

1 Cocolithophore calcification: Changing paradigms in changing oceans

2 Colin Brownlee^{a,b}, Gerald Langer^a, Glen L. Wheeler^a

3 a. Marine Biological Association, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK

4 b. School of Ocean and Earth Sciences, University of Southampton, Southampton,
5 SO14 3ZH, UK.

6 Abstract

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

17 Keywords

18 Cocolithophore, coccolith, transport, morphogenesis

19

20 1. Introduction

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

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

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

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

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

80 The cellular costs of calcification can be expressed in terms of energy costs, or nutrient
81 requirement for synthesis of transporter and other metabolic and structural molecules [12].
82 Overall the energetic costs of calcification can be broadly categorized into transport of
83 substrates (dissolved inorganic carbon (DIC) and Ca^{2+}) to the site of calcification in a
84 controlled manner as well as removal of the soluble products, namely H^+ , according to
85 physiological evidence that HCO_3^- is the external substrate for calcification [13,14]. It is often
86 useful to express these transport costs of calcification as the energetic cost, or ATP
87 requirement, relative to the cost of fixing an equivalent amount of CO_2 in photosynthesis.
88 Studies focussed mainly on the model species *E. huxleyi*, and *C. braarudii* have provided
89 estimates of costs for transport of DIC and Ca^{2+} , with certain assumptions about the
90 concentrations of Ca^{2+} and H^+ needed to allow calcite formation in the coccolith vesicle
91 [9,12,15]. Calculations that assume a calcification rate similar to that of organic carbon
92 fixation suggest that calcification requires 19-30% of the energy needed to fix an equivalent
93 mole of organic carbon in photosynthesis. The principle components of these costs are: 1)
94 the cost of bringing DIC into the coccolith compartment, 2) the cost of raising the
95 concentration of Ca^{2+} at the calcification site and 3) the cost of removing H^+ from the
96 coccolith vesicle and out of the cell.

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

104 calcification pathway in coccolithophores. [17-19]. The energy requirements of Ca^{2+}
105 transport are likely determined significantly by the nature of the Ca^{2+} transport pathway and
106 the need to keep free Ca^{2+} concentration very low in the cytosol. A simple consideration of
107 coccolith vesicle DIC and Ca^{2+} concentrations required to achieve CaCO_3 precipitation, making
108 certain assumptions about the pH of the coccolith vesicle, suggests that the cost of
109 delivering Ca^{2+} to the precipitation site may be as much as 20% of the cost of fixing an
110 equivalent amount of organic carbon by photosynthesis [20]. The mechanism of Ca^{2+} transport
111 into and H^+ removal from the site of calcification is supported by the observed strong up-
112 regulation of CAX-like $\text{H}^+/\text{Ca}^{2+}$ antiporters in calcifying *E. huxleyi* cells [21]. Essentially $\text{H}^+/\text{Ca}^{2+}$
113 antiport may underlie the required alkalinisation of a Ca^{2+} -rich acidocalcisome precursor
114 compartment that may develop into the coccolith vesicle or provide the source of Ca^{2+} -rich
115 vesicles [22,23] for calcite precipitation. The cost of H^+ removal (or HCO_3^- accumulation)
116 from this compartment required to elevate the saturation state of calcite ($(\Omega = [\text{Ca}^{2+}][\text{CO}_3^{2-}]$
117 $/K_{\text{sp}} > 1)$) has been estimated at approximately 5% of the total photosynthetic energy
118 requirement [12,20]. These considerations therefore put the combined transport costs for
119 Ca^{2+} , DIC and H^+ at around 25% of the total photosynthetic energy budget. A separate
120 detailed analysis of coccolithophore transport costs [10], taking into account ion:ATP
121 stoichiometry of transporters, with certain assumptions about the transporters involved, gave
122 a slightly lower transport cost of CaCO_3 precipitation of 19% of the cost of photosynthetic
123 carbon fixation.

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

140 10^{-8} mol cell s^{-1} would decrease cytosolic pH by 0.3 pH units/minute from resting pH values
141 if excess H^+ were not removed [24,27].

