Coccolithophore cell biology: chalking up progress

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Abstract

Coccolithophores occupy a special position within the marine phytoplankton due to their production of intricate calcite scales, or coccoliths. They are major contributors to global ocean calcification and long-term carbon fluxes. The intracellular production of coccoliths requires modifications to cellular ultrastructure and metabolism that are surveyed here. In addition to calcification, which appears to have evolved with a diverse range of functions, a number of other remarkable features that likely underpin the ecological and evolutionary success of coccolithophores have recently been uncovered. These include complex and varied life cycle strategies related to abiotic and biotic interactions, together with a range of novel metabolic pathways and nutritional strategies. Together with knowledge of coccolithophore genetic and physiological variability, these findings are beginning to shed new light on species diversity, distribution, and ecological adaptation. Further advances in genetics and functional characterization at the cellular level will likely to lead to rapid acceleration of this understanding.

1.0 Introduction to coccolithophores

The coccolithophores are an important group of marine phytoplankton, characterized by their covering of external calcium carbonate plates (coccoliths). They emerged relatively recently in evolutionary timescales (estimated at around 300 Ma) and have become major contributors to marine ecosystems and global biogeochemical cycles. The most abundant coccolithophore species in modern oceans is *Emiliania huxleyi* which can form massive blooms in temperate and subpolar regions, producing up to 10^8 cells L^{-1}. Together with other ecological significant species, the coccolithophores contribute up to half of the estimated ~1.6 Pg $year^{-1}$ $CaCO_3$ produced in the pelagic zone (Balch et al. 2007). Coccolithophores influence surface ocean biogeochemistry by fixing a significant amount of carbon through photosynthesis (biological C-pump) and through release of CO_2 during coccolith formation (carbonate counter pump) (Rost & Riebesell 2004). The ballast of sinking coccolithophore calcite increases the burial flux of organic matter (Ziveri et al. 2007). Coccolithophores also contribute to global sulphur cycling through their production of dimethylsulphoniopropionate (DMSP).

Coccolithophores exhibit remarkable metabolic features that underpin their ability to successfully complete with other species in the surface oceans. Most notably (and unlike most calcifying organisms) production of their calcite coccoliths occurs in an intracellular compartment, with subsequent secretion onto the cell surface (Figure 1) (Raven & Giordano 2009). A better understanding of their ecophysiology through studies of phenotypic and physiological plasticity, cell metabolism, microbial interactions, and mechanisms of calcification will lead to an improved understanding of their biogeochemical impacts and responses to environmental change (O'Brien et al. 2016). Here we highlight some of the most recent advances in these areas and offer suggestions of potential avenues for further research.

2.0 Evolution of coccolithophores

Coccolithophores belong the Haptophyta lineage of eukaryotes, the position of which in the eukaryote tree of life has been much debated. Recent multigene

phylogenies place the haptophytes as a sister group to the centrohelids in the Haptista, which shows some association to the Stramenopile-Alveolate-Rhizarian (SAR) supergroup but excludes cryptophytes (Burki et al. 2016). Haptophytes possess a plastid of red algal origin, although the mechanism through which this has been acquired is also the subject of considerable debate. A recent study suggests that plastids of red algal origin in many photosynthetic eukaryotes may have arisen from serial endosymbiotic events, with haptophytes acquiring their plastid from an ochrophyte (photosynthetic stramenopile) ancestor (Stiller et al. 2014). This hypothesis is in alignment with both ultrastructure and pigment associations between these two groups and presumably occurred early in the evolution of haptophytes as chloroplast acquisition by the aplastidic ancestral cell is estimated to be around ~1100Ma (De Vargas et al. 2007), although it is clear that much remains to be learnt about haptophyte evolution.

Calcification (the precipitation of calcium carbonate) occurs in diverse eukaryote lineages, suggesting that this trait has evolved on multiple independent occasions (Raven & Giordano 2009). It is likely that calcification emerged in the coccolithophores close to the divergence of the Calcihaptophycidae and Prymnesiales (around 310 Ma) (Liu et al. 2010), with the earliest fossil heterococcoliths and holococcoliths dated at 220 Ma and 185 Ma respectively (De Vargas et al. 2007). Calcification in the haptophytes may have evolved independently on more than one occasion, as the phylogenetic position of *Braaudosphaera,* which produces atypical pentagonal nannoliths, remains uncertain (Hagino et al. 2016). The elevated Mg content of the pentaliths of *Braarudosphaera* suggests that they are formed extracellularly (Hagino et al. 2016), although the inability to grow this species in culture has hampered more detailed investigations into its physiology and its evolutionary origins. Loss of calcification appears to have occurred at least once in the coccolithophores, as the Isochrysidales contain numerous non-calcifying lineages (e.g. *Isochrysis*). The calcifying members of the Isochrysidales (*Emiliania*, *Gephyrocapsa*) also lack holococcoliths in their haploid life cycle stages, suggesting either this trait evolved after the divergence of the Isochrysidales or it was lost in this lineage. Some members of the Coccolithales

(*Pleurochrysis*, *Hymenomonas*) also lack holococcoliths, supporting independent loss.

Whether there were strong co-evolutionary relationships between the emergence of calcification in haptophytes and the physico-chemical properties of the oceans remains unclear (Raven & Giordano 2009). A recent study revisited the hypothesis that intracellular calcification evolved as a strategy to avoid cytotoxicity of Ca^{2+} under the higher levels of Ca^{2+} in which coccolithophores arose. A calcifying strain of *E. huxleyi* showed resilience to increased levels of Ca^{2+} representative of the Cretaceous, whereas several non-calcifying phytoplankton and a non-calcifying strain of *E. huxleyi* were unable to tolerate these higher Ca^{2+} levels (Muller et al. 2015). This most likely demonstrates the efficiency of the Ca^{2+} transport and sequestration system in calcifying coccolithophores that can overcome the additional burden of Ca^{2+} influx imposed under these conditions. Whether the higher environmental Ca^{2+} in which coccolithophores evolved acted to select for the evolution of an entire intracellular calcification system remains highly speculative. As Raven and Crawfurd (2012) point out, low free cytosolic [Ca^{2+}] environment evolved early in eukaryote evolution, well before the emergence of intracellular calcification in coccolithophores.

3.0 Cell biology, life cycle and ecological niches

3.1 Life cycle transitions

Coccolithophores exhibit both calcified haploid and diploid life cycle phases that can reproduce asexually (Frada et al. 2009, Houdan et al. 2004, Nöel et al. 2004, Young et al. 2005). Diploid cells produce structurally complex calcite crystal heterococcoliths, and dominate natural populations. In many species the periodic haploid phase produces holococcoliths made up of simple calcite rhombohedra (Geisen et al. 2002, Young et al. 1999). Transitional coccospheres comprising holo- and heterococcoliths have been described for most major taxonomic groups, primarily from field specimens (Cros et al. 2000, Geisen et al. 2002, Young et al. 2005), suggesting coccolithophores readily undergo life phase transitions in natural populations. There are a few examples of species within the Pleurochrysidaceae and

Hymenomonadacae in which the heterococcolith bearing phase alternates with a non-calcifying haploid phase (Fresnel 1994, Nöel et al. 2004).

It has been proposed that nutrient poor pelagic waters favour motile haploid holococcolithophore assemblages, whereas diploid cells are better adapted for warmer, nutrient rich coastal waters (Oviedo et al. 2015). Nutrient driven diploid-haploid niche partitioning may also underlie the depth distributions of coccolithophores, as observed in the NW Mediterranean, with nutrient-depleted upper oligotrophic waters favouring haploid holococcolith-bearing cells and deeper nutrient rich waters favouring diploid heterococcolith-bearing cells (Cros & Estrada 2013, Oviedo et al. 2015). Accordingly, the diploid *Coccolithus braarudii* and *Calcidiscus leptoporus*, sustain higher growth rates than haploid motile cells under high inorganic nutrient levels (Houdan et al. 2006). Moreover, transitions from haploid cells to heterococcolith diploid cells of the oceanic *Calyptrosphaera sphaeroidea* could be induced by increasing trace-metals and vitamins in the culture medium (Nöel et al. 2004). Decreased temperature was also found to cause diploid-to-haploid transitions in this species (Nöel et al. 2004). Switching to a haploid, and potentially mixotrophic mode of nutrition presumably enables these cells to sustain growth rates that would otherwise not be possible under inorganic nutrient limitation (see below). Consistent with this, growth of haploid cells of *C. braarudii* and *C. leptoporus* is stimulated by addition of organic C, and they are known to actively phagocytose bacteria (Houdan et al. 2006). Increased turbulence inhibits growth of haploid motile *Coccolithus braarudii* and induces phase transitions to the diploid non-motile phase (Houdan et al. 2006). Finally, both laboratory and field experiments suggest a shift from susceptive diploid, to resistant (non-calcifying) haploid *E. huxleyi* is promoted by viral infection (Frada et al. 2008, Frada et al. 2012), implying alternating life history phases could be a crucial response to the presence of pathogens that ensures long term persistence of the resident population (Figure 2).

