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Experimental evidence for foraminiferal calcification under anoxia

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Abstract. Benthic foraminiferal tests are widely used for paleoceanographic reconstructions from a range of different environments with varying dissolved oxygen concentrations in the bottom water. There is ample evidence that foraminifera can live in anoxic sediments. For some species, this is explained by a switch to facultative anaerobic metabolism (i.e. denitrification). Here we show for the first time that adult specimens of three benthic foraminiferal species are not only able to survive, but are also able to calcify under anoxic conditions, at various depths in the sediment, and with or without nitrates. In fact, several specimens of *Ammonia tepida* (1–4 %), *Bulimina marginata* (8–24 %) and *Cassidulina laevigata* (16–23 %) were able to calcify at different redox fronts of sediment cores, under laboratory conditions. This demonstrates ongoing metabolic processes, even in micro-environments where denitrification is not possible. Earlier observations suggest that the disappearance of foraminiferal communities after prolonged anoxia is not due to instantaneous or strongly increased adult mortality. Here we show that it cannot be explained by an inhibition of growth through chamber addition either. Our observations of ongoing calcification under anoxic conditions mean that geochemical proxy data obtained from benthic foraminifera in settings experiencing intermittent anoxia have to be reconsidered. The analysis of whole single specimens or of their successive chambers may provide essential information about short-term environmental variability and/or the causes of anoxia.

1 Introduction

Oxygen depletion is one of the most severe environmental stressors in marine ecosystems. It is predicted to increase in the near future (Sarmiento et al., 1998; Keeling and Garcia, 2002; Meier et al., 2011) due to climate change, changes in circulation and enhanced eutrophication. There is therefore an urgent need to study the past variability of dissolved oxygen concentrations in benthic ecosystems in response to climate change. Foraminifera are among the most ubiquitous marine calcifying micro-organisms and among the most commonly used proxy carriers. The identification of the environmental conditions under which calcification takes place is of prime importance for the interpretation of proxies based on the geochemical composition of the foraminiferal tests. Although many foraminiferal species are known to be able to survive short to long periods of hypoxic or even anoxic conditions (Bernhard, 1993; Bernhard and Alve, 1996; Moodley et al., 1997; Langlet et al., 2013; Geslin et al., 2014), it is still an open question as to whether benthic foraminifera are able to calcify under anoxia. The ability of some species to calcify under hypoxia ($< 63 \mu\text{mol L}^{-1}$; Middelburg and Levin, 2009) was only recently shown experimentally, by Geslin et al. (2014). The discovery of a facultative anaerobic metabolism (i.e. denitrification; Risgaard-Petersen et al., 2006) by certain foraminiferal species, and the hypothesis of other possible anaerobic pathways suggested by several authors (e.g. Bernhard and Alve, 1996; Bernhard and Bowser, 2008), allowing them to survive and potentially be active in the absence of oxygen, suggested that calcification could eventually also take place under anoxia.

We designed an experiment that allowed us to study the calcification of three foraminiferal species in various

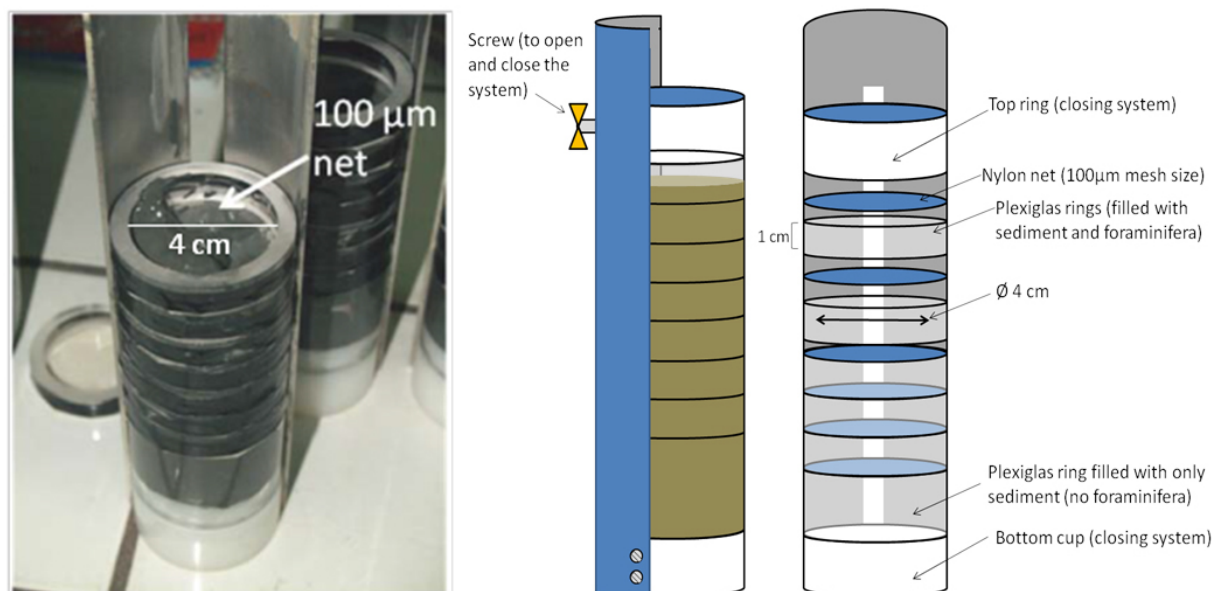


Figure 1. Experimental cores. Picture showing the filling up of the cores with sieved sediment (left) and the scheme of the experimental cores (right).

