Coccolithophore calcification: Changing paradigms in changing oceans

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Abstract

Coccolithophores represent a major component of the marine phytoplankton and contribute to the bulk of biogenic calcite formation on Earth. These unicellular protists produce minute calcite scales (coccoliths) within the cell, which are secreted to the cell surface. Individual coccoliths and their arrangements on the cell surface display a wide range of morphological variations. This review explores some of the recent evidence that point to similarities and differences in the mechanisms of calcification, focussing on the transport mechanisms that bring substrates to, and remove products from the site of calcification, together with new findings on factors that regulate coccolith morphology. We argue that better knowledge of these mechanisms and their variations is needed to inform more generally how different species of coccolithophore are likely to respond to changes in ocean chemistry.

Keywords

Coccolithophore, coccolith, transport, morphogenesis

1. Introduction

Coccolithophores represent a globally-distributed group of haptophyte marine phytoplankton, distinguished by the production of complex crystalline calcite scales (coccoliths). They are particularly widespread in temperate and sub-tropical oceans with low abundance in Polar regions. Coccolithophores account for around 20% of ocean productivity and for the bulk of global biological calcification in the ocean [1], making major contributions to global biogeochemical cycles, particularly the long-term removal of inorganic carbon from the surface ocean [1]. Uniquely amongst calcifying organisms, coccoliths are produced intracellularly, within Golgi-derived coccolith vesicles. This involves transport of substrates to the coccolith vesicle and nucleation of calcite at multiple sites on an organic baseplate within the vesicle. Regulated crystal growth leads to mature coccoliths, which are secreted via exocytosis to the cell surface [2] where they are arranged in an ordered manner that has been shown to be necessary for proper cell division and structural integrity of the coccosphere [3,4].

Coccolithophores show large variations in coccolith morphology and complexity (Figure 1) and many contemporary studies have focused on their responses to changing ocean conditions, particularly the changes in carbonate chemistry associated with ocean acidification [e.g. 5]. From a cellular perspective, key questions in coccolithophore biology relate to the role(s), costs and benefits of coccolith production, the underlying cellular mechanisms and the factors controlling coccolith morphology. Answers to these questions are fundamental to gaining an understanding of their global ecology and for predicting the impacts of climate change on marine phytoplankton more generally. As with other calcifying organisms, coccolithophore biomineralization may be vulnerable to ocean acidification, which is predicted to reduce average ocean pH by as much as 0.5 pH unit in the current century [6]. Moreover, their global abundance and widespread distributions may mean that perturbations at the cellular level leading to small changes in growth rate are likely to have strong ecological and biogeochemical impacts [7]. However, since they produce calcite intracellularly in isolation from the surrounding seawater, they may exert significant biological control of the process, effectively providing a buffer from the external environment. It is also clear that coccolithophore responses to changing carbonate chemistry are not uniform across, or even within species [8].

However, studies comparing responses of different species have revealed inconsistencies that need to be resolved. To date much of our knowledge of coccolithophore cell biology and the responses of calcification to changing ocean conditions has been gained from studies of a single species, *Emiliania huxleyi*. Even within this species, significant variation in calcification responses to reduced ocean pH have been observed, likely reflecting the diversity of strains and morphotypes studied [8,9]. One meta analysis study comparing different species responses suggested that decreases in ocean pH to values predicted to occur in the current century will have a negative impact on calcification in *E. huxleyi* and *Gephyrocapsa oceanica*, with the larger more heavily calcified species *Coccollithus braarudii (formerly C. pelagicus)* less affected [9]. However, a more recent analysis of calcification responses of different coccolithophore species [10] has revealed an interesting relationship between cellular particulate inorganic to organic carbon (PIC:POC) ratios. More heavily calcified species with higher PIC:POC ratios were shown to be more, rather than less sensitive to decreased pH than those with lower PIC:POC ratios. Moreover, this trend was apparent when comparing the responses of different strains of *E. huxleyi* with differing PIC:POC ratios. The authors interpreted these findings in terms of intracellular pH regulation and the greater need to dispose of calcification-derived H^+ by more heavily calcified species or strains in the face of decreasing external pH.

Understanding wider coccolithophore diversity of function requires a better understanding of underlying mechanisms and their costs and functional benefits. This review explores some of the common mechanisms underlying coccolithophore calcification and outlines exceptions that point to divergence and the need to reassess some preconceived ideas of coccolithophore biomineralization. The occurrence of specific coccolithophore biogeographical assemblages with distinct coccosphere architectures points to links between coccolith formation and ecological adaptation [11]. It is likely that grazing pressure represented a common evolutionary driver for coccolith production with functional diversity evolving into providing roles as diverse as protection from photodamage in surface waters, pathogens and even as light collection devices in deep waters [11].

