Calcification of selected coccolithophore species: strontium partitioning, calcium isotope fractionation and dependence on seawater carbonate chemistry

Dissertation

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"If entire coccospheres are examined ..... it will be found that numerically about 80 per cent. of them contain an internal oval colourless body. Closer examination reveals that this body is in many cases a complete and perfect coccolith....."

(Dixon 1900)

1 General Introduction

1.1 Coccolithophores

Oceans cover roughly seventy percent of the earth's surface. Unicellular photoautotrophic primary producers such as diatoms, dinoflagellates and coccolithophores are key organisms throughout the euphotic zone of this vast expanse. Despite their minute size these photosynthetic organisms form the basis of the marine food chain and play an important role in biogeochemical cycles, such as the marine carbon cycle. Coccolithophores are unicellular, marine algae belonging to the division Haptophyta, which have evolved the ability to produce a composite exoskeleton formed of minute, calcite platelets. These platelets were discovered by T. H. Huxley in a sediment sample from the Atlantic Ocean. In this sample he recognised "...very curious rounded bodies...", which he called coccoliths (Huxley 1868) (Figure 1).

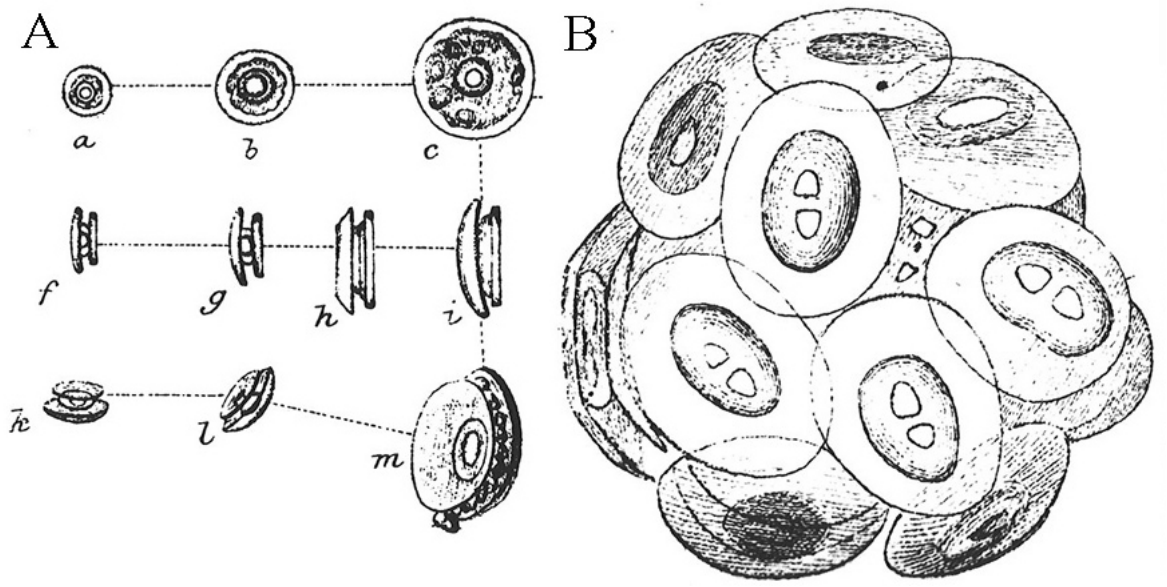

Figure 1. A) The coccoliths reported by T. H. Huxley. Shown are various aspects of single coccoliths from Atlantic mud. B) The coccosphere of T. H. Huxley. Reproduced from Pautard (1970)

Huxley considered coccoliths as of inorganic origin, because they are readily dissolvable in dilute acids. The first living coccolithophores were described by Wallich (1877). Coccolithophores first appear in the Late Triassic, ca. 220 Ma before present (Bown 1998) and as the first abundant calcareous microplankton they profoundly changed global

sedimentation through the formation of calcareous oceanic sediments. Together with planktonic foraminifera they still form the bulk of calcareous deep sea deposits (Baumann et al. 2004) and so play a major role in oceanic biogeochemical cycles (Rost and Riebesell 2004).

One of the most distinct features of coccolithophore biology is their haplo-diplontic life cycle. It is now clear that the typical life cycle of coccolithophores consists of independent haploid and diploid phases, both of which are capable of indefinite asexual binary fission, i.e. mitosis (Billard 1994). Both phases usually produce coccoliths but via distinctly different biomineralisation processes resulting in consistent structural differences (Geisen et al. 2002; Young et al. 1999). Two very different types of coccoliths occur: heterococcoliths, which consist of radial arrays of complex crystal units, and holococcoliths, which consist of large numbers of tiny morphologically simple crystallites. The diploid phase is characterised by heterococcoliths, whereas the haploid phase is characterized by holococcoliths (Figure 2).

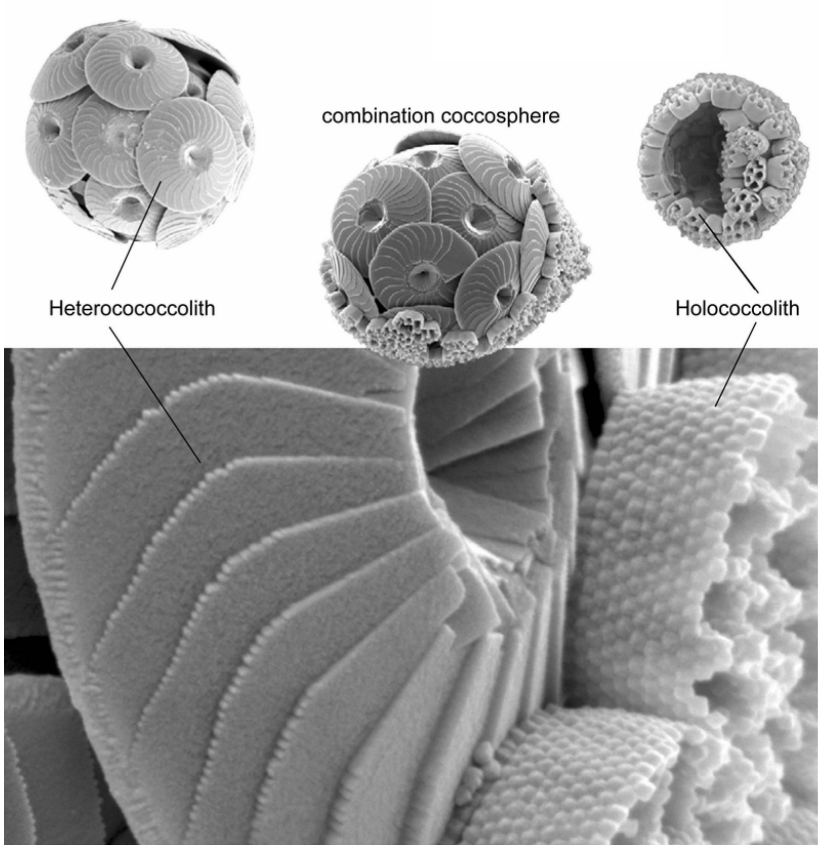

Figure 2. Coccolithophore life cycle phases. SEM images show coccospheres of the two phases of *Calcidiscus leptoporus* ssp. *quadriperforatus* and a combination coccosphere with both coccolith types. Reproduced from Young and Henriksen (2003)

It should be mentioned that, despite the existence of several hypothesis, the function of coccolith formation and coccoliths is unknown; for overview see Young (1994). It was suggested that coccoliths might act as light gatherers (Gartner and Bukry 1969) or reflect ultraviolet light (Braarud et al. 1952). Moreover, the coccosphere might protect the plasmamembrane (Manton 1986) and enclose a small volume of water, which can be controlled by the cell with respect to chemical composition (Sikes and Wilbur 1982).

Besides their function, another basic question concerning coccoliths is: how are they produced? Almost nothing is known about holococcolith formation, but the scarce observations at hand lead to the conclusion that calcification occurs outside the plasmamembrane (Rowson et al. 1986). Nevertheless, holococcolith formation is a highly regulated process with the final morphology being a product of biologically controlled mineralization. Biological control of the mineralization process is comparatively well understood in the heterococcolith phase and will be described in the following section.

1.2 Calcification of coccolithophores

The calcium carbonate polymorph of coccoliths is calcite (Young et al. 1991). This polymorph is comprised of identical rhomb units in a repeated pattern in three dimensions. The typical calcite rhomb is readily recognisable in inorganically precipitated calcite, whereas a coccolith looks noticeably different (Figure 3). However, crystallographic interpretation of atomic force microscopy images allows for relating a coccolith-crystal to the calcite rhomb indicating that inorganically stable calcite faces and directions are utilized in coccolith design (Figure 3 C).

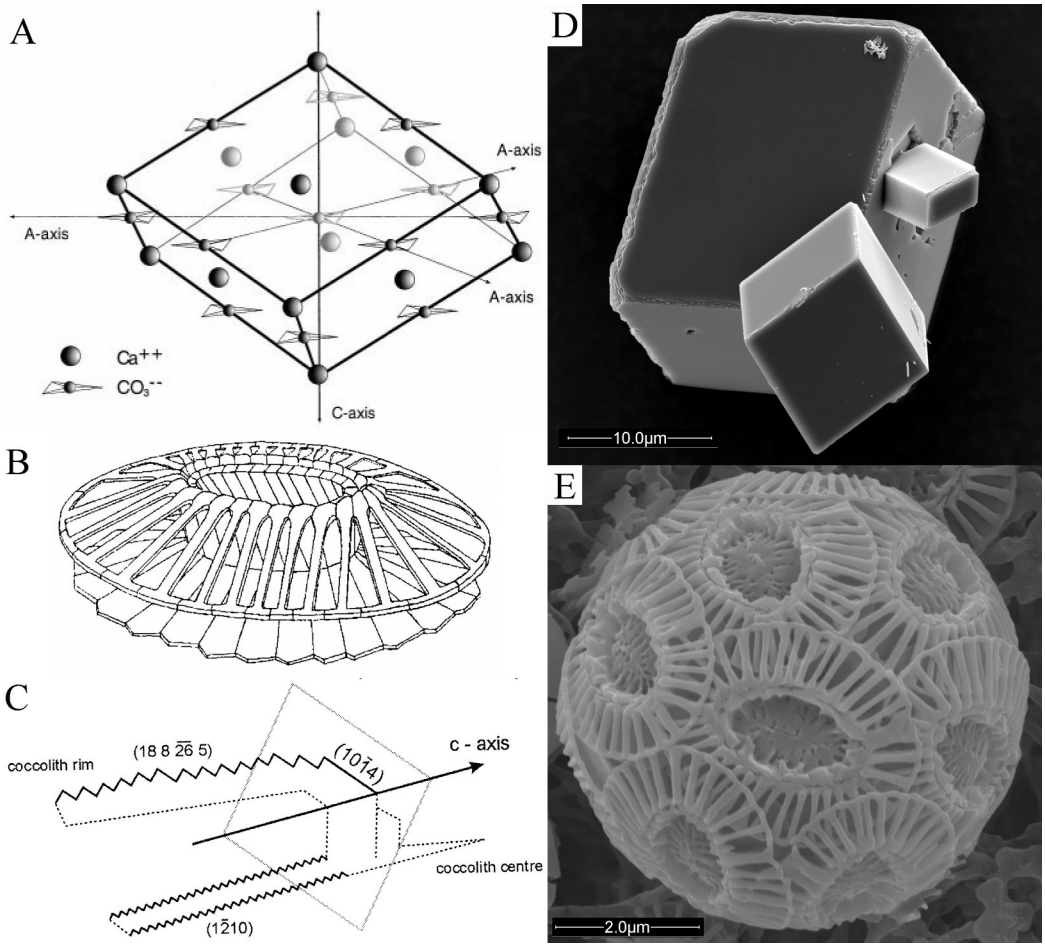

Figure 3. A) Sketch of a three dimensional array of a very small portion of $CaCO_3$ atoms in their spatial arrangement in calcite; reproduced from Young et al. (1999). B) Drawing of a coccolith of *Emiliania huxleyi*; reproduced from Young et al. (1999) C) Schematic cross-section through an *E. huxleyi* crystal showing its crystallographic relation to the calcite rhomb (grey); reproduced from Henriksen et al. (2004a) D) SEM image of inorganically precipitated calcite (courtesy of Gernot Nehrke, AWI-Bremerhaven) E) SEM image of *E. huxleyi*

The question arises how a coccolithophore cell accomplishes this most elaborately regulated shaping of a crystalline material. In this respect the regulation of three processes proves essential: 1. manipulating the chemical composition of the fluid from which the coccolith is precipitated 2. nucleation of the crystals 3. crystal growth.

Coccolith formation can be observed using transmission electron microscope analysis of microtome sections through cells. It was shown that calcite precipitation of heterococcoliths takes place inside a specialised vesicle, the coccolith vesicle (Klaveness 1972; Manton and Leedale 1969; Outka and Williams 1971). The coccolith vesicle is derived from the Golgi body (Westbroek et al. 1984; Young et al. 1999). Hence the site of calcification is separated from the surrounding seawater by at least two membranes. The precipitated calcite, i.e. the coccolith, is transported to the cell surface, extruded via exocytosis and finally becomes part of the coccosphere, which is the shell of a coccolithophore composed of interlocking platelets. This process was first described by Dixon more than a century ago (Dixon 1900).

Inside the coccolith vesicle coccolith production commences with the formation of an organic scale, called the base plate (Marsh 1999; Westbroek et al. 1984). Nucleation of calcite crystallites is located on the rim of this base plate where a complete closed ring of small rhomb-shaped crystallites (the proto-coccolith ring) develops. The proto-coccolith ring consists of alternating crystals having approximately radial (R-units) and vertical (V-units) ***c***-axis orientations. The V/R nucleation pattern is the most fundamental feature of heterococcolith formation (Young et al. 1992; Young et al. 1999). In terms of morphogenesis it is important that nucleation controls total crystallographic orientation, i.e. not simply ***c***-axis orientation but also ***a***-axis orientation (see also Figure 3 A) (Davis et al. 1995; Henriksen et al. 2003; Mann and Sparks 1988). As the rhomb shaped crystallites of the proto-coccolith ring grow, they expand outwards and upwards from the centre forming crystals of very complex shapes.

The regulation of crystal growth is to a considerable extent imparted by the interaction of the crystal surface with organic growth modifiers. The prime organic molecules involved are complex acidic polysaccharides, which were isolated from different coccolithophore species (Fichtinger-Schepman et al. 1981; Marsh 2000; Ozaki et al. 2001). Characterisation and localisation of these polysaccharides revealed species specific differences. Three types of polysaccharides were isolated from *P. carterae,* PS1, PS2 and PS3 (Marsh 2000). PS1 and PS2 bind calcium, form coccolithosomes (complexes of calcium ions and acidic polysaccharides) and are involved in calcium transport inside the cell. In addition PS2

probably plays an important role in nucleation of the proto-coccolith ring (Marsh and Dickinson 1997). PS3 is located between the crystals and the vesicle membrane and its function is believed to be shape regulatory. In *E. huxleyi* on the other hand only one type of polysaccharide, CAP, could be detected (Borman et al. 1986; Fichtinger-Schepman et al. 1981), which has the ability to inhibit calcite crystallisation (Borman et al. 1982) and to influence crystal morphology (Didymus et al. 1993). The interaction of CAP with the calcite surface was analysed by means of atomic force microscopy and it was shown how CAP can regulate crystal morphology by site-specific attachment to crystallographic steps (Henriksen et al. 2004a). After exocytosis of the coccolith, a polysaccharide-coating remains on its surface, which protects crystal faces from etching (Henriksen et al. 2004b).

In addition to this biochemical mean of crystal growth control the coccolithophore cell exerts a mechanical control. Cytological sections show that during growth the vesicle membrane stays in close contact with the crystals. Therewith the vesicle membrane could mould the growing coccolith, causing the crystals to grow in certain directions and inhibiting growth in others (Young et al. 1999). It is to date an unresolved question why the vesicle membrane adopts such a rigid shape. One possibility is that the cytoskeleton exerts the necessary force for a mechanical control of crystal growth.

Since coccolith formation takes place inside a specialised vesicle, i.e. completely isolated from other cellular processes the composition of the vesicle fluid can be optimized for calcite precipitation. Membrane transport proteins, i.e. ion channels and ion pumps, are the prime tools to control chemical composition of a cellular compartment. A calcium stimulated ATPase was found in the coccolith vesicle membrane of *P. carterae* (Kwon and Gonzalez 1994), which was subsequently discovered to bear enzymatic similarities to higher plant proton pumps of the V-type (Araki and Gonzalez 1998). Cloning of a gene that is homologous to the 16-kDa subunit c of the proton-pumping V-ATPase of higher plants and localisation of the encoded protein on the coccolith vesicle strongly suggests the existence of a proton pump in the coccolith vesicle membrane (Corstjens et al. 2001). In terms of membrane transporters nothing is known about calcification related inorganic carbon transport, but physiological research suggests that bicarbonate is the primary external carbon source for calcite precipitation in *E. huxleyi* (Paasche 2002). Intracellular ion concentrations provide indirect information about membrane transport systems. Values for cytosolic pH of approx. 7.0 were reported, which is close to values typically found in other eukaryotic cells (Anning et al. 1996; Dixon et al. 1989; Nimer et al. 1994). Anning et al. (1996) provided the one and only measurements of coccolith vesicle lumen pH. Using the fluorescent dye SNARF they

measured values ranging from 6.8 to 8.3. This is a considerably broad range and further research is clearly warranted to establish a better constraint value. Calcium concentrations in the coccolith vesicle, on the other hand, were not measured due to experimental difficulties in dye loading (Anning et al. 1996).

Cellular transport of calcium ions is still a matter of debate. Up to now endocytosis of seawater was not observed and it was concluded that transmembrane transport is the pathway by which calcium enters the cell (Berry et al. 2002). Patch clamp measurements using *C. pelagicus* suggest that a calcium selective ion channel resides in the plasmamembrane (Taylor and Brownlee 2003). Once inside the cell calcium has to be removed immediately from the cytoplasm, which is a compartment with a very low calcium concentration of approx. 0.1 µmol L^{-1} (Brownlee et al. 1995). Cytological observations indicate species specific differences in intracellular calcium transport related to coccolith formation (Figure 4).

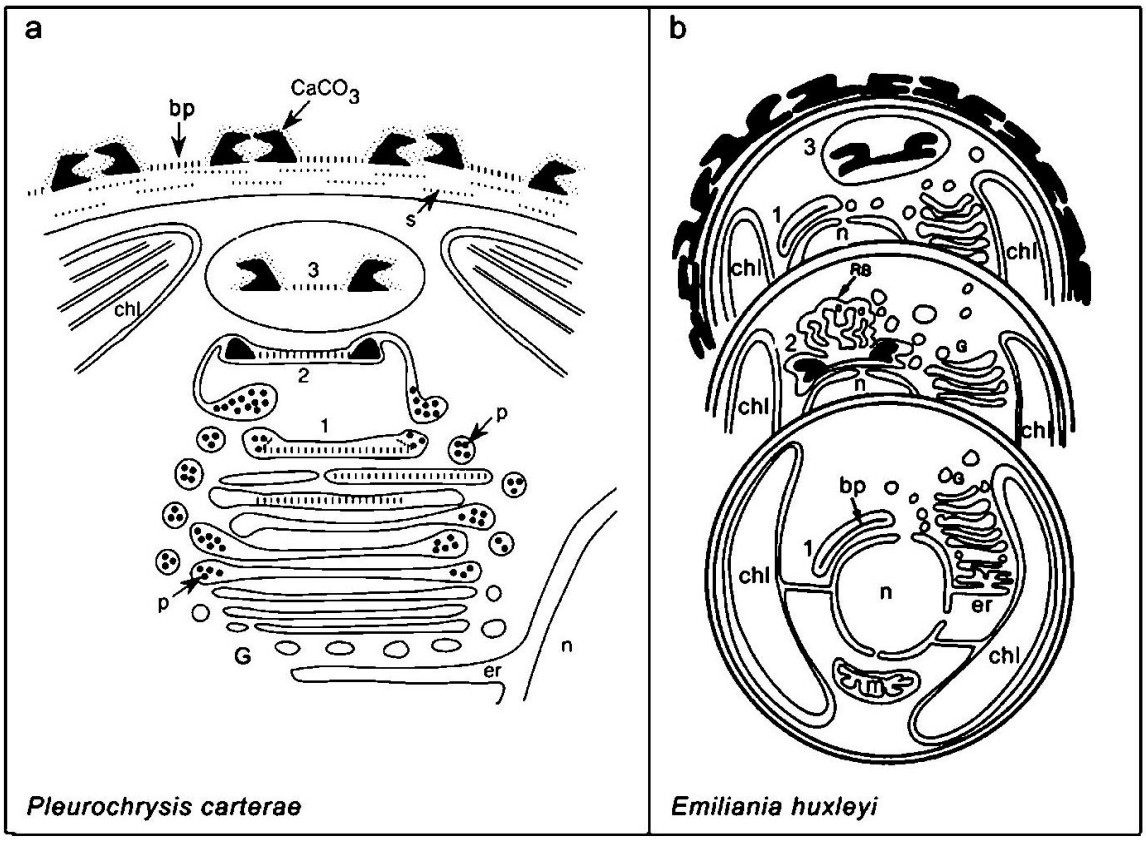

Figure 4. Intracellular pathway of coccolith formation in (a) *P. carterae* and (b) *E. huxleyi*. The coccolith vesicle is shown before (1) during (2) and after (3) mineral deposition. Polysaccharide and calcium containing particles (p), reticular body (RB), base plates (bp), chloroplasts (chl), Golgi stacks (G), endoplasmic reticulum (er), nuclei (n), mitochondrion (m), unmineralized scales (s). Reproduced from Marsh (2003)

In *Pleurochrysis carterae* large numbers of 20-nm particles known as coccolithosomes (Outka and Williams 1971), which are complexes of calcium ions and acidic polysaccharides (Marsh 1994), are formed in the Golgi-body and subsequently transported to the coccolith

vesicle via small vesicles. Hence calcium uptake from the cytosol maybe proceeds via calcium ATPases in the Golgi-membrane. In *Emiliania huxleyi* no coccolithosomes could be observed, but a labyrinthine membrane system known as the reticular body develops at the distal surface of the coccolith vesicle (Klaveness 1972). The reticular body has a large surface area to volume ratio and therewith is perfectly suited to the rapid transport of large quantities of calcium ions (Marsh 2000). To summarize, transmembrane transport of ions is an important feature of coccolithophore calcification, and its potential influence on coccolith trace element and stable isotope ratios has to be taken into account.

1.3 Biogenic calcium carbonate based proxies – a biased selection

It has been recognised for decades that the incorporation of trace elements and stable isotopes into biogenic calcium carbonate depends on environmental conditions. The isotopic composition and the trace metal to calcium ratio of biogenic calcium carbonate was therefore used to reconstruct environmental parameters, e. g. temperature. These are the target parameters. The proxy is a measurable descriptor, e.g. isotopic composition, that stands in for the target variable, which is unobservable. In order to transform measured proxy values into target values, a transforming algorithm has to be established by calibration. The calibration of the proxy relies on empirical relationships, which are determined in laboratory experiments or field studies.

The sea surface temperature (SST) provides the most important information for modelling of climate and ocean circulation and is therefore the most wanted target in paleoceanography. Unsurprisingly, a number of different proxies are used to reconstruct SST. Following pioneering theoretical (Urey 1947) and experimental (McCrea 1950) work, $\delta^{18}O$ became the first extensively applied SST-proxy (Emiliani 1955). The $\delta^{18}O$ / temperature relationship of foraminifera is well characterized (for review see (Bemis et al. 1998)) by calibrations obtained from culture experiments (Erez and Luz 1983) and core-top studies (Shackleton 1974). The influence of the oxygen isotopic composition of seawater on $\delta^{18}O$ values of biogenic calcite was first highlighted in a down-core study of benthic foraminifera (Shackleton 1967). The mean $\delta^{18}O$ of seawater is determined by the amount of continental ice (Dansgaard and Tauber 1969) but local variations can also occur due to differences in evaporation – precipitation ratio (Dansgaard 1961). A further caveat is the influence of seawater carbonate chemistry on $\delta^{18}O$ of planktonic foraminiferal shells (Bijma et al. 1999; Spero et al. 1997).

Oxygen isotopic variations of Pleistocene polyspecific coccolith-dominated sediment fine fractions co-vary with those measured in planktonic foraminifera, but the coccolith fraction isotopic composition mostly is offset from equilibrium values, e.g. Anderson and Cole (1975). Culture experiments show that oxygen isotope fractionation of coccolithophores correlates with temperature (Dudley et al. 1986) and that this apparent temperature effect is not caused by underlying growth rate changes (Ziveri et al. 2003). However, the especially wide range of vital effects in oxygen isotopic composition (Dudley et al. 1986; Ziveri et al. 2003) may hamper the application of coccolith $\delta^{18}O$ over major paleoceanographic events where there is significant change in the nannofossil assemblage (Bralower 2002). Oxygen isotopes in reef corals precipitating aragonite were also shown to respond to temperature (Weber and Woodhead 1972), but vital effects can offset this correlation to varying degrees (McConnaughey 1989a; McConnaughey 1989b). In a recent study such vital effects were interpreted as growth induced pH changes (Rollion-Bard et al. 2003).

Although subject to the influence of salinity changes the Mg/Ca ratio of foraminiferal calcite can be used as a SST proxy (Nürnberg et al. 1996) and has become an extensively studied one since the mid 1990s (Rosenthal et al. 1997). It has been known for decades that the Mg/Ca ratio of foraminiferal shells is influenced by partial dissolution (Lorens et al. 1977), which has to be taken into account when applying the proxy (Rosenthal and Lohmann 2002). Furthermore species specific effects and pH changes are of a considerable effect (Lea et al. 1999). Experiments with cultured coccolithophores suggest that temperature may be an important control on coccolith magnesium partitioning, but the low magnesium content of coccolith calcite (0.1 mmol mol^{-1}) combined with the difficulties in removing magnesium-rich organic phases probably precludes the application of the coccolith Mg/Ca ratio as SST proxy (Stoll et al. 2001). It was suggested that the Mg/Ca ratio of corals could be a promising SST proxy (Mitsuguchi et al. 1996), but variability not related to temperature was discovered shortly after the initial study (Fallon et al. 1999). The Sr/Ca ratio of coral skeletons was used to reconstruct SST (Beck et al. 1992), but here as well a caveat appeared, this time in the form of growth rate and symbiont activity (Cohen et al. 2002; de Villiers et al. 1994). This brief overview of SST proxies and the problems associated with their application illustrates the need for further independent SST proxies.

The latest achievement in SST proxy development is the possibility of using calcium isotopes. A temperature dependent calcium isotopic fractionation in foraminiferal calcite was suggested (Zhu and MacDougall 1998) and subsequently confirmed (Nägler et al. 2000). The last named study provides a temperature versus $\delta^{44}Ca$ calibration curve of the planktonic

foraminifer species *Globigerinoides sacculifer*. This introduced the $\delta^{44}Ca$ signal as a potential new SST proxy. In a case study using *G. sacculifer*, in which the common proxies $\delta^{18}O$ and Mg/Ca were substantially altered by other climatic factors, mainly salinity and chemical composition of the seawater, the applicability of $\delta^{44}Ca$ as a SST proxy was further reinforced (Gussone et al. 2004). However, the slope of the calibration curve of a second foraminifer, *Orbulina universa*, is by an order of magnitude smaller than the slope obtained from *G. sacculifer* (Gussone et al. 2003). Whereas the *G. sacculifer* slope allows for a resolution of roughly 0.5 °C, the *O. universa* slope allows for a resolution of only 5 °C and is therewith not suited for paleothermometry. Obviously species composition of the sediment is of great importance to the question whether foraminiferal calcium isotopes can be applied as SST proxy. Coccolithophores also formed a huge sedimentary archive that might as well be suited for calcium isotope based SST reconstructions. It was therefore one of the objectives of this study to take a first look at coccolith calcium isotope composition and its temperature dependence.

The most developed coccolith-based proxy in terms of elemental chemistry is the Sr/Ca ratio. In a pioneering study core-top samples from across a persistent productivity gradient were analysed (Stoll and Schrag 2000). In these samples high Sr/Ca ratios coincided with high surface productivity and high nutrient concentrations. It was concluded that the Sr/Ca ratio might serve as a proxy for coccolithophore growth and calcification rate. These initial findings were confirmed later on (Stoll et al. 2002c). Monospecific sediment samples revealed higher amplitudes of Sr/Ca variations in larger coccolithophores, e.g. *C. pelagicus*, than in smaller ones, e.g. *F. profunda*. This was interpreted as an ecological effect: *F. profunda* for instance lives in deeper waters with more constant nutrient levels and is therewith less likely to display nutrient related Sr/Ca variations. Two sets of laboratory experiments using the prominent species *Emiliania huxleyi* clearly support the notion that nutrient related growth rate changes result in Sr/Ca variability. Coccolith Sr/Ca ratios increase notably with growth rate, if growth is regulated by nitrate limitation (Rickaby et al. 2002), but only a modest increase is observed if growth is regulated by light intensity (Stoll et al. 2002b). Hence the factor limiting growth most likely is the agent that causes Sr/Ca ratios to vary and not the growth rate itself.

Besides temperature (Stoll et al. 2002a; Stoll et al. 2002b) the Sr/Ca ratio of seawater is the most important secondary influence affecting coccolith Sr/Ca ratios. The Sr/Ca of seawater is likely to have changed through time on both short and long timescales (Lear et al. 2003; Stoll and Schrag 1998). Therefore, it is mandatory to consider its influence on the Sr/Ca

of biogenic calcium carbonates. Recent studies investigating coccolith dominated Cenozoic and Cretaceous carbonates consequently account for changing seawater Sr/Ca (Billups et al. 2004; Stoll and Schrag 2001). Calculations in these studies rely on the premise that the strontium exchange coefficient is unaffected by the seawater Sr/Ca ratio. It is not known whether this premise holds true. It was therefore one objective of this study to obtain an empirical relationship between seawater Sr/Ca and coccolith Sr/Ca in *Emiliania huxleyi*.

Publications 1, 2 and 3 of this study deal with the assessment of coccolith-based proxies. In a second part (publication 4) the effects of changing carbonate chemistry on two prolific coccolithophore species are described. Since anthropogenic CO_2 emissions substantially change seawater carbonate chemistry (Sabine et al. 2004), this issue is of considerable interest.