geochemical microenvironments in the sediment (along a redox cline), eliminating bioturbation effects and inhibiting foraminifera from migrating to more favourable microhabitats, as was previously observed by several authors (Alve and Bernhard, 1995; Moodley et al., 1998a; Duijnste et al., 2003; Geslin et al., 2004). Two experiments were carried out using three benthic foraminiferal species: (1) *Ammonia tepida* (coastal species), and (2) *Bulimina marginata* and (3) *Cassidulina laevigata* (shelf to deep-sea species).

2 Materials and methods

2.1 Experimental design

For each experiment, five cores were filled with natural sediment, sampled at the same sites where each foraminiferal species was also collected (see the next paragraphs for details), and sieved ($< 38 \mu\text{m}$) without the addition of water by using a brush to press the sediment through the sieve. The cores were then placed in an aquarium filled with approximately 20 L of bubbled artificial seawater (ASW) (Red Sea salt in MilliQ water) and kept under controlled conditions of salinity and temperature in the dark, to avoid algal blooms and the consequent geochemical instability. For the same reason no fresh organic matter was added during the experiment, despite the potentially negative impact on survival and calcification rates. Two of these cores, with internal diameters of 8 cm, were used for geochemical analysis at the start (T_{start}) and at the end (T_{end}) of each experiment. Foraminifera were introduced in the three remaining special cores (Fig. 1), constituted of superposed Plexiglas rings (4 cm \varnothing , 1–3 cm high),

which were each separated by 100 μm mesh nylon nets to avoid foraminiferal migration. The cores were left 30 days in the aquarium before starting the experiment, in order to retrieve a relative stability of geochemical parameters of the cores.

After one month (T_{start}), the foraminiferal replicate cores were opened under an N_2 -flushed atmosphere to introduce calcein-labelled foraminifera following Bernhard et al. (2004). The cores were then returned to the aquaria after being introduced in plastic bags filled with sediment, to avoid any further possible lateral oxygen penetration. At this time the first geochemical core was removed from the aquarium and sliced under an N_2 -flushed atmosphere to obtain pore waters for geochemical analyses (see below for details).

At the end of the experiment, sixty days later (T_{end}), each sediment layer of the foraminiferal cores was sieved (100 μm) with ASW, and the foraminifera were picked. A two-step observation was performed on each specimen in order to avoid any possible bias related to the use of two fluorescent probes (calcein and fluorescein diacetate) that excite and emit at similar wavelengths (Fig. 2). First, the presence of newly formed chambers, not calcein labelled, was checked using epifluorescence microscopy (Nikon SMZ 1500, 460–490 nm excitations) for the specimens of a core layer (a process which took approximately 1 h). Only thereafter the foraminifera were incubated for 24 h in a 10 μM solution of fluorescein diacetate (FDA) in ASW (Bernhard et al., 1995) and assessed for vitality under epifluorescence microscopy. The much more intense fluorescence of FDA-labelled cells compared to dead calcein-labelled specimens allowed us to distinguish dead and live foraminifera easily, even in the

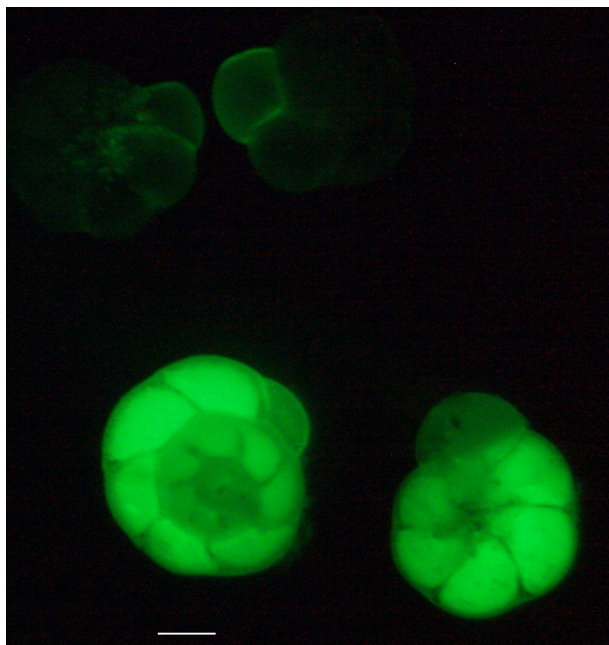


Figure 2. Example of calcein-labelled specimens of *Ammonia tepida* as they were observed before incubation in FDA (top), and two living specimens after FDA incubation (bottom). Photo exposure time: 1/4". Scale bar is 100 μm .

presence of calcein-labelled chambers (see Fig. 2). Moreover, specimens with new chambers were incubated separately from the others in an FDA solution, in order to avoid any mixing of samples. At the end of the experiment, the remaining geochemical core was sliced for pore water analyses at T_{end} .

