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Biomineralization in perforate Foraminifera

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Abstract

In this paper, we review the current understanding of biomineralization in Rotaliid foraminifera. Ideas on the mechanisms responsible for the flux of Ca^{2+} and inorganic carbon from seawater into the test were originally based on light and electron microscopic observations of calcifying foraminifera. From the 1980's onward, tracer experiments, fluorescent microscopy and high-resolution test geochemical analysis have added to existing calcification models. Despite recent insights, no general consensus on the physiological basis of foraminiferal biomineralization exists. Current models include seawater vacuolization, transmembrane ion transport, involvement of organic matrices and/or pH regulation, although the magnitude of these controls remain to be quantified. Disagreement between currently available models may be caused by use of different foraminiferal species as subject for biomineralization experiments and/ or lack of a more systematic approach to study (dis)similarities between taxa. In order to understand foraminiferal controls on element incorporation and isotope fractionation, and thereby improve the value of foraminifera as paleoceanographic proxies, it is necessary to identify key processes in foraminiferal biomineralization and formulate hypotheses regarding the involved physiological pathways to provide directions for future research.

1. Introduction

All foraminifera make tests although a number of different materials are used in their construction. The 'naked' foraminifera produce tests from organic matter, agglutinated foraminifera use sediment grains as building blocks and calcifying foraminifera use constituents dissolved in seawater to secrete calcium carbonate. Formation of $CaCO_3$ tests plays a significant role in ocean biogeochemical cycles and, more importantly, the fossil remains of calcifying foraminifera are widely used to reconstruct past ocean chemistry and

environmental conditions. Elemental and isotopic composition of foraminiferal calcite depends on a variety of environmental parameters such as temperature, salinity, pH and ion concentration (McCrea et al., 1950; Epstein et al., 1951; Boyle, 1981; Nürnberg et al., 1996). These physical and chemical variations are the foundation for developing geochemical proxies that quantify environmental changes through time (see Wefer et al., 1999; Zeebe et al., 2008; Katz et al., 2010 for reviews). For example, the magnesium concentration in foraminiferal calcite ($Mg/Ca_{calcite}$) varies primarily with seawater temperature (Nürnberg et al., 1996; Lea et al., 1999; Hönisch et al., 2013) and can be used to reconstruct past sea surface (Hastings et al., 1998; Lea et al., 2000) and deep-water (Lear et al., 2000) temperatures. Reliable application of these proxies requires calibration over a wide range of environmental conditions as well as a thorough understanding of the physiological parameters influencing test formation.

Studies calibrating foraminiferal test composition based on core-tops and controlled growth experiments show that both the chemical and isotopic compositions of these tests are not in equilibrium as defined by inorganic precipitation experiments (Lowenstam and Weiner, 1989; Dove et al., 2003). Microenvironmental controls related to foraminifera physiology have been implicated to explain disequilibrium fractionation in test chemistry (Figure 1). Most foraminiferal species incorporate Mg with one to two orders of magnitude lower concentration compared to non-biologically precipitated calcium carbonate (Bentov and Erez, 2006; Katz, 1973; Bender et al., 1975). The concentration of barium, on the other hand, is ~10 times higher in foraminiferal calcite (Lea and Boyle, 1991; Lea and Spero, 1992) compared to inorganic precipitation results (Pingitore and Eastman, 1984). Additionally, elemental concentrations between foraminiferal species can vary by several orders of magnitude (up to two orders of magnitude for Mg; Bentov and Erez, 2006). The biological controls on element

incorporation and isotope fractionation that cause these offsets are often summarized as 'the vital effect' (Urey et al., 1951; Weiner and Dove, 2003).

Figure 1: Minor and trace element composition of foraminiferal (left) and inorganically precipitated (right) calcite precipitated from seawater (middle). Concentrations are qualitative as they differ between foraminiferal species and depend on environmental conditions. Precipitation rates, ionic strength of the medium and presence of organic compounds are also known to affect partition coefficients. All values are in parts per million (ppm) and based on data in Kitano et al. (1975), Ishikawa and Ichikuni (1984), Rimstidt et al. (1998), Marriott et al. (2004), Morse et al. (2007), Tang et al. (2012) He et al. (2013) for inorganically precipitated calcium carbonates, and Lea and Boyle (1991), Rickaby and Elderfield (1999), Segev and Erez (2006), Terakado et al. (2010), Allen et al. (2011) for foraminiferal calcite composition.

Vital effects comprise 1) chemical alterations of the foraminifers' microenvironment due to physiological processes, 2) cellular controls on the composition of the fluid from which calcite is precipitated and 3) controls on nucleation and crystal growth (e.g. by presence of organic templates). Foraminiferal respiration and/or photosynthesis by symbiotic algae alter the foraminiferal microenvironment chemistry and thereby the conditions in which foraminiferal tests mineralize. Because habitat depth differences in the water column (planktonic species) or migration in the sediment and attachment to plant leaves (benthic species) also modify the calcification environment, these ecological factors are sometimes regarded as being part of the vital effect as well (e.g. Schmiedl and Mackensen, 2006). Ecology-based variability in element incorporation, however, can be accounted for when habitat preferences of foraminiferal species are known. Hence, the term "vital effects" should

100 only be used when discussing foraminiferal cellular processes that alter the chemistry of the
101 microenvironment during test mineralization.
102 To understand the physiological impact on element incorporation and isotope fractionation,
103 the (intra)cellular mechanisms which foraminifera employ to precipitate test $CaCO_3$ must be
104 identified. Biogeochemical mechanisms are involved in regulating concentrations of ions
105 and/or their activity at the site of calcification. Calcification from seawater can be promoted
106 using different mechanisms. Hence, multiple mechanisms have been proposed to explain test
107 calcification, including endocytosis of seawater, transmembrane ion transporters, ion-specific
108 organic templates, production of a privileged space and mitochondrial activity (Spero, 1988;
109 Erez, 2003; Bentov and Erez, 2006; Bentov et al., 2009).
110 A process-based framework for both inorganic and organismal control of foraminiferal test
111 formation is crucial for the development, calibration and application of geochemical proxies
112 in the geological record. At the same time, a mechanistic understanding of foraminiferal
113 biomineralization will also permit researchers to better interpret data from the fossil record as
114 well as predicting the response of foraminiferal calcification to future environmental changes
115 such as ongoing ocean acidification. Most of the initial observations of chamber formation
116 and calcification in planktonic foraminifera were published during the early period of
117 planktonic foraminifera culturing (e.g. Bé et al., 1977). Highlights of those observations can
118 be found summarized in the seminal text on "Modern Planktonic Foraminifera" (Hemleben et
119 al., 1989). More recently, studying living specimens under controlled conditions (e.g. Kitazato
120 and Bernard, 2014) has further propelled our understanding of foraminiferal growth,
121 reproduction and calcification.
122 Recent hypotheses on foraminiferal biomineralization are based mainly on experiments with
123 benthic species and although these ideas have to be tested for planktonic species, we will also
124 include the latter group in our discussion. Although a general model for foraminiferal

biomineralization is still lacking, and it is not yet clear that a single model fits all groups of foraminifera, details on the underlying mechanisms in different species have accumulated and are described here in the context of previously published biomineralization models.

2. Ions for calcification

2.1 Seawater as the direct source for Ca^{2+} and DIC

Foraminifera calcify by creating a microenvironment supersaturated with respect to $CaCO_3$, while overcoming inhibition by crystallization inhibitors such as Mg^{2+}. Hence, calcification requires a tight control on the concentration and/or ion activity at the site of calcification, commonly referred to as the "delimited" space (Erez, 2003) or "privileged" space. Elevated $[Ca^{2+}]$, $[CO_3^{2-}]$ and/or their ion activities have to be actively maintained in order for calcification to proceed. Simultaneously, the concentrations of crystal growth inhibitors have to be lowered even further. Although CO_3^{2-} needed for calcification may be partially derived from respired CO_2 (Erez, 1978; Grossman, 1987; Ter Kuile and Erez, 1991; Hemleben and Bijma, 1994; Bijma et al., 1999), the majority of the carbon and the Ca^{2+} needed for test formation must be derived from the seawater environment.

Calcification requires equal amounts of Ca^{2+} and CO_3^{2-}. Because seawater Ca^{2+} concentrations are approximately 5 times higher than that of DIC and often >50 times higher than that of CO_3^{2}, foraminifera have to spend more time and/or energy in taking up and concentrating DIC than they have to do for Ca^{2+}. A foraminifer needs to process several times the seawater equivalent of its own cell volume in order to acquire enough Ca^{2+} and inorganic carbon to calcify a new chamber. Although the exact amount needed depends on shape, size and the thickness of the chamber wall (e.g. Brummer et al., 1987), juveniles of some species need 50-100 times their own cell volume to extract the Ca^{2+} required to produce one new chamber (De

Nooijer et al., 2009b). Because seawater [CO_3^{2-}] is significantly lower than [Ca^{2+}], these individuals need the equivalent of ~3,000 times their own volume in order to take up the necessary [CO_3^{2-}] if this anion is used exclusively. However, observations of high pH at the site of calcification (Erez, 2003; De Nooijer et al., 2009a; Bentov et al., 2009) as well as oxygen isotope data from laboratory experiments (Spero et al., 1997; Zeebe, 1999) suggest that foraminifera can convert CO_2 and/or HCO_3^- into the CO_3^{2-} needed for calcification. Evidence that foraminifera concentrate inorganic carbon is also provided by experiments using ^{14}C tracer incorporation kinetics into the skeleton of perforate species (Ter Kuile and Erez 1987, 1988, Ter Kuile et al 1989b). A carbon concentrating mechanism would reduce the volume of seawater necessary for calcification by 50-90% (De Nooijer et al., 2009b). To concentrate the ions needed for calcification, foraminifera must either extract Ca^{2+} and dissolved inorganic carbon (CO_2, HCO_3^- and CO_3^{2-}, or DIC) or take up seawater and subsequently reduce the concentrations and/or activities of all other ions relative to Ca^{2+} and DIC (Figure 2). Removal of protons from (endocytosed) seawater is also a prominent feature in recently developed calcification mechanisms, but will be discussed in a separate section (2.2). In case of the second option, spontaneous nucleation of $CaCO_3$ crystals may be prevented by separation of Ca^{2+} and DIC into different vacuole groups.

Figure 2: Two different mechanisms to concentrate Ca^{2+} and DIC from seawater for calcification: a) Calcium- and bicarbonate-ions are specifically taken up from seawater, or b) the other ions are selectively removed, thereby increasing Ca and DIC concentrations.

Both processes transport ions either directly to the site of calcification or temporarily store these ions. In the case of uptake into some benthic foraminifers, Ca^{2+} and/ or DIC are thought to be present in so-called 'intracellular reservoirs' (also known as ‘pools’; Ter Kuile and Erez,

1988; Erez, 2003). These reservoirs may be seen as temporal storage compartments with high concentrations of ions that are either emptied upon calcification or provide a dynamic cycling of Ca^{2+} and DIC through the cell that is gradually used for calcification. Without an intracellular reservoir, Ca^{2+} and DIC could also be directly transported to the privileged space during calcification (Erez, 2003; Bentov and Erez, 2006). The relative importance of intracellular reservoirs versus direct transport among benthic and planktonic species remains a subject of debate and active research.

2.2 Internal reservoirs

Internal reservoirs may be important for foraminiferal calcification in certain groups. Conceptually speaking, one can envision Ca^{2+} or DIC being derived from internal reservoirs. With seawater as the basis for calcification, carbon reservoirs will have to be approximately 5 times larger than those for Ca^{2+} or have a 5 times faster turnover rate. Evidence suggests that different foraminifer groups employ different strategies. For instance, a time-lag has been observed between uptake and incorporation of labelled inorganic carbon in the large benthic foraminifera *Amphistegina lobifera* suggesting inorganic carbon may be stored in an internal reservoir (Ter Kuile and Erez, 1987; 1988; Ter Kuile and Erez, 1991). In pulse-chase experiments it was observed that ^{14}C was incorporated into the calcite during the chase period in ^{14}C free seawater, implying a large internal reservoir of DIC in the benthic *Amphistegina lobifera* but not in the milliolid *Amphisorus hemprichii* (Ter Kuile et al 1989b). Isotope labelling experiments with the planktonic foraminifer *G. sacculifer* and a number of benthic species using both ^{14}C and ^{45}Ca show that proportionally more labelled ^{45}Ca is incorporated into the shell compared to labelled ^{14}C (Erez, 1978; 1983). For the planktonic species *Orbulina universa* and *Globigerina bulloides*, on the other hand, Bijma et al. (1999) showed that the contribution from an internal carbon pool is insignificant in these species.

To determine whether planktonic foraminifera have an internal Ca-reservoir, Anderson and Faber (1984) grew *G. sacculifer* in artificial seawater spiked with ^{45}Ca. They showed that calcite formed during the first 24 hours contains significantly less ^{45}Ca than that produced in the second 24 hours. These data argue for the existence of an unlabeled intracellular Ca-reservoir that was filled prior to the introduction of the isotopic spike. Using pulse-chase experiments with both a 'hot' incubation period (10-15 days) and 'cold' chase period (10-20 days), Erez (2003) traced the uptake of ^{45}Ca over time in the benthic species *Amphistegina lobifera*, showing that as much as 75% of the Ca^{2+} used during chamber calcification resided in an intracellular reservoir. ^{48}Ca uptake data from experiments using *Orbulina universa*, supported the existence of a Ca-reservoir in a planktonic species, but demonstrated that it was completely flushed of labelled Ca^{2+} within the initial 6 hours of chamber formation and thickening (Lea et al., 1995). These latter observations could indicate that *O. universa* utilizes a small Ca^{2+} reservoir to assist with the initial chamber formation, but that much of the remaining chamber Ca^{2+} is derived from seawater without passing through an internal storage reservoir.

Toyofuku et al. (2008) reported formation of (incomplete) chambers in the benthic *Ammonia beccarii* maintained in seawater devoid of Ca^{2+}. These data clearly support the existence of a Ca^{2+}-reservoir of finite volume in benthic species. If Ca^{2+} and other divalent cations that co-precipitate in the $CaCO_3$ shell are derived from the same internal reservoir, one would expect cation concentrations to reflect Rayleigh fractionation if the reservoir is a closed system. Such a system has been used to partly explain minor and trace element distributions in foraminiferal calcite (Elderfield et al., 1996). However, a model using Rayleigh fractionation relies on a number of assumptions about the internal reservoir regarding its size and initial composition as well as refreshment rate and chamber calcification rate. These unknowns highlight the need to better constrain the size and extent of these reservoirs.