The alternating haploid-diploid life cycle of coccolithophores, combined with the quite distinct physiological capabilities between these two cell types, likely represents a successful niche partitioning strategy under varying abiotic and biotic

pressures. The haploid phase is also the precursor to sexual reproduction through syngamy, that potentially contributes to micro-adaptation in response to the prevailing conditions. The high genomic variability among diploid calcifying *E. huxleyi* strains (Read et al. 2013) points to a strong adaptation signal among extant isolates. Interestingly, a recent study showed the haploid gene content of diploid strains varied considerably among biogeographical isolates. Using sequencing and competitive genome hybridization Von Dassow and colleagues (2015) demonstrate the haploid genome content was diminished in some diploid strains. For example, 23% of a set of *E. huxleyi* genes associated with cilia or flagella function (including 3 of the critical dynein heavy chain proteins) are missing from the genome sequence of CCMP1516, suggesting that it has lost the ability to form functional flagella (von Dassow et al. 2015). Whether this loss occurred during the ~20 years this strain remained in culture under stable high nutrient conditions is unclear. However, a targeted PCR of 83 diploid strains isolated from different oceanographic regions showed that 37 strains associated with warmer oligotrophic waters lacked two dynein heavy chain genes essential for flagella motility. This suggests the ability to undergo phase transitions to motile haploid cells was not advantageous for *E. huxleyi* under these generally warmer, stable and low nutrient conditions (von Dassow et al. 2015). This finding is seemingly at odds with the studies in holococcolith-bearing species described above, that demonstrate oligotrophic conditions favor the transition to the haploid stage. A more detailed understanding of the ecological drivers for life-cycle transitions in coccolithophores is needed. It will be interesting to see whether strains that have lost the ability to produce motile haploid cells are susceptible to the *E. huxleyi* virus (see below).

3.2 Mixotrophy

Bacterivory in oligotrophic ecosystems is dominated by picoeukaryote algae (Hartmann et al. 2012) with small flagellated non-calcifying haptophyte taxa contributing up to 30% of bacterivory in oligotrophic coastal waters (Unrein et al. 2014). These haptophytes have been shown to acquire and incorporate C and N from labeled *Prochlorococcus* and *Synechococcus* implying they can be significant

grazers of picocyanobacteria, significantly redirecting C within marine food webs (Ward & Follows 2016). Given the mixotrophic origins of the haptophytes (De Vargas et al. 2007), it is not surprising that coccolithophores possess the genes associated with the maintenance of a phagosomal pathway. While phagocytotic behavior has generally been attributed to the haploid motile (and haptonemal-bearing) life phase of coccolithophores, transcripts of genes related to phagocytosis in *E. huxleyi* have been shown to be more abundant in diploid non-motile, calcifying cells (Rokitta et al. 2011). If phagocytosis occurs in these diploid cells, it does so in the absence of a haptonemal appendage and while the cell is covered with a layer of coccoliths.

Much is still be learned about the nutritional capability of coccolithophores, to determine whether they can be significant grazers of bacteria and to assess their ability to occupy alternate ecological niches in order to overcome inorganic nutrient limitation. This is of particular interest for climate change scenarios whereby increased sea surface temperature and stratification may favor mixotrophic modes of nutrition (Mitra et al. 2014, Wilken et al. 2013).

4.0 Biotic interactions

4.1 Bacteria

Although there are several well-described mutualistic interactions between eukaryote phytoplankton and bacteria (see (Cooper & Smith 2015) and references therein), functional interactions between bacteria and coccolithophores remain largely uncharacterized. A survey of bacteria associated with cultured *E. huxleyi* and *C. pelagicus f braarudii* highlighted a species rich community of α- and γ-proteobacteria with several taxa in common with other phytoplankton including *Marinobacter* and *Marivita* (Green et al. 2015). Of interest are bacteria that may be more specific to the unique coccolithophore 'phycosphere'. These include hydrocarbon degrading bacteria, and a Bacteroidetes diversity dominated by Sphingobacteria as opposed to the Flavobacteria that are more typical of diatoms and dinoflagellates. The presence of species of Acidobacteria known to be associated

with organisms that secrete carbonate biominerals, led to the proposal that these acid-secreting bacteria could degrade coccolith calcite and access CAPs as a source of organic carbon (Green et al. 2015).

How coccolithophore-bacteria associations influence nutrient exchange and carbon flow are unknown. However, comparing axenic and non-axenic cultures, Van Oostende et al. (Van Oostende et al. 2013) show the presence of bacteria in cultures of P-limited *E. huxleyi* results in altered composition of dissolved polysaccharides and a greater production of extracellular particulate organic matter. Thus, bacterial activity can modify the pattern of organic matter produced and released by coccolithophores, thereby influencing export production. Moreover, intracellular pools of lipids and alkenones are likely dependent on bacterial assemblages associated with coccolithophores (Segev et al. 2016), which warrants further investigation, given the importance of the alkenones as temperature paleoproxies.

Evidence of mutualistic interactions between bacteria and coccolithophores is limited. Seyedsayamdost and co-workers recently described a mutualism between a bloom-associated rosebacter *P. gallaceiensis* and *E. huxleyi* in which the bacterium produces antibiotics and auxin, which are presumed to support a growth enhancing relationship in which the bacterium derives C- and S- from DMSP produced by the alga (Seyedsayamdost et al. 2011). However, *P. gallaceiensis* opportunistically switches from mutualist to pathogen as *E. huxleyi* approach stationary or senescing stages. The cue for this so-called 'Jekyll-and-Hyde' transition by *P. gallaceiensis* is *p*-coumaric acid, a lignin-like compound released by the aging *E. huxleyi* cells. In response to *p*-coumaric acid, *P. gallaceiensis,* produces a suite of secondary metabolites including potent algicides (roseobacticides) (Seyedsayamdost et al. 2011). This complex metabolic interaction has an interesting twist as isotope labeling demonstrates the bacteria incorporates the *p*-coumaric acid into the biosynthetic pathway of the roseobacticides, resulting in a virulent hybrid molecule derived from both host and pathogen (Seyedsayamdost et al. 2014). Moreover, the DMSP derived from the alga during the mutualistic phase is important in providing a source of S for toxin production (Seyedsayamdost et al. 2014) (Figure 3).

The potential for detrimental interactions with bacteria is also demonstrated by the high sensitivity of *E. huxleyi* to the algicidal marine γ-Proteobacteria *Pseudoalteromonas piscicida* (Harvey et al. 2016). In this case, a soluble quorum sensing alkyl-quinolone was purified and found to mediate mortality in *E. huxleyi* at nM concentrations, whereas the green alga *Dunaliella tertiolecta* and the diatom *Phaeodactylum tricornutum* were insensitive to this compound. Nevertheless, all three species were susceptible to exudates of *P. piscicida* suggesting the production of a cocktail of compounds that confers broad algicidal activity (Harvey et al. 2016).

These recent developments demonstrate that coccolithophores have a complex and underexplored repertoire of symbiotic, mutualistic and antagonistic interactions with bacteria. Characterizing these interactions is important to determine the relative contributions of bacteria and viruses to coccolithophore population dynamics and associated biogeochemical cycles (Figure 3).

4.2 Viruses

The complete genome sequence and transcription profile of a large DNA virus (EhV-86) that infects *E. huxleyi* (Wilson et al. 2005) yielded important insights into the host-virus dynamic. Both mesocosm (Pagarete et al. 2011) and mesoscale studies have demonstrated the ability of the virus to regulate bloom dynamics in natural populations, especially under relatively stable physical conditions (Lehahn et al. 2014). Research by several groups over the last decade has focused on the viral infection mechanism (Figure 4).

An important early observation of the EhV-86 genome was the presence of a suite of genes, derived from host-virus horizontal gene transfer (Monier et al. 2009), coding for the biosynthesis of sphingolipids, which are expressed during the lytic infection cycle (Wilson et al. 2005). It has since been shown that the virus reprograms host lipid metabolism, stimulating production of highly saturated triacylglycerols (Malitsky et al. 2016), suppressing host glycosphingolipid pathway, and promoting production and incorporation of virus encoded sphingolipids (vGSLs) (Rosenwasser et al. 2014). At least in the early stages of infection, the

virions bud from the host and retain a lipid envelope derived from the host (Mackinder et al. 2009), but highly enriched in saturated triacylglycerols (Malitsky et al. 2016) and containing vGSLs (Fulton et al. 2014, Vardi et al. 2009).

The infection mechanism of EhV appears to involve recognition of components in lipid raft microdomains of the host membrane. Uninfected cells have a diverse lipid raft proteome the composition of which is rapidly altered on infection by EhV (Rose et al. 2014). Of particular interest is a Toll-like interleukin receptor protein present in host lipid rafts that may interact with a virus-associated C-type lectin, possibly mediating attachment and viral translocation across the membrane. Whether lipid rafts play a role in both viral entry and egress has not been fully resolved. Susceptibility to infection appears also to be strongly correlated with the presence of sialic-acid GSLs in diploid cells (Hunter et al. 2015). The absence of sGSLs from the lipidome of resistant haploid cultures suggests a mechanism that could explain the basis for the diploid-to-haploid 'escape strategy' during virus-induced bloom termination (Frada et al. 2008, Frada et al. 2012). The events that trigger a switch to haploid and resistant cells on viral infection are yet to be established.