2. Cellular transport during calcification: one set of rules for all?

The cellular costs of calcification can be expressed in terms of energy costs, or nutrient requirement for synthesis of transporter and other metabolic and structural molecules [12]. Overall the energetic costs of calcification can be broadly categorized into transport of substrates (dissolved inorganic carbon (DIC) and Ca^{2+}) to the site of calcification in a controlled manner as well as removal of the soluble products, namely H^+, according to physiological evidence that HCO_3^- is the external substrate for calcification [13,14]. It is often useful to express these transport costs of calcification as the energetic cost, or ATP requirement, relative to the cost of fixing an equivalent amount of CO_2 in photosynthesis. Studies focussed mainly on the model species *E. huxleyi*, and *C. braarudii* have provided estimates of costs for transport of DIC and Ca^{2+}, with certain assumptions about the concentrations of Ca^{2+} and H^+ needed to allow calcite formation in the coccolith vesicle [9,12,15]. Calculations that assume a calcification rate similar to that of organic carbon fixation suggest that calcification requires 19-30% of the energy needed to fix an equivalent mole of organic carbon in photosynthesis. The principle components of these costs are: 1) the cost of bringing DIC into the coccolith compartment, 2) the cost of raising the concentration of Ca^{2+} at the calcification site and 3) the cost of removing H^+ from the coccolith vesicle and out of the cell.

The pathway for Ca^{2+} delivery to the intracellular site of calcification has yet to be definitively established. Passive Ca^{2+} entry into the cell via cation channels, coupled with active transport, for example via a Ca^{2+}/H^+ antiporter into an endomembrane compartment is the most likely route of Ca^{2+} entry into the coccolith vesicle [15,16]. The presence of an endomembrane intermediary Ca^{2+} storage compartment has been proposed [15] and such compartments (acidicalcicosmes) are a common feature of both calcifying and non-calcifying haptophytes, leading to the hypothesis that these have been adapted as a component of the

calcification pathway in coccolithophores. [17-19]. The energy requirements of Ca^{2+} transport are likely determined significantly by the nature of the Ca^{2+} transport pathway and the need to keep free Ca^{2+} concentration very low in the cytosol. A simple consideration of coccolith vesicle DIC and Ca^{2+} concentrations required achieve $CaCO_3$ precipitation, making certain assumptions about the pH of the coccolith vesicle, suggests that the cost of delivering Ca^{2+} to the precipitation site may be as much as 20% of the cost of fixing an equivalent amount organic carbon by photosynthesis [20]. The mechanism of Ca^{2+} transport into and H^+ removal from the site of calcification is supported by the observed strong up-regulation of CAX-like H^+/Ca^{2+} antiporters in calcifying *E. huxleyi* cells [21]. Essentially H^+/Ca^{2+} antiport may underlie the required alkalinisation of a Ca^{2+}-rich acidocalcisome precursor compartment that may develop into the coccolith vesicle or provide the source of Ca^{2+}-rich vesicles [22,23] for calcite precipitation. The cost of H^+ removal (or HCO_3^- accumulation) from this compartment required to elevate the saturation state of calcite ($(\Omega = [Ca^{2+}]\,[CO_3^{2-}]\,/K_{sp}>1$) has been estimated at approximately 5% of the total photosynthetic energy requirement [12,20]. These considerations therefore put the combined transport costs for Ca^{2+}, DIC and H^+ at around 25% of the total photosynthetic energy budget. A separate detailed analysis of coccolithophore transport costs [10], taking into account ion:ATP stoichiometry of transporters, with certain assumptions about the transporters involved, gave a slightly lower transport cost of $CaCO_3$ precipitation of 19% of the cost of photosynthetic carbon fixation.

We can add to this analysis a consideration of the cost of removing H^+ from the cytosol across the plasma membrane to maintain cytosolic pH around pH 7.0. At external pH 8.3 and measured resting membrane potential of around -50 mV [24], H^+ are close to electrochemical equilibrium across the plasma membrane, requiring only small excursions of cytosolic pH or depolarization of the plasma membrane potential to favour H^+ efflux. Voltage-gated H^+ channels in the plasma membrane are activated by membrane depolarization [24]. These have been shown to be involved in pH regulation [24] and are likely to play a key role in alleviating H^+ load during calcification. This also implies a dynamic regulation of intracellular pH via membrane potential (Figure 2). Indeed coccolithophores are electrically excitable, displaying rapid action potential-like depolarisations of the plasma membrane [25]. Moreover, they possess both animal-like voltage-gated 4-domain cation channels (Helliwell et al, unpublished results) and a recently discovered class of single domain voltage gated cation channels (EukCats) [24]. We hypothesize that the co-ordinated activity of voltage-gated cation and H^+ channels provides a mechanism to allow rapid, high capacity regulation of cytosolic pH. Earlier calculations have shown that at typical calcification rates in *C. braarudii* or *E. huxleyi,* H^+ production from calcification at a rate of 5 x

10^{-8} mol cell s^{-1} would decrease cytosolic pH by 0.3 pH units/minute from resting pH values if excess H^+ were not removed [24,27].

