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An evaluation of Mg/Ca, Sr/Ca, and Ba/Ca ratios as environmental proxies in aragonite bivalve shells

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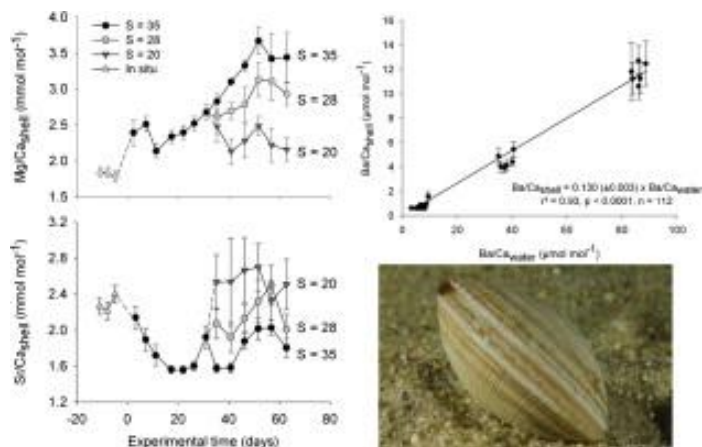
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Abstract :

The influence of salinity and water chemistry on Mg/Ca, Sr/Ca and Ba/Ca ratios in the aragonitic shells of the Manila clam were investigated. Clams were reared at constant temperature (20 °C) under different controlled conditions of salinity, commonly encountered in their natural habitat. Clams were held in three tanks with a constant salinity of 35 for the first 35 days and then two tanks were changed to lower salinities (20 and 28) for the next 29 days. Individual shell Mg/Ca, Sr/Ca and Ba/Ca ratios were studied through time. Despite stable conditions (temperature, salinity, and Mg/Cawater) for clams reared at salinity 35 during the experiment, Mg/Ca shell ratios increased through the time. Moreover the salinity decrease at t35 slowed the increase of Mg/Ca shell ratios at salinity 28 and resulted in a Mg/Cashell decrease at salinity 20, despite similar Mg/Cawater ratios in the different salinity treatments. Microprobe analyses illustrate that Mg varies along contemporaneous growth lines. The variable shell Mg/Ca ratios suggests that incorporation of magnesium into shell carbonate is strongly regulated by the organism and not by environmental conditions. Interestingly, microprobe analyses illustrated that Mg was not associated with shell sulfur as other studies have suggested. Sr/Ca shell ratios of clams reared at salinity 35 and under constant conditions were also not as constant as expected if Sr/Ca ratios were an environmental proxy. There was an inverse correlation between shell Sr/Ca and salinity despite a slight positive correlation between salinity and Sr/Ca ratios of the water, indicating that Sr/Ca ratios do not reflect environmental conditions. A strong inverse correlation between salinity and Ba/Ca shell ratios (and a positive correlation between Ba/Ca shell and Ba/Ca water) was observed. Therefore, Ba/Ca shell ratios seem to be a promising proxy of high-resolution (1 day) salinity variations in estuarine waters (via the relationship between Ba/Ca water and salinity). This study clearly illustrates that both Mg/Ca and Sr/Ca ratios in aragonite shells are not under environmental control and that Ba/Ca ratios are, with the later tracking high-resolution water Ba/Ca ratios and hence estuarine salinity variations.

Graphical abstract:



Highlights

► Aragonite clams were grown under constant temperature and Sr/Ca and Mg/Ca ratios. ► Ba/Ca water ratios varied with salinity. ► Shell Sr/Ca and Mg/Ca ratios varied significantly with stable environmental conditions. ► Shell Ba/Ca ratios were highly correlated with water Ba/Ca ratios. ► Shell Sr/Ca and Mg/Ca ratios are not good environmental proxies, while Ba/Ca is.

Keywords : *Ruditapes philippinarum*, shells, aragonite, barium, strontium, magnesium, estuarine water, sclerochronology, bivalve, mollusk

1. Introduction

Calibration of chemical and structural proxies in bivalve shells has attracted much attention in the context of paleo-climatic and paleo-environmental reconstruction (e.g. Richardson 2001, Schöne et al. 2002, Dettman et al. 2004, Wanamaker et al. 2008a). Bivalves can incorporate elements into their shells in amounts relative to the chemical concentrations or to the physical and biological properties of the surrounding seawater. However, the incorporation of elements in shell carbonate can also be controlled by bivalve physiology and uptake kinetics, processes usually called “vital effects” (Urey et al. 1951, Schöne 2008, Gillikin and Dehairs 2013). This often involves complications in the interpretation of chemical data obtained from shell carbonates. For example, strong vital effects have been suggested for Sr/Ca ratios in aragonite shells (Purton et al. 1999, Gillikin et al. 2005), whereas others have shown relationships between Sr/Ca ratios and water temperature (Hart and Blusztajn, 1998, Takesue and van Geen 2004, Schöne et al. 2011). Similarly, others have proposed that Mg/Ca ratios can be used to record water temperature (Ullmann et al. 2013, Bougeois et al. 2014), while there are many reports of strong vital effects in bivalve shells for this element (Lorrain et al. 2005, Wanamaker et al. 2008b, Surge and Lohmann 2008). One way to test if these element ratios are environmentally controlled is to grow bivalves under constant conditions and determine if shell ratios are also stable.

Not all elemental ratios in bivalve shells have been problematic as environmental proxies. Ba/Ca ratios in bivalve shells have been shown to track salinity (Gillikin et al. 2006, 2008). Ba/Ca profiles are typically characterized by a flat background signal interrupted by sharp peaks. Many authors suggested synchronization between these peaks and phytoplankton blooms (e.g. Stecher et al. 1996, Vander Putten et al. 2000, Lazareth et al. 2003, Elliot et al. 2009, Thébault et al. 2009a). Building on the work of Stecher et al. (1996), Thébault et al. (2009a) proposed two main hypotheses to explain the peaks: (1) ingestion of barite originating from assemblages of recently dead diatoms or (2) adsorption of barium onto iron oxyhydroxides associated with diatoms frustules. However, data from Gillikin et al. (2008) suggested that Ba/Ca peaks started nearly 40 days after the crash of a diatom bloom and were caused by an undetermined environmental forcing. Nevertheless, Ba/Ca background profiles in bivalve shells have been shown to be controlled by Ba/Ca ratios in the surrounding water (Gillikin et al. 2006). Considering the inverse relationship between Ba/Ca ratios of water and salinity, shell Ba/Ca background ratios could be used as an estuary specific (or relative) indicator of salinity (Gillikin et al. 2006).

According to Dodd and Crisp (1982), it seems that Sr/Ca and Mg/Ca shell ratios are unaffected by salinity above 10 because these ratios in surrounding water remain relatively constant, thereby opening the possibility of these ratios to act as salinity independent temperature proxies. However, shell precipitation rate may also have an influence on element incorporation such as strontium (e.g. Stecher et al. 1996, Purton et al. 1999, Takesue and van Geen 2004, Lorrain et al. 2005, Gillikin et al. 2005, Carré et al. 2006) and lithium (Thébaud et al. 2009b). Moreover, recent studies have suggested that a large amount of the Mg budget in the shells does not reside in the aragonite lattice, but is rather hosted by organic compounds (Takesue et al. 2008, Foster et al. 2008, Jacob et al. 2008, Schöne et al. 2010) and/or nanoparticles of the inorganic phase (Foster et al. 2008). In spite of these conflicting results, research on Sr/Ca and Mg/Ca ratios in aragonitic shells as paleotemperature proxies (Schöne et al. 2010, Ullmann et al. 2013, Bougeois et al. 2014) and Ba/Ca ratios as a paleosalinity proxy (Gillikin et al. 2006, 2008, Hatch et al. 2013) is still ongoing.