2.2 Experiment 1: *Ammonia tepida*

Superficial sediment ($\approx 2\text{ mm}$) was hand collected during the low tide period, at the intertidal area of Aiguillon Bay (Bay of Biscay, France; $46^{\circ}15.279'\text{ N}$, $-1^{\circ}8.410'\text{ O}$) in January 2013. The sediment was sieved ($63\text{ }\mu\text{m}$) on a field with natural seawater and stored in plastic jars filled with seawater until laboratory. There, the seawater in the jars was substituted with a solution of calcein (10 mg L^{-1}) in artificial seawater (ASW) for calcite labelling (Bernhard et al., 2004). At the same site, sediment samples to fill up the cores (both geochemical and foraminiferal ones) were also collected. Sub-superficial ($0.5\text{--}10\text{ cm}$) sediment, potentially poorer in organic matter content than superficial sediment, was used to avoid a compression of redox fronts towards the top of the core (Michaud et al., 2010). The sediment was sieved ($< 38\text{ }\mu\text{m}$) without addition of water, in order to remove foraminifera and most of the meiofauna, to avoid bio-turbation, and was homogenised before its introduction into the cores.

The top layer of the foraminiferal cores was only partially filled, with a sediment layer of 0.3 cm . The choice was made in order to obtain a first layer as high as the oxygen penetration depth in the core and therefore to separate the oxygenated layer 1 from the hypoxic to anoxic layers below.

The cores were placed in the dark in an aquarium filled with ASW at salinity 35 ± 0.1 and an ambient temperature between 16.5 and 18°C . An air bubbler was placed in the aquarium to oxygenate the water and to create a minimal circulation.

After thirty days, the sediment incubated in the calcein solution was sieved at $150\text{--}350\text{ }\mu\text{m}$ with ASW, and the calcein-labelled specimens were picked. The vitality of each specimen was tested on a thin layer of fine sediment as described in Koho et al. (2011). The foraminiferal specimens were randomly separated into groups of 50 individuals. Foraminiferal cores were opened under an N_2 atmosphere, to avoid re-oxygenation of deep sediments, and 50 specimens of *Ammonia tepida* were introduced at each layer with a Pasteur pipette. To avoid any possible sediment re-oxygenation, the ASW needed for the foraminiferal transfer from Eppendorf tubes to core layers (0.5 mL maximum) was first flushed with N_2 . Only for the superficial oxygenated layer were foraminifera introduced under an oxygenated atmosphere.

2.3 Experiment 2: *Bulimina marginata* and *Cassidulina laevigata*

Sediment sampling was performed with a box corer at site GF1 ($58^{\circ}19.284'\text{ N}$, $11^{\circ}32.902'\text{ E}$) at 117 m in depth, and site GF3 ($58^{\circ}16.042'\text{ N}$, $11^{\circ}28.901'\text{ E}$) at 51 m in depth, in the Gullmar Fjord, Sweden, in October 2012. The first millimetres of sediment were collected with a spoon and sieved ($63\text{ }\mu\text{m}$) with bottom waters sampled by a standard Niskin bottle (10 L) collection system to avoid thermal and salinity stress to foraminifera. Temperatures at the sampling site varied between 6 and 7.5°C . The samples were transported into ice boxes in order to maintain a constant temperature until the laboratory. The sediment was then incubated at $12 \pm 0.5^{\circ}\text{C}$ in a solution of calcein in seawater from the sample site (10 mg L^{-1}). The choice to incubate the samples and then to perform the experiment at 12°C for these species was taken because Barras et al. (2009) suggest that this is the optimal temperature favouring the growth of *Bulimina marginata*.

The sediment to fill the experimental cores was collected at site GF1. Three sediment layers, $0\text{--}5$, $5\text{--}10$ and $10\text{--}15\text{ cm}$, were collected separately from a box corer and sieved ($< 38\text{ }\mu\text{m}$) in the laboratory without addition of water. The cores were introduced in an aquarium filled with approximately 20 L of ASW at salinity $= 34 \pm 0.1$, air bubbled and incubated in the dark at $12 \pm 0.5^{\circ}\text{C}$.

After incubation in calcein solution, labelled specimens of *Bulimina marginata* and *Cassidulina laevigata* ($> 100\text{ }\mu\text{m}$) were picked. As these species are much less motile than *Ammonia tepida*, their vitality was tested by incubating ($\approx 24\text{ h}$)

the specimens in Petri dishes filled with ASW and a thin layer of a concentrated solution of lyophilised *Chlorella* sp. in ASW (ca. 0.5 g/50 mL) on the bottom. The specimens showing moving traces and/or dark green cytoplasm (due to algal ingestion) were considered to be alive.

Thirty days after the preparation of sediment cores, *Bulimina marginata* specimens ($n = 31$ or 32) were introduced at layers 1–5 (corresponding to 0–4.3 cm of the absolute sediment depth), while *Cassidulina laevigata* ($n = 32$ or 33) was only introduced at layers 1, 2 and 4, corresponding to absolute depths respectively of 0–0.3, 0.3–1.3 and 2.3–3.3 cm. The procedure to introduce foraminiferal specimens was the same described for experiment 1.

