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Effect of the fluorescent indicator calcein on Mg and Sr incorporation into foraminiferal calcite

Delphine Dissard and Gernot Nehrke
Alfred Wegener Institute for Polar and Marine Research, D-27570 Bremerhaven, Germany (delphine.dissard@awi.de)

Gert Jan Reichart
Faculty of Geosciences, Utrecht University, NL-3508 TA Utrecht, Netherlands

Also at Alfred Wegener Institute for Polar and Marine Research, D-27570 Bremerhaven, Germany

Julius Nouet
IDES, UMR 8148, Paris Sud 11 University, F-91405 Orsay, France

Jelle Bijma
Alfred Wegener Institute for Polar and Marine Research, D-27570 Bremerhaven, Germany

[1] The development of particular analytical methods like laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS) renders it possible to determine the composition of a single chamber of foraminifers tests. This is crucial in the investigation of benthic foraminifers since the growth of new chambers under laboratory conditions does not face the difficulties associated with experiments involving reproduction. The addition of chambers can be recognized by the incorporation of the fluorescent dye calcein. When added to the culture medium, previously formed chambers are not affected, and calcein is only incorporated in calcite that is formed in its presence. However, it has never been verified whether calcein affects the element incorporation into foraminiferal calcite. In order to investigate and quantify possible effects, specimens of the benthic foraminifer *Ammonia tepida* were cultured in the presence and absence of calcein (15°C, salinity 33), and Mg and Sr in newly formed chambers were analyzed with LA-ICP-MS. Magnesium concentrations of cross sections and longitudinal sections of foraminifera from the same experiment were also analyzed by electron microprobe measurements. Additionally, the impact of calcein on Mg and Sr incorporation in inorganically precipitated calcium carbonate crystals was quantified. Results show that presence of calcein does not impact the incorporation of Mg and Sr into biologically and inorganically precipitated calcium carbonate.

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1. Introduction

[2] The elemental composition of foraminiferal calcite provides widely used tools to reconstruct past oceanic conditions [e.g., *Boyle*, 1981; *Nürnberg et al.*, 1996; *Lea et al.*, 1999; *Marchitto et al.*, 1998; *Martin et al.*, 1999; *Rickaby and Elderfield*, 1999; *Russell et al.*, 2004; *Hall and Chan*, 2004; *Hall et al.*, 2005]. However, together with the increasing use of foraminiferal elemental proxies, several complications have been recognized as well. The, largely unquantified, consequences of physiological controls on calcite precipitation (so-called vital effects) may affect the elemental composition of biogenic calcium carbonate [*Havach et al.*, 2001; *Elderfield et al.*, 2002; *Erez*, 2003; *Hönisch and Hemming*, 2004; *Eggins et al.*, 2004; *Anand and Elderfield*, 2005; *Sadekov et al.*, 2005; *Bentov and Erez*, 2005, 2006; *Hintz et al.*, 2006]. Culture studies carried out under controlled physicochemical conditions together with recent analytical improvement allowing determination of elemental concentration on a single chamber, can provide information necessary to rule out microhabitat effects and to deconvolve potential environmental effects [e.g., *Reichart et al.*, 2003; *Eggins et al.*, 2004; *Hintz et al.*, 2006; *De Nooijer et al.*, 2007; *Sadekov et al.*, 2008]. Incubations with the fluorescent dye calcein (Bis [N,N bis(carboxymethyl) aminomethyl]-fluorescein), allows to discriminate between preexisting foraminiferal calcite and chambers added during experimental treatment [e.g., *Bernhard et al.*, 2004; *Hintz et al.*, 2004, 2006; *De Nooijer et al.*, 2007] as the newly calcium carbonate precipitated in presence of calcein fluoresced a yellow green when viewed with epifluorescence (470 nm excitation, 509 nm emission). A large number of studies on various organisms including fish [e.g., *Wilson et al.*, 1987; *Hernaman et al.*, 2000; *Leips et al.*, 2001], ascidians [*Lambert and Lambert*, 1997], echinoderms [e.g., *Medeirosbergen and Ebert*, 1995; *Rogers-Bennett et al.*, 2003], brachiopods [*Rowley and MacKinnon*, 1995], cnidarians [*Marschal et al.*, 2004] and mollusks [e.g., *Day et al.*, 1995; *Kaehler and McQuaid*, 1999; *Allen and Williams*, 2003; *Clarke et al.*, 2004] have reported on the use of calcein as a fluorescent marker in skeletons and shells. It has been demonstrated that calcein uptake is nonlethal and does not affect the survival rate of foraminifera [*Bernhard et al.*, 2004]. However, one cannot exclude that the incorporation of calcein impairs the incorporation of paleoceanographically relevant elements such as Mg and Sr. In this study the impact of calcein on the Mg and Sr incorporation into foraminifera shells and abiotic calcite was measured using laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS) analysis and as well electron microprobe (EM) measurements for Mg/Ca. Measurements were carried out on the symbiont barren, shallow water, benthic foraminifer *Ammonia tepida* cultured under controlled laboratory conditions and on calcium carbonate precipitated inorganically in the presence and absence of calcein.

