



# Foraminiferal survival after long-term in situ experimentally induced anoxia

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**Abstract.** Anoxia was successfully induced in four benthic chambers installed at 24 m depth on the northern Adriatic seafloor from 9 days to 10 months. To accurately determine whether benthic foraminifera can survive experimentally induced prolonged anoxia, the CellTracker™ Green method was applied and calcareous and agglutinated foraminifera were analyzed. Numerous individuals were found living at all sampling times and at all sampling depths (to 5 cm), supported by a ribosomal RNA analysis that revealed that certain benthic foraminifera were active after 10 months of anoxia. The results show that benthic foraminifera can survive up to 10 months of anoxia with co-occurring hydrogen sulfides. However, foraminiferal standing stocks decrease with sampling time in an irregular manner. A large difference in standing stock between two cores sampled under initial conditions indicates the presence of a large spatial heterogeneity of the foraminiferal faunas. An unexpected increase in standing stocks after one month is tentatively interpreted as a reaction to increased food availability due to the massive mortality of infaunal macrofaunal organisms. After this, standing stocks decrease again in cores sampled after 2 months of anoxia to then attain a minimum in the cores sampled after 10 months. We speculate that the trend of overall decrease of standing stocks is not due to the adverse effects of anoxia and hydrogen sulfides but rather due to a continuous diminution of labile organic matter.

## 1 Introduction

Over the last decade, numerous marine environments have been affected by hypoxia (Diaz and Rosenberg, 2008). The increase in the frequency of occurrence of hypoxia and the spatial distribution of such oxygen-depleted areas are assumed to be linked to eutrophication and climate change (Stramma et al., 2008; Rabalais et al., 2010). Hypoxia can dramatically affect pelagic and benthic biodiversity (Levin, 2003; Gooday et al., 2009; Stramma et al., 2010). The mechanisms leading to low-oxygen conditions differ between the open ocean and coastal enclosed basins. In many oceanic upwelling areas, hypoxia is permanent and the dissolved oxygen concentration remains below  $0.5 \text{ mL L}^{-1}$  ( $20 \mu\text{M}$ ; Helly and Levin, 2004; Middelburg and Levin, 2009). Hypoxia is caused by a combination of high pelagic productivity and slow renewal of oxygen in the water column. In most semi-enclosed coastal areas, hypoxia is related to continental inputs (of nutrients and organic matter) and seasonal water-column stratification. Pelagic production is a response to natural and/or anthropogenic nutrient input, and hypoxia is mainly caused by benthic oxygen consumption. In such coastal environments hypoxia appears seasonally.

Generally, bottom-water oxygen concentrations are strongly linked to several other environmental parameters. Increased organic matter supplies stimulate benthic production, leading to an increased sediment oxygen demand and ultimately to lowered bottom water oxygenation. In

extreme cases, when the seafloor becomes anoxic, organic matter degradation continues via anaerobic metabolic pathways, eventually leading to sulfate reduction near the sediment–water interface (Middelburg and Levin, 2009). In such conditions toxic hydrogen sulfide may diffuse into the bottom water.

Various terminologies have been proposed to describe the intensity of hypoxia, mainly based on the effect of oxygen concentration on marine biota (reviewed in Hofmann et al., 2011 and Altenbach et al., 2012). For the purpose of the present study we will use only three terms defined as follows (Tyson and Pearson, 1991; Bernhard and Sen Gupta, 1999; Middelburg and Levin, 2009): (1) oxic – the measured oxygen concentration above  $1.5 \text{ mL L}^{-1}$  ( $> 63 \mu\text{M}$ ); (2) hypoxic – concentration below  $1.5 \text{ mL L}^{-1}$  ( $< 63 \mu\text{M}$ ), which is the threshold at which an impact has been recorded on coastal metazoan biota; and (3) anoxic – no detectable dissolved oxygen present.

In the past, several field and laboratory studies have been conducted to better understand the response of benthic faunas to hypoxia (i.e., to low oxygen concentration and its co-occurring processes such as high organic matter availability and increased hydrogen sulfide concentration) in coastal environments. These studies demonstrate that for macrofauna (invertebrates larger than 1 mm) the first responses to low oxygenation are usually behavioral or physiological changes (e.g., body extension, shallowing of burial depth, emergence from the sediment; see Levin et al. (2009) for a review), and the organisms exhibit a high mortality rate after only two to seven days of anoxia (Riedel et al., 2008; Stachowitsch et al., 2012). Within the meiofauna (invertebrates measuring  $45 \mu\text{m}$  to 1 mm), copepods are among the taxa most sensitive to anoxia (Moodley et al., 1997; Diaz and Rosenberg, 2008), whereas several nematode species can survive up to 60 days of anoxia (Wieser and Kanwisher, 1961). Benthic foraminifera appear to be more tolerant to anoxia than most meiofaunal metazoans (Josefson and Widbom, 1988; Moodley et al., 1997; Levin et al., 2009).

In field studies, the foraminiferal density tend to decrease during anoxic and/or hypoxic events (e.g., Jorissen et al., 1992; Duijnsteet et al., 2004) and infaunal taxa migrate upward in hypoxic conditions (Alve and Bernhard, 1995; Duijnsteet et al., 2003). In the diatomaceous mud belt off Namibia, *Virgulinema fragilis* appear to be the only foraminiferal species that can survive anoxic and sulfidic conditions (Leiter and Altenbach, 2010). Laboratory studies show that foraminiferal assemblages can survive up to 78 days of anoxia (Moodley et al., 1997). Conversely, benthic foraminiferal densities respond positively to increased input of fresh organic matter if it is not accompanied by oxygen depletion (Heinz et al., 2001; Ernst and van der Zwaan, 2004; Ernst et al., 2005; Nomaki et al., 2005). Some evidence suggests that an exposure to hydrogen sulfide for up to 21 days does not significantly affect foraminiferal density, and that

after 66 days of exposure, some individuals were still found alive (Moodley et al., 1998).