1.4 The seawater carbonate system

To understand the consequences anthropogenic CO_2 emissions might have for marine calcifiers, some background in seawater carbonate chemistry is necessary. Gaseous CO_2 dissolves in seawater and according to Henry's law the concentration of aqueous CO_2 [CO_2] is proportional to the partial pressure of CO_2 in the atmosphere pCO_2:

$$[CO_2] = \alpha \times pCO_2 \quad (1)$$

where α is the temperature- and salinity dependent solubility coefficient. In contrast to other gases like oxygen and nitrogen CO_2 chemically reacts with water, so that the concentration of dissolved inorganic carbon (DIC) is considerably higher than that of dissolved CO_2. Dissolved CO_2 is hydrated to carbonic acid (H_2CO_3), which subsequently dissociates to bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and protons (H^+):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2\,H^+ \quad (2)$$

Since the concentration of carbonic acid [H_2CO_3] in seawater is very low, the sum of aqueous [CO_2] and [H_2CO_3] will be approximated as [CO_2] hereafter. The sum of all dissolved inorganic carbon species is defined as:

$$DIC = [CO_2] + [HCO_3^-] + [CO_3^{2-}] \quad (3)$$

The proportion of carbon species varies as a function of pH (Figure 5). Typical present day seawater has a pH of 8.2 and bicarbonate contributes ca. 90% of DIC, i.e. $[HCO_3^-]$ = 2000 µmol L^{-1}, whereas CO_2 accounts for only 1% (~13µmol L^{-1}). With increasing pH the chemical equilibrium shifts to a lower proportion of CO_2 and a higher proportion of CO_3^{2-} and vice versa. It should be noted that in natural seawater the relative concentrations of the dissolved carbon species determine the pH and not vice versa.

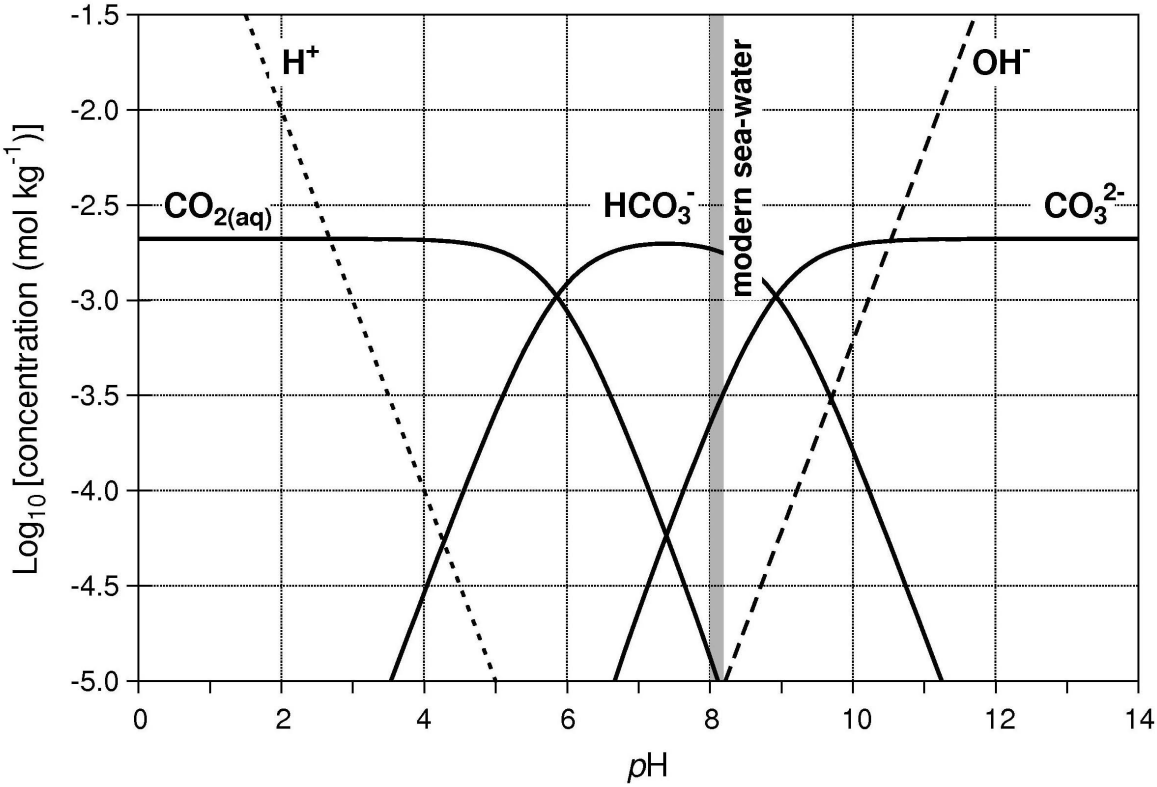

Figure 5. The concentrations of the dissolved carbon species as a function of pH. Reproduced from Ridgwell and Zeebe (2005)

A further measurable and conservative parameter of the carbonate system is the so called total alkalinity (TA), which is defined as TA:

$$\begin{aligned} TA = {} & [HCO_3^-] + 2\,[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] \\ & + [HPO_4^{2-}] + 2\,[PO_4^{3-}] + [H_3SiO_4^-] + [NH_3] + [HS^-] \\ & - [H^+]_F - [HSO_4^-] - [HF] - [H_3PO_4] \end{aligned} \qquad (4)$$

where $[H^+]_F$ is the free concentration of hydrogen ion. The most important summands of TA are HCO_3^- and CO_3^{2-}. TA can be measured by the titration of seawater with a strong acid; see Dickson (1981). For a discussion of TA compare also Zeebe and Wolf-Gadrow (2001).

The parameters of the carbonate system, $[CO_2]$, $[HCO_3^-]$, $[CO_3^{2-}]$, DIC, TA and pH, are interdependent and thus one cannot be altered without affecting at least one other. During

precipitation of calcium carbonate DIC and TA are reduced. Due to its double charge CO_3^{2-} contributes twice as much to TA than to DIC, the consequence being a shift of the chemical equilibrium towards higher [CO_2] and lower pH. Dissolution of calcium carbonate represents the reverse reaction, increasing TA, DIC and pH and decreasing [CO_2]. Whether calcium carbonate precipitates or dissolves depends on the saturation state Ω of the solution:

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}} \quad (5)$$

where [Ca^{2+}] is the calcium concentration and [CO_3^{2-}] the carbonate concentration in the solution and *Ksp* is the salinity, temperature and pressure dependent solubility constant of the respective calcium carbonate polymorph (calcite, aragonite or vaterite). Precipitation of calcium carbonate is thermodynamically favourable when Ω is greater than unity, whereas dissolution can occur at Ω<1. For further information on the carbonate system the reader is referred to Zeebe and Wolf-Gadrow (2001).

The carbonate system of seawater can be manipulated in various ways. In an open system changes in atmospheric pCO_2 cause changes in [CO_2], pH and DIC, while TA remains constant. The addition of HCl or NaOH to a closed system affects [CO_2], pH, and TA, while DIC remains constant. The latter procedure was applied in the present study.

1.5 Marine calcifiers and seawater carbonate chemistry

Since the mid 19th century fossil fuel burning has released more than 250 billion tons of carbon in the form of CO_2 into the atmosphere (Houghton et al. 2001). Unabated CO_2 emissions will cause a doubling in surface ocean pCO_2 levels over their pre-industrial values by the middle of this century, accompanied by a decrease in surface ocean pH three times greater than that experienced during the transition from glacial to interglacial periods. This rapid change in seawater carbonate chemistry may directly impact marine calcifying organisms. A number of recent studies have given rise to the notion that in benthic as well as planktonic organisms calcification is reduced due to seawater acidification, see Feely et al. (2004).

Laboratory experiments investigating the effect of HCl-induced seawater acidification on calcification of a scleractinian coral showed a strong reduction in calcification rate when pCO_2 is increased to 3980 µatm and $\Omega_{aragonite}$ is decreased to 0.28 (Marubini and Atkinson

1999). It should be noted that in this species a reduction in calcification rate due to seawater acidification occurs even when seawater remains supersaturated with respect to aragonite (Marubini et al. 2001). The observation that marine calcifiers are affected by changes in saturation state well above unity excludes dissolution of the shell as a possible explanation for observed adverse effects. The zooxanthellate coral *Stylophora pistillata* increases calcification rate when aragonite saturation state increases from unity to 3.9, i.e. close to the typical present saturation state of tropical seawater (Gattuso et al. 1998). A further increase in saturation state does not affect calcification rate. *Stylophora pistillata* is the only species that was shown to respond to calcium-induced (Gattuso et al. 1998) as well as pCO_2-induced (Reynaud et al. 2003) changes in saturation state. This suggests that indeed the aragonite saturation state is the parameter causing the changes in calcification rate and not some other parameter of the carbonate system. A similar result was obtained in a coral reef mesocosm study, where overall calcification of reef organisms was sensitive to changes in calcium concentration and carbonate concentration (Langdon et al. 2000). The calcification sensitivity of a coral community to changes in carbonate chemistry could also be observed when bubbling with air of different pCO_2 (Leclercq et al. 2000; Leclercq et al. 2002). Results from laboratory experiments revealed that several species of coralline red algae show a decrease in calcification rate with decreasing aragonite saturation state over a range in saturation state from 1 to 4 (Agegian 1985; Borowitzka 1981; Gao et al. 1993). Calculations based on the response of selected coralline algae, a coral species and a marine mesocosm predict that projected changes in oceanic aragonite saturation state will decrease aragonite precipitation of reef communities by 14 to 30 % by the middle of this century (Kleypas et al. 1999).

Unicellular calcite producers were also shown to be sensitive to changes in carbonate chemistry. In laboratory experiments cultured specimens of the planktonic foraminifera *Orbulina universa* and *Globigerinoides sacculifer* displayed decreasing shell weight in response to decreasing carbonate concentration (Bijma et al. 1999; Bijma et al. 2002). In *O. universa* the slope of the shell weight decrease is much steeper below extant carbonate concentrations than above. In comparison to *Orbulina universa* the shell weight of *G. sacculifer* shows a weaker dependence on carbonate concentration. In the case of *O. universa* different experimental procedures to manipulate the carbonate concentration were applied, but similar results were obtained (Bijma et al. 1999). It was concluded that the parameter causing shell weight to vary is not pH or CO_2 concentration, but carbonate concentration. However, since calcite saturation state was not altered by manipulating calcium concentration it remains an open question whether carbonate concentration or calcite saturation state is the agent

causing shell weight to vary. The positive correlation between shell weight and carbonate concentration was also observed in *Globigerina bulloides* shells from a sediment core (Barker and Elderfield 2002). In this study shell weight of *G. bulloides* correlates with known changes in glacial-interglacial carbonate chemistry of the past 50,000 years. Results from laboratory experiments using the benthic foraminifera *Amphistegina lobifera* and *Amphisorus hemprichii* showed a correlation between calcification rate and pH (Ter Kuile et al. 1989). In *A. hemprichii* calcification rate increases with increasing pH over a range from 7.5 to 9.5, whereas in *A. lobifera* calcification rate increases from 7.5 to 8.2, remains constant between 8.2 and 9.0 and decreases from 9.0 to 9.5.

Besides foraminifera, coccolithophores are the prime open ocean calcite producers and apparently calcification and coccolith morphogenesis of these algae are hampered by acidification of seawater. In laboratory batch culture experiments using *Emiliania huxleyi* and *Gephyrocapsa oceanica* a linear decrease in calcification rates with decreasing pH was observed, the slope of the linear regression being steeper for *G. oceanica* (Riebesell et al. 2000; Zondervan et al. 2001). The results for *E. huxleyi* were confirmed in a subsequent study by means of continuous culturing and it was shown that the decreased calcification can also be observed under nitrate limitation (Sciandra et al. 2003). However, the dependence of calcification rate on carbonate chemistry in *E. huxleyi* was not observed at low light intensities (Zondervan et al. 2002). The adverse effect of seawater acidification was also evident in a coccolithophore dominated North Pacific phytoplankton assemblage (Riebesell et al. 2000) and in a Norwegian mesocosm study (Delille et al. 2005). The mesocosm bloom was dominated by *E. huxleyi* and calcification rate increased when pCO_2 decreased from ca. 710 ppm to ca. 410 ppm, whereas no change in calcification rate occurred in response to a further decrease in pCO_2 to ca. 190 ppm (Delille et al. 2005). Since research so far has focused on only two closely related species further experiments are needed to assess the question whether there is a typical response of coccolithophores as a group. It was therefore one objective of this study to gain information about the response of *Coccolithus pelagicus* and *Calcidiscus leptoporus*, two of the most important calcite producers (Baumann et al. 2004; Broerse et al. 2000), which are moreover clearly separated from *E. huxleyi* and *G. oceanica* in terms of phylogenetic relationship (Sáez et al. 2003).

1.6 Outline of the thesis

This thesis investigates the response of two prominent coccolithophores, *Coccolithus pelagicus* and *Calcidiscus leptoporus*, to varying carbonate chemistry of seawater with special emphasis on calcification. The second issue of the present work is the assessment of coccolith-based proxies, in particular the Sr/Ca ratio and calcium isotope composition. This part focuses on *Emiliania huxleyi*.

Publication 1 establishes an empirical relationship between coccolith Sr/Ca ratios and seawater Sr/Ca ratios in *Emiliania huxleyi*. A conceptual model is presented, which reconciles the measured strontium partitioning coefficient with values obtained from inorganic precipitation experiments.
Publication 2 reports calcium isotope fractionation and its temperature dependence of selected coccolithophore species, namely *Helicosphaera carteri*, *Umbilicosphaera foliosa*, *Calcidiscus leptoporus* and *Syracosphaera pulchra*. The aim of this study is to assess species specific differences in calcium isotope fractionation.
Publication 3 investigates the temperature dependence of calcium isotope fractionation in *Emiliania huxleyi* in more detail. The primary aim of this study is to answer the question whether temperature itself or concomitant calcification and growth rate changes affect calcium isotope fractionation.
Publication 4 reports calcification rates and coccolith morphology of cultured *Calcidiscus leptoporus* and *Coccolithus pelagicus* in response to altered carbonate chemistry of seawater. The results are compared to morphological data from the sedimentary record and the potential for adaptation of coccolithophores to long-term changes in CO_2 levels is discussed.

In a concluding discussion main results of this study are summarized and discussed with respect to the possible consequences of anthropogenic CO_2 emissions on coccolithophores, the paleoceanographic potential of the coccolith sedimentary archive and the impact of calcification mechanisms on these issues.

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2 Publications

2.1 List of publications

This doctoral thesis is based on the following publications

I Langer, G., Gussone, N., Nehrke, G., Riebesell, U., Eisenhauer, A., Kuhnert, H., Rost, B., Trimborn, S., Thoms, S. 2005. Coccolith strontium to calcium ratios in *Emiliania huxleyi*: The dependence on seawater strontium and calcium concentrations. *Limnology and Oceanography*, Accepted

II Gussone, N., Langer, G., Geisen, M., Steel, B., Eisenhauer, A., Riebesell, U. Temperature dependent calcium isotope fractionation in coccoliths of cultured *Calcidiscus leptoporus, Helicosphaera carteri, Syracosphaera pulchra* and *Umbilicosphaera foliosa,* to be submitted to *Earth and Planetary Science Letters*

III Langer, G., Gussone, N., Nehrke, G., Riebesell, U., Eisenhauer, A., Thoms, S. Calcium isotope fractionation during coccolith formation in *Emiliania huxleyi*. submitted to *Limnology and Oceanography*

IV Langer, G., Geisen, M., Baumann, K.-H., Kläs, J., Riebesell, U., Thoms, S., Young, J. R. Coccolithophore calcification adapting to changes in oceanic CO_2 levels, submitted to *Proceedings of the National Academy of Sciences of the USA*

2.2 Declaration on the contribution of each publication

Publikation I

Die Laborexperimente wurden von mir geplant und durchgeführt. Ich habe die Daten ausgewertet und das Manuskript in Zusammenarbeit mit den Koautoren verfasst.

Publikation II

Die Laborexperimente wurden von Markus Geisen geplant und durchgeführt. Ich habe die Daten in Zusammenarbeit mit Nikolaus Gussone ausgewertet und das Manuskript in Zusammenarbeit mit den Koautoren verfasst.

Publikation III

Die Laborexperimente wurden von mir geplant und durchgeführt. Ich habe die Daten in Zusammenarbeit mit Nikolaus Gussone ausgewertet und das Manuskript in Zusammenarbeit mit den Koautoren verfasst.

Publikation IV

Die Laborexperimente wurden in Zusammenarbeit mit Markus Geisen geplant und von mir durchgeführt. Ich habe die Daten ausgewertet und das Manuskript in Zusammenarbeit mit den Koautoren verfasst.

Coccolith strontium to calcium ratios in *Emiliania huxleyi*: The dependence on seawater strontium and calcium concentrations

Gerald Langer, Nikolaus Gussone, Gernot Nehrke, Ulf Riebesell, Anton Eisenhauer, Henning Kuhnert, Björn Rost, Scarlett Trimborn and Silke Thoms

Limnology and Oceanography, accepted

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Abstract

In recent studies the Sr/Ca ratio of coccolithophore calcite was used as a proxy for past coccolithophore growth and calcification rates. Since Sr and Ca concentrations in seawater have not remained constant through time, interpretation of Sr/Ca data from the coccolith dominated sedimentary record requires knowledge about the incorporation of seawater Sr into coccolith calcite during coccolithogenesis. Here we show that Sr/Ca of *Emiliania huxleyi* coccoliths is linearly related to seawater Sr/Ca, meaning that the Sr exchange coefficient does not change with changing seawater Sr/Ca. The exchange coefficient for Sr in this study, 0.39, is close to values presented in the literature and is high compared to values obtained by inorganic precipitation experiments. This suggests a strong effect of cell physiology on biogenic calcite precipitation in coccolithophores. We present a conceptual model, based on the transmembrane transport of Sr and Ca, which explains the offset.

Introduction

The Sr/Ca of calcium carbonate producing organisms was widely used to reconstruct past oceanographic conditions. Coral Sr/Ca, for instance, was used as a temperature proxy (Smith et al. 1979). Sr/Ca of coccolithophore calcite was linked to coccolithophore growth and calcification rates (Stoll and Schrag 2000). It was suggested that despite temperature dependent Sr/Ca partitioning in coccolith calcite (Stoll et al. 2002a; Stoll et al. 2002b) it is possible to infer past coccolithophore productivity from the coccolith fossil record (Billups et al. 2004; Stoll and Schrag 2001).

The Sr/Ca of seawater is likely to have changed through time on both short and long timescales (Lear et al. 2003; Stoll and Schrag 1998). Therefore, it is mandatory to consider its influence on the Sr/Ca of biogenic calcium carbonates. Recent studies investigating coccolith dominated Cenozoic and Cretaceous carbonates consequently account for changing seawater Sr/Ca (Billups et al. 2004; Stoll and Schrag 2001). Calculations in these studies rely on the premise that the exchange coefficient is unaffected by the seawater Sr/Ca ratio. This was shown to be true in inorganically precipitated calcite (Lorens 1981; Tesoriero and Pankow 1996), while nothing is known about coccolithophores in this respect.

Exchange coefficients of coccolith calcite presented in the literature are high compared to values of inorganically precipitated calcite (Stoll et al. 2002b). Although kinetic effects can explain part of this offset (Stoll et al. 2002b) the origin of this discrepancy is yet not fully understood. Often the term “vital effects” is used to account for the fact that numerous biological processes control the precipitation process. Since the bulk of the coccolith is thought to be precipitated by an inorganic crystal growth mechanism (Young et al. 1999), the application of results from inorganic precipitation experiments to coccolithogenesis may provide a useful framework. However, such inferences are doubtful, because precipitation of coccolith calcite takes place within a specialised Golgi-derived vesicle, the so called coccolith-vesicle and is organically modulated (Henriksen et al. 2004; Young et al. 1999). Determination of exchange coefficients for coccoliths is problematic, because the Sr/Ca of seawater is used for calculation although the chemistry of seawater differs from that of the solution within the coccolith vesicle.

Here we present results from batch culture experiments using the dominant coccolithophore *Emiliania huxleyi*, a bloom forming species that occurs world wide in both coastal and open oceanic environments. In these experiments the dependence of coccolith

Sr/Ca on seawater Sr/Ca was examined. Our results are compared to literature data and a conceptual model for transport of calcium and strontium from seawater into the coccolith-vesicle, capable of explaining the observed high values of exchange coefficient, is discussed.

Material and Methods

Monospecific cultures of *Emiliania huxleyi* (strain PML B92/11) were grown in sterile-filtered (0.2 µm) artificial seawater enriched with 100 µmol L^{-1} nitrate and 6.25 µmol L^{-1} phosphate and with trace metals and vitamins according to F/2 (Guillard and Ryther 1962). The detailed composition of the artificial seawater is given in Table 2. Seawater Sr and Ca concentrations were varied to obtain a wide range in Sr/Ca in the growth medium. A 16/8 hour light/dark cycle was applied. Experiments were carried out at constant temperature of 15°C and various photon flux densities (Table 3), which were maintained by growing the cells in an adjustable incubator (Rubarth Apparate GmbH, Germany). Cells were pre-adapted to experimental conditions for approximately 12 generations and grown in dilute batch cultures. Low cell density at harvest ensured that less than 5% dissolved inorganic carbon (DIC) was consumed (i.e. DIC consumed by the cells at the end of experiment). The carbonate system was nearly constant during all experiments and did not vary significantly between different flasks. CO_2 concentrations [CO_2] were adjusted to an average value of 16.4 µmol L^{-1} through the addition of NaOH (1 mol L^{-1}). The cells were grown in duplicate in HCl-rinsed polycarbonate flasks.

Samples for alkalinity measurements were filtered (approx. 0.6 µm), poisoned with 1 ml of a $HgCl_2$ solution (35 g L^{-1}) and stored in 300 ml borosilicate flasks at 0°C. DIC samples were sterile-filtered (0.2 µm) and stored in 13 ml borosilicate flasks free of air bubbles at 0°C prior to analysis. Total alkalinity was calculated from linear Gran plots (Gran 1952) after duplicate potentiometric titration (Bradshaw et al. 1981; Brewer et al. 1986) and DIC was measured photometrically (Stoll et al. 2001) in triplicates. The carbonate system was calculated from temperature, salinity, the concentrations of DIC, total alkalinity and phosphate, using the program CO_2sys (Lewis and Wallace 1998). Equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987) were chosen. Samples for determination of total particulate carbon (TPC) and particulate organic carbon (POC) were filtered on pre-combusted (12 hours, 500°C) GF/F-filters (approx. 0.6 µm) and stored at -20°C. Prior to analysis, the POC filters were fumed for two hours with a saturated HCl solution to remove all inorganic carbon. TPC and POC were subsequently measured on a Carlo Erba® NA-1500 Analyzer. Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. For determination of cell density samples were taken daily

or every other day and counted immediately after sampling using a Coulter "Multisizer III". Cell growth rate (μ_{cocco}, unit: d^{-1}) was calculated as:

$$\mu_{cocco} = (\ln c_1 - \ln c_0) / \Delta t \quad (1)$$

where c_0 and c_1 denote the cell densities at the beginning and the end of experiment, and Δt represents the duration of incubation in days.

Calcification rate (P, pg calcite $cell^{-1}$ d^{-1}) was calculated according to

$$P = \mu_{cocco} \times (\text{cellular calcite content}) \quad (2)$$

Samples for Sr/Ca measurements were centrifuged in 50 ml Falcon® tubes, rinsed with Sr-free artificial seawater, centrifuged again in 1 ml tubes in order to remove seawater, thereafter dried at 60°C for 48 hours and stored at room temperature.

Sample preparation and determination of Sr/Ca ratios: Coccolith samples were transferred into acid-cleaned 1.5 ml PP (polypropylene) PCR-tubes. For removal of organic matter, the samples were bleached in a 10% dilution of concentrated NaOCl-solution for about 24 h. Afterwards the solution was removed and the samples were covered with ultrapure H_2O (pH 8-9, adjusted by the addition of NH_4OH, to prevent partial dissolution of the calcite samples), ultrasonicated for 2 minutes, and then centrifuged. The water was removed and new H_2O was added. This washing procedure was repeated 6 times. The cleaned coccoliths were dissolved in 2.5 mol L^{-1} HCl and evaporated. The samples were recovered in a HNO_3-H_2O_2 mixture and evaporated again. The samples were recovered in 2% HNO_3 and internal Sc and Y-standards were added prior to element analysis. Ca and Sr concentrations were determined on a Finnigan Element 2 inductively coupled plasma mass spectrometer at the Department of Geosciences at the University of Bremen. The reproducibility (2 SD) of the Sr/Ca ratios was determined to be about 0.038 mmol mol^{-1} by repeated measurements of a house standard during the course of the analyses.

Theoretical Background

The partition coefficient describes the distribution of an element between two phases. In aqueous systems these two phases are normally an aqueous solution and a mineral. Unfortunately the terminology in this field is incoherently used throughout the literature (Beattie et al. 1993), so that the terms “partition coefficient”, “distribution coefficient”, and “exchange coefficient” and the symbols D, K_d, and K_D are used interchangeably. We use the following terminology: The partition coefficient, and the symbol D_x (the subscript x stands for the element of interest) are defined as,

$$D_x = \frac{[\mathrm{x}]_s}{[\mathrm{x}]_l} \tag{3}$$

where $[\mathrm{x}]_s$ is the molar concentration of the element of interest in the solid and $[\mathrm{x}]_l$ its molar concentration in the solution. For most aqueous systems the partition coefficient is normalized to the partition coefficient of another element. The term exchange coefficient and the symbol $K_{D_{Tr}}$ will be used for the normalized value. From here on the subscript Tr (trace element) and M (major element) will be used instead of the previously used *x* since this is more consistent with the literature. The exchange coefficient is defined as,

$$K_{D_{Tr}} = \frac{D_{Tr}}{D_M} \tag{4}$$

D_{Tr} is the partition coefficient of the element of interest and D_M is the partition coefficient of the element used for normalization. Since in this work we focus on the element strontium (Sr) the exchange coefficient for Sr is defined as the partitioning of Sr between calcite and the solution normalized to the distribution of calcium (Ca) between calcite and solution (Equation (5)).

$$K_{D_{Sr}} = \frac{[\mathrm{Sr}]_S / [\mathrm{Sr}]_l}{[\mathrm{Ca}]_S / [\mathrm{Ca}]_l} \tag{5}$$

It has been shown that the distribution of trace elements at thermodynamical equilibrium is related to the solubility product of the mineral phases. McIntire (1963) showed

that the solubility products of MCO_3 and $TrCO_3$ are correlated to the exchange coefficient as follows (symbol explanations see Table 1):

$$K_{D_{Tr}} = \left(\frac{K_{MCO_3}}{K_{TrCO_3}} \right)^{\frac{1}{\nu_c}} \left(\frac{\gamma_{Tr}}{\gamma_M} \right) \left(\exp\left(\frac{-\Delta\mu}{\nu_a RT} \right) \right) \quad (6)$$

It is difficult to measure or calculate the values for all parameters of this equation. In particular, the difference of the chemical potential of the trace element in the pure $TrCO_3$ crystal and its chemical potential as a component in the MCO_3 crystal is not known. Rimstidt et al. (1998) tried to estimate the values for various elements by fitting a large dataset obtained from the literature. Their work, and many other experimental investigations (e.g. Lorens 1981; Tesoriero and Pankow 1996) showed that the exchange coefficient is in many cases influenced by kinetics. The degree of supersaturation has a strong influence on the growth processes at the crystal surface, which are complex and not trivial to investigate. However, the crystal growth rate generally increases with increasing levels of supersaturation (Nielsen 1964). Experiments with varying growth rates are easily performed and therefore ideal for the investigation of the kinetic effects on trace element incorporation during crystal growth. Numerous experiments showed that three different exchange behaviors of trace elements can be distinguished (e.g. Rimstidt et al. 1998; Tesoriero and Pankow 1996): 1. the trace element concentration is enriched within the crystal compared to its concentration in solution, $K_{D_{Tr}} > 1$, 2. the trace element concentration is depleted within the crystal, $K_{D_{Tr}} < 1$, and 3. no fractionation occurs $K_{D_{Tr}} = 1$.

Depending on this exchange behavior, the measured value for the exchange coefficient differs from the estimated equilibrium value the following way. For elements with a $K_{D_{Tr}} > 1$ measured $K_{D_{Tr}}$ values are lower than predicted and approach the predicted value with decreasing growth rate. For $K_{D_{Tr}} < 1$ the opposite behavior can be observed. This behavior is illustrated in Figure 1. It should be noted that strictly speaking equilibrium is only achieved under conditions of zero growth. In spite of this, the term “growth at equilibrium” is widely used in the literature and describes a behavior not influenced by kinetics. However, for our purposes it is important to notice that the concentration measured within the crystal can be influenced by two variables: the concentration in solution (Equation (3)) and the growth rate of the crystal. The concentration in solution in general refers to the solution the crystal is precipitated from. If crystal growth proceeds via biomineralization, the chemical composition

of the fluid at the actual growth site, located within an organism, is mostly unknown. Nevertheless, it is common to calculate an exchange coefficient by means of the element ratio in the growth medium (bulk medium). Values obtained in this way are not based on the real composition at the site of crystal growth. We suggest that exchange coefficients calculated based on a reference solution (if the solution composition at the site of crystal growth is not known) should be referred to as apparent exchange coefficient, $K^{B}_{D_{Tr}}$ (the superscript B denotes the use of the bulk medium concentration as the reference solution). Symbol explanations are given in Table 1.

Results

The Sr/Ca of coccolith calcite is linearly related to Sr/Ca of seawater (Figure 2). The slope of the regression curve (Figure 2) represents $K^{B}_{D_{Sr}}$, with a value of 0.39. Deviation of the y-axis intercept from zero is due to precision of the measurement. The linearity in the slope of coccolith Sr/Ca implies that our experimental practice to adjust the Sr and Ca concentrations in our growth medium by changing either the strontium or the calcium concentration relative to those in natural seawater had no influence on the exchange coefficient.

The highest Sr/Ca applied in our experiments, 90 mmol mol^{-1}, was achieved by lowering the calcium concentration to 1 mmol L^{-1}. The corresponding calcite saturation state (Ω), with

$$\Omega = \frac{[Ca][CO_3]}{K_{sp}} \tag{7}$$

was 0.4, indicating undersaturation of seawater with respect to calcite. SEM images of coccoliths produced under these conditions reveal partial dissolution of the rim and central area (Figure 3). Therefore, the decreased calcification rate occurring at 1 mmol L^{-1} external Ca can in part be attributed to dissolution.

Cell growth rates and calcification rate of *Emiliania huxleyi* varied between 0.32 to 0.91 d^{-1} and 2 to 58 pg calcite $cell^{-1}$ d^{-1} (see Table 3).

Discussion

Implications for proxy use

The Sr/Ca of *Emiliania huxleyi* coccoliths increased linearly with increasing Sr/Ca of seawater within the range tested. This range comprises three orders of magnitude and the lowest value corresponds to 1% of the modern seawater value. Because this range is much larger than the postulated changes of Sr/Ca in the seawater since the Cretaceous (Lear et al. 2003; Stoll and Schrag 2001) the assumption of a constant $K^B_{D_{Sr}}$ with changing seawater Sr/Ca (Billups et al. 2004; Stoll and Schrag 2001) is supported by the data presented here.