In field experiments and more particularly in variable environments such as estuarine water, it is generally difficult to deconvolve the effect of each environmental and physiological factor on elemental profiles. For these reasons, laboratory studies are particularly useful since they allow control of the environmental conditions and therefore to evaluate the impact of one environmental factor on the elemental uptake. The Manila clam, *Ruditapes philippinarum*, is an excellent species for experimental work, because this species is easy to grow in the laboratory and thrive in a wide range of environmental conditions (Poulain et al. 2010). During this experiment, we focused on Sr/Ca, Mg/Ca, and Ba/Ca ratios in the aragonitic shell of *R. philippinarum* (Adams and Reeve, 1850). Clams were reared at constant temperature (20°C) under controlled conditions of salinity and water chemistry, commonly encountered in their natural habitat. The main objectives of this study are to test if Sr/Ca, Mg/Ca, and Ba/Ca shell ratios are stable when clams are reared under constant environmental conditions, and to study the effect of salinity and water chemistry on elemental uptake and the response time necessary to induce a modification in the shell chemistry. Our main hypotheses are 1) Mg/Ca and Sr/Ca ratios will be constant in clams grown in water with constant temperature if these element ratios are a proxy of water temperature, and 2) Ba/Ca ratios will be different between clams grown in different salinities (and hence different water Ba/Ca ratios).

2. Materials and methods

2.1. Experimental bivalve: *R. philippinarum*

The Manila clam, *Ruditapes philippinarum*, was chosen for this laboratory experiment for several reasons: (1) it is a euryhaline bivalve living mainly at salinities ranging from 16 to 36 (Nie 1991), a typical salinity range in estuaries and at river mouths; (2) due to its importance for aquaculture and fisheries, the biology and physiology of the species are well studied (e.g. Goulletquer et al. 1989, Kim et al. 2001, Richardson 1987, Marin et al. 2003, Flye-Sainte-Marie et al. 2007); (3) annual growth bands and microgrowth bands can be identified in its shell (Richardson 1987, Poulain et al. 2011). Since the tidal periodicity of shell growth increment formation in *R. philippinarum* in intertidal flats is not lost even after several months in laboratory under constant conditions and without emersion (Richardson 1987), it is possible to assign a calendar date to each growth increment and (4) other members of the *Ruditapes* genus are frequently found in shell middens, and are therefore suitable candidates as archives of past coastal human settlements (e.g., Dupont and Marchand 2008).

2.2. Animal collection

A total of 240 Manila clams (*R. philippinarum*; two to three-years old; average length 27 ± 0.19 mm; \pm standard error) were collected by hand at low tide in an estuary located in the Gulf of Morbihan (Bay of Kerdréan, 47°37'N, 2°56'W; Brittany, France; semidiurnal tidal regime) on the 18th of August 2008. Clams were transferred on the 19th of August 2008 to IFREMER Argenton Shellfish Laboratory (North Finistère, France).

2.3. Experimental design

The experimental design was the same as described in Poulain et al. (2010). Clams were randomly divided into three batches of 80 individuals and each batch was placed into a 25 L tank without sediment. Water within the tanks was homogenized with an aquarium air pump. Each tank was supplied with UV sterilized water from a 300 L buffer tank allowing renewal rate of $25\% \text{ h}^{-1}$ and complete water changes were made once a week. The 300 L buffer tanks were filled every 2 to 3 days with 1 μm filtered, UV sterilized water at room temperature (20°C). Water flowing into the 25 L tanks was supplemented continuously with cultured microalgae (50% *Isochrysis affinis galbana* (T.iso) and 50% *Chaetoceros calcitrans*). The experiment was performed over 64 days, during which temperature (20°C) and photoperiod

(12h/12h) were kept constant. During the first 35 days, salinity of the three tanks was maintained constant at 35. From days 35 to 64, one tank was kept at 35, while salinity was changed to 28 and 20 in the other two tanks. Salinities of 20 and 28 were obtained by mixing seawater and tap water within the 300 L buffer tanks, and salinity was checked and adjusted using a conductivity meter (WTW, LF 197-S). Salinity and temperature were recorded every five minutes in the three tanks using an autonomous data logger (YSI-600 OMS) from day 7 (t_7) to the end of the experiment (t_{64}). Two calcein markings were used to establish a temporal scale in the shells. Clams in each salinity treatment were exposed to a 150 mg L^{-1} calcein solution for 4 h (Rowley and Mackinnon, 1995; Thébault et al., 2005) at days 15 (t_{15}) and 35 (t_{35}). At the end of the experiment (t_{64}), 30 clams from each salinity were sacrificed and their shells were used for growth rate determination and geochemical sampling.

2.4. Sampling

Once per week, water from each experimental tank was filtered through $0.6 \mu\text{m}$ polycarbonate filters (47 mm diameter, Nucleopore). The filtrate was transferred into a 30 mL HDPE Nalgene bottle, acidified with 200 μL of trace metal grade bi-distilled HNO_3 and stored at 4°C until analysis.

At the end of the experiment, thirty clams from each tank were sacrificed (the other clams were previously removed for another experiment, see Poulain et al., 2010). Five of the thirty shells per batch were selected based on their age (two-year old clams were selected to avoid any effect of ontogeny on elemental shell carbonate results) and on their high shell growth rate to obtain sufficient material for elemental analysis.

2.5. Elemental analysis

Water

For water elemental analysis, 150 μL of water was diluted 25 times with ultra-pure deionized water ($> 18 \text{ M}\Omega \text{ cm}^{-1}$) acidified to a concentration of 2.5 % with bi-distilled HNO_3 . Water elemental composition was analyzed on a Finnigan Element 2 High Resolution - Inductively Coupled Plasma - Mass Spectrometer (HR-ICP-MS) at the European Institute of Marine Studies (IUEM, Brest, France). Indium was used as internal standard to control instrument fluctuation. Instrumental drift was monitored by running a seawater solution every 5 samples and data then were corrected accordingly. The calibration was achieved using artificial multi-

elemental solutions and a Certified Reference Material of seawater (CRM–SW, Greyhound, High Purity Standards). The isotopes monitored were ^{25}Mg , ^{43}Ca , ^{86}Sr , and ^{135}Ba . The reproducibility of the seawater solution was better than 4 % (percent relative standard deviation; % RSD) for Sr, Ca, and Mg and better than 8 % for Ba, which had much lower concentrations.