A further innovation of EhV is the proviral utilization of the host autophagy pathway. Schatz and coworkers recently demonstrated the lytic phase of EhV infection corresponds to increased expression of autophagy-related genes (ATG genes) along with ultrastructural changes (multi membrane vesicles), increased lysosomal activity, and a protein marker (Atg8) for autophagy associated with the membranes surrounding viral particles (Schatz et al. 2014). Viral entry and DNA replication are unaffected by inhibitors of autophagy, whereas assembly and exit from the cell are suppressed, suggesting co-opting of the autophagy pathway is a late stage cellular interaction that promotes intracellular encapsulation of the virions in modified host membranes and propagation by burst release (Schatz et al. 2014)(Figure 4). Viral particles are coated with several layers of host membrane enriched with TAG and viral GSLs, and facilitate the envelope fusion of viruses particles with uninfected host membranes (Mackinder et al. 2009)

Terminal events following infection, and in response to increasing vGSLs include increased reactive oxygen production (Evans et al. 2006), rapid degradation of cellular components, reduction of photosynthetic efficiency, and induction of host metacaspases and caspase activity that is necessary to promote viral production (Figure 4). This suggests co-evolution of the host-virus resulted in strong selection for viruses that co-opt the phytoplankton PCD pathway in their infection strategy (Bidle et al. 2007, Vardi et al. 2009). These cellular events have since been confirmed in natural populations of *E. huxleyi* (Vardi et al. 2012).

How phytoplankton viruses propagate in natural ecosystems is not well understood, though the coccolithophore model has yielded important discoveries. Multiple viral transmission mechanisms likely play a critical role the ecology of *E. huxleyi*. Both zooplankton (Frada & Vardi 2015) and aerosolisation (Sharoni et al. 2015) have the potential to increase dispersal of competent EhV particles through the water column and over large scales respectively, facilitating rapid infection and termination of coccolithophore blooms. Local diffusion and encounter rates of viral particles determine infection of the host cell at the microscale. Zooplankton-mediated dispersal may be an important determinant of infection at the mesoscale due to their non-random diffusivity in a patchy prey landscape (Figure 4). The half-life of aerosolized virus particles exposed to sunlight/UV is only 20 min. Nevertheless, the 1000 x greater diffusivity of aerosols compared to particles in the water column suggest aerosols could be a highly effective transmission mechanism over larger oceanographic realms.

Advances in understanding the host-virus infection dynamic have enabled deeper ecological questions to be addressed. For example, the vGSLs have successfully been used as in-situ biomarkers for viral infection in natural populations (Vardi et al. 2012), allowing interrogation at the population level to reveal the degree of genetic and metabolic variability among natural host-virus populations. Such biological and ecological insights will enable viral impacts on nutrient fluxes, microbial food webs, and C export from the surface ocean to be more clearly defined and to be incorporated effectively into ecosystem models (Weitz et al. 2015).

5.0 Coccolithophore metabolism and physiological versatility

Coccolithophores exhibit unique metabolic traits that contribute to their physiological versatility. Genomic, proteomic, metabolomic and biochemical approaches have been largely restricted to *E. huxleyi* and may not reflect the full metabolic diversity of other coccolithophore lineages.

5.1 Carbon metabolism

In most photosynthetic organisms, the major carbon storage compounds are α-/β-glucans (e.g. starch in land plants and green algae). Although *E. huxleyi* produces a water soluble β-glucan, quantitative analyses of carbon fluxes during photosynthesis suggest that β-glucan is only a minor sink (<1%) for fixed carbon (Tsuji et al. 2015). Instead, carbon is predominately stored in low molecular weight compounds (such as mannitol), acidic polysaccharides, alkenones and other lipids (Obata et al. 2013, Tsuji et al. 2015). The absence of significant storage glucans and the primary accumulation of carbon into mannitol and alkenones are a distinct feature of carbon metabolism in *E. huxleyi* (Obata et al. 2013, Tsuji et al. 2015).

Other unique aspects of coccolithophore metabolism are evident from the presence of novel enzymes or their unusual localization. For example, pyruvate carboxylase is commonly found in the cytoplasm or mitochondria of eukaryotes, where it plays an important role in replenishing TCA cycle intermediates. However, in *E. huxleyi* pyruvate carboxylase was found to be plastid-localized, leading to the proposal that it plays a novel role by acting to regulate carbon flux to amino acid skeletons within the plastid (Tsuji et al. 2015). Transcriptomic studies suggest that *E. huxleyi*, like the diatoms, possesses an ornithine-urea cycle (OUC) playing a similar role in N-redistribution during N-limitation (Mckew et al. 2015, Rokitta et al. 2014). N-limitation also led to elevated expression of a mitochondrial malate-quinone oxidoreductase (MQO) in *E. huxleyi* (Rokitta et al. 2014). This enzyme enables direct transfer of electrons from malate to quinone, representing an alternative input to the mitochondrial electron transport chain that is not dependent on the activity of the TCA cycle. MQO is found in dinoflagellates and some

other alveolates, but is notably absent from diatoms (Danne et al. 2013, Rokitta et al. 2014). Interestingly, GC-MS based metabolite profiling studies in *E. huxleyi* could not detect malate, whereas malate accumulates significantly in the cells of land plants (up to 350 mM) (Obata et al. 2013). These findings suggest that malate plays a very different role in coccolithophore metabolism and that coccolithophores are much less reliant on the activity of the TCA cycle than land plants.

5.2 Osmoprotectants

Coccolithophores accumulate a range of metabolites that can act as osmoprotectants. These include polyols (mannitol), quaternary ammonium compounds (glycine betaine (GBT) and homarine), and the tertiary sulphonium compound dimethylsulphoniopropionate (DMSP) (Gebser & Pohnert 2013). DMSP is a major metabolite in many haptophytes, although it is absent from some haptophyte lineages such as *Pavlova*, which accumulate cyclitols (e.g. D-1,4/2,5-cyclohexanetetrol) instead (Kobayashi et al. 2007). DMSP production by coccolithophores and other marine phytoplankton plays an important role in the global sulphur cycle, as DMSP is the precursor of the climate active gas, dimethyl sulphide (DMS). Both DMSP and DMS act as powerful infochemicals that can influence a wide variety of biotic interactions at both the micro- (e.g. chemotaxis of bacteria and alteration of zooplankton trophic behavior) and macroscale (e.g. as chemoattractants for birds, turtles and fish) (Fredrickson & Strom 2009, Garren et al. 2014, Savoca & Nevitt 2014, Seymour et al. 2010, Steinke et al. 2006, Wolfe et al. 1997). Coccolithophores accumulate high amounts of DMSP (up to 400 mM in *E. huxleyi*), the concentration of which is influenced by light intensity, salinity, growth phase and diel cycle and also differs significantly between strains (Darroch et al. 2015, Franklin et al. 2010, Keller et al. 1999, Steinke et al. 1998). Gebser and Pohnert (2013) demonstrated that the major zwitterionic metabolites in *E. huxleyi* were DMSP, GBT and homarine and that the ratio of these osmolytes was remarkably constant over a range of salinities (ratios of approximately 100:6:10). This suggests that all three of these osmolytes are regulated by similar mechanisms in response to changes in salinity. N-limitation results in much lower cellular

concentrations of the quaternary ammonium compounds (GBT and homarine), although little or no compensatory increase in the cellular concentration of DMSP was observed under these conditions (Keller et al. 1999).

Bacteria and algae both contain enzymes (DMSP lyases) that can cleave DMSP to generate DMS which contributes a major flux of sulphur to the atmosphere. Significant progress in the past decade has led to the identification a range of bacterial DMSP lyases that are thought to play a major role in DMS production in the oceans (Moran et al. 2012). However, the recent identification of an *E. huxleyi* gene product, Alma1, as a specific and highly active algal DMSP lyase indicates that coccolithophores can directly cleave the DMSP they produce to generate DMS and acrylate (Alcolombri et al. 2015). The *E. huxleyi* enzyme shares no sequence similarity with the DMSP lyases found in bacteria, although related enzymes are found in marine phytoplankton that accumulate DMSP, including other haptophytes (e.g. *Phaeocystis antartica*, *Prymnesium parvum*) and dinoflagellates (e.g. *Symbiodinium*) (Alcolombri et al. 2015). Levels of *Alma1* gene expression and protein abundance in *E. huxleyi* correlated very closely with DMSP lyase activity. The discovery of Alma1 fills an important missing link in the marine sulphur cycle and will aid estimations of the relative contribution of phytoplankton and bacteria to global DMS production. The Alma1 protein has two conserved cysteines essential for enzyme activity and activity is sensitive to oxidants. This suggests that changes in cellular redox status may modulate DMSP lyase activity, which may be linked to the proposed antioxidant role for DMSP (Darroch et al. 2015, Sunda et al. 2002), as rates of DMSP cleavage would be lowered in response to oxidative stress.

6.0 Recent insights into functional roles of calcification

The most striking metabolic specialization in coccolithophores is calcification itself. Despite this, the functional role(s) of calcification in coccolithophores remain uncertain (Raven & Crawfurd 2012, Taylor & Brownlee 2016, Young 1994) with several hypotheses relating to nutrient uptake, photosynthesis and protection from biotic and abotic stressors. Nevertheless, some recent studies have provided intriguing new insights. A potential role for calcification in utilization of HCO_3^- as a

source of CO_2 for photosynthesis has been widely discussed (Berry et al. 2002, Raven & Crawfurd 2012). Although photosynthesis and calcification interact metabolically (see section 6.3), an obligatory dependence of photosynthesis upon calcification, at least in *E. huxleyi,* is not well supported by recent studies (Herfort et al. 2004, Leonardos et al. 2009, Trimborn et al. 2007). Indeed, under low DIC conditions photosynthesis may compete with calcification for HCO_3^- (Bach et al. 2013).