Electrophysiological studies of *C. braarudii* have revealed that, unusually, the outward current elicited on membrane depolarization is carried by H^+ rather than K^+, strongly suggesting that the repolarizing current of the action potential is carried by H^+ efflux from the cell [24]. This information allows a simple calculation of the effectiveness of this mechanism to remove calcification-derived H^+ from the cell. Repolarizing currents monitored in patch clamp recordings [24,25] are around 200 pA under normal seawater conditions. The estimated rate of calcification-derived H^+ production is equivalent to 0.3 pA of membrane current if all H^+ were removed from the cell through H^+ channels in the plasma membrane. This would require significantly less than one action potential per second to maintain cytosolic pH at 7.0 at normal seawater pH. The energetic costs associated with this mechanism relate to the cost of recharging the ionic gradients that are lost during the depolarization phase of the action potential.

If, by analogy with a typical animal-like action potential, Na^+ is the main carrier of the depolarization current, and assuming an approximately 10-fold Na^+ concentration difference across the plasma membrane, and a total depolarizing Na^+ influx equal to the repolarizing H^+ efflux (Figure. 2), the cost of maintaining the plasma membrane Na^+ gradient would be approximately 5% of the cost of carbon fixation. How would this mechanism cope with reduced extracellular pH in an acidifying ocean? From published electrophysiological data [24] the outward H^+ current would be significantly reduced at external pH around 7.5-7.8, due to the reduced H^+ electrochemical gradient and the voltage-dependency of the H^+ channels shifting to more positive potentials at lower external pH. The additional cost associated with this could have significant consequences for cell growth and competition in an ecological context, where costs and benefits need to be finely balanced. On the other hand, if the depolarizing phase of the action potential is carried by Ca^{2+} influx and if all the Ca^{2+} entering the cell is then subsequently used for calcification, there would potentially be little further cost of removing H^+ from the cell. Better understanding of these mechanisms underlying pH regulation will be required to address these important questions.

Key information needed to constrain cost-benefit analyses of calcification relates to the chemical conditions at the site of calcification in different coccolithophores. So far, coccolith vesicle pH is the only parameter to have been directly addressed. Earlier use of fluorescent indicators has given pH estimates from regions of the cell containing the coccolith vesicle ranging from 6.5 to 8.5, possibly reflecting pH changes as the calcifying compartment matures [20]. Limitations of optical resolution and the likely contribution of cytosolic dye to

the coccolith vesicle signal presented a limitation of this approach. Arguably, the advent of higher resolution confocal imaging and the future possibility of developing targeted genetic probes, following recent advances in genetic transformation of haptophytes [26] present opportunities for more accurate investigations.

An alternative approach to monitoring pH at the site of calcification involves monitoring boron/calcium (B/Ca) ratios of coccoliths [29,30]. Stoll et al [29] demonstrated the feasibility of this approach for investigating the regulation of pH or DIC in the coccolith vesicle in two coccolithophore species (*E. huxleyi* and *C. braarudii*). Borate is the only species of B incorporated into calcite and the ratio of borate to boric acid is dependent on pH at the site of precipitation. At constant seawater pH, the B/Ca ratio in the coccolith is determined by the pH and DIC concentration in the coccolith vesicle. Application to a wider range of species and experimental conditions is likely to provide significant insight into the regulation of conditions within the coccolith vesicle. This information is needed not only to constrain cost-benefit analyses of the calcification process but also to gain a deeper mechanistic understanding of how conditions at the site of calcification may be affected by changing ocean chemistry, particularly ocean pH. Such studies will also be critically important to understand how coccolith vesicle conditions vary between different coccolithophore types. Indeed, the coastal coccolithophore species, *Ochrosphaera neapolitana*, which is able to maintain relatively constant PIC:POC ratios at different seawater pH values in culture is also able to maintain constant coccolith vesicle pH conditions under differing CO_2-controlled seawater pH conditions [30]. Interestingly, *O. neapolitana* as well as *Pleurochrisis carterae* produce high Mg calcite [31,32], unlike most coccolithophores, which produce very low Mg calcite. *P. carterae* is also able to maintain constant PIC:POC ratios under varying seawater pH [32], suggesting fundamental differences in the calcification mechanism and ion transport pathways in these two species that may relate to mechanisms that control carbon allocation to calcification, setting them apart from other coccolithophores. Intriguingly, it has recently been shown that coccoliths of *S. apstenii* have an order of magnitude higher Sr content than those of other coccolithophores [33]. While this does not necessarily indicate a fundamental difference in the calcification pathway or machinery, it does point to further subtle differences between different coccolithophore species that will be important to understand.

