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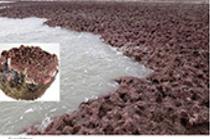
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## Calcification in the coralline red algae: a synthesis

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### ABSTRACT

A descriptive model of calcification in the coralline red algae is integral to understanding their contributions to marine geochemistry, the responses of seascapes to global change, and their own evolution and ecology. Yet, the development of a holistic model of this process has so far been limited by the intrinsic challenge of assembling multiple lines of experimental and observational evidence from varied disciplines. To resolve this synthetic gap, we integrated ultrastructural, geochemical, biochemical and physiological data against a backdrop of anatomy and morphogenesis, which provide context for the localized process at play within the algal thallus. This synthesis represents a comprehensive description of calcification in the coralline red algae.

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## INTRODUCTION

The coralline red algae (Rhodophyta, Corallinophycidae; Corallinales, Corallinapetrales, Hapalidiales and Sporolithales) are important calcifying organisms in marine ecosystems globally, from the tropics to the polar regions. Coralline algae provide a variety of ecological and biogeochemical ecosystem services, including the cementation of reef substrates and major contributions to coastal carbon cycles (reviewed in McCoy & Kamenos 2015; Cornwall *et al.* 2023). Research on the coralline algae has increased in recent years because of the possible impacts of ocean warming and ocean acidification on calcifying organisms (Kroeker *et al.* 2010, 2013a, b; Cornwall *et al.* 2018, 2021). Interest in these ecological impacts has resulted in a renewed focus on and better understanding of the mechanism of calcification in these algae (Cornwall *et al.* 2018; Bergstrom *et al.* 2022; Xue *et al.* 2022).

Although many models of the mechanism of calcification in coralline algae have been proposed (e.g. Borowitzka 1982; Okazaki & Furuya 1985; Borowitzka 1987, 1989; Adey *et al.* 2013; Cornwall *et al.* 2017; Nash *et al.* 2019), none of these is comprehensive. A complete model would incorporate morphological and developmental aspects of thallus growth, the dynamic nature of cell wall composition, the chemical environment in which calcification occurs, cell-mediated ionic fluxes in the context of photosynthesis and respiration, and CaCO<sub>3</sub> crystal nucleation, growth, morphology and composition. The last decade has seen significant advances in the understanding of biogenic CaCO<sub>3</sub> formation (Martone *et al.* 2010; Cornwall *et al.* 2018; DeCarlo *et al.* 2019; Xue *et al.* 2022). Studies of the physiological responses of coralline

algae to changes in the environment due to global warming and ocean acidification are providing new information relevant to this understanding. New methodologies that allow measurement of the environment within or near to the sites of calcification in the cell walls are also providing important new data. This paper attempts to integrate all the available information from diverse disciplines to provide a general model of the mechanism of coralline algal calcification, while also highlighting the main gaps in knowledge.

Biogenic calcification can be generally classified as biologically induced, organic-matrix mediated, or biologically controlled based on the site of calcification, crystal isomorph formed, and organisation of the CaCO<sub>3</sub> deposited (Lowenstam 1981; Borowitzka 1987). Calcification in the coralline algae is a form of 'organic matrix mediated' calcification (Borowitzka 1987, 1989), in which high-Mg calcite is precipitated extracellularly within the walls of vegetative cells in a chemically controlled space. The contribution of metabolic activity in coralline algal calcification is highlighted by recent studies of the effects of increased CO<sub>2</sub> on coralline algae, which show that calcification is affected by but not directly correlated with external seawater chemistry, because the algal metabolism can compensate somewhat to maintain calcification in the changed environment (Hofmann *et al.* 2013; McCoy & Ragazzola 2014; Cornwall *et al.* 2018; Sordo *et al.* 2019; Cornwall *et al.* 2020).

In the following sections, we will first consider the mechanisms of crystal nucleation, formation and growth, then review the anatomical and morphogenic features of coralline algae pertinent

to localized and biologically controlled decalcification. For clarity, we provide definitions of many anatomical features, terms and processes that have been used inconsistently in the literature (Table 1). This information is then integrated with ultrastructural, geochemical, biochemical, physiological and other information on calcification in coralline algae in a conceptual model of the mechanism of calcification. Specifically, we outline evidence that coralline algae actively control their chemistry at their site of calcification by producing an organic matrix that favours the precipitation of CaCO<sub>3</sub> and organizes cell growth. Finally, we identify major knowledge gaps in building a cohesive model of calcification in this group.

## CALCIFICATION BASICS

The formation of crystalline CaCO<sub>3</sub> requires Ca<sup>2+</sup> and CO<sub>3</sub><sup>2-</sup>, possibly in the form of HCO<sub>3</sub><sup>-</sup>, followed by CaCO<sub>3</sub> nucleation, crystal formation and subsequent crystal growth. This process is thermodynamically favoured when the mineral saturation state ( $\Omega$ ) is higher. In coralline algae, as in many other marine calcifiers such as echinoderms, Mg<sup>2+</sup> can be substituted for Ca<sup>2+</sup>, resulting in variable amounts of bulk Mg incorporated into crystalline CaCO<sub>3</sub> (roughly 10%–17% Mg<sup>2+</sup> by weight; Smith *et al.* 2012) and the general formation of high-Mg calcite in the coralline algae.

Nucleation is a fundamental and essential step in all crystal formation (Smeets *et al.* 2017; Gebauer *et al.* 2018). CaCO<sub>3</sub> nucleation proceeds slowly in seawater in the absence of biological mediation (Zuddas & Mucci 1998). In this synthesis, we present collective evidence that coralline algae induce nucleation by two mechanisms: 1) localized regulation of ion concentrations (i.e. the degree of supersaturation) as a consequence of metabolic activities, which include photosynthesis, respiration and ion transport; and 2) the production of polysaccharides or proteins (e.g. the organic matrix) that mediate the formation of solute pre-nucleation clusters, the nucleation process itself, the solubility of the initially precipitated phase and ultimately the CaCO<sub>3</sub> crystal form.

## ULTRASTRUCTURE

The ultrastructural studies reviewed below reveal three regions of calcification: (1) the middle lamella/interfilament

region, (2) the primary cell wall that is formed during cell growth, and (3) the secondary cell wall which is laid down inside the primary wall once the cell has stopped growing (refer to Table 1 for terminology; Figs 1–3). Secondary cell walls are generally absent in the red algae (Craigie 1990). In coralline algae, secondary cell walls have been documented only in uncalcified genicular cells (Martone *et al.* 2009).

## The middle lamella

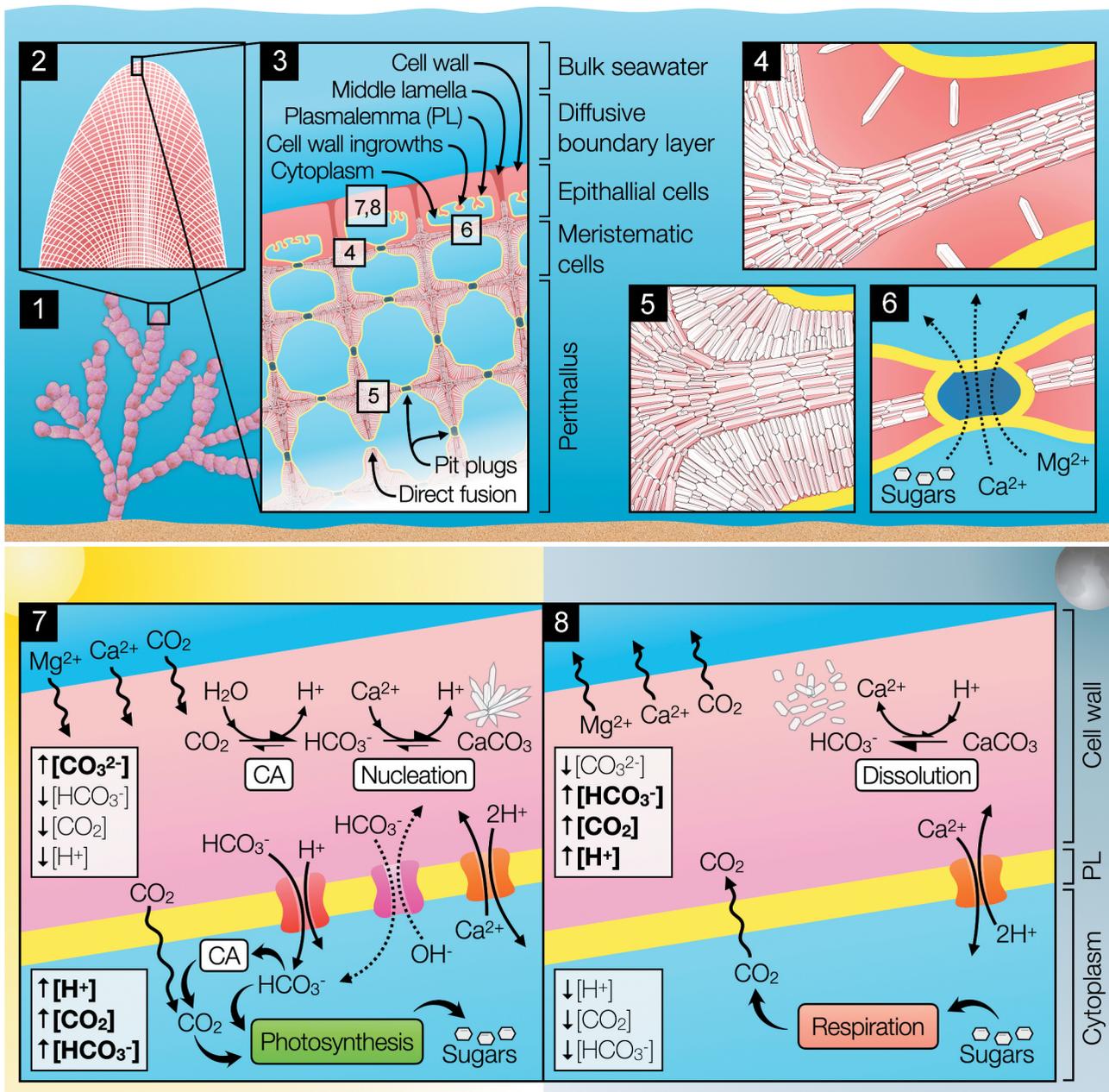
Calcification in coralline algae is observed first in the middle lamella region of sporelings as early as the 4-cell stage in *Jania rosea* (Lamarck) Decaisne [Vesk & Borowitzka 1984, as *Haliptilon cuvieri* (J.V. Lamouroux) H.W. Johansen & P.C. Silva], later at the 8–16 cell stage in *Lithophyllum corallinae* (P. Crouan & H. Crouan) Heydrich (de Carvalho *et al.* 2022), and at about the 32-cell stage in other species (Furuya 1960; Cabioch 1972; Jones & Moorjani 1973) in the region where two adjacent filaments touch (the interfilament region of Adey *et al.* 2013). We hypothesize that the earlier nucleation of CaCO<sub>3</sub> observed in the middle lamella is due to a higher affinity for Ca<sup>2+</sup> by the polysaccharides found in that region, which stain for ‘pectin’-like acid polysaccharides with Ruthenium Red (Matty & Johansen 1981), in comparison to the polysaccharides found in the primary cell wall. The morphology of CaCO<sub>3</sub> crystals in this region, which are usually elongated and arranged roughly tangentially to the cell surface, suggests that limitations on ion flux via diffusion distance and structure porosity occur during coralline algal crystal formation (Jo *et al.* 2020).