At 1 mmol L^{-1} external Ca, SEM images show partial dissolution of coccoliths, probably as a consequence of the calcite saturation state of 0.4. Lowered production of calcite is also likely to have occurred at 1mmol L^{-1} calcium in seawater (Paasche 1964), an interpretation which the lower growth rates render even more likely. Consequently, we are not able to report the exact proportion of calcite that was subject to dissolution. However, the Sr/Ca of the remaining calcite fits the data obtained from coccoliths not showing partial dissolution. We conclude that partial dissolution seems to have no significant effect on coccolith Sr/Ca. This conclusion is supported by dissolution experiments with carbonate-rich sediment samples (Stoll and Schrag 2000). Furthermore there are considerable differences in growth and calcification rates between the samples, which are due to differences in light intensities (Table 3). Apparently these differences in growth and calcification rates have no effect on $K^B_{D_{Sr}}$. The confidence level (r^2 = 0.99) of the linear regression curve (Figure 2) indicates a constant $K^B_{D_{Sr}}$ with changing seawater Sr/Ca. This is in accord with previous experiments with *Emiliania huxleyi* (Stoll et al. 2002b) and other species (Stoll et al. 2002c), which showed that irradiance-induced changes in growth rate did not affect strontium partitioning. Nutrient-stimulated changes in growth rate, on the other hand, affected strontium partitioning in *Emiliania huxleyi* (Rickaby et al. 2002). This distinction between irradiance-induced and nutrient-stimulated changes in growth rate strongly suggests that some physiological processes, rather than growth rate per se, are responsible for variable strontium partitioning in coccoliths.

$K^B_{D_{Sr}}$ values of coccolithophore calcite

The measured $K^B_{D_{Sr}}$ of 0.39 falls well within the range of previously reported values (Rickaby et al. 2002; Stoll et al. 2002b). $K^B_{D_{Sr}}$ values ranging from 0.1 to 0.6, depending on calcification rate, were measured by Rickaby et al. (2002), using the same strain as in our experiments. A different strain was used by Stoll et al. (2002b). Based on the data they published (seawater Sr/Ca 8.55 mmol mol^{-1}, coccolith Sr/Ca 2.92 mmol mol^{-1}) we calculated a $K^B_{D_{Sr}}$ of 0.34.

Inorganically precipitated calcite shows $K_{D_{Sr}}$ values ranging from 0.021 to 0.14 depending on the precipitation rate (Lorens 1981; Tesoriero and Pankow 1996), where the lowest value represents the thermodynamical equilibrium value. This value, however, is one order of magnitude smaller than the common $K^B_{D_{Sr}}$ value for coccolith calcite (see above).

A positive correlation between $K^B_{D_{Sr}}$ and growth rate is well documented for inorganically precipitated calcite (Lorens 1981; Tesoriero and Pankow 1996). The correlation was used by Carpenter and Lohmann (1992) in order to explain the high $K^B_{D_{Sr}}$ of biogenic calcite. This hypothesis was examined with regard to coccolithophore calcite by Stoll et al. (2002b). The authors used a surface enrichment model (Watson and Liang 1995) to fit the above mentioned data from inorganic precipitation experiments (Lorens 1981; Tesoriero and Pankow 1996). As concluded by the authors themselves the calculations of Stoll et al. (2002b) show that $K^B_{D_{Sr}}$ values of coccolith calcite cannot be explained by kinetic effects, i.e. simply by virtue of fast crystal growth rates. We perfectly agree with this conclusion, which is even underscored when considering the inevitable conversion of crystal growth rate units of the inorganic data (Tesoriero and Pankow 1996) in more detail. The relationship between seed crystal surface area and seed mass, i.e. the specific surface area, is the crucial number in this conversion. A careful review of the experiments described in Tesoriero and Pankow (1996) revealed that the value for specific surface area most likely is smaller than the one reported by Tesoriero and Pankow (1996). We put emphasis on the notion that this has no consequences at all for the results of Tesoriero and Pankow (1996). It has, however, consequences for $K^B_{D_{Sr}}$ values of coccolith calcite, which can be obtained using this number. Given a linear coccolith growth rate of ca. 1^x10^{-8} cm s^{-1} (Stoll et al. 2002b) a specific surface area of 0.1 m^2 g^{-1} (the value given by Tesoriero and Pankow (1996) is 0.6 m^2 g^{-1}) yields a $K^B_{D_{Sr}}$ for coccolith

calcite of 0.021, which represents the thermodynamical equilibrium value (Tesoriero and Pankow 1996). This calculation adds to the conclusion of Stoll et al. (2002b) that kinetic surface enrichment effects cannot explain the strontium partitioning in coccoliths.

The authors (Stoll et al. 2002b) also stated that the Sr/Ca of the coccolith vesicle solution is not known and that higher Sr/Ca of the calcifying fluid in comparison to seawater could possibly explain $K^B_{D_{Sr}}$ values. A mechanism that produces such elevated Sr/Ca of coccolith vesicle solution is still lacking.

Towards an explanation of coccolith $K^B_{D_{Sr}}$: a conceptual model

In order to understand why intracellular Sr/Ca should differ from seawater ratio it is necessary to briefly review the present knowledge of the calcification mechanism and cellular transport of calcium ions in *Emiliania huxleyi*.

Precipitation of calcite takes place within a membrane-delimited space completely isolated from the cytosol, the coccolith vesicle (e.g. Young et al. 1999). Thus seawater is separated from the site of calcification by at least two membranes, i.e. the plasmamembrane and the coccolith-vesicle membrane. Calcium uptake for calcification is thought to be accomplished by transmembrane transport via Ca-channels in the plasmamembrane and Ca-ATPases in the endomembrane system (Brownlee et al. 1995). It is still a matter of debate which compartment takes up calcium from the cytosol. It was suggested that the peripheral endoplasmic reticulum (ER) could play that role (Berry et al. 2002). Another structure that appears to be suited for the task is the reticular body, which is part of the coccolith vesicle in *Emiliania huxleyi*, because this structure combines large surface area with small volume (Marsh 2000). For reasons of simplicity, we assume the reticular body to be responsible for Ca uptake from the cytosol in our further discussion. Another premise of our conceptual model is the small volume of solution surrounding the coccolith inside the coccolith vesicle. The membrane of the coccolith vesicle closely envelopes the growing coccolith and so does not contain a substantial reservoir of fluid. Judging from transmission electron micrographs the vesicle volume is at most double the coccolith volume (Young, J. and Henriksen, K., pers. comm.) and is here assumed to have a maximum value of 1.8 μm^3.

Calcium homeostasis is accomplished by an arrangement of channels and pumps in the plasmamembrane and various endomembranes which provides a steep calcium gradient between the cytosol and its environment. From a thermodynamical point of view, a Ca-gradient of 10^4 to 10^5 can be achieved by the hydrolysis of ATP for a high ATP/ADPxPi ratio

in the cytosol with a ΔG of 15 kcal mol^{-1} (Meldolesi and Pozzan 1998) and due to the presence of macromolecules (acidic polysaccharides, proteins) near the cell surface and probably also in the ER. The polysaccharides may form complexes with Ca or Sr, as known for calreticulin, thereby decreasing the concentration of the free cations.

The cytosolic concentration of Ca is a rather universal constant in the inactivated cell with a value of 0.1 µmol L^{-1} (Brownlee et al. 1995). In case that Ca pumps in the plasmamembrane work near the thermodynamical limit, the gradient of 10^5 yields a calcium concentration of 10 mmol L^{-1} at the cell surface, which in fact is exactly the Ca concentration of seawater. Because of the calcium homeostasis, the Ca at the cell surface should be quite independent from the bulk Ca concentration in different experimental conditions. Ca transport near the thermodynamical limit would have the advantage that 1) pumping Ca from the cytosol to the environment proceeds with a minimum dissipation of energy in ATP consumption, and 2) a steep Ca gradient provides fast influx in cell signalling. We therefore postulate that a constant concentration of Ca at the cell surface represents a part of calcium homeostasis. The value of the surface Ca concentration we assume to be close to the thermodynamical limit, i.e. close to the Ca concentration of seawater (10 mmol L^{-1}).

Calcification takes place in vesicles derived from the Golgi-system. The kinetics of calcite formation can be optimised inside the cell, but biomineralisation can not bypass thermodynamics, e.g. calcite precipitation can not occur below the calcite saturation concentration. Here, we assume that thermodynamics is the only restriction for the vesicle calcification, i.e. the Ca concentration in the vesicle is determined by the saturation product. Hence, the calcite precipitation should start right after a supersaturation is obtained in the coccolith vesicle, see also Young et al. (1999). Moreover, the incorporation of Sr into calcite should proceed with the equilibrium $K_{D_{Sr}}$ of 0.021 (Tesoriero and Pankow 1996).

For an unconstrained inorganic precipitation of calcite in the coccolith vesicle, there are two possible scenarios for explaining the observed $K^B_{D_{Sr}}$. First scenario: Precipitation occurs in steady state for both Ca and Sr. Under this condition the Sr/Ca of the calcite is determined by the ratio of the Sr and Ca influx. In this case the observed $K^B_{D_{Sr}} < 1$ could be explained in terms of a strong fractionation of the Ca pump against Sr. However, there is no experimental evidence for a significant difference in the cellular transport kinetics for Ca and Sr, neither for pumps (SERCA) nor for channels (Allen and Sanders 1994; Berman and King 1990). Therefore, an unconstrained inorganic precipitation mechanism that operates in steady state appears to be unlikely.

Second scenario: Sr/Ca of the new calcite is changing with time. The calcite precipitation yields an accumulation of Sr in the coccolith vesicle (owing to $K_{D_{Sr}} < 1$) until steady state is achieved. Then, the observed Sr/Ca of the coccolith calcite would be an integral value which arises from the sum of Sr/Ca over the time required for the formation of one coccolith. This mechanism assumes a similar timescale for reaching steady state and formation of the coccolith. The time required to achieve steady state can be estimated by means of a one-compartment model for inorganic precipitation of calcite in the coccolith vesicle. Within this model the concentrations of Ca and Sr in the vesicle are described in terms of two simple differential equations:

$$V\frac{d[\mathrm{Ca}]}{dt} = f_{\mathrm{In}}^{Ca} - f_{\mathrm{Pr}}^{Ca}$$
$$V\frac{d[\mathrm{Sr}]}{dt} = R^B f_{\mathrm{In}}^{Ca} - K_{D_{Sr}}\frac{[\mathrm{Sr}]}{[\mathrm{Ca}]} f_{\mathrm{Pr}}^{Ca}, \qquad R^B = \left(\frac{\mathrm{Sr}}{\mathrm{Ca}}\right)_{bulk} \tag{8}$$

where V, f_{In}^{Ca}, f_{Pr}^{Ca}, are coccolith vesicle volume, Ca influx and Ca precipitation flux respectively. In this model we assume that the ratio of the Sr and Ca influx equals the Sr/Ca in the bulk medium (R^B). Typical parameter values for the coccolith vesicle volume and the calcification rate are given in Table 4. After the onset of calcite precipitation, the Ca concentration in the coccolith vesicle is assumed to be constant, where the value of Ca concentration is determined by the saturation product. Then, the solution of equation 8 yields the following expression for the time dependence of the Sr concentration:

$$[\mathrm{Sr}] = [\mathrm{Ca}]_{Sat}\frac{R^B}{K_{D_{Sr}}}\left[1-(1-K_{D_{Sr}})e^{-t/\tau}\right] \tag{9}$$

where the characteristic time constant, τ, is given by:

$$\tau = \frac{V}{K_{D_{Sr}}}\frac{[\mathrm{Ca}]_{Sat}}{f_{\mathrm{Pr}}^{Ca}} \quad = \quad 5.2s \tag{10}$$

The value of τ indicates that the steady state is obtained after a few seconds, which is negligible compared to the 0.7 hours required for the formation of one coccolith. Thus, the

$K^B_{D_{Sr}}$ of coccolith calcite is entirely determined by its steady state value. Therefore, we have to exclude also scenario 2 as a possible mechanism yielding the observed coccolith $K^B_{D_{Sr}}$.

Based on the previous discussion we speculate that there is a strong impact of the cell physiology on the coccolith Sr/Ca. We suggest that the Ca pumps in the endomembrane system provide an additional thermodynamical constraint on inorganic calcite precipitation: During the calcification, the concentration of Sr in the coccolith vesicle increases with time (see Equation 9 and Figure 4). However, the accumulation of Sr in the vesicle will stop at the thermodynamical limit existing for the gradient of Sr concentration between the vesicle and the cytosol (see Figure 4). At the thermodynamical limit, a gradient of 10^5 between the cytosol and the coccolith vesicle can be expected for Sr, corresponding to the situation at the plasmamembrane.

Hence, the achieved Sr concentration in the vesicle ($[Sr]_V$) is smaller than the steady state concentration of an unconstrained inorganic precipitation, and should be close to the Sr concentration at the cell surface ($[Sr]_S = [Sr]_V$). Therefore, we assume that $[Sr]_V$ mirrors all changes of $[Sr]_S$, whereas the value of the Ca concentration in the vesicle ($[Ca]_V$) is close to the saturation value ($[Ca]_{Sat}$) (see Fig. 5). Since the external CO_2 concentrations are the same for all our experiments, $[Ca]_{Sat}$ should be constant. Also, the Ca concentration at the cell surface ($[Ca]_S$) is assumed being a constant (10 mmol L^{-1}) owing to Ca homeostasis (see above). Then, in the expression for the $K^B_{D_{Sr}}$ of coccolith calcite, the Sr concentrations in the coccolith vesicle and at the cell surface cancel:

$$K^B_{D_{Sr}} = K_{D_{Sr}} \frac{[Sr]_V}{[Ca]_V} \frac{[Ca]_B}{[Sr]_B} = K_{D_{Sr}} \frac{[Sr]_V}{[Ca]_{Sat}} \frac{[Ca]_S}{[Sr]_S} = K_{D_{Sr}} \frac{[Ca]_S}{[Ca]_{Sat}} = \text{const.} \qquad (11)$$

Hence, we conclude, that the $K^B_{D_{Sr}}$ of coccolith calcite should have a constant value, given a constant external CO_2 concentration. This is in good agreement with our experimental finding.

Using for $[Ca]_{Sat}$ a typical value for [Ca] in the ER (500 µmol L^{-1}, see Table 4) yields a $K^B_{D_{Sr}}$ of 0.4, which is very close to the observed $K^B_{D_{Sr}}$ of 0.39. Based on the seawater saturation product ($K_{sp} = 10^{-6.37}$ mol^2 kg^{-2}, see Table 4), a $[Ca]_{Sat}$ of 500 µmol L^{-1} would correspond to a [DIC] in the vesicle (or $[CO_3^{2-}]$ at a strong alkaline pH) of 893 µmol L^{-1}.
This is probably a slightly overestimated value for [DIC] in the vesicle, since the ionic strength inside the coccolith vesicle is most likely lower than that of seawater (Anning et al.

1996). However, a [DIC] of 893 µmol L^{-1} is still in agreement with the observed mean cellular DIC of 500 µmol L^{-1} (Anning et al. 1996).

As mentioned above previous experiments with *Emiliania huxleyi* (Stoll et al. 2002b) and other species (Stoll et al. 2002c) showed that irradiance-induced changes in growth rate did not affect strontium partitioning, whereas nutrient-stimulated changes did (Rickaby et al. 2002). Since *Emiliania huxleyi* does not dispose of an efficient nitrogen uptake system (Riegman et al. 2000) it is possible that nitrogen availability affects DIC transport proteins. If DIC concentration in the coccolith vesicle and external nitrogen concentration are positively correlated this would have an effect on strontium partitioning. According to our model an increase in coccolith vesicle DIC concentration corresponds to a decrease in coccolith vesicle calcium concentration $[Ca]_{Sat}$. Hence an increase in external nitrogen concentration would lead to an increase in $K^B_{D_{Sr}}$ predicted by our model (Equation 11). This correlation was observed experimentally (Rickaby et al. 2002). In the latter study $K^B_{D_{Sr}}$ values ranging from 0.1 to almost 0.7 were measured. In order to cover this range in our model values for $[Ca]_{Sat}$ ranging from 279 µmol L^{-1} to 1950 µmol L^{-1} are required. These values fall well within the range of measured ER-values (Meldolesi and Pozzan 1998). An additional explanation for effects of nitrogen limitation can be given. Under nitrogen limitation *Emiliania huxleyi* produces excess polysaccharides, which are extruded onto the cell surface (Engel et al. 2004). Polysaccharides bind divalent cations by a complexation mechanism called egg-box model (Pellerin and O'Neill 1998) where strontium is bound more strongly to polysaccharides than calcium (Cohen-Shoel et al. 2002). Hence with increasing polysaccharide concentration at the cell surface the calcium to strontium ratio at the cell surface would also increase and become higher than that of the bulk medium. Mathematically this can be described by adding a factor γ to Equation 11:

$$K^B_{D_{Sr}} = K_{D_{Sr}} \frac{[Sr]_V}{[Ca]_V} \frac{[Ca]_B}{[Sr]_B} = K_{D_{Sr}} \gamma \frac{[Sr]_V}{[Ca]_{Sat}} \frac{[Ca]_S}{[Sr]_S} = K_{D_{Sr}} \gamma \frac{[Ca]_S}{[Ca]_{Sat}} \qquad (12)$$

The factor γ can assume values between 0 and 1. In case of no polysaccharides at the cell surface γ equals 1. It should be noted that a very small amount of polysaccharides, the coccolith associated polysaccharides (CAP) (Henriksen et al. 2004), is always present at the cell surface of *Emiliania huxleyi*. The amount of CAP, however, is negligible compared to the amount of polysaccharide produced and extruded additionally during nitrogen limitation (Engel et al. 2004).

Strontium partitioning in different coccolithophore species is not the same under identical conditions; $K^B_{D_{Sr}}$ values range from 0.257 to 0.356 among five species at 17 °C (Stoll et al. 2002a). According to our model this corresponds to a range of coccolith vesicle calcium concentrations $[Ca]_{Sat}$ from 548 µmol L^{-1} to 759 µmol L^{-1}. We propose that different species (or strains) have different coccolith vesicle calcium concentrations and that this is the reason why $K^B_{D_{Sr}}$ values differ among species (or strains). One explanation for species-specific coccolith vesicle calcium concentrations is that different species have different polysaccharides in the coccolith vesicle (Marsh 2000), which in turn requires different coccolith vesicle calcium concentrations $[Ca]_{Sat}$ in order to achieve supersaturation with respect to calcite.

The capability of the model can be further demonstrated by applying it to two independent datasets. The first set of data describes the temperature dependence of $K^B_{D_{Sr}}$ in *Emiliania huxleyi* (Stoll et al. 2002b) whereas the second shows the temperature dependence of $K_{D_{Sr}}$ in inorganic calcite precipitation experiments (Malone and Baker 1999). Both datasets show a linear dependence of the Sr incorporation into calcite. However, the two slopes differ by one order of magnitude. Inserting the expression for the dependence of $K_{D_{Sr}}$ obtained from inorganic precipitation experiments (slope: 1.85×10^{-4} °C^{-1}) into equation 11 yields the $K^B_{D_{Sr}}$ for Sr incorporation into *Emiliania huxleyi* coccoliths with a slope of 3.7×10^{-3} °C^{-1}. This model value is in agreement with the observed slope of 3.2×10^{-3} °C^{-1} (Stoll et al. 2002b).

We have shown experimentally that coccolith Sr/Ca of *Emiliania huxleyi* increases linearly with increasing seawater Sr/Ca over three orders of magnitude. This relationship in general provides the basis for correcting measured Sr/Ca from sediment samples for different composition of seawater with respect to these ions.

The high $K^B_{D_{Sr}}$ values measured for coccolith calcite can be explained by a simple conceptual model based on the channel/carrier mediated transport of calcium and strontium ions inside the cell. The model is solely based on thermodynamical constraints and does not assume a relationship between the exchange coefficient and the crystal growth rate.

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Table 1. Notation

d	day
D_x	partition coefficient for the element x
$K_{D_{Sr}}$	exchange coefficient for Sr
$K_{D_{Tr}}$	exchange coefficient for a trace element (*Tr*)
$K^{B}_{D_{Tr}}$	apparent exchange coefficient for a trace element (*Tr*)
K_{MCO_3}, K_{TrCO_3}	solubility product of MCO_3 and $TrCO_3$, respectively
K_{sp}	seawater calcite saturation product
R	gas constant
T	absolute temperature
γ_{Tr}/γ_M	ratio of activity coefficients for trace (*Tr*) and major (*M*) element in solution
$\Delta\mu$	difference of the chemical potential of the trace element in the pure $TrCO_3$ and in the MCO_3
ν_a	stoichiometry factor accounting for the number of anions in the carbonate structure
ν_c	stochiometry factor accounting for the number of cations in the carbonate structure
Ω	calcite supersaturation

Table 2. Composition of artificial seawater

Salt	reagent grade, producer	final concentration (mmol L^{-1})
NaCl	suprapur, Merck	395
$MgCl_2{}^x6H_2O$	fractopur, Merck	53.23
Na_2SO_4	suprapur, Merck	28.24
KCl	fractopur, Merck	10
$SrCl_2{}^x6H_2O$	p. A., Merck	0.004, 0.02, 0.09, 0.42
$NaHCO_3$	suprapur, Merck	2.33
NaBr	suprapur, Merck	0.84
H_3BO_3	suprapur, Merck	0.4
$CaCl_2{}^x4H_2O$	suprapur, Merck	1, 10, 20

Table 3. Experimental setup and results

Experiment No.	PFD (μmol m^{-2} s^{-1})	$[Ca]_B$ (mmol L^{-1})	$[Sr]_B$ (mmol L^{-1})	seawater Sr/Ca (mmol mol^{-1})	coccolith Sr/Ca (mmol mol^{-1})	apparent Sr exchange coefficient	growth rate μ_{cocco} (d^{-1})	calcification rate (pg calcite $cell^{-1}d^{-1}$)
1	100	1	0.09	90	37	0.411	0.54	8
2	100	10	0.09	9	5.44	0.605	0.86	23
3	100	20	0.09	4.5	2.67	0.592	0.86	24
4	300	1	0.09	90	37	0.411	0.66	5
5	300	10	0.09	9	5.48	0.608	0.85	26
6	300	20	0.09	4.5	2.67	0.594	0.78	22
7	500	1	0.09	90	36.2	0.402	0.65	3
8	500	10	0.09	9	5.63	0.625	0.89	26
9	500	20	0.09	4.5	2.9	0.645	0.91	29
10	800	1	0.09	90	33.8	0.376	0.71	2
11	800	10	0.09	9	5.32	0.591	0.87	25
12	800	20	0.09	4.5	2.6	0.578	0.77	24
13	30	10	0.004	0.4	0.17	0.427	0.56	18
14	30	10	0.02	2	0.64	0.320	0.53	17
15	30	10	0.42	42	13.7	0.325	0.55	17
16	300	10	0.004	0.4	0.16	0.390	0.87	55
17	300	10	0.02	2	0.68	0.340	0.80	51
18	300	10	0.42	42	15.2	0.363	0.87	58

Table 4. Model parameters

Parameter	Symbol	Unit	Values	References
Calcification rate	P	pg calcite $cell^{-1}$ d^{-1}	50	typical value, e.g. own results, (Riebesell et al. 2000; Stoll et al. 2002b; Zondervan et al. 2002; Zondervan et al. 2001)
Light Dark cycle	L/D	1	16/8	experimental setup
$CaCO_3$ content of one coccolith	$P_{coccolith}$	pg	2.2	(Young and Ziveri 2000)
Sr exchange coefficient	$K_{D_{Sr}}$	1	0.021	(Tesoriero and Pankow 1996)
volume of coccolith vesicle solution	V	μm^3	1.8	Young, J., Henriksen, K. pers. comm.
Ca precipitation flux	f_{Pr}^{Ca}	pmol h^{-1}	0.031	calculated
Apparent Sr exchange coefficient	$K_{D_{Sr}}^{B}$	1	0.39	exp. result
			0.4	model result
Ca concentration in the coccolith vesicle	$[Ca]_{Sat}$	$\mu mol\ L^{-1}$	500	(Meldolesi and Pozzan 1998)
Ca concentration in seawater	$[Ca]_S$	$mmol\ L^{-1}$	10	(Broecker and Peng 1982)
Calcite saturation product in seawater	K_{sp}	$mol^2\ kg^{-2}$	$10^{-6.37}$	(Mucci 1983)
free energy change for ATP hydrolysis	ΔG	kcal mol^{-1}	15	(Meldolesi and Pozzan 1998)

Figure captions

Fig. 1 Dependence of the exchange coefficient $K_{D_{Tr}}$ on the element ratio x_{Tr}/x_M in the bulk medium. For a $K_{D_{Tr}} > 1$ the value for x_{Tr}/x_M is higher in the crystal than the bulk value. A $K_{D_{Tr}}$ value < 1 shows the opposite exchange behaviour. If the incorporation into the crystal shows the same ratio as the bulk medium the $K_{D_{Tr}}$ equals 1. For $K_{D_{Tr}}$ values greater 1 experimental values are in general lower than predicted equilibrium value and approaches equilibrium values with decreasing growth rate. The opposite holds for experimental values in case of $K_{D_{Tr}} < 1$. Modified after Rimstidt et al. (1998).

Fig. 2 Experimental results. Shown is the dependence of Sr/Ca in coccolith calcite on Sr/Ca in bulk medium. The slope of the linear regression curve yields a $K^B_{D_{Sr}}$ of 0.39.

Fig. 3 SEM images of coccoliths produced under (A) 1 mmol L^{-1} Ca and (B) 10 mmol L^{-1} Ca in the bulk medium. The rim and central area show dissolution under 1 mmol L^{-1} Ca in the bulk medium.

Fig. 4 Concentration of Sr in the coccolith vesicle. The solid curve shows the increase of Sr concentration after onset of calcite precipitation as it would follow from an unconstrained inorganic precipitation mechanism. The dashed line indicates the thermodynamical limit imposed by the Ca pump in the coccolith vesicle membrane. Shown are the values corresponding to a bulk medium Sr/Ca of 0.01, i.e. the Sr/Ca of seawater.

Fig. 5 Proposed mechanism for the partitioning of Sr and Ca during calcification in *Emiliania huxleyi* yielding the $K^B_{D_{Sr}}$. Depicted are the relative concentrations of Sr and Ca in the two involved cellular compartments (coccolith vesicle and cytosol) and the cell surface (schematic drawing). The Sr concentration (dashed lines) at the cell surface and in the coccolith vesicle increases until the thermodynamical limit of the concentration gradient between the cytosol and adjacent compartments is reached. The same holds for the Ca concentration (solid lines) at the cell surface. On the other hand, the Ca concentration in the coccolith vesicle is determined by the saturation product. All variations of the Sr concentration at the cell surface are reflected by variations of the Sr concentration in the coccolith vesicle (as indicated by the double-headed arrows).

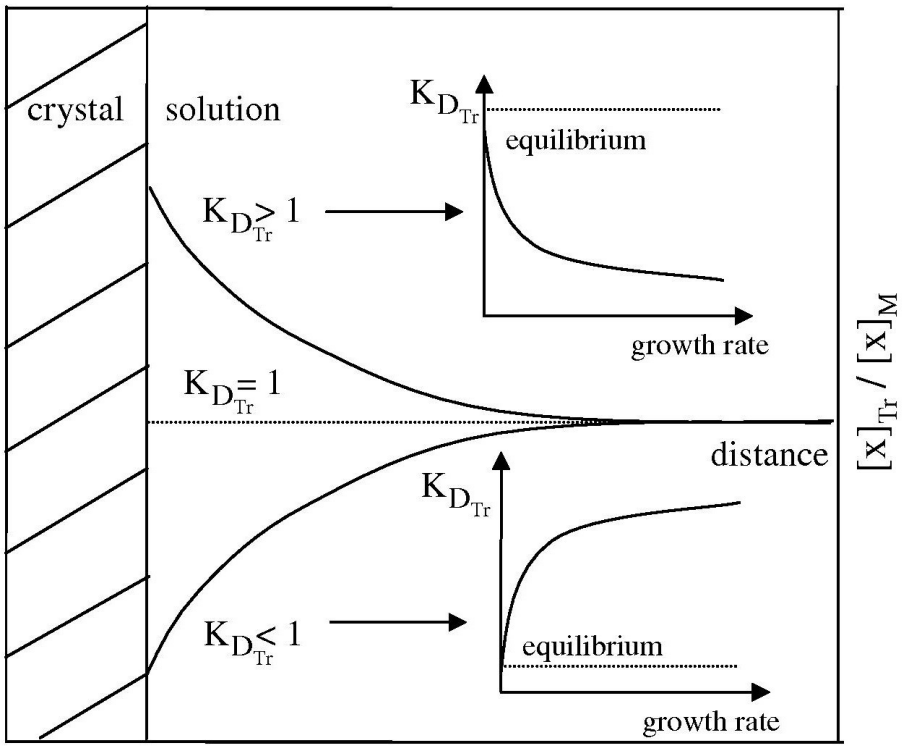
crystal
solution
$K_{D_{Tr}}$
equilibrium
$K_{D_{Tr}} > 1$
growth rate
$K_{D_{Tr}} = 1$
distance
$[x]_{Tr} / [x]_M$
$K_{D_{Tr}}$
$K_{D_{Tr}} < 1$
equilibrium
growth rate

Figure 1

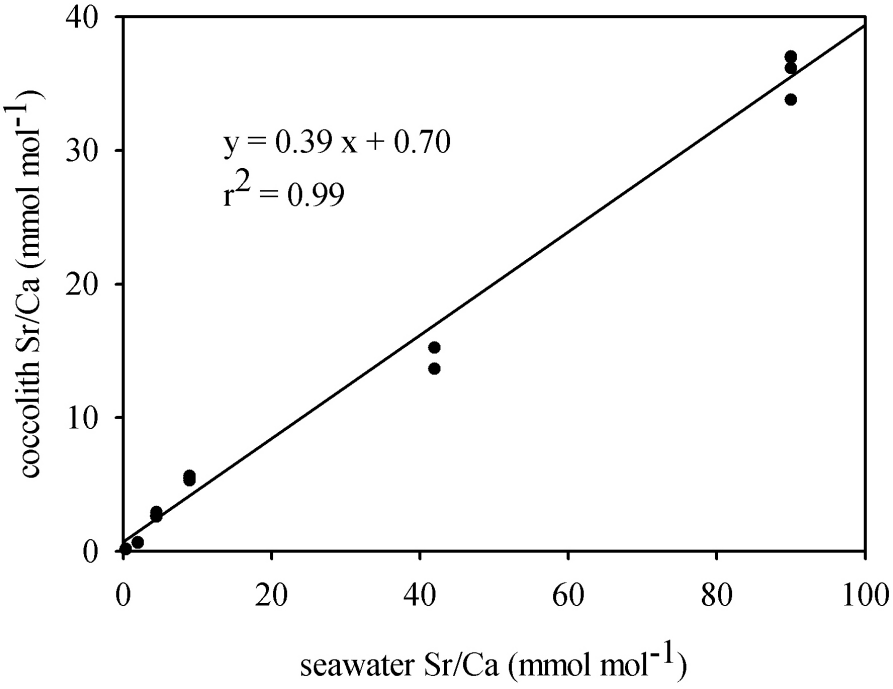
y = 0.39 x + 0.70
r2 = 0.99
coccolith Sr/Ca (mmol mol-1)
seawater Sr/Ca (mmol mol-1)
0
10
20
30
40
0
20
40
60
80
100