To what extent different coccolithophore species use the same transport mechanism and pathways for calcification? Gene expression analysis revealed a predominance of conserved anion and cation transport components associated with calcification in cells of *E. huxleyi* [21], suggesting that calcification-associated transport processes likely evolved from pre-existing transport components and that calcification-related transport systems may be more generally conserved across different coccolithophore species. However, there is insufficient

detailed genetic, structural or physiological information on calcification-specific transport pathways in a wider range of species to be able to make robust comparisons. Indeed, several lines of evidence suggest divergence from the general model. A study of carbon isotope fractionation in two different coccolithophore species, *C. braarudii* and *Gephyrocapsa oceanica*, a close relative of *E. huxleyi*, revealed differences in their isotope fractionation properties in relation to DIC supply [34]. It was proposed that *C. braarudii* may utilise calcification-derived H^+ produced for photosynthetic CO_2 production. In contrast, the smaller cells of *G. oceanica* may utilise H^+ removed from the cell to facilitate external CO_2 generation through the action of external carbonic anhydrase. This contrasts with earlier work showing that in *E. huxleyi*, inhibition of calcification by removal of Ca^{2+} from the external medium, did not affect the cells' abilities to acquire DIC for photosynthesis under short-term carbon-limiting conditions [35,36]. Moreover, multifactorial experiments examining the DIC species utilized for calcification in *E. huxleyi* provided good evidence that both photosynthesis and calcification can utilize external HCO_3^- and that they may even compete for DIC substrate under certain conditions [37].

The differential sensitivities of coccolithophore calcification to external pH perturbation shown by different species may also suggest some variability or specialization of transport pathways. However, significant variation in sensitivity to external pH can also found between strains within the same species [8], which are unlikely to reflect fundamental differences in calcification pathways or components. There are also important ultrastructural differences between species that likely relate to more fundamental differences in calcification mechanisms. For example, a membrane-rich organelle, known as the reticular body has been described in certain coccolithophore species, including *E. huxleyi* and *S. apstenii* [38-40] but is absent in other species, such as *Pleurochrysis (Chrysotilla)* spp. Since the reticular body has been proposed as a major component of the calcification transport pathway, this does imply substantial mechanistic differences between these species. Currently only limited genetic information is available to allow detailed exploration of these potential differences, with one fully sequenced genome and a handful of coccolithophore transcriptomes available. [41,42]

3. Coccolith morphology: divergence in regulatory factors

While it is relatively straightforward, given certain assumptions, to provide estimates of the on energetic costs of calcification and the likely impacts of ocean acidification, it is perhaps less obvious how changing ocean chemistry may affect the ability of cells to shape coccolith structure. As research on coccolith formation moves increasingly from descriptive to mechanistic, some recent studies of coccolith crystal morphology are beginning to shed light

on the requirement for proper coccolith morphology for cell function and the potential for environmental factors to alter this morphology. Many species of coccolithophore produce two types of coccoliths: Heterococcoliths, produced by diploid cells are complex multicrystalline structures while holococcoliths, produced by haploid life cycle stages are comprised of simple rhombic crystals and have been less studied. The factors that promote life cycle phase transitions are not well studied in most coccolithophore species. Nutritional preferences of haploid and diploid cells have been shown to give rise to shifts in dominance of particular life cycle phases in culture populations [43] and induction of life cycle switching has been observed in Calyptrosphaera shpaeroidea [44] there are few reports of directs phase transitions In *E. huxleyi,* which produces heterococcolith-bearing diploid cells but naked flagellate haploid cells, there is evidence that morphological transitions within the diploid phase occur in response to viral infection to produce cells that are morphologically similar to motile haploid cells. However, these transitions are de-coupled from life cycle transistions. [45]

During heterococcolith formation, initial nucleation events, most likely on an organic baseplate template determine the location of crystal elements and their orientation [46]. Surprisingly little is known about the nature of the organic baseplase, its composition and how this varies in different species or the underlying mechanism by which the elements of the baseplate that form the protococcolith ring are organised. However, recent *in vitro* studies offer new insights into the role of the organic baseplate in initiating coccolith crystal orientation and growth. In a breakthrough study, Gal et al [47], using isolated heterococcoliths of *P. carterae* showed that the differently oriented crystals (V and R type) had different structural associations with the baseplate. They also showed that specific interactions between soluble organic molecules and an organic backbone structure directs Ca^{2+}-dependent association of mineral components to specific sites on isolated baseplates. This provided strong evidence for a cooperative interaction between components of the organic template and soluble molecules in directing Ca^{2+} to the site of mineralization. Further insight into this mechanism was provided by Sukurada et al [48] who demonstrated, by chemical treatment of baseplates of *P. haptonemofera*, a requirement for both protein and a particular acidic polysaccharide (Ph-PS-2) for precipitation of calcium-rich aggregates around the baseplate rim.