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

154 If, by analogy with a typical animal-like action potential, Na^+ is the main carrier of the
155 depolarization current, and assuming an approximately 10-fold Na^+ concentration difference
156 across the plasma membrane, and a total depolarizing Na^+ influx equal to the repolarizing H^+
157 efflux (Figure. 2), the cost of maintaining the plasma membrane Na^+ gradient would be
158 approximately 5% of the cost of carbon fixation. How would this mechanism cope with
159 reduced extracellular pH in an acidifying ocean? From published electrophysiological data
160 [24] the outward H^+ current would be significantly reduced at external pH around 7.5-7.8,
161 due to the reduced H^+ electrochemical gradient and the voltage-dependency of the H^+
162 channels shifting to more positive potentials at lower external pH. The additional cost
163 associated with this could have significant consequences for cell growth and competition in
164 an ecological context, where costs and benefits need to be finely balanced. On the other
165 hand, if the depolarizing phase of the action potential is carried by Ca^{2+} influx and if all the
166 Ca^{2+} entering the cell is then subsequently used for calcification, there would potentially be
167 little further cost of removing H^+ from the cell. Better understanding of these mechanisms
168 underlying pH regulation will be required to address these important questions.

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

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

179 An alternative approach to monitoring pH at the site of calcification involves monitoring
180 boron/calcium (B/Ca) ratios of coccoliths [29,30]. Stoll et al [29] demonstrated the feasibility
181 of this approach for investigating the regulation of pH or DIC in the coccolith vesicle in two
182 coccolithophore species (*E. huxleyi* and *C. braarudii*). Borate is the only species of B
183 incorporated into calcite and the ratio of borate to boric acid is dependent on pH at the site of
184 precipitation. At constant seawater pH, the B/Ca ratio in the coccolith is determined by the
185 pH and DIC concentration in the coccolith vesicle. Application to a wider range of species
186 and experimental conditions is likely to provide significant insight into the regulation of
187 conditions within the coccolith vesicle. This information is needed not only to constrain cost-
188 benefit analyses of the calcification process but also to gain a deeper mechanistic
189 understanding of how conditions at the site of calcification may be affected by changing
190 ocean chemistry, particularly ocean pH. Such studies will also be critically important to
191 understand how coccolith vesicle conditions vary between different coccolithophore types.
192 Indeed, the coastal coccolithophore species, *Ochrosphaera neapolitana*, which is able to
193 maintain relatively constant PIC:POC ratios at different seawater pH values in culture is also
194 able to maintain constant coccolith vesicle pH conditions under differing CO₂-controlled
195 seawater pH conditions [30]. Interestingly, *O. neapolitana* as well as *Pleurochrisis carterae*
196 produce high Mg calcite [31,32], unlike most coccolithophores, which produce very low Mg
197 calcite. *P. carterae* is also able to maintain constant PIC:POC ratios under varying seawater
198 pH [32], suggesting fundamental differences in the calcification mechanism and ion transport
199 pathways in these two species that may relate to mechanisms that control carbon allocation
200 to calcification, setting them apart from other coccolithophores. Intriguingly, it has recently
201 been shown that coccoliths of *S. apstenii* have an order of magnitude higher Sr content than
202 those of other coccolithophores [33]. While this does not necessarily indicate a fundamental
203 difference in the calcification pathway or machinery, it does point to further subtle differences
204 between different coccolithophore species that will be important to understand.

