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**Physiology of  
*Ammonia tepida***

L. J. de Nooijer et al.

# Physiological controls on seawater uptake and calcification in the benthic foraminifer *Ammonia tepida*

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## Abstract

During the last decades conceptual models describing the calcification pathway of foraminifera and its physiological controls have been developed. These models are derived by combining data of tracer experiments and microscopic observations obtained from different species. Although vital for understanding their calcitic isotopic and trace elemental composition, direct observational evidence on e.g. seawater vacuolization and intracellular Ca-cycling is lacking for most species. To analyse the relation between seawater uptake and calcification, we incubated juveniles of the cosmopolitan benthic, intertidal foraminifer *Ammonia tepida* with various fluorescent probes. Visualizing the membranes of endocytosed vesicles was achieved by incubating specimens with the dye FM1-43, while Ca ions in the calcification vesicles were detected by the  $\text{Ca}^{2+}$ -indicator Fluo3-AM. Uptake of fluorescent latex-beads (0.5  $\mu\text{m}$  diameter) and subsequent transport to the site of chamber formation provided additional evidence that endocytosis is related to the calcification pathway and not merely involved in membrane cycling. Our results show for the first time that endocytosis of seawater is part of the calcification process in *Ammonia tepida*. Data on the intracellular calcium ion-cycling allowed for calculating a preliminary cellular Ca-budget during foraminiferal calcification.

## 1 Introduction

Fossil foraminiferal calcite carries imprints from past oceanic conditions since incorporation of many trace elements and stable isotopes is correlated to specific environmental conditions. Among the more popular tools, the  $\delta^{18}\text{O}$  of foraminiferal calciumcarbonate has been widely used to reconstruct seawater temperature (after correction for the global ice volume: Duplessy et al., 1970). More recently, foraminiferal Mg/Ca ratios have also been adopted to reconstruct past marine temperatures and are in good agreement with those reconstructed from calcitic  $\delta^{18}\text{O}$  (Lear et al., 2000). Calibrations

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for these relationships are based on culturing (e.g. Nürnberg et al., 1996) and field studies (e.g. Elderfield and Ganssen, 2000). Data presented in these studies have shown large differences to data from inorganic precipitation experiments (e.g. Mucci, 1996; Morse and Bender, 1990) and therefore imply that foraminiferal calcite is biologically controlled. This control is also reflected by differences in trace elemental and isotopic composition within (Hathorne et al., 2003; Kunioka et al., 2006) and between species (Blackmon and Todd, 1959; Lear et al., 2000). In addition, for some benthic species it is shown that there is an ontogenetic offset in their calcitic  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  (Schmiedl et al., 2004). The sources of these biological variabilities are commonly termed the “vital effect” and need to be accounted for to improve the accuracy of reconstructions based on foraminiferal proxy-relationships (Erez, 2003).

Besides improving foraminiferal-based paleoreconstructions, another reason that underscores the necessity for analyzing their calcification pathway is the importance of the response of foraminifera to ocean acidification. Culture experiments have shown that shell weights of foraminifera decrease with decreasing carbonate ion concentration of seawater (Bijma et al., 1999). Planktonic foraminifera from sediments deposited over glacial-interglacial cycles (Barker and Elderfield, 2002) and those deposited from pre-industrial to modern times (Moy et al., 2009) apparently confirm the trend found in cultured specimens. With a decreasing oceanic pH from a pre-industrial value of 8.2 to approximately 7.8 by the end of the century, changes in the amount of calcite precipitated or in the export of  $\text{CaCO}_3$  from the surface to the bottom of the ocean may have consequences for the capacity of the ocean to take up atmospheric  $\text{CO}_2$  (Heinze, 2004; Orr et al., 2005). To predict the response of foraminifera to ocean acidification, the sensitivity of the calcification pathway to changes in seawater alkalinity and pH need to be quantified.