Figure 2

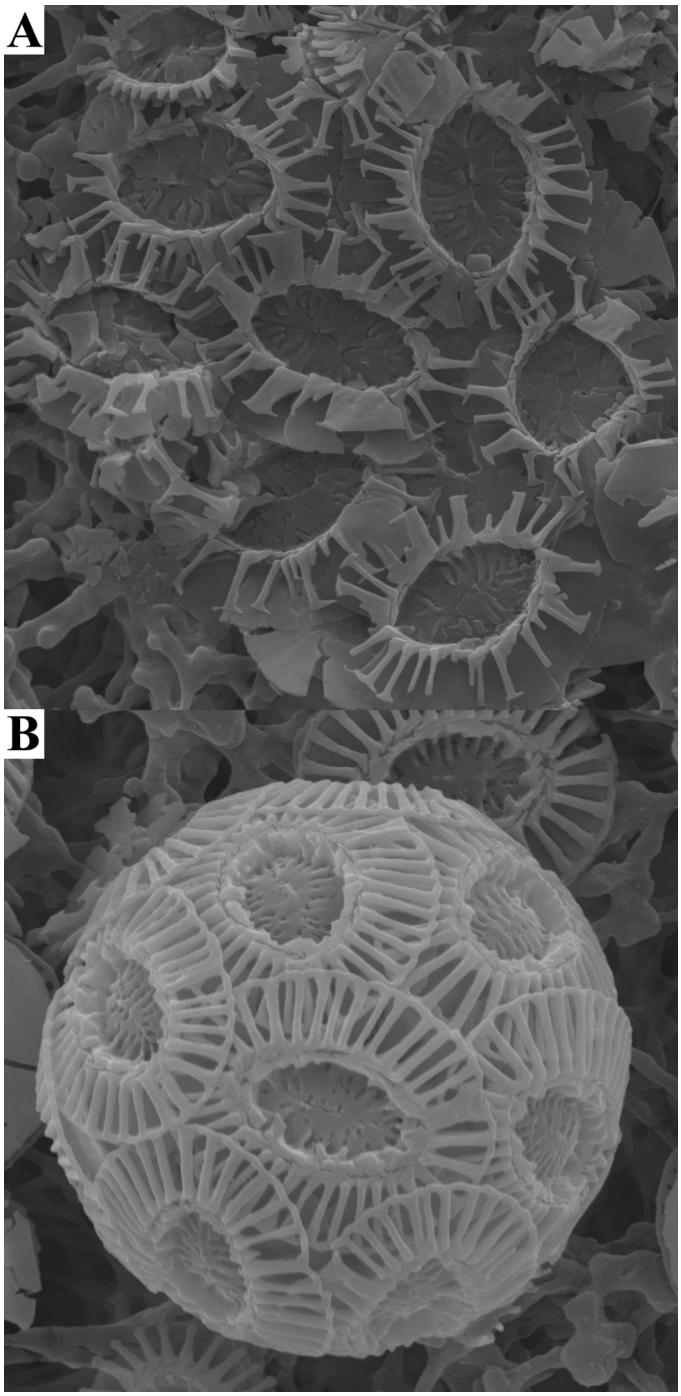

Figure 3

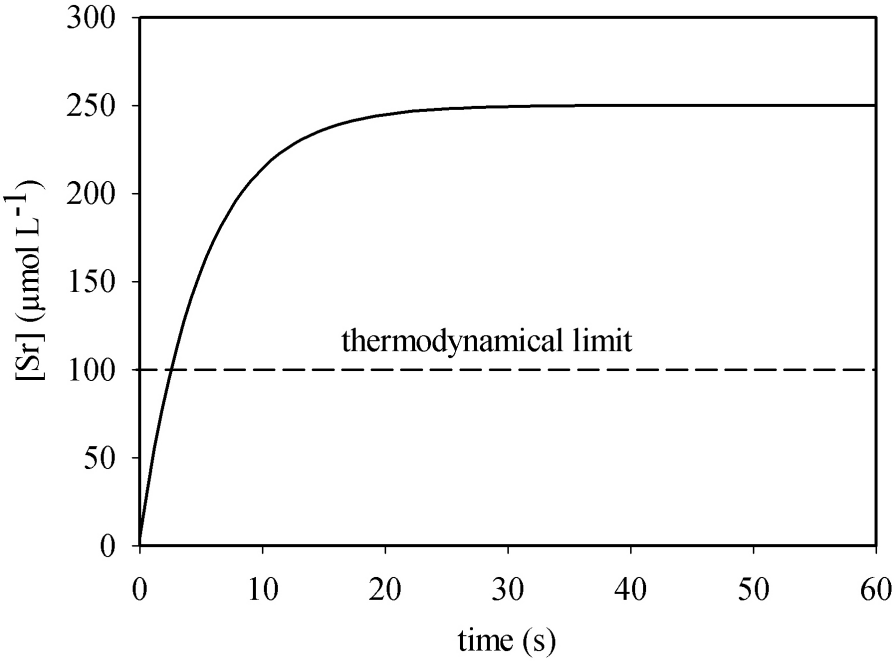
300
250
200
150
100
50
0
[Sr] (µmol L-1)
thermodynamical limit
0
10
20
30
40
50
60
time (s)

Figure 4

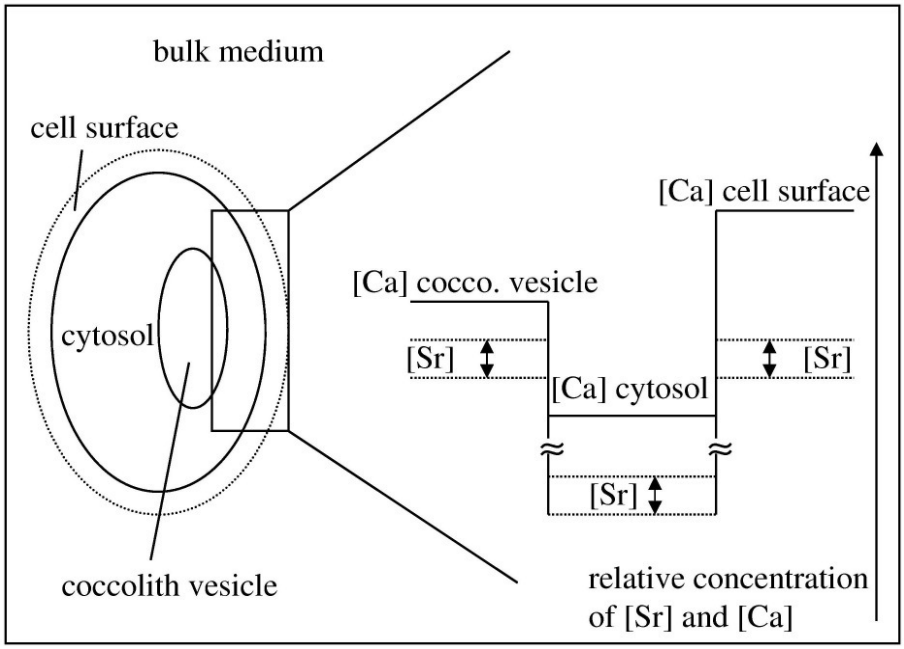
bulk medium
cell surface
cytosol
coccolith vesicle
[Ca] cocco. vesicle
[Sr]
[Ca] cytosol
[Sr]
[Ca] cell surface
[Sr]
relative concentration
of [Sr] and [Ca]

Figure 5

Temperature dependent calcium isotope fractionation in coccoliths of cultured *Calcidiscus leptoporus, Helicosphaera carteri, Syracosphaera pulchra* and *Umbilicosphaera foliosa.*

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Abstract

Four species of marine calcifying algae, the coccolithophores *Calcidiscus leptoporus, Helicosphaera carteri, Syracosphaera pulchra* and *Umbilicosphaera foliosa* were grown in laboratory cultures with temperature varying from 14 to 23°C. Calcium isotope composition of the coccoliths resembles both in absolute fractionation as well as in temperature sensitivity previous calibrations of marine calcifying species e.g. *Emiliania huxleyi* (coccolithophores) and *Orbulina universa* (planktonic foraminifera). The observed lack of a dependence of Ca isotope fractionation on the carbonate ion concentration in *C. leptoporus* also agrees with similar results for *E. huxleyi* and *O. universa*. Since coccoliths form a dominant part of the global ocean $CaCO_3$ export production, the limited range of Ca isotope fractionation in coccoliths is important for defining the isotopic composition of the Ca sink of the ocean, when modeling the changes of the marine Ca budget over time.

Keywords: Coccolithophores, Calcium isotopes, *Calcidiscus leptoporus, Helicosphaera carteri, Syracosphaera pulchra*, *Umbilicosphaera foliosa,* $\delta^{44/40}Ca$, $\delta^{44}Ca$, $\delta^{mu}Ca$

1 Introduction

Coccolithophores are important marine primary producers and contribute about half of the marine $CaCO_3$ production [1]. They are unicellular marine phytoplankton, belonging to the phylum Haptophyta and are characterized by an exoskeleton composed of numerous minute calcitic platelets, the coccoliths. As a consequence they are an important link between global Ca and C cycling. Because they act as a major Ca sink of the ocean they also play an important role for the isotopic Ca budget of the ocean. For modeling the Ca budget of the ocean [2-6], a detailed knowledge of the isotopic compositions of Ca sources and sinks is required. Since coccoliths are responsible for a major part of marine carbonate precipitation, their isotopic composition is of particular interest. However, previous studies on Ca isotopes of coccolith ooze reported considerably different values. De La Rocha and DePaolo [2] and DePaolo [7] reported Ca isotope values of coccolith oozes similar to culture experiments of *Emiliania huxleyi* (16°C: $1000\ln\alpha$=-1.3‰; with $\alpha = (^{44}Ca/^{40}Ca)_{coccolith}/(^{44}Ca/^{40}Ca)_{seawater}$) [2]. In contrast Zhu and MacDougall [8] observed considerably lighter Ca isotope values of Holocene coccolith oozes ($1000\ln\alpha$=-1.9 to -2.6).

Detailed investigation of the Ca isotope response to changing environmental parameters like salinity, pCO_2, illumination, Ca^{2+} concentration and temperature, identified the latter parameter, apart from the isotopic composition of the medium, as the only factor significantly influencing the Ca isotope composition of the coccoliths of *E. huxleyi* [9, 10]. The significant but small temperature dependence of 0.027±0.006‰/°C is not a possible explanation for the observed differences in Ca isotope values of Quarternary coccolith oozes [2, 7, 8].

In order to investigate whether the floral composition of different coccolithophore species might be responsible for the observed discrepancy in Ca isotope composition of marine carbonate oozes, we cultured different coccolithophore species at varying temperatures and different pCO_2 levels. These parameters were chosen, because previous studies revealed that they can have a large effect on Ca isotope fractionation. For instance different species of foraminifera exhibit different temperature dependent Ca isotope fractionation, with temperature sensitivities differing by a factor of about 13 [8, 11, 12]. In addition Lemarchand et al. [13] observed a large sensitivity of Ca-isotope fractionation of inorganically precipitated calcite in response to changes in the carbonate ion concentration of the fluid.

2 Material and Methods

2.1 Coccolithophore culturing

We cultured coccolithophore species from the CODENET culture collection maintained in the ALGOBANK laboratory at the University de Basse Normandie in Caen, France. Four species, *Syracosphaera pulchra* (strain GK7, GK17), *Calcidiscus leptoporus* (strains NS10-2, ASM31), *Umbilicosphaera foliosa* (strain ESP 6MI) and *Helicosphaera carteri* (strain NS10-8, NS8-4) were grown in monoclonal cultures covering a temperature range from 14 to 23 °C (table 1) at The Natural History Museum (NHM), London. In addition the *Calcidiscus leptoporus* strain AS31 was grown at different CO_2 levels at the Alfred Wegener Institute (AWI), Bremerhaven. SEM illustrations of the four investigated cocolithophore species are displayed in Plate 1.

The cultures used at the NHM were grown under sterile but not axenic conditions on a temperature-light gradient table. The mean irradiance level was 42 (±3) µmol photons·m^{-2}·s^{-1} and a 16/8 hours light/dark cycle was used. All the cultures were grown in 100 ml glass flasks with 75 ml medium. Medium was prepared from seawater collected from the French coast of the English Channel. The seawater was filtered and autoclaved at 120 °C for 15 min. After cooling nutrients (nitrate, phosphate, trace metals and vitamins) were added under a laminar flow cabinet. The chemical composition of the medium resembles media used by Guillard [14] and Keller et al. [15]. A detailed description of the medium used is given in table 3.

The *C. leptoporus* strain AS31 used at the AWI was grown in sterile filtered (0.2 µm) seawater enriched with 100 µM nitrate and 6.25 µM phosphate and with trace metals and vitamins according to f/2 [16]. The incident photon flux density was 350 µmol photons·m^{-2}·s^{-1} and a 16/8 hour light/dark cycle was applied. Experiments were carried out at 15 °C. Cells were acclimated to experimental conditions for approximately 10 generations and grown in dilute batch cultures [17]. Low cell density at harvest (in general less than 6000 cells per ml) resulted in less than 8% DIC (dissolved inorganic carbon) consumption (i.e. DIC consumed by the cells at the end of experiment) and a shift in pH of not more than 0.06 units. Ca^{2+} consumption was in all experiments less than 1%. Therefore, a significant subsequent shift of the Ca isotopic composition of the seawater during the experiment due to Raleigh fractionation caused by the preferential uptake of light Ca isotopes into the precipitated $CaCO_3$, as observed by Teichert et al. [18], can be excluded. CO_2 levels were adjusted by adding calculated amounts of 1 M HCl or 1 M NaOH to the medium. In order to prevent gas exchange with the atmosphere 2.4 l borosilicate flasks were filled without headspace and

closed with teflon lined screw caps. For the determination of growth rate, however, regular sampling for cell counts was required. The maximum headspace created by this sampling is 6 ml and the resultant air-water CO_2 equilibration of 3% shift in CO_2aq concentration is negligible. Samples for alkalinity measurements were filtered (approx. 0.6 µm), poisoned with 1 ml of a $HgCl_2$ solution (35 g l^{-1}) and stored in 300 ml borosilicate flasks at 0°C. DIC samples were sterile filtered (0.2 µm) and stored in 13 ml borosilicate flasks free of air bubbles at 0° C. Total alkalinity was calculated from linear Gran plots [19] after duplicate potentiometric titration [20, 21] and DIC was measured photometrically [22] in triplicate. The carbonate system was calculated from temperature, salinity, and the concentrations of DIC, total alkalinity and phosphate, using the program CO_2sys [23]. Equilibrium constants of Mehrbach et al. [24] refitted by Dickson and Millero [25] were chosen.

Culture material for calcium isotope measurements was harvested in the late exponential growth phase. To remove salt, samples were washed and centrifuged; pellets were dried at 60 °C for 48 hours and subsequently stored at room temperature.

2.2. Sample preparation

The coccolith samples were transferred into acid-pre-cleaned PP-reaction vials and bleached for 24 h in a 10% NaClO solution (~1% active chlorine). During the bleaching the samples were ultrasonicated several times. Then the bleach was removed and the samples were washed 6 times in ultra pure water, with pH adjusted to 8-9 by the addition of NH_4OH-solution to prevent partial dissolution of the coccolith carbonate. Afterwards the samples were dissolved in 2.5 N HCl. Aliquots of the solution, corresponding to 300-400 ng Ca were mixed with a ^{43}Ca-^{48}Ca-double spike for the correction of isotope fractionation during the data acquisition in the mass spectrometer.

2.3. Ca isotope analysis

Calcium isotope ratios were determined on a Finnigan Triton T1 thermal-ionization-mass-spectrometer at the IFM-GEOMAR Kiel, following the method described in [26]. The isotope variations of Ca are expressed as $\delta^{44/40}$Ca values ($\delta^{44/40}$Ca [‰ SRM915a] = $(({}^{44}Ca/{}^{40}Ca)_{sample}/({}^{44}Ca/{}^{40}Ca)_{SRM\ 915a} -1)\cdot 1000$), as proposed by [27] and as δ^{mu}Ca [ppm/amu SRM915a] = $(({}^{a}Ca/{}^{b}Ca)_{sample}/({}^{a}Ca/{}^{b}Ca)_{SRM\ 915a} -1)\cdot (a/((b+1)\cdot(a-b))\cdot 10^6)$ (with ^{a}Ca and ^{b}Ca being the respective Ca isotopes) [28], in order to increase the comparability with Ca isotope data based on Ca isotope ratios other than ^{44}Ca/^{40}Ca (e.g. ^{44}Ca/^{42}Ca). $\Delta^{44/40}Ca_{(Seawater\text{-}NistSRM\ 915a)}$=1.88‰, $\Delta^{mu}Ca_{(Seawater\text{-}NistSRM\ 915a)}$ = 470ppm/amu. The average $2\sigma_m$ of our samples is

0.12 ‰ (30 ppm/amu) determined by repeated aliquot measurements of various sample materials.

3. Results

The Ca isotope values of the four investigated coccolithophore species *Syracosphaera pulchra, Calcidiscus leptoporus, Umbilicosphaera foliosa, and Helicosphaera carteri* (figure 1, table 1) are in the range of published results of marine biogenic calcites, cf.[2, 3, 8, 28-31]. The relation of temperature dependent Ca isotope fractionation of *S. pulchra* and *H. carteri* are given in equations 1-3. The uncertainties in the equations represent 2 σ-errors. We calculated independent equations for two distinct arrays defined by *S. pulchra*, differing in temperature sensitivity by a factor of about 10. For *C. leptoporus* and *Umbilicophaera foliosa*, no equations are shown, because the temperature range is not large enough to define a reliable temperature dependence.

S. pulchra (filled triangles):

$\delta^{44/40}$Ca (‰SRM915a)= 0.05±0.22 + (0.029±0.013)·T (°C) ; R^2=0.84, p<0.001, n=6 (1a)

$10^6 \cdot \ln(\alpha_{mu})$ (ppm/amu)= -494±64 + (8.0±3.4)·T (°C) (1b)

S. pulchra (strong T dependence; open triangles)

$\delta^{44/40}$Ca (‰SRM915a)= -3.23±0.37 + (0.27±0.05)·T (°C) ; R^2=0.99, p<0.05, n=3 (2a)

$10^6 \cdot \ln(\alpha_{mu})$ (ppm/amu)= -1370±218 + (73±14)·T (°C) (2b)

Helicosphaera carteri:

$\delta^{44/40}$Ca(‰SRM915a) = 0.3±0.1 + (0.005±0.005)·T (°C) ; R^2=0.35, p<0.09, n=9 (3a)

$10^6 \cdot \ln(\alpha_{mu})$ (ppm/amu) = -425±25 + (1.4±1.4)·T (°C) (3b)

For calculation of equations 3a and 3b one data point was excluded (open square in figure 1) because this data point is most likely an analytical artifact. The temperature sensitivity of *Helicosphaera carteri* is small and not significant within the analytical uncertainty. The temperature dependence of *S. pulchra* is identical within analytical uncertainty to the previously determined temperature dependence of *E. huxleyi* (0.027 ± 0.003‰/°C) [9] and similar to the planktonic foraminifera *Orbulina universa* (0.019 ± 0.003‰/°C), inorganic aragonite (0.015±0.002‰/°C) [32], biogenic calcites (0.026±0.01 ‰/°C) and biogenic aragonite (0.017±0.006‰/°C) [28].

The response of calcium isotopes in *C. leptoporus* to changes in carbonate concentration is insignificant within analytical precision (Equation 4) (figure 2, table 2).

Calcidiscus leptoporus:

$1000 \ln(\alpha) = -1.22 \pm 0.14 + (-5.5 \pm 8.2) \cdot 10^{-4} \cdot CO_3^{2-}$ (µmol/kg); $R^2=0.31$, $p<0.25$; $N=6$ (4a)

$10^6 \cdot \ln(\alpha_{mu}) = -329 \pm 78 + (0.15 \pm 0.22) \cdot CO_3^{2-}$ (µmol kg^{-1}) (4b)

This result is similar to previous findings for *E. huxleyi* [9] and *O. universa* [32], but different from trends observed in inorganically precipitated calcite [13], which exhibits a strong $\delta^{44/40}$Ca-$[CO_3^{2-}]$-dependence.

4. Discussion

Coccoliths of the four investigated coccolithophore species *C. leptoporus*, *U. foliosa, S. pulcha* and *H. carteri* show Calcium isotope values lying in the same range as most previously published marine calcite samples. The observed small temperature dependences are similar to previously reported data on *E. huxleyi*. As well, the lack of a $[CO_3^{2-}]$ sensitivity of *C. leptoporus* (Fig. 2), in contrast to inorganic calcite [13], resembles observation on *E. huxleyi*. These findings might suggest that similar mechanisms are involved in Ca isotope fractionation in different coccolithophores. For *E. huxleyi* it was proposed that the chemical properties inside the coccolith vesicle are decoupled from the seawater and that inorganic Ca isotope fractionation processed at the crystal surface can not account for the observed Ca isotope values in coccoliths of *E. huxleyi*, because of Ca pathways and budget inside the cell [9]. Instead dehydration processes during Ca incorporation at the plasma membrane were found to be most likely the effective fractionation processes in *E. huxleyi*.

Beside this general mechanism, additional factors affecting Ca isotope fractionation might be indicated by the bimodal Ca isotope fractionation response to temperature in *S. pulchra*. The first defined temperature slope resembles most previously defined temperature dependences of marine carbonates like e.g. *Orbulina universa* or *E. huxleyi,* while the second array exhibits an about 10 fold stronger temperature dependence (0.27±0.05‰/°C), resembling the planktic foraminifer *Globigerinoides sacculifer* (0.24‰/°C) [12]. The array with the strong temperature dependence is marked by only two data points; it is therefore in principle possible and can not be completely ruled out that this array is an analytical artefact due to contamination during the cleaning procedure or sample preparation. Hence this

observation of a bimodal temperature slope is pending further verification. However, there is also no direct evidence which justify to completely ignoring these data, in particular because the presence of bimodal temperature response would have a rather large impact on the proxy use of coccolithophores and possibly other marine calcifiers. Large differences in intraspecific Ca isotope fractionation would strongly affect the reliability of proxy applications (not only Ca isotopes, but also element/Ca ratios, which are also like Ca determined by cellular ion transport mechanisms in coccolithophores [33]), but would also shed light on Ca transport and biomineralisation processes. The two clones of *S. pulchra* used for this experiment were isolated from the same water mass, therefore genetic differences seem to be unlikely for this strong intraspecific variation. Instead possible mechanisms might be associated with changes in the internal Ca usage including the use of internal Ca storage pools. The possible association of a steep Ca isotope-temperature slope in foraminifera and internal Ca pool was earlier proposed by Gussone et al. [32] and later supported by Marriott et al. [34].

The presence of a bimodal Ca isotope-temperature response in one species might help to explain discrepant observations made for temperature dependent Ca isotope fractionation in different previous studies. For example a strong temperature dependence in *G. sacculifer* was found in several studies [8, 11, 12] while other studies [30, 31] did not demonstrate such a dependence, although such a strong dependence should be visible in spite of limited analytical precision using $\delta^{44/42}$Ca on a MC-ICPMS as well as poorly defined temperatures due to e.g. the use of undated coretop samples.

The observation that coccolith Ca isotope composition of several coccolithophore species is similar to previously published Ca isotope values of marine carbonates and cover only a limited range supports earlier assumptions of a rather homogeneous Ca isotopic composition of marine carbonates of about -1.3‰ $1000 \cdot \ln\alpha_{44/40}$ (-325 ppm/amu) [2, 7]. This confirmation is important for a reliable estimation of the Ca sink in the ocean, to model its isotopic budget over time. As a consequence, species specific calcium isotope fractionation seems to be unlikely to cause the observed differences in calcium isotope values of Quarternary coccolith oozes [2, 6-8].

5. Summary and Conclusions

The $\delta^{44/40}Ca$ in the coccoliths of the five coccolithophore species tested so far, *Calcidiscus leptoporus, Helicosphaera carteri, Syracosphaera pulchra, Umbilicosphaera foliosa* and *Emiliania huxleyi,* is relatively uniform. The temperature dependences of Ca isotope fractionation resemble those of other carbonate precipitating organisms. *C. leptoporus* shows no significant $[CO_3^{2-}]$- $\delta^{44/40}Ca$ response similar to *E. huxleyi* and *O. universa*, which is in contrast to inorganic calcite. The similar calcium isotope values of the investigated coccoliths indicate that the calcification processes of the vesicle-based coccolith formation of these coccolithophores might be generally rather similar. Additional Ca transport and biomineralisation processes might be responsible for the development of a steep temperature dependence in *S. pulchra*. Apart from the possibly bimodal temperature response of *S. pulchra* the calcium isotope fractionation of coccolithophores does not show large species specific differences and thus coccolithophores might be regarded as a group with respect to calcium isotope fractionation. This finding helps better defining the isotopic composition of the Ca sink of the ocean.

6. Acknowledgements

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7. References

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Figure captions

Figure 1: Temperature dependent Ca isotope fractionation in different coccolithophore species.
Syracosphaera pulchra, Calcidiscus leptoporus, Umbilicosphaera foliosa, Helicosphaera carteri show similar Ca isotope values as *E. huxleyi* [9], [33]. Shaded areas mark the 95% confidence bands. One data point of *H. carteri* (open square) was not used for linear regression. *S. pulchra* seems to exhibit a bimodal temperature response behaviour, a weak temperature dependence (filled triangles), like *E. huxleyi* as well as a strong temperature dependence (open triangles) similar to *G. sacculifer* (slope indicated by the dashed line).

Figure 2: Ca isotope fractionation as a function of carbonate concentration of *C. leptoporus* (15°C) compared to published data of inorganic calcite (21°C) [13], *O. universa* (22°C) [32], *E. huxleyi* (20°C) [9]. The response of Ca isotope fractionation on changes in CO_3^{2-} of *C. leptoporus* is similar to *E. huxleyi* and *O. universa*, showing no significant dependence between CO_3^{2-} and Ca isotope fractionation, in contrast to the inorganic calcite of [13]. Shaded areas represent 95% confidence bands.

Plate I: Typical morphologies of the coccolithophore species examined in this study
1: Scanning electron micrograph of a *E. huxleyi* coccosphere. The coccoliths exhibit the A-type morphology. Water sample, N. Atlantic, R/V Meteor cruise 42-4B, station US 1B.
2: Scanning electron micrograph of a *S. pulchra* coccosphere. This typical specimen displays endothecal and exothecal coccoliths. Coccoliths surrounding the flagellar pole are spine bearing. Water sample, N. Atlantic, off the Canary Islands, R/V Poseidon cruise P233B, station 3.
3: Scanning electron micrograph of a *C. leptoporus* coccosphere. Culture sample (NS 10-2), S. Atlantic, off South Africa, R/V Agulhas cruise MARE 2.
4: Scanning electron micrograph of a *H. carteri* coccosphere. The helicoliths show the typical spiral arrangement and possess enlarged flanges in the circumflagellar coccoliths. The central area of this specimen shows the typical morphology with two aligned slits, which are separated by a bar. Water sample, N. Atlantic, Portuguese shelf, R/V Andromeda cruise CODENET 2, station 6.

5: Scanning electron micrograph of two *U. foliosa* coccospheres. U. foliosa cells are typically found in clusters of up to four cells. Water sample, western Mediterranean, Alboran Sea, R/V Hesperides cruise MATER 2, station 69.

Scalebar: Fig. 1 - 2µm, Figs 2-5 - 5µm.

Tables

Table 1: Temperature dependent Ca isotope fractionation of cultured coccolithophore species:

Sample	Origin	T [°C]	$\delta^{44/40}$Ca (‰SRM915a)	1000ln(α)	δ^{mu} Ca (ppm/amu)	$10^6 \ln\alpha_{mu}$
Helicosphaera carteri						
NS 10-8-1	S. Atlantic	13.7	0.38	-1.50	103	-402
NS 10-8	S. Atlantic	15.5	0.36	-1.52	96	-408
NS 10-8	S. Atlantic	16.7	0.38	-1.49	103	-401
NS 10-8	S. Atlantic	19.6	0.43	-1.44	117	-388
NS 10-8	S. Atlantic	23.1	0.41	-1.47	111	-394
NS 8-4	S. Atlantic	13.7	0.33	-1.55	90	-415
NS 8-4	S. Atlantic	15.5	0.39	-1.49	104	-401
NS 8-4	S. Atlantic	16.7	0.76	-1.12	204	-301
NS 8-4	S. Atlantic	19.6	0.43	-1.45	114	-390
NS 8-4	S. Atlantic	23.1	0.38	-1.49	103	-402
Syracosphaera pulchra						
GK 7	W. Mediterranean	13.7	0.49	-1.39	132	-373
GK 7	W. Mediterranean	15.5	1.04	-0.84	280	-224
GK 7	W. Mediterranean	16.7	0.51	-1.37	137	-368
GK 7	W. Mediterranean	19.6	0.57	-1.31	152	-352
GK 7	W. Mediterranean	23.1	0.78	-1.10	210	-294
GK 17	W. Mediterranean	15.5	0.51	-1.37	136	-369
GK 17	W. Mediterranean	16.7	1.30	-0.58	349	-156
GK 17	W. Mediterranean	19.6	0.59	-1.29	159	-345
Calcidiscus leptoporus						
NS 10-2	S. Atlantic	17	0.41	-1.47	109	-395
NS 10-2	S. Atlantic	19	0.48	-1.40	129	-376
NS 10-2	S. Atlantic	23	0.43	-1.45	115	-390
ASM 31	W. Mediterranean	19	0.42	-1.45	114	-391
Umbilicosphaera foliosa						
ESP6MI	W. Mediterranean	19	0.46	-1.42	124	-381
ESP6MI	W. Mediterranean	23	0.49	-1.38	133	-372

Column 5: fractionation factor $\alpha = ({}^{44}Ca/{}^{40}Ca)_{solid}/({}^{44}Ca/{}^{40}Ca)_{fluid}$. Fluid is seawater ($\delta^{44/40}$Ca: 1.88 ‰ SRM 915a).

Column 6: δ^{mu}Ca [ppm/amu SRM915a] = $(({}^{a}Ca/{}^{b}Ca)sample/({}^{a}Ca/{}^{b}Ca)SRM915a\ -1)\cdot(1/(a-b))\cdot 10^6)$ and a, b being the masses of the respective Ca isotopes.

Column 7: fractionation factor $\alpha_{mu}=\alpha^{a/((b+1)\cdot(a-b))}$ (assuming equilibrium isotope fractionation) with $\alpha=({}^{a}Ca/{}^{b}Ca_{cc})/({}^{a}Ca/{}^{b}Ca_{fluid})$ and a, b being the masses of the respective Ca isotopes.

Table 2: Ca isotope fractionation of *C. leptoporus* in response to $[CO_3^{2-}]$changes

Sample	Origin	$[CO_3^{2-}]$ (µmol/kg)	$\delta^{44/40}$Ca (‰SRM915a)	1000ln(α)	δ^{mu} Ca (ppm/amu)	$10^6 \ln\alpha_{mu}$
Calcidiscus leptoporus (15°C)						
1 ASM31 360	W. Mediterranean	214	0.53	-1.35	142	-362
2 ASM31 360	W. Mediterranean	219	0.54	-1.34	145	-360
1 ASM31 225	W. Mediterranean	162	0.63	-1.25	169	-336
2 ASM31 225	W. Mediterranean	166	0.51	-1.37	137	-368
1 ASM31 679	W. Mediterranean	106	0.62	-1.26	167	-338
2 ASM31 679	W. Mediterranean	105	0.57	-1.31	153	-352

Columns 5, 6, 7: details for calculation of α, δ^{mu}Ca and α_{mu} see captions of Table 1

Table 3: Composition of K medium. Stock solutions are numbered 1-5. Each stock is made such that the addition of 1ml/litre yields the final concentration in the medium.