205 To what extent different coccolithophore species use the same transport mechanism and
206 pathways for calcification? Gene expression analysis revealed a predominance of conserved
207 anion and cation transport components associated with calcification in cells of *E. huxleyi*
208 [21], suggesting that calcification-associated transport processes likely evolved from pre-
209 existing transport components and that calcification-related transport systems may be more
210 generally conserved across different coccolithophore species. However, there is insufficient

211 detailed genetic, structural or physiological information on calcification-specific transport
212 pathways in a wider range of species to be able to make robust comparisons. Indeed,
213 several lines of evidence suggest divergence from the general model. A study of carbon
214 isotope fractionation in two different coccolithophore species, *C. braarudii* and
215 *Gephyrocapsa oceanica*, a close relative of *E. huxleyi*, revealed differences in their isotope
216 fractionation properties in relation to DIC supply [34]. It was proposed that *C. braarudii* may
217 utilise calcification-derived H⁺ produced for photosynthetic CO₂ production. In contrast, the
218 smaller cells of *G. oceanica* may utilise H⁺ removed from the cell to facilitate external CO₂
219 generation through the action of external carbonic anhydrase. This contrasts with earlier
220 work showing that in *E. huxleyi*, inhibition of calcification by removal of Ca²⁺ from the
221 external medium, did not affect the cells' abilities to acquire DIC for photosynthesis under
222 short-term carbon-limiting conditions [35,36]. Moreover, multifactorial experiments
223 examining the DIC species utilized for calcification in *E. huxleyi* provided good evidence that
224 both photosynthesis and calcification can utilize external HCO₃⁻ and that they may even
225 compete for DIC substrate under certain conditions [37].

226 The differential sensitivities of coccolithophore calcification to external pH perturbation
227 shown by different species may also suggest some variability or specialization of transport
228 pathways. However, significant variation in sensitivity to external pH can also be found
229 between strains within the same species [8], which are unlikely to reflect fundamental
230 differences in calcification pathways or components. There are also important ultrastructural
231 differences between species that likely relate to more fundamental differences in calcification
232 mechanisms. For example, a membrane-rich organelle, known as the reticular body has
233 been described in certain coccolithophore species, including *E. huxleyi* and *S. apstenii* [38-
234 40] but is absent in other species, such as *Pleurochrysis (Chrysotilla)* spp. Since the
235 reticular body has been proposed as a major component of the calcification transport
236 pathway, this does imply substantial mechanistic differences between these species.
237 Currently only limited genetic information is available to allow detailed exploration of these
238 potential differences, with one fully sequenced genome and a handful of coccolithophore
239 transcriptomes available. [41,42]

240 **3. Coccolith morphology: divergence in regulatory factors**

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

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

260 During heterococcolith formation, initial nucleation events, most likely on an organic
261 baseplate template determine the location of crystal elements and their orientation [46].
262 Surprisingly little is known about the nature of the organic baseplate, its composition and
263 how this varies in different species or the underlying mechanism by which the elements of
264 the baseplate that form the protococcolith ring are organised. However, recent *in vitro*
265 studies offer new insights into the role of the organic baseplate in initiating coccolith crystal
266 orientation and growth. In a breakthrough study, Gal et al [47], using isolated
267 heterococcoliths of *P. carterae* showed that the differently oriented crystals (V and R type)
268 had different structural associations with the baseplate. They also showed that specific
269 interactions between soluble organic molecules and an organic backbone structure directs
270 Ca²⁺-dependent association of mineral components to specific sites on isolated baseplates.
271 This provided strong evidence for a cooperative interaction between components of the
272 organic template and soluble molecules in directing Ca²⁺ to the site of mineralization.
273 Further insight into this mechanism was provided by Sukurada et al [48] who demonstrated,
274 by chemical treatment of baseplates of *P. haptanemofera*, a requirement for both protein and
275 a particular acidic polysaccharide (Ph-PS-2) for precipitation of calcium-rich aggregates
276 around the baseplate rim.

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

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

287 3.1 Regulation of crystal growth and morphology: the role of polysaccharides.

288 As with the crystal nucleation process, little is known about what factors regulate crystal
289 growth and overall coccolith morphology following nucleation. Likely roles for the
290 involvement of polysaccharides and cytoskeleton have been proposed for some time [23,
291 52-55]. There is now substantial evidence that coccolith-associated polysaccharides
292 (CAPs) play an important role in the regulation of both coccolith growth and morphology.
293 CAPs are predominantly acidic polysaccharides and the negatively charged carboxyl groups
294 of uronic acid residues in solution can bind Ca^{2+} and potentially inhibit calcite precipitation.