Foraminifera precipitate their calciumcarbonate from modified seawater. Between uptake of seawater and calcite precipitation, the isotopic and trace elemental composition of the calcifying fluid is strongly modified (Erez, 2003). In particular, the original magnesium content of the seawater is heavily reduced by foraminifera, resulting in

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a low Mg-content (2–20 mol %  $\text{MgCO}_3$ ) of most species' calcite (Blackmon and Todd, 1959; Bentov and Erez, 2006). It is therefore assumed that foraminifera have  $\text{Mg}^{2+}$ -channels or pumps that remove magnesium from vacuolized seawater. An internal pool with a very low Mg/Ca ratio then remains and is used for calcite precipitation (Erez, 2003; Bentov and Erez, 2006). During calcification, the pH is increased to  $\geq 9.0$ . This results in the conversion of bicarbonate into carbonate and thereby increases the calcite saturation state during chamber formation greatly (de Nooijer et al., 2009). The speed at which foraminifera can produce a new chamber suggests that all or most of the  $\text{Ca}^{2+}$  and inorganic carbon must be accumulated within the individual prior to chamber formation (Erez, 2003). This could be stored either as crystalline  $\text{CaCO}_3$  (as in miliolids: Hemleben et al., 1986; Debenay et al., 1996), in separate pools (Anderson and Faber, 1984; Ter Kuile et al., 1989) or as amorphous  $\text{CaCO}_3$  (as suggested by Erez, 2003; Bentov and Erez, 2006).

To understand trace elemental/isotopic fractionation in foraminiferal calcite, a number of conceptual models of the calcification pathways have been developed that are based on experimental results and a suite of assumptions (Grossman, 1987; Ter Kuile et al., 1989; Ter Kuile and Erez, 1991; Elderfield et al., 1996; Erez, 2003). The ecological and physiological diversity among foraminiferal taxa spans symbiosis with and without photosynthetic organisms, benthic and planktic lifemodes, and precipitation of both calcitic and aragonitic test. This renders it very unlikely that the biomineralization process in foraminifera is similar among the taxa, which is well reflected in the substantial variety of Mg concentrations observed among foraminiferal  $\text{CaCO}_3$  (Bentov and Erez, 2006). Therefore, understanding biomineralization in foraminifera starts by obtaining all information on the calcification pathway from a single species, since it is not known to what extent observations from different species on e.g. seawater vacuolization can be generalized.

To put in perspective a number of recently obtained partition coefficients (particularly for Mg) for the benthic foraminifer *Ammonia tepida* and environmental controls on their fractionations (de Nooijer et al., 2007; Dissard et al., 2009) we chose to analyse

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various parts of their calcification pathway by using fluorescent probes and Confocal Laser Scanning Microscopy (CLSM). The obtained results are used to construct a basic model and calculate Ca and CO<sub>3</sub> budgets for calcification in *A. tepida*.

## 2 Methods

### 2.1 Collection and maintainance

Sediment containing foraminifera was collected at the intertidal flats near Dorum, Northwestern Germany in fall 2008. Upon return in the laboratory, material was sieved over a 1 mm-screen to remove the largest macrofauna. The remaining sediment was stored at 10°C and used to isolate living foraminifera. Before isolation, a small amount of sediment was sieved over a 250 µm-mesh and the remaining material was screened for individuals of *Ammonia tepida* containing bright yellow protoplasm. Isolated, living adults of *A. tepida* were incubated at 25°C in 3 ml of filtered seawater (0.2 µm) in petridishes with a glass bottom and were fed living *Dunaliella salina*. Every two days the medium was replaced and once a week new food was provided. Regularly, adults underwent asexual reproduction, resulting in 50–200 juveniles consisting of a megalo-spheric proloculus (diameter ≥ 40 µm). In the first week, these juveniles can grow a new chamber every day and were therefore selected to be incubated with several fluorescent probes and latex beads.

### 2.2 Fluorescent probes

Individuals with 3–5 chambers were incubated for 15 min with the fluorescent probe FM1-43 (Invitrogen, Molecular Probes), dissolved in dimethyl sulfoxide (DMSO) and diluted with filtered seawater to a final concentration of 10 µM. The final concentration of DMSO in the seawater was <5%. After incubation, individuals were washed several times with filtered seawater and immediately placed under a Leica TCS CLSM. Exicta-

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tion of the specimens was accomplished by a Kr/Ar laser, wavelength tuned to 488 nm. Emission wavelengths between 600 and 650 nm were recorded.