Additions	Final concentration in medium (µM)	Comments
(1) KNO_3	884	same as f/2
(2) NH_4Cl	10	addition to f/2
(3) Na_2 ortho-PO_4	36	same as f/2 (K uses organic form)
(4) Trace metals:		
FeEDTA *	11.7	f/2 uses $FeCl_3$
$MnCl_2.4H_2O$	0.9	same as f/2
$ZnSO_4.7H_2O$	0.03	same as f/2
$CoSO_4.7H_2O$	0.05	same as f/2
$Na_2MoO_4.2H_2O$	0.08	same as f/2
$CuSO_4.5H_2O$	0.01	one half f/2 level
$Na_2EDTA.2H_2O$	100	order of magnitude higher than f/2
Na_2SeO_3	0.01	addition to f/2
(5) Vitamins		
Thiamin-HCl	0.3	same as f/2
Biotin	0.0021	same as f/2
B12	0.00037	same as f/2
Seawater	to 1 litre	

1-5 made with reagent grade chemicals and HPLC grade water.

All solutions filter-sterilised through 0.2µm membrane filters.

1-4 stored at 4°C.

5 stored frozen at -20°C.

* ethylenediamine tetra-acetic acid.

Figure 1

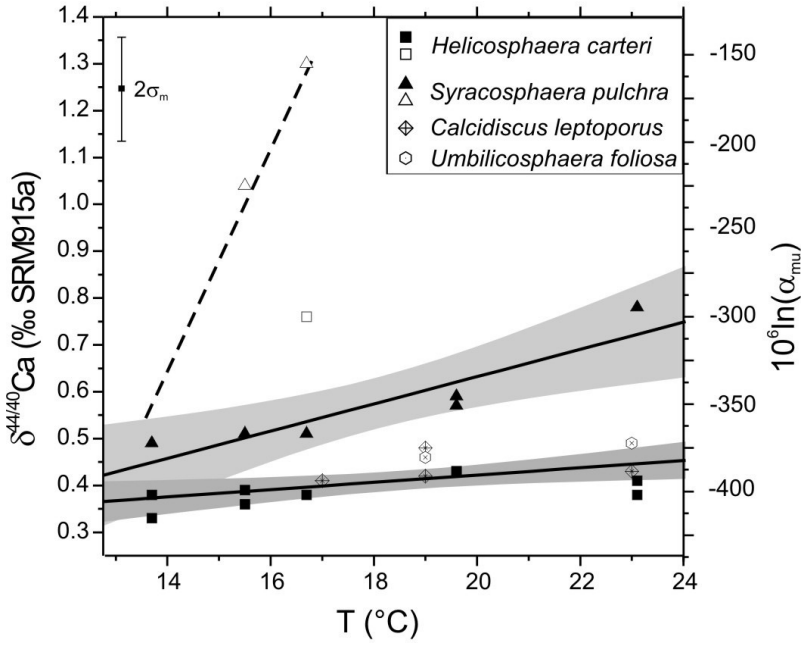


Figure 2

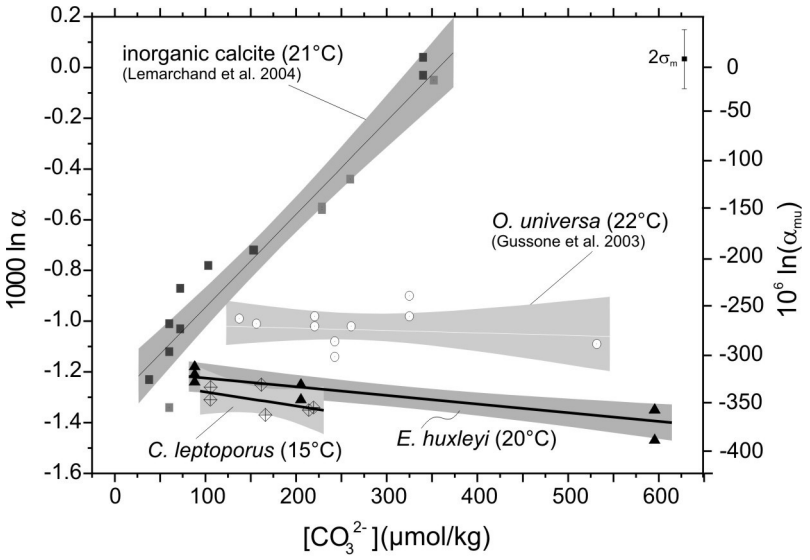
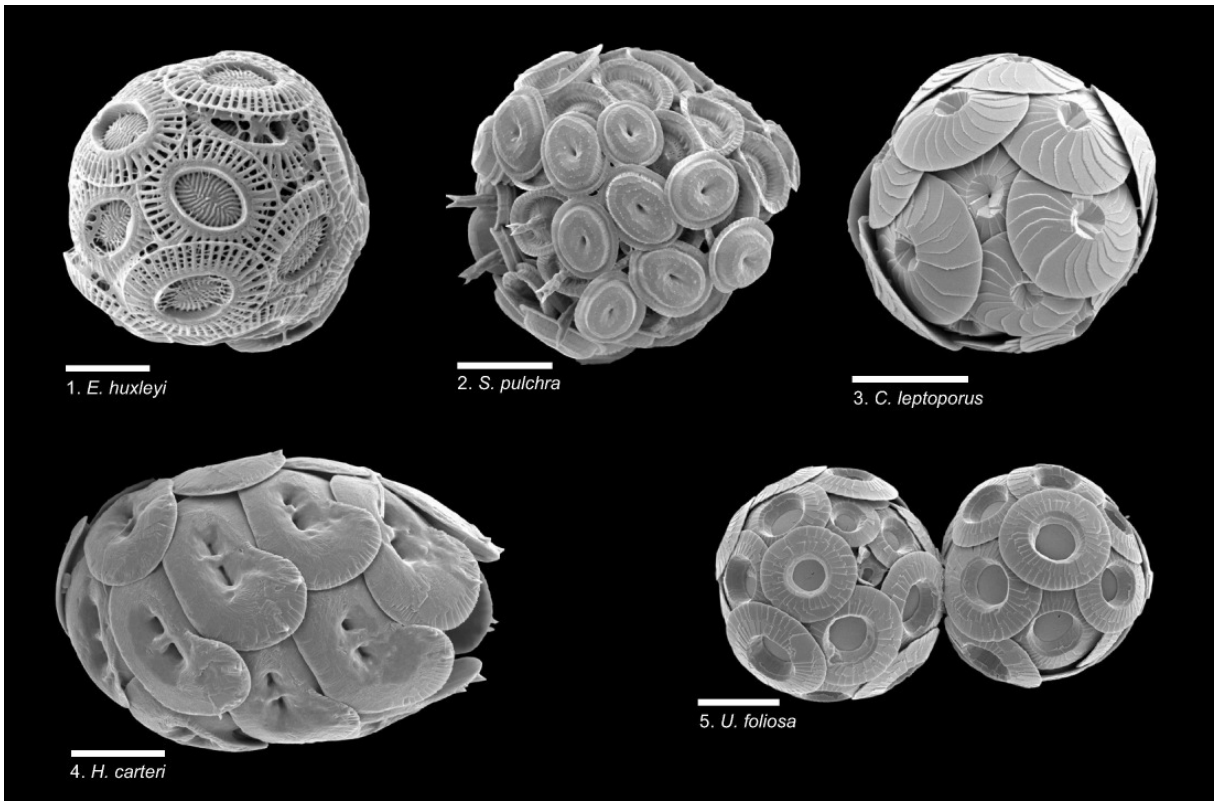


Plate I



Ca isotope fractionation during coccolith formation in *Emiliania huxleyi*

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Abstract

The calcium isotopic composition of marine calcifiers has gained special interest with respect to the global calcium cycle and its use as a sea surface temperature proxy. Research in this area has focused on foraminifera. Here we investigated the coccolithophore alga *Emiliania huxleyi*, one of the most productive pelagic calcite producers. The $\delta^{44/40}$Ca values of coccoliths from cultured specimens covered a range of 0.3-0.7‰ (relative to the NIST standard SRM915a). This range is similar to that observed in several foraminiferal species and coccolith oozes, suggesting that marine biogenic calcite possesses a rather homogeneous isotopic composition. Like other carbonates coccoliths of *E. huxleyi* are depleted in heavy ^{44}Ca (enriched in ^{40}Ca) compared to seawater. The calcium isotope fractionation in *E. huxleyi* decreases with increasing temperature (0.027‰ °C^{-1}), leading to a positive $\delta^{44/40}$Ca-temperature relation. This apparent temperature effect is not caused by temperature-induced changes in growth and calcification rates. The slope of the temperature curve is similar to that observed in the planktic foraminifer *Orbulina universa* (0.019‰ °C^{-1}) and inorganically precipitated aragonite (0.015‰ °C^{-1}) and calcite (0.015‰ °C^{-1}). In recently published inorganic precipitation experiments a strong dependence of calcium isotope fractionation on carbonate ion concentration was observed. In *E. huxleyi* we found only a weak correlation between $\delta^{44/40}$Ca and carbonate ion concentration of seawater. Possible mechanisms of calcium isotope fractionation and its dependence on temperature in *E. huxleyi* are discussed. It is concluded that the location of calcium isotope fractionation is not the coccolith surface. We propose two possible alternative locations: 1) the entrance of a calcium selective ion channel in the plasmamembrane and 2) cytosolic calcium buffer proteins.

Introduction

Calcium is one of the major divalent cations in seawater. Its importance for marine biota spans a great variety of aspects, from regulation of cell physiology to shell building. The formation of calcareous shells, as brought about by productive pelagic calcifiers like foraminifera and coccolithophores and the subsequent sedimentation of those shells links the marine calcifying biota to the global calcium cycle (Skulan et al. 1997).

Calcium has six stable isotopes, (^{40}Ca, ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{46}Ca and ^{48}Ca), with ^{40}Ca being the most abundant (96.941%). The calcium isotopic fractionation performed by marine biota has gained special interest in recent years. Skulan et al. (1997) for instance showed that marine organisms fractionate against the heavier isotopes. Moreover the study of Skulan et al. (1997) indicates that calcium becomes isotopically lighter as it moves through food chains.

A temperature dependent calcium isotopic fractionation in foraminiferal calcite was suggested (Zhu and MacDougall 1998) and subsequently confirmed (Nägler et al. 2000). The latter study provides a temperature versus $\delta^{44/40}$Ca calibration curve of the surface dwelling planktonic foraminifer species *Globigerinoides sacculifer*. This suggested that $\delta^{44/40}$Ca could be used as a sea surface temperature (SST) proxy. In a case study, in which the common proxies δ^{18}O and Mg/Ca were substantially altered by other climatic factors, mainly salinity and chemical composition of the seawater, the applicability of $\delta^{44/40}$Ca as a SST proxy was further reinforced (Gussone et al. 2004). However, there appears to be no uniform slope of the $\delta^{44/40}$Ca temperature relationship in foraminifera (Gussone et al. 2003; Nägler et al. 2000) and in core-top studies no significant temperature dependence of $\delta^{44/42}$Ca could be found for a variety of species (Chang et al. 2004; Sime et al. 2005).

Besides foraminifera coccolithophores are an important group of organisms playing a key role in the marine calcium cycle. They are major marine calcite producers and their calcareous plates, or coccoliths, are estimated to compose 60 to 90% of pelagic calcareous sediments, which are the main Ca sink of the ocean, since the Jurassic (Berger and Roth 1975). This means that it is critical to understand the controls on the isotopic composition of coccolith calcite when modeling the calcium isotope budget of the ocean (DePaolo 2004; Fantle and DePaolo 2005; Heuser et al. 2005; Schmitt et al. 2003; Skulan et al. 1997; Zhu and MacDougall 1998). In addition to that the coccolithophore sedimentary record could possibly provide useful information about long term changes in ocean chemistry (De La Rocha and DePaolo 2000; DePaolo 2004). However, $\delta^{44/40}$Ca values of pure coccolithophore calcite, obtained from controlled laboratory cultures or from environmental samples, are rare. The

only value published to date was measured at 16°C (De La Rocha and DePaolo 2000). It is unknown, therefore, whether coccolithophore $\delta^{44/40}Ca$ values are temperature dependent, as observed in foraminiferal calcite and inorganic precipitates (Gussone et al. 2003; Marriott et al. 2004; Nägler et al. 2000). Moreover the latter studies did not rule out a possible influence of growth and calcification rate on the $\delta^{44/40}Ca$ signal. Since growth and calcification rate change inevitably with temperature the question arises whether putative temperature effects are due to altered growth and calcification rate. The major objective of this study was to investigate whether coccolithophore $\delta^{44/40}Ca$ values are temperature dependent and whether there is a growth and calcification rate dependency.

In a recent study it was shown that calcium isotope fractionation in inorganically precipitated calcite is rate dependent and sensitive to changes in carbonate ion concentration (Lemarchand et al. 2004). *Emiliania huxleyi* decreases its calcification rate in response to decreasing carbonate ion concentration (increasing CO_2 concentration) (Zondervan et al. 2002). Hence, it appears likely that calcium isotope fractionation in coccolithophores is influenced by carbonate chemistry. We tested this possibility by growing *E. huxleyi,* one of the most prolific marine calcite producers, in seawater containing 88 to 600 µmol kg^{-1} carbonate ion. Besides playing a large role in the global calcium cycle, *E. huxleyi* has a process of biomineralisation that is relatively well understood and thus well suited for the investigation of calcium isotope fractionation by coccolithophores.

Material and methods

Monospecific cultures of *Emiliania huxleyi* (strain PML B92/11) were grown in sterile filtered (0.2 µm) seawater enriched with 100 µmol L^{-1} nitrate, 6.25 µmol L^{-1} phosphate and trace metals and vitamins as in F/2 media (Guillard and Ryther 1962). Cultures were grown under a 16/8 hour light/dark cycle. Experiments were carried out at various temperatures from 5 to 20 °C and various light intensities from 22 to 180 µmol photons m^{-2} s^{-1} in an adjustable incubator (Rubarth Apparate GmbH, Germany). Cells were pre-adapted to experimental conditions for approximately 12 generations and grown in dilute batch cultures in triplicate (Zondervan et al. 2002). Low cell densities even at the termination of the experiments resulted in the consumption of less than 5% dissolved inorganic carbon (DIC). Calcium consumption was less than 1 % and hence changes of calcium isotope composition of the fluid during the course of the experiment were negligible. Different CO_2 levels were attained by adding calculated amounts of 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH to the medium. In order to prevent gas exchange with the atmosphere, the 2.4 L borosilicate flasks were filled, leaving no headspace and closed with teflon lined screw caps.

Samples for alkalinity measurements were filtered (approx. 0.6 µm), poisoned with 1 ml 35 g L^{-1} $HgCl_2$, and stored in 300 ml borosilicate flasks at 0°C. DIC samples were sterile filtered (0.2 µm) and stored in 13 ml borosilicate flasks free of air bubbles at 0°C. Total alkalinity was calculated from linear Gran plots (Gran 1952) after potentiometric titration (in duplicate) (Bradshaw et al. 1981; Brewer et al. 1986). DIC was measured photometrically (Stoll et al. 2001) in triplicate. The carbonate system was calculated from temperature, salinity, and the concentrations of DIC, total alkalinity and phosphate, using the DOS-program CO_2sys (Lewis and Wallace 1998). Equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987) were used. Samples for determination of total particulate carbon (TPC) and particulate organic carbon (POC) were filtered on precombusted (12 hours, 500°C) GF/F-filters (approx. 0.6 µm) and stored at -20°C. Prior to analysis, the POC filters were fumed for two hours with a saturated HCl solution to remove all inorganic carbon. TPC and POC were subsequently measured on a Carlo Erba NA-1500 Analyzer. Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. For determination of cell density samples were taken at the beginning and the end of experiment and counted immediately after sampling using a Coulter “Multisizer II”. Growth rate (µ) was calculated as:

$$\mu = (\ln c_1 - \ln c_0)\, \Delta t^{-1} \qquad (1)$$

where c_0 and c_1 are the cell concentrations at the beginning and the end of experiment, respectively, and Δt is the duration of incubation in days.

Inorganic carbon production, i.e. calcification rate (P, pg PIC $cell^{-1}$ d^{-1}) was calculated according to

$$P = \mu * (\text{cellular inorganic carbon content}) \qquad (2)$$

Samples for calcium isotope measurements were filtered on acid rinsed polycarbonate filters (0.2 µm), dried at 60 °C for 48 hours and stored in plastic petri-dishes. Prior to calcium isotope analysis, coccolith samples were bleached in a sodium hypochlorite-solution (~1% active chlorine) to remove organic components. Afterwards, the samples were washed in ultra pure water adjusted to pH 8-9 by the addition of NH_4OH to prevent dissolution, in methanol (CH_3OH), and finally six times in ultra pure water.

The $^{44}Ca/^{40}Ca$-ratio of the coccolith $CaCO_3$ was determined on a Finnigan Triton T1 Thermal Ionization Mass Spectrometer at the Leibniz Institute for Marine Science, Kiel using a $^{43}Ca/^{48}Ca$-Double-Spike-technique, following the description of Heuser et al. (2002). Isotope values are reported as $\delta^{44/40}Ca$ (‰) relative to the SRM 915a carbonate standard provided by NIST: $\delta^{44/40}Ca$ (‰)= $((^{44}Ca/^{40}Ca)_{sample}/(^{44}Ca/^{40}Ca)_{standard}).-1)\cdot x\ 1000$. Average 2 σ_m of our data is about 0.12 ‰. It should be noted that the $\delta^{44/40}Ca$ notation is used for the sake of comparability with other studies. In addition to the $\delta^{44/40}Ca$ notation, we present isotope values as 1000 $\ln\alpha$ with $\alpha = (^{44}Ca/^{40}Ca)_{calcite}/(^{44}Ca/^{40}Ca)_{solution}$. The seawater the cultures were grown in had a $\delta^{44/40}Ca$ value of +1.88 ‰.

Results

The $\delta^{44/40}$Ca values measured in coccoliths show that *Emiliania huxleyi* fractionates against the heavier calcium isotope (Figure 1), producing calcite with a $\delta^{44/40}$Ca value that is more negative than that of the seawater it was grown from. The $\delta^{44/40}$Ca value of the seawater is +1.88 ‰, and the $\delta^{44/40}$Ca of coccolith calcite in the experiments ranged from +0.3 to +0.7 ‰. Coccolith $\delta^{44/40}$Ca values are also positively correlated to temperature (Figure 1). The following equation is obtained from linear regression:

$$\delta^{44/40}\text{Ca } ‰ = 0.027\ T\ (°C) + 0.20 \qquad (r^2 = 0.96) \qquad (3)$$

No relationship between calcium isotope fractionation and photon flux density was observed (Figure 2), however calcium isotope fractionation appeared to vary with carbonate ion concentration and the calcite saturation state (Figure 3), which itself varies with the carbonate ion concentration.

Since carbonate chemistry was altered by acid / base additions (see material and methods) the range in carbonate ion concentration from 88 to ~600 µmol kg^{-1} carbonate ion corresponds to a range in pH from 7.87 to 8.83 on the NBS scale. Cell growth rates increased with increasing temperature and with increasing photon flux density (Figure 4). Calcification rates also increased with increasing temperature and with increasing photon flux density (Figure 5). The calcification and growth rate data depicted in Figures 4 and 5 were obtained from the same experiments as the $\delta^{44/40}$Ca data depicted in Figures 1 and 2. Each data point is the mean value of three culture experiments.

Discussion

Implications for proxy use and budget calculations

Emiliania huxleyi fractionates against the heavier isotopes of calcium to a degree similar to that of other marine organisms c.f. (Skulan et al. 1997). The $\delta^{44/40}$Ca measured in this study (Figure 1) is also in good agreement with a previously published value of cultured *E. huxleyi* at 16 °C (De La Rocha and DePaolo 2000) and several carbonate oozes (De La Rocha and DePaolo 2000; DePaolo 2004). In contrast, other coccolith oozes show considerably lighter $\delta^{44/40}$Ca values (Zhu and MacDougall 1998). The reason for this discrepancy is not clear yet. Culturing artifacts, diagenetic alteration or interspecific variation in the calcium isotope composition of coccolithophores may account for this discrepancy.

E. huxleyi shows a clearly temperature dependent calcium isotope fractionation. The slope of the temperature curve (0.027‰ $°C^{-1}$) (Figure 1) is similar to that observed for the planktic foraminifer *O. universa* (0.019±0.003‰ $°C^{-1}$), for abiogenically precipitated aragonite (0.015±0.002‰ $°C^{-1}$) (Gussone et al. 2003), abiogenically precipitated calcite (0.015±0.013‰ $°C^{-1}$) (Marriott et al. 2004) and biogenic aragonite (0.017±0.006‰ $°C^{-1}$) (Gussone et al. 2005) (errors given are 2σ errors). Changing temperature introduces subsequent changes on growth and calcification rate (Figures 4 and 5). One basic question is to what extent temperature-induced changes in growth and calcification rates can account for the observed change in calcium isotope fractionation. In order to unravel temperature and rate effects we conducted experiments with varying light intensities (20-180 µmol m^{-2} s^{-1}) and constant temperature (17°C) (Figure 2). In these experiments changes in $\delta^{44/40}$Ca were not observed (Figure 2), although calcification and growth rates varied by factors of 10 and 6 respectively (Figures 4 and 5). This indicates that calcium isotope fractionation in *E. huxleyi* does not depend on growth and calcification rates, but rather directly on temperature.

A further mechanism which has to be considered is that temperature directly influences the carbonate ion concentration via speciation of the components of the carbonate system, i.e. CO_2, HCO_3^- and CO_3^{2-}. The carbonate ion concentration in seawater increases with rising temperature (Millero 1995), due to the temperature dependence of the dissociation constants. In inorganically grown calcite $\delta^{44/40}$Ca is positively correlated to carbonate ion concentration (Lemarchand et al. 2004). However, we observed only a negligibly small dependence of $\delta^{44/40}$Ca on carbonate ion concentration (Figure 3). This dependence is one order of magnitude smaller and opposite in sign compared to inorganically grown calcite (Lemarchand et al. 2004). The $\delta^{44/40}$Ca of this inorganically grown calcite increases by 1.3 ‰

when carbonate ion concentration is increased from 50 to 350 µmol L^{-1} (Lemarchand et al. 2004), whereas coccolith $\delta^{44/40}$Ca decreases by ca. 0.2 ‰ due to an increase in carbonate ion concentration from 88 to 600 µmol kg^{-1} (Figure 3).

Temperature proxies (based on element or isotope ratios in carbonates) currently in use, e.g. δ^{18}O and Mg/Ca, are also influenced by factors other than temperature. The main secondary influences complicating the use of δ^{18}O as temperature proxy are changes in the mean δ^{18}O of seawater, which is determined by the amount of continental ice and the local δ^{18}O due to oxygen isotope fractionation by evaporation / precipitation (Rostek et al. 1993; Shackleton 1974). Furthermore there are known effects of carbonate chemistry on foraminiferal δ^{18}O (Bijma et al. 1999; Spero et al. 1997; Zeebe 1999). The Mg/Ca ratio is affected by partial dissolution (Brown and Elderfield 1996) as well as salinity and pH changes (Lea et al. 1999). Therefore a more robust temperature proxy would help improving the reconstruction of past sea surface temperature. A good candidate could principally be the $\delta^{44/40}$Ca in coccoliths, because it is presumably solely controlled by temperature and the isotopic composition of the medium / seawater. However, given the shallow slope of the temperature dependence of $\delta^{44/40}$Ca in *E. huxleyi* and the current precision of 0.12 ‰ for replicate samples (see material and methods) this proxy would allow only for a temperature resolution of 5°C, which is not sufficient for the use as paleo-temperature proxy.

Apart from the use of $\delta^{44/40}$Ca as a paleo-temperature proxy, knowledge about calcium isotope fractionation in coccolithophores can help better defining the isotopic composition of the oceanic calcium sink. The data presented here confirm the average fractionation factor ($1000 \ln\alpha = -1.3$ ‰) that was observed to be associated with biological removal of calcium from the oceans (De La Rocha and DePaolo 2000; DePaolo 2004; Gussone et al. 2003; Skulan et al. 1997). The calcium isotopic composition of the inputs of calcium to the oceans resembles the composition of the outputs, i.e. biogenic calcium carbonate (DePaolo 2004). Therefore, the current calcium isotopic composition of seawater (De La Rocha and DePaolo 2000; Heuser et al. 2005; Schmitt et al. 2003) is assumed to represent a value close to steady state (DePaolo 2004). To further reinforce that assumption a survey of calcium isotopic composition in a range of cultured coccolithophore species is desirable, since coccolithophores are major marine calcite producers. Further studies using natural plankton samples and more sediment samples are clearly warranted as well.

Implications for cellular calcium transport and calcite precipitation

In inorganic calcium carbonate precipitation experiments it was shown that the fractionation of calcium isotopes is of the order of 1-2‰ (Gussone et al. 2003; Marriott et al.

2004). Fractionation by diffusion of calcium ions due to differences in the mass of the different calcium isotopes would yield values for isotope fractionation much larger than those measured, i.e. 20 ‰ (Marriott et al. 2004). To explain this Gussone et al. (2003) suggested that is was not Ca^{2+} that is diffusing, but instead much heavier calcium-aquocomplexes, suppressing the magnitude of isotope fractionation. However, this explanation requires a high number of coordinated water molecules surrounding each calcium ion (Lemarchand et al. 2004; Marriott et al. 2004).

Lemarchand et al. (2004) proposed a mechanism which describes the temperature dependence of the $\delta^{44/40}Ca$ of inorganically precipitated calcite in terms of a temperature dependence of the inorganic carbon species composition. Carbonate ion concentration in seawater increases with increasing temperature (Millero 1995). Thus, an increase in temperature leads to an increase in supersaturation with respect to calcite. This increase in supersaturation increases the rate of precipitation of calcite (Nielsen 1964) and was suggested to result in a decreased magnitude of isotope fractionation due to co-precipitation of un-equilibrated calcium from the solution (Lemarchand et al. 2004). The model of Lemarchand et al. (2004) predicts a strong dependence of $\delta^{44/40}Ca$ on supersaturation due to changes in carbonate ion concentration. However, this could not be observed in the case of *E. huxleyi*. On the other hand, since seawater is separated from the site of calcification by at least two membranes, the plasmamembrane and the coccolith-vesicle membrane (Brownlee and Taylor 2004) the carbonate chemistry of external seawater does not equal coccolith vesicle chemistry. However, we cannot currently monitor the chemical conditions of the coccolith vesicle, and such data are required for a direct comparison of our data (Figure 3) for the dependence of $\delta^{44/40}Ca$ on carbonate ion concentration with the data presented by Lemarchand et al. (2004).

A strong dependence of calcium isotope fractionation on precipitation rate was found for abiogenically precipitated calcite (Lemarchand et al. 2004). For such abiogenic calcites, the mineral precipitation rate is directly related to the chemical composition of the solution from which the crystal is precipitated. In calcite precipitation as it occurs in coccolithophores, intracellular crystal growth rates can also be influenced by additional factors such as light availability. We found no correlation between the $\delta^{44/40}Ca$ of produced coccoliths and light-induced changes in cell growth and calcification rates (Figures 2, 4 and 5). Since coccolithogenesis includes phases during which no crystal growth occurs (see also Stoll et al. (2002)), the calculated calcification rates are time averaged values. Hence, calcification rates as determined in this study are not directly comparable to growth rates obtained from

inorganic precipitation experiments. The above mentioned differences between abiogenic and vesicle-based calcite formation make it difficult to directly compare data obtained from culture experiments with data from inorganic precipitation experiments. Therefore our light induced growth rate experiment alone is not suitable to decide whether calcium isotope fractionation in *E. huxleyi* is controlled by the processes described by Lemarchand et al. (2004).

Most attempts to explain $\delta^{44/40}$Ca data of biogenic calcite are solely based on inorganic precipitation but because cellular processes play such a key role in calcification of coccoliths the impact of cell physiology on isotope fractionation must be taken into account. In the case of *E. huxleyi,* calcium uptake for calcification is thought to be accomplished by transmembrane transport of calcium from seawater into the cytosol via Ca-channels and from the cytosol into the coccolith vesicle via Ca-ATPases (Brownlee and Taylor 2004). The membrane of the coccolith vesicle closely envelopes the growing coccolith and so does not contain a substantial reservoir of fluid. Judging from transmission electron micrographs the coccolith vesicle volume is at most double the coccolith volume at any given time (Young, J. and Henriksen, K., pers. comm.) and is here assumed to have a maximum value of 1.8 μm^3 (estimated using a coccolith volume calculated by Young and Ziveri (2000)). We assume a calcium concentration inside the vesicle of 500 µM, which represents a typical value for the ER lumen (Meldolesi and Pozzan 1998). Thus the vesicle reservoir of calcium contains ca. $40*10^{-18}$ g calcium. The average coccolith, on the other hand contains ~2.3 pg $CaCO_3$ (0.9 pg Ca) (Young and Ziveri 2000), i.e. roughly 23,000 times more than is present in the reservoir from which it is precipitated. This reservoir needs continuous input of calcium to compensate for the output via precipitation. In case this operates in steady state the calcium isotopic composition of the precipitated calcium must equal the isotopic composition of the calcium influx via Ca-ATPases. Hence calcium isotope fractionation during calcite precipitation cannot account for the calcium isotope fractionation in *E. huxleyi.* Since the model proposed by Lemarchand et al. (2004) describes fractionation during calcium carbonate precipitation it is not suited to describe the fractionation imparted by *E. huxleyi*, which does not occur during precipitation. The fractionation we observed must occur prior to precipitation, i.e. during cellular calcium transport. One possibility is that Ca-ATPases in the coccolith vesicle membrane fractionate against ^{44}Ca. Nothing is known about the calcium isotope fractionation behaviour of Ca-ATPases and the available information on these transporters does not allow for the formulation of a reasonable hypothesis on their fractionation behaviour. Therefore, we assume, for the moment, that Ca-ATPases do not fractionate. However, besides Ca-ATPases

there are other components of cellular calcium transport, which might be involved in calcium isotope fractionation. This will be discussed in the following.

Since fractionation presumably does not occur during the transfer of calcium from the cytosol into the calcite, calcium isotope fractionation in *E. huxleyi* probably takes place during the preceding step, the transport of calcium from seawater into the cytosol. Transport of calcium ions from seawater into the cytosol proceeds via calcium-selective channels in the plasmamembrane. In order to enter a calcium channel, the calcium ion must strip off its water shell (Hille 2001). The calcium ion then is bound to the carboxyl groups of a ring of glutamate residues, which form the narrow selectivity filter of the channel. The dehydration of the calcium ion at the channel entrance is in principle comparable with the dehydration at the crystal surface.