Most previous studies were based on the analysis of foraminiferal assemblages stained by rose bengal. Rose bengal is a bulk stain that adheres to the proteins present in the protoplasm (Walton, 1952; Bernhard, 2000). However, protoplasm may be preserved in the foraminiferal shell several days to weeks after the death of the organism in well-oxygenated conditions (Boltovskoy and Lena, 1970; Bernhard, 1988; Murray and Bowser, 2000) and up to three months in anoxia (Hannah and Rogerson, 1997), leading to the staining of dead organisms. The expectation is that protoplasm degradation is especially slow in anoxic conditions (Burdige, 2006; Glud, 2008). Consequently, using rose bengal can lead to substantial overestimation of the living foraminiferal standing stocks, and probably does not reliably reflect the living faunas in low-oxygen settings. Although rose bengal staining may yield a rapid overview of living faunas in distributional studies, it is unsatisfactory for studies with a high temporal resolution, where the time between successive samplings may be shorter than the time needed for degradation of the foraminiferal protoplasm. Several techniques have recently been developed to more accurately identify the vitality of benthic foraminifera (reviewed in Bernhard, 2000). As noted above, proteins can still be present after the organism has died and all energetic and enzymatic activities have stopped. These activities are the basis for more accurate foraminiferal vitality discrimination techniques. For instance, based on an adenosine triphosphate (ATP) concentration, an absence of living individuals was determined in the Santa Barbara Basin after a 10-month anoxic period (Bernhard and Reimers, 1991). A laboratory experiment using the ATP concentration method determined, in four species from a shallow site (45 m) in Drammensfjord (Norway), that all these species survived up to three weeks of anoxia. Three species, however, showed a lower ATP concentration in anoxic conditions (Bernhard and Alve, 1996). A recent laboratory experiment (Piña-Ochoa et al., 2010b) using the enzymatic activity sensor fluorescein diacetate (FDA; Bernhard et al., 1995) showed that the deep infaunal species *Globobulimina turgida* can survive anoxia (with presence of nitrate) for up to 84 days (with a survival rate of 46%). A subsequent study, using the same method, demonstrated that the individuals do not migrate vertically during anoxia (Koho et al., 2011). CellTracker™ Green (CTG; Bernhard et al., 2006), another enzymatic activity probe, has been used to determine that Adriatic Sea foraminiferal assemblages can survive up to 69 days of hypoxia and that their vertical migrational behavior is largely linked to the labile organic matter availability (Pucci et al., 2009). Finally, it has been shown recently that the active status of foraminifera can be confirmed by sequencing a taxonomic marker extracted from the sediment metatranscriptome (Lejzerowicz et al., 2013).

In summary, previous laboratory and field studies suggest that foraminifera can survive strong hypoxia and anoxia

for prolonged periods, in most cases without a clear decrease in population density. Many of these studies, however, are based on the problematic rose bengal staining method, which may substantially overestimate the number of living individuals or even indicate “living” faunas where none are present, especially in anoxic conditions. This calls for testing the resistance of shallow-water foraminifera to anoxia with a more reliable vitality determination technique. Additionally, until now the foraminiferal response to strong hypoxia and/or anoxia has mainly been assessed by laboratory experiments and by ecological field studies lasting up to 3 months, but never (to our knowledge) by an in situ experiment over 10 months. The current paper applies such a long-term in situ experimental approach to document the foraminiferal response to anoxia, using the CTG technique. Our experimental setup was designed to address several complementary aspects of the benthic ecosystem:

1. In order to ensure long-term anoxia (up to ~10 months), several benthic chambers were positioned on the sea floor for different periods of time. After closure of the chambers, the respiration of the benthic organisms rapidly consumed all available oxygen, leading to anoxia. In order to quantify these processes, the chemical characteristics of the sediment and the overlying waters were monitored (Koron et al., 2013; Metzger et al., 2013).
2. An in situ experimental approach has the advantage of considerably reducing “laboratory effects” due to differences between experimental and natural conditions, as well as of reducing the natural temporal variability (in temperature, salinity, water transparency, food availability, etc.) that can affect faunas in field studies.
3. Our experimental setup allows us to apply the same method on different faunal elements. It has been successfully used to study the effect of anoxia on macrofauna (Riedel et al., 2008, 2012, 2013; Stachowitsch et al., 2012; Blasnig et al., 2013) and on different meiofaunal groups such as copepods, nematodes (De Troch et al., 2013; Grego et al., 2013) and foraminifera (this study and Langlet et al., 2013).

Our study was conducted in the northern Adriatic Sea, which is a typical coastal area impacted by seasonal hypoxia/anoxia. It combines many aspects commonly associated with low-oxygenation events: a semi-enclosed, shallow (< 50 m) basin with a fine-grained substrate, a high riverine input, high productivity (leading to “marine snow” events), and long water residence times in summer/autumn (Ott, 1992). Several studies have reported the occurrence of bottom-water hypoxia and anoxia here (e.g., Giani et al., 2012), with an increasing frequency from the 1970s to the mid-1990s. It appears that the frequency of marked hypoxia has decreased since the mid-1990s (Giani et al., 2012), likely

**Table 1.** Deployment and sampling dates as well as duration of the experimental period for the cores representing in situ conditions (“Normoxia”) and the various periods of anoxia in the four different benthic chambers

Sample ID	Chamber deployment day	Core sampling day	Incubation duration
Normoxia	–	3 Aug 2010	0 days
9 days	2 Aug 2010	11 Aug 2010	9 days
1 month	27 Jul 2010	25 Aug 2010	29 days
2 months	27 Jul 2010	23 Sep 2010	58 days
10 months	24 Sep 2010	5 Aug 2011	315 days

due to a reduction of anthropogenic nutrient supplies and a reduced riverine nutrient input into the northern Adriatic Sea.

In this context, we investigate the responses of the foraminiferal faunas to experimentally induced anoxia in benthic chambers. The present paper focuses on the foraminiferal survival and density response to long-term anoxia. Our main question was whether benthic foraminifera could survive 10 months of anoxia. In the case of a positive answer, our goal was to determine how the density varies as a function of the duration of anoxia. Finally, we studied the variation of density in the upper 5 cm of the sediment in order to identify potential vertical migration of the foraminiferal faunas in response to experimental conditions. The response of individual species to anoxia is treated in a second paper (Langlet et al., 2013).

## 2 Material and methods

### 2.1 Experimental setup and sampling

The experiment was conducted in the Gulf of Trieste (northern Adriatic Sea) near the oceanographic buoy of the Marine Biology Station Piran (45°32.90' N, 13°33.00' E) at 24 m depth on a poorly sorted silty sandy bottom. The experimental setup has been adapted from an earlier experiment conducted by Stachowitsch et al. (2007) and Riedel et al. (2008). Both experiments used the Experimental Anoxia Generating Unit (EAGU), which is a 0.125 m<sup>3</sup> fully equipped benthic chamber (a cube with all sides 0.5 m long) that enables experimentally inducing and documenting local-scale anoxia (see Stachowitsch et al., 2007).

In the current study, we used four different chambers to produce anoxia for four different durations (Table 1). The four chambers were installed on the sea floor, several meters apart, on substrates that were visually poor in macrofauna. At the beginning of each experiment, two to three brittle stars (*Ophiothrix quinque maculata*) were introduced into each chamber (that otherwise did not contain any visible macrofauna) to provide rapid, visible indication of the onset of anoxia in each chamber. For comparison, two cores

(termed “normoxia”) were sampled at the start of the experiment. In order to standardize the terminology for all articles of the present Biogeosciences Special Issue, the different sampling times (also named in the present paper “sample ID” for “sample identification”) have been termed “9 days”, “1 month”, “2 months” and “10 months”. The exact duration (in days) of each of the experiments is given in Table 1. The first chamber, fully equipped with the EAGU analytical devices, documented the onset of anoxia during a 9-day period. The other three chambers were used to study the development of the meiofauna after  $\sim 1$  month,  $\sim 2$  months and about 10 months of anoxia, respectively. Foraminifera, copepods and nematodes were analyzed for the same cores. Sediment cores were taken by scuba divers using a Plexiglas™ corer with a 4.6 cm inner diameter (16.6 cm<sup>2</sup> surface area). Two replicate cores were taken in each of the benthic chambers at each sampling time.