Shell laser ablation

A 8×10 mm piece of the shell, including the shell material formed during the experiment, was cut from the shell edge along the major growth axis and embedded in epoxy resin (Araldite 2020, DIL, France). A cross-section (800 μm thick) was then cut using a low speed diamond saw (Struers, Accutom-50) and glued on a glass slide with epoxy resin. Thick sections were then ground with wet sandpaper (1200, 2400 and 4000 μm grit size) and polished with a suspended diamond solution (3 μm) using a TegraPol 35 (Struers) automatic polisher. Shell sections were observed under a fluorescence microscope (OLYMPUS BX41) equipped with a 50 W high-pressure Hg lamp and a calcein filter. Photographs were acquired using a Hamamatsu C4742-95 digital camera fitted on the microscope. *R. philippinarum* shell consists of two aragonitic layers, an inner homogeneous layer and an outer prismatic layer. Four clear marks in the outer layer allowed assigning calendar dates on the shell sections: a cleft presumably due to the shell growth stop induced by the transfer from the field to the laboratory, the two calcein marks and the ventral margin which sets the date the experiment was ended (Fig. 1). Since the tidal periodicity of shell growth increment formation at intertidal flats is not lost in *R. philippinarum* even after several months in laboratory under constant conditions (Richardson, 1987; Poulain et al., 2011), it was possible to assign a calendar date to each growth increment (with a precision of ± 3 days due to small behavioral disturbances induced by handling). Some clams presented fewer increments than the number of tides between collection in the field and the first calcein marking. We hypothesize that handling and transfer from the field to the laboratory induced a shell growth stop, thus the hiatus was placed just after the collection in the field.

High-resolution geochemical sampling and analysis of shells were carried out on laser ablation ICP-MS (LA-ICP-MS). A Geolas Pro (COHERENT) equipped with a COMPexPro 102 excimer laser (193 nm) was used. Laser energy was 200 mJ and repetition rate was 4 Hz. Each analysis was done as a single spot (diameter of the spot: 90 μm , see Fig 1) and lasted 95

seconds with the first 8 seconds as background acquisition (gas blank). Data were normalized against the ^{43}Ca signal (internal standard) and calibrated using the NIST612 silicate standard with the values of Pearce et al. (1997). This standard was analyzed every 10 samples. The isotopes monitored were ^{25}Mg , ^{43}Ca , ^{45}Ca , ^{86}Sr , and ^{138}Ba . The reproducibility of the USGS MACS-1 carbonate standard (n=5) was better than 4 % (percent relative standard deviation; % RSD) for Sr/Ca, Mg/Ca, and Ba/Ca. The laser was shot in the middle of the prismatic outer layer (Fig. 1). For each shell, three ablations were performed before the cleft induced by clam collection in order to characterize shell elemental composition from growth in the field. For these three samples, the distance between the centers of two successive spots was approximately 250 μm , corresponding to two to three days of shell growth. Then, the distance between two spots was set to allow three samples between the cleft and the first calcein mark, four samples between the first and the second calcein mark and six samples between the second calcein mark and the ventral margin. The number of samples was reduced when the shell growth was too small to follow this procedure.

2D-mapping with WDS Microprobe

Transversal sections of two clamshells reared at salinity 35 were re-ground and re-polished after the laser ablation, to remove the ablation pits. Just before microprobe analysis, shell sections were carbon coated under vacuum. Ion microprobe analysis was performed using a Camebax SX 100 WDS microprobe (Cameca) at Ifremer Brest (France). 2D-mapping of calcium (Ca), sulfur (S), and magnesium (Mg) was performed. The samples were analyzed using a 200 nA beam current, a voltage of 20 kV, an acquisition time of 180 ms and a step-size of 5 μm .

2.6.Data analysis

Elements (Me) are typically reported as a ratio to calcium (as molar ratios). The partitioning between the water and shell is expressed as a partition coefficient (D_{Me}):

$$D_{\text{Me}} = (\text{Me/Ca})_{\text{carbonate}} / (\text{Me/Ca})_{\text{water}}$$

The partition coefficient allows the evaluation of elemental discrimination in shell against the ambient water chemistry.

All statistical analyses were performed using the software Statgraphics. Homoscedasticity was tested using Bartlett's test ($\alpha=0.05$). ANOVAs were performed to check the differences in mean shell growth of clams for the three salinity conditions between t_{35} and t_{64} . The differences in water elemental concentration, water elemental ratios, shell elemental ratios and partition coefficient between the three salinity conditions were tested by performing non-parametric Kruskal-Wallis test.

3. Results

Culture conditions

Water temperature and salinity (except the shift at t_{35}) were stable during the experiment in clam tanks. Mean temperature (in °C) of the three tanks was 20.21 ($1\sigma = 0.27$), 20.17 ($1\sigma = 0.32$) and 20.09 ($1\sigma = 0.31$). During the first 35 days, salinity of the three tanks was 35.42 ($1\sigma = 0.21$), 35.77 ($1\sigma = 0.11$) and 35.36 ($1\sigma = 0.10$) respectively. From days 35 to 64, the first tank was maintained at 35.28 ($1\sigma = 0.16$), while salinity was changed to 28.02 ($1\sigma = 0.14$) and 20.76 ($1\sigma = 0.13$) in the two other tanks.

The Ca, Ba, Mg, and Sr concentrations of the water were relatively constant through time per salinity treatment (Table. 1). For each element, there was a significant difference in average values between the three salinity treatments (Kruskal-Wallis, $p < 0.0001$). The Ca, Mg, and Sr concentrations of the water were positively correlated to salinity whereas Ba concentration of the water was inversely correlated to salinity, which mimics typical estuarine conditions.

Ba/Ca water ratios were very different between the three salinity treatments (Kruskal-Wallis, $p < 0.0001$), Ba/Ca increased with decreased salinity. Mg/Ca water ratios were slightly but significantly different between the three salinity treatments (Kruskal-Wallis, $p < 0.0001$). Sr/Ca water ratios at salinity 20 were significantly different than Sr/Ca ratios at salinity 28 and 35 (Kruskal-Wallis, $p < 0.022$). Both Mg/Ca and Sr/Ca water ratios slightly decreased with decreased salinity.

Shell growth

All clams selected for elemental analyses exhibited significant shell growth during the experiment. Harvesting clams in the field and transportation to the laboratory induced a

decrease of the daily growth from approximately 80 to 45 $\mu\text{m day}^{-1}$, however, after 35 days in the laboratory, daily shell growth increased to approximately 70 $\mu\text{m day}^{-1}$ (Fig. 2.A). The three salinity treatments tested between day 35 and the end of the experiment showed no significant differences on shell growth of the clams used for elemental analyses (ANOVA, $p = 0.18$), with a mean total shell growth of 2227 μm ($\pm 108 \mu\text{m}$, $n=5$), 1901 μm ($\pm 153 \mu\text{m}$, $n=5$) and 1800 μm ($\pm 203 \mu\text{m}$, $n=5$) at salinity 35, 28 and 20 respectively (Fig. 2.B).

Shell Mg/Ca, Ba/Ca, and Sr/Ca profiles

Profiles of Mg/Ca, Ba/Ca, and Sr/Ca in shells reveal different patterns (Fig. 3). Clam transplantation from the field to experimental tanks induced an increase of Mg/Ca shell ratios from 1.79 (± 0.07) to 2.39 (± 0.18) mmol mol^{-1} . After which, Mg/Ca shell ratios increased though time to 3.67 (± 0.18) mmol mol^{-1} at t_{51} and were nearly stable between t_{51} and the end of the experiment (t_{64}). The salinity change at t_{35} from salinity 35 to 28 resulted in less of an increase of Mg/Ca ratios compared to values at salinity 35, but caused Mg/Ca ratios to decrease in salinity 20. Except at the first date after the salinity change, all the values at salinity 20 are significantly lower than values at salinity 35 and values at salinity 20 are significantly lower than values at salinity 28 at t_{51} and t_{62} (Kruskal-Wallis, $p < 0.05$).