The only current genetic evidence to suggest a regulatory role for a specific coccolith-associated protein factor comes from correlations between the coccolith morphology motif of the *E. huxleyi* coccolith-associated protein GPA (glutamate, proline, alanine-rich) and coccolith morphotype [49,50]. Gene expression studies in *E. huxleyi* have also shown an inverse correlation between the level of GPA expression and degree of calcification [51],

suggesting that GPA may have a negative regulatory role. GPA is not found in other coccolithophore species, so its role appears to be specific for *E. huxleyi.* However, GPA is a low complexity protein with a high degree of repeated sequences and it remains to be seen whether other low complexity proteins with similar acidic residue content are found in other coccolithophore species.

3.1 **Regulation of crystal growth and morphology: the role of polysaccharides**.

As with the crystal nucleation process, little is known about what factors regulate crystal growth and overall coccolith morphology following nucleation. Likely roles for the involvement of polysaccharides and cytoskeleton have been proposed for some time [23, 52-55]. There is now substantial evidence that coccolith-associated polysaccharides (CAPs) play an important role in the regulation of both coccolith growth and morphology. CAPs are predominantly acidic polysaccharides and the negatively charged carboxyl groups of uronic acid residues in solution can bind Ca^{2+} and potentially inhibit calcite precipitation.

A number of *in vitro* studies have identified a range of mechanisms through which soluble organic molecules may interfere with calcite precipitation from solution. Soluble natural organic material was shown to inhibit calcite precipitation and to be more effective at lower carbonate/calcium ratios and lower pH values [56]. This could be described by a Langmuir adsorption model whereby adsorptive interactions between the organic molecule and calcite surface are driven by entropy change of the adsorptive reaction. Moreover, higher molecular weight molecules and those with high aromatic content were more effective in inhibiting calcite precipitation. Microkinetic modelling that considers adsorptive energy at the crystal step, together with experimental calcite growth studies have indicated that soluble inorganic ions and organic molecules may inhibit calcite precipitation by both complexing in solution and inhibition of ion incorporation at the crystal steps to varying degrees [57]. Ions, such as SO_4^{2-} were shown to act through direct crystal step blocking, while Mg^{2+} acted through both step-blocking together with an degree of solution complexing. Soluble carboxylic acids, such as benzoate and acetate, most likely acted to inhibit precipitation through solution complexing with a smaller degree of crystal step-blocking and were more effective at lower calcite saturation states.

In vitro studies have shown that CAPs are able to regulate calcite precipitation in a range of coccolithophore species [e.g. 58-60], though definitive evidence of their role *in vivo* is mainly restricted to demonstration of localization of polysaccharides with the growing coccolith [22,23]. CAPs that have been isolated from coccolithophores show substantial biochemical diversity between species. A single CAP was isolated from *E. huxleyi* [58] and three distinct CAPs were identified in *P. carterae* [61], with evidence also for a role for at least one of

these in the supply of Ca^{2+} to the calcification site [40]. Significant differences in the uronic acid content of CAPs have been noted, which may potentially influence the shaping of the calcite crystals [40] or reflect adaptations to differing calcite saturation states of the coccolith vesicle. In this respect Lee et al [62] showed that coccolithophore species, such as a heavily calcifying *E. huxleyi* strain, belonging to the order Isochrysidales, which is likely to have a high calcite saturation state in the coccolith vesicle, deduced from data on intracellular DIC pools and surface area/volume ratios, also has a CAP with high uronic acid content. In contrast, *Coccolithus spp*, belonging to the order Coccolithales, showed the lowest CAP uronic acid content. Lee et al proposed that with lower surface area/volume ratios and no evidence for the presence of DIC accumulation Coccolithales arguably maintained a lower calcite saturation state in the coccolith vesicle. This was consistent with the demonstration that polysaccharide substrates with high levels of carboxyl functional groups promote fast nucleation rates under conditions of high saturation states, whereas polysaccharides with lower carboxyl content promote nucleation at lower saturation states [63]. Indeed, Lee et al [62] concluded that by altering their polysaccharide charge density, coccolithophores may fine-tune calcite precipitation to varying degrees of internal supersaturation, which may allow them to achieve optimal calcification. The inference from these studies is that CAPs are involved in promoting calcite nucleation whereas earlier work on isolated polysaccharides suggests that CAPs tend to inhibit calcite precipitation, albeit *in vitro* [e.g. 58-60]. At first sight, this appears to be a paradox in need of resolution and it is likely that the regulation of calcite precipitation by polysaccharides is more subtle than simple promotion or inhibition. There may be differences between behaviour *in vivo* and *in vitro* and, as illustrated above, it is clear that soluble polysaccharides may behave differently from those that have roles as solid substrates for precipitation.