## 2.2 Meiofaunal analysis

To identify whether the collected meiofaunal organisms were alive, we used CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes, Life Technologies; heretofore called “CTG”). When living cells are incubated in CTG, this nonfluorescent probe passes through the cellular membrane and reaches the cytoplasm, where hydrolysis with nonspecific esterase produces a fluorogenic compound. This fluorogenic compound does not leak out of the cell after fixation and can thus enable identifying the individuals exhibiting enzymatic activity when sampled (Bernhard et al., 2006).

For meiofaunal analyses, entire sediment cores were sliced every half centimeter from 0 to 2 cm and every centimeter between 2 and 5 cm. Within one hour after retrieval, sediments were stored in 100 cm<sup>3</sup> bottles, which were filled with sea water and CTG-DMSO (dimethyl sulfoxide) at a final CTG concentration of 1  $\mu$ M (Bernhard et al., 2006; Pucci et al., 2009). Samples were gently shaken and immediately placed in a cold room at in situ temperature, where they remained for at least 10 h in order to obtain the hydrolysis of the CTG probe. After this reaction period, samples were fixed in 4 % formaldehyde buffered with sodium tetraborate. The samples were further stored at room temperature.

Samples were then centrifuged to separate the soft meiofauna (i.e., copepods, nematodes; results presented in De Troch et al., 2013 and Grego et al., 2013) from the sediment and the “hard-shelled” meiofauna (i.e., foraminifera – the present article and Langlet et al. (2013)). Centrifugation has two main advantages: first it permits working on the same sample for both copepods/nematodes and foraminifera. These two groups are easily separated because of their different densities; foraminifera are heavier because of their agglutinated or calcareous test. Secondly, it simplifies the foraminiferal analysis because all the soft organisms, which show a stronger fluorescence than foraminifera (the test tends

to diminish the observed intensity of the fluorescence), have been removed.

Prior to centrifugation, samples were first sieved over a 38  $\mu$ m mesh to remove small sediment particles and formaldehyde. We then added a Levasil density medium and distilled water to achieve a density of about 1.17 g cm<sup>-3</sup>, and finally added about 10 g of kaolin powder to promote the sedimentation of the heavier particles (McIntyre and Warwick, 1984; Burgess, 2001). Next, we centrifuged for 10 min at 3000 rpm at ambient room temperature. After this, the supernatant (composed of soft meiofauna) and the deposit (composed of the hard-shelled meiofauna and sediment) were separately sieved over a 38  $\mu$ m mesh to remove the kaolin and the Levasil medium. The two separated samples were stored in a borax-buffered formaldehyde solution until further analysis.

The present study analyzes only the samples containing the hard-shelled meiofauna (soft-walled foraminiferal taxa are excluded). Therefore, this contribution only considers calcareous and agglutinated foraminifera. These samples were subsequently sieved over 315, 150, 125 and 63  $\mu$ m meshes.

A major asset of the present study is that we also analyzed all of the > 63  $\mu$ m size fractions of the benthic foraminifera, and we systematically studied the complete sample without splitting. Many studies fail to analyze the smaller size fractions (63–125 or 63–150  $\mu$ m) because it is extremely time consuming. The smaller size fraction, however, may contain abundant living individuals and can provide important information about small opportunistic species and about potential reproduction during the experiment.

For the 63–125  $\mu$ m fractions it was necessary to use a concentration method for the foraminifera to prevent splitting the sediment (leading to statistical problems) and/or unrealistic working times. Thus, all foraminiferal samples of the fractions 63–125  $\mu$ m were washed to remove the borax-buffered formaldehyde solution and then dried in a compartment dryer (Memmert GmbH + Co.KG) at 30–40 °C. The foraminiferal tests were then separated by treating the sediment with tetrachloride under a laboratory fume (Hohenegger et al., 1989; Murray, 2006). The tetrachloride was carefully decanted and the floating foraminifera were collected on paper filters, quickly dried and then stored in small glass vials.

Foraminiferal counts were performed in all fractions > 63  $\mu$ m using an epifluorescence stereomicroscope (Olympus SZX12 with a fluorescent light source Olympus URFLT or Nikon SMZ 1500 with a PRIOR Lumen 200). Samples were observed at the accurate excitation (492 nm) and emission (517 nm) wavelengths. The samples from the 63–125  $\mu$ m fractions were dried and picked dry, while the samples from the > 125  $\mu$ m fractions were not dried and picked wet. Dried specimens still show clearly the fluorescence produced by incubation with CTG. Only specimens showing clear green fluorescence were picked and counted as living.

### 2.3 Ribosomal RNA analysis

Total RNA was extracted from ca. 0.5 g of sediment from the top centimeter of one single core collected in the “10 months” anoxia chamber using the PowerSoil Total RNA Isolation Kit according to the manufacturer’s instructions (MoBio). For each sample, a 30  $\mu$ L aliquot of RNA extract was purified from co-extracted DNA by incubating 2 units of TURBO DNase enzyme (Ambion) in the presence of 3  $\mu$ L of its 10X buffer for 25 min at 37 °C. An additional incubation with another 2 units of DNase was conducted in order to guarantee the total digestion of small DNA fragments. Then, the digestion was stopped by adding 5  $\mu$ L of DNase Inactivation Reagent (Ambion) and incubating 2 min at ambient temperature. After centrifugation for 90 s at 10 000 g, the supernatant was collected and the absence of double-stranded DNA was confirmed by PCR amplification. This control PCR reaction was conducted on 1  $\mu$ L of DNase-treated RNA in a total volume of 15  $\mu$ L containing 1 units of Taq Polymerase (Roche), 1X of buffer solution (Roche), 0.2  $\mu$ M of each primer s14F3 (5′-ACGCAMGTGTGAACTTG-3′) and s17 (5′-CGGTCACGTTTCGTTGC-3′), and 0.2 mM of each dNTP. The reaction consisted of a pre-incubation at 94 °C for 90 s, followed by 25 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 45 s, 10 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, and a final incubation at 72 °C for 120 s. The amplification results are presented in the Supplement, Fig. 1. The complementary strands of ca. 150 ng of RNA (i.e., cDNA) were synthesized for each sample using the SuperScriptIII<sup>®</sup> Reverse Transcriptase (Invitrogen). Present in a volume of 11  $\mu$ L of RNA were pre-mixed 1  $\mu$ L of 10 mM dNTPs and 1  $\mu$ L containing 250 ng of random primers (Promega). After an incubation for 5 min at 65 °C and then for 2 min on ice, 4  $\mu$ L of 5X RT buffer (Invitrogen), 1  $\mu$ L of 100 mM dithiothreitol (DTT) and 200 U of SuperScriptIII<sup>®</sup> Reverse Transcriptase (Invitrogen) were added to the pre-mix. The reverse transcription was conducted for 5 min at 25 °C, followed by 50 min at 55 °C and 15 min at 15 °C. A control with no RNA was included and amplified as described in the next section in order to monitor the occurrence of contamination during the cDNA synthesis. The resulting cDNA were used as template to amplify, clone and sequence the foraminifera-specific hypervariable region 37f of the small subunit of the ribosomal RNA gene (SSU rDNA) as in Pawlowski et al. (2011) but using the above primers and no re-amplification. The resulting sequences were filtered for chimeras using UChime and each sequence assigned to a foraminiferal species or genus based on the taxonomically informative 37f hypervariable region (Pawlowski and Lecroq, 2010). Briefly, each of the 61 sequences was aligned to 1003 curated reference sequences of the corresponding 37f hypervariable foraminiferal region using the Needleman–Wunsch algorithm and the best match(s) under a threshold of 20 % dissimilarity was retained for assignment. In those cases where more than one reference se-