2. Materials and Methods

2.1. Collecting and Culturing Foraminifera

[3] Surface sediment containing living specimens of *Ammonia tepida* (referred to as molecular type T6E by *Hayward et al.* [2004], here further referred to as *A. tepida*) were collected at an intertidal flat of the Wadden Sea (near Dorum, Northwestern Germany) during spring 2006. Back in the laboratory, the sediment was sieved over a 630 μm mesh to remove the largest meiofauna. Six living specimens were harvested, cleaned and dried (see section 2.3.1) in order to determine elemental ratios of field specimens. Live foraminifera were recognized based on pseudopodial activity after examination of specimens using an inverted microscope (Zeiss Axiovert 200M). The rest of the sediment containing foraminifera was kept in stock cultures. Less than 2 weeks after collection, living individuals of *A. tepida* were hand-picked from the stock cultures and transferred to aquaria. The aquaria containing 0.2 μm filtered seawater (salinity of 33.1 ± 0.6, pH of 8.12 ± 0.03; Table 1) were covered with a lid to minimize evaporation (with a small opening allowing air exchange) and placed in temperature controlled incubators at 15 ± 0.1°C. At the start of the experiment, the fluorescent indicator calcein was added to the culture media at a concentration of 5 mg/L. After one month, the media was replaced by seawater free of calcein, and foraminifera were allowed to calcify for another month. Foraminifera were fed at the beginning of the experiment and subsequently every 2 weeks with a mixture of air-dried algae (*Phaeodactylum triconortum*, *Dunaliella salina* and *Isochrisis galbana*). In order to keep the carbonate chemistry constant, air presaturated with water vapor (pCO_2 = 380 ppm) was bubbled through the growth media. Growth media was replaced every 2 weeks in order to minimize the impact of bacterial growth, changes in salinity due to evaporation and overall changes in carbonate

Table 1. Carbonate Chemistry and Elemental Concentration of the Foraminiferal Growth Media[a]

	Growth Medium[b]			
	Calcein		No Calcein	
	T_0	T_{fin}	T_0	T_{fin}
Carbonate Chemistry				
Total alkalinity (μeq kg^{-1})	2406 (±32)	2534 (±67)	2420 (±5)	2536 (±17)
DIC (μmol kg^{-1})	2241 (±21)	2301 (±64)	2257 (±38)	2340 (±17)
pH[c] (NBS)	8.12 (±0.03)		8.11 (±0.02)	
Average salinity[c]	33.1 (±0.6)		33.0 (±0.4)	
Elemental Concentration of the Growth Medium				
Mg/Ca (mol/mol) (±0.1)	5.4	5.3	5.4	5.3
Sr/Ca (mmol/mol) (±0.2)	8.3	8.4	8.5	8.5

[a] The growth media were changed every 2 weeks. T_0 represents average values of alkalinity, DIC, Mg/Ca, and Sr/Ca for each new medium. T_{fin} represents average values of the same parameters after 2 weeks. Error ranges are presented in parentheses.
[b] Salinity is 33, and temperature is 15°C.
[c] These values represent the average of measurements performed every 2 days during the whole course of the experiment.

chemistry. Salinity and pH were measured every second day using a WTW conductivity meter 330i with TetraCon 325 electrode and a pH 3000 with Schott BlueLine Electrodes calibrated with NIST buffers, respectively (Table 1). Water samples were taken from each aquarium before and after exchanging the incubation media for dissolved inorganic carbon (DIC), alkalinity and seawater elemental composition analysis (measured by means of inductively coupled plasma-optical emission spectroscopy, ICP-OES). Samples for DIC measurements were sterile filtered (0.2 μm) and stored in 13 mL borosilicate flasks free of air bubbles at 4°C until they were measured photometrically with an autoanalyzer (Technicon TRAACS 800 Bran + Lübbe, Norderstedt, Germany). Average precision of DIC measurements was 10 μmol kg^{-1} (based on triplicate analyses). Samples for alkalinity analyses were stored in 300 mL borosilicate flasks at 4°C. Samples were measured in triplicate by potentiometric titration (average precision of 8 μEq kg^{-1} [*Brewer et al.*, 1986]). Total alkalinity was calculated from linear Gran Plots [*Gran*, 1952] (Table 1). After termination of the experiment, specimens with an unlabeled final chamber (F) and a calcein-tagged penultimate chamber (F-1; Figure 1) were selected and divided in two groups for elemental ratios measurements of the newly formed calcite using LA-ICP-MS and EM, respectively.

2.2. Crystal Growth Experiments

[4] A flow through system was used to perform the inorganic calcite growth experiments. Two separate solutions containing Ca^{2+} and CO_3^{2-} ions were pumped through reactors containing a single 2 mm to 3 mm sized calcite crystal each. The solutions were prepared by dissolving Merck® Suprapure® $CaCl_2 \bullet 4H_2O$ and K_2CO_3

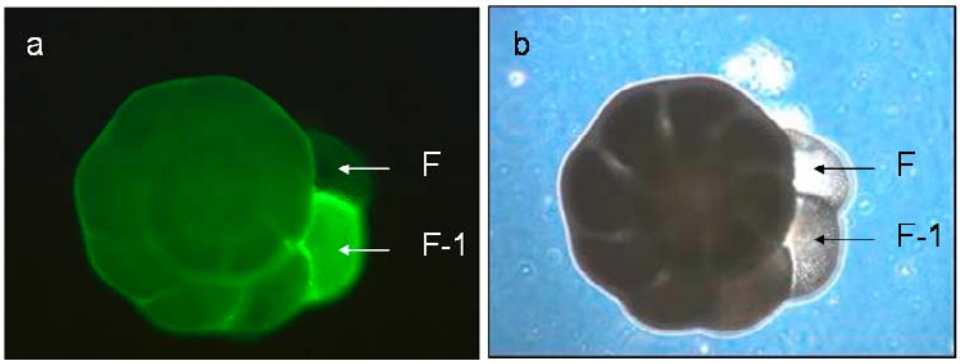


Figure 1. (a) *A. tepida* specimen showing the calcein labeled penultimate (F-1) and not labeled final (F) chambers under epifluorescent light. (b) Image of the same specimen taken with transmitted light.