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

311 *In vitro* studies have shown that CAPs are able to regulate calcite precipitation in a range of
312 coccolithophore species [e.g. 58-60], though definitive evidence of their role *in vivo* is mainly
313 restricted to demonstration of localization of polysaccharides with the growing coccolith
314 [22,23]. CAPs that have been isolated from coccolithophores show substantial biochemical
315 diversity between species. A single CAP was isolated from *E. huxleyi* [58] and three distinct
316 CAPs were identified in *P. carterae* [61], with evidence also for a role for at least one of

317 these in the supply of Ca^{2+} to the calcification site [40]. Significant differences in the uronic
318 acid content of CAPs have been noted, which may potentially influence the shaping of the
319 calcite crystals [40] or reflect adaptations to differing calcite saturation states of the coccolith
320 vesicle. In this respect Lee et al [62] showed that coccolithophore species, such as a heavily
321 calcifying *E. huxleyi* strain, belonging to the order Isochrysidales, which is likely to have a
322 high calcite saturation state in the coccolith vesicle, deduced from data on intracellular DIC
323 pools and surface area/volume ratios, also has a CAP with high uronic acid content. In
324 contrast, *Coccolithus spp*, belonging to the order Coccolithales, showed the lowest CAP
325 uronic acid content. Lee et al proposed that with lower surface area/volume ratios and no
326 evidence for the presence of DIC accumulation Coccolithales arguably maintained a lower
327 calcite saturation state in the coccolith vesicle. This was consistent with the demonstration
328 that polysaccharide substrates with high levels of carboxyl functional groups promote fast
329 nucleation rates under conditions of high saturation states, whereas polysaccharides with
330 lower carboxyl content promote nucleation at lower saturation states [63]. Indeed, Lee et al
331 [62] concluded that by altering their polysaccharide charge density, coccolithophores may
332 fine-tune calcite precipitation to varying degrees of internal supersaturation, which may allow
333 them to achieve optimal calcification. The inference from these studies is that CAPs are
334 involved in promoting calcite nucleation whereas earlier work on isolated polysaccharides
335 suggests that CAPs tend to inhibit calcite precipitation, albeit *in vitro* [e.g. 58-60]. At first
336 sight, this appears to be a paradox in need of resolution and it is likely that the regulation of
337 calcite precipitation by polysaccharides is more subtle than simple promotion or inhibition.
338 There may be differences between behaviour *in vivo* and *in vitro* and, as illustrated above, it
339 is clear that soluble polysaccharides may behave differently from those that have roles as
340 solid substrates for precipitation.

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

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

361

362 **3.2 Further determinants of coccolith morphology.**

363 The studies summarised above point to links between the cellular transport processes that
364 give rise to carbonate saturation conditions in the coccolith vesicle and regulatory processes
365 that determine calcite precipitation rate and morphology. Recently, two lines of evidence
366 have revealed further unexpected differences in control of coccolith morphology between
367 species. First, Ge a competitive inhibitor of Si transport in diatoms, was unexpectedly shown
368 to cause specific striking malformations in coccolith morphology in two members of the
369 Coccolithales (*C. braarudii* and *Calcidiscus leptoporus*) and in the Zygodiscales species
370 *Scyphosphaera apstenii* [68]. Growth in low Si medium produced similar results. In contrast,
371 Ge treatment had no effect at all on the Isochrysidales species *E. huxleyi*. Second, diatom-
372 like Si transporters (SITs) or SIT-like transporters were found in the transcriptomes of those
373 coccolithophore species that show sensitivity to Ge. Significantly, however, *E. huxleyi*, *G.*
374 *oceanica* and *P. carterae*, were shown to lack SITs and were completely insensitive to Ge
375 treatments. Moreover disruption of coccolith morphology in *C. braarudii* led to
376 disorganization of the coccosphere, which in turn resulted in an inability of cells to separate
377 following mitosis and the onset of cell cycle arrest [69]. These observations represent the
378 clearest physiological distinction to date between different coccolithophore groups and raise
379 a number of important questions: First, what is the mechanism by which Si is involved in the
380 regulation of proper coccolith morphology in those Si-requiring species? Some clues may
381 come from other studies showing interaction between Si and calcium carbonate. Silica has
382 been shown to be important in the deposition of amorphous calcium carbonate (ACC) in vitro
383 [70] and in mineralized cystoliths from plants [71]. However, there is so far no evidence that
384 ACC is involved in the precipitation of coccolith calcite. Second, the phylogeny of SITs
385 suggests that they were present in an ancestral lineage to coccolithophores [72]. We have
386 proposed that the Si requirement may be an evolutionary trait that has been lost in much