An additional *in vitro* approach [64] studied calcium carbonate precipitation in the presence of CAPs purified from *E. huxleyi* and *G. oceanica*, showing that CAPs from *G. oceanica*, but not those from *E. huxleyi* promoted the nucleation of calcite even under conditions that would normally promote precipitation as vaterite or aragonite. This suggests different functional roles for CAPS even between these closely related species. The study also showed that CAP is located within the grain boundaries rather than within the crystal lattice in both *in vitro* precipitation and in biogenic coccoliths. Since the coherence length data do not distinguish boundaries between individual crystals from boundaries within crystals an unanswered question concerns the location of the grain boundaries. This has an important bearing on the formation mechanism of coccolith crystals and whether they have a nano-structure similar to all other studied marine biogenic calcium carbonates.

A further complexity to the CAP story is the demonstration that extracellular coccoliths are coated with an organic layer that may have an important influence on the dissolution of coccoliths [48,65,66]. The use of fluorescent lectins [67] has shown that the polysaccharide layer covering external coccoliths differs significantly between species in structure and composition. Further significant differences were shown between species in monosaccharide composition and uronic acid content of these polysaccharides. Moreover, this study demonstrated an additional role of external polysaccharides in *C. braarudii* whereby polysaccharide extruded with, but not integral to the coccoliths, is important for the adhesion of the coccoliths to the cell surface and their overall organisation within the coccosphere.

3.2 Further determinants of coccolith morphology.

The studies summarised above point to links between the cellular transport processes that give rise to carbonate saturation conditions in the coccolith vesicle and regulatory processes that determine calcite precipitation rate and morphology. Recently, two lines of evidence have revealed further unexpected differences in control of coccolith morphology between species. First, Ge a competitive inhibitor of Si transport in diatoms, was unexpectedly shown to cause specific striking malformations in coccolith morphology in two members of the Coccolithales (*C. braarudii* and *Calcidiscus leptoporus*) and in the Zygodiscales species *Scyphosphaera apstenii* [68]. Growth in low Si medium produced similar results. In contrast, Ge treatment had no effect at all on the Isochrysidales species *E. huxleyi*. Second, diatom-like Si transporters (SITs) or SIT-like transporters were found in the transcriptomes of those coccolithophore species that show sensitivity to Ge. Significantly, however, *E. huxleyi, G. oceanica* and *P. carterae*, were shown to lack SITs and were completely insensitive to Ge treatments. Moreover disruption of coccolith morphology in *C. braarudii* led to disorganization of the coccosphere, which in turn resulted in an inability of cells to separate following mitosis and the onset of cell cycle arrest [69]. These observations represent the clearest physiological distinction to date between different coccolithophore groups and raise a number of important questions: First, what is the mechanism by which Si is involved in the regulation of proper coccolith morphology in those Si-requiring species? Some clues may come from other studies showing interaction between Si and calcium carbonate. Silica has been shown to be important in the deposition of amorphous calcium carbonate (ACC) in vitro [70] and in mineralized cystoliths from plants [71]. However, there is so far no evidence that ACC is involved in the precipitation of coccolith calcite. Second, the phylogeny of SITs suggests that they were present in an ancestral lineage to coccolithophores [72]. We have proposed that the Si requirement may be an evolutionary trait that has been lost in much

more recently evolving species, such as *E. huxleyi*. If so, we can ask what mechanisms did non-Si requiring coccolithophores evolve to regulate morphology during coccolith formation and growth? One possibility for further test is that certain classes of coccolith-associated polysaccharides may have substituted for Si in this respect. Third, does the differential Si requirement between species have any ecological relevance, such as explaining why *E. huxleyi* commonly forms blooms in Si-depleted waters following diatom blooms, or might it explain particular coccolithophore geographical distributions relating to Si availability? Finally, did the evolution of a Si-independent mechanism for coccolithogenesis correlate with the reduction in ocean silicate levels brought about by the rise of the diatom lineages? [73] (Figure 3).