## Primary and secondary cell walls

Morphological evidence suggests that calcification in the primary cell walls follows the initiation of calcification in the middle lamella (de Carvalho *et al.* 2022). The calcite crystals in the cell wall are arranged approximately perpendicular to the cell surface and sometimes appear fan-like, radiating from the plasmalemma surface (Fig. 5; Borowitzka & Vesk 1979; Giraud & Cabioch 1979; Nash *et al.* 2019; Auer & Piller 2020). In species with heavily calcified walls,

**Table 1.** Definitions as used in this paper.

Term	Definition
Middle Lamella Region	The region where the cell walls of two adjacent cells touch or almost touch (either adjacent filaments or adjacent cells within a filament). This term is broadly equivalent to the term ‘interfilament space’ as used by Adey <i>et al.</i> (2013) and Nash <i>et al.</i> (2019). It is analogous to, but not equivalent to, the middle lamella of higher plants.
Primary Cell Wall	The cell wall formed following cell division (Craigie 1990).
Secondary Cell Wall	Additional cell wall material laid down after the primary cell wall has been formed and cell growth has ceased (i.e. cell wall thickening) as occurs in the cells of the genicula (Martone <i>et al.</i> 2009).
Primary Calcification	Calcium carbonate formation immediately upon new cell wall formation or soon thereafter (occurs in both primary and secondary cell walls).
Secondary Calcification	Calcium carbonate deposition in cell spaces following cell death (infilling) or as part of a process of carbonate dissolution and reprecipitation in dead tissue or between the algal thallus and the substrate.
Epithallus	The outer cell layer(s) of the algal thallus, <i>sensu</i> Woelkerling (1988) and Irvine & Chamberlain (1994).
Perithallus	The cell layers making up the main part of the algal thallus, <i>sensu</i> Woelkerling (1988) and Irvine & Chamberlain (1994).
Hypothallus	The basal layer of the algal thallus, typically consisting of densely-packed filaments, <i>sensu</i> Woelkerling (1988) and Irvine & Chamberlain (1994).
Intercalary Meristem	Zone of active cell divisions.



**Figs 1–8.** Overview of calcification in coralline algae – structural, chemical and physiological aspects.

**Figs 1–3.** Calcification in coralline algae. The rate of calcification is highest in new growth, particularly the most superficial cells: the epithallial and meristematic cells. Calcification occurs exclusively in the middle lamella and cell wall, aided by the presence of a polysaccharide hydrogel.

**Figs 4–6.** As new epithallial cells form, calcification begins in the middle lamella (Fig. 4), with calcite crystals generally oriented parallel to the adjoining cell walls. Calcification then proceeds into the cell wall (Fig. 5), with calcite crystals generally oriented perpendicular to the plasmalemma (PL; yellow); the middle lamellae and cell walls of older, deeper cells are typically completely calcified. Pit connections among cells, both pit plugs and direct fusions allow for transport of ions and sugars needed for calcification, possibly by active transport (Fig. 6).

**Fig. 7.** During photosynthesis, calcification increases due to the pumping of  $H^+$  into the cell, increasing the extracellular pH. If carbonic anhydrase (CA) is active, the higher pH favours the conversion of  $CO_2$  and water to  $HCO_3^-$  and calcite ( $CaCO_3$ ) formation.  $CO_2$ ,  $Ca^{2+}$ , and  $Mg^{2+}$  can diffuse to the site of calcification and previously sequestered  $Ca^{2+}$  is actively transported out of the cell.

**Fig. 8.** During respiration, these processes reverse with  $H^+$  being actively transported out of the cell, causing a decrease in the pH outside the cell and favouring calcite dissolution. Speculative elements are indicated by dotted lines.

such as *Porolithon onkodes* (Heydrich) Foslie, the wall deposits show clear banding (Nash 2015) and generally, the calcite crystals in the cell walls appear to be much more densely packed than those in the middle lamella region (Figs 4, 5; Poeschel *et al.* 2005; de Carvalho *et al.* 2017; Nash *et al.* 2019).

The thickness of the cell walls varies between tissues, between species, and in response to seasonal changes in

temperature and water chemistry (Steneck & Paine 1986; Halfar *et al.* 2008; Kamenos & Law 2010; Adey *et al.* 2013; Ragazzola *et al.* 2013; McCoy & Ragazzola 2014). Additionally,  $CaCO_3$  crystallites exhibit a surprising variety of shapes and sizes. The complex arrangement and variable crystal shapes, sizes and orientation in different coralline taxa as seen in scanning electron microscopy (SEM) images (e.g. Nash *et al.* 2019; Auer & Piller 2020) clearly indicates that

coralline algal cells walls are neither structurally nor chemically uniform.

It is assumed that the initial nucleation step in primary cell wall calcification occurs in the vicinity of the cell plasma membrane. On gentle decalcification, the organic material of the cell walls shows similar orientation as the crystals in transmission electron microscopy (TEM) (Giraud & Cabioch 1979; Millson & Moss 1985; Wegeberg & Pueschel 2002), but this is not visible in light micrographs (Harvey & Bird 2008; Harvey *et al.* 2009). Whether the orientation of this organic material precedes calcification and is at least partially responsible for the orientation of the calcite crystals, or whether the organic material is moulded by the growing CaCO<sub>3</sub> crystals, remains unknown, although the latter seems more probable. Specifically, TEM images of non-calcified cell walls of both coralline and other rhodophytes indicate that the fibrils are never oriented at right-angles to the cell surface (Tsekos 1996).

Interestingly, SEM images of polished sections of coralline algae etched with EDTA solution or slightly acidic deionised water show structures presumed to be organic fibrils associated with the CaCO<sub>3</sub>, and these are sometimes arranged parallel to the cell in the primary cell wall (Flajs 1977; Nash & Adey 2018; Nash *et al.* 2019). Nash (2015) has suggested that these may block further crystal growth, thus leading to the banding observed on crystal deposits in the walls of some species of coralline algae (e.g. backscatter electron images of cell walls of *Porolithon onkodes* in Nash *et al.* 2011). Furthermore, the nanoSIMS images in Ragazzola *et al.* (2016) of the Ca<sup>2+</sup>, Mg<sup>2+</sup> and Sr<sup>2+</sup> distribution in the thick cell walls of *Lithothamnion glaciale* Kjellman show distinct banding in the wall around a single cell, suggesting that crystal deposition occurred in a sequential manner over time. By comparing SEM images of etched sections of coralline algal walls (Flajs 1977; Nash *et al.* 2019) with ones that have not been etched, it is obvious that the calcite deposits in the middle lamella region are more easily dissolved. TEM-viewed replicas of fractured, unetched material (Giraud & Cabioch 1979, fig. III d), SEM images of tissue where only the organic material has been removed by bleach (Pueschel *et al.* 2005), and TEM images of calcified or partially calcified material (figs 3, 11 in Borowitzka & Vesik 1978; fig. 2E in de Carvalho *et al.* 2017) suggest that the calcite crystallites in the middle lamella are less densely packed, thus allowing easier penetration of the etching solution (Figs 3–5).

## THE CELL WALL

Since the calcite crystals of coralline algae are formed within the cell walls, cell wall chemistry and structure are key to understanding the calcification process. It has been hypothesized that the organic components of the wall affect both CaCO<sub>3</sub> nucleation and control crystal morphology and growth (Borowitzka 1989). CaCO<sub>3</sub> formation (calcification) is most active in the meristematic region near the thallus surface where cell elongation followed by cell division occurs. This is clearly visualized in studies using Alizarin Red or Calcein staining where the stain is co-deposited with the CaCO<sub>3</sub> during thallus growth (Andrake & Johansen 1980;

Blake & Maggs 2003; Lewis & Diaz-Pulido 2017). Importantly, these stains are deposited in the same region as Calcofluor White (Martone 2010; Lewis & Diaz-Pulido 2017), a stain which binds to  $\beta$ -1,3- and  $\beta$ -1,4- polysaccharides of the cell wall (Anderson *et al.* 2010). In combination, these stains indicate that new cell wall polysaccharide formation and calcification occur together. Notably, the meristematic cell layers of coralline algae contain the most chloroplasts, and photosynthesis is most active in this region (Borowitzka 1981), indicating a direct link between cellular activity (energy and carbon fluxes) and the extracellular calcification space.