2.4 Geochemical analyses

2.4.1 Oxygen profiles

Oxygen profiles (up to 8) were performed on the geochemical cores daily during the first week and then weekly during the rest of the incubation time, using a Clark-type microelectrode with a 50 μm thick tip (OX50, Unisense, Denmark).

2.4.2 pH profiles

pH profiles were measured at 1000 μm depth increments on each core at T_{start} and T_{end} using a glassy microelectrode (Unisense, Denmark) with a 500 μm thick tip and a Ag / AgCl reference. The probes were calibrated using NBS buffer solutions (pH 4, 7 and 10). pH values are given as δpH , to remove errors due to the use of NBS standard buffers which do not have the same matrix of the analysed marine water samples (Metzger et al., 2007). The δpH was calculated as the difference between pH values at each sediment depth and the pH of overlying waters.

2.4.3 Analyses of pore waters

Geochemical cores at T_{start} and T_{end} of each experiment were cut under an N_2 -flushed atmosphere. Each sediment layer was centrifuged (10 min, 5000 r min^{-1}) to extract pore water. The water was then filtered (0.20 μm) and analysed for several geochemical species.

Total nitrate and nitrite (ΣNO_3^- , hereafter referred to as nitrates in the text) were analysed by flow injection analysis (FIA) following the method described by Anderson (1979).

Other geochemical species (alkalinity, sulfate, phosphate, calcium, magnesium, iron and manganese) were also analysed, and the obtained profiles are shown in the Appendix. They were measured for a more complete picture of the experimental cores' geochemistry, but they are not discussed in the present paper.

Total alkalinity was measured by the spectroscopic method modified after Sarazin et al. (1999). 250 μL of sample were added to 1 mL of reagent (300 μL of 0.1 M methanoic acid, 2 mL of 500 mg L^{-1} bromophenol blue, 2 mL of 2 M

NaCl and MilliQ water until 20 mL final volume). Absorption was measured at 590 nm (Podda and Michard, 1994; Sarazin et al., 1999).

Samples for sulfate analyses (250 μL) were fixed with 50 μL of zinc acetate 10–2 M and analysed by ionic chromatography after 1 : 800 dilution. Phosphate was analysed with the colorimetric method described by Murphy and Riley (1962), and calcium, magnesium, iron and manganese by ICP-AES (iCAP 6300 radial, ThermoFisher Scientific).

2.5 Statistical analyses

Statistical analyses were carried out using Past 2.17c (Hammer et al., 2001). ANOVA and Tukey's post hoc tests were performed in order to test the hypothesis of significant differences between layers in terms of both survival and calcification rates (p values < 0.05 or < 0.01 were considered to be significant). Percentages were transformed ($\arcsin X$) before performing ANOVA analyses.

3 Results

During the first experiment, performed with *Ammonia tepida*, the oxygen penetration depth did not exceed 0.3 cm, corresponding to the top layer of the "foraminiferal" cores (Fig. 3c). The first layer was the only one where both oxygen and nitrates were present (Fig. 3d). Nitrates were abundant down to about 8 mm in depth, with trace concentrations (up to 3.5 μM) detected until 2 cm in depth. Deeper sediment layers were characterised by low pH (δpH up to -1.8) (Fig. 3c). Layer 3 (2.3–3.3 cm in sediment depth) corresponded to the iron-reduction zone (Supplement Fig. S1), while still deeper layers showed increasing ammonium and phosphate concentrations and enhanced sulfate reduction (Supplement Fig. S1). After two months' incubation, average survival rates of *Ammonia tepida* ($n = 50$ in each layer) varied from 49 ± 9 to $90 \pm 6\%$, and did not display a significant difference between individual layers of the three replicate cores, down to 10.3 cm in depth (Fig. 3a; ANOVA, p value > 0.05). In all sediment layers except one, a small number (1–4 % on average) of the *Ammonia tepida* specimens added a single new chamber (Figs. 3b and 4a), irrespective of oxic or anoxic conditions.

In the second experiment, carried out with the open marine species *Bulimina marginata* ($n = 31$ to 32 in each layer) and *Cassidulina laevigata* ($n = 32$ to 33 in each layer), we obtained comparable results as in the first one. In this case the oxygen penetration depth ranged from 0.5 to 0.6 cm in depth, exceeding the depth of the top layer of the sediment (0–0.3 cm). However, oxygen concentrations in the second sediment layer were always lower than 50 $\mu\text{mol L}^{-1}$ (Fig. 5c), thereby representing hypoxic conditions (Middelburg and Levin, 2009). The δpH in the experiment showed different profiles compared to the first experiment. The decrease (δpH