6.1 Defense from grazers and pathogens,

While the coccosphere may be expected to have a protective role, the evidence currently available remains equivocal. For example, the presence of a coccosphere does not prevent ingestion of *E. huxleyi* by either copepods or microzooplankton predators (Harris 1994), although highly modified articulated coccoliths of members of the Syracosphaeraceae could act as a more direct mechanical deterrence (Young et al. 2009). Recent evidence showed that haploid, non-calcifying *E. huxleyi* cells possessed inducible grazing defense properties whereas calcifying cells did not, indicating complex relationships between prey and grazer activity (Kolb & Strom 2013). Recently, (Harvey et al. 2015) showed significant reduction in grazer growth rate feeding on calcified versus non-calcified *E. huxleyi* strains, the proposed mechanism being reduced digestion efficiency in the food vacuole/phagosome when feeding on calcified cells compared to non-calcifying prey. Thus calcification is integrated into a range of traits including production of DMSP and other undefined metabolites that can contribute to the degree of top-down control at the population level. In the case of pathogens, susceptibility and infection rates appear to be determined by a variety of metabolic interactions unrelated to calcification (see section 6.3). Indeed, diploid calcifying cells of *E. huxleyi* were shown to be suseceptible to viral infection whereas haploid cells were not, leading to the "Cheshire cat" diploid-to-haploid 'escape strategy' hypothesis (Frada et al. 2008).

6.2 Modulation of diffusion boundary layer

Comparison of isogenic calcifying and non-calcifying isolates of *E. huxleyi* showed that the non-calcifying strain exhibited higher growth rates than the calcifying strain under stable, nutrient-replete conditions (Bartal et al. 2015), consistent with the considerable energetic cost of calcification. However, under moderately turbulent growth conditions, the ability to produce coccoliths conferred mechanical resilience and improved affinity for nitrate acquisition, possibly via stabilization of the diffusion boundary layer at the cell surface. A similar role for diatom frustules has been proposed based on diffusional bias caused by their fine-scale architecture that enhances uptake in patchy nutrient environments (Mitchell et al. 2013). These observations emphasize the need to better understand the microenvironment between the coccosphere and cell membrane and how the coccoliths and associated structures can influence this.

6.3 Modulating the light field and energy balance

It has been speculated that coccoliths alter the light field experienced by the cell either in a photoprotective or photo-enhancing role (Nanninga & Tyrrell 1996, Quinn et al. 2005). Recent work on isolated *E. huxleyi* coccoliths suspended in solution and aligned in a magnetic field showed that both enhancement and inhibition of incident light scattering is possible although the effect on light intensity was less than 5% (Mizukawa et al. 2015). Similar conclusions can be drawn from experiments comparing photosynthetic parameters in calcifying diploid *E. huxleyi* with non-calcifying haploid cells (Houdan et al. 2005). Light saturation kinetics are similar in both cell types, although photoinhibition was only observed in the haploid non-calcifying strain.

The remarkable resistance to photoinhibition by calcified strains of *E. huxleyi* led to the hypothesis that calcification may provide an alternative energy sink in response to high light levels. Inhibition of calcification in low Ca^{2+} seawater led to down regulation of photosynthetic pigments and C-fixation (Xu & Gao 2012) and these cells were also more susceptible to UV radiation (Xu et al. 2011). Moreover, sudden increases in light intensity from sub-saturating growth irradiance enhanced calcification fixation within minutes in diploid *E. huxleyi* suggesting a mechanism for

rapid dissipation of excess energy additional to changes in light harvesting pigment content (Ramos et al. 2012). The relatively stable proteome of *E. huxleyi* during photoacclimation from sub-saturating to supra-saturating light levels (McKew et al. 2013a, McKew et al. 2013b) implies at steady-state, calcification machinery operates substantially below its maximum potential, and can respond rapidly to altered environmental conditions.

Regardless of the environmental drivers that may have led to the evolution of intracellular calcification, the selective advantage of calcite production in modern coccolithophores is likely to be multifarious, and remains enigmatic. Understanding these functional roles of calcification is important but will continue to be a challenge given the interdependency of the cellular and metabolic processes involved.

7.0 Opening the 'black box' of vital effects in coccolithophores

Well-preserved coccolithophore calcite and alkenones in ocean sediments are used to reconstruct physicochemical properties of the surface oceans. The carbonate structures produced by coccolithophores and foraminifera have been utilized to develop a range of geochemical proxies. This is because elements and isotopes in the mineral theoretically reflect their abundances in seawater, and allow for paleoreconstructions of environmental conditions in the surface oceans. Marine biogenic $CaCO_3$ proxies include Mg/Ca, Sr/Ca $^{18}O/^{16}O$ (paleothermometry) and $^{13}C/^{12}C$ (DIC and ocean productivity) and they are increasingly relevant tools to understand past climate events and to inform ecological scenarios that may arise through the predicted future Anthropocene climate (Levin et al. 2015).

The physiological processes that mediate biogenic $CaCO_3$ precipitation play a critical role in stable isotope incorporation that can dramatically deviate from thermodynamic predictions (de Nooijer et al. 2014). These so-called 'vital effects' are due to the biologically controlled transport of ions and organic compounds into the compartment that promotes a saturated state favoring nucleation and calcite precipitation. Foraminifera $CaCO_3$ proxies such as the Mg/Ca paleothermometer are well advanced and robust (Hermoso 2014, Levin et al. 2015) although considerable variation in Mg incorporation appears to be driven by cellular metabolic processes

(Spero et al. 2015), and isotopic fractionation of $\delta^{13}C$ and $\delta^{18}O$ is also significantly influenced by photo-symbionts (Takagi et al. 2015). Coccolithophore proxies have been covered extensively by Hermoso (Hermoso 2014). The multiple ion transporters and endomembrane compartments that are involved in the trans-cellular pathway of inorganic substrates for intracellular coccolith production have a significant, but largely uncharacterized influence on ion and isotope fractionation. Species differences in calcite precipitation, associated organic material, and coccolith digenesis are also poorly understood. Further implementation of robust coccolith proxies requires an improved mechanistic understanding of calcification.

8.0 Calcification Mechanism

The mechanism of coccolithophore calcification has been studied extensively since the pioneering work of Paasche more than 50 years ago (Paasche 1968) and advances in the intracellular model of calcification have been covered in several comprehensive reviews (Brownlee & Taylor 2004, Brownlee et al. 2015, Paasche 2001, Westbroek et al. 1989, Young et al. 1999, Young et al. 2005). Despite this, the mechanistic details of coccolith production are surprisingly incomplete.

8.1 Ultrastructure and role of intracellular membranes

Coccoliths are produced in an intracellular Golgi-derived vacuole (generally referred to as the coccolith vesicle, CV) that has a complex relationship with the endomembrane system. The basic sequence of events has been well described at the ultrastructural level in several species (see Figure 1b-d), but the mechanism by which coccoliths are produced and secreted is not fully understood. The endomembrane system most likely plays an important role in supply of the inorganic and organic substrates for calcification through targeted trans-Golgi trafficking and via the direct association with the CV (Figure 5). For example, a system of anastomosing tubules known as the reticular body is closely associated with the coccolith compartment and important for delivery of organic and inorganic

substrates for calcification and in determining fine-scale morphology of the mineral structure (Drescher et al. 2012, Taylor et al. 2007).

The scale of secretion of the coccolith itself - coccoliths are transferred to the cell surface via a single secretory event - suggests potentially novel features of exo- and endocytosis are required to co-ordinate the process and efficiently retrieve the membrane (see Figure 1b-d). Evidence of a modified membrane trafficking system (MTS) was recently found in the haptophyte complement of post-Golgi adaptor protein (AP) complexes (Lee et al. 2015). Losses of AP3, which targets multivesicular body (MVB) and lysosome, and AP5, which targets trans-Golgi network and MVB, but multiple expansions of AP4 that mediates trans-Golgi network trafficking to the plasma membrane and endosomes were common among calcifying and non-calcifying haptophytes (Lee et al. 2015), suggesting a specialized MTS may have been necessary for the genesis of haptophyte body scales prior to the evolution of calcified scales. A further unique AP4 expansion within calcifying haptophytes indicates additional specialization of the MTS specific for biomineralization. Moreover, diploid specific expression of several syntaxin/SNARE homologs that function in vesicle engagement and fusion with target membranes (Mackinder et al. 2011), implies that additional specificity in the MTS could be achieved through differential transcription and translation of MTS genes in calcifying cells. A closer analysis of expression patterns of MTS related genes and the localization of their proteins during calcification and viral infection could provide important information on the coordination of membrane dynamics during these processes.

Regulation of the cytoskeleton must play an important role in directing calcification because it actively interacts with the MTS, may influence the shape of the CV, and probably controls vesicle and cell movements associated with coccolith secretion. However, there is surprising little information on cytoskeletal dynamics of coccolithophores. Inhibitors of actin and microtubules unsurprisingly result in gross distortions of coccoliths (Langer et al. 2010) but the specific role these structures play in calcification have not been resolved.

8.2 Role of organic components in calcification

8.2.1 Coccolith associated polysaccharide:

While proteins are generally the predominant organic components of $CaCO_3$ biomineral structures (e.g. corals and molluscs), polysaccharides are the major organic components associated with calcite production in coccolithophores. In *P. caterae* the coccolith associated polysaccharides (CAP) are 2% of coccolith mass (Okumura et al. 2012) with up to 28% of ^{14}C incorporation dedicated to extracellular polysaccharide in *E. huxleyi,* reinforcing their importance in the calcification process (Kayano & Shiraiwa 2009).