To maintain an intracellular reservoir, a foraminifer needs to sustain a high cation flux rate by continuously vacuolizing, endocytosing and exocytosing large volumes of seawater. Tracing endo- and exocytosis in foraminifera is challenging and has yielded contrasting results. For instance, Bentov et al. (2009) showed that in *Amphistegina lobifera*, seawater is taken up in vacuoles that are subsequently transported to the site of calcification. This implies that seawater, internally modified or not, is directly involved in calcification. De Nooijer et al. (2009b) on the other hand, showed that endocytosis and subsequent exocytosis of seawater in *Ammonia tepida* are not directly related to chamber formation.

2.3 Direct uptake of ions

The ions needed for calcification may be derived from seawater during calcification without storage in an intracellular reservoir (Figure 3). A number of calcification models explicitly or implicitly assume that the ions for calcification are passively transported to the site of calcification through diffusion from the surrounding medium (Wolf-Gladrow et al., 1999; Zeebe et al., 1999). These models are able to explain the impact of photosynthetic symbionts on inorganic carbon chemistry in the vicinity of the foraminifer. Changes in pH and [DIC] due to photosynthesis affect the isotopic composition of the available carbonate (Wolf-Gladrow et al., 1999). However, diffusion of ions to the site of calcification without at least one additional mitigating mechanism, cannot account for the difference between seawater metal composition and Me/Ca ratios in foraminiferal calcite (Figure 1 and references in its caption).

Figure 3: Examples of possible involvement of internal reservoirs versus externally derived ions for calcification. A: Ca^{2+} and DIC are derived from internal reservoirs. B: Ca^{2+} and DIC are transported to the site of calcification without uptake and storage into reservoirs. C: DIC

is taken up directly and Ca^{2+} comes from an internal reservoir. D: Ca^{2+} is taken up during chamber formation and DIC is derived from an intracellular reservoir.

Ca^{2+} and DIC may be actively transported (through transmembrane pumps and/ or channels) to the site of calcification. Although such transport mechanisms are not yet identified in planktonic foraminifera, a number of studies support the existence of this mechanism in benthic species. Using radioactive labeling, Angell (1979) showed that the ions for calcification are taken up *during* chamber formation in the benthic species *Rosalina floridana*. Although this observation does not prove the absence of an internal reservoir *per se*, this observation reduces the turnover rate and/or size of such a reservoir considerably. Similarly, Lea et al. (1995) showed that the intracellular Ca-reservoir in the planktonic foraminifer *O. universa* is very small and/or has a fast turnover rate and does not significantly contribute to the total amount of Ca^{2+} during shell thickening. Results from the benthic *Ammonia* sp. show that intracellular vesicles containing elevated concentrations of Ca^{2+} are involved in chamber formation (Toyofuku et al., 2008), but that their amount within the cell is not sufficient for the production of a new chamber (De Nooijer et al., 2009b). Together, these studies suggest that the majority of the Ca^{2+} utilized for shell calcification is not stored in intracellular reservoirs prior to chamber formation in the species studied. If the internal reservoir refills after chamber formation within a relatively short period of time, it is critical that seawater labeling experiments should start directly after a chamber formation event to avoid underestimation of the true reservoir size. Studies addressing the issue of an intracellular reservoir are summarized in Table 1.

Table 1: Studies discussing internal reservoirs in perforate foraminifera.

3. Intracellular transport

3.1 Transmembrane ion transport

Due to the hydrophobic inner layer of cell membranes, molecules cannot freely move into or out of the cell's interior. Although the majority of ions and molecules diffuse across cell membranes, diffusion constants vary greatly. Small, uncharged molecules (CO_2, O_2, NO) diffuse easily down a concentration gradient whereas large molecules and ions require specialized transmembrane proteins to facilitate or energize membrane transport (Higgins, 1992). These transporter proteins can be divided into channels, carriers and pumps (Figure 4). Carrier proteins undergo substrate binding and transport. They show typical substrate affinities and follow Michaelis-Menten kinetics. Carrier transport is even effective against concentration gradients if a cosubstrate with a respective concentration gradient or charge is involved (secondary active transport). Pumps directly generate this energy for uphill transport from their ATPase activity. Transmembrane channels simply allow facilitated diffusion along electrochemical gradients by creating a selective pore through the cell membrane. For the uptake of inorganic carbon by foraminifera during calcification, a strong pH gradient (high inside; De Nooijer et al., 2009a; Bentov et al., 2009; low outside; Glas et al., 2012) may promote the influx of CO_2 and thus circumvent the need for specialized transmembrane proteins.

Figure 4: selective ion transporters. Ion pumps (left and middle) undergo structural changes that allow passage of ions from and to the binding sites. The example shown here is a simplified Na^+/K^+ exchanger that has specifically binds to Na-ions (blue squares) when in the first configurational state (left). After the structural change, affinity of the Na-binding sites decreases so that the Na-ions are released (middle). At the same time, K-ions (yellow circles)

bind to their binding sites after which the pump returns to state one and releases the K^+ to the cytosol. Ion channels (draw after the KcsA K^+ channel; right) consist usually of a narrow pore allowing certain ions to pass a cell membrane down the electro-chemical gradient. Another feature of some pumps and channels is the relatively large cavity that is created by the transmembrane protein-complex (here present in the cytosol-side of the channel). This can greatly reduce the distance that the ions have to be transported. The type of Ca^{2+}-transporters that are used by foraminifera are unknown, but determining their molecular structure is necessary to 1) know the extent of de-hydration during transport, 2) determine the rate of ion transport and 3) explain the selectivity for Ca^{2+}/ against other ions (e.g. Mg^{2+}) and their fractionation (e.g. Gussone et al., 2003).

3.2 Ca^{2+} transport in foraminifera

In foraminifera, most attention has been directed at ion transporters that might be responsible for the low Mg/Ca at the site of calcification. Logically, this may involve Mg^{2+}-transporters and/ or Ca^{2+} transporters. Because Ca^{2+} acts as a secondary messenger in most eukaryotic cells, cytosolic Ca^{2+} is kept low (< 1μM) by active removal out of the cell or into cytosolic compartments (ER, mitochondria). This makes Ca^{2+}-transporters one of the most ubiquitous and well-studied transmembrane ion transporters. From a variety of cell types, Ca^{2+}-ATPases, Ca^{2+}/H^+ and Ca^{2+}/Na^+ antiporters (e.g. Gonçalves et al. 1998) and Ca^{2+}/phosphate co-transporters (Ambudkar et al., 1984) have been described. Depending on the transporter's structure, ions may pass the membrane either with or without their hydration sphere (Gouaux and MacKinnon, 2005), although (partial) dehydration increases the selectivity greatly (see also Gussone et al., 2003).

The specificity of the transmembrane Ca-transporters varies greatly. For some Ca^{2+}/H^+-antiporters it has been reported that other cations with a small ionic radius (e.g. Zn^{2+}) can be

transported in a similar way as Ca^{2+} is transported (Gonçalves et al., 1999). For the same antiporter, the larger Ba^{2+} and Cs^{+} do not substitute for Ca^{2+}. An ion with intermediate size, Sr^{2+} (1.13 Å, compared to 0.99 Å for Ca^{2+}), appears to block the antiport and prevents transport of Ca^{2+} through the membrane. Studies concerning specificity for Ca^{2+} over Mg^{2+} are scarce, but some Ca-ATPases have been reported to have a 10^3-10^5 higher affinity for Ca^{2+} than for Mg^{2+} (Drake et al., 1996; Xiang et al., 2007).

In corals, calcium uptake is directly related to proton pumping (McConnaughey and Whelan, 1997; Sinclair and Risk, 2006). The efflux of H^{+} during calcification (Glas et al., 2012) may therefore help to constrain estimates of calcium pumping rates during calcification. Carbon dioxide uptake and proton efflux are also directly related in cyanobacteria (Ogawa and Kaplan, 1987). Ter Kuile et al. (1989b) suggested that Ca^{2+} is taken up by Ca^{2+}-ATPase and this mechanism was subsequently used by Zeebe and Sanyal (2002) and Zeebe et al. (2008) to show that H^{+} removal is far more energy-efficient than Mg^{2+}-removal during calcification. Such a mechanism would be consistent with a coupling of ion transporters (e.g. Ca^{2+} and H^{+}) during foraminifera calcification.

The amount of Ca^{2+} transported across a membrane depends on 1) transporter density in the membrane, 2) affinity for Ca^{2+} of the transporter and 3) the capacity of the transporter. For example, the Na^{+}/Ca^{2+} exchanger has a low affinity, but high capacity, resulting in transport of up to 5,000 ions per second (Carafoli et al., 2001). Such a transporter is useful when Ca^{2+} is present in high concentrations (e.g. as in seawater) and supply or removal rates of Ca^{2+} have to be high. Cell membrane calcium pumps, on the other hand have a high affinity, but low capacity, making it particularly suitable for transporting Ca^{2+} out of a medium or compartment with a low $[Ca^{2+}]$ (Wang et al., 1992). Finally, transport rates can be affected by the presence of inhibitors, high intracellular $[Ca^{2+}]$ (e.g. Pereira et al., 1993) or shortage of ATP (in case of e.g. Ca^{2+}-ATPase).

3.3 Inorganic carbon transport in foraminifera

Transport of inorganic carbon may be accomplished by bicarbonate-transporters. If seawater or metabolic CO_2 contributes to the inorganic carbon during calcification, diffusion rates across membranes would control the influx of inorganic carbon and thereby influence the rate of calcification. The diffusion rate is determined by the concentration gradient of CO_2, the membrane area over which CO_2 can diffuse, and the solubility of CO_2 in the membrane lipids. The concentration of CO_2 at the site of calcification or in internal reservoirs is determined by pH. Since foraminifera can control the pH in these compartments (Erez, 2003; Bentov et al., 2009; De Nooijer et al., 2009a; Glas et al., 2012), they can produce large CO_2 concentration gradients and hence promote the influx of DIC to the sites of calcification. The flux of ions can also be calculated from calcification rates, which is discussed in section 4.

In case of intracellular storage of ions, calcium and DIC are unlikely to be stored as free ions. Because the cytosol has very low concentrations of free Ca^{2+} and DIC, the cell volume will control the number of ions available for calcification. For the DIC-reservoir (if present) the additional problem is that CO_2 can easily diffuse across cell membranes and subsequent re-equilibration would thus result in net leakage of carbon out of the DIC-reservoir. To overcome this problem, DIC must be sequestered by mechanisms such as elevating the pH in the reservoir. Because there are usually no crystallites visible within the cells of hyaline species, Ca and DIC are likely sequestered together as non-crystalline $CaCO_3$ (i.e. amorphous calcium carbonate or ACC). Such a possibility may have consequences for the minor and trace element composition of the calcite precipitated, since it is known that formation of high-Mg calcite is accompanied by the formation of an amorphous precursor phase (Raz et al., 2000).

374 Regardless of the process concentrating Ca^{2+} and DIC from seawater, each would produce a
375 supersaturated solution at the site of calcification, with reduced levels of crystal inhibitors that
376 occur naturally in seawater (e.g. Mg^{2+} and PO_4^{2-}). The Ca^{2+} and CO_3^{2-} may form spontaneous
377 $CaCO_3$ crystals, but the specific morphology of foraminiferal chambers show that nucleation
378 and crystal growth is a tightly controlled process.
379
380 **4. Nucleation of calcification**
381
382 **4.1 Crystal nucleation energy and critical size**
383 Precipitation of a crystal from a solution occurs when free energy of the precipitate is lower
384 than that of the solution. Nucleation of a crystal requires even more energy since ions at the
385 surface of a crystal add to the free energy of the solid phase. This is caused by the fact that
386 ions at the surface of a crystal are not bound on all sides to other ions. The resulting
387 'interfacial energy' requires the formation of metastable clusters of a critical size to start
388 crystal growth (Figure 5). The interfacial free energy between the cluster and a solution is
389 usually larger than that between the cluster and a solid substrate, resulting in crystal
390 nucleation at solid surfaces rather than within the solution itself (De Yoreo and Vekilov,
391 2003). If the atomic structure of a substrate matches a particular plane of the nucleating phase
392 (e.g. calcite or aragonite), the interfacial free energy is reduced and nucleation is promoted
393 (De Yoreo and Vekilov, 2003).
394 In the case of nucleation of $CaCO_3$, presence of negatively charged groups at regular intervals
395 at the site of calcification may be able to bind Ca^{2+} and pre-form a part of the $CaCO_3$ lattice.
396
397 *Figure 5: relation between free energy changes (Δg) as a function of pre-nucleation sphere*
398 *(r), where Δg_s is the surface term and Δg_b the bulk term. The sum of Δg_s and Δg_b is the free*

energy barrier that can only be overcome by the formation of a nucleation sphere with a critical size (r_c). Biological control over crystal nucleation is often aimed at lowering of this energy barrier and can be achieved by increasing the concentrations of the solutes or the presence of an organic template.

4.2 Organic templates and nucleation of $CaCO_3$ in foraminifera

During biomineralization in foraminifera calcium carbonate nucleates at the site of calcification, likely involving an organic template. In all Rotaliid foraminifera, chamber formation starts with delineation of a finite environment that encompasses an inner chamber volume from the surrounding medium (Angell, 1979; Bé et al., 1979; Hemleben et al., 1986; Spero, 1988; Wetmore, 1999). Cytoplasmic activity by formation of a dense pseudopodial network transports vacuoles, mitochondria and organic particles to a defined zone in which the so-called Organic Primary Envelope, Primary Organic Lining, Anlage or Primary Organic Membrane (POM) is formed (e.g. Banner et al., 1973; Hemleben et al., 1977; Spero, 1988; not to be confused with inner and outer organic linings, nor with the outer protective envelope or cytoplasmic envelope: see section 4). The term POM is often used but may be confusing (Erez, 2003) since these organic templates are not technically membranes. Therefore, we recommend following the suggestion of Erez (2003) to rename the POM as the Primary Organic Sheet (POS). In a number of benthic species, the POS consists of unbranched polysaccharides such as glycosaminoglycans (Hottinger and Dreher, 1974; Langer, 1992). Proteins are also present in the organic lining of foraminifera, sometimes forming different classes based on their amino acid composition (Robbins and Brew, 1990). King and Hare (1972) showed that amino acids make up 0.02-0.04% of the weight of the calcite and that composition among planktonic species varies greatly. Interestingly, the largest compositional difference coincides with the planktonic foraminifera spinose/ non-spinose divide (King and

Hare, 1972), but differences in amino acid composition are also manifest at lower taxonomic levels (Robbins and Healy-Willliams, 1991).