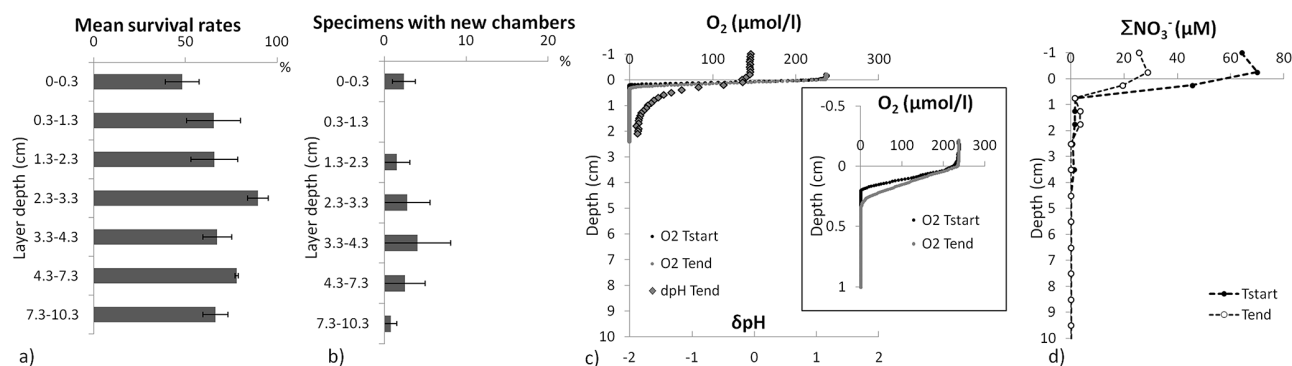


Figure 3. Main results of experiment 1 with *Ammonia tepida*. (a) Mean survival rates; (b) specimens that calcified new chambers; (c) oxygen and δpH profiles; (d) nitrate profiles. δpH is calculated as the difference between measured values at various sediment depths and the pH value measured in overlying water. Error bars represent the mean standard error.

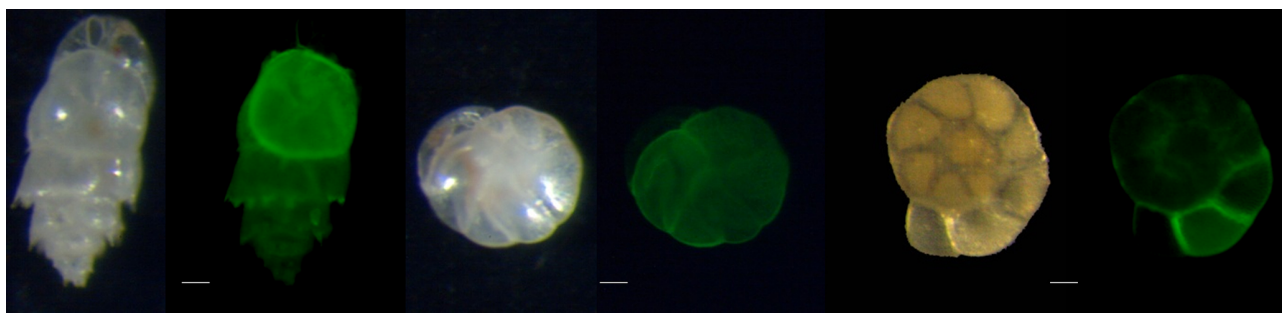


Figure 4. Example of new chambers, not calcein labelled, produced during the experiment under anoxic conditions. (a) *Ammonia tepida* from layer 4 (2.3–3.3 cm) under natural light (left) and epifluorescence (right); (b) *Bulimina marginata* from layer 3 (1.3–2.3 cm) under natural light (left) and epifluorescence (right); (c) *Cassidulina laevigata* from layer 4 (2.3–3.3 cm) under natural light (left) and epifluorescence (right). Photo exposure time: 1/2.5". Scale bar is 50 μm .

until -0.7 , Fig. 5c) observed in the first 0.5 cm in depth is due to organic matter degradation under oxic conditions, while the subsequent increase, below 0.5 cm (δpH until 1.3), is typical of sediments not subjected to sulfate reduction (Supplement Fig. S1). Nitrate profiles were also different from the first experiment (Fig. 5d). Although maximal concentrations (up to 100 μM) were observed in superficial layers (0–1.3 cm), nitrates were always present down to 4.3 cm in depth in the sediment.

After two months of incubation, the average survival rates of *Bulimina marginata* varied from 39 ± 4 to 34 ± 3 %, without significant differences (ANOVA, p value > 0.05) between the oxic, hypoxic and anoxic layers (Fig. 5a). *Bulimina marginata* was not only able to survive, but was also able to calcify in the first 3.3 cm of sediment, irrespective of oxygenation level (Figs. 5b and 4b). The ANOVA analysis (p value < 0.01) and Tukey's post hoc test (p value < 0.05) revealed a significant difference in calcification between the first two layers (0–1.3 cm) and the deepest one (3.3–4.3 cm), where none of the individuals added chambers. The difference between the topmost layer, where 31 ± 9 % of the specimens added new chambers, and the deeper layers

(0.3–3.3 cm), where 24 ± 3 % to 8 ± 4 % of the specimens calcified at least one chamber, was not significant. In most cases, only one new chamber was added. Four specimens grew more than one chamber (3 individuals from the top layer of one replicate produced 2 new chambers; 1 individual of the second layer of another replicate added 3 chambers). The absence of calcification in this lowermost layer does not seem to be related to changes in the analysed geochemical species (Fig. 5d and Supplement Fig. S1).