Rhodophyta are generally viewed as depositing only primary cell walls; however, convincing arguments have been made that the uncalcified, terminally differentiated genicular cells of articulated corallines have secondary cell walls (Martone *et al.* 2009, 2019). Recent hypotheses by Nash *et al.* (2019) rely on an interpretation that secondary cell walls are routinely deposited by ordinary cortex cells of articulated corallines and perithallial cells of crustose corallines, which differs from the conventional use of the term in algae or higher plants (Craigie 1990).

Cell walls in the Florideophyceae are mostly composed of sulphated galactans, (glyco)proteins and skeletal polysaccharides (e.g. cellulose, mannans, xylans). However, the cell walls of the coralline algae contain distinctive agarans with low to intermediate sulphate contents (Usov *et al.* 1997; Navarro & Stortz 2008; Martone *et al.* 2010; Navarro *et al.* 2011). The agarans of the non-calcified genicula have a highly methoxylated galactan with low levels of xylose side stubs, whereas the calcified intergenicular segments contain xylogalactans with high levels of xylose and low levels of methylated galactan units (Martone *et al.* 2010), suggesting that calcification is associated with the presence of the methylated galactans. Janot (2018) also found clear differences in the content and structure of polysaccharides between genicular and intergenicular cell walls, the latter being very similar to walls of non-geniculate coralline algae. The calcified intergenicular walls of articulated coralline algae and the walls of crustose coralline algae (all calcified) had a lower galactose and xylose content than the genicular walls (uncalcified) of the articulated coralline algae (Janot *et al.* 2022). Janot *et al.* (2022) also reported elevated glucose content (commonly a monosaccharide proxy for cellulose) in the calcified tissues of both crustose forms and the intergenicula of articulated forms relative to the non-calcified genicular tissue. Furthermore, alginic acids (linear polymers built up of the 1→4-linked residues of  $\beta$ -D-mannuronic and  $\alpha$ -L-guluronic acids) have been reported in some coralline algae (Okazaki *et al.* 1982; Usov *et al.* 1995; Usov & Bilan 1996). Alginic acids are characteristic of brown algae, very few of which calcify, and have not been found in any other group of Rhodophyta, thus additional inquiry could yield insights into the roles of additional organic compounds in calcification.

The cell walls of coralline algae have also been reported to contain cellulose (Turvey & Simpson 1966; Siddhanta *et al.* 2013; Janot *et al.* 2022), chitin (Rahman & Halfar 2014; Rahman *et al.* 2019), and chitin-associated glycoproteins (Rahman *et al.* 2019). Lignin is found also in the non-calcified secondary cell walls of the genicula (Martone *et al.*

2009; Xue *et al.* 2022). Cellulose synthesis in higher plants and diverse algae, including red algae (Tsekos 1996, 1999), is mediated by cellulose synthase. In higher plants, these enzyme complexes move within the plane of the cell membrane while guided by microtubules within the cortical cytoplasm (Bashline *et al.* 2014). As glucose monomers traverse the trans-plasmalemmal cellulose synthase, they are polymerized into microfibrils in the wake of the enzymes moving along the cell surface. The cellulose synthase complexes – which can be visualized by freeze-fracture TEM (e.g. Gunning & Steer 1996; Tsekos 1996, 1999), not by SEM – are trafficked to the plasmalemma in Golgi vesicles and have a residence time on the plasmalemma of only 15–40 min (Bashline *et al.* 2014). Consequently, the orientation of the microfibrils is necessarily parallel to the cell surface (e.g. Gunning & Steer 1996; Bashline *et al.* 2014; Martone *et al.* 2019), not perpendicular as was suggested in recent calcification models (Nash *et al.* 2019). Recent work by Xue *et al.* (2021) provides the functional characterization of a cellulose synthase from the articulated coralline *Calliarthron tuberculosum* (Postels & Ruprecht) E.Y. Dawson. Expanding on transcriptomic lines of inquiry (e.g. Page *et al.* 2019) will doubtless yield valuable insights to the functional roles of known synthetic pathways active within calcification.

### Soluble organic components of the cell wall

Evidence suggests that these soluble organic components rather than overall organic structure may catalyse CaCO<sub>3</sub> nucleation. Observations from multiple precipitation studies indicate that calcification in coralline algae is induced by the water-soluble components of the cell wall and that these also affect the observed crystallography (Borowitzka 1989; Raz *et al.* 2000; de Carvalho *et al.* 2017). In these studies, the water-insoluble components such as cellulose mainly play a structural role but have no role in nucleation, contrary to the model proposed by Nash *et al.* (2019). Cell wall polysaccharides differ between species, mediating biomineralization and species' differential responses to seawater chemistry and environment (Bergstrom *et al.* 2022).

We hypothesize that the cell walls of coralline algae act effectively as gel-like structures (hydrogels). A hydrogel is a three-dimensional network of interconnected macromolecular chains with the interspaces filled with water and electrolytes. In such systems, the critical parameters for crystal formation and precipitation are mostly determined by diffusivity and the local charge distribution (ionotropic effect; Kosanović *et al.* 2017). The role of hydrogels in the formation of CaCO<sub>3</sub> has been extensively studied in *in vitro* systems. The role of natural polysaccharides such as agarose, agar and chitosan as well as the effects of ions such as Mg<sup>2+</sup> have been examined; however, those studies have focused on calcification in invertebrates and not on the coralline algae (Asenath-Smith *et al.* 2012; Nindiyasari *et al.* 2015; Kosanović *et al.* 2017; Greiner *et al.* 2018). Studies of agar gels show that the density of the gel influences the calcite microstructure, and that crystal growth rate influences the degree of occlusion of agar in the crystals (Greiner *et al.* 2018). Furthermore, the rate of diffusion of the component

ions in the gel affects the morphology of the calcite formed (Jo *et al.* 2020).

Algal cell walls act as cation exchangers (Ritchie & Larkum 1982; Meichik *et al.* 2011). This means that the fixed ionic charges of the cell wall molecules allow exchange of ions between interstitial and external solutions (Figs 7, 8). Cation exchange is a pH-dependent process and thus may vary spatially according to the Donnan equilibrium in the cell wall, a process by which charged particles may not distribute equally on both sides of a semi-permeable membrane (Morvan *et al.* 1979; Smith 1985). Generally, preferential binding of Ca<sup>2+</sup> over Mg<sup>2+</sup> by acidic carboxylic and sulphate groups of cell wall polysaccharides creates localized environments in the cell wall with reduced Mg<sup>2+</sup> and enhanced Ca<sup>2+</sup> concentrations (Demarty *et al.* 1984; Wada *et al.* 1993). Since Mg<sup>2+</sup> ions inhibit calcite nucleation, localized reduction in Mg<sup>2+</sup> concentration may be sufficient to favour calcite nucleation (Morse *et al.* 1997; Evans *et al.* 2019). The surface charge of the polysaccharides and Ca<sup>2+</sup> binding may also result in spatially heterogeneous nucleation depending on the arrangement of surface charges. Mg<sup>2+</sup> incorporation into coralline algal skeletons is nevertheless correlated to ambient seawater Mg<sup>2+</sup> content, reflecting incorporation of Mg<sup>2+</sup> during initial skeletal growth (Stanley *et al.* 2002).

The effect of an ion-binding organic matrix on the type of crystal polymorph formed is illustrated by a study of electrocrystallization of CaCO<sub>3</sub> on indium tin oxide electrodes. Pavez *et al.* (2005) observed that the addition of alginic acid from the brown alga *Lessonia trabeculata* Villouta & Santelices strongly inhibited the effect of Mg<sup>2+</sup> on crystallization, favouring nucleation and growth of the calcite phase of CaCO<sub>3</sub>. Previous studies had shown that when the L-guluronic units of the alginate are more abundant than the D-mannuronic units, the affinity of the alginates for alkaline-earth metals is in the following order: Ba<sup>2+</sup> > Sr<sup>2+</sup> > Ca<sup>2+</sup> >> Mg<sup>2+</sup> (Smidsrød 1974). Alginic acids with a higher guluronic acid content thus present a smaller thermodynamic barrier to CaCO<sub>3</sub> nucleation (Giuffrè *et al.* 2013).

Thus, the various polysaccharides of the coralline cell wall play an important role in the process of calcification. However, no definitive roles can yet be assigned to any individual polysaccharide or other organic component of the cell wall, which likely play different roles in nucleation, crystal formation and crystal growth as has been found in the cocolithophorid algae (Taylor & Brownlee 2016). As in other rhodophytes, it is also likely that the different polysaccharides are not evenly distributed in the cell walls (e.g. Akatsuka & Iwamoto 1979; Cole *et al.* 1985). Together with nanostructural (TEM) and nanocrystallographic (SEM) evidence, low concentrations of both Ca<sup>2+</sup> and Mg<sup>2+</sup> found on the outward faces of cell walls of germinating spores even prior to calcification shows the biological control of calcification afforded by macromolecules such as cellulose fibrils (de Carvalho *et al.* 2022).

Polysaccharide synthesis requires photosynthesis to provide the basic carbon building blocks and energy. Different polysaccharides are synthesized and secreted from the cells during cell development and growth. The polysaccharide composition of red algal cell walls can vary with changes in

irradiance, temperature and nutrients (Lee *et al.* 2017; Véliz *et al.* 2017), which has recently been confirmed within the coralline algae (Bergstrom *et al.* 2022). We therefore hypothesize that the observed seasonal and spatial variations in calcification in coralline algal thalli are in part determined by the specific polysaccharides produced, their relative concentrations, and the effects of environmental factors on polysaccharide production and cell wall formation.