Table 2. Inflow Solution Reaction Compositions for the Inorganic Precipitation Experiment[a]

Experiment	[Ca] (mmol L^{-1})	[Mg] (μmol L^{-1})	[CO_3^{2-}] (mmol L^{-1})	pH (NBS)
Crystal 1 + 2	0.88 ± 0.02	0.9 ± 0.2	1.06 ± 0.02	10.2 ± 0.1

[a] The measured (ICP-EOS) total concentrations of [Mg] and [Ca], as well as the (free) ion concentration [CO_3^{2-}] and pH, calculated using Visual Minteq, are listed.

in filtered (0.2 μm pore size) reverse osmosis water (conductivity < 0.067 μS) with 0.1 M NaCl as background electrolyte. Magnesium was added to the Ca^{2+} solution as $MgCl_2$. The equilibrium chemical speciation of the inflow solution obtained after mixing of the Ca^{2+} and CO_3^{2-} solutions was calculated using the chemical speciation software Visual Minteq V. 2.40 (J. P. Gustafsson, VisualMINTEQ version 2.40. A chemical equilibrium model for the calculation of metal speciation, solubility equilibrium etc. for environmental systems, available at http://www.lwr.kth.se/English/OurSoftware/vMINTEQ/index.htm). The supersaturation (Ω) with respect to calcite was approximately 10 (solution composition is given in Table 2). The experimental setup was identical to those described by *Nehrke et al.* [2007], with the exception that no Sr was added. One crystal was grown in the presence of calcein (5 mg L^{-1}, added to the CO_3^{2-} ion containing solutions) and one without calcein. At the end of an experimental period of 30 days, crystals were removed from the reactors and dried in an oven for 1 h at 60°C. The Mg concentrations of the overgrowths were determined using LA-ICP-MS.

2.3. Measurements With LA-ICP-MS

2.3.1. Cleaning Procedures

[5] Individual foraminifera were soaked for 30 min in a 3–7% NaOCl solution to remove organic matter [*Gaffey and Brönnimann*, 1993]. Specimens were removed from the cleaning solution directly after complete bleaching, in order to avoid dissolution of the last chambers. Upon cleaning, samples were thoroughly rinsed with deionized water to ensure complete removal of reagent. Dried foraminifera were fixed on double-sided adhesive tape and mounted on plastic stubs for LA-ICP-MS analysis.

[6] The inorganically precipitated crystals were directly mounted for LA-ICP-MS analysis without any cleaning step.

2.3.2. LA-ICP-MS

[7] Newly formed chambers of *A. tepida* and inorganically grown calcium carbonate crystals, were ablated using an Excimer laser (Lambda Physik) with GeoLas 200Q optics inside a helium atmosphere flushed ablation chamber [*Reichart et al.*, 2003]. Pulse repetition rate was set at 6 Hz, with an energy density at the sample surface of 4 J/cm^2 and ablation craters set at 80 μm in diameter (Figures 2 and 3). The ablated material was carried on a He flow, which was diluted with Ar-He mixture before being analyzed as a function of time (and hence depth) on a quadrupole ICP-MS instrument (Micromass Platform ICP). Analyses were calibrated against National Institute of Standards and Technology SRM 610 glass, using concentration data of *Pearce et al.* [1997] with Ca as an internal standard. Calcium is ideal because the concentration is constant at 40 wt % in all calcitic foraminiferal tests, and because it allows direct comparisons with trace metals to Ca ratios from wet-chemical studies. A collision and reaction cell was used to minimize spectral interferences on the minor isotopes of Ca [*Mason and Kraan*, 2002].

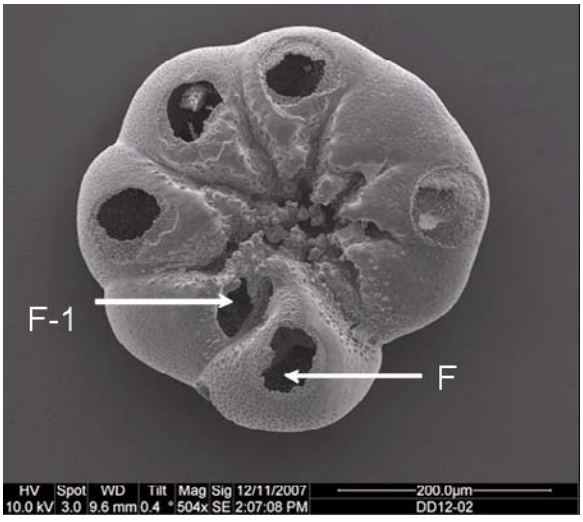


Figure 2. Scanning electron microscope (SEM) image of laser ablation craters in *A. tepida* (F-1, penultimate chamber; F, final chamber).