4. Concluding remarks

The evidence presented here points to an intricate system of interactions between the processes that drive calcification and those that regulate coccolith morphology and there is much to learn about both. It is clear that there are both subtle and stark differences between different types of coccolithophores, both in the physiological controls of coccolith production and the fine regulation of coccolith shape. It has been noted [2, 74,75] that the most commonly studied species *E. huxleyi* has a number of features, including its coccosphere structure, its biogeography and its ability to form huge blooms, which are atypical for coccolithophores. It is important, therefore to consider coccolithophore diversity and the ecological importance of other species, which together are at least equally globally abundant and biogeochemically important [e.g. 76,77] in order to fully understand their essential properties.

The varied mechanisms underlying key aspects of coccolithophore biology point to a need for better mechanistic understanding of the calcification process and its functions across the coccolithophore realm. Indications, outlined above, of differing transport processes, differing modes of regulation of the conditions at the site of calcification in the coccolith vesicle along with differing utilization of macromolecular components including proteins and polysaccharides call for a paradigm shift in approaches to understanding the diversity of cellular processes and mechanisms between and within species. This is particularly required in order to understand the varied impacts of changing ocean chemistry on the complex ecology of coccolithophores and their likely responses to acidifying and warming oceans that will in turn have important impacts on global biogeochemistry.

Our understanding of the roles and underlying mechanisms of calcification have been held back increasingly by the lack of genetic and other tools for coccolithophores and haptophytes more generally. While there will clearly be large obstacles to overcome, there

are encouraging signs that genetic manipulation of at least certain haptophytes is feasible [28,78], paving the way for gene knock-down and knock-out and the use of genetically-encoded reporters. Ongoing large-scale genome sequencing projects are likely to provide a wealth of information to help identify species-specific differences. These, together with the advent of new analytical approaches, such as cryo-EM and elemental analyses that are helping to define nanoscale processes in coccolith formation will ensure that our understanding of this important group of organisms will continue to improve.