## RELATIONSHIPS WITH PHOTOSYNTHESIS AND RESPIRATION

Cell metabolism is central to coralline algal calcification as it relates to three key processes (Figs 1–8):

- (1) Biosynthesis of cell wall polysaccharides and proteins;
- (2) Promoting elevated  $\Omega$  in the cell wall (e.g. by localized increases of dissolved inorganic carbon (DIC),  $\text{Ca}^{2+}$  and pH); and
- (3) Influencing the concentrations of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{CO}_3^{2-}$  at the site of calcification and thus affecting the crystal polymorph and incorporation of magnesium into calcite (Xto *et al.* 2019).

When considering the uptake of dissolved inorganic carbon (DIC) for photosynthesis and the supply of DIC to the site of calcification, it is important to consider how anatomy may affect these processes. As shown diagrammatically in (Figs 7, 8), uptake of  $\text{CO}_2$  and  $\text{HCO}_3^-$  for photosynthesis occurs either at the outer surface of the epithallial cells or from the intercellular region (cell walls) in the case of all other cells. Diffusion from external seawater in this intercellular cell wall region will be limited not only by the composition, structure and density of the organic components of the cell walls, but also by the degree of calcification in the cell walls. It must be noted that the supply of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and other ions (nutrients) face the same diffusion limitations. Some  $\text{CO}_2$  will be supplied to the intercellular region by respiration and  $\text{Ca}^{2+}$  may be transported from the cells into this region. However, in the case of  $\text{Ca}^{2+}$ , one needs to consider how this  $\text{Ca}^{2+}$  reached the cell in the first place – for example, by uptake from the intercellular region or from seawater by the epithallial cells, followed by transport through the pit connections to the cells below.

Numerous studies have shown that the rate of calcification is closely linked to photosynthesis and that the calcification rate is enhanced in the light; however, photosynthesis is not the only driver for calcification, because some calcification also occurs in the dark and in dead coralline algal thalli (Ikemori 1970; Pearse 1972; Böhm *et al.* 1978; Pentecost 1978; Borowitzka 1979, 1981; Smith & Roth 1979; Misonou *et al.* 1980; King & Schramm 1982; Chisholm 2000; El Haikali *et al.* 2004; Semesi *et al.* 2009; Kim *et al.* 2013; Martin *et al.* 2013b; McCoy *et al.* 2016). Decoupled responses of photosynthetic and calcification rates in response to latitudinal variation in temperature and light, and variation in carbon availability have also been shown (Kolzenburg *et al.* 2019). More extreme examples include reports of calcification in the dark in some Arctic species (Adey *et al.* 2013; Büdenbender

*et al.* 2011; Williams *et al.* 2018; Gould *et al.* 2022) and of non-photosynthetic parasitic coralline algae (e.g. *Kvaleya epilaeva* W.H. Adey & Sperapani) (Nash & Adey 2018). In low-light or extremely seasonal environments, coralline algae maintain photosynthetic efficiency by modification of their photosynthetic proteins (Bélanger & Gagnon 2021; Voerman *et al.* 2022). Ultimately, photosynthesis is essential to the long-term calcification in coralline algae by providing the DIC building blocks for the cell wall polysaccharides within which calcification occurs, and providing the energy required for metabolism.

Studies using Alizarin Red and Calcein, which stain newly deposited  $\text{CaCO}_3$ , clearly show that calcification is highest in the outer layers of the coralline thallus in growing regions where the bulk of the chloroplasts are located, and therefore where the photosynthetic activity is greatest (Blake & Maggs 2003; Kamenos & Law 2010; Lewis *et al.* 2017). In articulated coralline algae, calcification rates (on a dry weight basis) are highest in the youngest (apical, tip) segments and decrease in the older/lower segments, which only continue to calcify from their outermost cells (Pearse 1972; Pentecost 1978; Borowitzka 1981; McCoy *et al.* 2016). Although it is more difficult to do experiments along age gradients within the crustose coralline algal tissues, King & Schramm (1982) found that the apical sections of branches of *Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin calcified at a higher rate than the middle and basal sections, both on a per area and a per dry weight basis. The apical sections also had a higher surface area to dry weight ratio. Photosynthetic rates show the same age gradient (Pentecost 1978; Borowitzka 1981). The shapes of photosynthesis vs irradiance (P/E) and calcification rate vs irradiance (C/E) curves are also very similar (Chisholm 2000; Martin *et al.* 2013a; Egilsdottir *et al.* 2016). Among several studies of photosynthetic carbon uptake vs carbon uptake in calcification ( $C_{\text{Ps}}/C_{\text{Calc}}$ ), this ratio varies with species but is always higher than 2 (Table 2). The higher photosynthetic  $\text{CO}_2$  removal rate was comparable to the rate of  $\text{CaCO}_3$  formation.

Photosynthetic rate affects the rate of calcification, likely through the provision of sugars and other photosynthates to fuel metabolic process and local alkalinization of the medium. Further, photosynthetic  $\text{CO}_2$  uptake, accompanied by localized diffusion limitations, increases the pH and

**Table 2.** Photosynthetic C uptake to calcification C uptake ratio ( $C_{\text{ps}}/C_{\text{calc}}$ ) in coralline algae. Data are recalculated from the cited references.

Species		$C_{\text{ps}}/C_{\text{calc}}$	Reference
<i>Ellisolandia elongata</i>	Summer	8.25	Egilsdottir <i>et al.</i> (2016)
	Winter	6.29	
<i>Amphiroa foliacea</i>		6.3	Borowitzka (1981)
<i>Corallina officinalis</i>		1.71 <sup>a</sup>	Pentecost (1978)
<i>Lithophyllum cabiochae</i>		2.5	Martin <i>et al.</i> (2013a)
<i>Porolithon onkodes</i>		2.17 <sup>b</sup>	Chisholm (1988)
<i>Neogoniolithon fosliei</i>		2.5 <sup>b</sup>	Chisholm (1988)
<i>Paragoniolithon conicum</i>		4.8 <sup>c</sup>	Chisholm (1988)

<sup>a</sup>Mean of 3 measurements (range: 1.28–2.26)

<sup>b</sup>Using photosynthetic quotient of 1.2 (Chisholm 1988)

<sup>c</sup>Using photosynthetic quotient of 1.07 (Chisholm 1988)

shifts the chemistry towards higher local concentration of  $\text{CO}_3^{2-}$ , and thus locally increases the saturation of  $\text{CaCO}_3$  both in the diffusion boundary layer (Hurd & Pilditch 2011) and at the site of calcification (Comeau *et al.* 2019a). This proposed effect of photosynthetic carbon uptake on intercellular pH and  $\text{CaCO}_3$  saturation is supported by microelectrode measurements of the surface pH and  $\text{CO}_3^{2-}$  concentration, which showed that these were both higher than in bulk seawater in the light and decreased in the dark (Hurd *et al.* 2011; Hofmann *et al.* 2016, 2018). The effects of diffusion boundary layer thickness on both the carbonate saturation state of seawater at the surface of *Arthrocardia corymbosa* (Lamarck) Decaisne and the calcification rate (Cornwall *et al.* 2014) are consistent with this model, though these effects vary by environment (light and water velocity) and species morphologies (Comeau *et al.* 2019a). Using  $\delta^{11}\text{B}$  as a proxy for pH, Cornwall *et al.* (2017), Donald *et al.* (2017) and Anagnostou *et al.* (2019) have demonstrated that pH at the site of calcification of coralline algae is higher than the external pH (Cornwall *et al.* 2018) in a similar way to what has been found in corals (Comeau *et al.* 2018). Importantly, by pairing  $\delta^{11}\text{B}$  and microelectrode measurements, Comeau *et al.* (2019a) demonstrated that while pH in the calcifying space and the daytime pH in the diffusion boundary layer are correlated, pH in the calcifying space is much higher. Thus, conditions that lower photosynthetic modification of pH at the cell surface also decrease pH in the calcifying space (e.g. lower irradiance and higher water motion).

Bradassi *et al.* (2013) also provide indirect evidence that photosynthesis is important for calcification. When crusts of *Phymatolithon lenormandii* (Areschoug) W.H. Adey were grown on glass microscope slides at pH 8, new calcification was observed using Calcein labelling solely on the growing margin, whereas thalli raised in lower pH seawater (pH 7.55) showed Calcein labelling over the whole thallus. After transfer to Calcein-free water in the dark, the Calcein signal was unchanged in the pH 8 grown thalli, but the previously observed Calcein signal in the thalli grown at pH 7.55 had disappeared, indicating dissolution of the previously deposited  $\text{CaCO}_3$ . These observations suggest that, at low pH in the light, algal photosynthesis can raise the intercellular pH sufficiently for calcification to occur, whereas in the dark, the intercellular pH was sufficiently acidified to lead to complete  $\text{CaCO}_3$  dissolution.  $\text{CaCO}_3$  dissolution (negative calcification rate) in the dark has also been observed in some studies (Chisholm 2000), presumably due to acidification of the intercellular space by respiratory  $\text{CO}_2$ .