quence matched the environmental sequence at the lowest distance, the environmental sequence was assigned to the deepest consensus of taxonomy without conflict of these reference sequences, as in Lecroq et al. (2011). Pairwise global alignments between all assigned sequences were also used to form clusters or operational taxonomic units (OTUs) using the software mothur v1.28.0 (Schloss et al., 2009). A threshold of 4 % sequence dissimilarity was chosen as no further sequence clustered until a distance of 16 %. The resulting OTUs were assigned according to the consensus of the assignments for individual sequences. The analyzed sequences were submitted to GenBank under the accession numbers KF647255 to KF647315.

### 2.4 Data analysis

After the discrimination of the living individuals, they were counted and expressed as standing stock (number of living individuals per core in the 0–0.5 or in the 0–5 cm depth interval normalized for a 10 cm<sup>2</sup> surface area) and as density (number of living individuals per depth interval normalized for a 10 cm<sup>3</sup> sediment volume).

Three statistical procedures were used to identify the effect of several parameters on the foraminiferal standing stocks or density. All the procedures are linear models (Chambers and Hastie, 1992) in which the dependent variable (the response variable) is the log-transformed foraminiferal density or standing stock. The log-transformation ensures that the dependent variable shows a Gaussian distribution.

To be able to determine significant differences in the foraminiferal standing stocks between sampling times, two models test the effect of the sample ID (i.e., a qualitative expression of the sampling time) on standing stocks over two depths intervals. The first model tests the sample ID effect in the whole core (0–5 cm; Table 2, Model 1), the second only for the shallowest depth interval (0–0.5 cm; Table 2, Model 2).

To quantify the effect of anoxia on standing stocks in these two depth intervals (0–0.5 cm and 0–5 cm) we designed an analysis of covariance linear model. This model (Table 2, Model 3) tests the effect of sampling time (a quantitative expression of the duration of the experiment), depth interval (a qualitative expression of the selected depth intervals: “0–0.5 cm” and “0–5 cm”) and their interaction. This model was designed to identify whether the variation of the standing stock in function of sampling time is similar for the two depth intervals or not. Note that the sampling time has been log-transformed in order to avoid giving too much weight to the “10 months” chamber.

**Table 2.** Summary of the variables used in each of the three linear models, describing the name of the variable, its type (dependent or independent), its unit, nature (quantitative or qualitative) and the used transformation. For the quantitative variables the minimum and maximum values of the nontransformed data are presented in brackets, whereas for the qualitative variables the different categories are presented in brackets.

Name	Variable Type	Unit	Variable nature (values)	Transformation
Model 1: Analysis of variance				
Standing Stock (0–5 cm)	Dependent	Ind./10 cm <sup>2</sup>	Quantitative (min = 533; max = 1979)	log (1 + Standing Stock)
Sample ID	Independent	–	Qualitative (“normoxia”, “9 days”, “1 month”, “2 months” and “10 months”)	–
Model 2: Analysis of variance				
Standing Stock (0–0.5 cm)	Dependent	Ind./10 cm <sup>2</sup>	Quantitative (min = 115; max = 529)	log (1 + Standing Stock)
Sample ID	Independent	–	Qualitative (“normoxia”, “9 days”, “1 month”, “2 months” and “10 months”)	–
Model 3: Analysis of covariance				
Standing Stock	Dependent	Ind./10 cm <sup>2</sup>	Quantitative (min = 115; max = 1979)	log (1 + Standing Stock)
Depth Interval	Independent	–	Qualitative (“0–5 cm” and “0–0.5 cm”)	–
Time	Independent	days	Quantitative (min = 0; max = 315)	log (5 + Time)
Depth Interval × Time	Independent	–	Interaction	–
Model 4: Analysis of covariance				
Density	Dependent	Ind./10 cm <sup>3</sup>	Quantitative (min = 46; max = 1059)	log (1 + Density)
Depth	Independent	cm	Quantitative (min = 0.25; max = 4.5)	log (1 + Depth)
Depth <sup>2</sup>	Independent	cm	Quantitative (min = 0.25; max = 4.5)	(log (1 + Depth)) <sup>2</sup>
Sample ID	Independent	–	Qualitative (“normoxia”, “9 days”, “1 month”, “2 months” and “10 months”)	–
Depth × Depth <sup>2</sup>	Independent	–	Interaction	–
Depth × Sample ID	Independent	–	Interaction	–
Depth <sup>2</sup> × Sample ID	Independent	–	Interaction	–

**Table 3.** Total counts and standing stocks at every sampling time for every replicate core.

Sample ID	Time (days)	Replicate core	Total count (individuals)	Standing stock (indiv/10 cm <sup>2</sup> )
Normoxia	0	core1	3289	1979
Normoxia	0	core2	1368	823
9 days	9	core1	1422	856
9 days	9	core2	1241	747
1 month	29	core1	1834	1104
1 month	29	core2	1291	777
2 months	58	core1	1263	760
2 months	58	core2	1274	767
10 months	315	core1	886	533
10 months	315	core2	1131	681

Finally, to quantify the effect of the anoxia and of sediment depth on the foraminiferal density, we designed an analysis of covariance linear model. The first computed model (Table 2, Model 4) tests the effect of the sample ID, the sediment depth (i.e., here a quantitative expression of the depth interval, the value used being the midpoint of the depth intervals) and their first-order interaction with foraminiferal density. Note that to increase the quality of the fit, sediment depth has been log-transformed and has been introduced with a

