmol L^{-1}$  adversely affected 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 to that of 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  $mmol L^{-1}$  ( $0.67 \pm 0.01$  and  $0.72 \pm 0.01 \text{ d}^{-1}$  compared to  $0.68 \pm 0.01$  and  $0.71 \pm 0.01 \text{ d}^{-1}$  for the strains SO-5.25 and SO-8.04, respectively). The number of coccoliths per cell, however, increased remarkably from fewer than 2 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 have 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 extracellular 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 homeostasis and signalling (McAinsh and Pittmann, 2009; Webb, 2008; Brownlee and Hetherington, 2011), as well as in the differences between the Ca channels of eukaryotes, higher plants and mammalian cells (Wheeler and Brownlee, 2008). However, many unknowns remain about phytoplankton in-



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

tracellular ion regulation and the homeostasis of the major biological active cations like  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and their interaction and possible influence on each other. For example,  $\text{Ca}^{2+}$  has a higher ion-exchange capacity than  $\text{Mg}^{2+}$  (Harris, 2010) and when present in high concentrations might interfere with enzymatic reactions where  $\text{Mg}^{2+}$  acts as a co-factor (Moore et al., 1960; Legong et al., 2001). However, it remains speculative whether 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  $\text{Ca}^{2+}$  concentrations as the diatom and raphidophyte species (Fig. 3). This indicates that the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentrations. Reduced overall fitness triggered by high external  $\text{Ca}^{2+}$  concentrations is presumably associated with enhanced transmembrane  $\text{Ca}^{2+}$  influx, leading to higher energetic costs for cytosolic  $\text{Ca}^{2+}$  removal and might ultimately result in a disadvantage in resource competition between phytoplankton species. *Dunaliella*, a member of the class Chlorophyceae, is one of the most tolerant phytoplank-

ton 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  $\text{Ca}^{2+}$  concentrations and only forms blooms in hypersaline lakes when the upper water layer becomes sufficiently diluted with regard to its  $\text{Ca}^{2+}$  concentrations (Baas-Becking, 1931). This emphasizes the ecological importance of external  $\text{Ca}^{2+}$  concentrations for phytoplankton growth dynamics.

The remarkable tolerance of calcifying coccolithophores to elevated  $\text{Ca}^{2+}$  concentrations likely results from a tight control on transmembrane  $\text{Ca}^{2+}$  entry, intracellular transport, and deposition. Seawater  $\text{Ca}^{2+}$  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  $\text{CaCO}_3$  (Mackinder et al., 2010). Precipitation of  $\text{Ca}^{2+}$  in the form of calcite changes the ion to a biochemically inert state. Large amounts of  $\text{Ca}^{2+}$  can thereby be sequestered in a finite space and time. For *Emiliania huxleyi* to sustain a typical rate of calcification, an uptake of  $5 \times 10^6 \text{ Ca}^{2+} \text{ ions s}^{-1}$  is required (Mackinder et al., 2010). The fact that this massive intracellular  $\text{Ca}^{2+}$  flux needs to be achieved at a cytosolic concentration of only  $100 \text{ nmol Ca}^{2+} \text{ L}^{-1}$  without disturbing the cell's delicate  $\text{Ca}^{2+}$  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  $\text{CaCO}_3$  precipitation inside the coccolith vesicle and the regulation of cellular  $\text{Ca}^{2+}$  entrance and distribution) also allows for the observed tolerance to external  $\text{Ca}^{2+}$  concentrations. The absence of  $\text{Ca}^{2+}$ -stimulated calcification at levels above modern ocean  $\text{Ca}^{2+}$  concentrations (Fig. 2f) is in line with previous findings, which indicate saturation of calcification in *E. huxleyi* and *C. braarudii* at  $\sim 10 \text{ mmol Ca}^{2+} \text{ L}^{-1}$  (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  $\text{Ca}^{2+}$  concentration may limit  $\text{CaCO}_3$  precipitation at higher than ambient  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  homeostasis at modern  $\text{Ca}^{2+}$  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) 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  $\text{Ca}^{2+}$  concentrations (Fig. 4b) and, furthermore, might prevent cellular  $\text{Ca}^{2+}$  poisoning as seen in the non-calcifying *E. huxleyi* strain (Figs. 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  $\text{Ca}^{2+}$  concentrations. Although numerous hypotheses have been proposed concerning the precise function of coccolithophore calcification, including ballasting and protection from viruses, grazers and damaging irradiance, so far none of these is conclusively supported by experimental evidence (Raven and Crawford, 2012; Barcelos e Ramos et al., 2012).

