

Chapter 10

Survival, Reproduction and Calcification of Three Benthic Foraminiferal Species in Response to Experimentally Induced Hypoxia

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Abstract An experiment was conducted to test the survival rates, growth (calcification), and reproduction capacities of three benthic foraminiferal species (*Ammonia tepida*, *Melonis barleeanus* and *Bulimina marginata*) under strongly oxygen-depleted conditions alternating with short periods of anoxia. Protocols were determined to use accurate methods (1) to follow oxygen concentrations in the aquaria (continuously recorded using microsensors), (2) to distinguish live foraminifera (fluorogenic probe), (3) to determine foraminiferal growth (calcein-marked shells and automatic measurement of the shell size). Our results show a very high survival rate, and growth of *A. tepida* and *M. barleeanus* in all experimental conditions, suggesting that survival and growth are not negatively impacted by hypoxia. Unfortunately, no reproduction was observed for these species, so that we cannot draw firm conclusions on their ability to reproduce under hypoxic/anoxic conditions. The survival rates of *Bulimina marginata* are much lower than for the other two species. In the oxic treatments, the presence of juveniles is indicative of reproductive events, which can explain an important part of the mortality. The absence of juveniles in the hypoxic/anoxic treatments could indicate that these conditions inhibit reproduction. Alternatively, the perceived absence of juveniles could also be due to the fact that the juveniles resulting from reproduction (causing similar mortality rates as in the oxic treatments) were not able to calcify, and remained at a propagule stage. Additional experiments are needed to distinguish these two options.

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10.1 Introduction

Oxygen deficiency is one of the most widespread harmful effects for aerobic organisms in the marine environment. Over the past 10 to 15 years the number of coastal areas affected by seasonal hypoxia in the bottom waters has spread rapidly, mainly due to anthropogenically induced eutrophication (Diaz and Rosenberg 1995; Diaz and Rosenberg 2008). While hypoxic and anoxic events (hypoxic <math>< 63 \mu\text{M O}_2</math>, anoxic = below the detection limit of microsensors, following Middelburg and Levin 2009) existed throughout geological time, their occurrence in shallow marine and estuarine areas is clearly increasing. Global warming will probably enhance these effects and will enlarge the affected areas (Justic et al. 2003).

In most marine environments, only the top centimeter or millimeter of the sediment contain oxygen (Jørgensen and Revsbech 1989), because it is quickly consumed by biological and chemical processes (Wenzhöfer and Glud 2004). The oxygen content, which usually shows an exponential downward decrease, is the result of the equilibrium between downward oxygen diffusion from the bottom water into the sediment and the consumption of the oxygen used for the degradation of organic matter by aerobic organisms (Glud 2008). Oxygen deficiency may be caused by increased organic matter supplies and/or a decrease of the bottom water ventilation. At greater water depths, less organic matter reaches the seafloor, and less carbon is degraded in the sediment. Consequently, in general, in fined-grained organic-rich sediments in marine coastal areas, oxygen penetration is only a few millimeters (e.g. Jørgensen 2005) compared to several centimeters in the deep sea (Wenzhöfer and Glud 2004). In addition, other important factors controlling oxygen availability in sediments are photosynthetic benthic microorganisms (Revsbech et al. 1986), such as diatoms which form biofilms in the intertidal zone and burrowing macrofauna which increase oxygen penetration into the sediment (bio-irrigation) in deeper ecosystems (e.g. Aller 1994).

In environments with hypoxic bottom waters, oxic organic matter degradation is decreased but remineralization will continue using nitrates, sulfates and metal oxides. In permanently anoxic or sulfidic settings, most oxidants have been exhausted and early diagenetic processes in the sediments are dominated by sulfate reduction, methanogenesis and anaerobic oxidation of methane (Treude 2012).

Marine faunas are strongly affected by hypoxic and anoxic events, particularly the benthic fauna living at the sediment-water interface and within the superficial sediment. In general meiofauna is less sensitive to hypoxia and anoxia than macrofauna and megafauna (Josefson and Widbom 1988; Moodley et al. 1998). Obligate or facultative anaerobes occur in some meiofaunal protists groups including

flagellates, ciliates and foraminifera (Fenchel 2012). The diversity of anaerobic metabolic pathways applied by eukaryotes is much more restricted than the wide range of processes that enable prokaryotes to live and grow in the absence of free oxygen. Still, new findings show from time to time that eukaryotic organisms have developed some unexpected strategies to exert all basic life functions (survival, growth, reproduction) under anaerobic conditions (Oren 2012). However, anaerobic respiration using oxidized compounds of nitrogen, sulfur and other elements remain very rare among eukaryotes. Following observations that foraminifera may proliferate in anoxic environments (reviews in Bernhard and Sen Gupta 1999; Geslin et al. 2004), Risgaard-Petersen et al. (2006) discovered that a foraminiferal species (*Globobulimina turgida*) that usually lives in the oxygen-free zone of the sediment can use nitrate as electron acceptor and perform full denitrification forming N_2 as end product. This species is able to accumulate a large quantity of nitrate in its cell, allowing it to employ anaerobic metabolism for at least 3 months (Piña-Ochoa et al. 2010b). Piña-Ochoa et al. (2010a) recently showed that many other benthic foraminiferal species store nitrate in their cell, suggesting that nitrate reduction can potentially be applied by a wide range of species. A gene for nitrate reduction has recently been found in *Bolivina argentea* (Bernhard et al. 2012), suggesting that in this species denitrification could at least partially be performed by the foraminifera themselves, and not entirely by symbiotic bacteria, such as has been shown for allogromiid foraminifera (Bernhard et al. 2011). However, some foraminiferal species known to be tolerant to hypoxic and anoxic conditions do not store nitrate in their cell, strongly suggesting that they are not able to denitrify (Piña-Ochoa et al. 2010a). For example, this is the case of *Ammonia tepida*, a dominant shallow water species, often observed in deeper layers in hypoxic or anoxic sediments (e.g. Kitazato 1994; Bouchet et al. 2009). Also *Bulimina marginata* and *Melonis barleeanus*, which have often been observed in deeper sediment layers, and are therefore suspected to be facultative anaerobes, do not show elevated concentrations of nitrate in their cell (Piña-Ochoa et al. 2010a).

In the present paper, we want to focus on the response of these somewhat enigmatic species to alternating hypoxic and anoxic conditions. We were especially interested to determine their survival rate and growth capacity under hypoxic conditions and their ability to shift to nitrate reduction during short periods of anoxia.

To answer these questions, we studied in the laboratory the behavior of these three species (*Ammonia tepida*, *Bulimina marginata* and *Melonis barleeanus*) under different oxygen regimes, using adequate methods to distinguish live specimens, to observe the growth of living individuals, and to identify reproduction events. Three different treatments were applied: (1) oxic conditions with available nitrate, (2) strongly hypoxic to anoxic (nitrogen-flushed) conditions with nitrate and (3) strongly hypoxic to anoxic (nitrogen-flushed) conditions without nitrate. We expected that the third treatment would allow us to observe the ability of the three species to survive short periods of anoxia without having the possibility to store nitrate and/or to denitrify.

10.2 Ecological Characteristics of the Three Tested Species

10.2.1 *Ammonia tepida*

Ammonia tepida is a cosmopolitan species occurring in intertidal mudflats, brackish lagoons, estuaries and shallow marine areas that are extremely variable environments both temporally and spatially (Debenay et al. 2000; Murray 2006). It is a common species, which is able to survive a wide range of temperature, salinity and other environmental parameters varying on seasonal or daily scales (e.g. Bradshaw 1961; Schnitker 1974; Walton and Sloan 1990). It is a free living superficial to infaunal species in muddy to silty or fine sandy sediments (e.g. Debenay et al. 1998).

Ammonia tepida has been extensively studied for various attributes, such as geographic distribution, ecology, biology, life-cycles, morphology, structure, and dependence of environmental parameters such as temperature and salinity (e.g., Bradshaw 1957, 1961; Schnitker 1974; Poag 1978; Jorissen 1988; Walton and Sloan 1990; Goldstein and Moodley 1993; Geslin et al. 1998; Stouff et al. 1999a, b; Pascal et al. 2008). *Ammonia tepida* is considered as a deposit feeder and has been found feeding on algae (e.g. Moodley et al. 2000), bacteria (Goldstein and Corliss 1994; Langezaal et al. 2005; Pascal et al. 2008) and other meiofaunal groups (Dupuy et al. 2010). The congener *Ammonia* sp. shows a rapid uptake of freshly deposited algal carbon (Moodley et al. 2000).