The CAPs are acidic in nature, comprising a backbone of D-mannose residues with a series of side chains that include D-ribose, L-arabinose, D-xylose, L-rhamnose and D-galacturonic acid residues and ester-bound sulphate groups (Fichtinger-Schepman et al. 1981, Kayano & Shiraiwa 2009). The steric arrangement of the carboxyl groups of the acidic residues confers the ability to bind both free Ca^{2+} and Ca^{2+} of calcite crystal surfaces (Henriksen et al. 2004). The type of CAP produced appears to be species specific (Borman et al. 1982, Hirokawa et al. 2005, Marsh et al. 1992, Ozaki et al. 2007), and even within a single species, CAPs may play distinct roles during coccolith production. For example, in *Pleurochrysis carterae* acidic polysaccharides (PS1 and PS2) are predominantly associated with excreted coccoliths and thought to play a role in Ca^{2+} transport and crystal growth, whereas a mannose-, xylose-, and sulphate-rich polysaccharide (PS3), is proposed to play a role in governing morphology during later stages of coccolith development (Marsh et al. 2002). The biosynthetic pathway for CAP production is unknown, although it is assumed to occur via the endomembrane and Golgi system, with delivery to the developing coccolith compartment via trans-Golgi vesicle transport (Marsh 1994).

How CAP regulates coccolith morphology is also poorly understood, although they may in part determine coccolith crystal shape through inhibition of calcite growth at acute steps of calcite crystals (Henriksen et al. 2004, Kayano et al. 2011). Inorganic precipitation experiments demonstrate that pH and ionic composition of the medium strongly affect the interaction of the CAP with the mineral surface. Selective binding of CAP to the acute step edges of rhombic calcite crystals is

promoted between pH 3.4-7.7 and in presence of K^+ Na^+ Sr^{2+} and Ca^{2+} ions. CAP attachment to the acute step edges of calcite drives crystal morphology away from the rhombic form, allowing extension along the c-axis (Kayano et al. 2011). Basic pH and presence of Mg^{2+} prevents site-specific absorption of CAP (Henriksen & Stipp 2009), therefore favoring the rhombic calcite morphology. In *E. huxleyi* cultures, elevated Mg^{2+} in the growth medium results in aberrant coccoliths (Herfort et al. 2004), whereas elevated Sr^{2+} does not significantly alter coccolith morphology (Langer et al. 2006). Although it is not possible to extrapolate the concentration of these ions in the medium to the site of calcification, these results are consistent with inorganic experiments that show CAP interactions with calcite are strongly influenced by Mg^{2+} ions (Henriksen & Stipp 2009, Henriksen et al. 2004).

A future challenge is to understand the ontogenetic and temporal chemistry of the CV. It is conceivable that regulation of calcite morphology is achieved through temporal modulation of CAP interactions with the calcite surface by fluctuations in CV pH, as well as Ca^{2+}, carbonate species, and other metal cations such as Mg^{2+}. Given the very close association of the of the CV and endomembranes and the developing calcite crystal (Figure 5), it would be interesting to consider the glycolipid and glycoprotein complement of these membranes, to assess whether membrane associated oligosaccharide residues could play a direct role in altering crystal growth that leads to fine-scale morphological features such as pores (Drescher et al. 2012).

In addition to the role of CAPs in the mineralization process itself, their integration into the coccolith structure has important biogeochemical implications. Hassenkam and coworkers (2011) argue the notable lack of thermodynamically favored Ostwald ripening of calcite crystals in coccolith-dominated chalk deposits is due to the large amount of organic material associated with them, a striking observation that illustrates the geological influence of CAP. Indeed, intra-crystalline CAP can be recovered from fossil coccoliths -70 Ma and retains its ability to interact with calcite surfaces in inorganic experimental systems (Sand et al. 2014). CAPs are also critical in resisting coccolith dissolution, significantly influencing digenesis and the burial flux of inorganic carbon (Hassenkam et al. 2011). Moreover, the stable

and recalcitrant and intra-crystalline organic C in ancient coccolith deposits suggests a significant fraction is unavailable for remineralization. Given that up to ~15% of cellular organic C may be allocated to CAP, this is an important to consider when assessing inorganic/organic C export. CAP and precursors could also play important role in coagulation of cells and coccoliths, affecting the ballasting of calcite (Chow et al. 2015).

8.2.2 Coccolith associated proteins

Although the role of matrix proteins in coccolith production appears to be limited, and likely confined to the baseplate scale, a gene encoding a glutamic acid, proline and aspartic acid rich protein (GPA) with Ca^{2+} binding motifs is associated with coccolith morphology in *E. huxleyi* and *G. oceanica* (Corstjens et al. 1998). The GPA protein was isolated from coccolith associated polysaccharide fractions suggesting a role in coccolith growth and morphology. Quantitative PCR shows strong regulation of the GPA gene with up-regulation in non-calcifying haploid cells and in calcifying diploid cells in which calcification has been suppressed by low Ca^{2+} treatment (Mackinder et al. 2011). Although counter-intuitive, a number of possibilities could explain this observation, including an inhibitory role for GPA at high concentrations. Without a clearer understanding of how calcification is regulated by organic components in general, it is difficult to draw firm conclusions. The GPA gene has not been detected in the transcriptomes of any other coccolithophore species to date, suggesting pelagic, bloom-forming species in the family Noëlaerhabdaceae may have unique organic regulatory components that underlie mechanistically distinct calcification processes among coccolithophores (See section 8.5).

8.3 Ion transport

The calcification process (Figure 5) presents a remarkable case of transport physiology requiring some of the highest sustained trans-cellular fluxes of Ca^{2+}, HCO_3^- and H^+ of any known eukaryote cell (Brownlee & Taylor 2004, Brownlee et al. 2015). Comparative transcriptomics have identified transport genes likely to be

specifically associated with calcification (Mackinder et al. 2011, von Dassow et al. 2009). Of particular relevance are Ca^{2+}/H^+ exchangers (CAX3), a vacuolar H^+ ATPase and a Na^+-dependent K^+/Ca^{2+} exchanger (NCKX). Inorganic carbon fluxes are likely mediated by a HCO_3^- transporter(s) in the SLC4 family. Additional constitutive transporters such as Ca^{2+} channels and Ca^{2+}-ATPases (SERCA-like) likely facilitate transcellular transport of Ca^{2+} (Figure 5)

The coccolithophore cell faces the challenge of maintaining a large trans-cellular flux of Ca^{2+} from seawater to the coccolith-forming compartment, without disturbing the low cytosolic [Ca^{2+}]. Likewise a mechanism of removal of H^+ generated by calcification that avoids catastrophic acidosis of the cytosol is required. The endomembrane pathway and its arrangement with the coccolith-vesicle offer solutions to this problem that also meet some of the necessary charge-balancing (Raven & Crawfurd 2012) (Figure 5). Moreover, the presence of a closely associated endomembrane system may also explain the paradox presented by biochemical purification of a V-type H^+-ATPase from CV-enriched membranes in *Pleurochrysis* (Corstjens et al. 2001). The orientation of a V-type H^+-ATPase is the reverse to that required to remove H^+ from the CV, but its association with the CV may be due to co-purification of closely associated endomembranes in which a V-type H^+-ATPase could act to sequester H^+ released from the CV (Figure 5).

Based on gene expression studies a model for Ca^{2+} accumulation has been proposed whereby Ca^{2+} is concentrated in a CV precursor compartment prior to delivery to the calcification site (Mackinder et al. 2010, Mackinder et al. 2011). Consistent with this is the recent demonstration of a vacuolar-like compartment in calcifying *E. huxleyi* cells that concentrates a disordered Ca^{2+} phase and makes close contact with the CV (Sviben et al. 2016). Some of the features of the precursor vesicles associated with coccolith production are reminiscent of Ca^{2+} and P-rich acidocalcisomes that have been identified in a variety of microorganisms, including apicomplexan parasites (Rohloff et al. 2011). Whether such a compartment plays a direct role in coccolithophore calcification remains to be determined.

8.4. The Problem of Protons

The formation of $CaCO_3$ from Ca^{2+} and HCO_3^- external substrates (Bach et al. 2013) necessitates the production of H^+, most likely at the site of $CaCO_3$ precipitation that needs to be removed from the CV and ultimately the cytosol to prevent acidosis (Brownlee et al. 2015). Evidence from both gene expression studies (Mackinder et al. 2011) and flux modeling (Holtz et al. 2013) is consistent with the operation of Ca^{2+}/H^+ antiporters with a stoichiometry of at least $2H^+$:$1Ca^{2+}$, together with V-type H^+-ATPases in recycling of H^+ into the endomembrane system and in the accumulation of Ca^{2+} in a precursor calcification compartment (Brownlee et al. 2015, Taylor et al. 2011). The finding that the coccolithophore plasma membrane has a high permeability for H^+ (Suffrian et al. 2011) due to the activity of voltage-dependent H^+ channels (Taylor et al. 2011) that activate upon cytosolic acidification and/or depolarization of membrane potential, provides an effective high capacity H^+ efflux pathway that can alleviate transient imbalances in H^+ production between calcification and H^+ consumption though metabolism and buffering. This role for H^+ channels in cellular pH homeostasis represents a unique and highly novel aspect of coccolithophore biology.