Since we will refer to the equilibrium fractionation model by Marriott et al. (2004) in the following this approach to explain calcium isotope fractionation in inorganically precipitated calcium carbonate should be introduced. Incorporation of calcium ions into the calcite lattice requires the dehydration of calcium-aquocomplexes. It is assumed that the Ca-O bond in the calcium-aquocomplex is stronger than in a carbonate mineral (Marriott et al. 2004). This leads to the preferential partitioning of the heavier calcium isotope to the calcium-aquocomplex and the lighter calcium isotope to the calcite lattice (Marriott et al. 2004). These processes occur during precipitation. As argued for the model proposed by Lemarchand et al. (2004) a fractionation during precipitation cannot account for calcium isotope fractionation observed in *E. huxleyi*.

We suggest that the fractionation proposed to occur during dehydration of the calcium-aquocomplexes at the calcite surface (Marriott et al. 2004) could take place at the channel entrance instead. Along the lines of the hypothesis of (Marriott et al. 2004) we assume that the Ca-O bond in the calcium-aquocomplex is stronger than in the calcium channel, the oxygen atoms here being provided by the carboxyl groups of the glutamate residues. Since the heavier isotope accumulates in the stronger bond (Hoefs 1997) a mass dependent binding of calcium isotopes could favour the preferential partitioning of the heavier calcium isotope to the calcium-aquocomplex. Consequently, the calcium which moves through the channel is enriched in the lighter calcium isotope compared to the bulk medium. As a rule this effect is weakened with rising temperature (Mook and de Vries 2001), which is in agreement with our experimental findings (Figure 1).

Processes in the cytosol provide another possibility to explain calcium isotope fractionation. Calcium entering the cell is immediately bound to a variety of cytosolic calcium

buffer proteins, which bind 99% of the incoming calcium (Neher and Augustine 1992) (Figure 6). These proteins impart calcium complexation by so called EF-hand calcium binding motifes as exemplified for Troponin C (Yumoto et al. 2001) in Figure 6. Calcium buffer proteins contain very high affinity calcium binding sites with –log K_d values of 9 (Gross et al. 1987). Binding of calcium in a $CaCO_3$ (aq) complex or in a complex with organic dicarbolxylate ligands typically found in the cytosol is much weaker, e.g. $CaCO_3$ (aq) has a –log K_d of 3.22 (Plummer and Busenberg 1982) and calcium-oxalate 3.19 (Nancollas and Gardner 1974). This could favour the preferential partitioning of the heavier calcium isotope to the EF-hand, and the lighter isotope to the dissolved complexes, such as $CaCO_3$ (aq). Although so far nothing is known about calcium isotope partitioning of calcium binding proteins, it seems plausible that the mass dependent binding strength of calcium to EF-hands could principally drive isotope fractionation: the presence of cytosolic calcium buffer proteins enriches ^{40}Ca in the aqueous solution and ^{44}Ca in the protein fraction. Ca-ATPases in the vesicle membrane take up calcium from the cytosolic solution, i.e. calcium which is enriched in ^{40}Ca. It is well known that Ca-ATPases in the plasmamembrane of eukaryotes transport calcium out of the cell (Evans and Williams 1998). This “calcium leakage” prevents accumulation of ^{44}Ca inside the cytosol and is therewith a prerequisite for calcium isotope fractionation. Otherwise no fractionation would be measured, because all the calcium would be incorporated into the coccolith.

At present it is not clear which of the discussed processes accounts for calcium isotope fractionation nor do the data at hand allow for an answer to the question whether one or more processes are involved. However, the discussed cytological and physiological features of *E. huxleyi* strongly suggest that the location of calcium isotope fractionation is not the coccolith surface. We propose two possible alternative locations: 1) the entrance of a calcium selective ion channel in the plasmamembrane or 2) cytosolic calcium buffer proteins. In order to shed light on the question whether cytosolic calcium buffer proteins play an important role in calcium isotope fractionation processes of intracellular calcifiers it will be necessary to measure the fractionation factor of isolated proteins and the ratio of calcium efflux to influx. Fractionation at the channel entrance does not require “calcium-leakage”, whereas fractionation in the cytosol requires a “calcium-leakage”, which could be produced by calcium-ATPases in the plasmamembrane. However, in both cases, i.e. fractionation at the channel entrance or fractionation by cytosolic buffer proteins, fractionation would decrease with increasing temperature and be independent of carbonate ion concentration in the bulk medium and growth and calcification rate. This is in agreement with our data.

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Figure captions

Figure 1 The dependence of $\delta^{44/40}Ca$ of coccolith calcite on temperature. The linear regression curve yields a slope of 0.027 ‰ $°C^{-1}$. In this experiment growth and calcification rates varied with temperature as can be seen from Figure 4 and Figure 5 (filled black circles). Photon flux density was 150 µmol m^{-2} s^{-1}. Each data point is the mean value of three culture experiments.

Figure 2 The dependence of $\delta^{44/40}Ca$ of coccolith calcite on photon flux density. In this experiment growth and calcification rates varied with photon flux density as can be seen from Figure 4 and Figure 5 (open circles). Temperature was 17° C. Each data point is the mean value of three culture experiments.

Figure 3 The dependence of $\delta^{44/40}Ca$ of coccolith calcite on carbonate ion concentration and calcite saturation state respectively. pH increases from 7.87 to 8.83 over the range of carbonate ion concentrations depicted. Temperature was 20°C. Each data point is the mean value of three culture experiments.

Figure 4 The dependence of cell growth rate on temperature (filled black circles) and photon flux density (open circles). Each data point is the mean value of three culture experiments.

Figure 5 The dependence of calcification rate on temperature (filled black circles) and photon flux density (open circles). Each data point is the mean value of three culture experiments.

Figure 6 Calcium complexes in the cytosol. A: Calcium ions enter the cell through an open calcium channel in the plasmamembrane. In the cytosol the calcium ion forms several complexes including water and carbonate complexes (I) and complexes with calcium buffer proteins (II). B: Examples for typical calcium-complexes found in the cytosol of eukaryotic cells. In aqueous solution the calcium ion is surrounded by a first water shell which consists of six to ten water molecules (Kaufman Katz et al. 1996). A $CaCO_3$ (aq) complex (Plummer and Busenberg 1982) is depicted as an example for calcium complexes with non-protein ligands (I). The most important calcium complex with respect to binding strength is the complex which calcium forms with an EF-hand motif of a calcium-binding protein. Depicted here is an EF-hand from Troponin C (II) (modified after (Yumoto et al. 2001)).

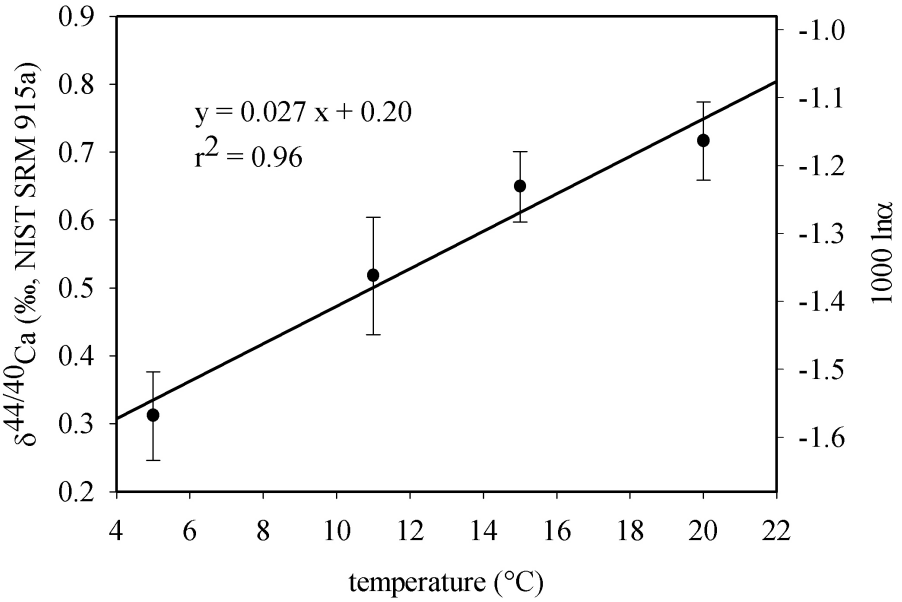
y = 0.027 x + 0.20
r2 = 0.96
δ44/40Ca (‰, NIST SRM 915a)
1000 lnα
temperature (°C)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
-1.6
4
6
8
10
12
14
16
18
20
22

Figure 1

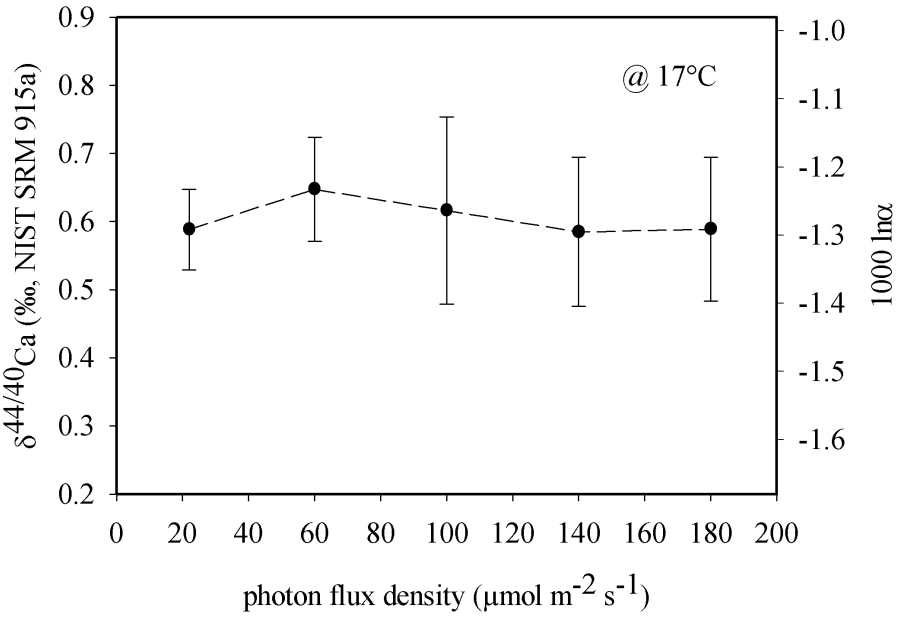
@ 17°C
δ44/40Ca (‰, NIST SRM 915a)
1000 lnα
photon flux density (µmol m-2 s-1)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
-1.6
0
20
40
60
80
100
120
140
160
180
200

Figure 2

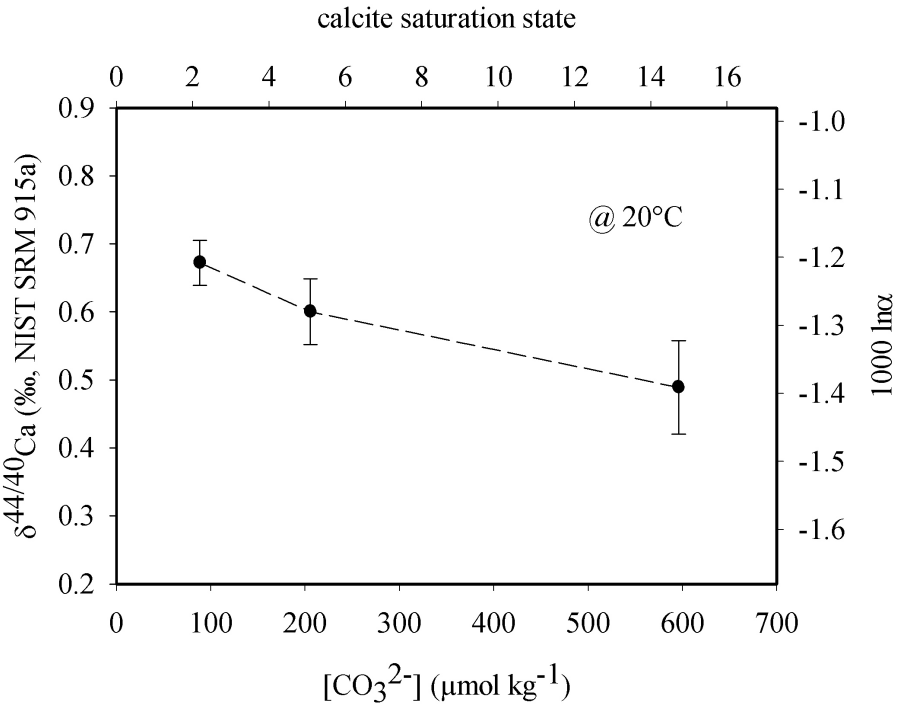
calcite saturation state
0
2
4
6
8
10
12
14
16
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
-1.6
@ 20°C
δ44/40Ca (‰, NIST SRM 915a)
1000 lnα
0
100
200
300
400
500
600
700
[CO3 2-] (µmol kg-1)

Figure 3

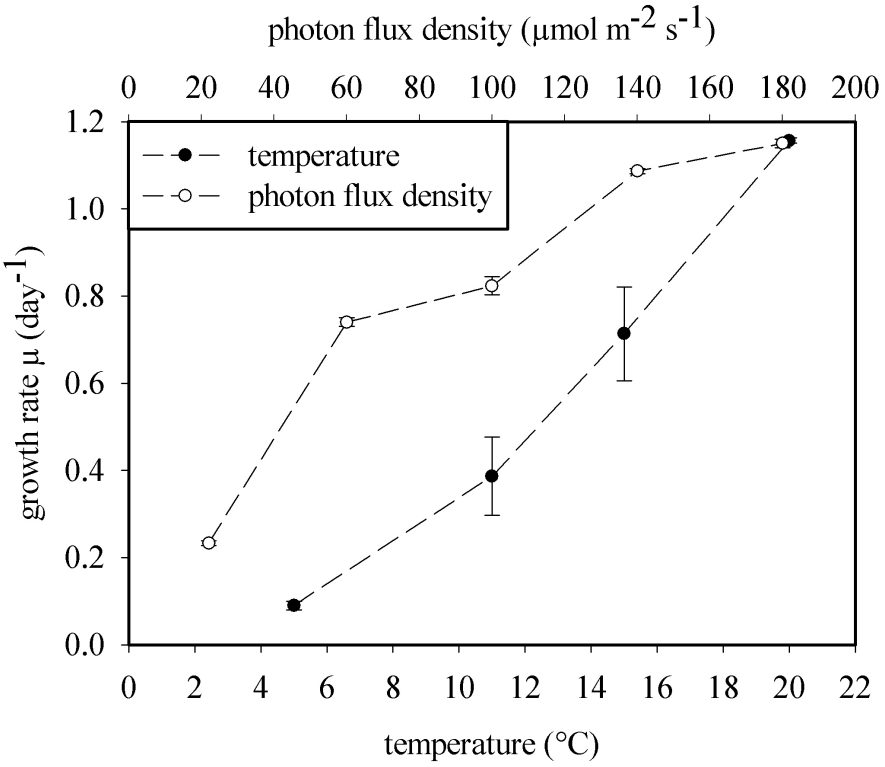
photon flux density (µmol m-2 s-1)
0 20 40 60 80 100 120 140 160 180 200
1.2
1.0
0.8
0.6
0.4
0.2
0.0
growth rate µ (day-1)
temperature
photon flux density
0 2 4 6 8 10 12 14 16 18 20 22
temperature (°C)

Figure 4

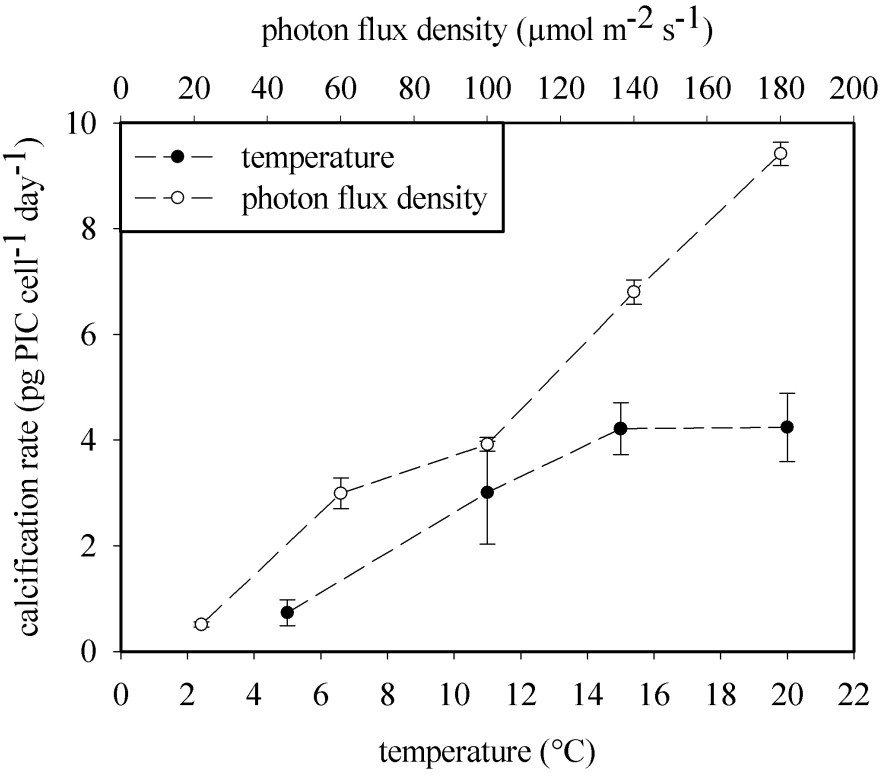
photon flux density (µmol m-2 s-1)
0 20 40 60 80 100 120 140 160 180 200
calcification rate (pg PIC cell-1 day-1)
0 2 4 6 8 10
temperature
photon flux density
0 2 4 6 8 10 12 14 16 18 20 22
temperature (°C)

Figure 5

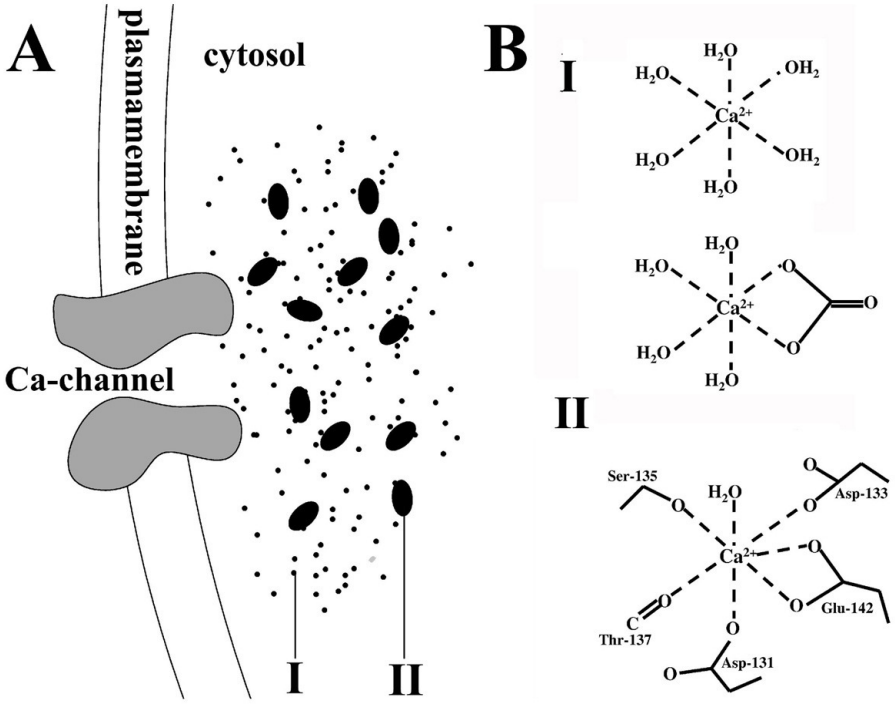
A
plasmamembrane
cytosol
Ca-channel
I
II
B
I
II
Ser-135
Asp-133
Glu-142
Thr-137
Asp-131

Figure 6

Coccolithophore calcification adapting to changes in oceanic CO_2 levels

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Abstract

Uptake of half of the fossil fuel CO_2 into the ocean causes gradual seawater acidification (1). This has been shown to slow down calcification of major calcifying groups, such as corals, foraminifera and coccolithophores (2-4). Here we show that two of the most productive marine calcifying species, the coccolithophores *Coccolithus pelagicus* and *Calcidiscus leptoporus*, do not follow the CO_2 related calcification response previously found. The two investigated species respond differently to changes in the seawater carbonate chemistry highlighting the need to consider species-specific effects when evaluating whole ecosystem responses. Comparison of *C. leptoporus* experimental data with sedimentary records of pre-industrial times and the last glacial maximum reveals calcification to optimize at prevailing CO_2 levels. These results may be explained by evolutionary adaptation to changing CO_2 concentrations.

Introduction

Since the mid 19th century fossil fuel burning has released more than 250 billion tons of carbon in the form of CO_2 into the atmosphere (5). Nearly 50% of the fossil fuel CO_2 emitted into the atmosphere was subsequently taken up by the ocean, 80% of which is stored in the upper 200 m of the water column (1). While ocean uptake of anthropogenic CO_2 provides an invaluable service by mitigating CO_2-related global warming, it leads to a continuous acidification of surface ocean seawater. Unabated CO_2 emissions will cause a doubling in surface ocean pCO_2 levels over their pre-industrial values by the middle of this century, accompanied by a decrease in surface ocean pH three times greater than that experienced during the transition from glacial to interglacial periods. Ocean acidification and the related changes in seawater chemistry may directly impact marine organisms and ecosystems.

Recent laboratory and field studies have revealed a profound effect of seawater acidification and the related decrease in carbonate saturation state on the calcification rates of individual species and communities in both planktic and benthic habitats (I-III see 2-4, 6). Corals (I), foraminifera (II), and coccolithophores (III) each showed a decrease in calcification with increasing CO_2 concentration. A CO_2 doubling from pre-industrial values of 280 ppm pCO_2 to 560 ppm pCO_2 caused a decreased in calcium carbonate precipitation by 5-25% in all calcifying organisms investigated to date (7). This rather uniform response appears enigmatic considering that major differences exist between the calcifying groups with regard to (I) the mineral phase of $CaCO_3$ produced, (II) the location of $CaCO_3$ precipitation, and (III) the pathways of the associated ion transport. Whereas the aragonitic structures of corals are formed at external surfaces providing microenvironments favouring $CaCO_3$ precipitation, the calcitic shells and platelets of foraminifera and coccolithophores are produced internally in calcifying compartments through vacuolization of seawater (8) and via transmembrane ion transport (9).

Despite the apparent consistency in the observed CO_2 effects on biogenic calcification, predictions about the fate of calcifying organisms in an increasingly acidified

ocean are problematic. Most results presently available are based on short-term experiments with calcifying organisms dominant in the present ocean. This may have created a bias for species and clones best adapted to modern CO_2 levels and raises questions regarding the general validity of the observed responses. It is also unknown whether calcifiers are capable to adapt to decreasing carbonate saturation states and at what rate. Aside from testing a wider range of calcifying species, there is obviously a need to examine long-term effects of CO_2 related changes in seawater chemistry.

In the present study we have combined experimental results of two previously untested coccolithophore species, *Calcidiscus leptoporus* and *Coccolithus pelagicus*, with the examination of coccolith morphology in glacial and pre-industrial marine sediments as well as from laboratory assay. *C. leptoporus* and *C. pelagicus* are two of the heaviest calcifying extant coccolithophores and, alongside with the bloom forming cosmopolitan *Emiliania huxleyi*, the most important species in terms of calcite export to the sediment (10). Comparison of short-term responses of calcification to abrupt CO_2 perturbations as observed in the laboratory with coccoliths originating from sedimentary records of periods with different CO_2 levels provides first evidence for adaptation of coccolithophore calcification to changing CO_2 concentration.

Material and methods

Experimental design

Monospecific cultures of *Calcidiscus leptoporus* (strain AC365, from South Atlantic off South Africa, CODENET culture collection, ALGOBANK http://www.nhm.ac.uk/hosted_sites/ina/CODENET/caencultures.htm) and *Coccolithus pelagicus* (strain AC400, from South Atlantic off Namibia, CODENET culture collection, ALGOBANK) were grown in sterile filtered (0.2 µm) seawater enriched with 100 µM nitrate and 6.25 µM phosphate and with trace metals and vitamins according to f/2 (11). The incident photon flux density was 350 µmol/m^2*s and a 16/8 hour light/dark cycle was applied. Experiments were carried out at optimum growth temperature, i.e. 20° C for *C. leptoporus* and 17° C for *C. pelagicus.* Cells were acclimated to experimental conditions for approximately 10 generations and grown in dilute batch cultures (12). For a summary of all measured parameters see Table 1. Low cell density at harvest (in general less than 6000 cells per ml) resulted in less than 8% DIC consumption (i.e. DIC consumed by the cells at the end of experiment) and a shift in pH of not more than 0.06 units. CO_2 levels were adjusted by adding calculated amounts of 1 M HCl or 1 M NaOH to the medium. In order to prevent gas exchange with the atmosphere 2.4 l borosilicate flasks were filled without headspace and closed with teflon lined screw caps. Determination of growth rate, however, required regular sampling for cell counts, thereby creating a maximum headspace of 6 ml. The resultant air-water CO_2 equilibration of 3% shift in CO_2aq concentration is negligible. Samples for alkalinity measurements were filtered (approx. 0.6 µm), poisoned with 1 ml of a $HgCl_2$ solution (35 g/l) and stored in 300 ml borosilicate flasks at 0° C. DIC samples were sterile filtered (0.2 µm) and stored in 13 ml borosilicate flasks free of air bubbles at 0° C. Total alkalinity was calculated from linear Gran plots (13) after duplicate potentiometric titration (14, 15) and DIC was measured photometrically (16) in triplicate. The carbonate system was calculated from temperature, salinity, and the concentrations of DIC, total alkalinity and phosphate, using the program CO_2sys (17). Equilibrium constants of Mehrbach et al. (18) refit by Dickson and Millero (19) were chosen. Samples for determination of total particulate carbon and particulate organic carbon were filtered on

precombusted (12 hours, 500°C) GF/F-filters (approx. 0.6 µm) and stored at -20° C. Prior to analysis, the POC filters were fumed for two hours with a saturated HCl solution to remove all inorganic carbon. TPC and POC were subsequently measured on a Carlo Erba NA-1500 Analyzer. Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. For determination of cell density samples were taken daily or every other day, stored at 0° C and counted within 3 hours after sampling using a Sedgwick Rafter counting cell. Cell density was plotted against time and growth rate (µ) was calculated by means of exponential regression. Inorganic and organic carbon production (P, pg C/cell*d) was calculated according to P = µ * (cellular carbon content).

Coccolith morphology

Samples for scanning electron microscope (SEM) analysis were filtered on cellulose nitrate filters (0.45 µm pore size), dried in a drying cabinet at 60° C for 24 hours, then sputter-coated with gold-palladium. Imaging was performed with a Philips XL-30 digital scanning field-emission electron microscope. Four categories were used to describe the morphology of *C. leptoporus*: normal, malformed, incomplete, and incomplete and malformed coccoliths. An average of approx. 500 coccoliths was analysed per CO_2 treatment. The relative numbers of coccoliths in the four categories were constant when counting more than 50 coccoliths, so that the relative numbers obtained from counting 500 coccoliths can safely be regarded as statistically significant. In order to get a morphological index of reduced cellular calcite content we combined the two categories containing incompletely grown coccoliths, since an incompletely grown coccolith contains less calcite than a completely grown one (including the malformed coccoliths). The relative number of all incompletely grown coccoliths is given in Table S1.

Sediment core samples

Six cores were selected for the geological control study representing a range of latitudes through the Atlantic Ocean, all cores were from well above the lysocline. From each core one sample was taken from the last glacial maximum (LGM) when CO_2 levels are believed to have been ca. 180ppmv (20), and a second from the sediment top (i.e. upper 1 cm of the core). Due to sampling disturbance and bioturbation core-top sediments are generally regarded as representing deposition over the past 500-1000 years and hence

predominantly pre-industrial sedimentation at CO_2 levels of ca. 280ppmv (21). Modern CO_2 values are ca. 370ppmv.

For the counting of core-top and LGM core samples the same procedure of dividing the coccoliths into categories and determining the relative numbers of coccoliths in the respective categories was applied. This allowed a quantitative comparison of coccolith morphology.

Results

The laboratory assay consisted of batch culture incubations of *C. leptoporus* and *C. pelagicus* grown over a range of CO_2 concentrations (for details see supporting online material) in which cellular particulate inorganic carbon (PIC), particulate organic carbon (POC), growth rate, and coccolith morphology (22) were monitored. In *C. leptoporus* the cellular inorganic carbon content changes with increasing CO_2 concentration in a non-linear relationship (Fig. 1). An optimum curve is obtained, with a maximum value at modern surface ocean pCO_2 levels (~360 ppm CO_2). With the cellular organic carbon content remaining constant over the range of CO_2 concentrations, the PIC/POC ratio also shows an optimum curve. Since the growth rate does not change with pCO_2, rates of POC- and PIC-production show the same pattern as POC- and PIC-content per cell (Table S1). Scanning electron microscopy analysis reveals highly variable degrees of coccolith malformation and proportions of incomplete coccoliths between CO_2 treatments (Fig. 3). At 360 ppm CO_2 most coccoliths show normal morphology, at higher and lower CO_2 concentrations the proportion of coccoliths showing incomplete growth and malformation increases notably.
Changes of calcification rate are highly correlated to changes in coccolith morphology. This raises the question whether decreasing calcite content per cell is due to a lowering of the calcite content per coccolith or to a reduction in the number of coccoliths per cell. Incomplete growth implies less calcite per coccolith. Plotting the relative number of incomplete coccoliths against CO_2 concentration yields an optimum curve of similar shape to the inorganic carbon content per cell curve. Therefore it is likely that the changing inorganic carbon content per cell is due to altered calcite content per coccolith. This

interpretation is supported by the fact that no differences in the number of visible coccoliths per cell could be observed (Table S1).

In the *Coccolithus pelagicus* cultures neither PIC nor POC content per cell changes significantly over the CO_2 range tested (Fig. 2, Table S1), yielding a stable PIC/POC ratio. Because growth rate does not change, PIC and POC production also remain constant with CO_2. Scanning electron microscope analysis of *C. pelagicus* coccoliths showed no significant change in the proportions of malformed or incomplete coccoliths over the range of CO_2 concentrations (Fig. 2 and Fig. 3).

Analysis of sediment samples was performed on six cores obtained from location well above the lysocline and covering a range of latitudes throughout the Atlantic Ocean (for details see supporting online material). From each core one sample was taken from the last glacial maximum (LGM) when CO_2 levels are believed to have been ca. 180 ppmv (20), and a second from the sediment top (i.e. upper 1 cm of the core), representing pre-industrial times. Scanning electron micrograph analysis of coccolith morphologies did not reveal any evidence for significant numbers of incomplete or malformed coccoliths of *C. pelagicus* and *C. leptoporus* in LGM and Holocene sediments (Table 1).