The large morphological variability of the genus *Ammonia* has led to considerable difficulties in species identification and more than 40 species and subspecies (or varieties) of recent *Ammonia* have been described worldwide under the generic names *Ammonia*, *Streblus*, and *Rotalia* (Ellis and Messina 1940). Recent studies have shown that the total number of genetically distinct and morphologically separable living species of *Ammonia* worldwide is likely to exceed 25–30 (Hayward et al. 2004). Comparing the morphology of *Ammonia tepida* collected in the Aiguillon Bay with the pictures presented by Hayward et al. (2004), our morphological range contains morphotypes close to molecular type T1.

Ammonia tepida is reported to be able to live in oxic and hypoxic conditions (Moodley and Hess 1992; Koho and Piña-Ochoa 2012). It has been found both in epifaunal and infaunal microhabitats (Kitazato 1994; Debenay et al. 1998). Bouchet et al. (2009) found Rose Bengal stained specimens below 20 cm in the sediment. However, it is still not 100 % clear whether this species is able to live in anoxic sediments, and, if this is the case, whether it uses an anaerobic metabolism or if it is able to survive in oxic microniches created by bioturbation. We expected our experiment to confirm or discount the ability of *A. tepida* to survive and to calcify under strongly hypoxic conditions lasting for two months and to clarify its capacity to denitrify during short periods of anoxia.

10.2.2 *Bulimina marginata*

Bulimina marginata is a cosmopolitan species found in various oceans around the world (e.g. Jorissen 1988; Corliss 1991; Jannink et al. 1998; Jorissen et al. 1998; De Rijk et al. 1999, 2000; Schmiedl et al. 2000; Mojtahid et al. 2006, 2008, 2010; Mojtahid 2007; Pucci et al. 2009). *Bulimina marginata* lives at a wide range of water depths, from the continental shelf between 10 and 150 m water depth (e.g. Barmawidjaja et al. 1992; Fontanier et al. 2002; Langezaal et al. 2005) to continental slope and bathyal environments, down to 2,200 m water depth (e.g. Jorissen et al. 1998; De Rijk et al. 1999, 2000). Abundant faunas of this species have also been described in submarine canyon environments (Schmiedl et al. 2000; Hess et al. 2005; Koho et al. 2007; Hess and Jorissen 2009).

Several studies indicate that *B. marginata* is a eutrophic taxon typical of fine grained muddy sediments with high food availability (e.g., Jorissen 1988; De Rijk et al. 2000). *Bulimina marginata* is generally considered as a detritivore. It occurs alive in shallow infaunal microhabitats in the topmost centimeters of the sediment, but abundant Rose Bengal stained assemblages have also been described in much deeper sediment layers (e.g. Kitazato 1989; Jorissen et al. 1998; Jorissen 1999). The presence of such deep assemblages may be related to macrofaunal burrows.

In several older studies, *B. marginata* has been considered as a good marker of low oxygen conditions (e.g. Phleger and Soutar 1973; van der Zwaan and Jorissen 1991; Sen Gupta and Machain-Castillo 1993; Bernhard and Sen Gupta 1999). It is supposed to support periodic episodes of anoxia or hypoxia (Pucci et al. 2009).

10.2.3 *Melonis barleeanus*

Melonis barleeanus is a typical species of open marine settings from the lower continental shelf to bathyal environments (e.g. Schmiedl et al. 2000; Fontanier et al. 2002, 2008). Rich assemblages have also been described in submarine canyons (Koho et al. 2008; Nardelli et al. 2010; Phipps et al. 2012).

Melonis barleeanus is commonly described as an intermediate infaunal species (Jorissen et al. 1998). This species is most often found in hypoxic sediments where pore-water nitrate concentrations are maximal (Jorissen et al. 1998; Fontanier et al. 2005; Koho et al. 2008; Mojtahid et al. 2010), and is assumed to feed on degraded organic detritus (e.g. Caralp 1989; Fontanier et al. 2002). Although only very low nitrate concentrations have been measured in *Melonis barleeanus*, (intracellular NO_3^- content: 0.6 ± 0.2 mM; Piña-Ochoa et al. 2010a), it has been considered as a potential denitrifier because of its systematic appearance in nitrate-rich sediment layers (Koho and Piña-Ochoa 2012).

10.3 Methodology

10.3.1 Experimental Design

10.3.1.1 Foraminiferal Collection and Storage in the Laboratory

Ammonia tepida

Superficial sediment was sampled at low tide on the 9th of February 2011 in the intertidal area of the Bay of Aiguillon (French Atlantic coast). This area is known for its very rich live faunas of *Ammonia tepida* (Pascal et al. 2008). In the field, the sediment was immediately sieved, using a 150- μm mesh and local seawater. The fraction $>150\ \mu\text{m}$, containing large amounts of foraminifera, was transported to the culture laboratory at the University of Angers and maintained at 14 °C until the beginning of the experiment. On the 15th of April 2011 (one month before the beginning of the experiment), *Ammonia tepida* was incubated in a calcein solution (Bernhard et al. 2004).

Bulimina marginata

Sediment cores were sampled in the Bay of Biscay at station K (Lat. 43°38'N, Long. 1°43'W, water depth 650 m depth) on the 4th of February 2011 with a classical Barnett multi-tube corer (Barnett et al. 1984). The two first centimeters were sliced from the subsurface, placed in containers with seawater and transported to the laboratory where they were stored at 10 °C until the beginning of the experiment.

Melonis barleeanus

Sediment cores were sampled with a MUC8+4 multi-tube corer in Nazaré Canyon at station 161 (Lat. 39°35'N, Long. 9°24'W, water depth 918 m) on the 20th of March 2011. The two first centimeters were sliced from the subsurface, placed in containers with seawater and transported to the laboratory where they were stored at 12 °C until the beginning of the experiment.

10.3.1.2 Identification and Collection of Living Organisms

One week before the start of the experiment, sediments from the 3 different locations were sieved with filtered (0.45 μm) seawater over a 150- μm mesh and living foraminifera were picked. To ascertain the vitality of the selected specimens, each foraminifer was placed in a thin layer of fine-grained ($<38\ \mu\text{m}$) sediment for approximately 12 h. Only the specimens that had moved, and had produced a burrow or trace on the sediment were considered alive and selected for the experiment.

10.3.1.3 Experimental Set-Up

Three different conditions were tested in three different aquaria with the same temperature (12.0 ± 0.3 °C) and salinity (35.4 ± 0.2).

Aquarium “OxN” (Oxic + Nitrate) contained well oxygenated artificial seawater (ASW) with $50 \mu\text{M NO}_3$. High oxygen concentrations were maintained by continuously bubbling seawater with air. Aquarium “HypN” (low $[\text{O}_2]$ + Nitrate) and aquarium “Hyp” (low $[\text{O}_2]$ without nitrate) were both maintained under low oxygen conditions and contained ASW with $50 \mu\text{M NO}_3$ for HypN or nitrate-free water for Hyp. Hypoxic conditions were obtained by a continuous introduction of N_2 gas mixed with 0.04 % CO_2 (to keep the pH stable (Piña-Ochoa et al. 2010b)) into the overlying water. Oxygen concentrations were measured at least once a day with a Clark-type microelectrode with a $100 \mu\text{m}$ thick tip (Revsbech 1989). The sensors were regularly 2-point calibrated in air-saturated ASW (temperature 12 °C, salinity 35) and in anoxic alkaline ascorbate.

Specimens were introduced into the aquaria in glass vials (20 mL each) with a thin layer of fine-grained sieved sediment ($<38 \mu\text{m}$) filled up with ASW, in order to create more ‘natural’ conditions for the foraminifera. Each vial was closed with a net ($100 \mu\text{m}$ mesh) to keep the foraminifera within the vials.

10.3.1.4 Survival Experiment

Living adult specimens of the three selected species *Ammonia tepida*, *Melonis barleeanus* and *Bulimina marginata* were introduced in 45 species-specific glass vials (one vial per sampling time per species per aquarium): 20 specimens per vial for *A. tepida*, 20 for *M. barleeanus* and 11 for *B. marginata*. Each aquarium contained 5 vials with *A. tepida*, 5 vials with *M. barleeanus* and 5 vials with *B. marginata*. The vial position in the aquaria was randomly defined to avoid any spatial effect on the foraminiferal survival rates.

The experiment started on the 16th of May 2011 and ran for 56 days. Three vials (one per species) were sampled in each aquarium after 7 days (T=1), 14 days (T=2), 29 days (T=3), 45 days (T=4) and 56 days (T=5). The contents (sediment + foraminifera) of the vials were transferred to Petri dishes and foraminifera were picked out. Nets and vials were checked under a stereomicroscope to pick also the specimens that had migrated upward (along the walls of the vials).