The organic matrix of the benthic *Heterostegina depressa* is shown to contain an EDTA-soluble and -insoluble fraction (Weiner and Erez, 1984). The insoluble fraction contains over-sulphated glycosaminoglycans and a small portion of non-polar proteins, forming the inner organic lining. The soluble fraction contains a number of proteins containing amino acids with acidic residues. Polar groups in both fractions may be involved in biomineralization since they may bind Ca^{2+} ions and thereby overcome the free energy barrier (Figure 5). If such groups are regularly spaced, they may help nucleation further by placing the Ca^{2+} ions in a regular grid with just enough space for the CO_3^{2-} ions to fit in between them. To test this hypothesis, the tertiary structures of the biomolecules (e.g. proteins and saccharides) that are involved in $CaCO_3$ nucleation need to be analyzed.

The presence of polysaccharides and proteins has led to the hypothesis that the POS has two functions in the process of calcification. The carbohydrates may form a structure determining the overall shape of the new chamber. The proteins associated with the polysaccharides, on the other hand, form the 'active' part of the POS by providing charged sites for nucleation of $CaCO_3$ (Towe and Cifelli, 1967). Since the chemical composition of the POS varies between species (Banner et al., 1973), its role in nucleation of calcium carbonate may differ between foraminiferal species (Bé et al., 1979; Hemleben et al., 1986; Spero, 1988; Wetmore, 1999). In some benthic species, the POS coincides with the location of the pores prior to calcification (Wetmore, 1999), suggesting that there are structural differences in the POS within a single chamber that determine where calcite does and does not nucleate. In planktonic species such as *Globorotalia truncatulinoides* and *G. hirsuta*, calcification begins in small nucleation zones at finite locations across the POS, where calcite forms centers of crystal growth that interlock to form the initial calcified chamber (Towe and Cifelli, 1967; Angell, 1979; Bé et al., 1979;

449 Hemleben et al., 1986). A similar pattern has been observed in *Orbulina universa*, where
450 small islands of calcite form on the POS, followed by calcite island fusion to produce the
451 spherical chamber (Spero, 1988).
452 Nucleation (and subsequent crystal growth) is also determined by the physico-chemical
453 conditions at the site of calcification. These conditions are only partly known in benthic
454 species (e.g. Erez, 2003; Bentov and Erez, 2005) and have only been modeled in planktonic
455 species (Zeebe et al., 1999; Zeebe and Sanyal, 2002). The volume between the crystal surface
456 and the shielding cytoplasmic envelope or pseudopodial network is extremely small, limiting
457 interpretation from light microscopic observations. However, TEM images of initial
458 calcification in *Orbulina universa* and other planktonic species suggests the privileged space
459 between rhizopodia and calcifying surfaces may be <10 nm (Bé et al 1979; Spero 1988).
460 Little is known about the chemical composition of the fluid from which $CaCO_3$ nucleates, but
461 high concentrations of Ca^{2+} and CO_3^{2-} need to be actively maintained, while the $[Mg^{2+}]$ needs
462 to be reduced to satisfy observations and ensure calcification (Zeebe and Sanyal, 2002).
463 Elevated pH at the site of calcification would promote the conversion of CO_2 and HCO_3^- to
464 CO_3^{2-}, thereby enhancing $CaCO_3$ nucleation and growth. Elevated concentrations of Mg^{2+}
465 around the POS in *Pulleniatina obliquiloculata* (Kunioka et al., 2006) may indicate that in
466 this species, the composition of the calcifying fluid is different during the first stage of
467 chamber formation, possibly due to a different rate or efficiency of the process that locally
468 reduces $[Mg^{2+}]$ vs $[Ca^{2+}]$. The participation of a small volume of seawater at the beginning of
469 chamber formation may explain the elevated Mg in the first calcite precipitated, although this
470 pattern does not hold for other planktonic species (e.g. such as *Orbulina universa*; Eggins et
471 al., 2004) where the lowest Mg/Ca ratios are associated with the intrashell zone that
472 corresponds to the POS. The above observations of inter species differences in chamber wall

elemental composition underscore the need to unravel the mechanisms controlling test calcification.

5. Chamber growth

After initial crystal nucleation, calcification proceeds by addition of calcite on both sides of the POS. Additional layers of $CaCO_3$ are added on top of pre-existing chamber calcite during each chamber formation event in perforate foraminifera (Reiss, 1957; 1960; Bé and Hemleben, 1970; Erez, 2003). Together, the primary and secondary layers of calcite are termed 'lamellar' calcite (Erez, 2003). Most observations on calcification are based on the first stage of chamber formation in which a thin-walled chamber is produced within 1-3 hours (Spero, 1988). Subsequent thickening of the chamber wall proceeds during the next 24-48 hours until a new chamber is formed. Thickening of earlier formed chambers occurs by addition of a calcite layer with each new chamber formation event (e.g. Bentov and Erez 2005, Nehrke et al., 2013). Future studies will need to show whether the timing of the start and end of chamber formation and thickening of previously formed chambers are coincidental, or whether thickening is a continuous process.

Future biomineralization research should also take into account the possibility that cellular controls on calcification may vary over time and location across the foraminifera shell. An example of the potential complexity and diversity of calcification within one specimen is provided by Bentov and Erez (2005). Their research demonstrated that the benthic *Amphistegina lobifera* recovering individuals produce at least three types of calcium carbonate: elongated, intracellular birefringent granules with a high magnesium and phosphorus content, extracellular microspheres with a high Mg concentration and extracellular spherulites with a low Mg content. These spherulites represent the lamellar

498 calcite while the microspherulites represent the initial presipitation over the POS in *A.*
499 *lobifera.*
500 During chamber formation, ions could be supplied to the site of calcification (SOC) from
501 internal reservoirs (Figure 3, Table 1) or by transport from the surrounding seawater. The
502 latter can be accomplished by transmembrane ion transporters (section 2), by direct exchange
503 of the calcifying fluid with seawater and/ or by diffusion from ambient seawater. The inner
504 and outer surfaces of newly formed chambers of the benthic *Heterostegina depressa* are
505 covered by thin layer of cytoplasm (Spindler, 1978), suggesting the SOC may be separated
506 from the surrounding medium. In a number of studies (Angell, 1979; Bé et al., 1979), a fan-
507 like arrangement of the pseudopodial network is observed in a zone outside the site of
508 calcification. Although the relation between this arrangement and calcification remains to be
509 investigated, it is likely to play a role in biomineralization since this dense network is not
510 observed between chamber formation events. Also in the planktonic species *G. hirsuta* and *G.*
511 *truncatulinoides*, calcification proceeds adjacent to a cytoplasmatic envelope (or outer
512 protective envelope) that may play a role in maintaining SOC integrity and shape, and
513 promoting initial calcification (Bé et al., 1979). In the benthic *Ammonia* sp., a pH gradient of
514 >2 pH units is observed across several μm distance and is maintained for hours between the
515 site of calcification (De Nooijer et al., 2009a) and the specimen's microenvironment (Glas et
516 al., 2012). These observations suggest that in *Ammonia* sp., the SOC is separated from the
517 outside environment. Spero (1988) on the other hand, presented transmission electron
518 micrographs that showed the site of calcification in *O. universa* is not shielded by a
519 continuous membrane. Nehrke et al. (2013) recently suggested that the site of calcification in
520 *Ammonia aomoriensis* is largely closed from the surrounding medium, but that a small
521 percentage of the fluid at the SOC is derived from leakage of the cell membranes separating it
522 from the outside medium, explaining observed Mg/Ca for the species studied.

523 The extent to which the site of calcification is open or closed, in combination with the
524 presence or absence of intracellular ion reservoirs, is an important unknown in understanding
525 foraminiferal calcification (Figure 6). For example, a site of calcification that is physically
526 separated from the surrounding seawater, together with the absence of intracellular ion
527 reservoirs, prescribes the need for transmembrane ion transporters (e.g. Ca^{2+}-APTase; section
528 II) that selectively pump ions from seawater to the SOC. A SOC that is open, on the other
529 hand, will experience relatively high concentrations of Mg and require an active Mg^{2+}-
530 removal mechanism.
531
532 *Figure 6: summary of the most important parts of the calcification mechanism in*
533 *foraminifera, including Ca-ion transport, active Mg-removal and contribution from internal*
534 *reservoirs. See text for description of the individual processes.*
535
536 Potential ion transport pathways to the site of calcification can be constrained from
537 calcification rates during chamber formation. It is important to distinguish between the overall
538 growth rate of a foraminifer and calcite precipitation rate during biomineralization. The
539 difference between these processes results from the episodic nature of growth (chamber
540 addition) in foraminifera. Some planktonic species have been reported to increase the weight
541 of their shell by 13-15% a day (*G. sacculifer*; Erez, 1983), but this may vary with
542 environmental conditions (Ter Kuile and Erez, 1984 and references therein). Secondly,
543 chamber addition rates vary over a foraminifer's lifetime, decreasing as the individual ages
544 (Ter Kuile and Erez, 1984). Calcite precipitation rates during chamber addition, on the other
545 hand, are much higher and vary between 0.4-0.9 µg/h in the planktonic foraminifer *G.*
546 *sacculifer* (Anderson and Faber, 1984), 0.06-0.32 µg/h in *O. universa* (Lea et al., 1995) and
547 ~10 µg/h in the benthic *A. tepida* (De Nooijer et al., 2009b). Since such rates are rarely

548 quantified, it is difficult to generalize these values to other species or other conditions.
549 Moreover, calcite precipitation rates can be variable between day and night calcification
550 periods (Erez, 1983; Spero, 1988; Lea et al., 1995). Since incorporation of some elements
551 may depend on precipitation rate (e.g. DePaolo, 2011), it is necessary to quantify these rates
552 across a diurnal time frame when chamber formation is occurring in order to assess the
553 kinetics of element incorporation and thereby proxy-relationships.
554 Mitochondrial activity may play an important role at the site of calcification and thereby
555 affect trace element incorporation. Besides providing energy, mitochondria pump cytosolic
556 Ca^{2+} and Mg^{2+}, and therefore modulate the cell's [Ca^{2+}] and [Mg^{2+}] (Carafoli et al., 2001).
557 This may be particularly important during calcification when the concentration of these ions
558 increases locally. Spero (1988) shows that calcification in *O. universa* around the POS is
559 associated with pseudopodia containing mitochondria, and hence possibly modulate [Mg^{2+}] at
560 the SOC. Similar results can be found in Bé et al (1979) for *Globorotalia truncatulinoides*.
561 Bentov et al (2009) discuss the possible role of mitochondria in producing metabolic CO_2 that
562 eventually accumulate in the alkaline vacuoles as DIC.
563 Photosynthesis by symbionts may also affect calcification rates. The relative concentrations of
564 DIC species are influenced by symbiont photosynthesis and CO_2-uptake during the day (or
565 release in the dark) and the resulting diurnal differences in microenvironment pH (Jørgensen
566 et al., 1985; Rink et al., 1998; Köhler-Rink and Kühl, 2000; 2005), thereby influencing uptake
567 and availability of inorganic carbon species. In some large benthic foraminifera (Wetmore,
568 1999), the symbionts are positioned near the POS prior to calcification, suggesting that their
569 activity could enhance calcification. Elimination of symbionts in *G. sacculifer* resulted in
570 reduced chamber formation rates and early gametogenesis or death of the foraminifera (Bé et
571 al., 1982). Reseeding the aposymbiotic foraminifera with symbionts from donor specimens
572 produced individuals that continued to add chambers and mature at a normal rate. These data

suggest that symbiont photosynthesis is critical to both nutrition and chamber calcification. Elevated light intensity promotes growth in *G. sacculifer* (Caron et al., 1982) but not in the benthic foraminifera *Amphistegina lobifera* in which both photosynthesis and calcification are optimal at relatively low light intensities that are found at 20-30 m water depth (Erez 1978, Ter Kuile and Erez, 1984).

Ter Kuile et al. (1989a), on the other hand, suggested that symbionts and foraminifera compete for inorganic carbon. Erez (1983) and Ter Kuile et al. (1989b) showed that inhibition of photosynthesis in both planktonic and benthic species by the photosystem II inhibitor DCMU, does not affect calcification rates and suggested that it is not photosynthesis itself, but rather light which directly promotes calcification. Finally, Ter Kuile et al (1989a) have shown that there is competition for CO_2 between the symbionts and their host in the benthic foraminiferan *A. lobifera*. Clearly, the relationship between symbioses and foraminifera calcification requires additional study.

Pore formation provides important information on foraminiferal biomineralization. In species producing macropores, we observe a pore plate that is continuous with the POS and separates the cytoplasm from the outside medium (Hemleben et al., 1977). In benthic, symbiont-bearing species, symbionts can be found in close proximity to the pores (e.g. Lee and Anderson, 1991) suggesting that respiratory gases such as CO_2 and O_2 may be able to diffuse through the pore plates. In symbiont-barren species, diffusion of gases between cytoplasm and environment could be enhanced by the permeability of a pore plate. Some have suggested that dissolved organic matter may be taken up through the pores in the benthic *Patellina* (Berthold, 1976). In *G. sacculifer*, pseudopodia appear to penetrate through the pore plates (Anderson and Bé, 1976). Pores in the benthic species *Patellina corrugata* have been reported to exist from the beginning of chamber formation (Berthold, 1976) and pores are observed in the *O. universa* sphere once initial calcification has locked in the spherical morphology of the chamber

598 (Spero, 1988). Some species of planktonic foraminifera have micro- instead of macropores
599 (often in species with secondary apertures; *Globigerinata glutinata*, *Candeina nitida*), ranging
600 from 0.3-0.7 μm (Brummer and Kroon, 1988). These micropores do not appear to have a pore
601 plate, and their function, formation and morphology is less well understood than those for
602 macropores.
603
604 **6. Overgrowth and encrusting**
605 The primary and secondary layers of calcite in perforate foraminifera are together referred to
606 as 'ontogenetic' or 'lamellar' calcite (Erez, 2003). Additional $CaCO_3$ can be present as
607 ornamentations (pustules, spines, ridges, tooth plates, etc.) or as layers of calcite covering the
608 whole test (crust or gametogenic (GAM) calcite). Whereas ornamentation is present
609 throughout the entire life cycle of a foraminifer (Hemleben, 1975), GAM calcite is exclusive
610 to planktonic foraminifera and is added after the last chamber is formed and just prior to
611 meiotic division of the nucleus and gametogenesis.
612 In some planktonic species, a calcite crust can be formed after formation of the final chamber
613 (Bé and Ericson, 1963; Bé and Lott, 1964; Bé, 1965; Bé and Hemleben, 1970; Olsson, 1976).
614 The morphology of this calcite is markedly different from that of either ontogenetic or GAM
615 calcite and its element and isotopic composition can differ from that of the ontogenetic calcite
616 because it forms under different environmental conditions of temperature and/or salinity. For
617 instance, crust Mg/Ca is generally lower than that of ontogenetic calcite in *Globorotalia*
618 *truncatulinoides* (Duckworth, 1977) and *Neogloboquadrina dutertrei* (Jonkers et al., 2012).
619 These lower element concentrations are partly a consequence of conditions deeper in the
620 water column (i.e. lower temperature), but it should be noticed that the observed partitioning
621 for Mg indicates that crust calcification is a biologically controlled process. Interestingly,