### Carbonic anhydrase and ion transport

Coralline algae acquire inorganic carbon for photosynthesis through passive  $\text{CO}_2$  diffusion and/or  $\text{CO}_2$ -concentrating mechanisms (CCMs). CCMs facilitate  $\text{CO}_2$  uptake by diffusion and  $\text{HCO}_3^-$  uptake by active transmembrane transport (Borowitzka 1981; Hofmann *et al.* 2016; Hofmann & Heesch 2018). Recently, Bergstrom *et al.* (2020) showed that field-

collected data of diffusive  $\text{CO}_2$  use and CCM capacity vary significantly across crustose coralline algal species. *Neogoniolithon fosliei* (Heydrich) Setchell & L.R. Mason had lower diffusive  $\text{CO}_2$  use (c. 30%) whereas *Porolithon cf. onkodes*, *Lithothamnion proliferum* Foslie and *Melyvonnea madagascariensis* (Foslie) Athanasiadis & D.L. Ballantine showed higher (c. 60%)  $\text{CO}_2$  use. Carbonic anhydrases, enzymes that accelerate the intrinsically slow equilibrium conversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$ , have important roles in almost all biological calcification systems (Hentunen *et al.* 2000). Coralline algae have an extracellular carbonic anhydrase (Mercado *et al.* 1997a; Hofmann *et al.* 2013, 2016), and the inhibition of photosynthesis by 4,4'-diisocyanostilbene-2,2'-disulphonate (DIDS) also indicates the presence of direct  $\text{HCO}_3^-$  uptake by anion exchange transport (Hofmann *et al.* 2016). However, this ratio varies with latitude; high latitude rhodoliths rely more on diffusive  $\text{CO}_2$  uptake than low latitude rhodoliths (Hofmann & Heesch 2018), like seaweeds in general (Raven *et al.* 2002). Local temperature variability also has been found to increase reliance on CCMs (Stepien 2015). CCM use allows a more efficient access to DIC and facilitates faster DIC acquisition (Stepien *et al.* 2016). Respiratory  $\text{CO}_2$  provides another carbon source, accounting for c. 16%–37% of the carbonate deposited (Lee & Carpenter 2001).

Several lines of evidence suggest that carbonic anhydrase may play a direct role in calcification. In *Corallina officinalis* Linnaeus, elevated  $\text{CO}_2$  stimulates total (internal and external) carbonic anhydrase activity (Hofmann *et al.* 2012, 2013), unlike what has been observed in non-calcareous algae where elevated  $\text{CO}_2$  downregulates carbonic anhydrase activity (García-Sánchez *et al.* 1994; Mercado *et al.* 1997b). Interestingly, Tambutté *et al.* (2007) showed that an extracellular carbonic anhydrase had an important role in the calcification process in the azooxanthellate coral *Tubastrea aurea* (i.e. it played no role in photosynthetic carbon uptake, but was involved in the carbon supply for calcium carbonate precipitation). Finally, a recent study by Rodríguez-Navarro *et al.* (2019) has shown that carbonic anhydrase can also play a direct role in the evolution of calcite crystal (nano)structure and morphology, interacting with and being incorporated into the newly formed  $\text{CaCO}_3$  precipitates.

There are multiple carbonic anhydrases in the rhodophytes (Razzak *et al.* 2019; Wang *et al.* 2020a), of which four  $\alpha$ -type carbonic anhydrases are extracellular. Two possible hypotheses for the role of carbonic anhydrases in calcification, like what has been proposed for coral calcification (Bertucci *et al.* 2013), include:

- a. If  $\text{CO}_2$  is the main source of DIC for calcification, then carbonic anhydrase can enhance the conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$ . The 'removal' of  $\text{CO}_2$  from the intercellular wall region will enhance the rate of diffusion of  $\text{CO}_2$  from the external seawater to the site of calcification by increasing the concentration gradient between it and the external seawater (i.e. facilitated diffusion *sensu* Occhipinti & Boron 2019). Moreover,  $\text{CO}_2$  diffusion in seawater is much faster than that of  $\text{HCO}_3^-$  (Zeebe 2011) and  $\text{CO}_2$  can diffuse through cell membranes, in contrast to  $\text{HCO}_3^-$ . However, the two

protons produced in the formation of  $\text{CaCO}_3$  need to be removed from the site of calcification. Under sufficient illumination, this could be balanced by  $\text{CO}_2$  uptake by the cells for photosynthesis. In coralline algae, photosynthetic DIC fixation has been observed to be always more than  $2\times$  higher than calcification (Table 2). In addition,  $\text{Ca}^{2+}/\text{H}^+$  antiports, as suggested by Hofmann *et al.* (2016), could remove protons and provide additional  $\text{Ca}^{2+}$  necessary to maintain elevated  $\text{CaCO}_3$  saturation state.

Or

- b. If  $\text{HCO}_3^-$  is the source of DIC for calcification, carbonic anhydrase may then accelerate the conversion to  $\text{CO}_2$  to buffer the proton produced by the conversion of  $\text{HCO}_3^-$  into  $\text{CO}_3^{2-}$  (Fig. 7). This requires a low  $\text{CO}_2$  concentration and pH above 8 which is likely the case at the site of calcification in coralline algae (Cornwall *et al.* 2018).

Cellular carbonic anhydrases can indirectly affect intracellular pH and thus the relative concentrations of  $\text{CO}_2$  and  $\text{HCO}_3^-$ , which in turn can affect the diffusive uptake of  $\text{CO}_2$ . Using the carbonic anhydrase inhibitor acetazolamide, photosynthesis was inhibited and the degree of alkalization seen at the surface of the coralline algal thallus in the light was reduced (Hofmann *et al.* 2016, 2018). Acetazolamide inhibits photosynthesis and calcification in *Halimeda* (Borowitzka & Larkum 1976; De Beer & Larkum 2001; Zuñiga-Rios *et al.* 2021). Unfortunately, no data are yet available on the effect of acetazolamide in coralline algae, and thus the exact role of carbonic anhydrase on calcification. We also know very little about any specific ion transporters, such as  $\text{Ca}^{2+}$  ATPases,  $\text{Na}/\text{H}^+$  and  $\text{HCO}_3^-/\text{Cl}^-$ , that are likely involved in the calcification process. Here again, recent developments in coralline algal genomics and transcriptomics that have identified transcriptomic responses of carbonic anhydrase pathways and thallus growth to environmental variation provide exciting avenues for the incorporation of gene expression in our understanding of these intracellular processes (Page *et al.* 2019; Yang *et al.* 2021; Xue *et al.* 2022).

## **CA<sup>2+</sup> AND OTHER ION TRANSPORT**

Ion transport mechanisms in the Rhodophyta are almost completely unknown, but their importance in calcification and pH control in other calcifying organisms such as corals has been long recognized (Tambutté *et al.* 2011; Capasso *et al.* 2021). On the basis of pH,  $\text{O}_2$  and  $\text{Ca}^{2+}$  measurements with microelectrodes at the surface of thalli of *Porolithon* sp. as well as the effects of inhibitors, Hofmann *et al.* (2016) suggested the presence of a  $\text{Ca}^{2+}$ -ATPase and  $\text{Ca}^{2+}$  channels. This hypothesis builds on important characterizations of  $\text{Ca}^{2+}$ - and  $\text{H}^+$ -dependent ATPases by Okazaki (1977) and Mori *et al.* (1996). They also provided evidence for a non-photosynthetic light-dependent  $\text{H}^+$  pump responsible for  $\text{H}^+$  uptake in the light while photosynthesis is inhibited. While evidence for  $\text{Ca}^{2+}$ - $\text{H}^+$  exchangers is equivocal, it is probable that these exist in coralline algae. Genes for  $\text{Ca}^{2+}$ -ATPase and  $\text{Ca}^{2+}$

- $\text{H}^+$  exchangers have been reported for several rhodophytes whose genomes have been sequenced (Schönknecht 2013). Recently, Xue *et al.* (2022) identified genes for two  $\text{Ca}^{2+}$ - $\text{H}^+$  antiporters and other candidate genes related to calcification. Furthermore,  $\text{Ca}^{2+}$  efflux has been associated with adventitious branch formation in *Gracilaria* (Wang *et al.* 2016) and Wang *et al.* (2020b) have also proposed the presence of a  $\text{Ca}^{2+}$ -dependent  $\text{Na}^+/\text{H}^+$  antiporter system in *Pyropia*. Finally, metabolically derived phosphate ( $\text{PO}_4^{3-}$ ) acts as a calcification inhibitor by acting as a ‘crystal poison’, which thermodynamically interferes with the mineralization process (Simkiss 1964), and therefore must be locally removed to facilitate calcification. Photosynthesis actively reduces phosphate accumulation within cell wall boundaries (Walker & Sivak 1986; Gauthier & Turpin 1997; Sharkey 2019).

## **GEOCHEMICAL SIGNATURES**

High-Mg calcite is a characteristic feature of coralline algae, with the Corallinales generally having the highest  $\text{Mg}^{2+}$  content, followed by the Sporolithales and the Hapalidiales (Smith *et al.* 2012). Coralline algae may also contain small amounts of magnesite ( $\text{MgCO}_3$ ) and aragonite; these are mainly secondary depositions as infill of empty cells and conceptacles in the older parts of the thallus (Smith *et al.* 2012; Nash *et al.* 2013; Diaz-Pulido *et al.* 2014; Kravesky-Self *et al.* 2016). In the tropical *Porolithon onkodes*, very-high-magnesium calcite ( $\text{Mg}_{0.5}\text{Ca}_{0.5}\text{CO}_3$ ; dolomite) has also been observed lining old hypothallial cell walls and much of skeletal structures due to secondary infilling (Nash *et al.* 2011; Nash 2015).

Mg content can be directly influenced by the Mg/Ca mole ratio of the seawater, as shown by Stanley *et al.* (2002) with *Amphiroa* spp, and by Ries (2006) with *Neogoniolithon* sp. The extremely low  $\text{Mg}^{2+}$  content (mean of 1.1%) in the calcite of the freshwater coralline alga *Pneophyllum cetinaense* Kaleb, Zuljević & V. Peña also reflects the low Mg/Ca ratio in the waters of its habitat, the Cetina River, southern Croatia (Ragazzola *et al.* 2020). However, analysis of the Mg distribution within the cell walls at a finer scale shows further variation in the Ca/Mg ratio. Thus, Nash & Adey (2017) showed that the calcite deposited in the middle lamella region of *Kvaleya epilaeve* had a lower  $\text{Mg}^{2+}$  content than the adjacent primary cell wall calcite. They hypothesize that calcification and the associated variations in the Ca/Mg ratio is controlled by the organic substrate present. The variation in  $\text{Mg}^{2+}$  content observed around a single cell by Ragazzola *et al.* (2016) is consistent with our hypothesis: that the organic cell wall constituents modify the relative ion activities within the cell wall matrix and thus affect the Ca/Mg ratio of the calcite crystals formed.