The majority of introduced foraminifera were recovered at the end of the experiment (80–100 % with one exception of 61 % at T=3 in the HypN condition for *B. marginata*). In some cases, juveniles were born during the experiment; they have been counted as well. Living specimens were recognized using a fluorescence technique. The fluorescence technique is a powerful, reliable and quick method to identify living individuals in experimental setups (Bernhard et al. 1995; Pucci et al. 2009; Morigi and Geslin 2009; Piña-Ochoa et al. 2010b; Koho et al. 2011; Heinz and Geslin 2012), particularly under anoxic conditions. Incubation in fluorogenic probes causes live specimens to become fluorescent. The fluorogenic probe used in

this experiment was fluorescein diacetate (FDA; Bernhard et al. 1995), which was diluted in a dimethyl sulfoxide solution and ASW in order to obtain a final solution of 100 μM FDA. Specimens were picked out at the end of experiment directly in the vials when it was possible or after sieving ($>63 \mu\text{m}$). They were incubated in ASW-FDA solution for 10–20 h. Foraminifera were then picked out and placed in ASW with no FDA. Specimens were inspected individually for fluorescence using an Olympus SZX12 with a light fluorescent source Olympus URFL-T. Survival rates were calculated for each species and for each experimental condition.

10.3.1.5 Growth Experiment

To study their growth ability under the various experimental conditions, one vial per species was added to each aquarium (Ox, HypN, Hyp). For *A. tepida* and *B. marginata*, calcein-marked living specimens were added; 50 semi-adults of *A. tepida* at $T=0$ and 20 juveniles (3 chambers) of *B. marginata* at $T=1$. Thanks to the calcein stain of the introduced tests, the newly formed chambers, calcified under controlled conditions, could be easily identified (Bernhard et al. 2004; Barras et al. 2009) and counted. Additionally, at the end of the experiment, all specimens were measured using an automatic particle analyzer (see description below). Specimens that did not calcify new chambers were used to estimate the initial size whereas specimens that calcified new chambers allowed us to determine the average size increase of *A. tepida* and *B. marginata* in each condition.

For *M. barleeanus*, specimens were not calcein-marked beforehand. To evaluate their growth during the experiments, the size of all introduced specimens (10 adult individuals per condition) was measured at the start ($T=0$) and the end ($T=5$) of the experiment using an automatic particle analyzer (see below).

10.3.2 Size Measurements Using an Automatic Particle Analyser

Automated particle analysis has been carried out with a fully automated incident light microscope system. Images are acquired and particles are analyzed with analySIS FIVE (SIS/Olympus[®]) software supported by a software add-in developed by MAS[®]. Samples are measured automatically under a Leica ZI6APO monocular microscope (plan-apochromatic objective). Images are captured with a CC12 colour camera (SIS[®]). Constant illumination of samples is provided by a Leica[®] CLS100X light source and a Leica[®] ring-light. This setup is the second generation of a particle analyzing system developed by Jörg Bollmann (Bollmann et al. 2004; Movellan et al. 2012).

10.3.3 Metabolism: Oxygen Respiration Rates

In order to estimate the amount of oxygen needed for foraminiferal aerobic metabolism, respiration rates of *M. barleeanus* were measured using a Clark type O₂ microsensor (Revsbech 1989) according to the methodology of Geslin et al. (2011), so that our results are totally comparable to their data. Four measurements were performed for *M. barleeanus* with 4 to 7 specimens per analysis. Aerobic respiration rates of *A. tepida* from Aiguillon Bay and *B. marginata* from Bay of Biscay were not measured because data are available in Geslin et al. (2011).

10.3.4 Internal Nitrate Contents in Foraminifera

In order to follow the intracellular nitrate contents in the foraminiferal cells during the experiment, living specimens of the 3 studied species were selected in each condition at each sampling time (except for *A. tepida* at T=4 and T=5). One specimen was required for intracellular nitrate measurement. A total of 147 specimens were analyzed with 10–24 specimens per condition and per time. Each specimen was rinsed in nitrate-free ASW, transferred to PCR tubes and stored at –20 °C. They were analyzed for nitrate content using the VCl₃ reduction method (Braman and Hendrix 1989) on a chemiluminescence detector (Model CLD 86, Eco Physics AG) as described by Risgaard-Petersen et al. (2006) and Høglund et al. (2008). Biovolumes were determined according to Geslin et al. (2011) in order to calculate the nitrate concentration in each individual.

10.3.5 Statistical Analyses

Two different parametric statistical procedures were used to test the effect of the experimental conditions (OxN, HypN and Hyp) and of time on the foraminiferal survival rates and growth. Due to the binomial nature of the response variable “survival” (i.e. dead or alive; 0 or 1) we used generalized linear model (GLM, Nelder and Wedderburn, 1972) procedures of R (R Development Core Team, 2011). In these GLM procedures we tested the effect of the quantitative independent variable “Time” and the qualitative independent variables “Conditions” (OxN, HypN and Hyp) and “Species” (*M. barleeanus*, *A. tepida* and *B. marginata*). This procedure allowed us to determine for each of the variables whether it had a significant effect on survival (expressed as a Deviance and a Chi probability) and if so, to quantify the effect (giving an estimate of a coefficient for which the significance was expressed with z and p statistics).

Because of the normality of the response variable (the dependant variable), we used linear models (LM, Chambers 1992) to quantify the effect of both “Conditions” and “Time” and their interaction on the size of the foraminifera (the Maximum Diameter or Length in μm according the species). In order to identify the effect of the conditions on each species we ran three models, one for each species. This procedure consists of two steps. The first one allows us to determine whether one or several independent variables (depending on the species: Time, Conditions or the Time*Conditions interaction) has a significant effect on the individual size (after an analysis of variance, expressed with F and the associated probability statistics). The aim of the second step was to quantify how each variable affects the foraminiferal size (expressed with an estimated coefficient, for which a Student’s t-test is realized to see if the estimate is significantly different from zero, associated with a t and its probability statistics). As the method used to determine the growth is slightly different for the three species we have to use different independent variables for each species. Both for *A. tepida* and *B. marginata*, the initial size was not measured at the beginning of the experiment but was estimated on the basis of the final size of the individuals that did not grow during the experiment. For these two species, we tested the effect of the qualitative dependent variable coded with 4 terms: “T5.NoGrowth”, “T5.OxN”, “T5.HypN” and “T5.Hyp”. For *M. barleeanus*, we tested the effect of Time, Conditions and of the interaction Time*Conditions on the dependant variable Maximum Diameter. For all procedures we used a 0.05 significance level.

10.4 Results

10.4.1 Monitoring of the Oxygen Conditions

During all the experiment in aquaria Hyp and HypN, oxygen concentrations were lower than $90 \mu\text{mol L}^{-1}$ ($\sim 2 \text{ mL L}^{-1}$) and several short anoxic periods occurred (up to 6 days). Average O_2 concentrations are 10 ± 13 and $14 \pm 18 \mu\text{mol L}^{-1}$ in aquaria HypN and Hyp, respectively.

10.4.2 Survival Rates of Adult Specimens

10.4.2.1 Influence of Incubation Time Under Oxidic Conditions

Under oxidic conditions (OxN), the survival rates of *A. tepida* (84–100 %) and *M. barleeanus* (87–95 %) are very high; whereas the survival rate of *B. marginata* (36–64 %) is much lower (Figs. 10.1, 10.2 and 10.3). GLModels show that there is a significant “Species” effect with *B. marginata* exhibiting a lower survival rate (Table 10.1). There is no significant effect of the incubation time and of the interaction Time \times Species on the survival rates of the three species (Table 10.1).

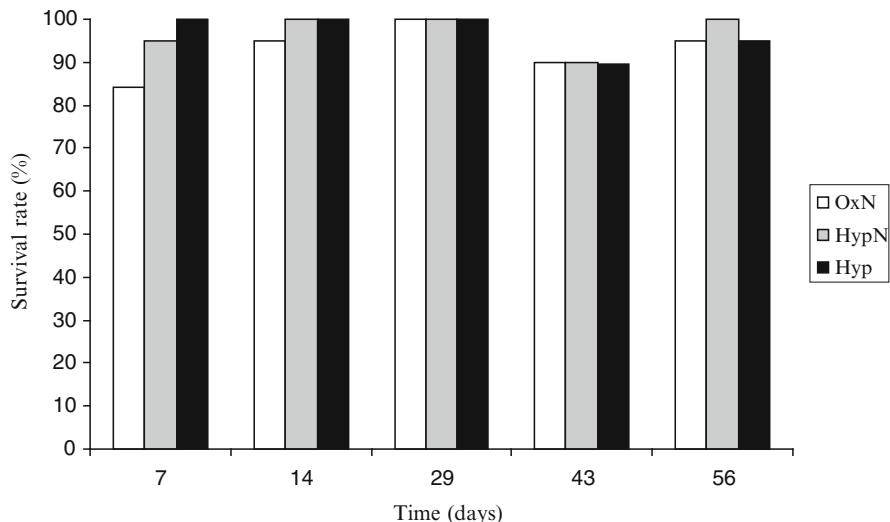


Fig. 10.1 Survival rates of *Ammonia tepida* after 7, 14, 29, 43 and 56 days under three experimental conditions