622 Nürnberg et al. (1996) found that crusts formed in culture can have a higher Mg/Ca than the
623 ontogenetic calcite.
624 In a number of species such as *G. sacculifer*, gametogenesis is preceded by the production of
625 a layer of calcite covering spine holes and the terrace-like structures of inter-pore rims (Towe
626 and Cifelli, 1967; Bé, 1980; Hemleben et al., 1985; Brummer et al., 1987). This GAM calcite
627 veneer gives the foraminifera a smooth appearance by covering the rough topography of the
628 shell surface and it has been suggested that it is enriched in some trace elements compared to
629 the ontogenetic calcite (Hathorne et al., 2009). Whether this observation holds for all
630 foraminifera forming GAM calcite, however, remains to be investigated.
631 From the perspective of biomineralization, variability in the types of $CaCO_3$ that are formed
632 may indicate that foraminifera do not have one single way to produce shell calcite. Rather, the
633 physiological tools to achieve calcite precipitation as discussed in sections 2 and 4, are likely
634 used in different combinations by different species of foraminifera. Moreover, the variability
635 in calcite within single specimens suggests a degree of flexibility of these physiological tools
636 even within single species. Identification of seawater vacuolization, transmembrane ion
637 transport, nucleation promoting organic templates, etc. across species and their contribution to
638 calcification within a foraminifer's life time are critical aspects of foraminiferal biology and
639 keys to understanding foraminiferal biomineralization from a mechanistic perspective.
640

641 **7. Future directions**

642 A complete mechanistic description of foraminiferal biomineralization and chamber
643 formation does not yet exist. Hence, the biological and environmental interplay that controls
644 the element composition and isotope fractionation of chamber calcite is only partly
645 understood. Literature on foraminiferal calcification is both qualitative and quantitative but on

646 occasion, contradictory. This leaves us with a number of outstanding questions that need to be
647 addressed in order to move this area of foraminifera biology forward. These include:
648
649 1. Which foraminiferal species use vacuolized seawater as the primary source for
650 calcification and which use transmembrane transport of Ca^{2+} and DIC during
651 calcification? The investigation into the transport of ions to the site of calcification
652 may be solved by answering a number of more practical questions, including:
653 - What is the relation between transmembrane transport and vaculization on the one
654 hand, and production of intracellular calcium and/ or carbon reservoirs on the other
655 hand?
656 - What is the biochemical basis of these processes? Which transmembrane transporters
657 are involved (e.g. Ca-ATPases, proton-Ca^{2+} antiporters)? By which mechanism is
658 inorganic carbon concentrated (e.g. involvement of Carbonic Anhydrase)?
659 - When characterized, can these (transport) mechanisms explain observed element
660 incorporation and isotopes fractionations. If yes, can these mechanisms explain
661 foraminiferal chemistry for (all) these elements and isotopes *at the same time*?
662 - Is there a general difference between planktonic and benthic species in production of
663 vacuolized seawater, internal reservoirs and/or direct ion transport?
664 - Do foraminifera employ both mechanisms to calcify and if yes, what is the balance
665 between these two pathways?
666 2. What is the tertiary structure of the organic matrix/ matrices (e.g. POS, organic
667 linings) involved in biomineralization? Which compounds help to lower the free
668 energy barrier, thereby promoting calcite nucleation? When identified, do these
669 organic compounds have an impact on the partition coefficient of elements and
670 fractionation of isotopes at the first stage of chamber formation?

3. To what extent is the site of calcification in contact with surrounding seawater? If seawater directly contributes (part of) the ions for calcification, can this source explain observed fractionation factors and partition coefficients?
4. What is the role of mitochondria in calcification? Do mitochondria (help to) regulate the Mg/Ca at the site of calcification?

Finally, a more detailed understanding of foraminiferal biominiralization will also allow researchers to compare calcification strategies accross marine calcifiers. Compared to foraminifera, biomineralization in corals (Al-Horani et al., 2003; Sinclair and Risk, 2006; Venn et al., 2013), coccolithophores (Marsh, 2003; Taylor et al., 2011; Ziveri et al., 2012; Bach et al., 2013), gastropods (e.g. Nehrke et al., 2011) and bivalves (Nudelman et al., 2006; Nehrke et al., 2012; Shi et al., 2013) are understood in greater detail. Identification of differences and similarities between these marine calcifying taxa will allow studying (convergent) evolutionairy patterns, help to understand differences in their response to (future) environmental perturbations and facilitate comparison of paleoceanographic information obtained across taxa.

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697
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Biomineralization in perforate Foraminifera

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Abstract

In this paper, we review the current understanding of biomineralization in Rotaliid foraminifera. Ideas on the mechanisms responsible for the flux of Ca^{2+} and inorganic carbon from seawater into the test were originally based on light and electron microscopic observations of calcifying foraminifera. From the 1980's onward, tracer experiments, fluorescent microscopy and high-resolution test geochemical analysis have added to existing calcification models. Despite recent insights, no general consensus on the physiological basis of foraminiferal biomineralization exists. Current models include seawater vacuolization, transmembrane ion transport, involvement of organic matrices and/or pH regulation, although the magnitude of these controls remain to be quantified. Disagreement between currently available models may be caused by use of different foraminiferal species as subject for biomineralization experiments and/ or lack of a more systematic approach to study (dis)similarities between taxa. In order to understand foraminiferal controls on element incorporation and isotope fractionation, and thereby improve the value of foraminifera as paleoceanographic proxies, it is necessary to identify key processes in foraminiferal biomineralization and formulate hypotheses regarding the involved physiological pathways to provide directions for future research.

1. Introduction

All foraminifera make tests although a number of different materials are used in their construction. The 'naked' foraminifera produce tests from organic matter, agglutinated foraminifera use sediment grains as building blocks and calcifying foraminifera use constituents dissolved in seawater to secrete calcium carbonate. Formation of $CaCO_3$ tests plays a significant role in ocean biogeochemical cycles and, more importantly, the fossil remains of calcifying foraminifera are widely used to reconstruct past ocean chemistry and

environmental conditions. Elemental and isotopic composition of foraminiferal calcite depends on a variety of environmental parameters such as temperature, salinity, pH and ion concentration (McCrea et al., 1950; Epstein et al., 1951; Boyle, 1981; Nürnberg et al., 1996). These physical and chemical variations are the foundation for developing geochemical proxies that quantify environmental changes through time (see Wefer et al., 1999; Zeebe et al., 2008; Katz et al., 2010 for reviews). For example, the magnesium concentration in foraminiferal calcite ($Mg/Ca_{calcite}$) varies primarily with seawater temperature (Nürnberg et al., 1996; Lea et al., 1999; Hönisch et al., 2013) and can be used to reconstruct past sea surface (Hastings et al., 1998; Lea et al., 2000) and deep-water (Lear et al., 2000) temperatures. Reliable application of these proxies requires calibration over a wide range of environmental conditions as well as a thorough understanding of the physiological parameters influencing test formation.

Studies calibrating foraminiferal test composition based on core-tops and controlled growth experiments show that both the chemical and isotopic compositions of these tests are not in equilibrium as defined by inorganic precipitation experiments (Lowenstam and Weiner, 1989; Dove et al., 2003). Microenvironmental controls related to foraminifera physiology have been implicated to explain disequilibrium fractionation in test chemistry (Figure 1). Most foraminiferal species incorporate Mg with one to two orders of magnitude lower concentration compared to non-biologically precipitated calcium carbonate (Bentov and Erez, 2006; Katz, 1973; Bender et al., 1975). The concentration of barium, on the other hand, is ~10 times higher in foraminiferal calcite (Lea and Boyle, 1991; Lea and Spero, 1992) compared to inorganic precipitation results (Pingitore and Eastman, 1984). Additionally, elemental concentrations between foraminiferal species can vary by several orders of magnitude (up to two orders of magnitude for Mg; Bentov and Erez, 2006). The biological controls on element

incorporation and isotope fractionation that cause these offsets are often summarized as 'the vital effect' (Urey et al., 1951; Weiner and Dove, 2003).

Figure 1: Minor and trace element composition of foraminiferal (left) and inorganically precipitated (right) calcite precipitated from seawater (middle). Concentrations are qualitative as they differ between foraminiferal species and depend on environmental conditions. Precipitation rates, ionic strength of the medium and presence of organic compounds are also known to affect partition coefficients. All values are in parts per million (ppm) and based on data in Kitano et al. (1975), Ishikawa and Ichikuni (1984), Rimstidt et al. (1998), Marriott et al. (2004), Morse et al. (2007), Tang et al. (2012) He et al. (2013) for inorganically precipitated calcium carbonates, and Lea and Boyle (1991), Rickaby and Elderfield (1999), Segev and Erez (2006), Terakado et al. (2010), Allen et al. (2011) for foraminiferal calcite composition.

Vital effects comprise 1) chemical alterations of the foraminifers' microenvironment due to physiological processes, 2) cellular controls on the composition of the fluid from which calcite is precipitated and 3) controls on nucleation and crystal growth (e.g. by presence of organic templates). Foraminiferal respiration and/or photosynthesis by symbiotic algae alter the foraminiferal microenvironment chemistry and thereby the conditions in which foraminiferal tests mineralize. Because habitat depth differences in the water column (planktonic species) or migration in the sediment and attachment to plant leaves (benthic species) also modify the calcification environment, these ecological factors are sometimes regarded as being part of the vital effect as well (e.g. Schmiedl and Mackensen, 2006). Ecology-based variability in element incorporation, however, can be accounted for when habitat preferences of foraminiferal species are known. Hence, the term "vital effects" should

100 only be used when discussing foraminiferal cellular processes that alter the chemistry of the
101 microenvironment during test mineralization.
102 To understand the physiological impact on element incorporation and isotope fractionation,
103 the (intra)cellular mechanisms which foraminifera employ to precipitate test $CaCO_3$ must be
104 identified. Biogeochemical mechanisms are involved in regulating concentrations of ions
105 and/or their activity at the site of calcification. Calcification from seawater can be promoted
106 using different mechanisms. Hence, multiple mechanisms have been proposed to explain test
107 calcification, including endocytosis of seawater, transmembrane ion transporters, ion-specific
108 organic templates, production of a privileged space and mitochondrial activity (Spero, 1988;
109 Erez, 2003; Bentov and Erez, 2006; Bentov et al., 2009).
110 A process-based framework for both inorganic and organismal control of foraminiferal test
111 formation is crucial for the development, calibration and application of geochemical proxies
112 in the geological record. At the same time, a mechanistic understanding of foraminiferal
113 biomineralization will also permit researchers to better interpret data from the fossil record as
114 well as predicting the response of foraminiferal calcification to future environmental changes
115 such as ongoing ocean acidification. Most of the initial observations of chamber formation
116 and calcification in planktonic foraminifera were published during the early period of
117 planktonic foraminifera culturing (e.g. Bé et al., 1977). Highlights of those observations can
118 be found summarized in the seminal text on "Modern Planktonic Foraminifera" (Hemleben et
119 al., 1989). More recently, studying living specimens under controlled conditions (e.g. Kitazato
120 and Bernard, 2014) has further propelled our understanding of foraminiferal growth,
121 reproduction and calcification.
122 Recent hypotheses on foraminiferal biomineralization are based mainly on experiments with
123 benthic species and although these ideas have to be tested for planktonic species, we will also
124 include the latter group in our discussion. Although a general model for foraminiferal

125 biomineralization is still lacking, and it is not yet clear that a single model fits all groups of
126 foraminifera, details on the underlying mechanisms in different species have accumulated and
127 are described here in the context of previously published biomineralization models.
128
129 **2. Ions for calcification**
130
131 **2.1 Seawater as the direct source for Ca^{2+} and DIC**
132 Foraminifera calcify by creating a microenvironment supersaturated with respect to $CaCO_3$,
133 while overcoming inhibition by crystallization inhibitors such as Mg^{2+}. Hence, calcification
134 requires a tight control on the concentration and/or ion activity at the site of calcification,
135 commonly referred to as the "delimited" space (Erez, 2003) or "privileged" space. Elevated
136 $[Ca^{2+}]$, $[CO_3^{2-}]$ and/or their ion activities have to be actively maintained in order for
137 calcification to proceed. Simultaneously, the concentrations of crystal growth inhibitors have
138 to be lowered even further. Although CO_3^{2-} needed for calcification may be partially derived
139 from respired CO_2 (Erez, 1978; Grossman, 1987; Ter Kuile and Erez, 1991; Hemleben and
140 Bijma, 1994; Bijma et al., 1999), the majority of the carbon and the Ca^{2+} needed for test
141 formation must be derived from the seawater environment.
142 Calcification requires equal amounts of Ca^{2+} and CO_3^{2-}. Because seawater Ca^{2+} concentrations
143 are approximately 5 times higher than that of DIC and often >50 times higher than that of
144 CO_3^2, foraminifera have to spend more time and/or energy in taking up and concentrating DIC
145 than they have to do for Ca^{2+}. A foraminifer needs to process several times the seawater
146 equivalent of its own cell volume in order to acquire enough Ca^{2+} and inorganic carbon to
147 calcify a new chamber. Although the exact amount needed depends on shape, size and the
148 thickness of the chamber wall (e.g. Brummer et al., 1987), juveniles of some species need 50-
149 100 times their own cell volume to extract the Ca^{2+} required to produce one new chamber (De

150 Nooijer et al., 2009b). Because seawater [CO_3^{2-}] is significantly lower than [Ca^{2+}], these
151 individuals need the equivalent of ~3,000 times their own volume in order to take up the
152 necessary [CO_3^{2-}] if this anion is used exclusively. However, observations of high pH at the
153 site of calcification (Erez, 2003; De Nooijer et al., 2009a; Bentov et al., 2009) as well as
154 oxygen isotope data from laboratory experiments (Spero et al., 1997; Zeebe, 1999) suggest
155 that foraminifera can convert CO_2 and/or HCO_3^- into the CO_3^{2-} needed for calcification.
156 Evidence that foraminifera concentrate inorganic carbon is also provided by experiments
157 using ^{14}C tracer incorporation kinetics into the skeleton of perforate species (Ter Kuile and
158 Erez 1987, 1988, Ter Kuile et al 1989b). A carbon concentrating mechanism would reduce the
159 volume of seawater necessary for calcification by 50-90% (De Nooijer et al., 2009b). To
160 concentrate the ions needed for calcification, foraminifera must either extract Ca^{2+} and
161 dissolved inorganic carbon (CO_2, HCO_3^- and CO_3^{2-}, or DIC) or take up seawater and
162 subsequently reduce the concentrations and/or activities of all other ions relative to Ca^{2+} and
163 DIC (Figure 2). Removal of protons from (endocytosed) seawater is also a prominent feature
164 in recently developed calcification mechanisms, but will be discussed in a separate section
165 (2.2). In case of the second option, spontaneous nucleation of $CaCO_3$ crystals may be
166 prevented by separation of Ca^{2+} and DIC into different vacuole groups.
167
168 *Figure 2: Two different mechanisms to concentrate Ca^{2+} and DIC from seawater for*
169 *calcification: a) Calcium- and bicarbonate-ions are specifically taken up from seawater, or b)*
170 *the other ions are selectively removed, thereby increasing Ca and DIC concentrations.*
171
172 Both processes transport ions either directly to the site of calcification or temporarily store
173 these ions. In the case of uptake into some benthic foraminifers, Ca^{2+} and/ or DIC are thought
174 to be present in so-called 'intracellular reservoirs' (also known as ‘pools’; Ter Kuile and Erez,

1988; Erez, 2003). These reservoirs may be seen as temporal storage compartments with high concentrations of ions that are either emptied upon calcification or provide a dynamic cycling of Ca^{2+} and DIC through the cell that is gradually used for calcification. Without an intracellular reservoir, Ca^{2+} and DIC could also be directly transported to the privileged space during calcification (Erez, 2003; Bentov and Erez, 2006). The relative importance of intracellular reservoirs versus direct transport among benthic and planktonic species remains a subject of debate and active research.