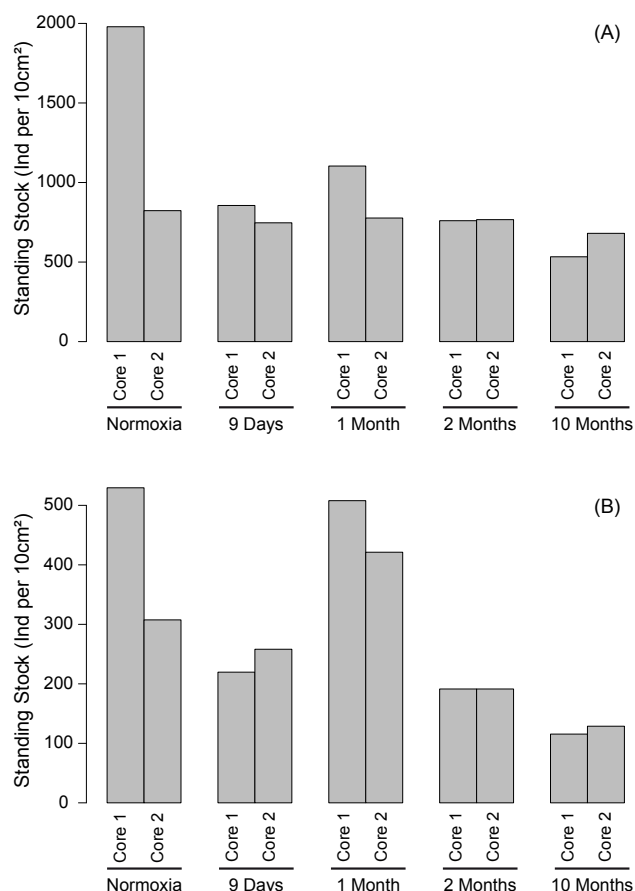
polynomial component (the log-transformed depth has been squared). The depth times squared depth interaction and the squared depth times sample ID interaction have also been added to the model.

Table 4 shows only the tested variables that have a significant effect. Table 2 in the Supplement shows how each of the variables affects the dependent variable.

### 3 Results

#### 3.1 Foraminiferal survival under various experimental conditions

Living benthic foraminifera (i.e., positively labeled with CTG) were present from the beginning of the experiment to the end, after about 10 months of anoxia. Relatively large standing stocks were recorded from the surface down to 5 cm depth (Fig. 1, Table 3). In the 10 studied cores, the total number of individuals in the top 5 cm varied from ~1980 individuals per 10 cm<sup>2</sup> in “normoxia” core 1 to ~530 individuals per 10 cm<sup>2</sup> in the “10 months” core 1. Over all sampling times, the living assemblages were dominated by *Reophax nanus*, *Eggerella scabra* and *Bulimina aculeata*, whereby *Textularia agglutinans*, *Quinqueloculina seminula*,

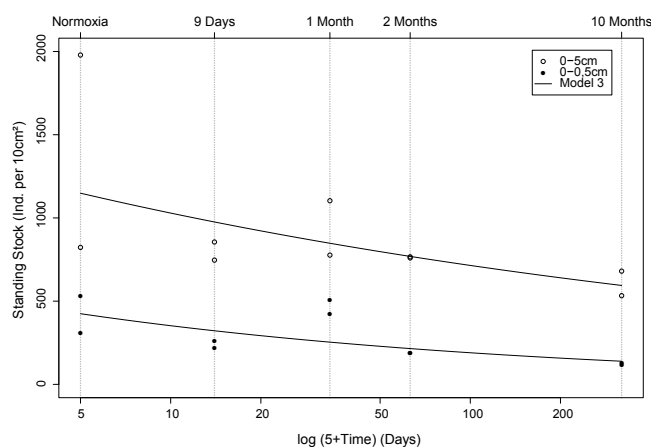


**Fig. 1.** Foraminiferal standing stocks in all sampled cores for the whole cores (0–5 cm, panel A) and for the 0–0.5 cm depth intervals (panel B).

*Lagenammia atlantica*, *Hopkinsinella glabra*, *Bolivina pseudoplicata* and *Quinqueloculina stelligera* were other conspicuous faunal elements. Census data are presented in the Supplement, Table 1; a more complete description and analysis of the faunal composition is presented in Langlet et al. (2013).

### 3.2 Density variation with time

Figure 1 shows that living benthic foraminifera (i.e., CTG-stained) were present at every sampling time. The standing stocks in the whole cores (0–5 cm depth interval; Fig. 1, Panel A) varied only slightly with incubation time. Although a clear maximum was found in “normoxia” core 1, no significant differences existed between the pairs of replicate cores of the five sampling times (Table 4, Model 1). When only the 0–0.5 cm depth interval is considered, the highest standing stocks occurred in “normoxia” core 1 and very similar values were found in both “1 month” cores, whereas the values were lowest ( $\sim 30$  individuals per  $10\text{ cm}^3$ ) in the two “10 months” cores.



**Fig. 2.** Foraminiferal standing stocks plotted against deployment time. Open circles represent standing stock in the 0–5 cm depth interval, filled black circles correspond to the standing stock in 0–0.5 cm depth. Solid black lines represent the modeled standing stocks plotted against time, for the two depth intervals, after Model 3. Note that incubation time is represented on a transformed log scale ( $5 + \text{time}$ ).

Model 2 (Table 4) was used to test whether the foraminiferal densities in the 0–0.5 cm depth interval (Fig. 3) were significantly different at different sampling times. The test shows that the standing stocks of the “normoxia” and the “1 month” cores are not significantly different, but both are significantly higher than the 0–0.5 cm densities of the “9 days” and “2 months” cores. The latter cores are not significantly different from each other (Table 4, Model 2 and Supplement Table 2). Finally, the standing stocks of the 0–0.5 cm layer of the “10 months” cores are significantly lower than those observed in all other cores (Table 4).

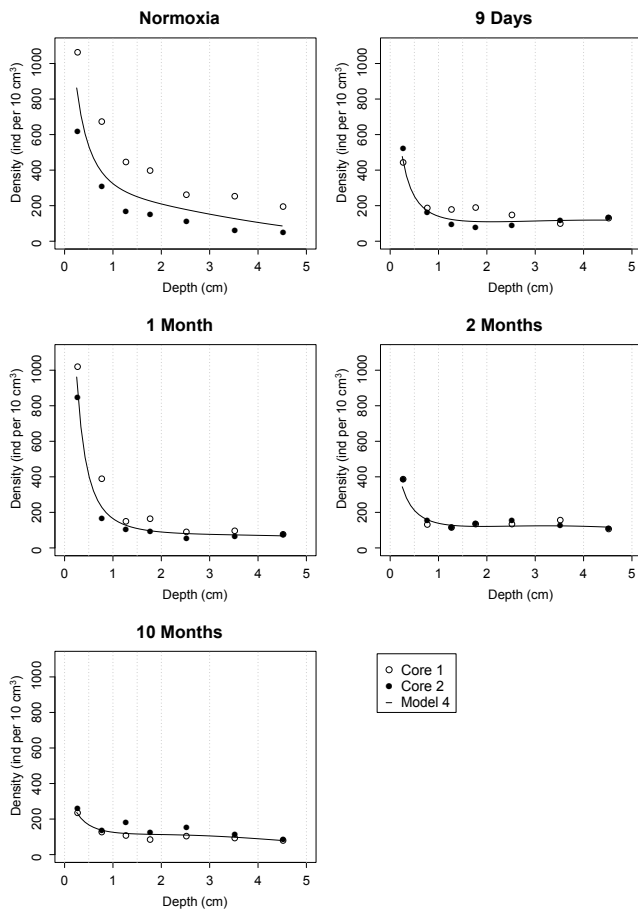
The analysis of covariance reveals that the total standing stocks showed a significant exponential decrease both for the total cores (0–5 cm) and for the 0–0.5 cm level in function of log-transformed experimental time (Fig. 2; Table 4, Model 3). The density was significantly lower in the 0–0.5 cm depth interval than in the whole core (Table 4, Model 3), indicating that a significant part of the living fauna was present in deeper sediment levels.