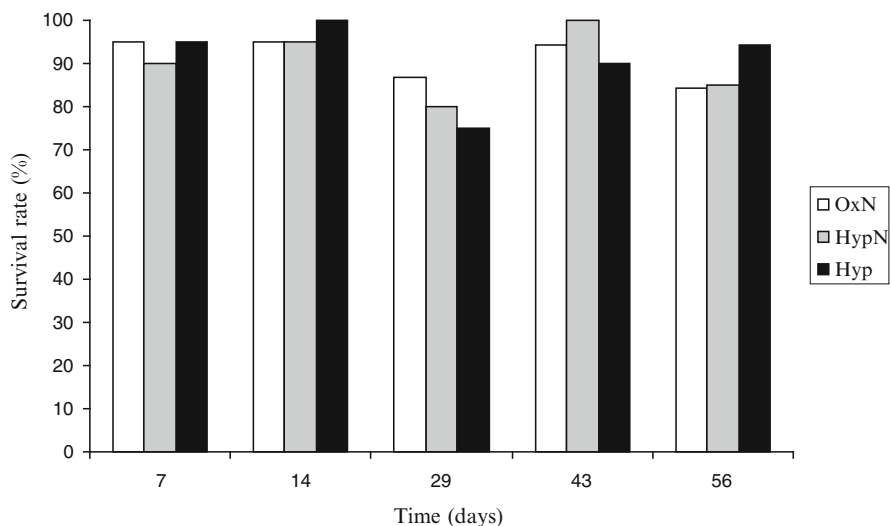


Fig. 10.2 Survival rates of *Melonis barleeanus* after 7, 14, 29, 43 and 56 days under three experimental conditions

10.4.2.2 Influence of Hypoxic Conditions

Specimens of the three tested species were found alive in both hypoxic conditions, until the end of the experiment. The survival rates (SR) are very high in both tested conditions for *A. tepida* (Fig. 10.1, 90–100 % for HypN, 89–100 % for Hyp,

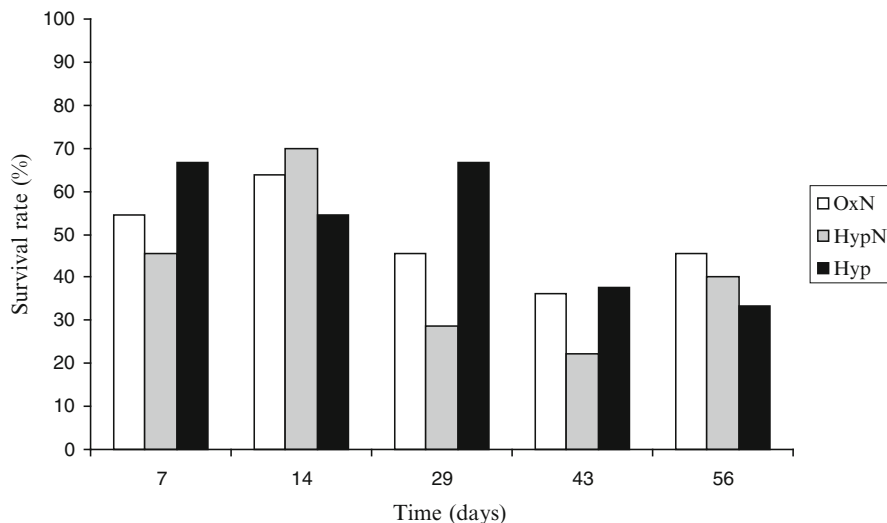


Fig. 10.3 Survival rates of *Bulimina marginata* after 7, 14, 29, 43 and 56 days under three experimental conditions

Table 10.1 Effect of the experimental conditions on survival rates of the three species under oxitic conditions (upper panel) and under all conditions (lower panel)

	Df	Deviance	p(Chi)
<i>Influence of incubation time under oxitic conditions</i>			
Species effect	2	46.9	6.6×10^{-11}
Incubation time effect	1	0.7	0.39
Interaction Incubation time \times Species	2	1.8	0.40
<i>Influence of hypoxic conditions</i>			
Species effect	2	151.9	$<2 \times 10^{-16}$
Incubation time effect	1	2.7	0.10
Conditon effect	2	0.2	0.92

The Degrees of freedom (Df), the Deviance and the probability (p(Chi)) statistic values are presented for every tested variables

respectively) as well as for *M. barleeanus* (Fig. 10.2, 80–100 % for HypN, 75–100 % for Hyp) (Table 10.1). For *B. marginata*, the survival rates are significantly lower than for the other two species (22–70 % for HypN, 33–67 % for Hyp, respectively) (Table 10.1, Fig. 10.3). None of the three studied species exhibit significant differences in their survival rates between the different sampling times in hypoxic conditions alternating with short (up to 6 days) periods of anoxia with or without nitrates. No significant difference is observed between the aquaria with and without nitrate (Table 10.1).

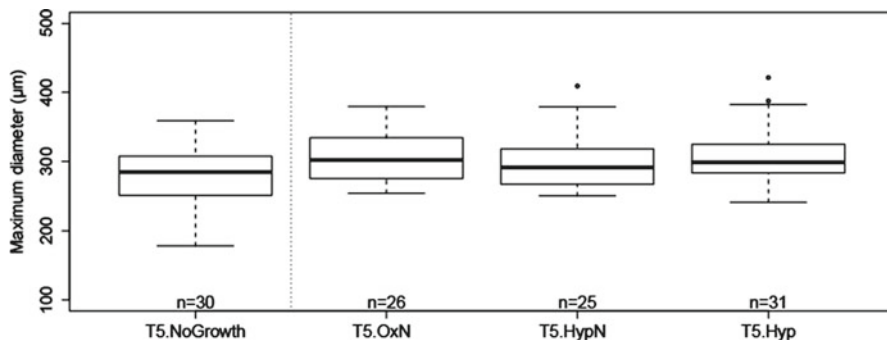


Fig. 10.4 Growth of *Ammonia tepida*: comparison of the size of the individuals that did not add new chambers (T5.NoGrowth) and those that calcified one or more new chambers (T5), for each tested condition (OxN, HypN, Hyp). On the box-and-whisker plot the two borders of each box are the first and third quartile while whiskers are 1.5 times the interquartile range of the lower and higher quartiles

10.4.3 Reproduction

No juveniles were obtained for *A. tepida* and *M. barleeanus*, whereas reproduction events occurred in the vials containing adult specimens of *B. marginata*. Juveniles were only found in the OxN aquaria: 80, 37 and 142 juveniles were counted at T= 1, T=3 and T=4 respectively.

10.4.4 Growth

At T=5 (56 days), 64–84 % of the *A. tepida* semi-adult specimens introduced in the vials for the growth experiment were found. For this species, growth was assessed through the observation of additional chambers calcified during incubation (use of calcein labeling). The percentage of specimens that added at least one chamber in OxN, HypN and Hyp aquaria was 81 %, 60 % and 82 %, respectively (n=50 for each condition). They added 1 or 2 chambers with the exception of a single specimen that added 3 new chambers. In order to study the size increase for *A. tepida*, the average size of all the individuals that did not add any new chambers was compared with the average size of individuals that calcified new chambers (Fig. 10.4).

According to the size measurements, the individuals of *A. tepida* that added new chambers were significantly wider than the ones that did not exhibit newly formed chambers (Df=3, F=3.05, p=0.03). The average difference of maximum diameter between specimens that added new chambers and those who did not is +27.4, +17.5 and +28.2 µm in OxN, HypN and Hyp conditions, respectively (Fig. 10.4, Table 10.2). The size difference between individuals with and without chamber addition was significant in both the OxN and Hyp aquaria, but was not significant in the HypN aquaria (Table 10.2).