Similar juveniles were placed in seawater with dissolved latex beads (Invitrogen, Molecular Probes). The beads had a diameter of 0.5  $\mu\text{m}$ , an excitation optimum of 575 nm and an emission optimum of 610 nm. Individuals were allowed to take up the beads for 24 h, washed with seawater and scanned under the CLSM after excitation at 568 nm.

Finally, juveniles were incubated with the fluorescent  $\text{Ca}^{2+}$ -indicator Fluo3-AM (8  $\mu\text{M}$ : Toyofuku et al., 2008) for 24 h. To track the  $\text{Ca}^{2+}$  utilization during chamber formation, individuals that were starting to make a new chamber were washed several times and placed under the CLSM and excited by 488 nm every minute until chamber formation was finished.

### 3 Results

#### 3.1 Cell membranes-vacuolization

After 15 min of incubation with FM1-43, only cell membranes of the pseudopodia and around the aperture are stained. In the hours following, fluorescence spreads throughout the individual, starting with membranes in the outer chambers. In a number of specimens, this reveals a quasi-circular organization of cell membranes in the final chamber (Fig. 1a,b). This structure consists of an outer and inner sphere, with a number of strands of membranes connecting them. The shape of the outer sphere sometimes resembles the form of the final chamber rather than a geometrical sphere. The spaces inbetween the membrane strands, as well as the space within the inner sphere appeared not to be filled with cytoplasm. Rather, the cytoplasm was confined to thin films bordered by labelled cell membranes. Occasionally, small vesicles (diameter app. 3  $\mu\text{m}$ ) are transported from the edge of the outer towards the inner sphere (Fig. 1c–f).

During chamber formation, fluorescence of membranes near the site of calcification

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shows that pseudopodia extend radially from the former aperture towards the new chamber wall. Around this new wall, these pseudopodia converge and form a zone of cytoplasm delining the site of calcification (Fig. 1g).

### 3.2 Fluorescent beads

5 In the hours preceding chamber formation, foraminifera form a protective cyst around themselves, in which many latex beads in the individual's vicinity are incorporated (Fig. 2). A number of beads, however, are ingested prior to cyst formation and circulate through all chambers of the specimen. During calcification, some of these beads are transported from the cytoplasm into the final chamber towards the new chamber wall  
10 (Fig. 2).

### 3.3 $\text{Ca}^{2+}$ during chamber formation

Before and during chamber formation in juveniles of *Ammonia tepida*, two types of fluorescence by Fluo3-AM are present. As reported before (Toyofuku et al., 2008), a weak background fluorescence exists throughout the cytoplasm, and in a number of  
15 chambers brightly fluorescent vesicles circulate through the cytoplasm. From the start until the end of chamber formation, a number of these bright vesicles are transported towards the site of calcification (Fig. 3). Over 90% of the  $\text{Ca}^{2+}$ -containing vesicles do not participate in chamber formation and remain in the cytoplasm after the new chamber is completed. In several individuals that were observed, the amount of such  
20 vesicles that are transported to the site of calcification in a new chamber does not exceed 20.

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## 4 Discussion

### 4.1 Seawater uptake

Theoretically, the internal Ca- and carbonate-pools from which foraminifera produce new chambers can be formed by transporting Ca-ions and dissolved inorganic carbon via trans-membrane transport from the medium into the cytoplasm (as in coccolithophores; Brownlee and Taylor, 2004). It has been reported, however, that their internal pools are essentially modified seawater (e.g. Erez, 2003). Our results are the first direct evidence that *A. tepida* vacuolizes seawater and that the internal pools containing  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$  are very likely modified seawater. The vacuolization of seawater and its participation in the calcification pathway were inferred from the following observations. The spherical organization of cell membranes in the final chamber and the transport of small vesicles (Fig. 1) strongly suggest that seawater vacuolization takes place. An alternative explanation for the occurrence of these vesicles could be membrane recycling unrelated to calcification. This, however, appears to be unlikely, because the fluorescent beads that cycle through the cytoplasm of incubated individuals (Fig. 2) can only be taken up by vacuolization. This also indicates that the observed endocytosis serves the purpose of taking up ions from seawater and is supported by two observations. First, some of the vesicles containing beads were transported from the cytoplasm into the final chamber towards the new chamber wall (Fig. 2). This indicates that these vesicles transport material, i.e. Ca and/or carbonate ions for calcification. In addition, transport of high-Ca vesicles to the site of calcification (Fig. 3) shows that the Ca necessary for calcification comes from within the individual. This shows that *A. tepida* uses endocytosis-vesicles to build up an internal Ca reservoir that is subsequently used for calcification: below we will use this mechanism to calculate a Ca-budget for this species.