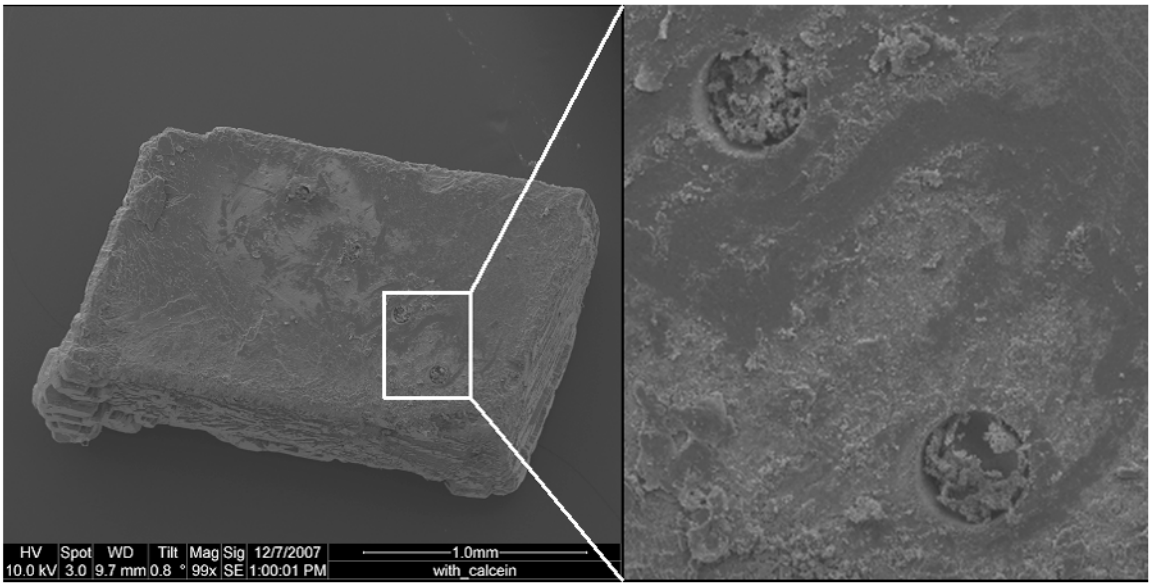


Figure 3. (left) SEM image of an inorganically precipitated calcium carbonate crystal (precipitated in the presence of the fluorescent compound calcein). (right) Ablation craters performed with the LA-ICP-MS.

^{44}Ca was used as an internal standard, monitoring ^{42}Ca and ^{43}Ca to check for consistency. The offset between the three isotopes was always less than 2%. Concentrations of Mg and Sr were calculated using ^{24}Mg, ^{26}Mg, ^{88}Sr, ^{27}Al, ^{55}Mn. An in-house (matrix matched) carbonate standard was used to check for a possible offset due to different ablation rates on glass and carbonate. No systematic offset was observed. The small interspecimen variability measured on foraminifera grown under similar conditions (see error bars in Figures 4a and 4b) confirms reproducibility of the data. Between isotopes measurements enough time was scheduled to allow the collector to reach background values, avoiding the possible impact of tailing of the more abundant isotopes on minor isotopes. Relative precision for ^{24}Mg, ^{88}Sr and ^{55}Mn was less than 6.5%.

2.4. Electron Microprobe

[8] Individual foraminiferal tests were embedded in a resin (Araldite XW396/XW397) under vacuum and dried under pressure (6 bar) at a temperature of 60°C. This procedure allowed the pores to be filled by the resin to obtain the required stability for the subsequent polishing (Figure 6). After polishing, resin blocks were carbon coated. The EM measurements were performed at a 15 kV accelerating voltage and a 12 nA beam current (5 s on peak position, 2.5 s for background and a 2μm electron beam). USNM Dolomite, USNM Strontianite, USNM Calcite and USNM Siderite where used as standards for Mg, Sr, Ca and Fe, respectively. Strontium concentrations in *A. tepida* are too low to be measured by the instrument used in this study (JEOLJXA 8900).

3. Results and Discussion

3.1. LA-ICP-MS Measurements

3.1.1. Elemental Concentration in Foraminifera

[9] To test for significance of elemental concentration differences in between F and F-1 chambers of specimens presented in Figure 4, statistic tests were performed with the statistic program R [*R Development Core Team*, 2009]. For data presented in Figures 4a–4d, normalities were checked by the means of a Shapiro-Wilk-test for each data set. No significant deviations from normality were detected (all $p > 0.27$). Subsequently, a pairwise *t* test was applied for each data set (Figures 4a–4d) and no significant differences were found (all $p > 0.29$). Finally, additional pairwise exact Wilcoxon test [*Hollander and Wolfe*, 1973] and pairwise Kolmogorov-Smirnov test were applied to the data showing no significant differences as well (all $p > 0.62$ and all p values > 0.77 for Wilcoxon test and Kolmogorov-Smirnov test, respectively). It can be concluded that the distributions in elemental concentration between F and F-1 chambers of specimens presented in Figures 4a–4d are not