8.5 Silicon and new paradigms for calcification

Calcification in the coccolithophores evolved at a time (c. 300 Ma) when the dissolved Si concentrations (DSi) of the surface ocean were much greater than they are today. The subsequent expansion of the diatoms at the beginning of the Cenozoic (from 66 Ma) led to a dramatic decline in the concentrations of DSi in the surface ocean (Siever 1992), which in turn resulted in a decrease in extent of silica produced by other silicified organisms, such as the heavily silicified sponges and radiolarians (Lazarus et al. 2009, Maldonado et al. 1999). The ability of diatoms to draw down DSi is due to high-affinity Na^+-coupled Si transporters (known as SITs) in their plasma membrane that facilitate uptake of silicic acid against a concentration gradient, leading to its eventual depletion from the surrounding seawater. Until recently, SITs were identified only in stramenopiles (diatoms and chrysophytes) and siliceous choanoflagellates (Marron et al. 2013). However, a SIT homologue was recently described in *Prymnesium neolepis*, an unusual silicifying

haptophyte (Durak et al. 2016). Remarkably, a SIT was also found in the coccolithophore, *Scyphosphaera apsteinii* and closely related SIT-like proteins (SITL) were also discovered in three coccolithophore species (*S. apsteinii*, *C. pelagicus* and *C. leptoporus*). Each of these species was shown to be highly sensitive to germanium (Ge), an analogue of Si that acts as competitive inhibitor of Si uptake. Growth of these coccolithophores in low Si seawater amended with 5 µM Ge resulted in highly aberrant coccoliths and this inhibitory effect was reversed by the addition of 100 µM Si. Prolonged growth at very low Si (<0.1 µM) also resulted in the production of aberrant coccoliths, indicating that Si is required for calcification. In stark contrast, no inhibitory effects of Ge (up to 20 µM) were observed in *E. huxleyi* and *G. oceanica*, bloom forming species that do not possess SITs or SITLs.

The role of Si in coccolithophore calcification remains to be determined. Small amounts of Si can be detected in the coccoliths of *S. apsteinii*, suggesting that Si may play a direct role in coccolith formation (Drescher et al. 2012). Recent advances have shown that Si can act to stabilise amorphous $CaCO_3$ (ACC)(Ihli et al. 2014, Kellermeier et al. 2010). It is therefore possible that Si stabilises an otherwise labile amorphous $CaCO_3$ phase (ACC) in coccolith development, which can then undergo transition to the crystalline calcite in combination with the coccolith-associated organic components. There is currently no conclusive evidence for the involvement of ACC in coccolithophore calcification, although small Ca^{2+}-rich membrane bound granules known as coccolithosomes appear to be an integral part of calcification at least in the early stages of coccolith production in *Hymenomonas carterae* and *Pleurochrysis carterae*. A transition from amorphous ACC present in the CV to calcite at the onset of calcification is difficult to reconcile with the fact that the very first $CaCO_3$ that appears to precipitate onto the baseplate scale is in the form of a highly ordered ring of rhomboid calcite crystallites, the protococcolith ring (Young et al. 1999), although this ontogenetic model coccolith growth is derived from the non-Si requiring *E. huxleyi*, and it is premature to rule out a contribution of ACC to calcification in all groups. High-resolution analytical measurements of coccolithophore cells and their calcite coccoliths through development, comparing Si-requiring and non-requiring species is now required to determine the mechanism

of Si-regulation of calcification, whether ACC is involved in the process and to what degree Si is incorporated into the calcite.

The long-held concept that the ecological niche of coccolithophores is partially defined by their lack of a requirement for Si is largely derived from studies on *E. huxleyi* (Tyrrell & Merico 2004). The presence of SIT/SITL transporters and Ge-sensitivity in a broader range of coccolithophores indicates there is considerable physiological diversity in their Si requirements. Although the Si quota of coccolithophores is likely to be small, the ability of certain species (i.e. *E. huxleyi* and *G. oceanica*) to avoid a requirement for Si entirely may confer a competitive advantage in specific environments, such as in the Si-depleted waters following a diatom bloom. A wider phylogenetic analysis should reveal whether a requirement for Si is an ancestral trait in coccolithophores and identify whether the dramatic depletion of DSi from surface waters during the Cenozoic provided selective pressure to uncouple calcification from Si in some coccolithophore lineages such as the Noelarhabdaceae. The identification of this major mechanistic difference between ecologically important coccolithophore species again highlights the need to study a multitude of species in laboratory-based studies in order to address how the differing requirements for Si influence competitive interactions of coccolithophores with their ecosystem.

9.0 Coccolithophore distribution, diversity and adaptation

Much of our understanding of coccolithophore physiology relates to *E. huxleyi*, although it is becoming clear that other coccolithophore lineages may exhibit considerably different physiological attributes (Durak et al. 2016, Rickaby et al. 2010). Whilst *E. huxleyi* is the most abundant coccolithophore species in modern oceans, many of the other larger coccolithophores such as *Coccolithus pelagicus* and *Calcidiscus leptoporus* contribute significantly to global calcite production (Daniels et al. 2014). Coccolithophore species exhibit distinct vertical and latitudinal zonation (Boeckel & Baumann 2008, Okada & Honjo 1973, Winter et al. 1994) with species diversity greatest in the stable, low nutrient environments found at low latitudes. In more variable regimes with higher nutrients found at higher latitudes,

coccolithophore species diversity is lower and assemblages are often dominated by *Emiliania* (Brun et al. 2015). Vertical zonation is pronounced in the communities found at higher latitudes. For example, in the equatorial Atlantic, the characteristic coccolithophores of the oligotrophic surface waters are *Umbellosphaera irregularis* and *Umbellosphaera tenuis*, whereas the typical coccolithophores of the lower photic zone are *Florisphaera profunda* and *Gladiolithus flabellatus* (Kinkel et al. 2000). *Emiliania* is distinct from many other species as it is common in all photic zones. The pronounced vertical zonation of coccolithophore species may be driven by factors such as light, temperature and nutrients, which are all likely to contribute to diversity in coccolithophore physiology. Many coccolithophore species, particularly those from the lower photic zone, have not yet been isolated in laboratory culture and so it is likely that the true breadth of coccolithophore physiology is yet to be discovered.

Intra-specific genetic diversity in coccolithophores also contributes to their physiological and morphological diversity. Strains of *E. huxleyi* can be assigned to a series of different morphotypes, based on the morphology of their coccoliths (Young & Westbroek 1991). Strain-specific differences in pigments and composition of lipid biomarkers such as alkenones and alkenes have also been observed, although these could not be assigned to different morphotypes (Conte et al. 1995). The sequencing of the *E. huxleyi* genome revealed pronounced genetic variability between strains, even those that have been isolated from similar geographical locations (Read et al. 2013). *E. huxleyi* strains possess a core genome that is common to all strains, as well as an additional complement of genes that differ markedly between strains. This 'pan-genome' is proposed to have enabled physiological plasticity and contributed to the ecological success of *E. huxleyi* in diverse marine environments (Read et al. 2013). Recently, detailed phylogenetic studies have provided insight into potential mechanisms underlying the genetic diversity exhibited by *Emiliania*. For example, *Emiliania* shows evidence for introgressive hybridization with older *Gephyrocapsa* clades, a process that would result in extensive genetic mixing (Bendif et al. 2015).

The predicted changes in ocean carbonate chemistry have led to considerable interest in the ability of coccolithophores to adapt to changes in their

environment. A full discussion of the implications of environmental change on coccolithophore biology is beyond the scope of this review (the reader is directed to several excellent recent reviews (Meyer & Riebesell 2015, Ridgwell et al. 2009, Rost et al. 2008)) but it is important to note the capacity for adaptation when considering genetic and physiological diversity between strains. Recent evidence suggests that the physiological properties of *E. huxleyi* strains isolated from differing geographical locations relates to the carbonate chemistry of the seawater from which they were isolated (Rickaby et al. 2016). This could reflect the ability of *E. huxleyi* to adapt to its environment or represent the selection of strains that exhibit a competitive advantage from a standing genetic stock. Laboratory experimental evolution approaches that address this question suggest that both processes are likely to contribute to adaptive evolution within *E. huxleyi* populations over relevant timescales (Lohbeck et al. 2012).

11.0 Concluding remarks

Remarkable new discoveries of cell physiology, microbial interactions, metabolism, and biomineralization continue to emerge. These have important implications for understanding ecosystem linkages and the role coccolithophores play in marine biogeochemical cycles. To date, advances have largely been achieved through a combination of genomics, transcriptomics, proteomics and metabolomics approaches together with targeted functional characterization of specific genes. The rapidly increasing haptophyte and coccolithophore genomic and transcriptomic resources provides unprecedented opportunity to understand the molecular basis of physiological versatility and diversity. The lack of stable transformation and reverse genetic systems is a bottleneck that now limits progress in understanding specific processes such as calcification. A multidisciplinary approach that combines functional characterization of genes with high-resolution ultrastructure, and analytical chemistry promises to yield answers to some of the most pressing questions in coccolithophore calcification.

Much of our understanding of coccolithophore biology comes from studies with *E. huxleyi*, but it is clear there is considerable physiological diversity between

ecologically important coccolithophore species, as well as genetic diversity within species. It is important to understand how these differences influence the distribution and ecological role of coccolithophores. This requires comparative physiology, ecology and genomics of a broader range of coccolithophore species representing the four major families.