Table 10.2 Effect of the experimental conditions on the maximum diameter of *A. tepida*: estimation of the coefficient, its standard error (Std. error) and the t and p statistics for the linear model

	Maximum diameter (μm)		Statistical parameter	
	Estimate	Std. error	t	p
Intercept (T5.NoGrowth)	280.0	7.5	37.6	$<2.1 \times 10^{-16}$
T5.OxN	27.4	10.9	2.5	0.01
T5.HypN	17.5	11.1	1.6	0.12
T5.Hyp	28.2	10.5	2.7	0.01

Note that the estimated Intercept corresponds to average of the reference category (here T5.NoGrowth) whereas all other estimated values are indicative of the average difference between the experimental and the reference population

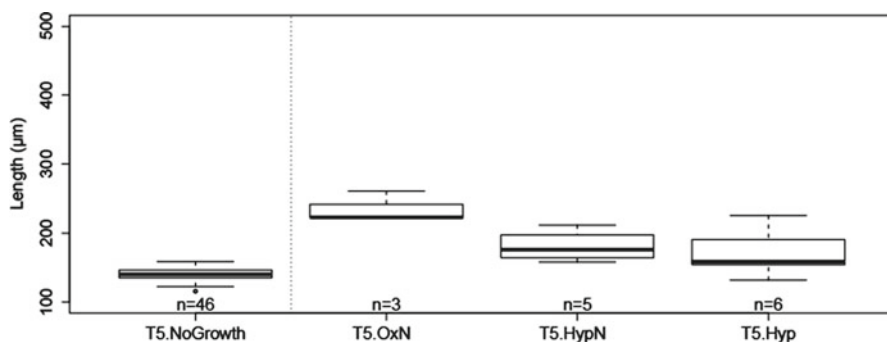


Fig. 10.5 Growth of *Bulimina marginata*: comparison of the size of the individuals that did not add new chambers (T5.NoGrowth) and those that calcified one or more new chambers (T5), for each tested condition (OxN, HypN, Hyp). On the box-and-whisker plot the two borders of each box are the first and third quartile while whiskers are 1.5 times the interquartile range of the lower and higher quartiles

All specimens of *B. marginata* introduced in the aquaria at T=1 (7 days) for the growth experiment (20 three-chambered juveniles for each treatment) were found at T=5 (56 days). The percentage of specimens that added at least one chamber in OxN, HypN and Hyp aquaria was 15, 25 and 30 %, respectively. Although only three specimens calcified in oxic conditions, each of these specimens added 2 or 3 new chambers. Conversely, the 11 specimens from hypoxic aquaria (5 in HypN and 6 in Hyp) that added new chambers added a single chamber, with the exception of a single specimen that added 2 chambers. In all three experimental conditions, at T=5, specimens that added new chambers had a significantly larger maximum length than specimens that did not add new chambers (Fig. 10.5, Df=3, F=49.7, p=8.35e-16 and Table 10.3). The average difference in maximum length between specimens with and without chamber addition was 95.7, 41.7 and 30.0 μm for OxN, HypN and Hyp, respectively (Table 10.3). A complementary statistical analysis shows that the size of the individuals that grew in the OxN conditions is significantly

Table 10.3 Effect of the experimental conditions on the maximum diameter of *B. marginata*: estimation of the coefficient, its standard error (Std. error) and the t and p statistics for the linear model

	Maximum diameter (μm)		Statistical parameter	
	Estimate	Std. error	t	p
Intercept (T5.NoGrowth)	140.0	2.2	62.9	$<2.1 \times 10^{-16}$
T5.OxN	95.7	9.0	10.6	4.5×10^{-15}
T5.HypN	41.7	7.1	5.9	2.5×10^{-7}
T5.Hyp	30.0	6.6	4.6	2.6×10^{-5}

Note that the estimated Intercept corresponds to average of the reference category (here T5.NoGrowth) whereas all other estimated values are indicative of the average difference between the experimental and the reference population

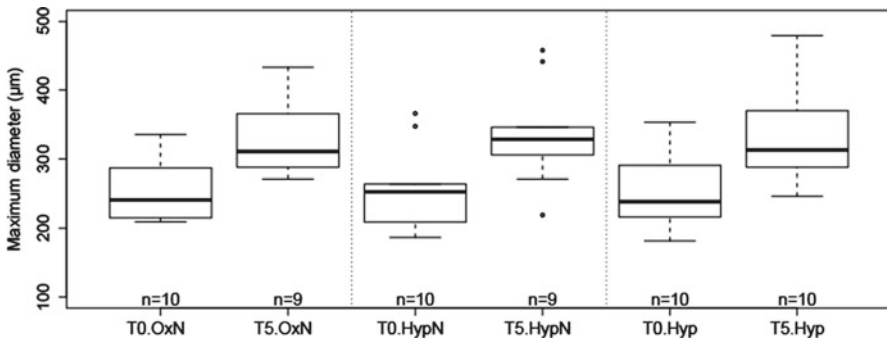


Fig. 10.6 Growth of *Melonis barleeanus* between T0 and T5 for each tested condition (OxN, HypN, Hyp). On the box-and-whisker plot the two borders of each box are the first and third quartile while whiskers are 1.5 times the interquartile range of the lower and higher quartiles

higher than the size of the individuals that grew in both hypoxic conditions. Summarizing, it appears that hypoxic conditions do not have a negative effect on the number of specimens that add new chambers. Conversely, for specimens that added new chambers, more chambers were added in oxic conditions, and consequently, the size difference was larger. Finally, the presence/absence of nitrate in the seawater has no significant impact on chamber addition.

All 10 incubated specimens of *M. barleeanus* were found in the Hyp aquarium, whereas 9 from the 10 individuals were found in the OxN and HypN aquaria. In the case of *M. barleeanus*, the same specimens were measured at T=0 and T=5, so that the obtained values can be directly compared. In all three experimental conditions, the size increase between T=0 and T=5 (Fig. 10.6) was statistically significant ($Df=1$, $F=25.2$, $p=6.32 \cdot 10^{-6}$), with an average increase of 81.6 μm , 85.9 μm and 80.3 μm for OxN, HypN and Hyp, respectively. No significant difference was found between the three experimental conditions (OxN, HypN, Hyp) ($Df=2$, $F=0.05$, $p=0.95$). It appears that, just as for *A. tepida*, strong hypoxic conditions alternating with short periods of anoxia do not inhibit the growth of *M. barleeanus*.

Table 10.4 Respiration rates (average value and standard error of the replicate measurements) of benthic foraminifera studied in the present paper with detailed data on their size (average size of maximal diameter or length and estimated biovolume)

Species	Location	Average size of max. Diam/Length	Biovolume (μm^3): Mean (SE)	RR (nL O ₂ cell ⁻¹ h ⁻¹)			References
				Mean	SE	n	
<i>Melonis barleeanus</i>	Portugal Continental Margin	347	8.6E+06 (7.2E+06)	0.32	0.26	3	Our data
<i>Ammonia tepida</i>	Aiguillon Bay	541	3.1E+07 (3.2E+06)	2.01	0.07	4	Geslin et al. (2011)
<i>Bulimina marginata</i>	Bay of Biscay	369	8.0E+06 (6.4E+05)	0.42	0.07	3	Geslin et al. (2011)

10.4.5 Respiration Rates

Oxygen respiration measurements were performed for *M. barleeanus*. The observed values were $0.32 \text{ nL} \pm 0.26 \text{ O}_2 \text{ cell}^{-1} \text{ h}^{-1}$ (Table 10.4).

10.4.6 Intracellular Nitrate Content in Foraminifera

In the following part, data of intracellular nitrate content of the 3 studied species are introduced without taking into account the different tested conditions. In a second part, data are presented as a function of time for each species.

Nitrate contents were measured in 29, 57 and 61 specimens of *A. tepida*, *M. barleeanus* and *B. marginata*, respectively. Nitrate was detected within the cell of individuals of all three species, but about 40 % of the measured *B. marginata* and *A. tepida* individuals show no measurable nitrate in their cell (Table 10.5). The average values of nitrate contents are 16 ± 5 , 61 ± 10 and 40 ± 7 pmol NO₃⁻ per cell for *A. tepida*, *M. barleeanus* and *B. marginata* respectively. The values of intracellular nitrate contents show a large intra-specific variability. For example, the average value of all measured *A. tepida* is $16 \text{ pmol NO}_3^- \text{ cell}^{-1}$ with a standard error of the mean (SEM) of 5 pmol cell^{-1} , a minimum value of 0 and a maximum value of $114 \text{ pmol cell}^{-1}$ (n=31) (Table 10.5). Taking into account the biovolume of each specimen, the concentrations in the cell can be calculated. The average concentrations of intracellular nitrate are varying between 3 and 12 mM according to the species (Table 10.5). These values are much higher than in the surrounding environments (max. $50 \mu\text{M NO}_3^-$), underlining the ability for each of these three species to store nitrate.

Figure 10.7 shows the evolution of nitrate contents with time for each condition and each species. No significant trend is detectable except for *B. marginata* in the hypoxic aquarium without additional nitrate ($p < 0.05$). In this case, nitrate contents decreased with time.