2.2 Internal reservoirs

Internal reservoirs may be important for foraminiferal calcification in certain groups. Conceptually speaking, one can envision Ca^{2+} or DIC being derived from internal reservoirs. With seawater as the basis for calcification, carbon reservoirs will have to be approximately 5 times larger than those for Ca^{2+} or have a 5 times faster turnover rate. Evidence suggests that different foraminifer groups employ different strategies. For instance, a time-lag has been observed between uptake and incorporation of labelled inorganic carbon in the large benthic foraminifera *Amphistegina lobifera* suggesting inorganic carbon may be stored in an internal reservoir (Ter Kuile and Erez, 1987; 1988; Ter Kuile and Erez, 1991). In pulse-chase experiments it was observed that ^{14}C was incorporated into the calcite during the chase period in ^{14}C free seawater, implying a large internal reservoir of DIC in the benthic *Amphistegina lobifera* but not in the milliolid *Amphisorus hemprichii* (Ter Kuile et al 1989b). Isotope labelling experiments with the planktonic foraminifer *G. sacculifer* and a number of benthic species using both ^{14}C and ^{45}Ca show that proportionally more labelled ^{45}Ca is incorporated into the shell compared to labelled ^{14}C (Erez, 1978; 1983). For the planktonic species *Orbulina universa* and *Globigerina bulloides*, on the other hand, Bijma et al. (1999) showed that the contribution from an internal carbon pool is insignificant in these species.

To determine whether planktonic foraminifera have an internal Ca-reservoir, Anderson and Faber (1984) grew *G. sacculifer* in artificial seawater spiked with ^{45}Ca. They showed that calcite formed during the first 24 hours contains significantly less ^{45}Ca than that produced in the second 24 hours. These data argue for the existence of an unlabeled intracellular Ca-reservoir that was filled prior to the introduction of the isotopic spike. Using pulse-chase experiments with both a 'hot' incubation period (10-15 days) and 'cold' chase period (10-20 days), Erez (2003) traced the uptake of ^{45}Ca over time in the benthic species *Amphistegina lobifera*, showing that as much as 75% of the Ca^{2+} used during chamber calcification resided in an intracellular reservoir. ^{48}Ca uptake data from experiments using *Orbulina universa*, supported the existence of a Ca-reservoir in a planktonic species, but demonstrated that it was completely flushed of labelled Ca^{2+} within the initial 6 hours of chamber formation and thickening (Lea et al., 1995). These latter observations could indicate that *O. universa* utilizes a small Ca^{2+} reservoir to assist with the initial chamber formation, but that much of the remaining chamber Ca^{2+} is derived from seawater without passing through an internal storage reservoir.

Toyofuku et al. (2008) reported formation of (incomplete) chambers in the benthic *Ammonia beccarii* maintained in seawater devoid of Ca^{2+}. These data clearly support the existence of a Ca^{2+}-reservoir of finite volume in benthic species. If Ca^{2+} and other divalent cations that co-precipitate in the $CaCO_3$ shell are derived from the same internal reservoir, one would expect cation concentrations to reflect Rayleigh fractionation if the reservoir is a closed system. Such a system has been used to partly explain minor and trace element distributions in foraminiferal calcite (Elderfield et al., 1996). However, a model using Rayleigh fractionation relies on a number of assumptions about the internal reservoir regarding its size and initial composition as well as refreshment rate and chamber calcification rate. These unknowns highlight the need to better constrain the size and extent of these reservoirs.

To maintain an intracellular reservoir, a foraminifer needs to sustain a high cation flux rate by continuously vacuolizing, endocytosing and exocytosing large volumes of seawater. Tracing endo- and exocytosis in foraminifera is challenging and has yielded contrasting results. For instance, Bentov et al. (2009) showed that in *Amphistegina lobifera*, seawater is taken up in vacuoles that are subsequently transported to the site of calcification. This implies that seawater, internally modified or not, is directly involved in calcification. De Nooijer et al. (2009b) on the other hand, showed that endocytosis and subsequent exocytosis of seawater in *Ammonia tepida* are not directly related to chamber formation.

2.3 Direct uptake of ions

The ions needed for calcification may be derived from seawater during calcification without storage in an intracellular reservoir (Figure 3). A number of calcification models explicitly or implicitly assume that the ions for calcification are passively transported to the site of calcification through diffusion from the surrounding medium (Wolf-Gladrow et al., 1999; Zeebe et al., 1999). These models are able to explain the impact of photosynthetic symbionts on inorganic carbon chemistry in the vicinity of the foraminifer. Changes in pH and [DIC] due to photosynthesis affect the isotopic composition of the available carbonate (Wolf-Gladrow et al., 1999). However, diffusion of ions to the site of calcification without at least one additional mitigating mechanism, cannot account for the difference between seawater metal composition and Me/Ca ratios in foraminiferal calcite (Figure 1 and references in its caption).

Figure 3: Examples of possible involvement of internal reservoirs versus externally derived ions for calcification. A: Ca^{2+} and DIC are derived from internal reservoirs. B: Ca^{2+} and DIC are transported to the site of calcification without uptake and storage into reservoirs. C: DIC

is taken up directly and Ca^{2+} comes from an internal reservoir. D: Ca^{2+} is taken up during chamber formation and DIC is derived from an intracellular reservoir.

Ca^{2+} and DIC may be actively transported (through transmembrane pumps and/ or channels) to the site of calcification. Although such transport mechanisms are not yet identified in planktonic foraminifera, a number of studies support the existence of this mechanism in benthic species. Using radioactive labeling, Angell (1979) showed that the ions for calcification are taken up *during* chamber formation in the benthic species *Rosalina floridana*. Although this observation does not prove the absence of an internal reservoir *per se*, this observation reduces the turnover rate and/or size of such a reservoir considerably. Similarly, Lea et al. (1995) showed that the intracellular Ca-reservoir in the planktonic foraminifer *O. universa* is very small and/or has a fast turnover rate and does not significantly contribute to the total amount of Ca^{2+} during shell thickening. Results from the benthic *Ammonia* sp. show that intracellular vesicles containing elevated concentrations of Ca^{2+} are involved in chamber formation (Toyofuku et al., 2008), but that their amount within the cell is not sufficient for the production of a new chamber (De Nooijer et al., 2009b). Together, these studies suggest that the majority of the Ca^{2+} utilized for shell calcification is not stored in intracellular reservoirs prior to chamber formation in the species studied. If the internal reservoir refills after chamber formation within a relatively short period of time, it is critical that seawater labeling experiments should start directly after a chamber formation event to avoid underestimation of the true reservoir size. Studies addressing the issue of an intracellular reservoir are summarized in Table 1.

Table 1: Studies discussing internal reservoirs in perforate foraminifera.

3. Intracellular transport

3.1 Transmembrane ion transport

Due to the hydrophobic inner layer of cell membranes, molecules cannot freely move into or out of the cell's interior. Although the majority of ions and molecules diffuse across cell membranes, diffusion constants vary greatly. Small, uncharged molecules (CO_2, O_2, NO) diffuse easily down a concentration gradient whereas large molecules and ions require specialized transmembrane proteins to facilitate or energize membrane transport (Higgins, 1992). These transporter proteins can be divided into channels, carriers and pumps (Figure 4). Carrier proteins undergo substrate binding and transport. They show typical substrate affinities and follow Michaelis-Menten kinetics. Carrier transport is even effective against concentration gradients if a cosubstrate with a respective concentration gradient or charge is involved (secondary active transport). Pumps directly generate this energy for uphill transport from their ATPase activity. Transmembrane channels simply allow facilitated diffusion along electrochemical gradients by creating a selective pore through the cell membrane. For the uptake of inorganic carbon by foraminifera during calcification, a strong pH gradient (high inside; De Nooijer et al., 2009a; Bentov et al., 2009; low outside; Glas et al., 2012) may promote the influx of CO_2 and thus circumvent the need for specialized transmembrane proteins.

Figure 4: selective ion transporters. Ion pumps (left and middle) undergo structural changes that allow passage of ions from and to the binding sites. The example shown here is a simplified Na^+/K^+ exchanger that has specifically binds to Na-ions (blue squares) when in the first configurational state (left). After the structural change, affinity of the Na-binding sites decreases so that the Na-ions are released (middle). At the same time, K-ions (yellow circles)

bind to their binding sites after which the pump returns to state one and releases the K^+ to the cytosol. Ion channels (draw after the KcsA K^+ channel; right) consist usually of a narrow pore allowing certain ions to pass a cell membrane down the electro-chemical gradient. Another feature of some pumps and channels is the relatively large cavity that is created by the transmembrane protein-complex (here present in the cytosol-side of the channel). This can greatly reduce the distance that the ions have to be transported. The type of Ca^{2+}-transporters that are used by foraminifera are unknown, but determining their molecular structure is necessary to 1) know the extent of de-hydration during transport, 2) determine the rate of ion transport and 3) explain the selectivity for Ca^{2+}/ against other ions (e.g. Mg^{2+}) and their fractionation (e.g. Gussone et al., 2003).

3.2 Ca^{2+} transport in foraminifera

In foraminifera, most attention has been directed at ion transporters that might be responsible for the low Mg/Ca at the site of calcification. Logically, this may involve Mg^{2+}-transporters and/ or Ca^{2+} transporters. Because Ca^{2+} acts as a secondary messenger in most eukaryotic cells, cytosolic Ca^{2+} is kept low (< 1μM) by active removal out of the cell or into cytosolic compartments (ER, mitochondria). This makes Ca^{2+}-transporters one of the most ubiquitous and well-studied transmembrane ion transporters. From a variety of cell types, Ca^{2+}-ATPases, Ca^{2+}/H^+ and Ca^{2+}/Na^+ antiporters (e.g. Gonçalves et al. 1998) and Ca^{2+}/phosphate co-transporters (Ambudkar et al., 1984) have been described. Depending on the transporter's structure, ions may pass the membrane either with or without their hydration sphere (Gouaux and MacKinnon, 2005), although (partial) dehydration increases the selectivity greatly (see also Gussone et al., 2003).

The specificity of the transmembrane Ca-transporters varies greatly. For some Ca^{2+}/H^+-antiporters it has been reported that other cations with a small ionic radius (e.g. Zn^{2+}) can be

transported in a similar way as Ca^{2+} is transported (Gonçalves et al., 1999). For the same antiporter, the larger Ba^{2+} and Cs^{+} do not substitute for Ca^{2+}. An ion with intermediate size, Sr^{2+} (1.13 Å, compared to 0.99 Å for Ca^{2+}), appears to block the antiport and prevents transport of Ca^{2+} through the membrane. Studies concerning specificity for Ca^{2+} over Mg^{2+} are scarce, but some Ca-ATPases have been reported to have a 10^3-10^5 higher affinity for Ca^{2+} than for Mg^{2+} (Drake et al., 1996; Xiang et al., 2007).

In corals, calcium uptake is directly related to proton pumping (McConnaughey and Whelan, 1997; Sinclair and Risk, 2006). The efflux of H^{+} during calcification (Glas et al., 2012) may therefore help to constrain estimates of calcium pumping rates during calcification. Carbon dioxide uptake and proton efflux are also directly related in cyanobacteria (Ogawa and Kaplan, 1987). Ter Kuile et al. (1989b) suggested that Ca^{2+} is taken up by Ca^{2+}-ATPase and this mechanism was subsequently used by Zeebe and Sanyal (2002) and Zeebe et al. (2008) to show that H^{+} removal is far more energy-efficient than Mg^{2+}-removal during calcification. Such a mechanism would be consistent with a coupling of ion transporters (e.g. Ca^{2+} and H^{+}) during foraminifera calcification.

The amount of Ca^{2+} transported across a membrane depends on 1) transporter density in the membrane, 2) affinity for Ca^{2+} of the transporter and 3) the capacity of the transporter. For example, the Na^{+}/Ca^{2+} exchanger has a low affinity, but high capacity, resulting in transport of up to 5,000 ions per second (Carafoli et al., 2001). Such a transporter is useful when Ca^{2+} is present in high concentrations (e.g. as in seawater) and supply or removal rates of Ca^{2+} have to be high. Cell membrane calcium pumps, on the other hand have a high affinity, but low capacity, making it particularly suitable for transporting Ca^{2+} out of a medium or compartment with a low [Ca^{2+}] (Wang et al., 1992). Finally, transport rates can be affected by the presence of inhibitors, high intracellular [Ca^{2+}] (e.g. Pereira et al., 1993) or shortage of ATP (in case of e.g. Ca^{2+}-ATPase).

3.3 Inorganic carbon transport in foraminifera

Transport of inorganic carbon may be accomplished by bicarbonate-transporters. If seawater or metabolic CO_2 contributes to the inorganic carbon during calcification, diffusion rates across membranes would control the influx of inorganic carbon and thereby influence the rate of calcification. The diffusion rate is determined by the concentration gradient of CO_2, the membrane area over which CO_2 can diffuse, and the solubility of CO_2 in the membrane lipids. The concentration of CO_2 at the site of calcification or in internal reservoirs is determined by pH. Since foraminifera can control the pH in these compartments (Erez, 2003; Bentov et al., 2009; De Nooijer et al., 2009a; Glas et al., 2012), they can produce large CO_2 concentration gradients and hence promote the influx of DIC to the sites of calcification. The flux of ions can also be calculated from calcification rates, which is discussed in section 4.