The slopes of the regression curves between the 0–5 cm and 0–0.5 cm intervals were not significantly different (Table 4, Model 3). This means that the density decrease over time was not significantly different between the whole core (0–5 cm) and the topmost sediment (0–0.5 cm).

### 3.3 Density variation with sediment depth

Figure 3 shows that foraminiferal density decreased strongly with sediment depth in the five pairs of replicate cores. In fact, all cores exhibited a significant exponential decrease of density in function of log-transformed sediment depth (Table 4, Model 4). Also the sample ID (i.e., a qualitative



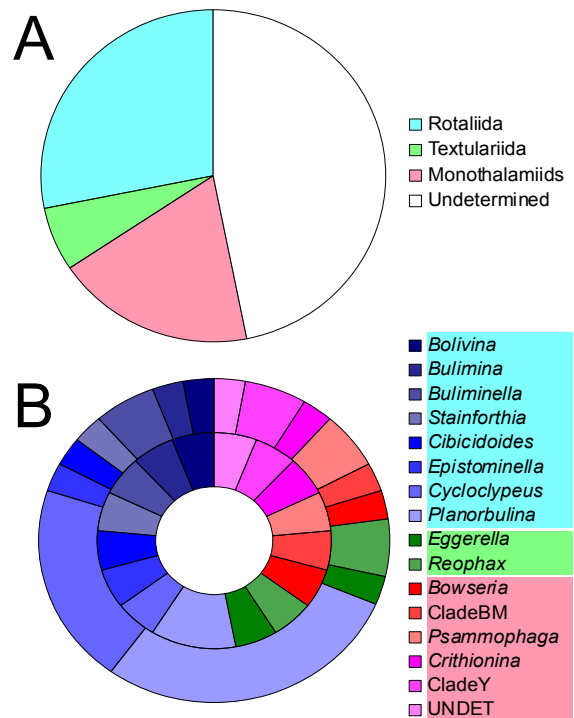


**Fig. 3.** Foraminiferal density plotted against sediment depth for all sampled cores. Open circles represent the density in replicate core 1, filled black circles that in core 2. Solid black line represents the modeled density according to model 4.

expression of time) effect is significant (Table 4, Model 4). When the various depth levels are considered individually, the densities in the “10 months” cores were significantly lower than those of the other four pairs of replicate cores (Table 4, Model 4 and Supplement Table 2). Additionally, the slopes of the density decrease with depth were not significantly different except for the one observed for the “1 month” cores, which is significantly steeper, mainly due to the very high density in the topmost level and the very strong decrease in the 0.5–1 cm level.

### 3.4 Ribosomal RNA analysis

A total of 61 filtered foraminiferal SSU rDNA sequences were obtained from the metatranscriptomic sediment extract of the “10 months” anoxic chamber. After clustering, 32 OTUs were obtained, that included 15 unassigned OTUs (Fig. 4a). The remaining OTU were assigned to the calcareous order Rotaliida (9 OTUs), the agglutinated Textulariida (2 OTUs) and to the soft-walled monothalamiids (6 OTUs).



**Fig. 4.** Taxonomic composition of the foraminiferal ribosomal RNA OTUs (operational taxonomy units) clustered at 96 % sequence identity from the top centimeter of the sediment in the “10 months” chamber. **(A)** Relative proportions of the 32 OTUs identified to the order level or left unassigned after taxonomic assignment based on Needleman-Wunsch global alignments of the 37f hypervariable region as in Lejzerowicz et al. (2013). **(B)** Relative proportion of OTUs (inner circle) and sequences (outer circle) assigned to the genus level.

One OTU represented by one sequence perfectly matched the reference sequence of a specimen that was morphologically determined as *Eggerella scabra* and collected in the same area (Fig. 4b).

## 4 Discussion

### 4.1 Pore water geochemistry and macrofaunal behavior during the experiment

Parallel to the meiofaunal and macrofaunal analyses, sediment geochemical analyses were conducted to control whether the experimental conditions indeed led to anoxia in both the overlying water and in the pore water (Koron et al., 2013; Metzger et al., 2013). This paragraph summarizes the findings relevant to our study; for more details about the data and methodology, refer to these papers. Metzger et al. (2013) show that the oxygen concentration in the waters overlying the sediment (measured by two oxygen microsensors placed a few millimeters above the sediment–water interface in the “9 days” chamber) started to decrease as soon as the chamber



**Table 4.** Results of the models 1, 2, 3 and 4. For each model, the degrees of freedom (Df), the sum and mean of squares (SumSq. and MeanSq.), the  $F$  value ( $F$ ) and its  $p$  value ( $p$ ) are given for each variable. The  $p$  values indicate whether the variable has a significant effect ( $p < 0.05$ ) or not ( $p > 0.05$ ).

	Df	SumSq.	MeanSq.	$F$	$p$
Model 1					
Sample ID	4	0.6	0.2	1.6	0.31
Residuals	5	0.5	0.1		
Model 2					
Sample ID	4	2.4	0.6	16.2	0.005
Residuals	5	0.2	0.0		
Model 3					
log(5+Time)	1	1.8	1.8	16.3	0.001
Depth Interval	1	7.3	7.3	66.4	< 10 <sup>3</sup>
log(5+Time) × Depth Interval	1	0.1	0.1	1.1	0.32
Residuals	16	1.7	0.1		
Model 4					
Sample ID	4	3.5	0.9	7.1	< 10 <sup>3</sup>
log(1+Depth)	1	16.7	16.7	134.6	< 10 <sup>3</sup>
log(1+Depth) <sup>2</sup>	1	2.7	2.7	22.0	< 10 <sup>3</sup>
Sample ID × log(1+Depth)	4	3.1	0.8	6.3	< 10 <sup>3</sup>
log(1+Depth) × log(1+Depth) <sup>2</sup>	1	0.5	0.5	4.1	0.048
Sample ID × log(1+Depth) <sup>2</sup>	4	1.0	0.3	2.0	0.10
Residuals	54	6.7	0.1		