Discussion

The assessment of the CO_2 sensitivity of marine biogenic calcification is primarily based on short-term experimental studies using single clones of selected species. The observed deterioration of coccolith production above as well as below present-day CO_2 concentrations in *C. leptoporus* and the lack of a CO_2 sensitivity of calcification in *C. pelagicus* refute the notion of a linear relationship of calcification with the carbonate ion concentration and carbonate saturation state, as previously suggested e.g. (7). Consequently, species-specific effects have to be taken into account when assessing whole ecosystem responses to changing carbonate chemistry.

In case of *C. leptoporus,* changes in coccolith morphology as occurring in response to the abrupt CO_2 perturbation applied in the experimental treatments are not mirrored in the sedimentary record. This suggests that in the natural environment *C. leptoporus* has

adjusted to the 80 ppm CO_2 and 180 ppm CO_2 difference between present, pre-industrial and glacial times, respectively.

An alternative explanation for observing only normal coccoliths in the fossil record would be selective removal through dissolution of incomplete or malformed coccoliths. This is not inconceivable, considering water-column dissolution of coccoliths and assuming that malformed morphotypes may be more vulnerable to dissolution. Since dissolution is a continuous process (23) however, complete removal of incomplete and malformed coccoliths cannot occur without inflicting visible changes to normal coccoliths. Such changes were not observed. This reasoning was confirmed in a thorough field study in the Indonesian Seas, where the coccolith morphology of a number of different species was investigated in both surface water- and sediment trap samples (24). This study conclusively demonstrated that in sediment trap samples both malformed and normal coccoliths were subject to dissolution and hence should be observable in the fossil record. These findings support our hypothesis that selective dissolution can not explain our results from fossil material.

Thus, it is reasonable to assume that *C. leptoporus* has adapted its calcification mechanism to the change in carbonate chemistry having occurred since the last glacial maximum. A possible explanation for such adaptation is that the population is genetically diverse, containing strains with diverse physiological and genetic traits, as already demonstrated for *E. huxleyi* (25-31). Our experimental data for *C. leptoporus* merely reflect the physiological response of one particular strain to short term changes of pCO_2. If genetic diversity is the basis of adaptation to changing CO_2 concentrations, this is not likely to be confined to *C. leptoporus* but can be assumed to play a role in other coccolithophore species as well. The sexual life-cycle of coccolithophores ensures extensive genetic recombination, resulting in natural populations with significant ecophysiological variability (25, 32).

On the cellular level the influence of changing carbonate chemistry can be interpreted in terms of a pH effect. It is well known that in many species changes in extracellular pH exert an influence on various physiological parameters, e.g. intracellular pH (33), nitrate uptake (34) and membrane potential (35). A constant membrane potential is

the result of a sophisticated ion balance mechanism. Hence it is likely that sudden external pH changes can upset this ion balance and consequently alter ionic composition and ionic strength of the cell. Intracellular ionic strength in turn determines the conformation of polyelectrolytes, e.g. cytoskeletal elements and coccolith-associated polysaccharides. According to polymer theory (36, 37) changes in ionic strength can turn a rod-like polyelectrolyte into a coil (Fig. 4), thereby disturbing its specific function. Both the cytoskeleton and the coccolith associated polysaccharides are thought to play an important role in coccolith morphogenesis (38, 39). Conformational changes of these polyelectrolytes may have caused the observed coccolith malformation. On longer timescales however, the population may be able to evolve so that the optimal CO_2 level for calcification of the species tracks the environmental value.

This study provides the first examination of possible effects of changing pCO_2 on coccolithophore calcification that does not entirely rely on experimental, i.e. very short, timescales. For planktic foraminifera there are both experimental (3) and geological (40) observations suggesting a decline of calcification rates in response to decreasing calcium carbonate saturation state of seawater. Because these two studies use different species, a combination of the two approaches, as presented in this study, is not possible. Nonetheless, the foraminiferal data support the notion of a decrease in calcification on geological timescales whereas our coccolithophore data do not. It is generally accepted that in coccolithophores there is no obvious correlation between coccolith morphology, in terms of size and degree of calcification, and CO_2 levels (calcium carbonate saturation state) on geological timescales. The Pleistocene, for example, an epoch of low pCO_2 levels and slightly higher values of calcite saturation state compared to the Eocene and the Late Cretaceous (41-44) is characterised by small lightly calcified coccoliths. In contrast, the Eocene and the late Cretaceous are times of large and heavily calcified coccoliths (45). Further research is clearly warranted to elucidate the possible differences between the various groups of marine calcifiers with respect to the CO_2 sensitivity of their calcification processes.

Our experimental data suggest a marked alteration in coccolith morphology of *C. leptoporus* with high levels of malformed and incomplete coccoliths when CO_2 levels are

reduced to last glacial maximum values. A survey of *C. leptoporus* coccoliths from the last glacial maximum (LGM) at various sites in the Atlantic Ocean did not reveal altered morphology of *C. leptoporus* coccoliths. Since the LGM, CO_2 levels have risen from 180 to 370 ppm. The time interval of ca. 18000 years arguably provides ample time for adaptation via evolutionary processes. Malformed or incompletely grown specimens were also not observed in natural assemblages (screened prior to the industrial revolution and in the course of the last 150 years) as well as in Holocene samples (for details see supporting online material). Since the industrial revolution the rate of CO_2 increase was 10 and possibly 100 times faster than at any other time in the past 420,000 years (46). The absence of a detectable alteration in coccolith morphology in natural samples suggests that adaptation to changing CO_2 levels occurred on much shorter timescale (i.e. hundreds of years). Whether adaptation to the projected future increase in CO_2-related ocean acidification will be possible, is presently unknown. The lack of CO_2 sensitivity observed in *C. pelagicus*, however, suggests that the dependence of calcification on carbonate saturation state is less uniform than previously thought. Genetic diversity, both between and within species, may allow calcifying organisms to prevail in a high CO_2 ocean as long as seawater remains supersaturated with respect to calcite and aragonite.

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Figure legends

Figure 1: Data from *C. leptoporus* culture experiments plotted against carbonate chemistry in the culture medium. A) Production of inorganic (dashed line) and organic (solid line) carbon per cell and day B) Ratio of inorganic to organic carbon content. C) Production of inorganic carbon per cell and day (as in A, dashed line) and relative number of incompletely grown and incompletely grown / malformed coccoliths (solid line).

Figure 2: Data from *C. pelagicus* laboratory culture experiments plotted against carbonate chemistry in the culture medium. A) Production of inorganic (dashed line) and organic (solid line) carbon per cell and day B) Inorganic to organic carbon ratio. C) Production of inorganic carbon per cell and day (as in A, dashed line) and relative number of incompletely grown and incompletely grown / malformed coccoliths (solid line).

Figure 3: Representative specimens of *C. leptoporus* (a-d) and *C. pelagicus* (e-h) from natural samples (a, e) and laboratory culture experiments grown at different CO_2 concentrations (b-d and f-h). Note the increase in malformation and/or incomplete growth (narrow rims) with elevated or reduced pCO_2 in *C. leptoporus,* where incomplete growth dominates samples from lower than present-day pCO_2 values, whereas malformation and incomplete growth dominated the sample with higher pCO_2 values.

Figure 4: Behaviour of polyelectrolytes (PEs) in relation to ionic strength of the solution. PEs are polymers that have ionizable groups (such as SO_3^- or CO_2^-). When dissolved in solutions, they dissociate into charged polymer chains (macroions) and free, mobile counterions. The delicate balance between chain configurational entropy and long range electrostatic monomer-monomer repulsion determines the structure of charged chains in dependence on the ionic strength of the solution. In the limit of low ionic strength PEs are usually stretched and assume as a rod-like conformation (L ~ N, N is the degree of polymerization). Increasing the ionic strength (a) can significantly reduce the rigidity of the chain, since the electrostatic interaction is screened over the Debye-Hückel screening length (which typically

varies from less than 1nm to more than 100nm). At very high ionic strength, the distant parts of a charged chain do not interact, and the chain behaves as a random coil with short-ranged repulsive interactions ($R \sim N^{\nu}$, $0.5 < \nu < 1$). PEs can adsorb onto a charged surface (b) mainly caused by electrostatic effects. At low ionic strength adsorption is very strong, and the PEs assume a conformation where the chain essentially lies flat on the surface. As the ionic strength is increased (a), the polymer chain can gain conformational entropy by extending somewhat into the solution. In the limit of high ionic strength PEs can desorb from the surface (c).

Tables

					C. leptoporus				*C. pelagicus*			
Sediment Core	Latitude	Longitude	Water Depth (m)	Age	total observations	normal in %	malformed in %	incomplete in %	total observations	normal in %	malformed in %	incomplete in %
Equatorial Atlantic												
GeoB1105	1°40'N	12°26'W	3232	surface	42	98	2	0				
GeoB2204	8°32'S	34°02'W	2085	surface	21	100	0	0				
				LGM	53	100	0	0				
SW-Africa												
GeoB1214	24°41'S	7°14'E	3220	surface	121	99	1	0				
				LGM	231	100	0	0				
GeoB1710	23° 26'S	11° 42'E	2983	surface					34	94	3	3
				LGM					54	96	4	0
GeoB3603	35°07'S	17°33'E	2851	surface	100	99	1	0				
				LGM	100	96	4	0				
NW-Africa												
GeoB5546	27°32'N	13°44'W	1071	surface	26	100	0	0	31	97	0	3
				LGM	57	96	4	0	36	97	3	0
North Atlantic												
ODP site 980B	55°30'N	15°42'W	2172	surface	154	97	2	1	71	99	0	1
ODP site 982A	57°51'N	15°52'W	1135	LGM	111	99	1	0	32	97	3	0
K 708-7	53°56'N	24°05'W	3502	surface					43	100	0	0
Norwegian-Greenland Sea												
GIK 23062	68°43'N	0°11'E	2243	surface					32	100	0	0
				LGM					14	93	7	0
GIK 23199	68°23'N	5°19'E	1968	surface					68	99	1	0

Table 1: Sample locations and dataset derived from the geological samples. The data displayed is showing the morphology of *C. leptoporus* and *C. pelagicus* coccoliths in samples from Holocene age (surface) and from the last glacial maximum (LGM). Note the low number of abnormally formed coccoliths in all samples. Samples are from the University of Bremen (GeoB), the Ocean Drilling Program (both at the ODP core repository at University Bremen), core K 708-7 is from a 1970 cruise of R/V *Kane* (subsamples are at the University Bremen), and cores designated GIK are from the geological institute of the University Kiel.

Figures

Figure 1

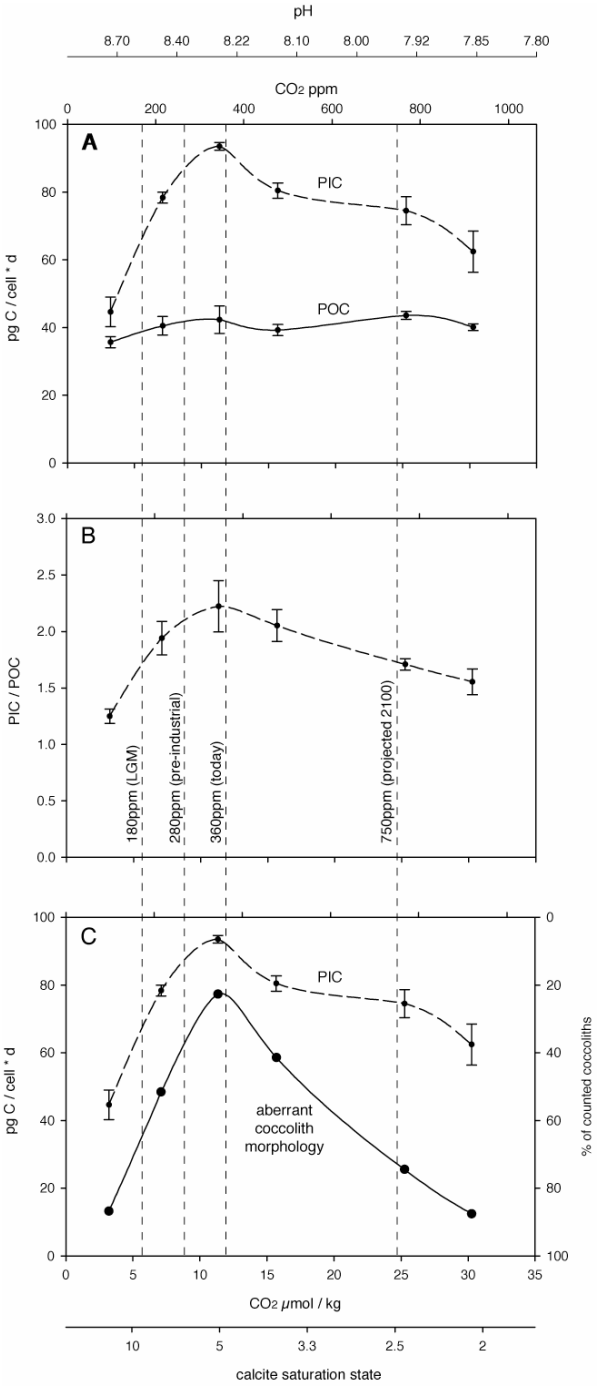


Figure 2

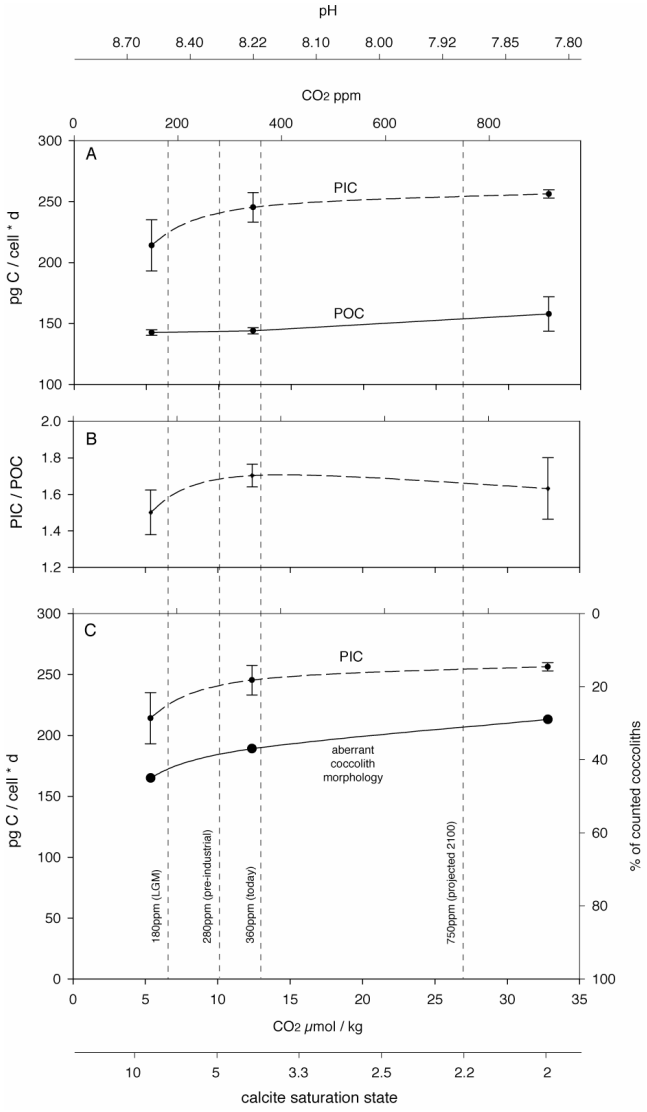

pH
8.70
8.40
8.22
8.10
8.00
7.92
7.85
7.80
CO2 ppm
0
200
400
600
800
A
PIC
POC
pg C / cell * d
B
PIC / POC
C
PIC
aberrant coccolith morphology
% of counted coccoliths
180ppm (LGM)
280ppm (pre-industrial)
360ppm (today)
750ppm (projected 2100)
CO2 µmol / kg
calcite saturation state

Figure 3

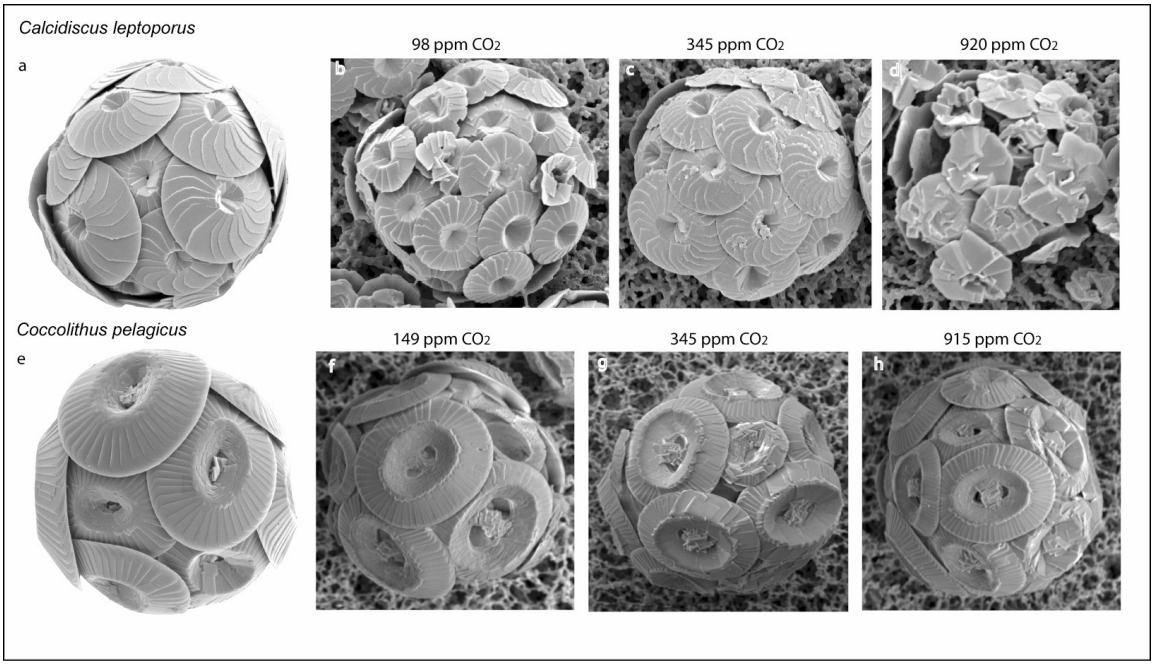
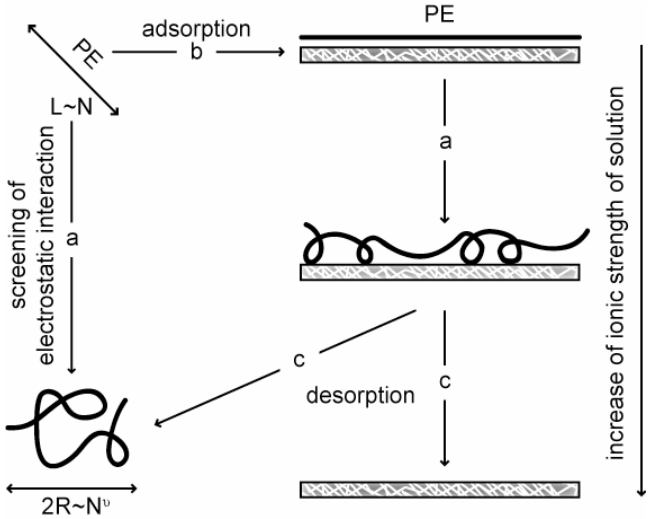


Figure 4



Supporting online Material for Langer et al. - Coccolithophore calcification adapting to changes in oceanic CO_2 levels

Pre-industrial plankton samples

If incomplete and malformed coccoliths of *C. leptoporus* were more common in pre-industrial conditions but were removed by dissolution during sedimentation then there might be evidence for them in observations of 19th century and early 20th century researchers. These researchers worked primarily qualitatively and used light microscopy, but they included outstanding microscopists who made detailed drawings and reliable notes. In addition to Wallich´s original drawings, which are stored in the Botany department of The Natural History Museum in London, we have surveyed the relevant literature (1-6) and have not found any drawings of coccoliths displaying incomplete growth and/or malformation, nor were such features described by the authors. Sample material is rarely preserved from this time, one exception are the fixed slides of Wallich (4), collected in 1857 which are available in the Natural History Museum, London. Some light micrographs from this material are presented in figure S1.

Discussion of timescale of ecophysiological adaptation

Experimental results suggest that reduction of CO_2 levels to last glacial maximum values results in marked alteration of coccolith morphology, with high levels of malformed and incomplete coccoliths. A survey of *C. leptoporus* coccoliths from the last glacial maximum (LGM) at various sites in the Atlantic Ocean, did not reveal altered morphology of *C. leptoporus* coccoliths. Since the LGM CO_2 levels have risen from 180 to 370 ppm. This time interval of ca. 18,000 years arguably provides ample time for ecophysiological adaptation via evolutionary processes. So it can be safely concluded that the timescale of ecophysiological adaptation does not exceed some thousand years. Also we have observed no malformed or incompletely grown specimens in both natural assemblages (screened prior to the industrial revolution and in the course of the last 150 years) and Holocene samples. Since the industrial revolution the rate of the CO_2 rise was 10 and possibly 100 times faster than at any other time in past 420,000 years (7). Hence the absence of any

change in morphology between pre-industrial, core-top and modern coccoliths is critical. This lack of change suggests that ecophysiological adaptation to changing CO_2 levels can occur on the timescale of decades.

Reproducibility of laboratory experiments

One year after our initial experiments the reproducibility of our morphological results was tested in a different laboratory (Fig. S2) using the same strain of *C. leptoporus.* Results from both experiments closely agree showing a marked alteration of coccolith morphology under pCO_2 levels below and above present day values. Due to a somewhat higher resolution in CO_2 levels, the optimum of the morphological curve has shifted to slightly more alkaline carbonate chemistry compared to the first experiment. In fact, the optimum value reflects the typical present-day seawater carbonate chemistry of 320ppm CO_2 in the South Atlantic Ocean (off South Africa) (8) from where the clone was isolated.

In contrast to natural plankton samples that generally do not show variation in coccolith morphology there is a base level of aberrant coccolith morphology in cultured strains. Our two independent and successive experiments (Fig. S2) show different levels of culture artefacts. Nevertheless, the effects of changing carbonate chemistry are the same, i.e. the shapes of the two curves depicted in Fig. S2 do not differ. The different base levels of culture artefact in coccolith morphology do not affect the interpretation of the experimental data presented here.

References

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Figure legends

Figure S1: Light micrographs of *C. leptoporus* taken from samples collected by Wallich (4) in 1857. This is a rare example where well-preserved pre-industrial plankton samples were available for analysis. Images 1-5 are taken with cross polarised light und in different focal depth to image both proximal and distal shield. Images 7-9 are phase contrast micrographs. No signs of malformation and/or incomplete growth were observed in this sample.

Figure S2: Comparison of the relative numbers of incompletely grown and incompletely grown / malformed coccoliths in two independent experiments with the same clone of *C. leptoporus*. The solid line represents data already shown in figure 1.

Tables

	medium chmistry					**sample parameters**						**coccolith morphology**					
	pCO2 in ppm	CO2 µmol/kg	pH (NBS)	CO3	Omega	µ (per day)	POC/cell (pgC/cell)	PIC/cell (pgC/cell)	PIC/POC	P (µ) POC (pgC/cell*day)	P (µ) PIC (pgC/cell*day)	sphere size µm	Liths/cell	normal coccoliths (1)	malformed coccoliths (2)	incompletely grown coccoliths (3)	malformed & incompletely grown coccoliths (4)
C. leptoporus AC365																	
	98	3.22	8.74	515.73	12.83	0.48	74.36	92.97	1.25	35.70	44.67	12.6	15	11	2	41	46
	216	7.11	8.44	306.43	7.50	0.64	63.17	121.94	1.94	40.53	78.38	x	14	45	3	33	18
	345	11.34	8.27	202.57	5.04	0.64	66.12	146.21	2.22	42.36	93.51	14.7	14	52	25	6	17
	477	15.70	8.13	159.93	3.92	0.62	63.44	129.83	2.05	39.29	80.48	x	14	47	11	9	32
	768	25.27	7.93	104.10	2.55	0.63	68.91	117.81	1.71	43.61	74.53	x	14	19	6	5	69
	920	30.26	7.86	81.80	2.04	0.62	66.87	100.85	1.55	40.16	62.47	13.2	16	4	8	2	86
C. pelagicus AC400																	
	149	5.36	8.56	339.90	8.29	0.78	183.92	276.42	1.50	142.52	214.12	x	x	50	5	22	23
	345	12.37	8.22	169.03	4.12	0.73	198.21	337.66	1.70	144.04	245.42	x	x	54	9	12	25
	915	32.80	7.81	67.87	1.66	0.73	217.59	353.62	1.63	157.82	256.34	x	x	53	18	14	16

Table S1: Dataset derived from the experiments.

Figures

Figure S1

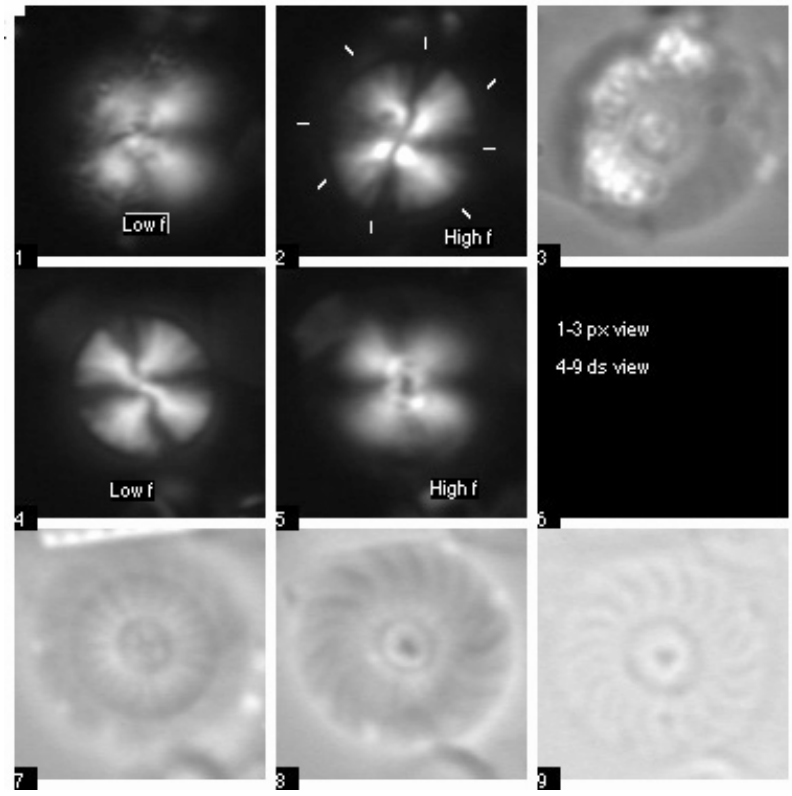
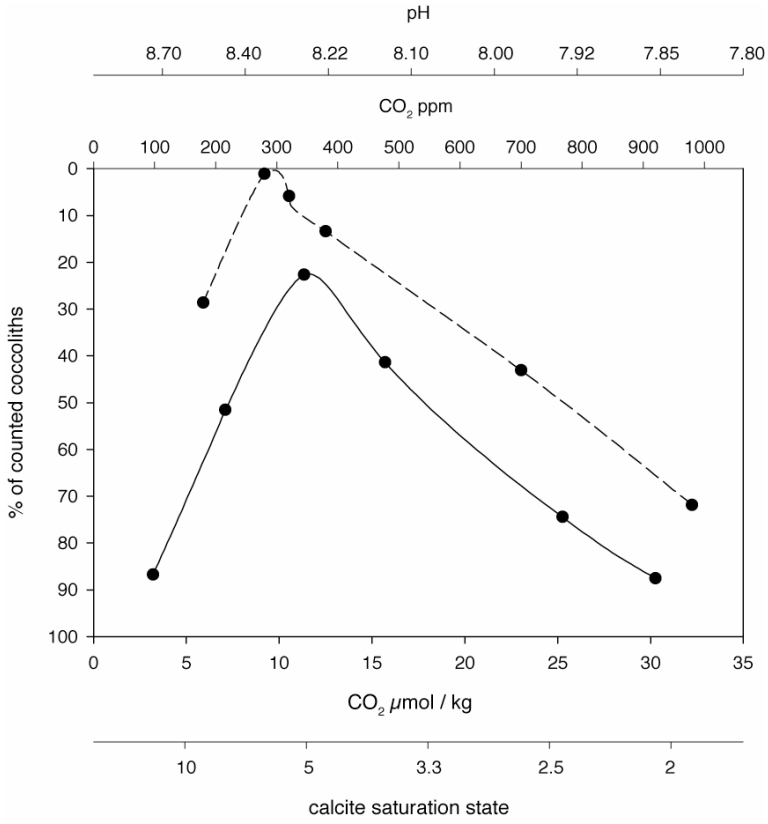


Figure S2


pH
8.70
8.40
8.22
8.10
8.00
7.92
7.85
7.80
CO2 ppm
0
100
200
300
400
500
600
700
800
900
1000
% of counted coccoliths
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
CO2 µmol / kg
10
5
3.3
2.5
2
calcite saturation state

3 General Discussion

Coccoliths are elaborate structures produced in a specialised vesicle inside the cell, the coccolith vesicle. The processes involved in coccolith production are an essential part of coccolithophore physiology. The coccoliths themselves constitute an important part of marine biogenic calcium carbonate and provide an extensive sedimentary archive. This study investigated the dependence of coccolith morphology and chemical composition on various environmental parameters in selected coccolithophore species. Laboratory experiments performed under controlled conditions delivered insight into the dependence of the coccolith Sr/Ca ratio on the Sr/Ca ratio of seawater in *Emiliania huxleyi*. Moreover, the first data from coccolithophores on the dependence of $\delta^{44/40}$Ca on temperature, growth and calcification rate and carbonate chemistry of seawater are presented. The impact of changing carbonate chemistry of seawater on calcification rate and coccolith morphology in two productive species, *Calcidiscus leptoporus* and *Coccolithus pelagicus*, was studied using both geological and experimental samples. In the following sections the results obtained in this study are discussed with regard to the use of coccoliths as proxy archives, the calcification mechanism and the consequences, which CO_2 related ocean acidification might have for coccolithophore calcification. Perspectives for future research are given at the end.

3.1 Sr/Ca of coccoliths – implications for proxy use and biomineralisation

In a pioneering study Stoll and Schrag (2000) hypothesised that the Sr/Ca ratio of coccoliths might record calcification rate, highly correlated with cell growth rates. Subsequent studies have confirmed that it is possible to infer past coccolithophore productivity, i.e. growth and calcification rates, from the coccolith fossil record (Billups et al. 2004; Stoll and Schrag 2001). The analysis of core top sediments showed that the Sr/Ca ratio of different coccolithophore species is sensitive to nutrient gradients across upwelling zones, where there are strong and easily identifiable gradients in productivity (Stoll et al. 2002b). Although Sr/Ca is one of the most promising coccolith-based proxies there are secondary influences to be considered, one of which is the Sr/Ca ratio of seawater. The Sr/Ca of seawater is likely to have changed through time on both short and long timescales (Lear et al. 2003; Stoll and Schrag 1998). Therefore, it is mandatory to consider its influence on the Sr/Ca of biogenic calcium carbonates. Recent studies investigating coccolith dominated Cenozoic and Cretaceous carbonates consequently account for changing seawater Sr/Ca (Billups et al. 2004;

Stoll and Schrag 2001). Calculations in these studies rely on the premise that the exchange coefficient is unaffected by the seawater Sr/Ca ratio. This was shown to be true for *Emiliania huxleyi*, one of the most productive marine calcifiers (Publication I). If other species also display a linear relationship between Sr/Ca ratios of seawater and that of coccoliths variable seawater Sr/Ca ratios would not hamper the application of this proxy as long as seawater chemistry can be constrained by independent means. Monitoring Sr/Ca ratios in another biogenic calcium carbonate with more constant Sr partitioning (Lear et al. 2003) is such an independent mean, that could prove helpful.