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## 4.2 Ca-utilization during chamber formation

The observation of calcium-rich vesicles during chamber formation (Fig. 3) was also reported by Toyofuku et al. (2008), but the time-lapse recordings reported here allow estimating the fluxes of intracellular  $\text{Ca}^{2+}$  in relation to chamber formation. To do so, we propose a conceptual model for Rotallid foraminiferal chamber formation (Fig. 4). In this simplification, a small individual consists of one spherical chamber and is building a second, small chamber with the shape of half a sphere. Whether the first, inner chamber is really one chamber or consists of a number of chambers (as the individuals in Figs. 2 and 3), does not affect the volume ratios between the new and older chambers significantly. For simplicity, we assumed that pore volume (reducing the volume of calcite in a new chamber) and the amount of calcite precipitated as a layer onto older chambers (increasing the volume of calcite precipitated; Erez, 2003) are in the same range and therefore do not need to be corrected for. We also assume that all  $\text{Ca}^{2+}$  for this new chamber is located in an intracellular Ca-pool formed as described above. In our simplified model, the new chamber has a wall thickness of 3  $\mu\text{m}$ , as estimated from embedded and cross-sectioned specimens (results not shown here).

The volume of the calcite precipitated to form this new chamber equals  $1.33 \times 10^{-8} \text{ cm}^3 \text{ CaCO}_3$ . With a density of  $2.71 \text{ g/cm}^3$ , this new chamber contains  $3.60 \times 10^{-8} \text{ g}$  of  $\text{CaCO}_3$ . With 40 wt% Ca, this means that  $1.44 \times 10^{-8} \text{ g}$  (or  $3.6 \times 10^{-10} \text{ mol}$ ) of  $\text{Ca}^{2+}$  must be inside the foraminifer at the onset of chamber formation. Assuming that all calcium is ultimately derived from seawater and that 100% of the calcium taken up is utilized for calcification, a volume of  $3.50 \times 10^{-8} \text{ l}$  of seawater ( $[\text{Ca}^{2+}] = 10.3 \text{ mmol/l}$ ) is needed.

In our simplified model, the individual has a total volume of  $5.24 \times 10^{-10} \text{ l}$  (Fig. 4), excluding the volume of the new chamber. This means that this foraminifer has to process a volume of seawater that is app. 75 times its own volume to build up the entire internal Ca-pool. Assuming that an individual could use 100% of its cytoplasm to store Ca-vesicles, this Ca-pool should have an average Ca-concentration of 0.6 M. In a pre-

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vious study the intracellular Ca-pool of *A. lobifera* was estimated to contain  $\text{Ca}^{2+}$  with a concentration of 2–20 M (Erez, 2003). Considering the uncertainties in the methods and assumptions used, our results can be regarded as complementing the previous estimations. The Ca-concentration of the putative pool cannot represent free Ca-ions, because Ca-ATPases are not able to build up the respective Ca-gradient between cytosol and vesicle-interior (for a more detailed discussion see: Langer et al., 2006). If we would assume that the Ca-pool is basically seawater, i.e. Ca in an aqueous solution, the consequence is that the Ca-pool does not contain enough Ca-ions to produce a new chamber, implying that more Ca must be taken up during chamber formation. We have observed, however, that on average only 20 vesicles (with a diameter of 5  $\mu\text{m}$ ) were consumed in the production of a new chamber. Supposing that all  $\text{Ca}^{2+}$  necessary for the new chamber is stored in these 20 vesicles, its concentration would equal approximately 50 000 M. This estimation clearly shows that Ca cannot be transported to the site of calcification in vesicles containing seawater (i.e.  $\sim 10 \text{ mM Ca}$ ).