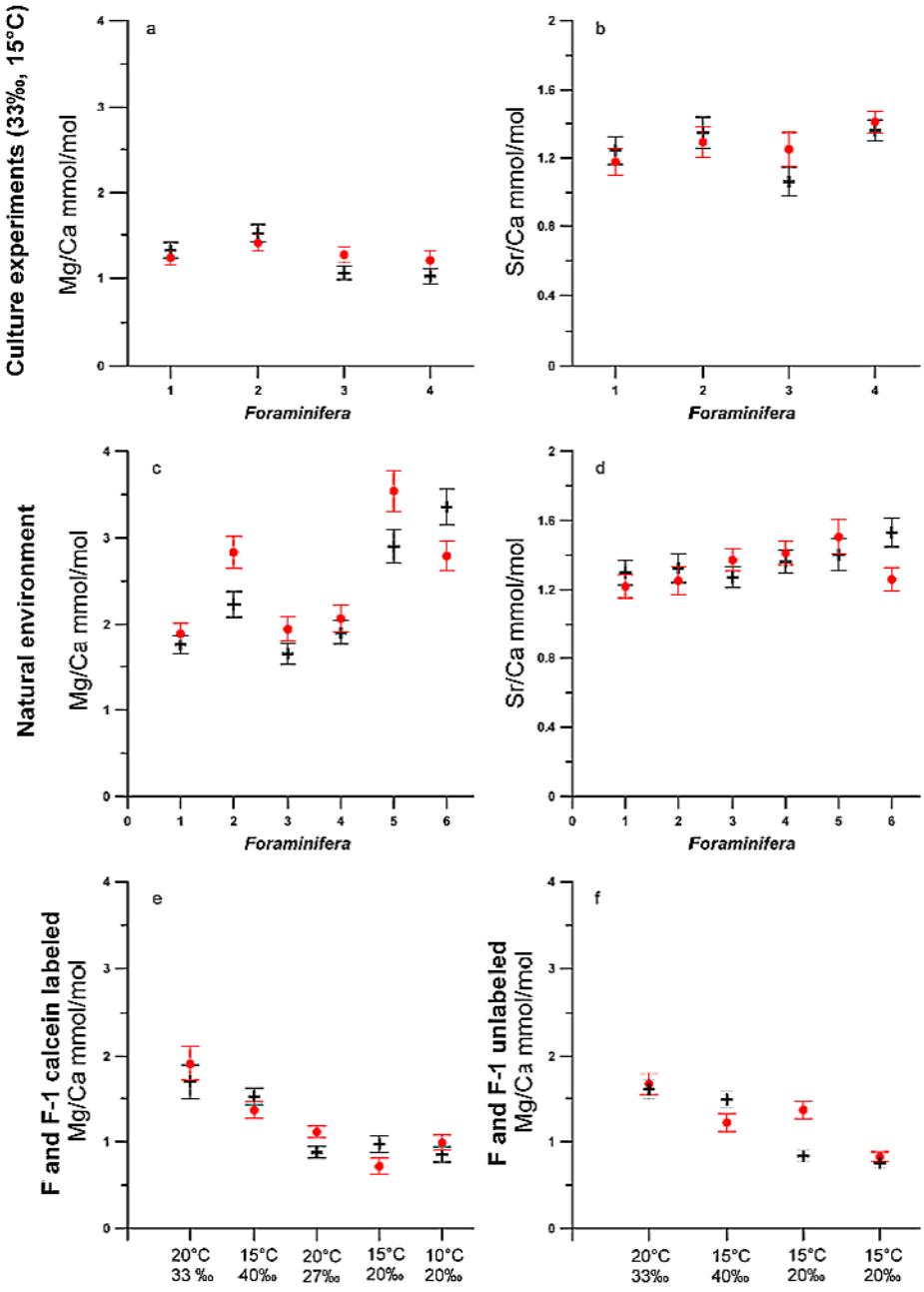


Figure 4. (a) Mg/Ca and (b) Sr/Ca ratios in four *A. tepida* specimens grown at 15°C and salinity 33‰. Crosses correspond to values measured on the F (final) not labeled chambers. Filled circles correspond to values measured on the F-1 calcein labeled chambers of the same specimens. (c) Mg/Ca and (d) Sr/Ca ratios measured in the F-1 chambers (filled circles) and F chambers (crosses) of six specimens of *A. tepida* directly after collection in their natural environment. (e) Mg/Ca ratios measured in five specimens of *A. tepida* grown under various culture conditions and presenting two calcein labeled chambers: F (crosses) and F-1 (full circles). (f) Mg/Ca ratios measured in four specimens of *A. tepida* grown under various culture conditions and presenting two unlabeled chambers: F (crosses) and F-1 (full circles), F-2 being calcein-tagged.

Table 3. Average ALK, DIC, pH, and Salinity of the Foraminiferal Growth Media of Five Culture Experiments[a]

	Experiments				
	20°C, 33‰	15°C, 40‰	20°C, 27‰	15°C, 20‰	10°C, 20‰
Average total alkalinity (μeq kg^{-1})	2434 (±57)	2925 (±70)	2042 (±29)	1595 (±72)	1565 (±49)
Average DIC (μmol kg^{-1})	2219 (±35)	2688 (±71)	1932 (±95)	1497 (±96)	1530 (±99)
Average pH (NBS)	8.04 (±0.09)	8.11 (±0.02)	8.01 (±0.08)	7.96 (±0.02)	7.93 (±0.08)
Average salinity	32.9 (±0.64)	33.0 (±0.16)	27.0 (±0.17)	19.9 (±0.15)	20.0 (±0.15)

[a] Experiments were run following the same protocol as described in section 2.1 but at various salinity and temperature conditions.

significantly different. The Mg/Ca ratio of cultured specimens of *A. tepida* is low (1.06–1.52 mmol/mol) (Figure 4a and Table 4a), relatively constant, and shows no significant differences between F (not labeled) and F-1 (calcein labeled) chambers (range 1.20–1.41 mmol/mol and 1.02–1.52 mmol/mol, respectively). Field specimens (Figure 4c and Table 4a) show a higher and more variable Mg/Ca ratio (1.65–3.54 mmol/mol) with a difference of ±0.40 mmol/mol between F and F-1 chambers. For specimens grown in culture, the difference between F and F-1 chambers is much lower (±0.14 mmol/mol, Figure 4a). Most of the field specimens have lower Mg concentrations in the final chamber than in their penultimate chamber (Figure 4c). For specimens grown in culture no clear trend exists (Figure 4a). This observation raises the question if foraminifera grown in the natural environment have an ontogenetic trend toward lower concentration in newer chambers. Indeed, although statistical tests performed on data from field specimens (Figure 4c) show no significant differences between F and F-1 chambers, the strong variance with large overlap of this data set makes it difficult to perform reliable statistical tests. Since it has been shown that ontogeny can modify Mg incorporation in benthic foraminiferal species [*Hintz et al.*, 2006] it is possible that the observed variations in Mg concentration between the two last chambers of specimens grown in the natural environment are caused by ontogeny. To test this, we compared specimens of *Ammonia tepida* presenting F and F-1 calcein-labeled chambers (Figure 4e) with specimens presenting F and F-1 unlabeled chambers (F-2 being calcein tagged) (Figure 4f). Since no such specimens were present at the end of the 15°C and 33‰ experiment, these data were obtained on specimens grown in different culture experiments (investigating the impact of salinity on Mg incorporation). These experiments were performed following the same protocol as described in section 2.1, but under various salinity and temperature conditions (Table 3). Although data presented in Figures 4e and 4f were not measured on specimens cultured under identical conditions, a pairwise test was performed for each data set. A Wilcoxon exact test was applied to test for significance of Mg/Ca ratio differences in between the F and F-1 chambers (Figure 4e, F and F-1 calcein labeled; Figure 4f, F and F-1