Shell Ba/Ca was relatively constant at salinity 35 during the experiment, with a mean value of 0.77 (± 0.04) $\mu\text{mol mol}^{-1}$. The decrease of salinity at t_{35} induced an immediate increase of shell Ba/Ca ratios to 4.03 (± 0.45) $\mu\text{mol mol}^{-1}$ at salinity 28 and 11.30 (± 1.32) $\mu\text{mol mol}^{-1}$ at salinity 20. Between t_{35} and the end of the experiment, Ba/Ca ratios were nearly constant in each salinity treatment, with a mean value of 4.49 (± 0.20) $\mu\text{mol mol}^{-1}$ at salinity 28 and 11.74 (± 0.58) $\mu\text{mol mol}^{-1}$ at salinity 20. At each sampling date after t_{35} , Ba/Ca ratios were significantly different between the three salinity conditions (Kruskal-Wallis, $p < 0.005$). Ba/Ca shell ratios and Ba/Ca water ratios were strongly positively correlated:

$$\text{Ba/Ca}_{\text{shell}} = 0.136 (\pm 0.005) \times \text{Ba/Ca}_{\text{water}} - 0.365 (\pm 0.203)$$

($r^2 = 0.88$, $p < 0.0001$, $n=112$). The intercept was not significant in the regression ($p=0.075$) and therefore was removed resulting in the regression equation:

$$\text{Ba/Ca}_{\text{shell}} = 0.130 (\pm 0.003) \times \text{Ba/Ca}_{\text{water}},$$

($r^2 = 0.93$ $p < 0.0001$, $n=112$, Fig. 4).

Shell Sr/Ca ratios were $2.3 (\pm 0.1)$ mmol mol⁻¹ in the field. Transplantation from the field to experimental conditions resulted in a decrease of shell Sr/Ca ratios through time to $1.5 (\pm 0.04)$ mmol mol⁻¹ at t_{17} . After t_{35} , Sr/Ca ratios increased with decreased salinity. However, Sr/Ca shell ratios were not significantly different between the three salinity conditions (Kruskal-Wallis, $p > 0.05$), except at t_{36} (Kruskal-Wallis, $p = 0.027$, the values at salinity 20 are significantly higher than values at salinity 35). The inter-individual variability of Ba/Ca_{shell} and Sr/Ca_{shell} increased at lower salinities (Fig. 3).

2D-Mapping of Mg and S with WDS microprobe

Results from microprobe Mg mapping for the two clams reared in constant conditions at salinity 35 were similar to those obtained using laser ablation; with both methods Mg concentration increased through experimental time (Fig. 5 and Fig. S1). Furthermore, Mg mapping revealed that Mg concentrations exhibited disparities along the prismatic layer height, Mg concentration increased with increasing distance from the periostracum. S mappings appeared to be different between the two clams analyzed. In one clam, a decrease of S was observed through time, whereas S was more homogeneous through time in the other clam (See Fig. 5 and S1). Moreover, higher S concentrations were found when the clam grew slower following the clam transplantation between t_0 and t_{15} , corresponding to dark growth lines. No evident correlation between Mg and S concentration appeared from visual comparison of the mapping for the two shells (Fig. 5 and S1).

4. Discussion

Our results show that shell Mg/Ca and Sr/Ca ratios varied through time despite constant temperature and nearly constant water elemental ratios. Changing salinity and water chemistry induced a significant variation of Mg/Ca, Sr/Ca, and Ba/Ca shell ratios. This experiment highlights the complexity of Mg and Sr incorporation into bivalve shell carbonate even under controlled conditions with stable environmental parameters. Under such conditions, we clearly show that highly variable Mg/Ca and Sr/Ca ratios occur within the

aragonite prismatic outer shell layer despite constant environmental conditions. These results are in agreement with number of studies which suggested that the uptake of these two elements are under strong biological control (e.g. Gillikin et al. 2005, Lorrain et al. 2005, Carré et al. 2006, Lazareth et al. 2007). However, Ba/Ca varied in a somewhat predictable manner, tracking Ba/Ca ratios of the water as has also been reported in other studies (e.g., Gillikin et al. 2006, 2008).

Mg/Ca shell profiles

Our experimental protocol allows investigation of shell elemental ratios through time. At salinity 35, with stable temperature (20°C) and water chemistry ($\text{Mg} \approx 1319 \text{ ppm}$; Table 1), stable $\text{Mg/Ca}_{\text{shell}}$ ratios were expected. Nevertheless, an increase in $\text{Mg/Ca}_{\text{shell}}$ ratios of nearly $1.5 \text{ mmol mol}^{-1}$ (a ~75% increase) was observed through time with both analytical methods (i.e. laser ablation and WDS microprobe). These Mg/Ca variations were as large as seasonal variations in Mg/Ca found in most aragonitic bivalve shells from coastal environments (e.g. Carré et al. 2006 in *Mesodesmadonatum* and *Chionesubrugosa*; Foster et al. 2008 in *Arctica islandica*; Takesue et al. 2008 in *Corbula amurensis*). These results illustrate that $\text{Mg/Ca}_{\text{shell}}$ ratios do not record variations in environmental parameters.

The decrease from salinity 35 to 20 at t_{35} induced a significant decrease in shell Mg/Ca ratios, but the Mg/Ca shift did not immediately follow the salinity shift. This lag in shell Mg/Ca ratios suggests that Mg^{2+} is not directly transferred from seawater to the site of calcification (extrapallial fluids) and that Mg^{2+} might have a certain metabolic residence time in the bivalve's tissues. This suggests that the amount of magnesium in shell carbonate is highly regulated by the organism during shell precipitation (cf. Lorens and Bender, 1980), providing further evidence for metabolic influences on shell Mg/Ca ratios.

Lorens and Bender (1980) have suggested that transplantation from field to laboratory induced precipitation of new shell carbonate with higher Mg/Ca ratios, possibly due to stress caused by handling and adaptation to laboratory conditions. Transplantation of clams and associated changes (e.g. handling, absence of sediment in tank and quality, quantity and elemental composition of food) may disturb the clams and induce a significant reduction in growth, which usually also results in higher shell organic matrix (Foster et al. 2008). Recent studies have also indicated that a large amount of Mg in shells does not reside in the aragonite

lattice, but instead is associated with organic compounds (Takesue et al. 2008, Foster et al. 2008, Jacob et al. 2008, Schöne et al. 2010) and/or nanoparticles of the inorganic phase (Foster et al. 2008). These higher shell organic contents have been proposed to be associated with high sulfur concentrations in biogenic mollusk carbonates (Rosenberg et al. 2001, Dauphin et al. 2003, 2005, Lazareth et al. 2007). The observed increase in Mg/Ca shell ratios in our study for the salinity 35 treatment could therefore be due to a reduction in shell growth induced by transplantation, which would have presumably resulted in higher shell organic content and subsequently in higher magnesium and sulfur concentrations. During the experiment, we observed a decrease of daily shell growth just after transplantation to the laboratory, and 2D-Mapping showed that this lower accretion rate was associated with high S and Mg concentrations in the shell. However, although this hypothesis can explain the increase of Mg during the first days of the experiment, it cannot explain the increase of Mg/Ca_{shell} ratios through the entire experiment at salinity 35 for two reasons: (1) the accretion rate is nearly constant until t_{35} and even increased after day 35 (Fig. 2), and (2) there is no correlation between the concentration of sulfur and magnesium in these shells, except at the beginning of the experiment, and therefore there does not seem to be a link between Mg content and shell organics (Fig. 5). Consequently, the increase of Mg/Ca shell ratios does not seem to be linked to an increase of the quantity of shell organic matter, but is probably linked to the stress of growing outside their natural habitat, maybe related to changes in internal physiological pH changes. Shifts in pH have been shown to control Mg incorporation into carbonates (Dove et al. 2014), and changes in physiology are known to alter internal pH (Crenshaw 1972).