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Figures

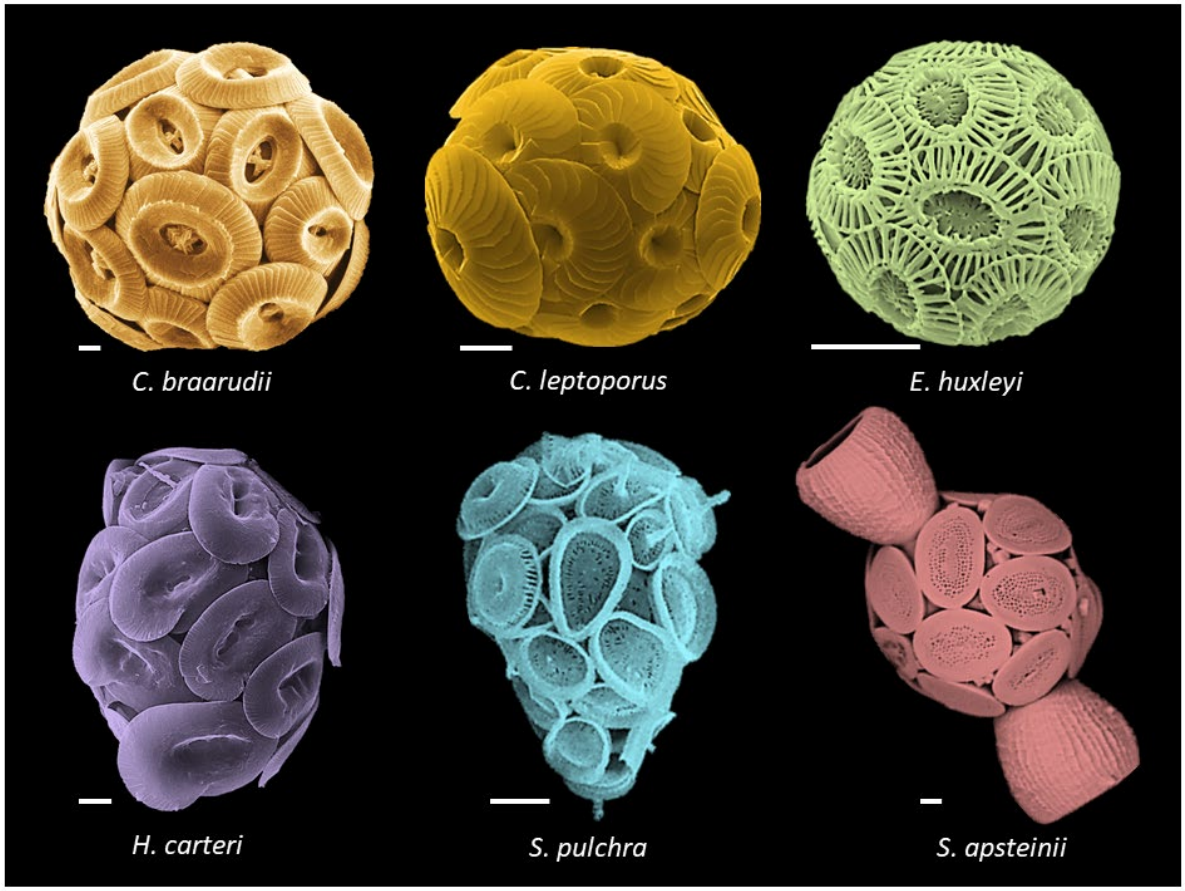

Figure 1. Examples of coccolithophore morphologies. Top (left to right): *Coccolithus braarudii, Calcidiscus leptoporus, Emiliania huxleyi.* Bottom: *Helicosphaera carteri, Syracosphaera pulchra, Scyphosphaera apstenii.* Scale bars, 2 μm.

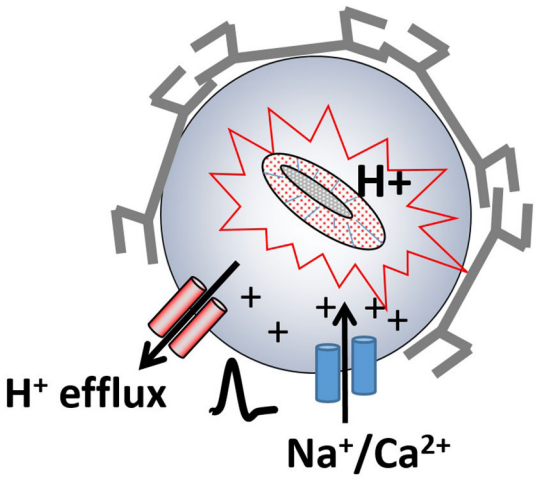

Figure 2. Schematic of hypothetical role of electrical control of cellular pH through the action of voltage-gated H+ channels that are in turn regulated by action potential-like

711 depolarization of the membrane potential brought about by the influx of cations through
712 voltage-gated cation channels.
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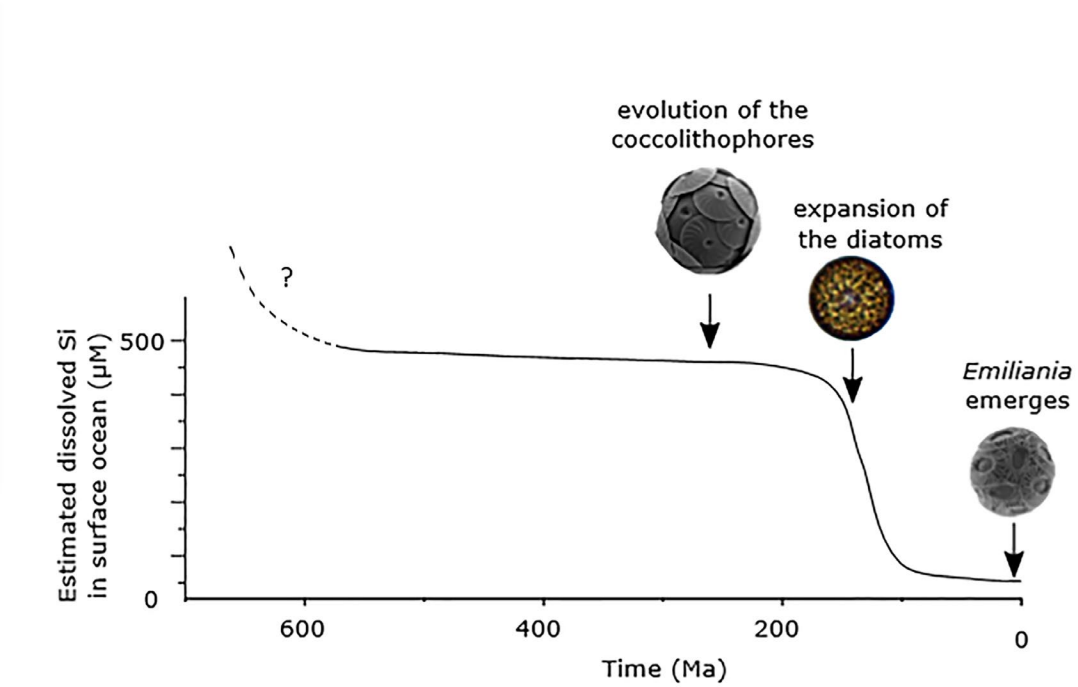

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715 **Figure 3**. Approximate dissolved Si levels [73] through geological time in relation to the
716 emergence of coccolithophores, diatoms and the much more recent emergence of *E. huxleyi*
717 in the paleo-oceanographic record. The dotted line indicates uncertainty around early ocean
718 Si levels.