As observed for *Bulimina marginata*, *Cassidulina laevigata* did not display significantly different survival rates (ANOVA, p value > 0.05) between oxic (top layer) and hypoxic (0.3–1.3 cm in depth) sediment layers, with average survival rates of 35 ± 9 % and 26 ± 4 % respectively. However, all specimens of *Cassidulina laevigata* introduced in the anoxic layer (2.3–3.3 cm in depth) died during the experiment (Fig. 5a).

Chamber addition was observed for *Cassidulina laevigata* at all incubation depths (Figs. 5b and 4c), including the anoxic layer (2.3–3.3 cm in depth), without significant differences between layers (ANOVA, p value > 0.05). The average percentage of specimens that calcified was 33 ± 8 ,

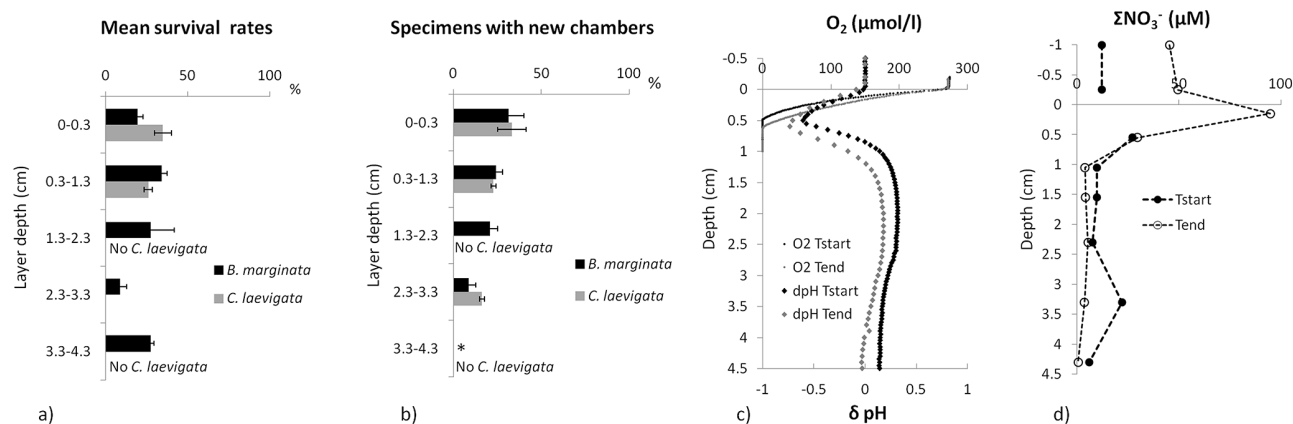


Figure 5. Main results of experiment 2 with *Bulimina marginata* and *Cassidulina laevigata*. **(a)** Mean survival rates; **(b)** specimens that calcified new chambers; **(c)** oxygen and δpH profiles; **(d)** nitrate profiles. δpH is calculated as the difference between measured values at various sediment depths and the pH value measured in overlying water. Error bars represent the mean standard error. In the 1.3–2.3 and 3.3–4.3 cm layers, *Cassidulina laevigata* were not introduced (no *Cassidulina laevigata*). Star (*) indicates a significant p value for Tukey's post hoc test (< 0.01).

23 ± 1 and 16 ± 1 %, respectively in the first (0–0.3 cm), second (0.3–1.3 cm) and fourth (2.3–3.3 cm) layers, and none of the specimens calcified more than a single chamber.

4 Discussions

4.1 Survival and calcification under anoxia

Ammonia tepida generally lives in superficial microhabitats (e.g. Debenay et al., 1998). In some cases it is also described in deeper sediments (Bouchet et al., 2009), but the use of rose Bengal staining in these studies may have overestimated or falsely indicated the presence of living individuals in anoxic sediments (Hannah and Rogerson, 1997). The absence of significantly different survival rates at different depth intervals, independent of oxygen conditions, is consistent with the results of the experiments carried out on *Ammonia tepida* by Geslin et al. (2014) under hypoxic to short-term anoxic (maximum 6 days) conditions. Their study shows that survival rates of this species are not affected by hypoxic conditions. Our experiment confirms their high tolerance to oxygen-depleted conditions (below 0.3 cm), and goes further, by reporting, for the first time, unaffected survival rates for this species until 60 days under anoxic conditions. Our results strongly suggest that the preference of *Ammonia tepida* for superficial microhabitats (in natural environments) is a response to the quantity and quality of the organic supplies rather than a response to dissolved oxygen concentrations. In our experiment, we used homogenised subsurface (0.5–10 cm) sediment to avoid large quantities of organic matter that could have slowed down the stabilisation of geochemical fluxes. The lack of fresh organic matter could therefore explain why the survival rates observed in the well-oxygenated top layer of our experiments are lower than the ones reported

by Geslin et al. (2014) under oxygenated laboratory conditions.