The strontium exchange coefficient measured in our study is 0.39 (Publication I), close to values presented in the literature, e.g. Stoll et al. (2002a). However, from inorganic precipitation experiments considerably lower exchange coefficients were obtained, e.g. (Lorens 1981). Since a positive correlation between strontium exchange coefficient and growth rate is well documented for inorganically precipitated calcite (Lorens 1981; Tesoriero and Pankow 1996), it was hypothesised that the high strontium exchange coefficients of biogenic calcite can be explained by kinetic effects (Carpenter and Lohmann 1992). In a thorough study Stoll et al. (2002a) showed that strontium exchange coefficient values of coccolith calcite cannot be explained by kinetic effects, i.e. simply by virtue of fast crystal growth rates. Alternatively, biological, rather than crystal kinetic factors may exert the dominant control over coccolith Sr/Ca ratios. Rickaby et al. (2002) suggested that there may be a rate dependent discrimination between strontium and calcium associated with transport through ion channels or carrier proteins. This hypothesis invokes a selectivity against strontium in channel or carrier transport, which is reduced at higher transport rates. Given that higher transport rates are correlated with higher calcification rates the Sr/Ca of the calcifying fluid could increase and produce coccoliths with higher Sr/Ca ratios at higher calcification rates. Previous experiments with *E. huxleyi* (Stoll et al. 2002a) and other species (Stoll et al. 2002b) showed that irradiance-induced changes in growth rate did not affect strontium partitioning, whereas nutrient-stimulated changes did (Rickaby et al. 2002). This distinction between irradiance-induced and nutrient-stimulated changes in growth rate strongly suggests that some physiological processes, rather than growth rate per se, are responsible for variable strontium partitioning in coccolithophores. Consequently, the actual mechanism of strontium partitioning in coccolithophores must be different from the model proposed by Rickaby et al. (2002).

However, a mechanism taking into account transmembrane transport of calcium and strontium is likely the most useful conceptual framework for further study. The high

strontium exchange coefficients measured for coccolith calcite can, in the case of *E. huxleyi,* be explained by a simple conceptual model based on the channel/carrier mediated transport of calcium and strontium ions inside the cell (Publication I). The model is solely based on thermodynamic constraints and does not assume a relationship between the exchange coefficient and either the crystal growth rate or the pumping rate of transport ATPases. Moreover a presumed dependence of the strontium exchange coefficient on external nitrogen concentration does not contradict the basic idea of the model, but appears quite plausible (Publication I). This is important, since nitrogen availability most likely is the factor inducing growth rate associated changes in strontium partitioning (as argued above).

Another factor, which was shown to influence strontium partitioning in *E. huxleyi* is temperature (Stoll et al. 2002a). Temperature also controls strontium partitioning in inorganically precipitated calcium carbonate (Malone and Baker 1999). In both cases linear relationships were observed, but the slopes differ by one order of magnitude. Our model reconciles this discrepancy (Publication I), which further demonstrates the capability of the model, and is also of considerable value for proxy use. The influence of temperature on strontium partitioning seems to be a ubiquitous effect in all calcium carbonates. Sr/Ca ratios were also found to be temperature dependent in corals (Smith et al. 1979), sclerosponges (Haase-Schramm et al. 2003) and foraminifera (Lea et al. 1999).

3.2 $\delta^{44/40}$Ca of coccoliths – implications for proxy use and biomineralisation

Besides foraminifera, coccolithophores have produced a huge calcareous sedimentary archive. This large repository of calcium isotopes constitutes a potential record of changes in the cycling of calcium at the earth's surface (De La Rocha and DePaolo 2000; DePaolo 2004; Zhu and MacDougall 1998). To understand the record held in carbonate sediments it is mandatory to document any calcium isotopic fractionation that occurs between calcium in seawater and biogenic calcium carbonate. Up to now studies dealing with these issues focused on foraminifera and it has become increasingly clear that the isotopic composition of foraminiferal calcite does not depend on the isotopic composition of seawater alone. It was suggested that temperature might influence calcium isotope fractionation in foraminiferal calcite (Zhu and MacDougall 1998), and in a subsequent study it was shown that the foraminiferal $\delta^{44/40}$Ca signal might indeed serve as a new sea surface temperature (SST) proxy (Nägler et al. 2000). The applicability of $\delta^{44/40}$Ca as a SST proxy was further demonstrated in a recent case study (Gussone et al. 2004).

Several coccolithophore species also display a temperature dependent calcium isotope fractionation (Publication II, Publication III). In contrast to foraminifera (Gussone et al. 2003) no significant species specific differences in temperature dependence could be observed. In view of the potential application of coccolith $\delta^{44/40}$Ca as a sea surface temperature proxy this observation is of interest. To use foraminiferal $\delta^{44/40}$Ca it is necessary to isolate a single species from the sediment (Gussone et al. 2004). This task imposes no problems since the large foraminifera can be handpicked. Not until recently, however, it became possible to isolate monospecific coccolith samples for geochemical analysis from the sediment (Stoll and Ziveri 2002). If the coccolithophore species contributing significantly to the sedimentary archive display a similar $\delta^{44/40}$Ca -temperature dependence the interpretation of $\delta^{44/40}$Ca with respect to temperature from bulk coccolith oozes may be possible.

In the case of *Emiliania huxleyi* it was shown that the $\delta^{44/40}$Ca signal is not influenced by growth and calcification rate (Publication III). Since the latter variables change inevitably with temperature this finding is crucial to the potential use of $\delta^{44/40}$Ca as temperature proxy. Whether this insensitivity is also valid for other species remains to be tested. The use of a temperature proxy is often further hampered by the disturbing influence of factors other than and independent from temperature. The main secondary influences complicating the use of δ^{18}O as temperature proxy are the mean δ^{18}O of seawater, which is determined by the amount of continental ice and local variations in δ^{18}O of seawater due to differences in the evaporation / precipitation ratio (Rostek et al. 1993; Shackleton 1974). Furthermore there are known effects of carbonate chemistry on foraminiferal δ^{18}O (Bijma et al. 1999; Spero et al. 1997; Zeebe 1999). The Mg/Ca ratio is affected by partial dissolution (Brown and Elderfield 1996; Rosenthal et al. 2000) and salinity and pH changes (Lea et al. 1999; Nürnberg et al. 1996). Carbonate chemistry was shown to have a negligible effect on $\delta^{44/40}$Ca of two coccolithophore species (Publication II, Publication III). Moreover, in *E. huxleyi* salinity seems to have no significant effect on calcium isotope fractionation (Langer et al., unpublished data). Whether partial dissolution affects $\delta^{44/40}$Ca is not known, but it can be hypothesised that it does not, because calcium is a major component of calcite. Judging from these considerations the $\delta^{44/40}$Ca of coccolith calcite could be a promising temperature proxy. However, given the current analytical precision the measured slope of the $\delta^{44/40}$Ca temperature relationship allows for a resolution of approximately 5 °C (Publication II, Publication III). This is not sufficient, but a future increase in analytical precision could render the $\delta^{44/40}$Ca of coccolith calcite a reliable temperature proxy.

This weak temperature dependence and the lack of other dependencies result in a limited range of calcium isotope fractionation in *E. huxleyi* (Publication III). Also different species display similar calcium isotope fractionation behaviour (Publication II). Since coccolithophores contribute about half of the marine calcium carbonate production (Milliman 1993) this narrow range of $\delta^{44/40}$Ca in coccoliths is important for defining the isotopic composition of the calcium sink of the ocean, i.e. biogenic calcium carbonate. The calcium isotope fractionation between seawater and coccoliths might therefore be assumed constant when reconstructing the oceanic calcium budget over time (De La Rocha and DePaolo 2000).

The origin of the absolute calcium isotope fractionation as well as its temperature dependence in both biogenic and inorganically precipitated calcium carbonates is still a matter of debate (Gussone et al. 2005). For inorganically precipitated calcium carbonates three models are discussed: a kinetic calcium isotope fractionation model based on the diffusion of heavy calcium-aquocomplexes (Gussone et al. 2003) as opposed to an equilibrium fractionation model assuming that the Ca-O bond in the calcium-aquocomplex is stronger than in a carbonate mineral (Marriott et al. 2004) and an equilibrium fractionation model including effects of co-precipitation (Lemarchand et al. 2004). The kinetic model of Gussone et al. (2003) was criticised for the use of calcium-aquocomplexes with an unrealistically high number of coordinated water molecules (Lemarchand et al. 2004; Marriott et al. 2004). The model of Lemarchand et al. (2004) is able to explain the slope of the temperature dependence of $\delta^{44/40}$Ca in inorganically precipitated calcite and aragonite as well as the one observed in the foraminifer *O. universa* (Gussone et al. 2003) and coccolithophore species (Publication II, Publication III). However, a strong argument against the applicability of the model (Lemarchand et al. 2004) to *E. huxleyi* stems from the calcification mechanism of this species. Based on the cytological and physiological features of this mechanism (for discussion see Publication I, Publication III) it was concluded that calcium isotope fractionation cannot occur at the crystal surface as required by the model (Lemarchand et al. 2004), but must occur during cellular calcium transport instead. It can be hypothesised that the dehydration of the calcium-aquocomplex at the entrance of a calcium channel residing in the plasmamembrane might be the process responsible for fractionation (Publication III). Another possible mechanism of fractionation is the enrichment of ^{44}Ca in the protein fraction of the cytosol (Publication III). In any case, these considerations highlight the influence of cell physiology on calcium isotope fractionation in coccolithophores.

3.3 Coccolithophore calcification and ocean acidification

The carbonate saturation state in the ocean is largely determined by the seawater carbonate concentration. A doubling of surface ocean pCO_2, which is predicted to occur by the middle of this century (Houghton et al. 2001), will be accompanied by a decrease in the average carbonate concentration of surface water of 35 %. Recent laboratory and field studies have revealed a profound effect of seawater acidification and the related decrease in carbonate saturation state on the calcification rates of individual species and communities in both planktic and benthic habitats (Wolf-Gladrow et al. 1999). Corals (Kleypas et al. 1999; Leclercq et al. 2000), foraminifera (Bijma et al. 1999; Spero et al. 1997), and coccolithophores (Delille et al. 2005; Riebesell et al. 2000; Zondervan et al. 2001) each showed a decrease in calcification with increasing CO_2 concentration.

However, *Coccolithus pelagicus*, a productive marine calcifier, is insensitive to changes in the carbonate system over a range including last glacial maximum values and a scenario predicted for the year 2100 (Publication IV). This observation stresses the importance to consider species specific effects in assessing whole ecosystem responses to rising atmospheric CO_2. Moreover the behaviour of *C. pelagicus* reflects the fact that the solution from which the coccolith is precipitated is not seawater, but coccolith vesicle solution (Young and Henriksen 2003). The composition of this fluid certainly differs from that of seawater and so does the calcite saturation state. Hence it is not plausible to assume a linear relationship between calcite saturation state of the bulk medium and coccolith precipitation rate as was found for inorganically precipitated calcite (Nielsen 1964). Taking into account the response of *Calcidiscus leptoporus* to altered carbonate chemistry (Publication IV) the roles of cell physiology and species specific effects are emphasised. In contrast to *C. pelagicus,* in this species calcification rate and coccolith morphology are strongly dependent on carbonate chemistry of seawater. Unlike *Emiliania huxleyi*, *Gephyrocapsa oceanica* and inorganically precipitated calcite, however, the response of *C. leptoporus* shows an calcification optimum at present day CO_2 levels. Alkalisation as well as acidification of seawater hampers calcification rate and morphogenesis of coccoliths. This response indicates that regulatory processes of the cell are disturbed by altered carbonate chemistry and excludes an explanation solely based on knowledge of inorganic precipitation. Recently it has become evident that the response of corals to elevated pCO_2 might also be more complex than previously assumed. It was shown that pCO_2 and temperature significantly interact to control calcification in scleractinian corals (Reynaud et al. 2003).

Another important challenge, which has received little attention so far, is whether long-term adaptation has the potential to mitigate acute physiological responses (Gattuso and Buddemeier 2000). With one exception (Barker and Elderfield 2002) studies showing adverse effects of CO_2-related ocean acidification on calcification of marine organisms were based on short-term experiments applying abrupt CO_2 perturbations. Recent results from a mycorrhizal fungal community (Klironomos et al. 2005) clearly show that the effect of an abrupt increase in CO_2 concentration differs from that observed as a consequence of a gradual increase over several years. In our study (Publication IV) we have compared new experimental results of two prominent coccolithophores with sedimentary records of pre-industrial times and the last glacial maximum. This approach allowed us to assess the potential for long-term adaptation in *C. leptoporus,* an organism responding strongly to short-term experimental CO_2 perturbations. We inferred that *C. leptoporus* is able to adapt to long-term changes in carbonate chemistry of seawater.

In order to address the question whether *C. leptoporus* will be able to adapt to projected future changes in CO_2 levels the following need to be considered. Since the industrial revolution the rate of CO_2 increase was considerably faster than at any other time in the past 420,000 years (Falkowski et al. 2000). This rapid increase requires a fast adaptation process in evolutionary terms. Judging from the survey of natural samples from the last glacial maximum, the Holocene, the last 150 years and the present it appears likely that the timescale of adaptation for *C. leptoporus* is in the region of decades (Publication IV). An unicellular green alga evolved CO_2 related physiological features in the course of 1000 generations (Collins and Bell 2004). Adaptive changes in the beak morphology of Darwin's finches were observed in a 30 year period (Grant and Grant 2002) and in four species of Australian snakes collected over 80 years the invasion of toxic prey induced morphological changes (Phillips and Shine 2004). These examples show that even in vertebrates, which have considerably slower reproductive rates than phytoplankton, evolutionary processes do not necessarily occur on the timescale of at least thousands of years. The sexual life-cycle of coccolithophores ensures extensive genetic recombination, resulting in natural populations with significant ecophysiological variability (Brand 1982; Geisen et al. 2004; Paasche 2002). This genetically and physiologically diverse offspring could provide a sufficient amount of "raw material" for natural selection to occur on comparatively short timescales, i.e. decades. However, having adapted to an increase in CO_2 concentration since the last glacial maximum (Publication IV) it is presently unknown whether *C. leptoporus* will be able to adapt to a further increase in CO_2 levels. These uncertainties and the question whether other species

have the potential to adapt to such environmental changes are a major challenge for future research. Whatever precise function a coccosphere might have (Young 1994) it can be assumed that this carefully crafted structure actually is beneficial for the cell, otherwise this feature would not have been preserved during coccolithophore evolution. The ability to sustain a well formed coccosphere in an increasingly acidified ocean may prove essential for evolutionary success of coccolithophores; see also Smetacek (2001).

3.4 Perspectives for future research

This study revealed species specific reactions of coccolithophores to varying carbonate chemistry of seawater (Publication IV). Since only four species were investigated to date further experiments are needed to assess the question whether there are species showing other types of responses. Representative species, which also contribute significantly to carbonate export production should be used in order to address that important question. Moreover, it is presently unknown, which parameter of the carbonate system, i.e. pH, CO_2, CO_3^{2-} and calcite saturation state, exerts an influence on coccolithophore calcification rate and coccolith morphology. In order to answer that question experimental procedures shall be adapted insofar that different parameters of the carbonate system can be changed independently. While using organic buffers, bubbling with CO_2 results in altered CO_2 concentration without changing pH. By manipulating the calcium concentration information on the effect of calcite saturation state could be gained. Another important finding of this study concerns the potential of long-term adaptation to changing carbonate chemistry of seawater in *Calcidiscus leptoporus* (Publication IV). In order to gain more detailed information about the adaptation potential of *C. leptoporus* long-term experiments applying a gradual increase of CO_2 concentrations over several years as described in a recent study (Klironomos et al. 2005) should be conducted.

The experimental results obtained from *Coccolithus pelagicus* and *C. leptoporus* (Publication IV) highlight the need to understand the calcification mechanisms operating in coccolithophores. Not only is such an understanding required with regard to the question how ocean acidification will affect coccolithophore calcification, moreover it will significantly improve the use of coccolith-based paleo-proxies. In general, the incorporation of divalent cations into biogenic calcite can be used as a tool for investigating calcification mechanisms. In particular, the conceptual model (Publication I) predicts that barium most likely displays a behaviour similar to that of strontium if the Ba/Ca ratio in the seawater is altered. On the other

hand magnesium should behave in a different manner, i.e. no linear relationship between seawater Mg/Ca and coccolith Mg/Ca is expected. These questions shall be addressed experimentally by conducting further batch culture experiments using artificial seawater. However, in the long run, an interpretation of the results from those experiments can only be given on the cellular level. The vesicle-based calcification mechanism of coccolithophores will make it necessary to investigate the transmembrane transport of calcium, other divalent cations and CO_2/bicarbonate/carbonate in detail. Evidence from calcium isotope fractionation experiments (Publication II, Publication III) reveals the importance of these transport systems. Furthermore the cytosol could possibly be the location of calcium isotope fractionation in *Emiliania huxleyi* (Publication III). Hence cytosolic calcium transport has to be characterised. Measuring the calcium isotope fractionation of isolated cytosolic buffer proteins is the first step in this direction. In addition to this other cellular compartments like the endoplasmic reticulum might be involved in calcification related cation transport (Berry et al. 2002) and have to be taken into account as well. To summarize, the complete ion transport pathway has to be unravelled. Besides trace metal partitioning and stable isotope fractionation, combined biochemical, genetic and physiological approaches are the means to this end.

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4 Summary

The response of the two coccolithophores, *Calcidiscus leptoporus* and *Coccolithus pelagicus*, to changing carbonate chemistry of seawater was investigated in a combined laboratory and geological approach. The laboratory assay consisted of batch culture incubations of these species grown over a range of CO_2 concentrations in which cellular particulate inorganic carbon (PIC), cellular particulate organic carbon (POC), growth rate, and coccolith morphology were monitored. In *C. leptoporus* PIC changes with increasing CO_2 concentration in a non-linear relationship. A PIC-optimum curve is obtained, with a maximum value at present day surface ocean pCO_2 levels (~360 ppm CO_2). With POC remaining constant over the range of CO_2 concentrations, the PIC/POC ratio also shows an optimum curve. In the *C. pelagicus* cultures neither PIC nor POC changes significantly over the CO_2 range tested, yielding a stable PIC/POC ratio. Since growth rate in both species did not change with pCO_2, POC- and PIC-production show the same pattern as POC and PIC. Changes of calcification rate (PIC-production) were highly correlated to changes in coccolith morphology. Based on morphological analysis and counts of coccoliths per cell it was concluded that changing PIC is due to altered calcite per coccolith and not altered number of coccoliths per cell. Analysis of sediment samples was performed on six cores obtained from location well above the lysocline and covering a range of latitudes throughout the Atlantic Ocean. Scanning electron micrograph analysis of coccolith morphologies did not reveal any evidence for significant numbers of incomplete or malformed coccoliths of *C. pelagicus* and *C. leptoporus* in last glacial maximum (LGM) and Holocene sediments. Comparison of *C. leptoporus* experimental data with sedimentary records of pre-industrial times and the LGM reveals calcification to optimize at prevailing CO_2 levels. These results may be explained by evolutionary adaptation to changing CO_2 concentrations.

In recent studies the Sr/Ca ratio of coccoliths was used to infer past coccolithophore growth and calcification rates from the coccolith fossil record. These reconstructions are based on the assumption that the strontium exchange coefficient is not affected by changing seawater Sr/Ca. In the present study it was shown that the Sr/Ca of *Emiliania huxleyi* coccoliths increases linearly with increasing Sr/Ca of seawater within the range tested. This range comprises three orders of magnitude, the lowest value corresponds to 1% of the modern seawater value. Since this range is much larger than the postulated changes of Sr/Ca in the seawater since the Cretaceous the assumption of a constant strontium exchange coefficient with changing seawater Sr/Ca is supported by the data presented here. The strontium

exchange coefficient of coccolith calcite measured in this study is close to values presented in the literature but is high compared to values of inorganically precipitated calcite. This discrepancy can be explained by a simple conceptual model based on the channel/carrier mediated transport of calcium and strontium ions inside the cell. The model is solely based on thermodynamic constraints and does not assume a relationship between the exchange coefficient and the crystal growth rate. The calcite precipitation yields an accumulation of strontium in the coccolith vesicle until a steady state with respect to vesicle strontium concentration is achieved. It was shown that the strontium exchange coefficient of coccolith calcite is entirely determined by its steady state value. Introducing an additional constraint on the vesicle strontium concentration, i.e. the thermodynamic limit for the gradient of strontium concentration between the vesicle and the cytosol, the measured strontium exchange coefficient could be calculated.

Four coccolithophore species, *Calcidiscus leptoporus, Helicosphaera carteri, Syracosphaera pulchra* and *Umbilicosphaera foliosa* were cultured in the laboratory covering a temperature range from 14 to 23 °C. Calcium isotope composition of the coccoliths resembles in absolute fractionation as well as in temperature sensitivity previous calibration of planktonic foraminifer *Orbulina universa.* The as well determined lack of a CO_3^{2-}-$\delta^{44/40}$Ca dependence of *C. leptoporus* also agrees with *O. universa*. In a further set of experiments *Emiliania huxleyi* was examined in more detail. Temperature dependent calcium isotope fractionation in *E. huxleyi* resembles previous calibration of *O. universa* and inorganic calcite and aragonite. Temperature itself, and not concomitant calcification and growth rate changes, affects calcium isotope fractionation in *E. huxleyi.* Despite this advantage the calcium isotope composition of *E. huxleyi* coccoliths is not suited for paleoceanographic temperature reconstructions, because the resolution of 5 °C is too low. Unlike for inorganically precipitated calcite, the CO_3^{2-} concentration of the medium has no significant effect on the calcium isotope composition of the coccoliths. This result indicates a decoupling of the chemical properties of the bulk medium and the calcifying vesicle. Cellular calcium pathways and cytological features of *E. huxleyi* indicate that fractionation cannot occur at the crystal surface as during inorganic precipitation. The dominant process, leading to the observed calcium isotope fractionation pattern in *E. huxleyi* probably is the dehydration of the calcium-aquocomplex at the plasmamembrane and attaching dissolved calcium to amino acid residues of calcium channels. Another possibility is the preferential partitioning of the heavier calcium isotope to cytosolic calcium buffer proteins. The lack of species specific calcium isotope fractionation in the studied coccolithophore species, the independence of calcium isotope

fractionation from [CO_3^{2-}] in *E. huxleyi* and *C. leptoporus* and the small temperature dependence of the $\delta^{44/40}$Ca signal in the species studied principally allow for reconstructions of the calcium isotopic composition of seawater based on coccolithophores.

5 Zusammenfassung

Die Reaktion zweier Coccolithophoriden, *Calcidiscus leptoporus* und *Coccolithus pelagicus*, auf sich ändernde Karbonatchemie des Meerwassers wurde in einem kombinierten Ansatz bestehend aus Laborversuchen und der Analyse geologischer Proben untersucht. Die genannten Arten wurden im Labor in geschlossenen Flaschen mit unterschiedlichen CO_2 Konzentrationen inkubiert, wobei partikulärer anorganische Kohlenstoff (PIC), partikulärer organischer Kohlenstoff (POC), Wachstumsrate und Coccolithen-Morphologie aufgezeichnet wurden. Im Fall von *C. leptoporus* zeigen PIC und CO_2 Konzentration einen nicht-linearen Zusammenhang. Es ist eine PIC-Optimumskurve zu beobachten, welche einen Maximalwert bei heute herrschenden CO_2 Konzentrationen aufweist (~360 ppm CO_2). Da POC über den gesamten Bereich an CO_2 Konzentrationen konstant bleibt, zeigt das PIC/POC Verhältnis ebenfalls eine Optimumskurve. Im Falle von *C. pelagicus* ändert sich weder PIC noch POC im getesteten CO_2 Bereich, was zu einem konstanten PIC/POC Verhältnis führt. Da sich die Wachstumsrate bei beiden Arten nicht ändert zeigen PIC- und POC-Produktion die gleichen Muster wie PIC und POC. Änderungen in der Kalzifizierungsrate (PIC-Produktion) korrelieren mit Änderungen in der Coccolithenmorphologie. Basierend auf morphologischen Analysen und Zählungen der Coccolithen pro Zelle wurde geschlossen, dass verändertes PIC auf veränderten Kalzitgehalt pro Coccolith zurückzuführen ist und nicht veränderte Anzahl von Coccolithen pro Zelle. Sedimentproben aus sechs Kernen aus verschiedenen Breitengraden im Atlantik wurden analysiert, wobei alle Proben aus Tiefen über der Lysocline stammten. Rasterelektronenmikroskopische Untersuchungen der Coccolithenmorphologie zeigten keinen Hinweis auf signifikante Anzahlen unvollständig ausgebildeter oder missgestalteter Coccolithen bei *C. pelagicus* und *C. leptoporus* aus dem letzten glazialen Maximum (LGM) und dem Holozän. Vergleiche experimenteller Daten von *C. leptoporus* und Sedimentproben aus vorindustrieller Zeit und dem LGM lassen darauf schliessen, dass die Kalzifizierung für momentan herrschende CO_2 Konzentrationen optimiert ist. Diese Ergebnisse könnten mit einer evolutiven Anpassung an wechselnde CO_2 Konzentrationen erklärt werden.

In neueren Arbeiten wurde das Sr/Ca Verhältnis in Coccolithen benutzt um Wachstums- und Kalzifizierungsrate von Coccolithophoriden in der Vergangenheit zu ermitteln. Diese Rekonstruktionen basieren auf der Annahme, dass der Strontium-Verteilungskoeffizient nicht durch ein verändertes Sr/Ca Verhältnis im Meerwasser beeinflusst wird. In der vorliegenden Arbeit wurde gezeigt, dass das Sr/Ca Verhältnis von

Emiliania huxleyi Coccolithen über einen grossen Bereich mit dem Sr/Ca Verhältnis des Meerwassers linear ansteigt. Der getestete Bereich umfasst drei Grössenordnungen, wobei der niedrigste Wert 1% des heutigen Meerwasserwertes entspricht. Da dieser Bereich sehr viel grösser ist als angenommene Änderungen im Sr/Ca Verhältnisses des Meerwassers seit der Kreide, wird die Annahme eines konstanten Strontium-Verteilungskoeffizienten mit variablem Sr/Ca Verhältnis des Meerwassers durch die hier vorgelegten Daten unterstützt. Der in dieser Arbeit gemessene Strontium-Verteilungskoeffizient des Coccolithenkalzits ist Werten aus der Literatur sehr ähnlich, allerdings ist er hoch verglichen mit Werten anorganisch gefällten Kalzits. Dieser Unterschied kann mit Hilfe eines einfachen konzeptionellen Modells erklärt werden, das auf dem Kanal/Transporter vermittelten Transport von Kalzium- und Strontium-Ionen in der Zelle beruht. Das Modell basiert ausschliesslich auf thermodynamischen Randbedingungen und bedient sich nicht eines Verhältnisses zwischen dem Verteilungskoeffizienten und der Kristallwachstumsrate. Die Kalzitfällung führt zu einer Akkumulation von Strontium im Coccolithenvesikel bis ein Fliessgleichgewicht in Bezug auf die Vesikelstrontiumkonzentration erreicht ist. Es wurde gezeigt, dass der Strontium-Verteilungskoeffizient vollständig durch seinen Fliessgleichgewichtswert bestimmt ist. Unter Einbeziehung einer zusätzlichen Randbedingung, d.h. der thermodynamischen Grenze für den Gradient der Strontiumkonzentration zwischen Vesikel und Cytosol konnte der Strontium-Verteilungskoeffizient errechnet werden.

Vier Coccolithophoridenarten, *Calcidiscus leptoporus, Helicosphaera carteri, Syracosphaera pulchra* und *Umbilicosphaera foliosa* wurden im Labor in einem Temperaturbereich von 14 bis 23 °C kultiviert. Die Kalziumisotopenzusammensetzung der Coccolithen ähnelt sowohl in absoluter Fraktionierung als auch in der Temperaturabhängigkeit einer bestehenden Kalibrierung der planktischen Foraminifere *Orbulina universa*. Auch das Fehlen einer $[CO_3^{2-}]$-$\delta^{44/40}$Ca Abhängigkeit stimmt mit *O. universa* überein. In weiteren Experimenten wurde *E. huxleyi* im Detail untersucht. Die Temperaturabhängigkeit der Kalziumisotopenfraktionierung von *E. huxleyi* ähnelt der bekanten Kalibrierung von *O. universa* und anorganisch gefälltem Kalzit und Aragonit. Die Temperatur selbst, und nicht temperatur-induzierte Kalzifizierungs- und Wachstumsratenänderungen beeinflussen die Kalziumisotopenfraktionierung in *E. huxleyi*. Trotz dieses Vorteils ist die Kalziumisotopenzusammensetzung von *E. huxleyi* Coccolithen nicht zur palaeozeanographischen Temperaturrekonstruktion geeignet, da die Auflösung von 5 °C zu gering ist. Ungleich anorganisch gefälltem Kalzit, hat die

Karbonationenkonzentration des Mediums keinen signifikanten Einfluss auf die Kalziumisotopenzusamensetzung der Coccolithen. Dieses Ergebnis deutet auf eine Entkopplung der chemischen Eigenschaften des Mediums und der des Coccolithenvesikels hin. Zelluläre Kalziumtransportwege und cytologische Eigenschaften von *E. huxleyi* deuten darauf hin, dass die Fraktionierung nicht an der Kristalloberfläche, wie bei anorganischer Fällung, ablaufen kann. Der vorherrschende Prozess, der zum beobachteten Kalziumisotopenfraktionierungsmuster von *E. huxleyi* führt, ist wahrscheinlich die Dehydrierung des Kalzium-Aquokomplexes an der Plasmamembran und die Bindung von gelöstem Kalzium an Aminosäurereste von Kalziumkanälen. Eine andere Möglichkeit ist die Anreicherung des schwereren Kalziumisotopes in cytosolischen Kalziumpufferproteinen. Das Fehlen einer artspezifischen Kalziumisotopenfraktionierung in den untersuchten Coccolithophoridenarten, die Unabhängigkeit der Kalziumisotopenfraktionierung von der Karbonationenkonzentration in *E. huxleyi* und *C. leptoporus* und die schwache Temperaturabhängigkeit des $\delta^{44/40}$Ca Signals in den untersuchten Arten gestatten prinzipiell die Rekonstruktion der Kalziumisotopenzusammensetzung des Meerwassers anhand von Coccolithophoriden.

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

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