This conclusion only holds if most of the Ca used for a new chamber can be detected by our method. For example, it could be that most of the Ca-transport is through very small vesicles that bud off from the larger ones, but are invisible even when using confocal microscopy. Another possibility is that Ca is sequestered by specialized organic compounds in the Ca-pool that would lower the free calcium-concentration. Release, however, from these organics at the site of calcification should then be visible as a fluorescent zone at the new chamber wall, which was not observed. It may also be that most calcium is stored in the form of amorphous calciumcarbonate (ACC) prior to chamber formation. The Ca in amorphous  $\text{CaCO}_3$  would not bind to Fluo3 and thus not cause fluorescence to be emitted from the Ca-containing vesicles. Furthermore, the ACC would not be detectable by examining these individuals with crosspolarized light. The ACC-storage suggestion may fit with the recent discovery that stable prenucleation clusters can form in a supersaturated medium prior to calciumcarbonate precipitation (Gebauer et al., 2009; Pouget et al., 2009). Contrary to classical theory, loose Ca- and carbonate-ions may not directly participate in crystal growth, but rather calcite and

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aragonite grow by the addition of stable clusters of ACC. Foraminifera may be able to control these conversions precisely so that very little free calcium is observed to participate in chamber formation. In addition, the size of these ACC clusters may explain the observation that most foraminifera's tests consist of small crystallites (Debenay et al., 1996).

### 4.3 Implications for trace metal partitioning

Compared to most elements, magnesium is incorporated into the calcite of many foraminiferal species with a partitioning coefficient much lower than the one determined for inorganic calcite formation (e.g. Blackmon and Todd, 1959; Nürnberg et al., 1996; Rosenthal et al., 1997). For *Ammonia tepida*/A. *beccarii* the  $Mg/Ca_{\text{calcite}}$  is 1–10 mmol/mol (Allison and Austin, 2003; de Nooijer et al., unpublished data), suggesting that this species precipitates its calcite from an internal Ca-reservoir with a much lower Mg/Ca than that of seawater. The mechanism that discriminates  $Mg^{2+}$  from  $Ca^{2+}$  is not known, but our results imply that they are separated *after* mutual vacuolization. An obvious possibility is that  $Mg^{2+}$  is pumped out of the seawater vacuoles and transported back into the surrounding medium. In that case the fluid of Ca-transporting vesicles would be essentially Mg-depleted seawater. Another possibility is that  $Ca^{2+}$  is pumped out of these vacuoles into an intracellular Ca-reservoir, i.e. another type of vesicle. In that case the fluid of Ca-transporting vesicles would have a composition radically different from seawater. For many trace elements, such as Sr, Ba, and Cd, the fractionation between seawater and foraminiferal calcite can be explained by Raleigh fractionation during  $CaCO_3$  precipitation from an internal reservoir (Elderfield et al., 1996). This model implies that for these trace elements, the internal reservoirs are comparable to seawater and thus, the Mg-pumping mechanism, as opposed to the Ca-pumping mechanism, is more likely to be responsible for the low Mg/Ca ratio in the internal Ca-pool of most foraminifera. This mechanism is further favoured by the fact that the vacuolized fluorescent beads are not egested after endocytosis (Fig. 2). If the Ca would be pumped out of the seawater vacuoles and the remaining fluids would

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be transported back into the medium, most of the fluorescent beads would not remain inside the cell.

Given that Sr partitioning can be explained by Raleigh fractionation (Elderfield et al., 1996), the question arises what that internal reservoir actually is and what amount of calcite is precipitated from it. Assuming that an entire chamber is precipitated from this reservoir, one would expect an area of elevated Ca-concentration (in comparison to cytosolic background) in the vicinity of the primary organic sheet. Since we have not observed this during chamber formation (Fig. 3), we conclude that the reservoir is much smaller and that it is impossible to precipitate an entire chamber from a single reservoir. This conclusion fits the possibility of the involvement of ACC-cluster transformation in chamber formation (see above). Since the Sr partitioning coefficients for ACC precipitation are unknown, but may be higher than for calcite precipitation from an aqueous solution, a Raleigh fractionation does not necessarily be the mechanism of trace metal partitioning.