The absence of significant differences in survival rates in anoxic layers below the nitrate front, including layers with extreme chemical conditions (i.e. the occurrence of sulfate reduction), suggests that *Ammonia tepida* is able to shift to lower metabolic rates or to metabolic pathways other than denitrification. Nitrate storage, which has not yet been demonstrated for this species (Piña-Ochoa et al., 2010), and/or a drastically lowered metabolism, may not be the only response mechanisms to anoxia. The occurrence of calcification (Figs. 3b and 5b) under anoxia, down to at least 7.3 cm in depth (well below the nitrate reduction front), could be indicative of other, as yet unknown, metabolic pathways, which would supply the necessary energy (ATP) for calcification (de Nooijer et al., 2009). This aspect will be discussed further in the next paragraph. Alternatively, the energy remaining in the foraminiferal cell when it was introduced into the experiment could have been enough to ensure the calcification of one extra chamber.

Bulimina marginata has been described from a wide range of marine environments, and has been considered in several studies as an indicator species of low oxygen conditions (Phleger and Soutar, 1973; Van der Zwaan and Jorissen, 1991; Sen Gupta and Machain-Castillo, 1993; Bernhard and Sen Gupta, 1999). In the present study, statistical analysis did not show significant differences in survival between layers, which confirms the tolerance of this species to anoxic conditions, and is consistent with the earlier results of Langlet et al. (2014). This species is known to be able to store nitrates in the cell (Piña-Ochoa et al., 2010), but so far its ability to denitrify has not been demonstrated. Our results suggest that this metabolic pathway could eventually allow *Bulimina marginata* to survive down to 4.3 cm in the sediment (where

nitrates are still present), without any apparent negative influence of oxygen depletion, not even in anoxia. Denitrification could also supply the energy needed for calcification under hypoxic to anoxic conditions.

Finally, the different survival rates of *Cassidulina laevigata*, with 100 % mortality in the 2.3–3.3 cm layer, suggest that this species is able to tolerate hypoxia but not anoxia. The observation agrees partly with observations reported from several natural systems, where *Cassidulina laevigata* is generally described as shallow infaunal in well-oxygenated systems and as generally declining under low-oxygen conditions (Nordberg et al., 2000; Gustafsson and Nordberg, 2001; Filipsson and Nordberg, 2004). However, Sen Gupta and Machain-Castillo (1993) listed *Cassidulina laevigata* as a species resistant to moderate oxygen depletion in bottom and pore waters, which is consistent with our findings. *Cassidulina laevigata* is able to store nitrates (Piña-Ochoa et al., 2010), although it is unknown whether it has the ability to denitrify. The observed sensitivity of the species to anoxia, even in the presence of nitrates (Fig. 5a and d), suggests, however, that it did not use denitrification as a facultative anaerobic metabolism. Interestingly, a rather surprising result was obtained for the calcification of *Cassidulina laevigata*: although none of the *Cassidulina laevigata* specimens introduced into the anoxic layer survived the experiment, some of them (6, 4 and 5 specimens of the 32 introduced into each of the three replicate cores) were able to calcify one chamber. Hence, in this anoxic layer, calcification occurred before the death of all specimens. This is a particularly interesting observation, indicating both that the specimens did not die immediately after their introduction into anoxic conditions and also that some energy was still allocated to calcification under these conditions.

4.2 Metabolic activity under extreme oxygen conditions

The occurrence of calcification in deep anoxic sediments not only highlights the fact that specimens were able to survive anoxic conditions, but also the fact that foraminifera were metabolically active. The existing biomineralisation models in foraminifera postulate that calcification is an energetically expensive process (Erez, 2003; de Nooijer et al., 2009; Nehrke et al., 2013). No eukaryotic organisms have been observed so far to calcify in the absence of oxygen. Our study demonstrates, for the first time, that three benthic foraminiferal species are able to calcify under anoxic conditions and at different redox conditions (e.g. in the presence or absence of nitrates), opening the way to a series of important new questions, insights and implications.

A major question is how these organisms can simultaneously support the absence of oxygen and produce the energy needed for calcification. Denitrification is so far the only known alternative metabolic pathway utilised by some benthic foraminiferal species under anoxic conditions (Risgaard-Petersen et al., 2006). Denitrification provides a lower ATP

production than oxic respiration, since oxygen is a much better electron acceptor for both bioenergetic and kinetic reasons (Strohm et al., 2007). However, even supposing that denitrification may be energetically sufficient to support calcification, for several reasons, this process cannot explain all our observations. First, the ability to store nitrates and/or to denitrify has not been demonstrated for the three tested species (Piña-Ochoa et al., 2010). Next, *Ammonia tepida* survived and calcified also in deeper sediment levels, where nitrates were absent. Therefore, while for *Bulimina marginata* denitrification could explain our results even though it remains to be demonstrated, other processes have to be envisaged for the other two investigated species: *Ammonia tepida* and *Cassidulina laevigata*. For *Ammonia tepida*, a possible explanation for its ability to survive and calcify under anoxic conditions could be a shift towards anaerobic metabolic pathways other than denitrification, eventually mediated by ecto- or endo-biotic bacteria, as hypothesised by Bernhard et al. (2012) for some foraminiferal species. Anaerobic metabolic pathways that do not involve electron chain transport are generally much less efficient in ATP production (Vazquez et al., 2010). Therefore, even if such metabolic pathways could eventually explain foraminiferal survival, it is puzzling that the foraminifera would have enough energy to calcify.