Table 10.5 Intracellular nitrate contents recorded in the present study compared to the previous published data for the same species. n is the number of measured specimens

Species	Reference	Location	Nitrate Content (pmol per cell)			NO ₃ ⁻ (mM) ^a	n
			Min	Max	Average		
<i>Ammonia tepida</i>	Our data	Aiguillon Bay	0	114	16 (5)	3 (1)	29
<i>Melonis barleeanus</i>	Our data	Portugal Margin	0	462	61 (10)	8 (1)	57
<i>Bulimina marginata</i>	Our data	Bay of Biscay	0	220	40 (7)	12 (2)	61
<i>Ammonia tepida</i>	Piña-Ochoa et al. (2010a, b)	Aiguillon Bay	0	58	13	0	15
<i>Melonis barleeanus</i>	Piña-Ochoa et al. (2010a, b)	North sea	1	27	9	1	7
<i>Melonis barleeanus</i>	Piña-Ochoa et al. (2010a, b)	Rhone delta	0	0	0	0	2
<i>Bulimina marginata</i>	Piña-Ochoa et al. (2010a, b)	Bay of Biscay	40	60	40	4	14

^aValues are mean values. Standard Error of the Mean (SEM) is given in parentheses

10.5 Discussion

10.5.1 Species Responses to Hypoxic Conditions

The high survival rates obtained under oxic conditions during long-term laboratory incubation for *Ammonia tepida* are not really surprising. In fact, this species is known to be well adapted to laboratory conditions (e.g. Bradshaw 1957, 1961; Schnitker 1974; Goldstein and Moodley 1993; Schmidt et al. 1957; Stouff et al. 1999a, b; Le Cadre et al. 2003; Dissard et al. 2010a, b). According to the literature, this species is resistant to various types of environmental stress (Murray 2006). *Ammonia tepida* is a common species in intertidal mud-flats where the oxygen penetration depth is very low (few millimeters). Rose Bengal stained specimens have been observed either on top of the sediment (e.g. Alve and Murray 2001) or deeper in the sediment where conditions are mainly anoxic (Frankel 1975; Buzas 1977; Kitazato 1994; Bouchet et al. 2007). Consequently, *A. tepida* is often considered as a species resistant to oxygen-depleted conditions (Moodley and Hess 1992). However, it is still not clear if *A. tepida* inhabits the anoxic part of the sediment or occupies oxic microhabitats created by burrowing macrofauna. Our experiment confirms the capacity of *A. tepida* to survive hypoxic conditions (at least 2 months) alternating with short anoxic periods (up to 6 days).

Similar experimental observations were reported by Moodley and Hess (1992) for *Ammonia beccarii*. They observed the activity of this species after 6 days of incubation under low oxygen conditions (12 µM) and 24 h of anoxia. After a longer anoxic incubation experiment, Moodley et al. (1997) noted that specimens of the genus *Ammonia* were found alive (Rose Bengal stained) after 78 days of putative anoxia.

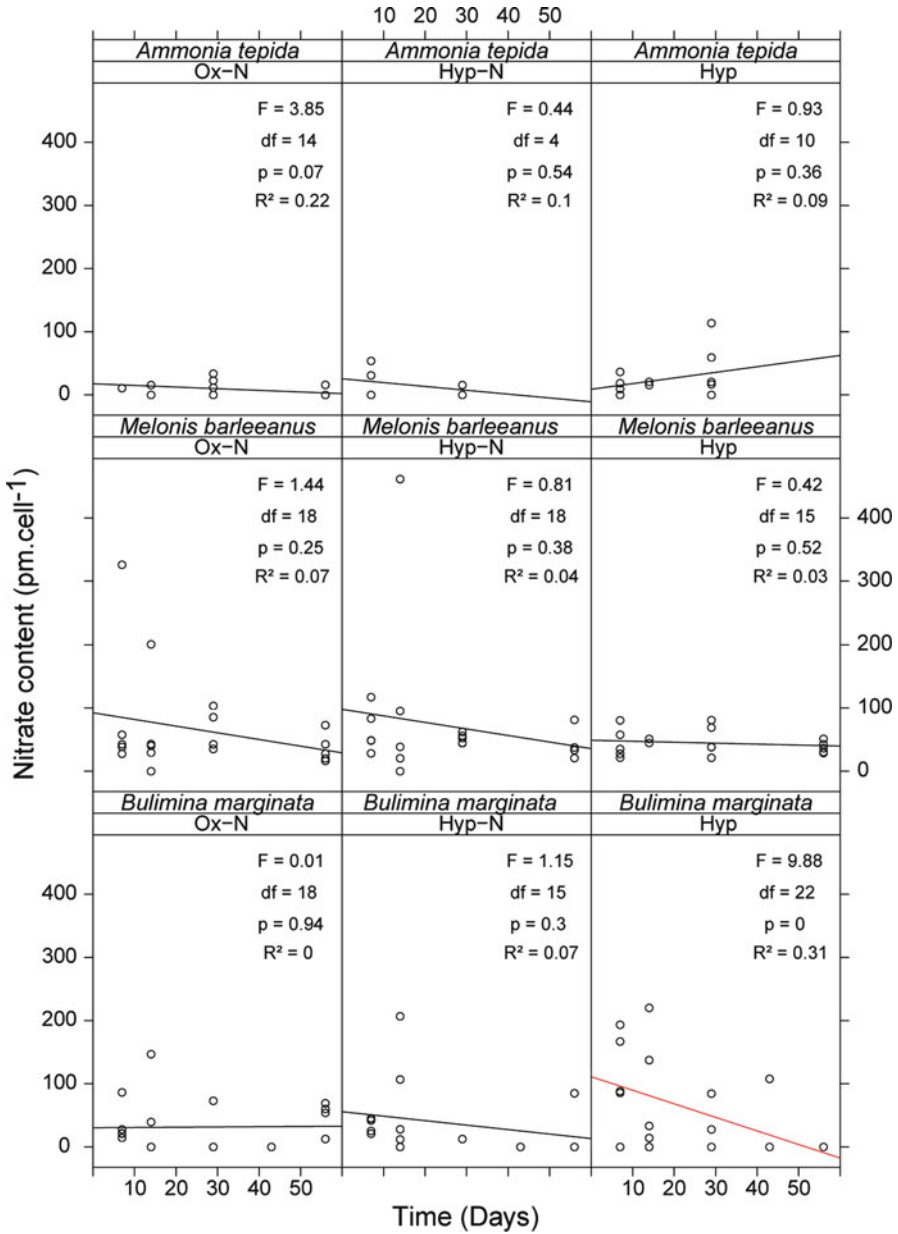


Fig. 10.7 Nitrate content (pmol cell⁻¹) recorded in the cell of the three species along the incubation time. The red line is statistically significant (only the case for *B. marginata*, Hyp)

However, using the Rose Bengal staining method in experimental studies is problematic; the number of living individuals may be totally unrealistic, since cytoplasm may stain for prolonged periods of time after the death of the organism, especially in anoxic conditions (Bernhard 1988).

Ammonia tepida is also known to be able to add chambers under laboratory condition (e.g. Bradshaw 1957, 1961; Goldstein and Moodley 1993; Stouff et al. 1999a, b; de Nooijer et al. 2009). In our case, growth was proven in all tested conditions by the observations of additional chambers not marked by calcein. Bradshaw (1961) reported growth rates of *Ammonia tepida* collected mainly in mud-flats (California, USA). He noted a growth of approximately 40 μm for adult specimens after 60 days at 15 °C. The values presented by Bradshaw (1961) are of the same order of magnitude as ours. Under oxic conditions, specimens of *A. tepida* that added new chambers (lack of calcein marking) were, on average, 27 μm larger in diameter than specimens that did not add chambers after 56 days of incubation. Our slightly lower values may be due to the fact that unlike Bradshaw (1961), we did not add food before or during the experiment. However, according to the large range of morphological and molecular types of *Ammonia tepida*, the specimens studied by Bradshaw (1961) were not necessarily conspecifics with our specimens.

Asexual reproduction has often been observed in *A. tepida* under laboratory conditions (Bradshaw 1957, 1961; Schnitker 1974; Goldstein and Moodley 1993; Stouff et al. 1999a; Diz et al. 2012). However, in our case, no reproduction (production of juveniles) was observed, not even in the oxic treatment. This may be due to low food availability in our experimental setup. In fact, Bradshaw (1961) has experimentally shown that *A. tepida* reproduces when food is added. Because of this absence of reproduction under oxic conditions, the results are inconclusive.

According to the literature, this is the first time that *M. barleeanus* has been used for an experimental study. The high survival rates obtained under oxic conditions show the ability of this species to survive under laboratory conditions. This result is very promising for future laboratory experiments using *M. barleeanus*. The majority of the specimens also survive 56 days of strong hypoxia alternating with short anoxic periods, with or without added nitrates. *Melonis barleeanus* is one of the most typical intermediate infaunal foraminiferal species (Jorissen 1999). It normally lives in a specific microhabitat at some millimeters or centimeters in the sediment, where oxygen is strongly diminished, and where nitrate reduction is maximal (Jorissen et al. 1995, 1998; Fontanier et al. 2005; Koho et al. 2008; Mojtahid et al. 2010).

Our comparison of tests sizes at the beginning and end of the experiment indicates that *M. barleeanus* is able to calcify under the three imposed laboratory conditions. Oxygen concentration had no significant effect on the growth rate of *M. barleeanus*, so that calcification does not seem to be affected by such conditions. The fact that no reproduction was observed may be explained, like with *A. tepida*, by the fact that no food was added during the experiment. However, since reproduction of this species has never been observed before in laboratory conditions, it is difficult to draw firm conclusions on this subject.