The 2D-Mapping on two clams reared at salinity 35 illustrate that Mg/Ca shell ratios were not homogenous along the contemporaneous outer layer growth lines, but rather increased with increasing distance from the periostracum (Fig. 5). As laser ablations were always performed in the middle of the prismatic layer, the heterogeneity of the Mg concentration along the outer layer growth lines cannot explain the increase of Mg/Ca ratios through experimental time. While it is clear that shell Mg/Ca ratios are not under environmental control, the mechanism for Mg incorporation is not straightforward.

The Mg partition coefficients were low, with a mean value of nearly 0.00054 (Tab. 2, Fig. S2). Similar Mg partition coefficients were found by Gillikin (2005) in the aragonitic shell of *Saxidomus giganteus* and by Strasser et al. (2008) in the aragonitic shell of *Mya arenaria* ($D_{Mg} = 0.00022$ and 0.00013 respectively). These low partition coefficient values are characteristic

of inorganic aragonite as well ($D_{\text{Mg}} \approx 0.00016$; Oomori et al. 1987). Nevertheless, it is clear Mg/Ca ratios are not behaving as in inorganic aragonite.

Sr/Ca shell profiles

Sr/Ca ratios have been proposed as a salinity independent temperature proxy in aragonite shells (e.g., Hart and Blusztajn, 1998), because Sr/Ca ratios are generally constant in the ocean and even in estuaries down to a salinity of ~ 10 (Dodd and Crisp 1982). More recent studies, however, have illustrated that Sr/Ca is under biological control in many aragonite species (e.g., Purton et al. 1999; Takesue and van Geen 2004, Gillikin et al. 2005, Carre et al. 2006). In our experiment, $\text{Sr/Ca}_{\text{water}}$ were indeed almost constant, and we observed only a slight decrease by lowering salinity (Table 1). Conversely, $\text{Sr/Ca}_{\text{shell}}$ increased in lower salinities and was very unstable (Fig. 3). Variations of shell Sr/Ca ratios from clams reared at salinity 35 and at 20°C in this study (for 64 days; values ranged from 1.55 to $2.13 \text{ mmol mol}^{-1}$) were as large as seasonal Sr/Ca variations observed in the shell of aragonitic clams from the field (Stecher et al. 1996, Gillikin et al. 2005, Surge and Walker 2006, Foster et al. 2009). Moreover, Sr partition coefficients in these shells ($D_{\text{Sr}} \approx 0.25$; Table 2) are lower than expected for inorganic aragonite ($D_{\text{Sr}} \approx 1$; Kitano et al. (1971)), but match previously reported values from aragonite bivalve shells (e.g., $D_{\text{Sr}} \approx 0.25$; Gillikin et al., 2005; also see this study for an in-depth discussion on D_{Sr} in aragonite). This evidence provides strong support that shell Sr/Ca ratios are not under environmental control, but are under strong biological control and should not be used as an environmental proxy.

Ba/Ca shell profiles

Shell Ba/Ca ratios are clearly controlled by water Ba/Ca ratios (Fig. 3 and 4). Our results confirm and add confidence to the use of shell Ba/Ca as a proxy of environmental Ba/Ca ratios (which are also correlated with salinity). As previously discussed, shell Ba/Ca ratios in bivalves are typically characterized by stable background Ba/Ca shell ratios punctuated by sharp peaks (e.g. Stecher et al. 1996, Vander Putten et al. 2000, Gillikin et al. 2008, Thébault et al. 2009a, Hatch et al. 2013, Goodwin et al. 2013). Our experimental conditions were chosen to avoid these enigmatic Ba/Ca peaks (see Gillikin et al. 2008) and to work only on the background signal. Gillikin et al. (2006) have demonstrated that *Mytilus edulis* shell background Ba/Ca ratios are directly related to dissolved Ba concentration in the surrounding water. Similarly, we observed a strong relationship between shell Ba/Ca ratios and water

Ba/Ca ratios ($Ba/Ca_{shell} = 0.133 \times Ba/Ca_{water}$, $r^2 = 0.89$, $p < 0.0001$, $n=177$). The partition coefficient calculated for the aragonite shells of *R. philippinarum* shells (0.13) is similar to that reported for the calcite shells of *M. edulis* (~0.10; Gillikin et al. 2006) and aragonite and calcite shells from other bivalve species (0.16 and 0.18; Gillikin et al. 2008; we also refer to these papers for a more in-depth discussion on Ba partition coefficients). Our results also demonstrate that the change of Ba^{2+} concentrations in the water was reflected in the shell in a very short time (1 day, see Fig. 3). As a result, background Ba/Ca ratios of the aragonitic shell of *R. philippinarum* could be an indicator of dissolved Ba^{2+} in the surrounding water at a very high temporal resolution (daily). Considering the inverse relationship between Ba/Ca water and salinity (e.g., Coffey et al. 1997; Gillikin et al., 2006), background Ba/Ca shell could be used to reconstruct high-resolution salinity fluctuations in estuarine and nearshore waters.

5. Conclusion

This experimental study suggests that Mg/Ca and Sr/Ca ratios in the aragonitic shells of *R. philippinarum* are not good environmental proxies. However, background Ba/Ca shell ratios seems to be a promising proxy of Ba/Ca ratios in the surrounding water with rapid response times (1 day) and therefore are a proxy of high-resolution salinity fluctuations in estuarine waters.