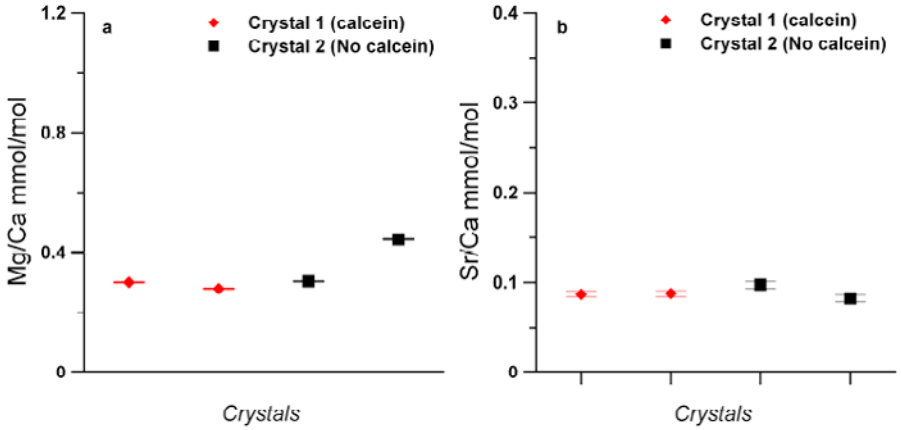


Figure 5. (a) Mg/Ca and (b) Sr/Ca ratio in two calcium carbonate crystals precipitated inorganically under controlled laboratory conditions. The diamonds represent values measured on the crystal grown in the presence of calcein (crystal 1), the squares represent values measured on the crystal grown without calcein (crystal 2), and error bars represent the precision of the instrument.

Table 4a. Row Data of the LA-ICP-MS Measurements Performed on Foraminifera

	Mg/Ca (mmol/mol)	
Foraminifera	F (No Calcein)	F-1 (Calcein)
Figure 4a		
1 (15°C, 33‰)	1.32 (±0.091)	1.23 (±0.083)
2 (15°C, 33‰)	1.52 (±0.102)	1.41 (±0.095)
3 (15°C, 33‰)	1.06 (±0.077)	1.27 (±0.093)
4 (15°C, 33‰)	1.02 (±0.090)	1.20 (±0.105)
Figure 4c		
1 (natural environment)	1.76 (±0.112)	1.89 (±0.116)
2 (natural environment)	2.23 (±0.148)	2.83 (±0.184)
3 (natural environment)	1.65 (±0.121)	1.95 (±0.141)
4 (natural environment)	1.90 (±0.143)	2.07 (±0.154)
5 (natural environment)	2.90 (±0.193)	3.54 (±0.235)
6 (natural environment)	3.36 (±0.206)	2.79 (±0.171)
	Mg/Ca (mmol/mol)	
Foraminifera	F (Calcein)	F-1 (Calcein)
Figure 4e		
1 (20°C, 33‰)	1.70 (±0.202)	1.91 (±0.230)
2 (15°C, 40‰)	1.52 (±0.095)	1.36 (±0.083)
3 (20°C, 27‰)	0.88 (±0.067)	1.11 (±0.305)
4 (15°C, 20‰)	0.97 (±0.095)	0.72 (±0.069)
5 (10°C, 20‰)	0.85 (±0.088)	0.99 (±0.102)
	Mg/Ca (mmol/mol)	
Foraminifera	F (No Calcein)	F-1 (No Calcein)
Figure 4f		
1 (20°C, 33‰)	1.61 (±0.116)	1.67 (±0.122)
2 (15°C, 40‰)	1.49 (±0.095)	1.22 (±0.102)
3 (15°C, 20‰)	0.83 (±0.067)	1.37 (±0.102)
4 (15°C, 20‰)	0.75 (±0.051)	0.83 (±0.056)
	Sr/Ca (mol/mol)	
Foraminifera	F (No Calcein)	F-1 (Calcein)
Figure 4b		
1 (15°C, 33‰)	1.24 (±0.081)	1.18 (±0.077)
2 (15°C, 33‰)	1.35 (±0.089)	1.29 (±0.087)
3 (15°C, 33‰)	1.06 (±0.084)	1.25 (±0.098)
4 (15°C, 33‰)	1.36 (±0.061)	1.41 (±0.064)
Figure 4d		
1 (natural environment)	1.30 (±0.071)	1.22 (±0.067)
2 (natural environment)	1.32 (±0.083)	1.25 (±0.079)
3 (natural environment)	1.27 (±0.059)	1.37 (±0.64)
4 (natural environment)	1.36 (±0.065)	1.41 (±0.067)
5 (natural environment)	1.40 (±0.092)	1.50 (±0.100)
6 (natural environment)	1.53 (±0.082)	1.26 (±0.068)

Table 4b. Row Data of the LA-ICP-MS Measurements Performed on Calcium Carbonate Crystals

Crystals	Mg/Ca (mmol/mol)	Sr/Ca (mol/mol)
Figures 5a and 5b		
Crystal 1 (calcein)	0.300 (±0.0004)	0.087 (±0.0033)
Crystal 1 (calcein)	0.279 (±0.0004)	0.088 (±0.0034)
Crystal 2 (no calcein)	0.304 (±0.0004)	0.098 (±0.0040)
Crystal 2 (no calcein)	0.444 (±0.0003)	0.082 (±0.0035)

unlabeled). No significant differences were found for any of the two data sets (both p values > 0.62) (Wilcoxon exact test was done in R package exactRankTests). Differences in Mg/Ca ratios between F and F-1 chambers of specimens grown under laboratory conditions can be considered as not significant, regardless if zero, one or two of the last two chambers are calcein–tagged. Thus, it can be concluded that the high variation in Mg concentration measured in the field samples reflects the variation in environmental conditions in an intertidal flat and is not related to the fluorescent compound calcein.