Surprisingly, *Bulimina marginata* was strongly affected by all our laboratory conditions, with low survival rates, both under oxic and hypoxic conditions. This result is surprising because, unlike many other deep-sea foraminiferal taxa, *B. marginata* previously showed a good adaptation to laboratory conditions (Wilson-Finelli et al. 1998; Havach et al. 2001; Hintz et al. 2004, 2006a, b; McCorkle et al. 2008; Barras et al. 2009, 2010; Filipsson et al. 2010).

Nevertheless, our results show no significant difference between the three different treatments. The survival rates of *B. marginata* are not significantly lower in the aquaria with long-term hypoxia alternating with short anoxic periods, with or without nitrate. This result is in agreement with the literature. *Bulimina marginata* is known to be able to live under hypoxic and anoxic conditions in the natural environment (Bernhard and Sen Gupta 1999). In many older studies, *B. marginata* has been considered as a good marker of low oxygen conditions (e.g. Phleger and Soutar 1973; van der Zwaan and Jorissen 1991; Sen Gupta and Machain-Castillo 1993; Bernhard and Sen Gupta 1999). However, some experimental studies have shown that oxygen-depleted conditions can have a negative effect on this species. In a 24-day experiment, the survival rate of *B. marginata* in nitrogen-flushed waters was significantly lower than in the oxic controls (Bernhard and Alve 1996). According to Ernst et al. (2005), *B. marginata* was hardly influenced by hypoxia, but higher abundances were observed in oxygenated microcosms. Alve and Bernhard (1995) noticed that specimens migrated to the sediment surface and occurred on polychaete tubes when the oxygen condition became lower than 0.2 mL L^{-1} . They interpreted this migration as behavior to avoid decreasing oxygen contents within the sediment. Conversely, Moodley et al. (1998) performed laboratory experiments to test the viability of foraminifera in anoxia; *Bulimina marginata* was still found alive (Rose Bengal stained) after a maximum of 21 days of anoxic incubation.

In our study, growth of juveniles of *B. marginata* was recorded in oxic as well as hypoxic conditions. Although the percentage of individuals that added new chambers is lower in oxic conditions (15 % versus 25 % and 30 % in the hypoxic aquaria), the number of added chambers was higher than in hypoxic conditions. Unfortunately, our dataset is too limited to draw any firm conclusion.

Bulimina marginata is able to reproduce under oxic conditions, producing juveniles which have grown and calcified. Such reproductions were observed at T1, T3 and T4 with 80, 37 and 142 counted juveniles, respectively. In experiences described by Barras et al. (2009), in which low quality food was added, adult specimens of *B. marginata* produced an average of 30 juveniles per reproduction event. If the number of juveniles per reproduction had been the same in our experiments, we can estimate the numbers of adults that reproduced (3 at T1, 1 at T3 and 4 to 5 at T4). The apparent absence of reproduction in our hypoxic aquaria contrasts with results obtained by Alve and Bernhard (1995). During a 4-week mesocosm experiment with low oxygen conditions, these authors observed Rose Bengal stained juveniles of *B. marginata*, suggesting that reproduction took place.

Two main facts stand out in the data obtained for *B. marginata*: (1) the survival rates of *B. marginata* are low in all tested conditions, and (2) juveniles were observed under oxic conditions but not under hypoxic conditions. According to the literature, *Bulimina marginata* is relatively easy to keep alive, and reproduction can be obtained under laboratory condition by feeding them with fresh or frozen diatoms or green algae (Hintz et al. 2004, 2006a, b; McCorkle et al. 2008; Barras et al. 2009; Filipsson et al. 2010). However, no reproduction was observed when no additional food was added to the cultures (C. Barras, Pers. Com). To explain our surprising observations, three contrasted hypotheses can be envisaged.

Hypothesis 1

In our experiment, high mortality rates of *B. marginata* could be explained by unfavorable conditions in our aquaria due to the lack of fresh organic matter. The only source of foraminiferal food was the organic matter existing in the fine sediment added. We chose not to add food in order to avoid complex geochemical processes in the vials. However, reproduction occurred under oxic conditions. Boltovskoy and Wright (1976) discussed how reproduction could be a stress response or a response to favorable conditions. Our results may lead to contradictory hypothesis. Either food is lacking and could explain high mortality rate and reproduction events as a response to stress condition; or food is not a limiting factor and reproduction occurred in response to favorable condition. In this case, another parameter may be responsible for the death of *B. marginata*.

Hypothesis 2

The absence of juvenile specimens in the hypoxic/anoxic treatments suggests that hypoxic conditions have a negative impact on reproduction. We could hypothesize that foraminifera did not reproduce under hypoxia, which could lead a decrease of the metabolic rate. Such a diminished metabolic rate could be sufficient for cell growth and calcification, but insufficient for reproduction.

Hypothesis 3

Asexual reproduction of foraminifera usually results in the death of the parent individual, creating the presence of empty adult tests (Lee et al. 1991; Barras et al. 2009). For *Bulimina marginata*, Barras et al. (2009) observed that all adults died after reproduction. In our study, reproduction occurred in oxic conditions explaining a large part of the empty adult tests, thereby contributing in a significant way to the low survival rates observed for *B. marginata*. Very similar survival rates were observed under oxic and hypoxic conditions. We may therefore wonder if the low survival rates in hypoxic conditions could also be partly explained by reproduction events. The only evidence we have that no reproduction took place in the hypoxic aquaria is the absence of juveniles. However, Alve and Goldstein (2003) demonstrated by experimental approaches that reproduction of foraminifera produces propagules (uncalcified chamber smaller than 63 μm) which are able to stay in the sediment for at least 4 months without any growth and calcification. This phenomenon may also have occurred in our experiment. Specimens of *B. marginata* may have reproduced and, perhaps, the produced propagules never calcified. At the end of the experiment, sediment and water containing foraminifera were sieved over a 63 μm mesh and $>63 \mu\text{m}$ fractions were observed. With this protocol, it is very well possible that propagules $<63 \mu\text{m}$ were present at the end of the hypoxic experiments.

A very important consequence of this hypothesis would be that hypoxia does not have an impact on reproduction but does have a large impact on propagule calcification, which did not take place. Our results, obtained for the growth rates

(chamber addition and calcification) of *B. marginata*, support this idea (lower calcification rate under hypoxia than under oxic conditions). The ultimate consequence of this hypothesis would be that the successive disappearance of foraminiferal species after long term hypoxia/anoxia (as observed in Mediterranean sapropels, e.g., Jorissen 1999) would not be explained by adult mortality, but rather because juveniles are unable to calcify. Unfortunately, our present data set does not allow us to make firm conclusions at this moment; additional experiments are needed to confirm or refute this hypothesis.

10.5.2 *Metabolism in Hypoxic Conditions*

Aerobic respiration rates that were measured in this study for *Melonis barleenus* ($0.32 \text{ nL O}_2 \text{ cell}^{-1} \text{ h}^{-1}$ (± 0.26)) are in the same range as those published by Geslin et al. (2011), who reported a minimum respiration rate value of $0.09 \text{ nL O}_2 \text{ cell}^{-1} \text{ h}^{-1}$ (± 0.02) for *Rectuvigerina phlegri* and a maximum value of $5.27 \text{ nL cell}^{-1} \text{ h}^{-1}$ (± 0.52) for *Ammonia beccarii*. As it has been shown previously, respiration rates of foraminifera vary in function of cell size following the equation $R = 3.98 \cdot 10^{-3} \text{ BioVol}^{0.88}$ (with respiration rate (R) expressed in $\text{nL O}_2 \text{ h}^{-1}$ and biovolume (BioVol) in μm^{-3}), for the 17 species studied (Geslin et al. 2011). The same authors showed that benthic foraminifers have lower oxygen respiration rates than other groups of meiofauna, even when standardized for biovolume. This suggests that foraminifera have a relatively low oxygen demand, which may explain why their aerobic metabolism is less affected by low oxygen contents (our hypoxic treatments). The lower respiration rate may reflect a lower metabolic rate; which could in turn be explained by the low degree of activity of the foraminifera. A relatively low metabolic rate is advantageous for organisms exposed to environmental stress (Theede et al. 1969). In this specific case of oxygen deficiency, a low metabolic rate may help the foraminifer to preserve its energy pool during short anoxic periods.