Acknowledgements

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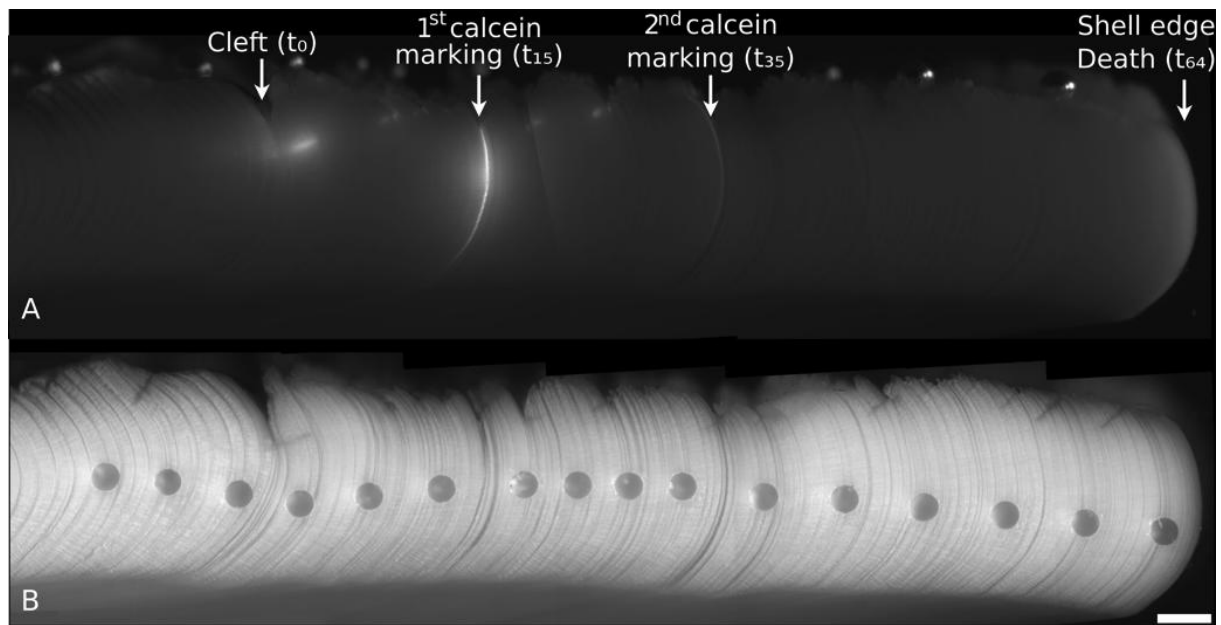
Figures:

Fig. 1. Photographs of shell transversal section. Growth direction is left to right, the outside of the shell is toward the top of the pictures. A: The cleft corresponds to a growth stop due to harvesting in the field on the 18th of August 2008. Clams were kept in laboratory for 64 days. The two calcein stains at t_{15} and t_{35} are visible. Shell edge corresponds to the end of the experiment. B: Laser ablation holes (90 μm diameter) made in the middle of the outer layer. Scale bar = 200 μm .

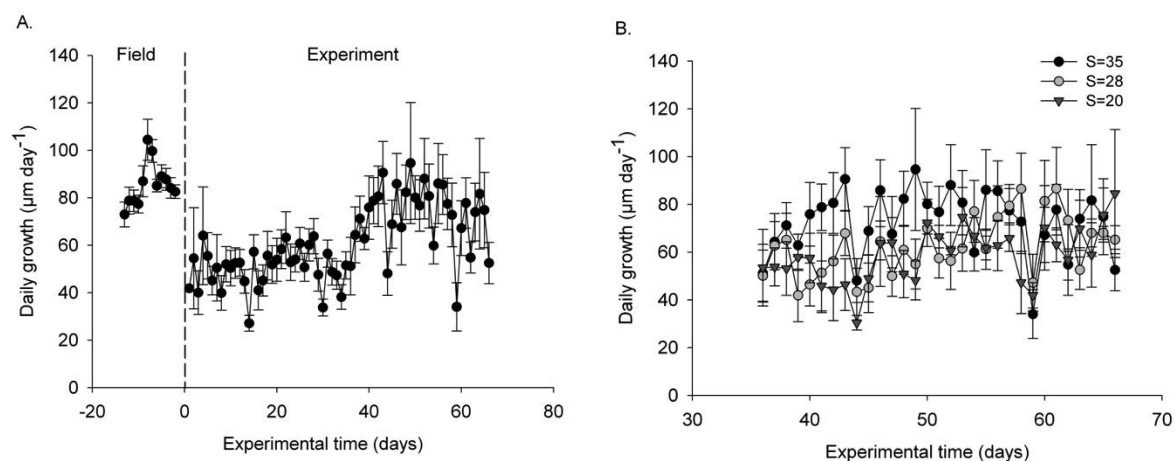


Fig. 2. A. Daily shell growth of clams *in situ* (field) and during the experiment at salinity 35 ($n = 15$ until the day 35 and $n = 5$ from day 35 to the end of the experiment). B. Daily shell growth of clams reared at salinity 20, 28 and 35 between t_{35} and the end of the experiment ($n=5$ for each salinity treatment). Values are expressed as means \pm SE.