#### 4.1 Palaeoecological implications

Palaeoceanographic studies have indicated that the oceanic conditions of the Cretaceous were quite different from those in the modern ocean (e.g. see Zeebe, 2001; Hay, 2008). Besides elevated seawater  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentrations seem to be a major environmental aspect promoting coccolithophore over non-calcifying phytoplankton growth.

It remains an open question whether the onset of calcification in coccolithophores (approx. 225 Ma) at relatively low seawater  $\text{Ca}^{2+}$  concentrations evolved primarily to efficiently regulate cellular  $\text{Ca}^{2+}$  homeostasis or whether cal-

cification had other functions at that time. If calcification in coccolithophores evolved as a  $\text{Ca}^{2+}$  detoxification mechanism, it was presumably an additional instrument to regulate intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 led us to suggest that these two species in the modern ocean do not rely on cellular  $\text{Ca}^{2+}$  detoxification by biomineralization.

#### 5 Concluding remarks

The concept of biocalcification as a  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentrations (Arp et al., 2001), suggesting similarities in the benefits of calcification in fossil cyanobacteria and coccolithophores. It remains speculative to extend the “ $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  concentration during Earth’s history and the observed  $\text{Ca}^{2+}$  sensitivity of dominant marine phytoplankton species, the ocean’s  $\text{Ca}^{2+}$  ion concentration should be considered a potential factor influencing the evolution of marine life on Earth.

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## 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 swarms of *Dunaliella viridis* 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  $\text{CaCO}_3$  to foraminifera  $\text{CaCO}_3$  in the late Holocene deeper-sea sediments, *Paleoceanogr.*, 24, PA3205, doi:10.1029/2009PA001731, 2009.
- Brownlee, C. and Hetherington, A.: Introduction to a Virtual Special Issue on calcium signalling in plants, *New Phytol.*, 192, 786–789, 2011.
- Brownlee, C., Wood, J. W., and Britton, 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’Institut océanographique de Monaco*, 14, 19–35, 1995.
- Case, R. M., Eisner, D., Gurney, A., Jones, O., Muallem, S., and Verkhatsky, 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  $\text{CO}_2$  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), *Environ. 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 & Company, 7th Edition, 899 pp., 2010.
- Hay, W. W.: Evolving ideas about the Cretaceous climate and ocean circulation, *Cret. Res.* 29, 725–753, 2008.
- Herfort, L., Lose, E., Meldrum, 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. Ac.*, 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’Institut océanographique de Monaco no. spec., Monaco, 13, 61–117, 1994.
- Kester, D. R., Duedall, I. W., Connors, 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. Geosys.*, 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 *Emiliana huxleyi* (Haptophyta), *J. Phycol.*, 45, 1046–1051, 2009.
- Loeblich, A. R. 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 Phytol.*, 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.-Methods*, 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 *Emiliana 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. Ac.*, 75, 2088–2102, 2011.
- Müller, M. N., Trull, T. W., and Hallegraeff, G. M.: Differing responses of three Southern Ocean *Emiliana huxleyi* ecotypes to changing seawater carbonate chemistry. *Mar. Ecol.-Prog. Ser.*, 331, 81–90, 2015.
- Oren, A.: Halophilic Microorganisms and their Environment, Kluwer Academic Publishers, Dordrecht, The Netherlands, 171 pp., 2002.
- Oren, A.: A hundred years of Dunaliella research: 1905–2005, *Saline Systems*, 1, 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 *Emiliana huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions, *Phycologia*, 40, 503–529, 2002.
- Raven, J. A. and Crawford, 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, *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, 360 pp., 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 desmadies 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 *Emiliana 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. Ac.*, 68, 3521–3530, 2004.
- Webb, A. A. R.: The chloroplast as a regulator of  $Ca^{2+}$  signalling, *New Phytol.*, 179, 568–570, 2008.
- Wheeler, G. L. and Brownlee, C.:  $Ca^{2+}$  signalling in plants and green algae—changing channels, *Trends Plant Sci.*, 13, 506–14, 2008.
- Zeebe, R. and Wolf-Gladrow, D.:  $CO_2$  in Seawater: Equilibrium, Kinetics, Isotopes, Elsevier Science B.V., Amsterdam, Netherlands, 360 pp., 2001.
- Zeebe, R. E.: Seawater pH and isotopic paleotemperatures of Cretaceous oceans, *Palaeogeogr. Palaeoclimatol.*, 170, 49–57, 2001.