In case of intracellular storage of ions, calcium and DIC are unlikely to be stored as free ions. Because the cytosol has very low concentrations of free Ca^{2+} and DIC, the cell volume will control the number of ions available for calcification. For the DIC-reservoir (if present) the additional problem is that CO_2 can easily diffuse across cell membranes and subsequent re-equilibration would thus result in net leakage of carbon out of the DIC-reservoir. To overcome this problem, DIC must be sequestered by mechanisms such as elevating the pH in the reservoir. Because there are usually no crystallites visible within the cells of hyaline species, Ca and DIC are likely sequestered together as non-crystalline $CaCO_3$ (i.e. amorphous calcium carbonate or ACC). Such a possibility may have consequences for the minor and trace element composition of the calcite precipitated, since it is known that formation of high-Mg calcite is accompanied by the formation of an amorphous precursor phase (Raz et al., 2000).

374 Regardless of the process concentrating Ca^{2+} and DIC from seawater, each would produce a
375 supersaturated solution at the site of calcification, with reduced levels of crystal inhibitors that
376 occur naturally in seawater (e.g. Mg^{2+} and PO_4^{2-}). The Ca^{2+} and CO_3^{2-} may form spontaneous
377 $CaCO_3$ crystals, but the specific morphology of foraminiferal chambers show that nucleation
378 and crystal growth is a tightly controlled process.
379
380 **4. Nucleation of calcification**
381
382 **4.1 Crystal nucleation energy and critical size**
383 Precipitation of a crystal from a solution occurs when free energy of the precipitate is lower
384 than that of the solution. Nucleation of a crystal requires even more energy since ions at the
385 surface of a crystal add to the free energy of the solid phase. This is caused by the fact that
386 ions at the surface of a crystal are not bound on all sides to other ions. The resulting
387 'interfacial energy' requires the formation of metastable clusters of a critical size to start
388 crystal growth (Figure 5). The interfacial free energy between the cluster and a solution is
389 usually larger than that between the cluster and a solid substrate, resulting in crystal
390 nucleation at solid surfaces rather than within the solution itself (De Yoreo and Vekilov,
391 2003). If the atomic structure of a substrate matches a particular plane of the nucleating phase
392 (e.g. calcite or aragonite), the interfacial free energy is reduced and nucleation is promoted
393 (De Yoreo and Vekilov, 2003).
394 In the case of nucleation of $CaCO_3$, presence of negatively charged groups at regular intervals
395 at the site of calcification may be able to bind Ca^{2+} and pre-form a part of the $CaCO_3$ lattice.
396
397 *Figure 5: relation between free energy changes (Δg) as a function of pre-nucleation sphere*
398 *(r), where Δg_s is the surface term and Δg_b the bulk term. The sum of Δg_s and Δg_b is the free*

energy barrier that can only be overcome by the formation of a nucleation sphere with a critical size (r_c). Biological control over crystal nucleation is often aimed at lowering of this energy barrier and can be achieved by increasing the concentrations of the solutes or the presence of an organic template.

4.2 Organic templates and nucleation of $CaCO_3$ in foraminifera

During biomineralization in foraminifera calcium carbonate nucleates at the site of calcification, likely involving an organic template. In all Rotaliid foraminifera, chamber formation starts with delineation of a finite environment that encompasses an inner chamber volume from the surrounding medium (Angell, 1979; Bé et al., 1979; Hemleben et al., 1986; Spero, 1988; Wetmore, 1999). Cytoplasmic activity by formation of a dense pseudopodial network transports vacuoles, mitochondria and organic particles to a defined zone in which the so-called Organic Primary Envelope, Primary Organic Lining, Anlage or Primary Organic Membrane (POM) is formed (e.g. Banner et al., 1973; Hemleben et al., 1977; Spero, 1988; not to be confused with inner and outer organic linings, nor with the outer protective envelope or cytoplasmic envelope: see section 4). The term POM is often used but may be confusing (Erez, 2003) since these organic templates are not technically membranes. Therefore, we recommend following the suggestion of Erez (2003) to rename the POM as the Primary Organic Sheet (POS). In a number of benthic species, the POS consists of unbranched polysaccharides such as glycosaminoglycans (Hottinger and Dreher, 1974; Langer, 1992). Proteins are also present in the organic lining of foraminifera, sometimes forming different classes based on their amino acid composition (Robbins and Brew, 1990). King and Hare (1972) showed that amino acids make up 0.02-0.04% of the weight of the calcite and that composition among planktonic species varies greatly. Interestingly, the largest compositional difference coincides with the planktonic foraminifera spinose/ non-spinose divide (King and

Hare, 1972), but differences in amino acid composition are also manifest at lower taxonomic levels (Robbins and Healy-Willliams, 1991).

The organic matrix of the benthic *Heterostegina depressa* is shown to contain an EDTA-soluble and -insoluble fraction (Weiner and Erez, 1984). The insoluble fraction contains over-sulphated glycosaminoglycans and a small portion of non-polar proteins, forming the inner organic lining. The soluble fraction contains a number of proteins containing amino acids with acidic residues. Polar groups in both fractions may be involved in biomineralization since they may bind Ca^{2+} ions and thereby overcome the free energy barrier (Figure 5). If such groups are regularly spaced, they may help nucleation further by placing the Ca^{2+} ions in a regular grid with just enough space for the CO_3^{2-} ions to fit in between them. To test this hypothesis, the tertiary structures of the biomolecules (e.g. proteins and saccharides) that are involved in $CaCO_3$ nucleation need to be analyzed.

The presence of polysaccharides and proteins has led to the hypothesis that the POS has two functions in the process of calcification. The carbohydrates may form a structure determining the overall shape of the new chamber. The proteins associated with the polysaccharides, on the other hand, form the 'active' part of the POS by providing charged sites for nucleation of $CaCO_3$ (Towe and Cifelli, 1967). Since the chemical composition of the POS varies between species (Banner et al., 1973), its role in nucleation of calcium carbonate may differ between foraminiferal species (Bé et al., 1979; Hemleben et al., 1986; Spero, 1988; Wetmore, 1999). In some benthic species, the POS coincides with the location of the pores prior to calcification (Wetmore, 1999), suggesting that there are structural differences in the POS within a single chamber that determine where calcite does and does not nucleate. In planktonic species such as *Globorotalia truncatulinoides* and *G. hirsuta*, calcification begins in small nucleation zones at finite locations across the POS, where calcite forms centers of crystal growth that interlock to form the initial calcified chamber (Towe and Cifelli, 1967; Angell, 1979; Bé et al., 1979;

449 Hemleben et al., 1986). A similar pattern has been observed in *Orbulina universa*, where
450 small islands of calcite form on the POS, followed by calcite island fusion to produce the
451 spherical chamber (Spero, 1988).
452 Nucleation (and subsequent crystal growth) is also determined by the physico-chemical
453 conditions at the site of calcification. These conditions are only partly known in benthic
454 species (e.g. Erez, 2003; Bentov and Erez, 2005) and have only been modeled in planktonic
455 species (Zeebe et al., 1999; Zeebe and Sanyal, 2002). The volume between the crystal surface
456 and the shielding cytoplasmic envelope or pseudopodial network is extremely small, limiting
457 interpretation from light microscopic observations. However, TEM images of initial
458 calcification in *Orbulina universa* and other planktonic species suggests the privileged space
459 between rhizopodia and calcifying surfaces may be <10 nm (Bé et al 1979; Spero 1988).
460 Little is known about the chemical composition of the fluid from which $CaCO_3$ nucleates, but
461 high concentrations of Ca^{2+} and CO_3^{2-} need to be actively maintained, while the $[Mg^{2+}]$ needs
462 to be reduced to satisfy observations and ensure calcification (Zeebe and Sanyal, 2002).
463 Elevated pH at the site of calcification would promote the conversion of CO_2 and HCO_3^- to
464 CO_3^{2-}, thereby enhancing $CaCO_3$ nucleation and growth. Elevated concentrations of Mg^{2+}
465 around the POS in *Pulleniatina obliquiloculata* (Kunioka et al., 2006) may indicate that in
466 this species, the composition of the calcifying fluid is different during the first stage of
467 chamber formation, possibly due to a different rate or efficiency of the process that locally
468 reduces $[Mg^{2+}]$ vs $[Ca^{2+}]$. The participation of a small volume of seawater at the beginning of
469 chamber formation may explain the elevated Mg in the first calcite precipitated, although this
470 pattern does not hold for other planktonic species (e.g. such as *Orbulina universa*; Eggins et
471 al., 2004) where the lowest Mg/Ca ratios are associated with the intrashell zone that
472 corresponds to the POS. The above observations of inter species differences in chamber wall

elemental composition underscore the need to unravel the mechanisms controlling test calcification.

5. Chamber growth

After initial crystal nucleation, calcification proceeds by addition of calcite on both sides of the POS. Additional layers of $CaCO_3$ are added on top of pre-existing chamber calcite during each chamber formation event in perforate foraminifera (Reiss, 1957; 1960; Bé and Hemleben, 1970; Erez, 2003). Together, the primary and secondary layers of calcite are termed 'lamellar' calcite (Erez, 2003). Most observations on calcification are based on the first stage of chamber formation in which a thin-walled chamber is produced within 1-3 hours (Spero, 1988). Subsequent thickening of the chamber wall proceeds during the next 24-48 hours until a new chamber is formed. Thickening of earlier formed chambers occurs by addition of a calcite layer with each new chamber formation event (e.g. Bentov and Erez 2005, Nehrke et al., 2013). Future studies will need to show whether the timing of the start and end of chamber formation and thickening of previously formed chambers are coincidental, or whether thickening is a continuous process.

Future biomineralization research should also take into account the possibility that cellular controls on calcification may vary over time and location across the foraminifera shell. An example of the potential complexity and diversity of calcification within one specimen is provided by Bentov and Erez (2005). Their research demonstrated that the benthic *Amphistegina lobifera* recovering individuals produce at least three types of calcium carbonate: elongated, intracellular birefringent granules with a high magnesium and phosphorus content, extracellular microspheres with a high Mg concentration and extracellular spherulites with a low Mg content. These spherulites represent the lamellar

calcite while the microspherulites represent the initial presipitation over the POS in *A. lobifera.*

During chamber formation, ions could be supplied to the site of calcification (SOC) from internal reservoirs (Figure 3, Table 1) or by transport from the surrounding seawater. The latter can be accomplished by transmembrane ion transporters (section 2), by direct exchange of the calcifying fluid with seawater and/ or by diffusion from ambient seawater. The inner and outer surfaces of newly formed chambers of the benthic *Heterostegina depressa* are covered by thin layer of cytoplasm (Spindler, 1978), suggesting the SOC may be separated from the surrounding medium. In a number of studies (Angell, 1979; Bé et al., 1979), a fan-like arrangement of the pseudopodial network is observed in a zone outside the site of calcification. Although the relation between this arrangement and calcification remains to be investigated, it is likely to play a role in biomineralization since this dense network is not observed between chamber formation events. Also in the planktonic species *G. hirsuta* and *G. truncatulinoides*, calcification proceeds adjacent to a cytoplasmatic envelope (or outer protective envelope) that may play a role in maintaining SOC integrity and shape, and promoting initial calcification (Bé et al., 1979). In the benthic *Ammonia* sp., a pH gradient of >2 pH units is observed across several μm distance and is maintained for hours between the site of calcification (De Nooijer et al., 2009a) and the specimen's microenvironment (Glas et al., 2012). These observations suggest that in *Ammonia* sp., the SOC is separated from the outside environment. Spero (1988) on the other hand, presented transmission electron micrographs that showed the site of calcification in *O. universa* is not shielded by a continuous membrane. Nehrke et al. (2013) recently suggested that the site of calcification in *Ammonia aomoriensis* is largely closed from the surrounding medium, but that a small percentage of the fluid at the SOC is derived from leakage of the cell membranes separating it from the outside medium, explaining observed Mg/Ca for the species studied.

523 The extent to which the site of calcification is open or closed, in combination with the
524 presence or absence of intracellular ion reservoirs, is an important unknown in understanding
525 foraminiferal calcification (Figure 6). For example, a site of calcification that is physically
526 separated from the surrounding seawater, together with the absence of intracellular ion
527 reservoirs, prescribes the need for transmembrane ion transporters (e.g. Ca^{2+}-APTase; section
528 II) that selectively pump ions from seawater to the SOC. A SOC that is open, on the other
529 hand, will experience relatively high concentrations of Mg and require an active Mg^{2+}-
530 removal mechanism.
531
532 *Figure 6: summary of the most important parts of the calcification mechanism in*
533 *foraminifera, including Ca-ion transport, active Mg-removal and contribution from internal*
534 *reservoirs. See text for description of the individual processes.*
535
536 Potential ion transport pathways to the site of calcification can be constrained from
537 calcification rates during chamber formation. It is important to distinguish between the overall
538 growth rate of a foraminifer and calcite precipitation rate during biomineralization. The
539 difference between these processes results from the episodic nature of growth (chamber
540 addition) in foraminifera. Some planktonic species have been reported to increase the weight
541 of their shell by 13-15% a day (*G. sacculifer*; Erez, 1983), but this may vary with
542 environmental conditions (Ter Kuile and Erez, 1984 and references therein). Secondly,
543 chamber addition rates vary over a foraminifer's lifetime, decreasing as the individual ages
544 (Ter Kuile and Erez, 1984). Calcite precipitation rates during chamber addition, on the other
545 hand, are much higher and vary between 0.4-0.9 µg/h in the planktonic foraminifer *G.*
546 *sacculifer* (Anderson and Faber, 1984), 0.06-0.32 µg/h in *O. universa* (Lea et al., 1995) and
547 ~10 µg/h in the benthic *A. tepida* (De Nooijer et al., 2009b). Since such rates are rarely

548 quantified, it is difficult to generalize these values to other species or other conditions.
549 Moreover, calcite precipitation rates can be variable between day and night calcification
550 periods (Erez, 1983; Spero, 1988; Lea et al., 1995). Since incorporation of some elements
551 may depend on precipitation rate (e.g. DePaolo, 2011), it is necessary to quantify these rates
552 across a diurnal time frame when chamber formation is occurring in order to assess the
553 kinetics of element incorporation and thereby proxy-relationships.
554 Mitochondrial activity may play an important role at the site of calcification and thereby
555 affect trace element incorporation. Besides providing energy, mitochondria pump cytosolic
556 Ca^{2+} and Mg^{2+}, and therefore modulate the cell's [Ca^{2+}] and [Mg^{2+}] (Carafoli et al., 2001).
557 This may be particularly important during calcification when the concentration of these ions
558 increases locally. Spero (1988) shows that calcification in *O. universa* around the POS is
559 associated with pseudopodia containing mitochondria, and hence possibly modulate [Mg^{2+}] at
560 the SOC. Similar results can be found in Bé et al (1979) for *Globorotalia truncatulinoides*.
561 Bentov et al (2009) discuss the possible role of mitochondria in producing metabolic CO_2 that
562 eventually accumulate in the alkaline vacuoles as DIC.
563 Photosynthesis by symbionts may also affect calcification rates. The relative concentrations of
564 DIC species are influenced by symbiont photosynthesis and CO_2-uptake during the day (or
565 release in the dark) and the resulting diurnal differences in microenvironment pH (Jørgensen
566 et al., 1985; Rink et al., 1998; Köhler-Rink and Kühl, 2000; 2005), thereby influencing uptake
567 and availability of inorganic carbon species. In some large benthic foraminifera (Wetmore,
568 1999), the symbionts are positioned near the POS prior to calcification, suggesting that their
569 activity could enhance calcification. Elimination of symbionts in *G. sacculifer* resulted in
570 reduced chamber formation rates and early gametogenesis or death of the foraminifera (Bé et
571 al., 1982). Reseeding the aposymbiotic foraminifera with symbionts from donor specimens
572 produced individuals that continued to add chambers and mature at a normal rate. These data

suggest that symbiont photosynthesis is critical to both nutrition and chamber calcification. Elevated light intensity promotes growth in *G. sacculifer* (Caron et al., 1982) but not in the benthic foraminifera *Amphistegina lobifera* in which both photosynthesis and calcification are optimal at relatively low light intensities that are found at 20-30 m water depth (Erez 1978, Ter Kuile and Erez, 1984).