There is also a close correlation between growth rate, the degree of calcification and the Mg content of the calcite, which can be clearly seen in the form of growth bands in some of the massive non-geniculate coralline algae such as *Clathromorphum compactum* (Kjellman) Foslie, *C. nereostratum* Lebednik and *Lithothamnion glaciale* Kjellman (Kamenos *et al.* 2008, 2009; Nash *et al.* 2019). When the algae are growing faster (i.e. in summer at higher

temperatures, higher irradiances etc.), the density of calcite in the cell walls is reduced and more Mg is incorporated in the calcite (Kamenos *et al.* 2009; Kamenos & Law 2010; Sletten *et al.* 2017). The greater growth in summer is achieved by the formation of significantly longer cells compared to those formed in winter.

### Impacts of seawater carbonate chemistry and geochemical evidence

Major variability exists within geniculate and non-geniculate types in skeletal density, overall thallus thickness, and whether thallus thickness or segment lengths are determinate or indeterminate in nature (e.g. the indeterminate growth and annual banding in *Clathromorphum* spp vs determinate thallus thickness in most crustose forms). Environmental conditions dictate additional variability in some generalizable cases, as in the example of seasonal changes in growth described above. Seawater carbonate chemistry often affects the density of calcite in the cell walls in addition to the  $Mg^{2+}$  content (Cornwall *et al.* 2021), and therefore is difficult to disentangle from temperature effects, which also affect those parameters (Pauly *et al.* 2015).

Exposure to seawater with low carbonate saturation states has led to changes in the thickness of coralline algal cell wall calcite, with variation between morphotypes (McCoy & Ragazzola 2014) and timescales of exposure (Ragazzola *et al.* 2012, 2013), and thus may be related to environmental controls on thallus growth rather than direct controls of calcification.  $Mg^{2+}$  content declines in around one in three cases where coralline algae have been grown under lower pH seawater that simulates the effects of ocean acidification (Cornwall *et al.* 2022). However, the mechanisms are currently poorly understood at a physiological level. Whether less  $Mg^{2+}$  is incorporated, slower overall calcification rates are the causal mechanism, or whether  $Mg^{2+}$  is preferentially dissolved have all been invoked as potential mechanisms, and more research is needed in this area.

Additionally,  $\delta^{11}B$  declines in most species under low pH, which is important as this tracks the pH of the fluid in which coralline algal calcite is formed (Cornwall *et al.* 2017; Donald *et al.* 2017; Comeau *et al.* 2019a, b). At low pH, the mineralogical organization (mineral lattice positional disorder and full width half maximum, FWHM, *sensu* Bischoff *et al.* 1985; Pauly *et al.* 2015) of coralline algal calcite appears to be more stable than changes in pH within the calcification space (Comeau *et al.* 2018). However, mineralogical responses are variable by species (McCoy & Kamenos 2018) and carbonate chemistry parameters (Cornwall *et al.* 2018), likely reflecting the multiple species-specific processes discussed in the previous sections. Positional disorder is related to, but not solely driven by,  $Mg^{2+}$  or other trace metal content (Bischoff *et al.* 1985; DeCarlo 2018), though both mineralogical features may affect the structural integrity of calcification in coralline algae.

The mechanisms by which low seawater pH relates to the suite of B and Raman spectroscopy proxies are not fully understood, limiting our interpretation of implications for calcification (Cusack *et al.* 2015). For example, inorganic seed experiments with aragonite have shown that this positional disorder is related to the saturation state of the fluid in which the aragonite was formed, which correlates well with

calculations from other independent proxies ( $\delta^{11}B$  and B/Ca; DeCarlo *et al.* 2017; McCulloch *et al.* 2017). However, similar relationships (B/Ca) do not apply to coralline algal calcite, likely due to the high  $Mg^{2+}$  content of their calcite. Calibrating the relationship between positional disorder, other geochemical proxies and different components of seawater carbonate chemistry would allow us to quantify DIC and saturation state in the calcification site. Experimental work that decouples carbonate chemistry parameters points to  $H^+$  and DIC as the most probable drivers of coralline algal calcification, likely occurring via multiple processes described above (Comeau *et al.* 2018). This geochemical evidence collectively informs us that chemistry in the cell wall thermodynamically favours precipitation. If the same proxies that work in aragonite also function in high Mg calcite, they collectively indicate that as well as pH being much higher than seawater, so too is DIC (from B/Ca) and  $\Omega$  (FWHM) at the site of calcification.

In summary, both the chemistry at the calcification site and the impacts of changing seawater carbonate chemistry are very species-specific. Collectively, elevated pH and DIC would favour speciation of DIC towards  $CO_3^{2-}$ , while also allowing faster conversion and ready supply of  $HCO_3^-$  to renew  $CO_3^{2-}$  that is precipitated. Though not yet assessed, it is also very likely that the concentration of  $Ca^{2+}$  would be higher than that of seawater if any direct pumps were involved.  $Mg^{2+}$  has traditionally been seen as an inhibitor of calcification (Morse *et al.* 1997; Evans *et al.* 2019). Therefore, coralline algae must have specific biological control that allows  $Mg^{2+}$  incorporation in the calcification site, as well as probably having controls that elevate both pH and DIC in the same space.

### ANATOMICAL EVIDENCE FOR LOCALIZED, BIOLOGICALLY CONTROLLED DECALCIFICATION

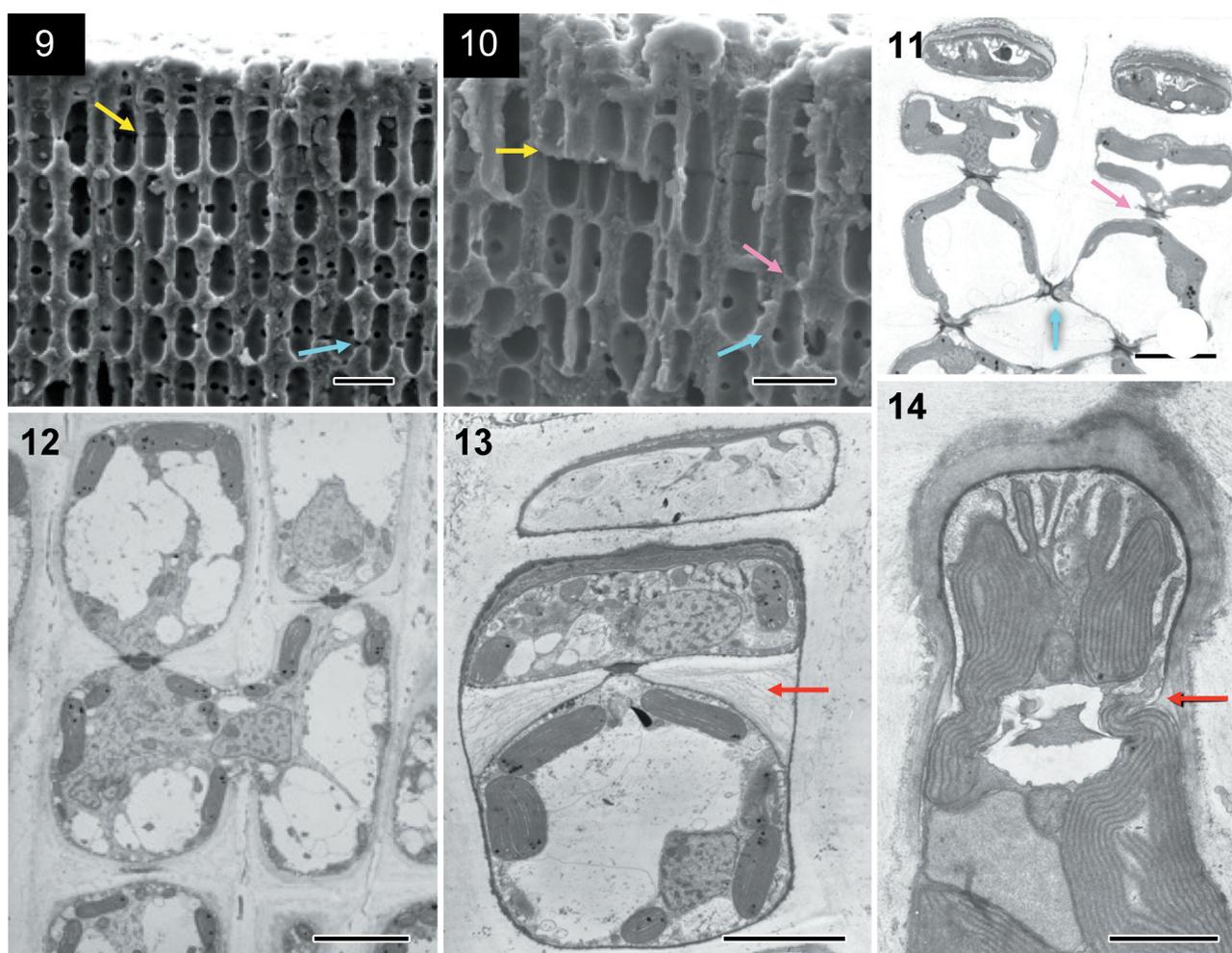
Coralline algal anatomy is complex, and it is important to consider the morphogenetic events which lead to the various cell types when studying calcification. During thallus development, some cells calcify, and later decalcify and calcify again. Some cells, such as the genicular cells, can have both calcified and non-calcified regions. The anatomy of the thallus also defines the pathways for ion fluxes to the sites of calcification. While mechanisms of decalcification are poorly understood, evidence summarized below points to clear biological control of this process. Electron microscopic studies have shown individual  $CaCO_3$  crystals and a low density of crystal aggregations in the cell walls adjacent to epithallial cells and sometimes at the surface of epithallial cells (Borowitzka & Vesik 1978; Pueschel *et al.* 2005). Deeper in the thallus, the cell walls are essentially completely filled with  $CaCO_3$  crystals (Nash *et al.* 2019) and new  $CaCO_3$  formation would be spatially impossible without some prior decalcification.