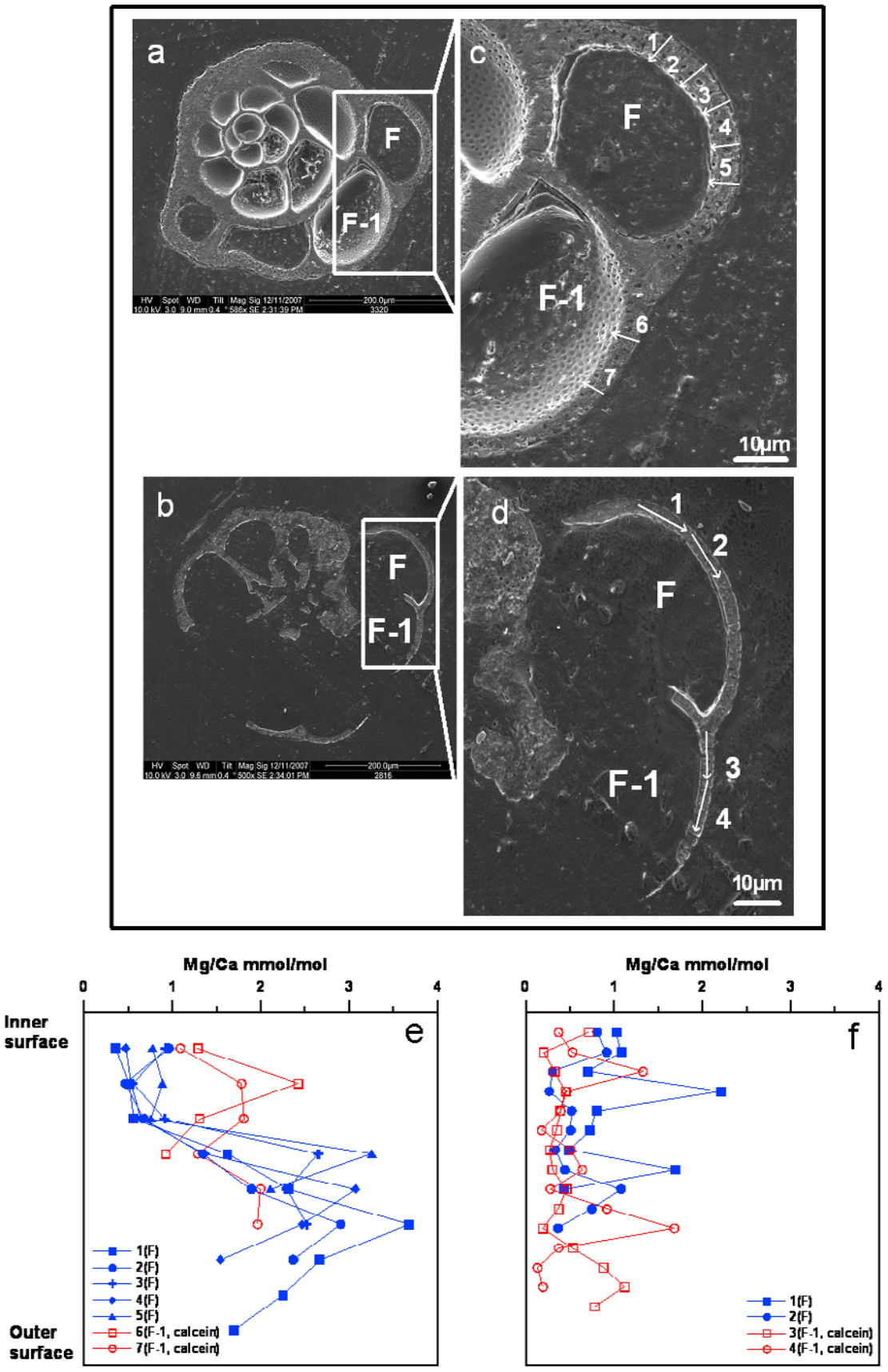
[10] The Sr/Ca ratios of foraminifera grown in culture (1.1–1.4 mmol/mol, Figure 4b and Table 4a) are similar to those measured in the field (1.2–1.5 mmol/mol, Figure 4d and Table 4a). Furthermore, as concluded from statistical tests described above, no significant differences of the Sr/Ca ratio is seen between F-1 (labeled) and F (unlabeled) chambers of the cultured foraminifera (ranges 1.18–1.41 mmol/mol and 1.06–1.37 mmol/mol, respectively). Similarly, no significant differences of the Sr/Ca ratios are observed between F and F-1 chambers of field specimens (ranges 1.27–1.53 mmol/mol and 1.22–1.50 mmol/mol, respectively). The fluorescent compound calcein, therefore, does not impact Sr incorporation into *A. tepida* foraminiferal calcite.

3.1.2. Elemental Concentration in Calcium Carbonate Crystals

[11] No obvious variation in Mg/Ca ratios was observed between the crystals grown in the presence (0.27–0.30 mmol/mol) or absence (0.30–0.44 mmol/mol) of calcein (Figure 5 and Table 4b). Even though no extra Sr was added during the

Table 4c. Row Data of the Electron Microprobe Measurements Performed on Foraminifera