Ter Kuile et al. (1989a), on the other hand, suggested that symbionts and foraminifera compete for inorganic carbon. Erez (1983) and Ter Kuile et al. (1989b) showed that inhibition of photosynthesis in both planktonic and benthic species by the photosystem II inhibitor DCMU, does not affect calcification rates and suggested that it is not photosynthesis itself, but rather light which directly promotes calcification. Finally, Ter Kuile et al (1989a) have shown that there is competition for CO_2 between the symbionts and their host in the benthic foraminiferan *A. lobifera*. Clearly, the relationship between symbioses and foraminifera calcification requires additional study.

Pore formation provides important information on foraminiferal biomineralization. In species producing macropores, we observe a pore plate that is continuous with the POS and separates the cytoplasm from the outside medium (Hemleben et al., 1977). In benthic, symbiont-bearing species, symbionts can be found in close proximity to the pores (e.g. Lee and Anderson, 1991) suggesting that respiratory gases such as CO_2 and O_2 may be able to diffuse through the pore plates. In symbiont-barren species, diffusion of gases between cytoplasm and environment could be enhanced by the permeability of a pore plate. Some have suggested that dissolved organic matter may be taken up through the pores in the benthic *Patellina* (Berthold, 1976). In *G. sacculifer*, pseudopodia appear to penetrate through the pore plates (Anderson and Bé, 1976). Pores in the benthic species *Patellina corrugata* have been reported to exist from the beginning of chamber formation (Berthold, 1976) and pores are observed in the *O. universa* sphere once initial calcification has locked in the spherical morphology of the chamber

598 (Spero, 1988). Some species of planktonic foraminifera have micro- instead of macropores
599 (often in species with secondary apertures; *Globigerinata glutinata*, *Candeina nitida*), ranging
600 from 0.3-0.7 μm (Brummer and Kroon, 1988). These micropores do not appear to have a pore
601 plate, and their function, formation and morphology is less well understood than those for
602 macropores.
603
604 **6. Overgrowth and encrusting**
605 The primary and secondary layers of calcite in perforate foraminifera are together referred to
606 as 'ontogenetic' or 'lamellar' calcite (Erez, 2003). Additional $CaCO_3$ can be present as
607 ornamentations (pustules, spines, ridges, tooth plates, etc.) or as layers of calcite covering the
608 whole test (crust or gametogenic (GAM) calcite). Whereas ornamentation is present
609 throughout the entire life cycle of a foraminifer (Hemleben, 1975), GAM calcite is exclusive
610 to planktonic foraminifera and is added after the last chamber is formed and just prior to
611 meiotic division of the nucleus and gametogenesis.
612 In some planktonic species, a calcite crust can be formed after formation of the final chamber
613 (Bé and Ericson, 1963; Bé and Lott, 1964; Bé, 1965; Bé and Hemleben, 1970; Olsson, 1976).
614 The morphology of this calcite is markedly different from that of either ontogenetic or GAM
615 calcite and its element and isotopic composition can differ from that of the ontogenetic calcite
616 because it forms under different environmental conditions of temperature and/or salinity. For
617 instance, crust Mg/Ca is generally lower than that of ontogenetic calcite in *Globorotalia*
618 *truncatulinoides* (Duckworth, 1977) and *Neogloboquadrina dutertrei* (Jonkers et al., 2012).
619 These lower element concentrations are partly a consequence of conditions deeper in the
620 water column (i.e. lower temperature), but it should be noticed that the observed partitioning
621 for Mg indicates that crust calcification is a biologically controlled process. Interestingly,

622 Nürnberg et al. (1996) found that crusts formed in culture can have a higher Mg/Ca than the
623 ontogenetic calcite.
624 In a number of species such as *G. sacculifer*, gametogenesis is preceded by the production of
625 a layer of calcite covering spine holes and the terrace-like structures of inter-pore rims (Towe
626 and Cifelli, 1967; Bé, 1980; Hemleben et al., 1985; Brummer et al., 1987). This GAM calcite
627 veneer gives the foraminifera a smooth appearance by covering the rough topography of the
628 shell surface and it has been suggested that it is enriched in some trace elements compared to
629 the ontogenetic calcite (Hathorne et al., 2009). Whether this observation holds for all
630 foraminifera forming GAM calcite, however, remains to be investigated.
631 From the perspective of biomineralization, variability in the types of $CaCO_3$ that are formed
632 may indicate that foraminifera do not have one single way to produce shell calcite. Rather, the
633 physiological tools to achieve calcite precipitation as discussed in sections 2 and 4, are likely
634 used in different combinations by different species of foraminifera. Moreover, the variability
635 in calcite within single specimens suggests a degree of flexibility of these physiological tools
636 even within single species. Identification of seawater vacuolization, transmembrane ion
637 transport, nucleation promoting organic templates, etc. across species and their contribution to
638 calcification within a foraminifer's life time are critical aspects of foraminiferal biology and
639 keys to understanding foraminiferal biomineralization from a mechanistic perspective.
640

641 **7. Future directions**

642 A complete mechanistic description of foraminiferal biomineralization and chamber
643 formation does not yet exist. Hence, the biological and environmental interplay that controls
644 the element composition and isotope fractionation of chamber calcite is only partly
645 understood. Literature on foraminiferal calcification is both qualitative and quantitative but on

646 occasion, contradictory. This leaves us with a number of outstanding questions that need to be
647 addressed in order to move this area of foraminifera biology forward. These include:
648
649 1. Which foraminiferal species use vacuolized seawater as the primary source for
650 calcification and which use transmembrane transport of Ca^{2+} and DIC during
651 calcification? The investigation into the transport of ions to the site of calcification
652 may be solved by answering a number of more practical questions, including:
653 - What is the relation between transmembrane transport and vaculization on the one
654 hand, and production of intracellular calcium and/ or carbon reservoirs on the other
655 hand?
656 - What is the biochemical basis of these processes? Which transmembrane transporters
657 are involved (e.g. Ca-ATPases, proton-Ca^{2+} antiporters)? By which mechanism is
658 inorganic carbon concentrated (e.g. involvement of Carbonic Anhydrase)?
659 - When characterized, can these (transport) mechanisms explain observed element
660 incorporation and isotopes fractionations. If yes, can these mechanisms explain
661 foraminiferal chemistry for (all) these elements and isotopes *at the same time*?
662 - Is there a general difference between planktonic and benthic species in production of
663 vacuolized seawater, internal reservoirs and/or direct ion transport?
664 - Do foraminifera employ both mechanisms to calcify and if yes, what is the balance
665 between these two pathways?
666 2. What is the tertiary structure of the organic matrix/ matrices (e.g. POS, organic
667 linings) involved in biomineralization? Which compounds help to lower the free
668 energy barrier, thereby promoting calcite nucleation? When identified, do these
669 organic compounds have an impact on the partition coefficient of elements and
670 fractionation of isotopes at the first stage of chamber formation?

3. To what extent is the site of calcification in contact with surrounding seawater? If seawater directly contributes (part of) the ions for calcification, can this source explain observed fractionation factors and partition coefficients?
4. What is the role of mitochondria in calcification? Do mitochondria (help to) regulate the Mg/Ca at the site of calcification?

Finally, a more detailed understanding of foraminiferal biominiralization will also allow researchers to compare calcification strategies accross marine calcifiers. Compared to foraminifera, biomineralization in corals (Al-Horani et al., 2003; Sinclair and Risk, 2006; Venn et al., 2013), coccolithophores (Marsh, 2003; Taylor et al., 2011; Ziveri et al., 2012; Bach et al., 2013), gastropods (e.g. Nehrke et al., 2011) and bivalves (Nudelman et al., 2006; Nehrke et al., 2012; Shi et al., 2013) are understood in greater detail. Identification of differences and similarities between these marine calcifying taxa will allow studying (convergent) evolutionairy patterns, help to understand differences in their response to (future) environmental perturbations and facilitate comparison of paleoceanographic information obtained across taxa.

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Figure 1

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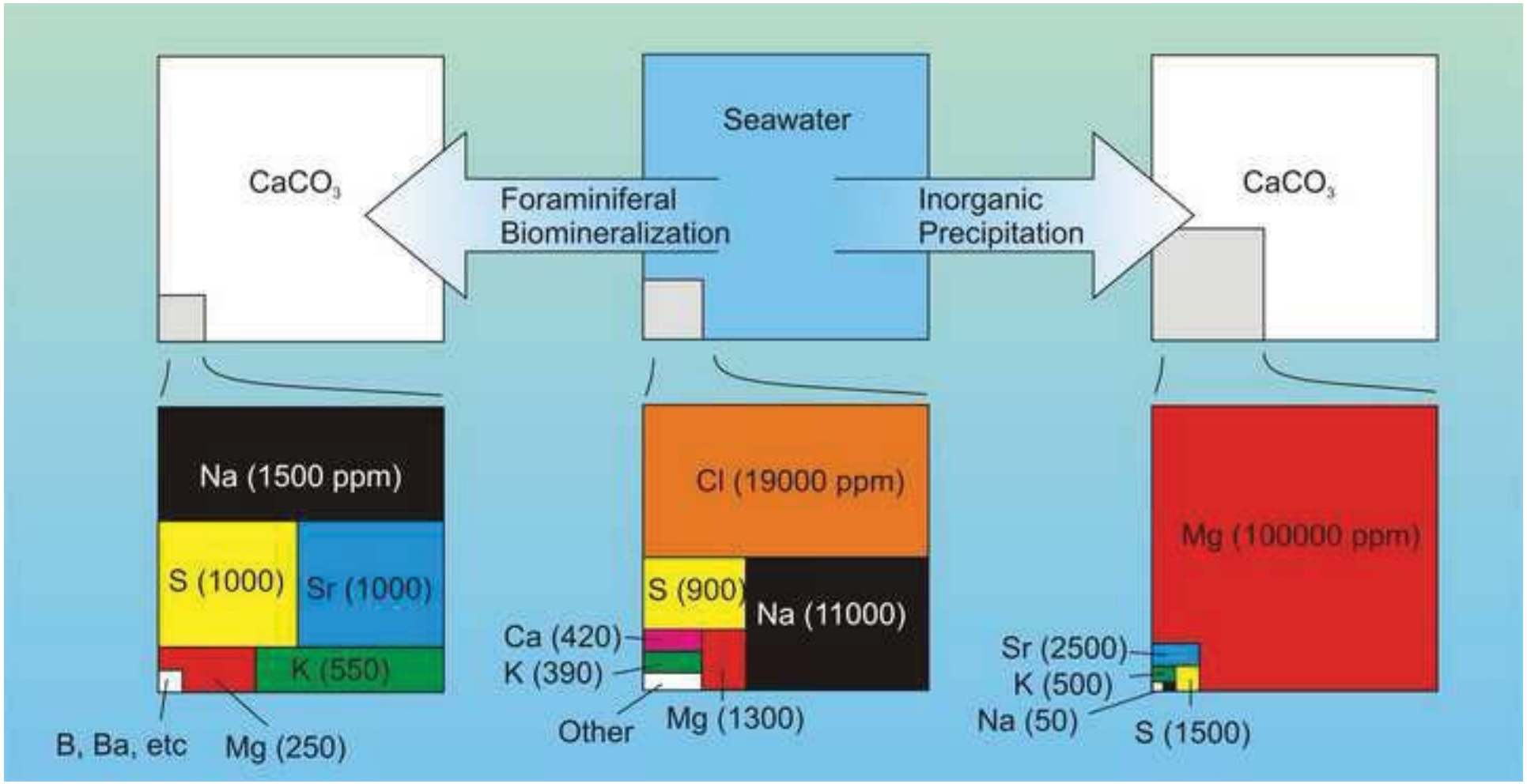