10.5.3 *Intracellular Nitrate Content*

10.5.3.1 *Average Data of Intracellular Nitrate Content*

The intracellular nitrate contents recorded in the present study are in the same order of magnitude as the data published by Piña-Ochoa et al. (2010a). The average of intracellular nitrate contents calculated for the numerous measurements made for the three studied species varies between 16 to 61 pmol NO_3^- per cell (Table 10.4) whereas measurements reported by Piña-Ochoa et al. (2010a) range between 3 to 40 $\text{pmol NO}_3^- \text{ cell}^{-1}$. However, the nitrate concentrations of the three studied species are still very low (max. value 462 pmol NO_3^- per cell) compared to the much higher values reported by Piña-Ochoa et al. (2010a) for *Cyclammina cancellata*

(45,500 pmol NO_3^- per cell) or *Globobulimina turgida* (18,000 pmol NO_3^- per cell). The high concentrations of intracellular nitrates allow these two species to respire nitrates under anoxia for at least 3 months (Piña-Ochoa et al. 2010a).

Ammonia tepida was reported as non nitrate-storage species by Piña-Ochoa et al. (2010a). In our case, a higher number of specimens was measured ($n=29$) and, although 13 specimens showed no intracellular nitrate, the other had intracellular nitrate with a maximum value of 114 pmol NO_3^- per cell (23.3 mM of NO_3^-). Knowing that the maximum concentration of nitrate in the surrounding sea water is 50 μM , these new data show that also *A. tepida* is able to store nitrate in their cells. However, these results do not imply that this species is also actively denitrifying. In Piña-Ochoa et al. (2010a), no positive denitrification rates were obtained for *Ammonia tepida*. However, it is probable that this negative result is explained by the fact that the specimens used by Piña-Ochoa et al. (2010a) did not contain any intracellular nitrate. In order to clarify this point, denitrification rates should be measured using specimens with elevated intracellular nitrate contents.

The specimens of *M. barleeanus* measured in the present study show higher nitrate contents than those reported by Piña-Ochoa et al. (2010a). However, the nitrate contents of *M. barleeanus* are still very low compared to other species such as *Uvigerina elongatastriata* (5,400 pmol NO_3^- per cell), which occupies the same intermediate infaunal microhabitat as *M. barleeanus* (e.g., Koho et al. 2008; Mojtahid et al. 2010). Piña-Ochoa et al. (2010a) suggested that *M. barleeanus* would not be able to denitrify because of the insufficient amount of nitrate available in the cell. In the light of our new data, this conclusion should be reconsidered. However, in order to ascertain that *M. barleeanus* is indeed able to respire nitrate under anoxia, it is absolutely necessary to measure denitrification rates. The fact that roughly all analyzed specimens of *M. barleeanus* collected on the continental Margin off Portugal contain intracellular nitrate ($n=55$, our study) whereas specimens collected in the Rhone delta ($n=2$, Piña-Ochoa et al. 2010a) did not (Table 10.4), suggests that environmental parameters influence the nitrate content of this species.

10.5.3.2 Impact of the Three Tested Conditions on Intracellular Nitrate Contents

The intra-specific variability of intracellular nitrate concentrations within a population is high. The same observation was made by Koho et al. (2011) who reported that the nitrate concentration in single individuals of living *G. turgida* ranged from 0 to 32,541 pmol N per cell, corresponding to average concentrations of $3,929 \pm 4,590$ and $8,999 \pm 9,023$ pmol N per cell (SEM higher than the mean) in two replicate cores. A similarly high intra-population variability in nitrate concentrations has also been noted by Piña-Ochoa et al. (2010a, b) and Bernhard et al. (2011, 2012). Because of this high variability, it is not easy to follow the story of the intracellular nitrate in individual foraminifera. Nevertheless, our data show a single statistically significant trend.

Intracellular nitrate contents do not show significant changes with time for *Ammonia tepida* and *Melonis barleeanus* (Fig. 10.7). If these species are able to

denitrify, we could expect that they should use nitrate at least during the short periods of anoxia. This should lead to a decrease of their intracellular nitrate concentration in the hypoxic aquaria without nitrate during anoxia. Furthermore, if these 2 species would shift to nitrate respiration in case of anoxia, in the hypoxic aquaria without nitrate they should die after the exhaustion of their pools of intracellular nitrate. Therefore, all our data suggest that *A. tepida* and *M. barleeanus* did not denitrify but nevertheless survived hypoxia and short periods of anoxia.

The only significant trend is the decrease of nitrate contents in *B. marginata* with time in hypoxic conditions without nitrate which could be explained by the fact that this taxon has indeed used nitrate for foraminiferal denitrification during the short periods of anoxia. It is possible that such a decreasing trend in nitrate content has not been observed in the hypoxic aquaria with nitrate because the foraminifera have recharged their intracellular nitrate pool after each of the anoxic periods. Our data therefore suggest the capacity of *B. marginata* to denitrify. However, the significant trend could be discussed because of the high variability of nitrate content. The previous suggestions should be resolved by additional studies with measurements of denitrification rates.

10.5.4 Discussion Regarding Experimental Methodologies

10.5.4.1 Survival Determination

Working with foraminifera in oxygen-depleted environments can be problematic because of the determination of living foraminifera during the experiment. Observation of Rose Bengal stained cytoplasm inside the test is not sufficient to judge vitality when incubations are performed under oxygen-depleted conditions or for short periods of time, because cytoplasm can be retained in dead specimens for weeks to months (e.g. Bernhard 1988; Hannah and Rogerson 1997). Accurate methods to distinguish living foraminifera are needed. We chose to use fluorogenic probes which are non fluorescent compounds that produce a fluorescent product after modification by intracellular esterases that are only active in living individuals. Two different fluorogenic probes may be used: CTG (Cell Tracker Green) and FDA (Bernhard et al. 1995, 2006). CTG is a fluorogenic probe producing fluorescence which can be fixed with formalin (Bernhard et al. 2006; Pucci et al. 2009). Fixed samples may be observed after the fixation (weeks to months) using an epifluorescence binocular. On the opposite, FDA, another fluorogenic probe, cannot be fixed with formalin (Bernhard, 2000) so that samples treated with FDA have to be observed few hours after the incubation. Consequently, CTG is more practical when the amount of samples to observe is too high to be treated within a few hours. The inconvenience of this probe is its prohibitive price. In our case, we observed the fluorescence of FDA incubated specimens a few hours after the end of the experiment to assess vitality. The same protocol was used by Piña-Ochoa et al. (2010b) and Koho et al. (2011).

10.5.4.2 Growth Observations

In our study, two methodologies were used to determine growth of the incubated specimens: introduction of marked-shell specimens using calcein (Bernhard et al. 2004) and automatic measurements of the foraminiferal shell size before and after the experiment. The advantage of the calcein method is to be able to detect growth of specimens even if growth is very small (e.g. one chamber) and to determinate the number of additional chambers (unmarked) calcified during the incubation. Determination of growth is also possible with precise size measurements when considering each specimen separately at the beginning and at the end of the experiment. However, when considering the average size of a pool of individuals, which is the case in our study (practically impossible to have 1 specimen per vial and a statistically significant numbers of individuals per condition), a slight growth of a limited number of individuals of this pool might be hidden in the standard error of the average size of all specimens.

10.5.4.3 Reproduction

In many experiments, reproduction events are identified thanks to the production of juveniles (e.g. Le Cadre and Debenay 2006; Barras et al. 2009). The first step of the foraminiferal life is a single uncalcified chamber called a “propagule” (Alve and Goldstein 2003). These propagules may calcify chambers in order to become juvenile foraminifera, but they also may remain as uncalcified propagules for many weeks to months (Alve and Goldstein 2003). Consequently, reproduction events may occur without the production of calcified juveniles. To prove accurately the occurrence of reproduction events during experiments, it is therefore necessary to observe the entire samples (no sieving) and particularly the small fraction (< 63 µm) with a microscope in order to avoid loss of propagules.

10.6 Conclusion

Ammonia tepida and *Melonis barleeanus* show a similar response to hypoxic conditions alternating with short periods of anoxia. We have recorded similarly very high survival rates under oxic and hypoxic conditions as well as similar growth rates. No reproduction was observed, which may be due to the lack of added food. Therefore, additional experiments are needed to demonstrate their ability to reproduce under low oxygen conditions.

Bulimina marginata shows a very different response. The survival rates are much lower, but there was no significant difference in survival between the oxic and hypoxic/anoxic treatments.

It is possible that the high mortality is due to unfavorable experimental conditions (e.g. lack of food). Alternatively, the observation of juveniles in the oxic treatment suggests that the high mortality rate may be at least partially due to reproduction. The absence of calcified juveniles in the hypoxic/anoxic treatments can result from an absence of reproduction (in which case hypoxia would inhibit reproduction), but could also be explained by the incapacity of the juveniles to calcify, forcing them to stay at a propagule stage.

Intracellular nitrate contents suggest that *B. marginata* may be able to denitrify during short periods of anoxia.

This study allows us to propose future experimental work with interesting species such as *M. barleeanus* which was not previously used for laboratory experiments.

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