was closed. The consumption rate was  $950 \text{ nL cm}^{-2} \text{ h}^{-1}$  (i.e.,  $0.0115 \text{ nmol cm}^{-2} \text{ s}^{-1}$  or  $9.93 \text{ mmol m}^{-2} \text{ day}^{-1}$ ). Microsensor data show that hypoxia was reached after two to six days and anoxia after seven days. The overlying water remained anoxic until the end of the deployment (nine days). To estimate the contribution of the foraminiferal assemblages to the oxic respiration in our benthic chambers, we compared the oxygen consumption rate with the total foraminiferal respiration estimated using the power relation between foraminiferal biovolume and respiration rate (Geslin et al., 2011). The application of this relation, taking into account the foraminiferal densities in the oxygenated sediment layer (the 0–0.5 cm interval of the “normoxia” cores) in four distinct size classes (63–125, 125–150, 150–315 and > 315  $\mu\text{m}$ ) yields a total foraminiferal respiration rate of  $4.57 \text{ nL cm}^{-2} \text{ h}^{-1}$ . In comparison, the value is  $950 \text{ nL cm}^{-2} \text{ h}^{-1}$  for total benthic respiration (Metzger et al., 2013). Accordingly, foraminifera are responsible for about 0.5 % of the total benthic respiration in the chamber. These values are similar to those estimated for assemblages inhabiting the Rhone prodelta (0.6 and 1.2 % at 37 and 60 m depth, respectively; Geslin et al., 2011; Goineau et al., 2011). Nevertheless, as these estimations are based on the standing stock of foraminifera larger than 63  $\mu\text{m}$ , and thus neglect all the smaller individuals, these values probably underestimate the respiration rates of the whole foraminiferal community.

At the beginning of each experiment, 2 to 3 brittle stars (*Ophiothrix quinquemaculata*) were placed into each chamber, which otherwise contained no visible macroepifauna. *Ophiothrix* shows typical behavioral reactions to decreasing oxygen concentrations, such as arm tipping at the transition from normoxia to hypoxia, probably to raise the position of the respiratory organs in the water column (see Riedel et al., 2008, 2012, 2013). This macrofaunal behavior (including emerging infaunal species) was used as an additional in situ indicator for the onset of hypoxia in each chamber. The brittle stars and the other macrofaunal organisms died after 7 to 15 days of deployment. Shortly after the brittle stars died, the normally greyish-brown sediment turned black a few centimeters around the dead organisms. About 2 days later (i.e., after 9 to 17 days of deployment), the whole surface of the sediment in the chambers was completely black.

After this 9-day period, direct oxygen measurements were no longer technically possible (due to the limited battery capacity of the data-logging system), but other chemical indicators indicate that reductive conditions occurred and remained present until the opening of the “10 months” chamber. Upward shifts of the manganese, iron and sulfate reduction zones were observed during the experiment, confirming a shift from oxic to anoxic conditions at the sediment–water interface as well as in the overlying water and in the sediment column (Metzger et al., 2013). Whereas this change seems to

be progressive in the sediment column, the chemical composition of the overlying waters shows a more complex pattern. The “1 month” probes showed a considerable increase of free sulfide hydrogen above the sediment–water interface. No such intensive sulfide production was observed in the “2 months” and “10 months” samples. This particular pattern caused an inverse sulfide gradient and consequently a downward sulfide flux into the sediment. In the “1 month” sample, sulfide was still detected 2 cm below the interface. These observations have been interpreted to reflect the degradation of the dead macrofaunal remains concentrated at the sediment surface. Previous studies at the same site, using the same experimental protocol, have shown that the macrofaunal organisms suffer massive mortality during the first week of anoxia (Riedel et al., 2008, 2012). The geochemical results (Metzger et al., 2013) and especially the decrease in biogeochemical activity suggest that the abundant labile organic matter resulting from macrofaunal mortality (the already present infaunal organisms and the introduced brittle stars) is consumed in the first month(s) of the experiment. Finally, the geochemical analyses also indicate that nitrates were present both in the overlying water and in the pore waters at all times, without any major changes in vertical distribution (Koron et al., 2013).

To summarize, the pore water chemistry indicates that the oxygen concentration decreased during the first days of the experiment. Anoxia was reached after 7 days, and was maintained until the end of the experiment after 315 days. Sediment geochemistry also suggests that a nonnegligible amount of fresh organic matter was added to the system due to the mortality of the macrofauna. This newly available labile organic matter was consumed in the first month(s) of the experiment.

#### 4.2 Methodological strategy of the study

The present study is original for combining an in situ experiment with the use of the very accurate CTG labeling method (instead of the traditional rose bengal staining method) and for the very long duration of the anoxia.

We believe our experimental approach to have three advantages compared to laboratory experiments. The first advantage is that it circumvents undesirable laboratory effects, such as stress from transport and/or culture maintenance, reproduction after incubation (e.g., Geslin et al., 2014; Ernst et al., 2006; Murray, 2006), and slightly differing environmental conditions (temperature, light, salinity, etc.). In comparison to nonexperimental field studies, our experimental setup allowed us also to prolong the anoxic period and to avoid the effect of seasonal variation of food supplies. Conversely, our closed setup blocked food supply from the outside, and toxic components such as sulfides are not dispersed in the water column. Moreover, deployment by scuba divers to 24 m depth limits the available manpower and manipulation time precluding sampling every month and obtaining a

higher temporal resolution. Finally, our cores were taken a few decimeters from each other, potentially resulting in important variation related to spatial patchiness, especially in the distribution of macrofaunal organisms and burrows, and the related biogeochemical processes. Laboratory studies can avoid the effects of patchiness by sieving and homogenizing the sediment, which could in turn affect the results pertaining to more sensitive species (Langezaal et al., 2004). Nonetheless, we think that the advantages of our in situ approach outweigh the disadvantages.

A second advantage of our approach is avoiding the use of rose bengal, a bulk stain that adheres to the proteins in the foraminiferal protoplasm. Since the protoplasm can be preserved weeks to months after death (Boltovskoy and Lena, 1970; Bernhard, 1988; Corliss and Emerson, 1990), faunal inventories based on rose bengal can substantially overestimate foraminiferal densities, especially in anoxic conditions, where organic matter degradation will be slow (Hannah and Rogerson, 1997; Glud, 2008). It can also yield false positives in experiments in anoxic conditions, calling for caution in interpreting the results of many earlier studies (e.g., Moodley et al., 1997; Heinz et al., 2001; Duijnsteet et al., 2003). Our goal of following the faunal variation on a weekly to monthly scale required using a more accurate vitality discrimination technique. CTG is a fluorescent probe that is hydrolyzed within any cytoplasm by foraminiferal or bacterial (or any) metabolism. Since such enzymatic activity should stop after the death of the organism, the CTG method should very accurately determine living specimens (Bernhard et al., 2006). This assumption has been confirmed during a comparative study on copepods and nematodes (Grego et al., 2013) using the same experimental design as our study; these authors found that the two methods yielded similar estimations of copepod density but that rose bengal yielded higher densities of nematodes in well-oxygenated conditions. After seven days of anoxia, the rose bengal method overestimated (compared to CTG) the density of living nematodes and copepods by about 30 and 50 %, respectively (Grego et al., 2013). Accordingly, especially in anoxic conditions and in the case of a high temporal resolution, the CTG method produces considerably more reliable results. Nevertheless, recognizing pink tests using rose bengal and fluorescent tests using CTG both have a subjective component and call for observer expertise. Several parameters can affect the observer's judgment: the various test types (hyaline, porcelaneous, agglutinated) influence the way the fluorescence of the cytoplasm is seen through the test, the presence of other fluorescent organic material close to the object (e.g., nematodes and copepods are more fluorescent than foraminifera) can “hide” the foraminiferal fluorescence (we removed these organisms from our samples by centrifugation to avoid such a negative effect), the natural fluorescence of the sediment, and the interspecific and interindividual variability in the fluorescence intensity. Finally, prior to the use of CTG, several tests were performed to calibrate the identification of living