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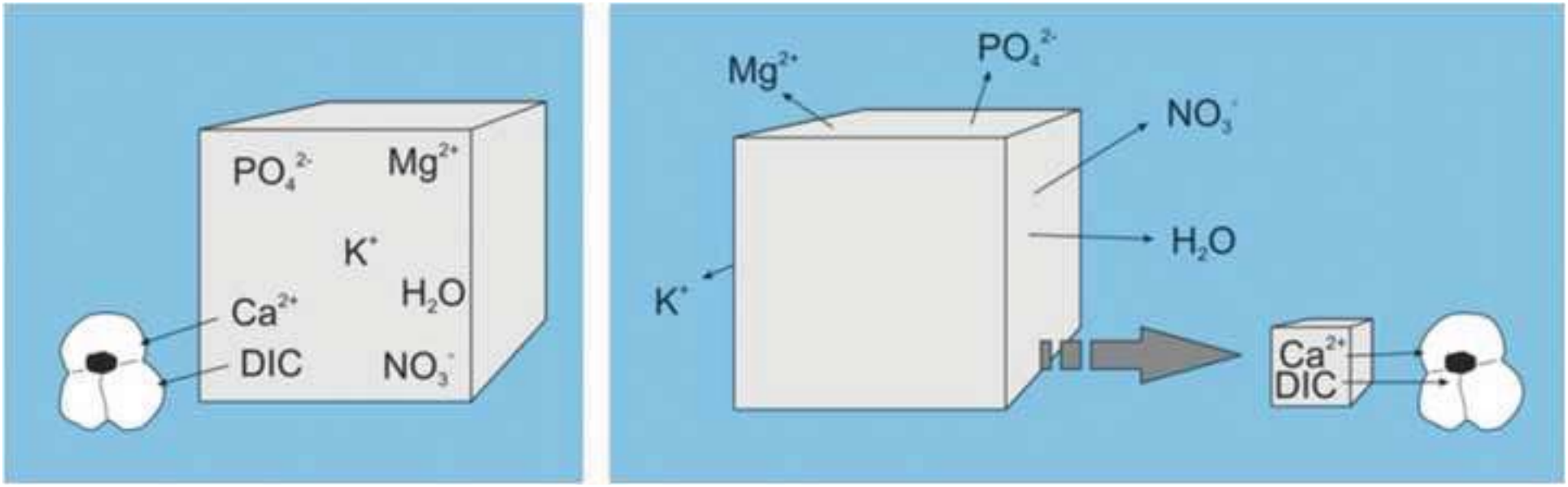

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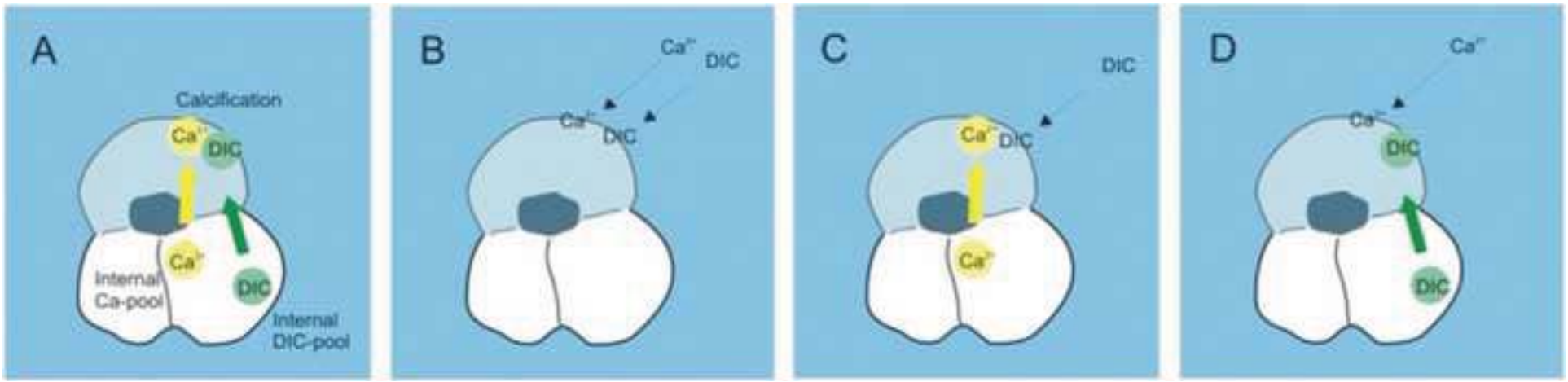

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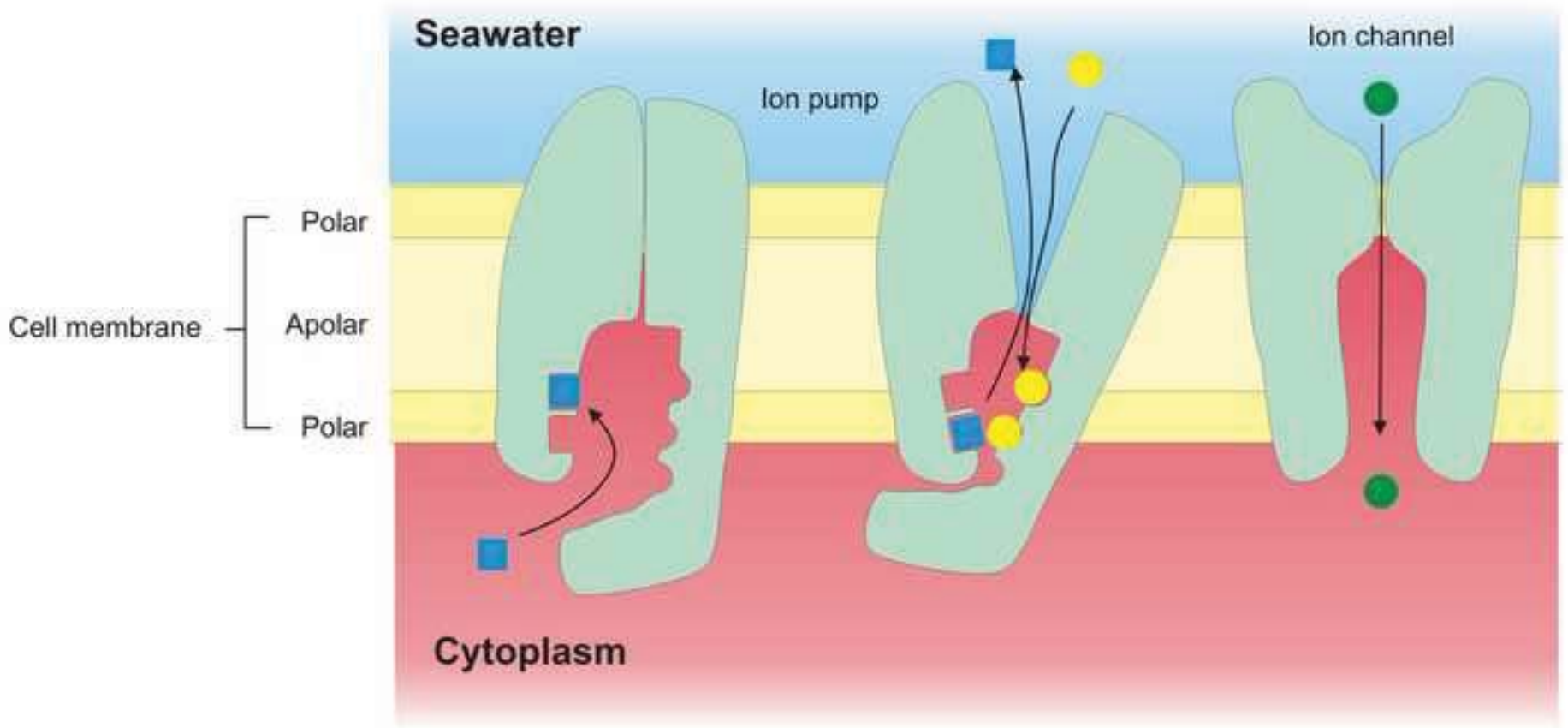

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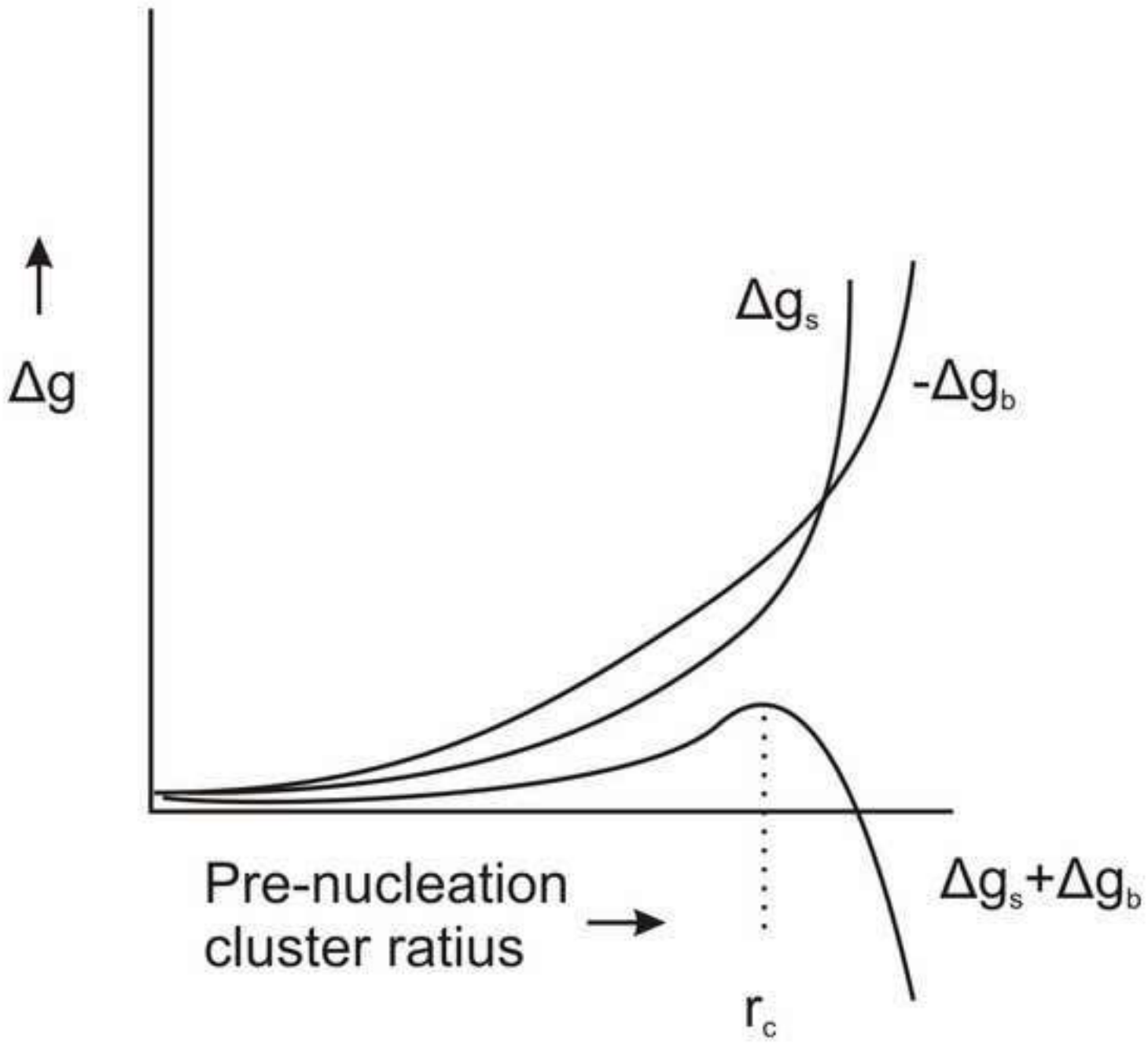

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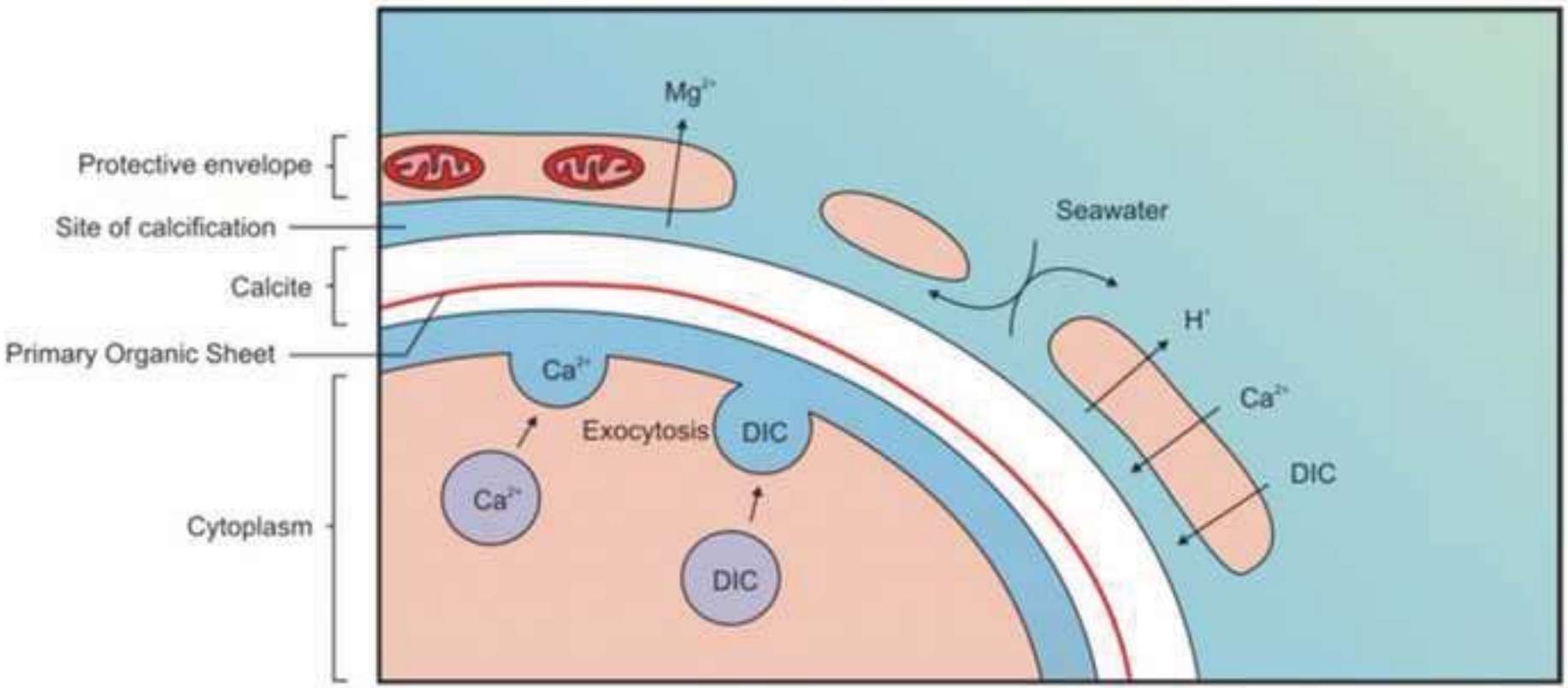

Table 1: Studies discussing internal reservoirs in perforate foraminifera.

	Ca^{2+} reservoir	DIC reservoir
Large volume reservoirs	Anderson and Faber (1984) Erez (2003) Toyofuku et al. (2008)	Ter Kuile and Erez (1987; 1988; 1989b; 1991) Erez (1978; 1982) Bentov et al. (2009)
No or small volume reservoirs	Angell (1979) Lea et al. (1995) Nehrke et al. (accepted)	Angell (1979)