#### 4.4 Inorganic carbon-utilization during chamber formation

To cover the amount of carbonate needed during chamber formation, our hypothetical individual (Fig. 4) would need an even greater volume of seawater. Assuming that all inorganic carbon in vacuolized seawater can be converted into carbonate by increasing the pH (de Nooijer et al., 2009), a juvenile would still need approximately  $17.5 \times 10^{-8}$  l seawater (more than 300 times its own volume). This amount of seawater could be reduced in case the intracellular carbonate-pool is enriched with metabolic  $\text{CO}_2$  (upto 10%; Grossman, 1987; Ter Kuile, 1991). Tracer experiments with  $^{14}\text{C}$  have shown that the hyaline species *Amphistegina lobifera* has an intracellular carbon pool with an estimated concentration of app. 2 M (Ter Kuile and Erez, 1988). This matches the estimated Ca-concentration of the intracellular Ca-pool (Erez, 2003). From inorganic calcite precipitation experiments it is known that a 1:1 Ca: $\text{CO}_3$  ratio results in highest calcite growth rates (Nehrke et al., 2007) and it is not unlikely that foraminifera have evolved a mechanism to benefit from this fact.

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Direct measurements on the concentrations of intracellular Ca and inorganic carbon need to be made to improve the model for calcification as described above. In addition, the phase in which they are present in the internal pools (i.e. free, sequestered by organic compounds or as ACC) need to be complemented by our results to understand the effect that foraminiferal physiology has on trace element and isotope fractionation during chamber formation in *A. tepida*.

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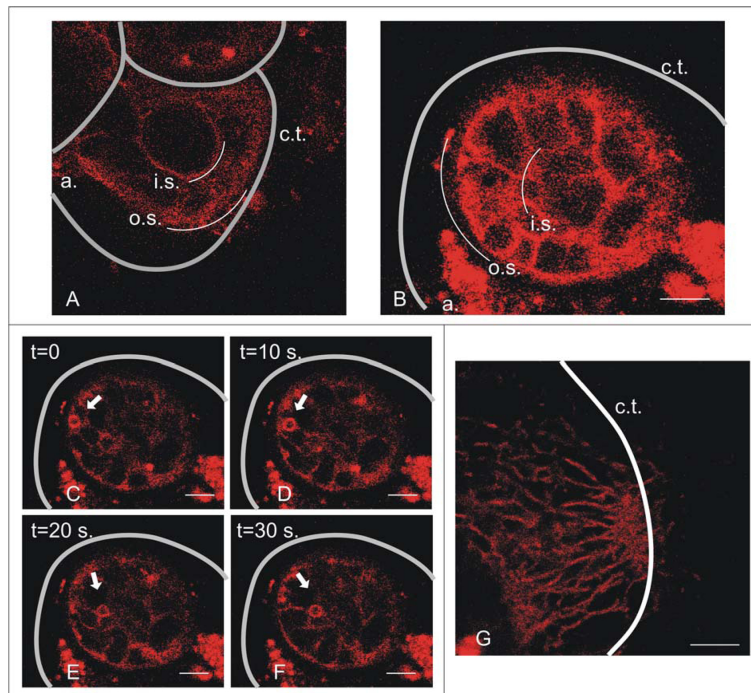
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**Fig. 1.** Distribution of cell membranes in the final chamber of two juveniles of *Ammonia tepida*. **(A)** Location of the two spheres with a number of cytoplasmic strands inbetween them. **(B)** Another individual with a similar organization of these spheres. **(C–F)** Same individual as in (B), scanned every 10 s. A small vesicle (arrows) is transported from the outer towards the inner sphere after which the vesicle disappears. **(G)** Pseudopodial distribution in a third individual during chamber formation. For all figures, the outline of the test of the final chamber is drawn to facilitate recognition of the position of the cell membranes and the calcite. a.=aperture, c.t.=calcitic test, i.s.=inner sphere, o.s.=outer sphere. Scale bars=8 μm.