An alternative explanation could be that essential life processes (calcification, nutrition, etc.) were continued at the beginning of the experiment, using energy reserves present in the cell, and were progressively abandoned later in the experiment. Such a mechanism would explain why the large majority of specimens calcified only a single new chamber, even *Cassidulina laevigata*, which died later in the experiment, probably due to a lower tolerance to anoxia.

Another question: why would foraminifera spend energy to calcify under anoxic conditions? Based on the results, we hypothesise that for the species which did not demonstrate significant changes in survival rates under anoxic conditions (*Ammonia tepida* and *Bulimina marginata*), it is possible that calcification, meaning energy consumption, occurred because the life cycle was not affected by the scarcity or absence of oxygen. However, the case of *Cassidulina laevigata*, which was able to calcify under all tested conditions, from oxic to anoxic, but which did not survive 60 days of anoxia, suggests that although calcification occurred, not all life processes could be ensured. This seems to indicate that, at least for this species, calcification occurred only early in the experiment, and was sustained by energy reserves present in the cell when foraminifera were introduced in the experiment.

Finally, it has to be noted that in our experiments, the initial homogenisation of the sediment column produced an organic matter gradient probably different from natural environments, with the presence of a larger quantity of labile organic carbon in layers deeper than in natural settings. This could have enhanced foraminiferal activity (including calcification) in deeper layers. It is therefore not

obvious that in nature calcification takes place in deeper anoxic sediment layers. However, our experimental results clearly show that the lack of oxygen does not inhibit calcification, and that during anoxic periods with durations of up to 60 days, foraminifera will continue to calcify, at least at the sediment–water interface.

4.3 Implications and new perspectives

Several laboratory (e.g. Moodley et al., 1997, 1998b; Geslin et al., 2014) and field studies (e.g. Leiter and Altenbach, 2010; Langlet et al., 2013) have shown that foraminifera can survive anoxia, in some cases up to 10 months (Langlet et al., 2013). The present study demonstrates that calcification may continue under these conditions, at least in the early stages of anoxia. However, the foraminifera disappear after prolonged anoxia, as shown for instance by Mediterranean sapropel records (Jorissen, 1999). Experimental results suggest that the ultimate disappearance of the foraminiferal communities cannot be explained either by adult mortality, as high survival rates have been noted in several studies (e.g. Langlet et al., 2013; Duijnsteet et al., 2003), or by inhibition of calcification (this study), or by a possible lack of reproduction, as reproduction was observed under experimental anoxia by Alve and Bernhard (1995). We speculate that the final disappearance may be explained by a possible higher sensitivity to anoxia in the juveniles, who may no longer be capable of ensuring essential life functions such as calcification.

Our observations of calcification under anoxia and different redox conditions may also have important consequences for paleo-proxy interpretations, especially in settings with intermittent anoxia. Until today it was generally assumed that no foraminiferal tests are produced during anoxic periods, and consequently, foraminiferal samples representing an alternation of short-term oxic and anoxic periods would only contain foraminiferal tests formed during the oxic phases. This is especially relevant in coastal areas with seasonal anoxia, or the upper limits of intense oxygen minimum layers, which may also show important seasonal variability. In view of our results, it is possible that in such settings, contrary to earlier ideas, continuous records may be obtained.

Previous studies on the geochemical composition of individual foraminiferal tests have shown very large intraspecific differences between specimens from single samples (e.g. Duplessy et al., 1970; Rathburn et al., 2003). It appears that these differences are in many cases not due to different environmental parameters, but rather due to still poorly understood vital effects. Our results suggest that in settings with short-term anoxia, part of this intraspecific variability could be due to the fact that different individuals represent periods with strongly contrasting oxygen concentrations, as suggested by Groeneveld and Filipsson (2013). The study of individual specimens may in such cases add important information about seasonal variability, not only of bottom water oxygenation, but also of other environmental parameters

such as temperature or salinity. This information may also help us to understand the onset of anoxia better.

Similarly, by analysis of elemental ratios in successive chambers of single specimens (for instance by laser ablation ICP-MS), it may be possible to obtain highly detailed reconstructions of ecosystems characterised by short-term (foraminiferal lifetime-scale, seasonal) changes in bottom water oxygenation.

Finally, although our data show calcification in anoxia for all three investigated species, they demonstrate clear interspecific differences in tolerance of anoxia, with *Cassidulina laevigata* showing much lower survival rates than *Ammonia tepida* and *Bulimina marginata*. This suggests that some species will produce better (more continuous) records in areas affected by short-term anoxia than other ones. A thorough knowledge of foraminiferal ecology, which will allow the selection of the best proxy carriers, remains therefore a prerequisite for successful geochemical paleoceanography studies.

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