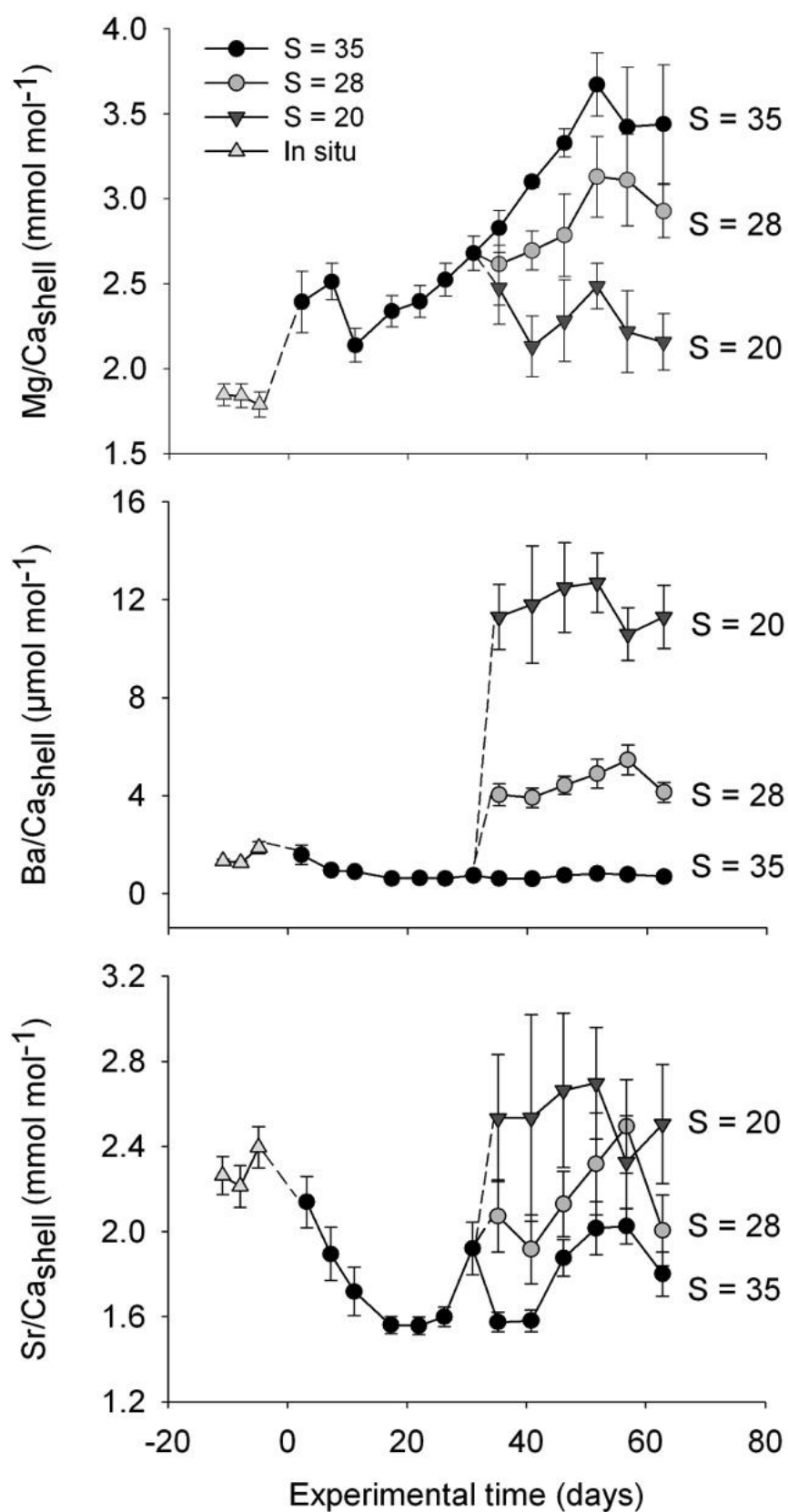


Fig. 3. Mg/Ca, Ba/Ca, and Sr/Ca ratios of shells cultured at salinity 35 (black circles), 28 (grey circles) and 20 (grey triangles). Values are expressed as means and error bars represent

standard errors. For each point, $n = 15$ until the day 35 and $n = 5$ from day 35 to the end of the experiment, except for the first two points of the experiment where $n = 8$ due to cessation of growth of some clams after collection.

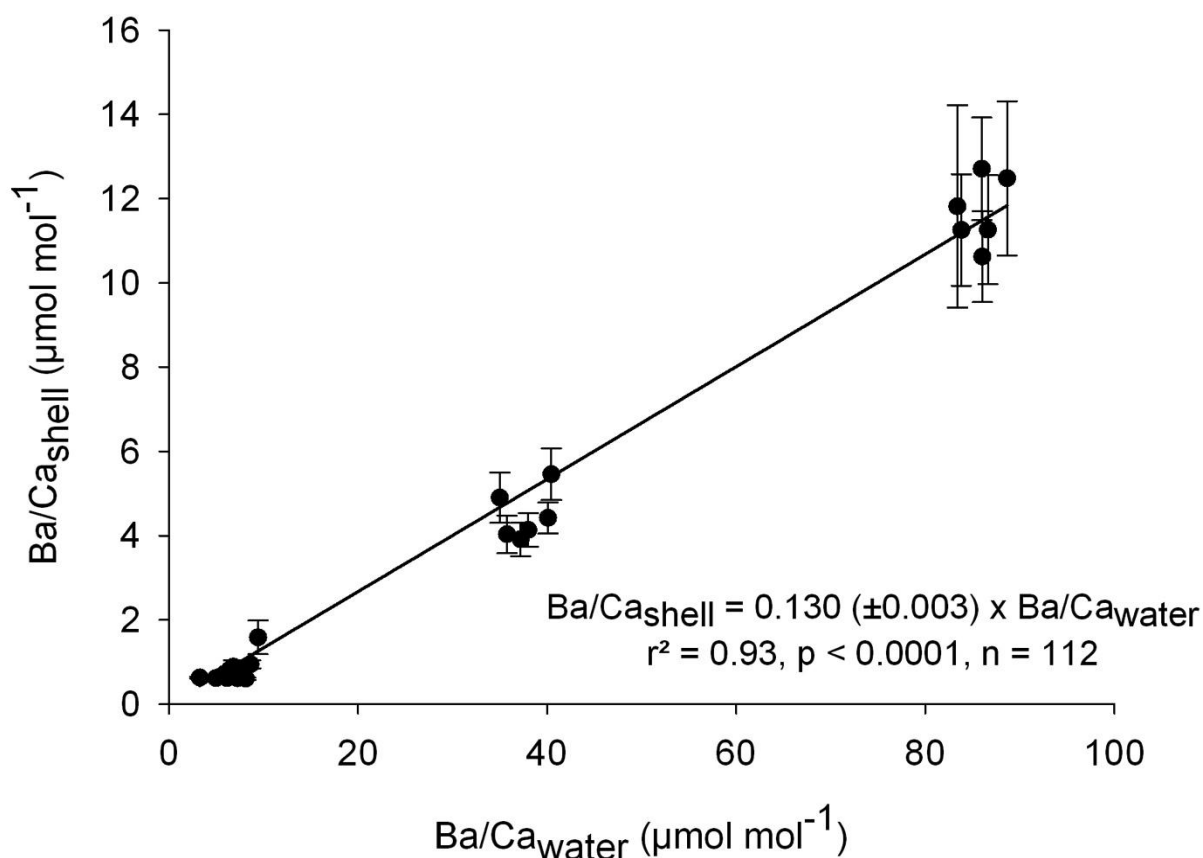


Fig. 4. Mean shell Ba/Ca ratios per sampling date (\pm SE) versus Ba/Ca ratios of water during the laboratory experiment. The solid line shows the linear squares regression calculated with all the data obtained during this experiment.