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

397 **4. Concluding remarks**

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

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

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

422 are encouraging signs that genetic manipulation of at least certain haptophytes is feasible
423 [28,78], paving the way for gene knock-down and knock-out and the use of genetically-
424 encoded reporters. Ongoing large-scale genome sequencing projects are likely to provide a
425 wealth of information to help identify species-specific differences. These, together with the
426 advent of new analytical approaches, such as cryo-EM and elemental analyses that are
427 helping to define nanoscale processes in coccolith formation will ensure that our
428 understanding of this important group of organisms will continue to improve.

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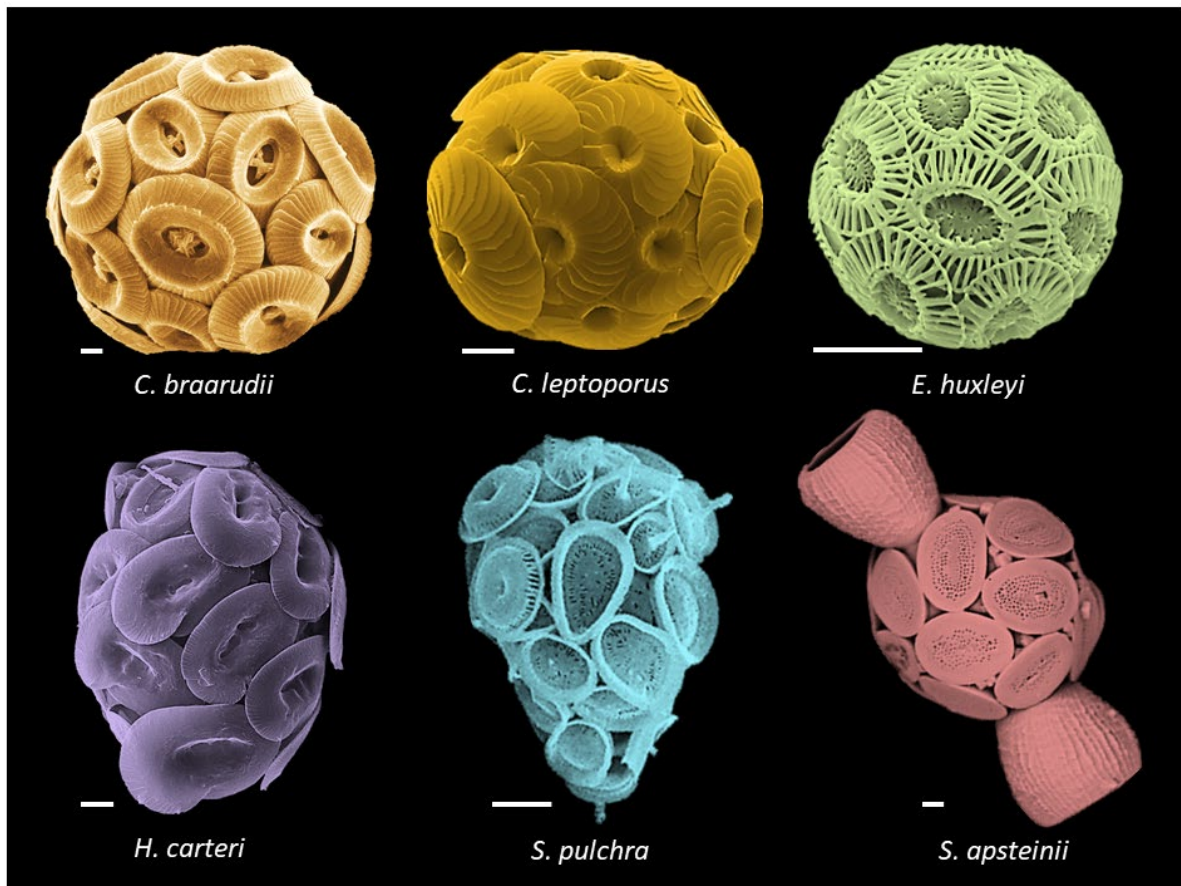
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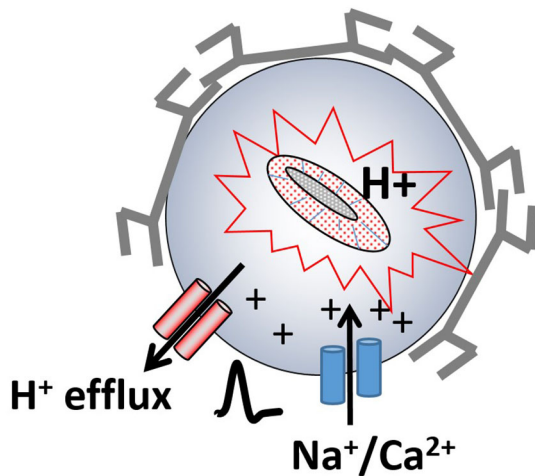
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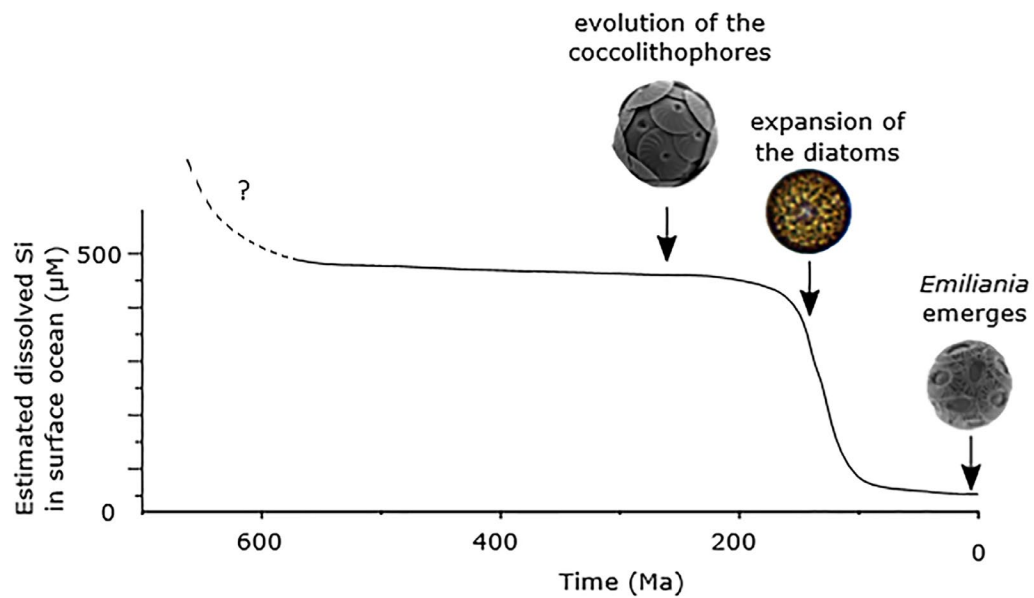
703
 704 **Figure 1.** Examples of coccolithophore morphologies. Top (left to right): *Coccolithus*
 705 *braarudii*, *Calcidiscus leptoporus*, *Emiliana huxleyi*. Bottom: *Helicosphaera carteri*,
 706 *Syracosphaera pulchra*, *Scyphosphaera apsteinii*. Scale bars, 2 μm .

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 709 **Figure 2.** Schematic of hypothetical role of electrical control of cellular pH through the
 710 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.
713



714
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.