#### Epithallial cells

The epithallial cell never divides in coralline algae. Instead, meristematic activity resides in the cells that subtend and produce new epithallial cells distally. The meristem cells also generate cells proximally that contribute to growth of thallus thickness. Epithallial cells have a full complement of

organelles when first formed. This includes chloroplasts with abundant thylakoids that indicate that the cells are photosynthetically competent. However, epithallial cells do not deposit starch grains; instead, starch accumulates in cells below the layer of meristem cells, though protein bodies are sometimes formed in epithallial cells and then later mobilized (Giraud & Cabioch 1983; Pueschel 1996). Epithallial cells undergo a programmatic pattern of differentiation, during some stages of which they are clearly highly metabolically active, as indicated by the presence of chloroplasts and the dense cytoplasm. The irregular deposition of abundant organic material on the upper end of the cell forms wall projections, sometimes branching, that penetrate deep into the cell lumen (Borowitzka & Vesik 1978; Bressan *et al.* 1981; Pueschel & Keats 1997; Pueschel *et al.* 2005). As a consequence, the surface area of the distal face of the epithallial cell is greatly enlarged.

New growth can often only occur in crustose coralline algae by first moving or altering existing cells (Figs 9–14). The epithallial cell appears to degrade in a programmatic pattern of senescence eventually. As this occurs, the pit plug shared by the senescent epithallial cell and its subtending cell is sealed off by the secretion of wall material beneath the proximal end of the pit plug (Pueschel *et al.* 1996). After the degenerating epithallial cell is shed, the wall material that was deposited beneath the pit plug becomes a feature of the distal wall of the replacement epithallial cell (Wegeberg & Pueschel 2002). Secondary calcification by individual small, usually needle-like crystals is sometimes visible within dense organic material on the distal wall (Pueschel *et al.* 1996; Wegeberg & Pueschel 2002). The proximal wall of the dead cell may persist long after cellular contents are lost. This process of epithallial cell senescence and regeneration may occur numerous times. The cells may be lost from the thallus surface individually, or shedding epithallial cells may peel off in a sheet (i.e. epithallial sloughing; Adey 1964; Giraud & Cabioch 1976).



**Figs 9–14.** Ultrastructure of thalli of coralline algae. Figures 9, 10 by S. McCoy and C. Pueschel; Figs 11–14 by C. Pueschel.

**Figs 9, 10.** *Lithophyllum impressum*, SEM. Yellow arrows point to a striation of decalcification allowing the expansion of meristem cells. Blue arrows point to secondary pit connections, pink arrow to a primary pit connection. Scale bars = 10  $\mu$ m.

**Fig. 11.** *Amphiroa rigida* J.V. Lamouroux, TEM. Blue arrow points to perithallial cells with secondary pit connections, below initial cells and a single epithallial cell layer. Pink arrow indicates a primary pit connection. Scale bar = 5  $\mu$ m.

**Fig. 12.** *Neopolyporolithon arcticum* (Kjellman) P.W. Gabrielson, S.C. Lindstrom & Hughey [= *Clathromorphum loculosum* (Kjellman) Foslie], TEM, showing cell fusion, binucleate cells. Scale bar = 5  $\mu$ m.

**Fig. 13.** *Corallina officinalis*, TEM, showing an initial cell, a new epithallial cell and a dead epithallial cell; epithallial cells have distal wall ingrowths (red arrow). Scale bar = 5  $\mu$ m.

**Fig. 14.** *Pneophyllum keatsii* Y.M. Chamberlain, TEM, showing an initial cell just beginning to cleave off an epithallial cell distally. Distal wall ingrowths are already present, with lobes of chloroplasts visible between ingrowths. Scale bar = 5  $\mu$ m.

Movement of a newly formed epithallial cell into a position to replace the older, senescent cell requires decalcification of the wall distal to the new cell, as well as calcification of the wall between the meristem cell and new epithallial cell (Wegeberg & Pueschel 2002). It has been proposed that the elaborated plasma membrane associated with the distal wall ingrowths of epithallial cells may have a role in the decalcification of the mineralized wall between the replacement epithallial cell and the thallus surface (Pueschel *et al.* 2005). Although the epithallial filaments maintain a stable length, which can vary by species from one to over a dozen cells, the epithallus is in flux as cells treadmill from their formation by meristem cells to the thallus surface, where the terminal cell of each filament undergoes senescence and shedding (Wegeberg & Pueschel 2002). The crosswalls between epithallial cells are calcified; therefore, the calcified matrix surrounding all the epithallial filaments must undergo a parallel pattern of adding new calcification at the base of the epithallus while losing calcified walls around the cells being shed from the thallus surface. These cells generally are not calcified at the distal (upper) surface; however, in the process of epithallial cell turnover, the calcified proximal wall of a senescing cell becomes the distal wall of its replacement (Wegeberg & Pueschel 2002). In those species with multicellular epithallial filaments (i.e. *Clathromorphum circumscriptum* (Strömfelt) Foslie, *Lithophyllum neoatalayense* T. Masaki), all epithallial cells are calcified. The formation of new epithallial cells necessarily involves localized decalcification of the cell walls and subsequent recalcification (Wegeberg & Pueschel 2002; Pueschel *et al.* 2005).

The increased plasmalemma surface area due to the invaginations allows greater membrane transport activity, an important feature considering that the sides and base of these cells are embedded in  $\text{CaCO}_3$  (Wegeberg & Pueschel 2002), which would greatly limit the diffusion of ions (including nutrients) from the external seawater. Borowitzka & Vesik (1978) pointed out that the structure of these cells is analogous to that of 'transfer cells' in land plants which are specialized for solute transport (Offler *et al.* 2003). In the aquatic angiosperm *Ranunculus*, transfer cells on the lower leaf surface have been shown to be the location of light-activated plasmalemma  $\text{H}^+$ -ATPase activity whose proposed function is to acidify the apoplast (cell wall) and thus enhance the production of  $\text{CO}_2$  from  $\text{HCO}_3^-$  (Rascio *et al.* 1999). Ligrone *et al.* (2011) have also demonstrated a high level of light-dependent ATPase activity in the wall ingrowths of the transfer cells in the aquatic angiosperm *Elodea*. A similar function has been shown for the reticulate plasmalemma invaginations known as charasomes which occur in characean algae (Ray *et al.* 2003). The acidification produced by the  $\text{H}^+$ -ATPase activity could lead to localized decalcification.

The distinctive features of epithallial cell differentiation and senescence have given rise to several hypotheses for the function of these cells: (1) wall ingrowths increase membrane surface area, enhancing nutrient uptake or ion fluxes, (2) the epithallus provides mechanical protection of the meristem cells layer from grazers, or (3) turnover of the epithallus provides a means of removing epiphytes (Keats *et al.* 1997). These various proposals are not mutually exclusive, but only

the proposals involving physiological processes address the highly unusual morphology of epithallial cells.

### Deep-layer sloughing

Deep-layer sloughing is a process with much greater anatomical consequences than the epithallial cell sloughing described above. For example, this has been recorded in *Spongites yendoi* (Foslie) Y.M. Chamberlain, where as much as half the thallus thickness was shed, including the entire epithallus, conceptacles, the layer of meristem cells, and many perithallial cells (Keats *et al.* 1993). Ultrastructural study of *Lithophyllum neoatalayense* showed that the typical mechanism of shedding of individual epithallial cells was operational, but thalli also underwent deep-layer shedding (Pueschel & Keats 1997). With the dead cells held in place within the intact carbonate skeleton, the uppermost living cells deposited wall material that sealed off primary and secondary pit plugs that once connected them to the now moribund cells. The development of a layer of mature epithallial cells with typical distal wall projections and thick organic wall layer preceded the sloughing of the still-mineralized dead tissue (Pueschel & Keats 1997). Regardless of the cue for the initiation of this process, which may arise from ecophysiological cues (Keats *et al.* 1997), this mechanism further supports localized control of both calcification and decalcification by the alga.

### Pit connections and cell fusions

Intercellular connections provided by cell fusions and both primary and secondary pit connections are important when considering the transport of nutrients and photosynthate between cells. Diffusion of nutrients and DIC from the thallus surface past the epithallial cells to cells below must be more restricted in the coralline algae than in other rhodophytes due to cell wall calcification, whereby DIC uptake for photosynthesis and nutrient uptake for cell growth is more limited. However, cells of the intercalary meristem and perithallus are clearly photosynthetic, as evidenced by the presence of many chloroplasts and starch grains. Whether ion transport occurs through the pit connections has been hotly debated (e.g. Wetherbee 1979; Pueschel 1980), and most evidence for transport through pit connections has been circumstantial. For example, no alternative transport route can be found during the development of the gametophyte and in the nutrition of non-photosynthetic parasitic algae (Wetherbee & Quirk 1982; Kamiya & Kawai 2002). Experimental evidence for transport of solutes through pit connections has been extremely limited and sometimes equivocal. Transport of  $^{14}\text{C}$  labelled compounds in *Delesseria sanguinea* (Hudson) J. V. Lamouroux (Hartmann & Eschrich 1969; Pearse 1972; LaVelle 1979) and *Gracilaria cornea* J. Agardh (Gonen *et al.* 1996) probably occurs via the pit connections. A recent study has demonstrated cell-to-cell movement of fluorescent tracers through pit connections (Kim *et al.* 2022). Therefore, it is highly likely that pit connections play important roles in transporting dissolved substances and metabolic products between cells in corallines. This is an important area that requires much further research.