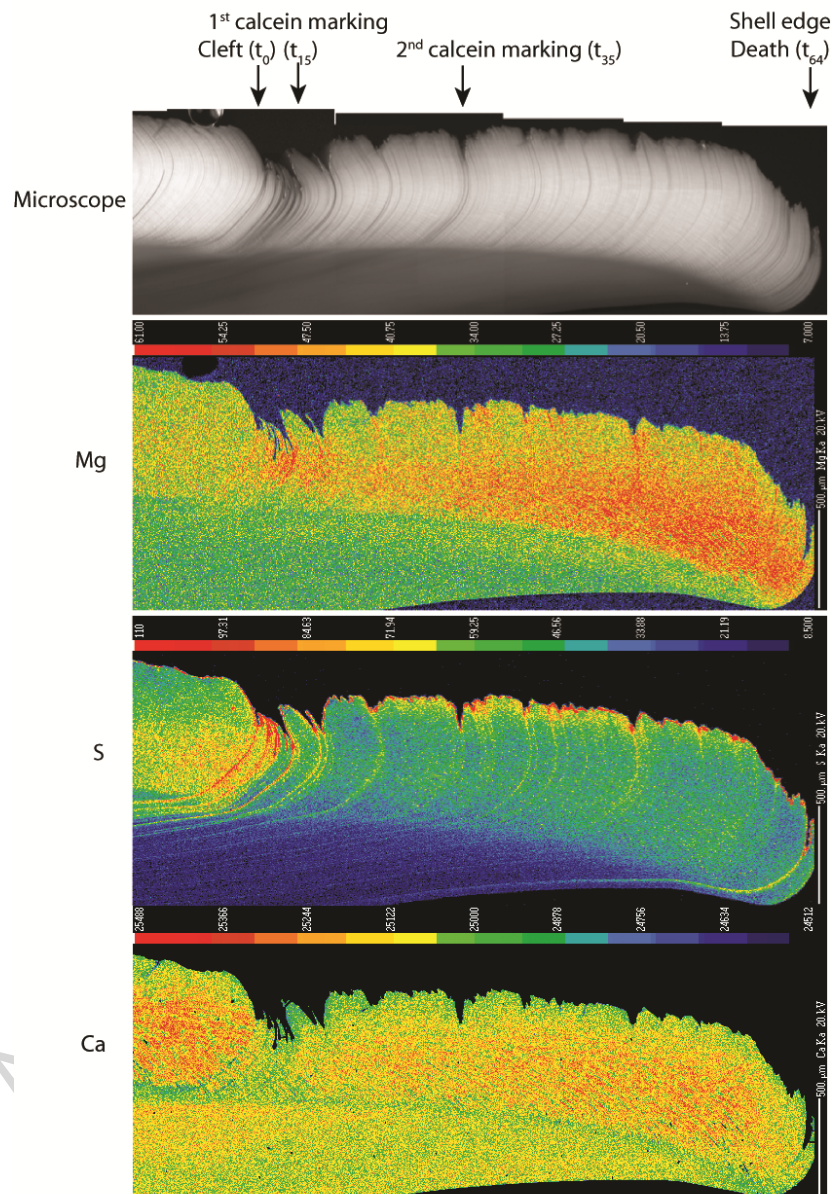


Fig. 5. Reflected microscope photograph and WDS Microprobe ionic mapping of Mg, S, and Ca of shell transversal section of a clam reared at salinity 35 during the experiment (clam 1). High concentrations are presented in red and low concentrations in blue.

Table 1. Chemical properties of the water in tanks during the experiment. Values are expressed as means \pm SE.

	t_0 to t_{35}	t_{35} to t_{64}		
	All tanks	Tank 1	Tank 2	Tank 3
	S = 35	S = 35	S = 28	S = 20
$[\text{Ca}^{2+}]$ (ppm)	398 (\pm 10)	407 (\pm 5)	322 (\pm 4)	250 (\pm 3)
$[\text{Ba}^{2+}]$ (ppb)	9.4 (\pm 1.3)	8.9 (\pm 0.3)	40.0 (\pm 2.7)	73.0 (\pm 1.4)
$[\text{Mg}^{2+}]$ (ppm)	1306 (\pm 36)	1326 (\pm 16)	1006 (\pm 12)	756 (\pm 11)
$[\text{Sr}^{2+}]$ (ppm)	7.73 (\pm 0.18)	8.01 (\pm 0.16)	6.27 (\pm 0.09)	4.69 (\pm 0.07)
Ba/Ca ($\mu\text{mol mol}^{-1}$)	6.9 (\pm 1.0)	6.4 (\pm 0.3)	36.2 (\pm 2.4)	85.6 (\pm 1.1)
Mg/Ca (mmol mol^{-1})	5422 (\pm 21)	5360 (\pm 24)	5150 (\pm 36)	4979 (\pm 35)
Sr/Ca (mmol mol^{-1})	8.90 (\pm 0.15)	8.97 (\pm 0.11)	8.89 (\pm 0.07)	8.57 (\pm 0.08)

Table 2: Partition coefficient of Ba, Mg, and Sr per salinity treatment (20, 28 and 35) between t_{35} and the end of the experiment.

Salinity treatment	D_{Ba}	D_{Mg}	D_{Sr}
20	0.137 (\pm 0.007)	0.00046 (\pm 1.55×10^{-5})	0.298 (\pm 0.014)
28	0.120 (\pm 0.006)	0.00056 (\pm 1.79×10^{-5})	0.242 (\pm 0.009)
35	0.107 (\pm 0.005)	0.00062 (\pm 1.78×10^{-5})	0.202 (\pm 0.006)

Supplementary data

Fig. S1. Reflected microscope photograph and WDS microprobe ionic mapping of Mg, S, and Ca of shell transversal section of a clam reared at salinity 35 (clam 2) during the experiment. High concentrations are presented in red and low concentrations in blue.

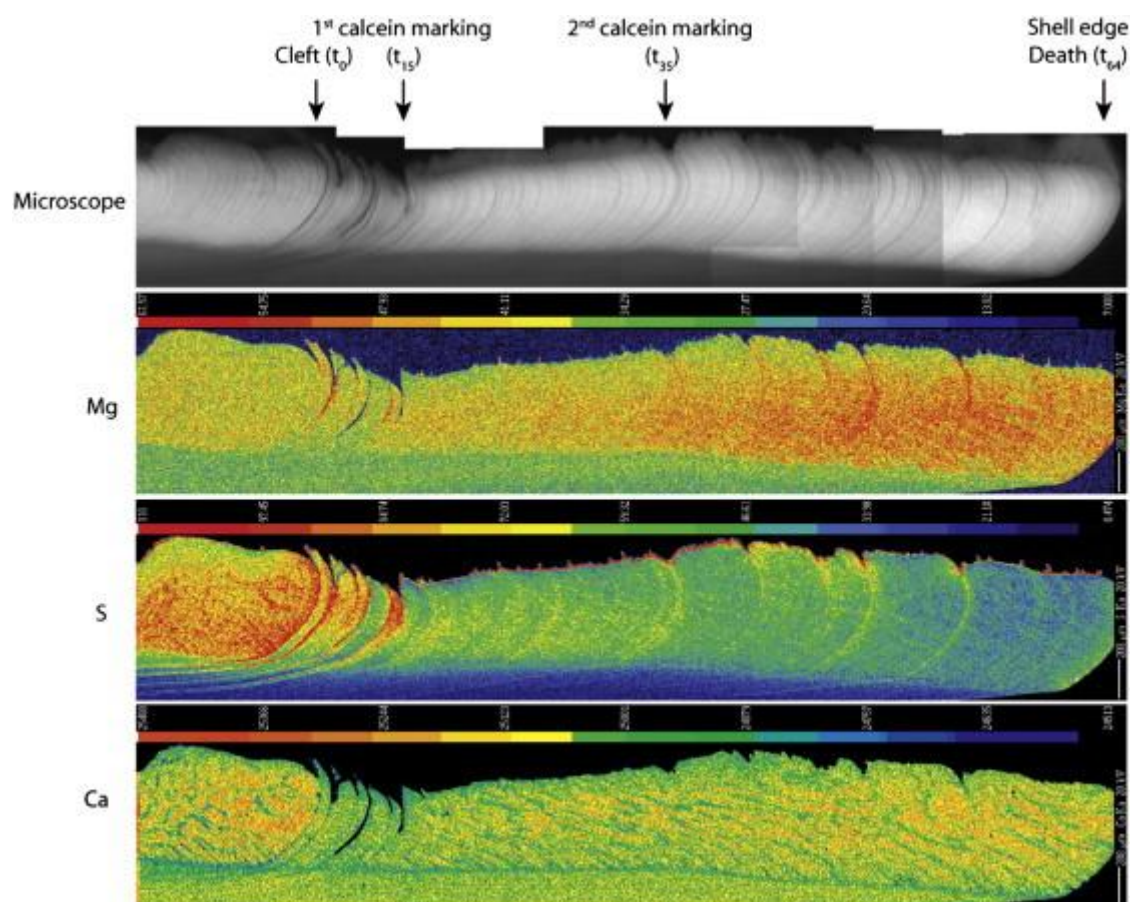


Fig. S2. Partition coefficients (DMg, DBa, and DSr) of shells cultured at salinity 35 (black circles), 28 (grey circles) and 20 (grey triangles). Values are expressed as means and error bars represent standard errors. For each point, $n = 15$ until the day 35 and $n = 5$ from day 35 to the end of the experiment, except for the first two points of the experiment where $n = 8$ due to cessation of growth of some clams after collection.