With a better understanding of the unique physiology of coccolithophores it will be possible to provide inputs compatible with trait-based ecosystem models that have great potential for describing biogeography of phytoplankton and their responses to environmental variables (Follows & Dutkiewicz 2011). The future is promising with the community increasingly adopting an interdisciplinary approach from bench to field in order to understand how the unique physiological versatility and metabolic repertoire of coccolithophores defines their ecology and responses to climate change.

Author financial interests:

The authors have no competing or financial interests related to the publication of this article.

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Figures

Figure 1. Coccolithophores and intracellular calcification

(a) Scanning electron micrographs of several coccolithophores reproduced to the same scale. From left to right: *Scyphosphaera apsteinii*, *Emiliania huxleyi*, *Calcidiscus leptoporus*, *Gephyrocapsa oceanica* and *Coccolithus braarudii*. (b-c) Model illustrating the sequence of intracellular coccolith production. (b) The process starts with nucleation of peripheral calcite crystals onto the organic baseplate that is produced in a Golgi-derived coccolith vesicle. Trans-Golgi vesicle trafficking provides organic components. (c) As the calcite coccolith matures the endomembranes associated with the CV become more complex, likely playing an important role in ion transport and coccolith morphology. (d) Coccoliths are released to the cell surface through exocytosis. Recycling of the membrane components is likely required for new coccolith production.

Figure 2. Coccolithophore life cycle transitions; environmental factors and niche partitioning.

A cartoon summarizing the major abiotic and biotic factors that appear to influence life cycle phase transitions in coccolithophores. See section 3.0 for details.

Figure 3. Microbial interactions with coccolithophores and DMSP metabolism

Summary of recent findings related to bacterial and virus interactions with *Emiliania huxleyi*. These interactions also intersect with the DMSP metabolism of coccolithophores. Aside from its osmoprotective properties, DMSP serves other functional roles. These include anti-grazing activity, antioxidant capacity and sustaining bacterial mutualisms. The recently discovered DMSP-lyase Alma1 in *E. huxleyi* indicates endogenous control of DMSP pools can be dynamically regulated in coccolithophores. Release of DMSP on cell death leads to rapid turnover and increased DMS production through bacteria-mediated cleavage. See section 4.2 and 5.2 for details.

Figure 4. *E. huxleyi* virus dynamics; a timeline of major cellular events

A diagram that summarizes recent research findings on the cellular dynamics of EhV infection of diploid *E. huxleyi*. Upper panel shows main stages of infection of *E. huxleyi* by viral particles in the ocean, and indicates the potential of viral transmission mechanisms that have been identified. The lower panel indicates a timeline of hours post infection (hpi) and the main cellular events that have been examined in laboratory cultures of *E. huxleyi*. See section 4.2 for details.

Figure 5. Ion transport during calcification in coccolithophores

A cartoon illustrating how the ion transport mechanisms, known to be related to calcification, may be integrated in this process. Of particular importance is the efficient movement of Ca^{2+} and H^{+} without compromising cytoplasmic homeostasis of these ions. The endomembrane system that surrounds the coccolith vesicle likely plays a critical role. The role of Si in coccolith production has yet to be determined, but may be important in stabilizing an intermediate form of $CaCO_3$. See Section 8.3 for details.