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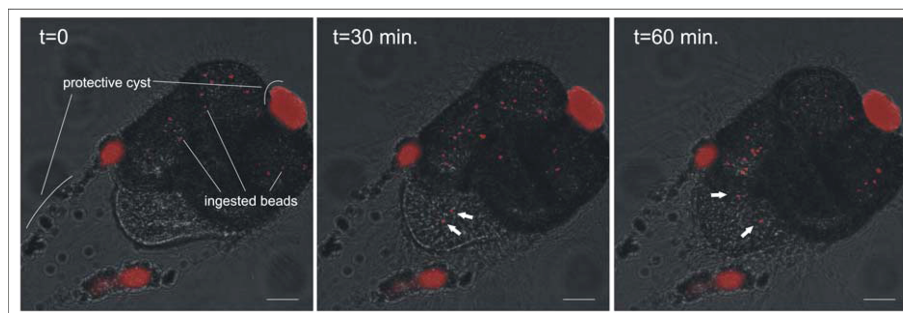
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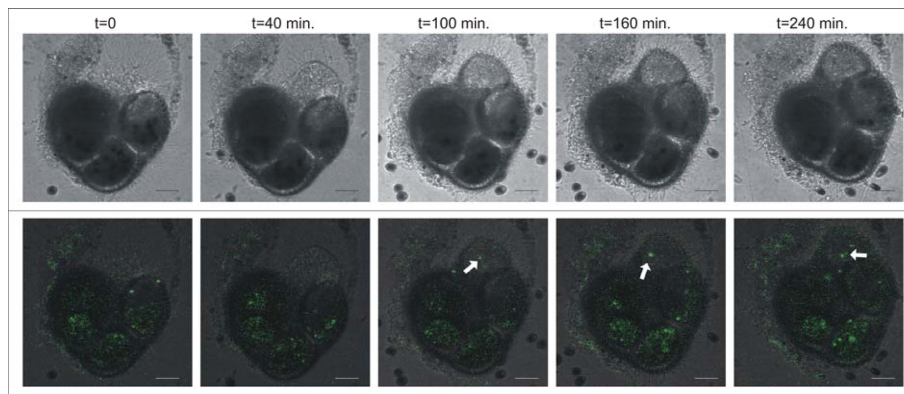


**Fig. 2.** Distribution of fluorescent beads in a calcifying juvenile of *Ammonia tepida*. Many beads are incorporated in the protective cyst that an individual forms prior to chamber formation and result in fluorescence at the surface of the foraminiferal test. The ingested beads, however, cycle within and between all five chambers of this individual. A number of them are transported into the newly formed chamber (arrows). Scale bars=8  $\mu$ m.

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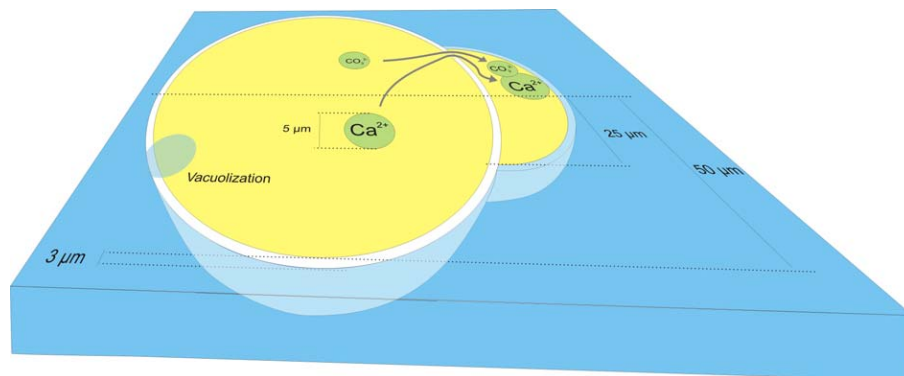
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**Fig. 3.** Distribution of  $\text{Ca}^{2+}$  in a calcifying juvenile of *Ammonia tepida*. The upper row shows the extending pseudopodia at the onset of chamber formation ( $t=0$ ), the production of the primary organic sheet ( $t=40$  min) and calcification onto this sheet ( $t=100$ – $240$  min). In the bottom row of figures, the confocal scans are superimposed on the pictures of the upper row. Arrows indicate  $\text{Ca}^{2+}$ -containing vesicles that are transported to the site of calcification. Scale bars= $20\text{ }\mu\text{m}$ .

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**Fig. 4.** Simplified representation of calcification in a Rotallid juvenile, based on our observations of chamber formation in *Ammonia tepida*. See text for detailed description.

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