	Mg/Ca (mmol/mol)						
	F (No Calcein)					F-1 (Calcein)	
	Transect 1	Transect 2	Transect 3	Transect 4	Transect 5	Transect 6	Transect 7
1 (15°C, 33‰) (Figure 6e)							
Position 1	0.355	0.956	0.918	0.468	0.770	1.284	1.085
Position 2	0.508	0.464	0.545	0.521	0.881	2.418	1.778
Position 3	0.557	0.673	0.907	0.635	0.755	1.302	1.805
Position 4	1.617	1.339	2.647	1.359	3.252	0.919	1.282
Position 5	2.312	1.890	2.288	3.066	2.104		1.992
Position 6	3.674	2.900	2.514	2.462			1.958
Position 7	2.659	2.364		1.538			
Position 8	2.247						
Position 9	1.686						
2 (15°C, 33‰) (Figure 6f)							
Position 1	1.022	0.803	0.710	0.366			
Position 2	1.077	0.910	0.196	0.524			
Position 3	0.694	0.297	0.322	1.321			
Position 4	2.203	0.262	0.445	0.458			
Position 5	0.798	0.513	0.385	0.391			
Position 6	0.722	0.503	0.347	0.175			
Position 7	0.486	0.329	0.270	0.502			
Position 8	1.684	0.435	0.294	0.635			
Position 9	0.431	1.072	0.457	0.272			
Position 10		0.743	0.368	0.912			
Position 11		0.359	0.187	1.676			
Position 12			0.528	0.368			
Position 13			0.874	0.127			
Position 14			1.112	0.188			
Position 15			0.776				
Position 16			0.151				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6(F-1, calcein)
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Mg/Ca mmol/mol
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Figure 6

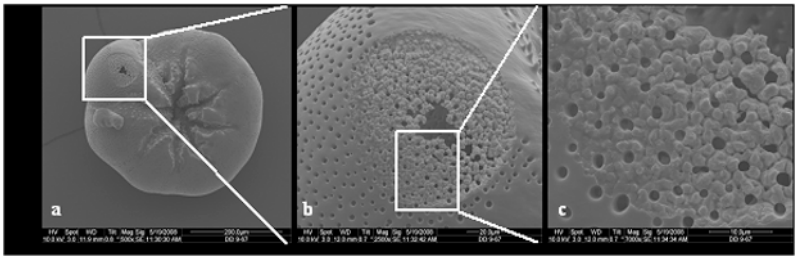

Figure 7. (a–c) SEM image of a laser ablation crater in *Ammonia tepida*. On the periphery of the crater (Figure 7b) only the secondary calcite is removed by the ablation allowing observation of primary calcite characterized by a granulated honeycomb structure (Figure 7c).

precipitation experiment, its presence as an impurity in the reagent grade chemicals was sufficient to result in a concentration within the calcite crystal which could be measured with the analytical method used. However, as in living *A. tepida* ratios, no differences in Sr/Ca ratios were found between crystals grow with or without calcein (Table 4b).

3.2. Electron Microprobe Measurements

[12] Mg/Ca ratios in cross and longitudinal sections of foraminifera chamber walls measured using an electron microprobe (EM) are given in Figure 6 and Table 4c. Average Mg/Ca ratios of EM cross sections (Table 4c) do not show any significant variations between calcein labeled F-1 (range 1.48–1.64 mmol/mol) and unlabeled F chambers (range 1.43–1.73 mmol/mol). Moreover, average values measured using EM are similar to LA-ICP-MS measurements performed on specimens from the same experiment (range 1.02–1.52 mmol/mol and 1.20–1.41 mmol/mol for F-1 and F chambers, respectively). However, profiles obtained using EM cross sections reveal a variation in Mg/Ca spatial distribution between the F and F-1 chambers (Figure 6e). Measurements on the F chamber indicate an increase in Mg/Ca ratios at the inner part of the test while, the profiles obtained from F-1 chamber show an increase occurring in the opposite direction with higher values closer to the outer surface. Differences in Mg/Ca ratios with calcite type (primary and secondary calcite) have been previously reported in foraminiferal tests studies [*Allison and Austin*, 2003; *Eggins et al.*, 2004; *Sadekov et al.*, 2005; *Bentov and Erez*, 2005, 2006]. Primary and secondary calcite can also be seen in the walls of *A. tepida* (Figure 7). To verify whether these different calcite morphologies or whether the florescent compound calcein is responsible for differences in the spatial Mg/Ca distribution, longitudinal transects were performed (Figure 6f and Table 4c). Longitudinal electron microprobe transect display low and constant Mg concentrations (0.46–1.01 mmol/mol) (Figure 6f), and do not reveal significant variations between F (unlabeled) and F-1 (calcein labeled) chambers (0.78 mmol/mol and 0.51 mmol/mol for F and F-1 chambers, respectively). It can thus be concluded that the fluorescent compound calcein does not impact the incorporation in Mg into *Ammonia tepida* calcite.

[13] Since calcein appears not to impact significantly foraminiferal calcite elemental composition, the calcein-tagging method could be used directly during experiments, allowing that way a significant shortage of culture experiments duration.

4. Conclusions

[14] In this study, we investigated the effect of calcein, a fluorescent dye that allows to identify newly deposited calcite in living organisms, on the Mg/Ca and Sr/Ca ratios in foraminifera *A. tepida* and in inorganically precipitated calcite. Our results show no significant impact of calcein on the relative Mg and Sr incorporation in *A. tepida.* Similarly, no obvious effect of calcein on the

Figure 6. (a and b) SEM image of two *A. tepida* specimens embedded in resin and subsequently polished for electron microprobe analysis. The white boxes indicate positions of the F-1 chamber labeled with calcein and the F chamber (not labeled) shown at higher magnification in Figures 6c and 6d. Numbers and positions (white arrows) of (c) cross sections and (d) longitudinal sections measured using EM. Mg/Ca values in mmol/mol from electron microprobe measurements along several (e) cross sections and (f) longitudinal sections. Numbers in the legends refer to the numbers of Figures 6e and 6f and positions given in Figures 6c and 6d, respectively.

elemental composition of inorganically grown calcium carbonate crystals was observed. Hence, the use of the fluorescent marker calcein is a powerful tool to study the effect of environmental conditions on Mg/Ca and Sr/Ca ratios in living foraminifera by shortening significantly the duration of incubation experiments.

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