Figure 1 Coccolithophores and intracellular calcification

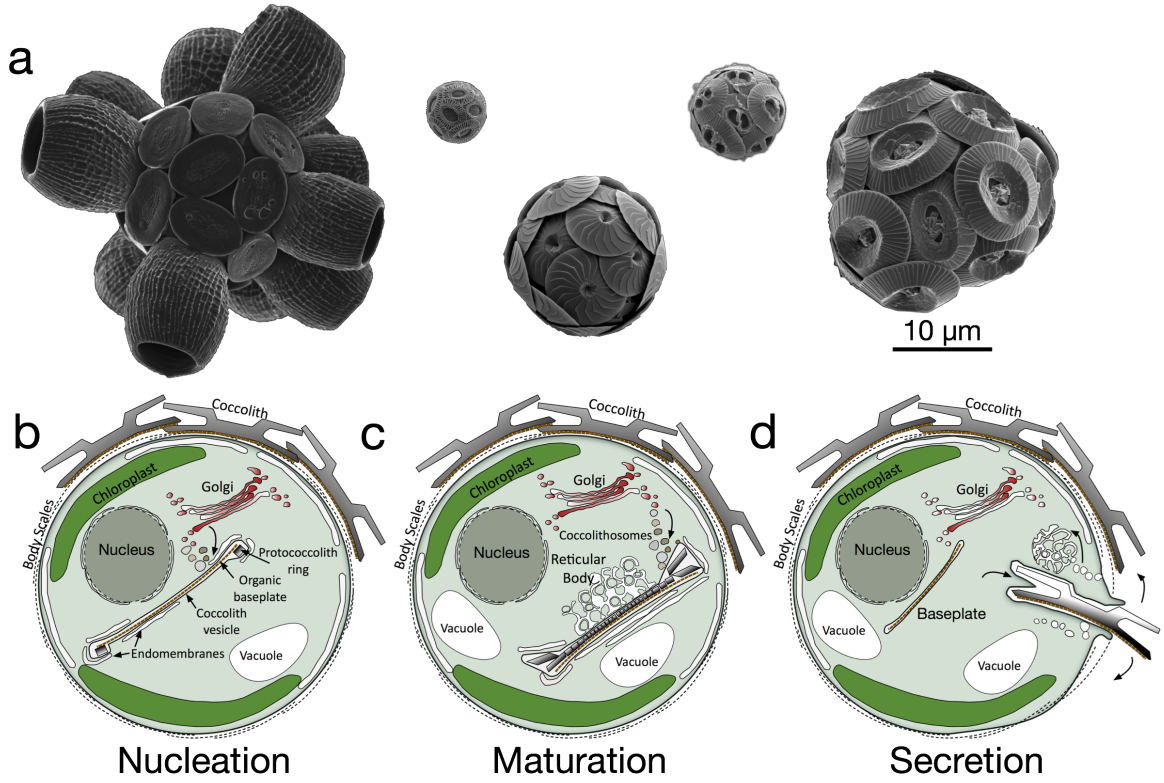


Figure 2. Life cycle transitions- environmental factors and niche partitioning

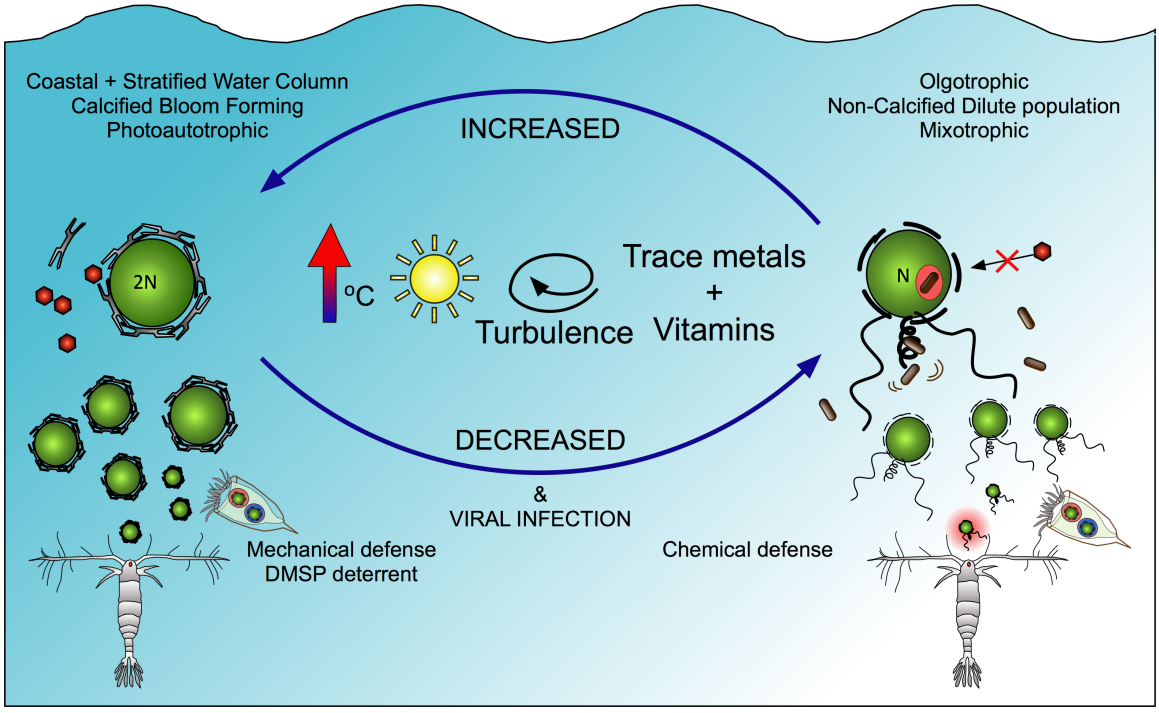


Figure 3. Microbial interactions and DMSP metabolism

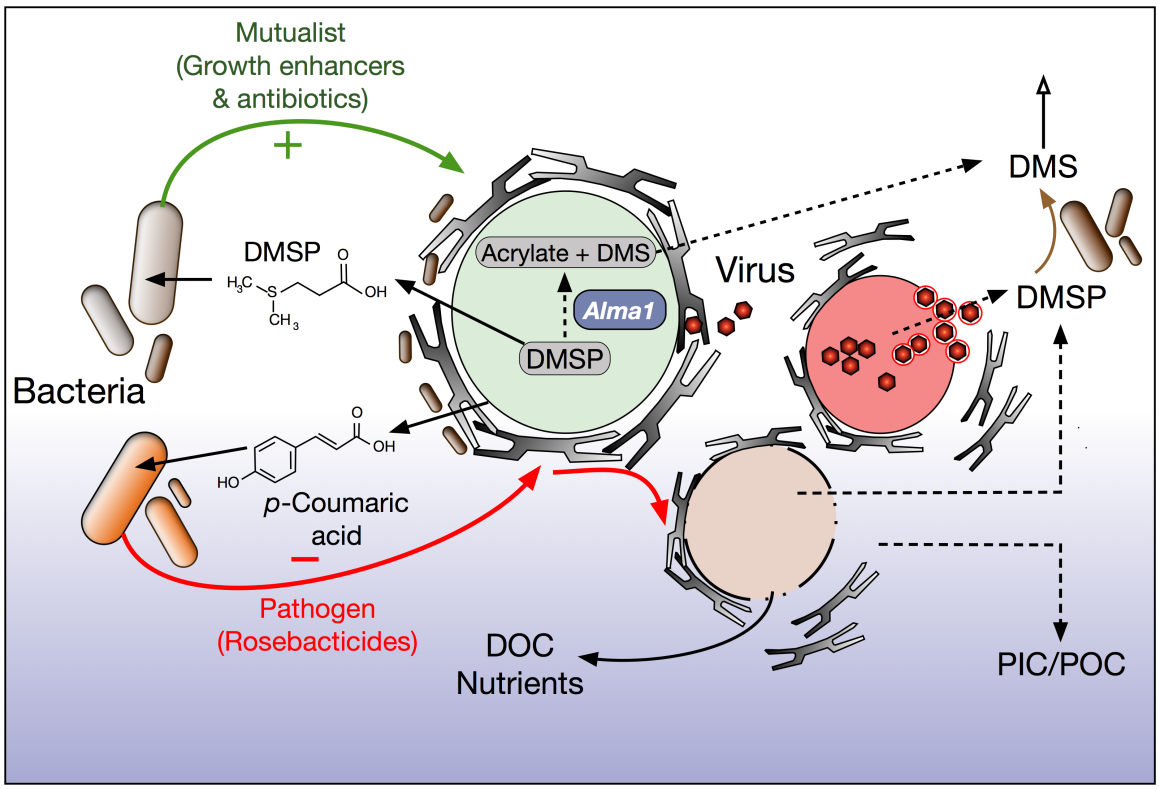


Figure 4 *E. huxleyi* virus; timeline of major cellular events.

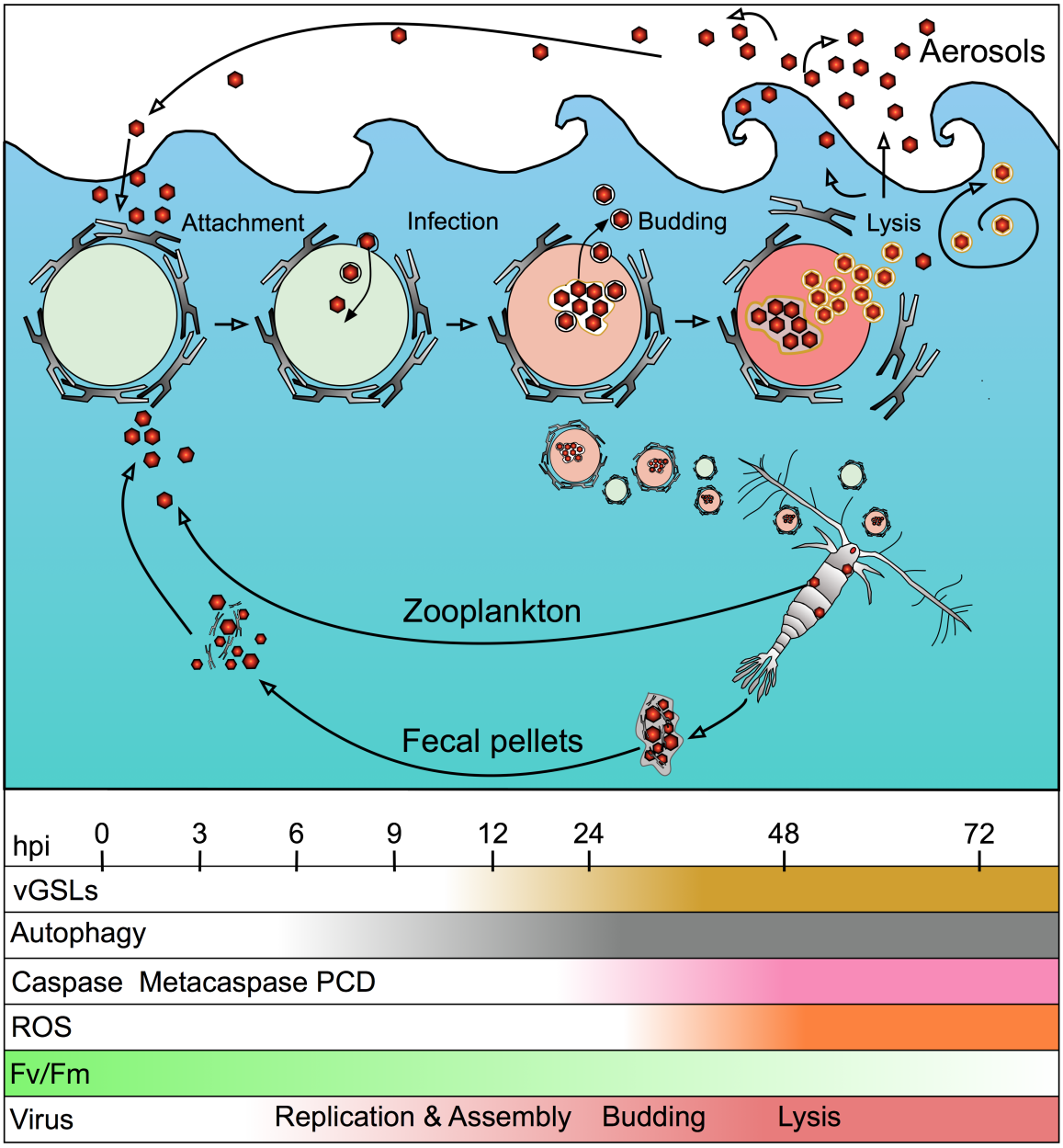
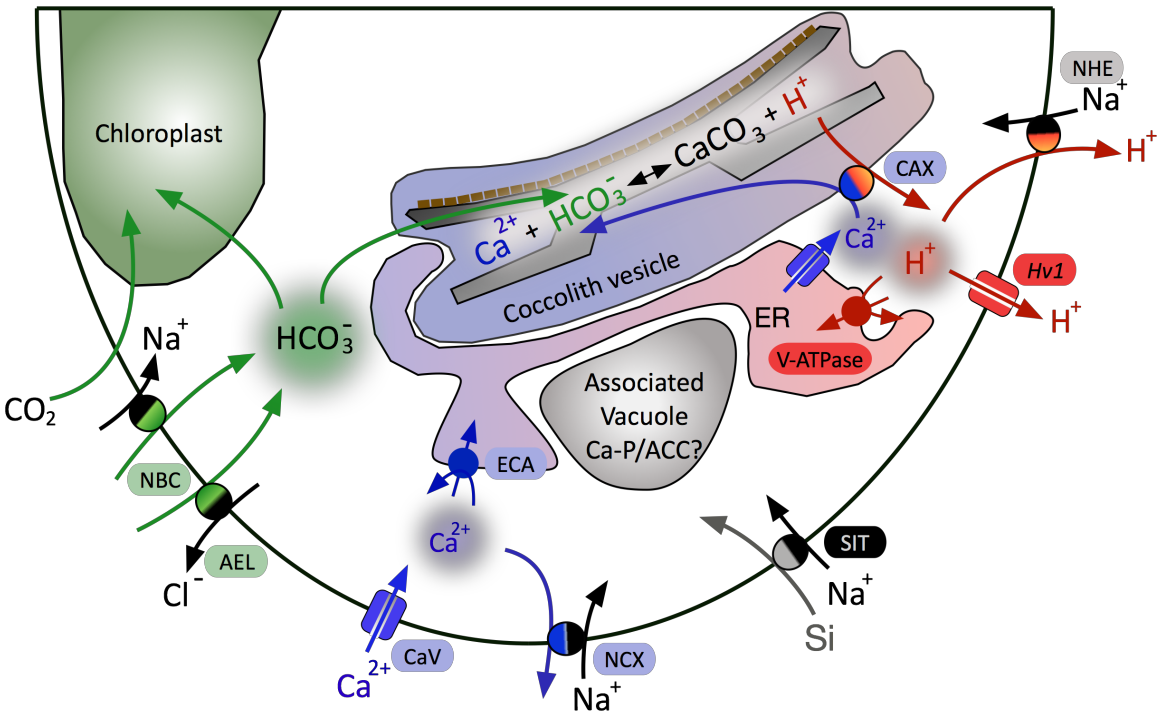


Figure 5 Ion transport during calcification in coccolithophore



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