As in the vast majority of multicellular red algae, the lineage of cells in coralline algae is, in principle, traceable from the apical cells of the mature thallus to the first-formed cells of the sporeling by the presence of pit connections shared by kindred cells. However, two types of secondary processes, cell fusion and secondary pit connection formation, can form connections between non-kindred vegetative cells. One or both of these processes occur in particular subgroups of coralline algae. Both processes require decalcification of existing cell walls to establish the intercellular connections, and a single cell may share fusions (Pueschel 2021b) or secondary pit connections (Pueschel 2021c) with several adjacent cells.

## Genicula

Coralline algal thalli consist of laterally coherent branching filaments that grow by cell division and enlargement near the top of the filaments. This fundamental construction is responsible for producing a wide variety of forms, the most common being crusts growing on the substratum and expanding at their margins (Cabioch 1988). Only in a few endophytic parasites does the thallus consist of uniseriate filaments, and even these give rise to anatomically complex conceptacles that house gametes or spores. Crustose forms have repeatedly given rise evolutionarily to erect axes that may be highly branched with independently evolved non-calcified joints (genicula) (Janot & Martone 2016).

Genicula are uncalcified joints that allow flexing under the mechanical forces of wave action, as opposed to non-geniculate forms that lack this feature. Genicula have evolved independently many times (Bailey & Chapman 1998; Bailey 1999), and some species have also lost the geniculate morphology, e.g. in *Bossiella* and *Crusticorallina* (Hind *et al.* 2016, 2018). Comparing genicular anatomy in geniculate groups reveals profoundly different developmental patterns that reflect their independent origins. The greatest amount of anatomical specialization associated with genicula is found in Corallinoideae. The role of decalcification in geniculum formation varies considerably: little or none in Metagoniolithoideae, a modest amount in Corallinoideae, and the greatest amount in Lithophylloideae (Johansen 1981; Janot & Martone 2018).

Genicula of *Amphiroa* (Lithophylloideae) are created by localized decalcification of the anatomically differentiated axis, so the cellular patterns of genicula and intergenicula are nearly identical and both regions have secondary pit connections (Johansen 1969). Decalcification begins at the midpoint of the axis and progresses towards the surface, often irregularly, so that all cells of some tiers of medullary cells are completely decalcified, but other tiers have both calcified and uncalcified cells. After decalcification, additional secretion thickens the walls of each genicular cell, medullary and cortical alike, strengthening the now pliable joints. Formation of genicula in *Lithothrix* is different. Tiers of short and long medullary cells alternate with one another. The short medullary cells are fully calcified and stay short; these constitute the intergenicula. Cells of the alternating tiers elongate locally; the distal portion remains embedded in mineral and elongation without calcification and

deposition of additional cell wall material occurs in the proximal portion of the cells, with the proximal portion of this tier of cells collectively serving as a geniculum (Borowitzka & Vesik 1979). Downward growth of the cortex partially covers the uncalcified genicula, giving the outward appearance of a much larger intergeniculum.

The formation of genicula in Metagoniolithoideae is unique in that the apical genicula cells are natively uncalcified and covered by a thick polysaccharide ‘cap’ (Ducker 1979). All types of cells, medullary, cortical and epithallial, are part of the genicula and cell fusions occur within the genicula. Furthermore, the central cells of intergenicula occur in tiers and this tiered construction is not present in the genicula. Another distinctive feature of this group is that branches can arise from genicula (Ducker 1979).

Genicula of the Corallinoideae consist of a single tier of highly elongated medullary cells, up to 0.5 mm long. Only the ends of the medullary cells are calcified. As the medullary cells elongate, the overlying cortex ruptures and some decalcification is associated with this process. Genicula of *Calliarthron* have been particularly well studied. The walls of its genicular cells after full elongation are reinforced by as much as three-fold thickening and this production of additional wall layers after elongation resembles secondary wall deposition in embryophytes (Martone 2007). The cellulose microfibrils do not exhibit a particular orientation within the plane of the wall layers, an arrangement that adds strength while preserving flexibility; these features are favourable in a high-drag environment (Martone *et al.* 2019). An even more distinctive feature of coralline genicular cell walls is the presence of lignin, a wall strengthening material otherwise found only in land plants (Martone *et al.* 2009; Xue *et al.* 2022).

## Trichocytes

Some corallines form unicellular hairs, commonly called trichocytes. Although the base of the trichocyte cell is partially surrounded by calcified wall, the shaft is uncalcified. When the size and contents of former trichocyte bases differ from those of normal vegetative cells, visible evidence of past trichocytes can be seen in thallus anatomy. This is especially common in non-geniculate corallines and these anatomical features gave rise to such descriptive terms as megacell and heterocysts. Cabioch (1971) described three types of trichocytes, and Townsend & Adey (1990) reviewed the diverse terminology that has been applied. However, the variations in trichocyte development may be largely the consequence of differences in anatomical context, such as where they are borne, and thallus morphology, rather than in the trichocyte dynamics themselves.

An ultrastructural study of *Jania adhaerens* J.V. Lamouroux revealed that the primary trichocyte is subapical and subtends a small cell in the epithallial layer, termed a crown cell (Judson & Pueschel 2002). The developing hair passes through the lumen of the crown cell and emerges from the thallus surface. After the primary trichocyte dies, a subtending meristem cell divides to generate a new trichocyte. Remnants of old walls and discarded pit plugs provided evidence that the regeneration process occurred at least 20

times from a single meristem cell in this alga (Judson & Pueschel 2002) and at least 17 times in *Amphiroa fragilissima* (Linnaeus) J.V. Lamouroux (Pueschel 2021a). When no longer called upon to produce trichocytes, the meristem cell may become a more typical vegetative cell, although, as stated above, sometimes distinguishable by its unusual size or numerous wall layers.

Studies have shown that trichocytes are produced in seasonal conditions of elevated temperature and illumination (e.g. Chamberlain 1985), which suggests a role of hairs in reducing light intensity. However, these environmental conditions correlate with, or may serve as cues of, seasonally lower nutrient availability. A role for trichocytes in enhancing nutrient uptake by increasing the thallus surface area directly exposed to the seawater also has been proposed (Basso *et al.* 2014). However, trichocytes also increase the width of the diffusion boundary layer and thus the concentrations of ions at the thallus surface, which could affect both calcification and photosynthesis. Thus, epithallial growth, epithallial or deep layer sloughing, and the development of genicula and trichocytes all point to fine spatial and temporal control of both calcification and decalcification within the coralline algal thallus.

## CALCIFICATION MECHANISM – SUMMARY

We have examined in detail various factors that affect the rate and nature of calcification. Here, we attempt to distil the key aspects of the process of calcification in simplified form. Morpho-anatomical evidence of localized decalcification and recalcification during development provides strong arguments for the importance of biological control of calcification in this group. We summarize the possible mechanisms of biological control below.

**Calcification occurs within the cell wall.** Calcification occurs in the vicinity of meristematic activity in the outer layers of the thallus together with cell wall formation. Calcification is a rapid process and its early stages have only been distinguished in juvenile crusts formed following tetraspore germination. The first signs of calcification appear in the region where two cells touch (i.e. the middle lamella region) after this region is sufficiently isolated from the external seawater. This initial calcification is soon followed by  $\text{CaCO}_3$  deposition within the cell wall.

**Cell wall composition is important.**  $\text{CaCO}_3$  nucleation and crystal formation requires specific water-soluble polysaccharide cell wall components that make up the hydrogel structure of the wall. These organic components also determine the  $\text{CaCO}_3$  crystal isomorph deposited. Although there is no existing evidence, non-water-soluble cell wall components such as cellulose may also have a structural function and may affect the crystal orientation. Based on TEM and SEM observations, it appears that calcification occurs soon after the deposition of the organic cell wall components. In species with thick cell walls, such as *Porolithon onkodes* and *Lithothamnion glaciale*, this results in a banded crystal structure observed with layered variations in the  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Sr}^{2+}$  content of the calcite around the cells.

**Links to photosynthesis.** As elaborated, photosynthesis plays a major role in calcification by affecting the pH surrounding the cells, by providing the building blocks for the cell wall polysaccharides and proteins, and by providing the energy for cell wall formation and active transport of ions. Carbonic anhydrases are likely to play important roles, both in conjunction with the CCM increasing  $\text{CO}_2$  provision for photosynthesis and in regulating the speciation of inorganic carbon in the cell walls. Inorganic carbon is supplied to the calcification sites in the cell walls by diffusion from the external seawater as well as by respiratory  $\text{CO}_2$ . Similarly,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  diffuse to the calcification site and  $\text{Ca}^{2+}$  transporters at the plasmalemma are likely. The pH at the site of calcification is more elevated than the bulk seawater and this is due to photosynthetic  $\text{CO}_2$  uptake, the very likely active transport of ions, and the long diffusion distance from the external seawater to the calcification site that reduces diffusive exchange. There is also some indirect evidence of  $\text{H}^+$  transport at the plasmalemma.

**Biological control.** Localized decalcification occurs in multiple features of coralline algal anatomy, pointing to developmental cues and control of that process. Further, geochemical evidence points to control of  $\Omega$  at the calcification (and likely decalcification) site.

## FUTURE DIRECTIONS

The greatest uncertainty in our model revolves around the degree to which coralline algae control or regulate conditions at the sites of calcification, and the molecular mechanisms by which they predominantly do so. While we describe candidate mechanisms of control via cell wall polysaccharide composition and hypothetical anion exchange transport mechanisms, the following themes warrant targeted research:

- (1) Additional experimental description of coralline algal regulation of the calcification site, both of  $\Omega$  and organic matrix deposition.
- (2) Environmental and taxonomic drivers of variability in cell wall polysaccharide composition.
- (3) Abiotic experimentation of calcification nucleation by biogenic polysaccharides.
- (4) Description of the activities of  $\text{H}^+$ -ATPase,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{PO}_4^{3-}$ ,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  transport within the algal thallus.

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