individuals. To do so, active (moving), nonactive (nonmoving) and clearly dead individuals were incubated in CTG. This calibration tests shows clear differences in the fluorescence intensity and patterns. It has been previously evidenced that foraminiferal cytoplasm can be (partially) overtaken by bacteria and still positively stain with rose bengal (Bernhard et al., 2010). Such a possibility cannot be entirely excluded either with the CTG method, since living bacteria also react positively to CTG. In our material, for some specimens the fluorescence intensity was rather low, and appeared patchy and “milky”. We assume that such a patchy/milky fluorescence could be due to the presence of bacteria, and did not count such individuals as living. Fortunately, this type of fluorescence can be easily distinguished from actual foraminiferal fluorescence. Because of our very strict selection of fluorescent individuals (with a clear and bright fluorescence), we are confident that the large majority of the inventoried fluorescent individuals were alive at the time of sampling.

It is possible that centrifugation in the Levasyl solution at 300 rpm may have destroyed some fragile foraminiferal specimens. However, in spite of this treatment, fragile species such as *Leptohalysis scottii* or *Reophax nanus* were found in relatively large numbers in the centrifuged samples, indicating that these species can resist such a treatment. The most problematic part of the centrifugation protocol is probably the potentially low extraction efficiency for foraminifera estimated at 88% (Burgess, 2001). The application of our protocol could therefore lead to an underestimation of the foraminiferal standing stock in the 63–125  $\mu\text{m}$  fraction.

The third advantage of the experimental setup is the long-term duration of the chamber deployment. While previous anoxia incubation experiments on foraminifera lasted up to 3 months, we extended this period to more than 10 months. Although it cannot be excluded that deeper-burrowing macrofauna may eventually find their way into the chambers, our geochemical analyses indicate that the conditions were strongly reductive and anoxic at the end of the evaluated deployment.

### 4.3 Foraminiferal survival under the experimental conditions

#### 4.3.1 Foraminiferal survival under anoxia in the presence of hydrogen sulfides

Abundant CTG-labeled specimens were found in all cores. Thus, either many foraminifera survived from 9 days to more than 10 months of anoxia, in all sediment layers down to 5 cm depth, or offspring were produced during that time period. To confirm that foraminifera were indeed alive after almost one year of anoxia, a complementary foraminiferal ribosomal RNA analysis was carried out. Ribosomal RNA marker sequences were successfully recovered from total sediment RNA extractions and assigned to diverse foraminifera. As in

a recent study of deep-sea foraminifera realized at a comparably low sequencing effort, sequences originating from the RNA material corresponded to the species that were also found stained with rose bengal (Lejzerowicz et al., 2013). Sequences recovered in anoxic conditions in the present study were assigned to several groups of foraminifera (Rotaliida, Textulariida and monothalamiids), including to the textulariid *Eggerella scabra*, which was shown to survive prolonged anoxia (Langlet et al., 2013). This foraminiferal diversity was reported based on the RNA material found from the first centimeter of sediments collected after 10 months of anoxia, i.e., from sediment exposed to prolonged anoxia. RNA molecules are short lived, although one study reported the existence of extracellular RNA molecules particularly well preserved after 2.7 million years in subsurface sediments (Orsi et al., 2013). Nevertheless, the use of RNA as a proxy for describing active species has already proved successful in numerous studies of modern micro-eukaryotic communities (Sørensen and Teske, 2006; Stoeck et al., 2007; Alexander et al., 2009; Frias-Lopez et al., 2009; Takishita et al., 2010; Logares et al., 2012; Stock et al., 2012), although the differences in the activity levels of identified species cannot be inferred from sequence data (Logares et al., 2012). Ribosomal RNA marker sequences were assigned to species also revealed by CTG (Langlet et al., 2013), leaving few doubts that these species were alive and metabolically active after 308 days of anoxia (315 days of experiment – 7 days of oxic and hypoxic conditions). Our molecular results further support the accuracy of the CTG method for detecting living foraminifera.

Notwithstanding the caveats of the rose bengal approach, available data strongly suggest that foraminifera are tolerant to anoxia (Bernhard and Alve, 1996; Moodley et al., 1997; Leiter and Altenbach, 2010; Piña-Ochoa et al., 2010b). It is nonetheless surprising that benthic foraminifera are still alive in considerable numbers after 308 days of anoxia ( $\sim 530$  and  $\sim 680$  individuals per  $10\text{ cm}^2$  down to 5 cm in the two replicate cores). We assert that we have presented the first conclusive evidence that such a long-term foraminiferal survival in anoxic conditions could occur.

With the development of anoxia, hydrogen sulfides were produced. The concentration has been roughly estimated at  $> 100\ \mu\text{M}$  in the overlying water of the “1 month” chamber (Metzger et al., 2013). Despite the potential toxicity (Giere, 1993; Fenchel and Finlay, 1995), the foraminiferal faunas survived this strong exposure.

#### 4.3.2 Implications of the experiment for our understanding of foraminiferal metabolism

We show that foraminifera remain alive in anoxic conditions, despite the occurrence of sulfides, for at least 308 days. Prior to this study, foraminifera were known to survive relatively short periods in hypoxic and anoxic conditions (Alve and Bernhard, 1995; Piña-Ochoa et al., 2010b)

and rose-bengal-stained organisms were found under constant anoxic conditions (Leiter and Altenbach, 2010). Several experimental and ultrastructural observations reviewed by Bernhard (1996) and Bernhard and Sen Gupta (1999) identified the sequestration of chloroplasts, encystment, dormancy, aggregation of endoplasmic reticulum and peroxisomes or the presence of symbiotic bacteria as potential mechanisms to survive anoxia. Several authors showed that certain taxa can respire nitrates in anoxic conditions (Risgaard-Petersen et al., 2006; Høglund et al., 2008; Piña-Ochoa et al., 2010a). 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 by symbiotic bacteria, such as has been shown for allogromiid foraminifera (Bernhard et al., 2011). Based on denitrification budget calculations, Risgaard-Petersen et al. (2006) estimated that the most efficient species could survive anoxia for two to three months by using their intracellular nitrate stock. In our experiment, however, all species, including some that do not store nitrate in large quantities (*Bulimina aculeata* – see Piña-Ochoa et al., 2010a, or *Eggerella scabra* – see Langlet et al., 2013), survived almost one year of anoxia. Two explanations can be proposed to explain this discrepancy: some species may indeed denitrify and continuously renew their nitrate stocks (nitrates are always available in pore and bottom waters; Koron et al., 2013). Others may shift to other survival strategies, such as drastically decreasing their metabolic rates, or use as yet undescribed metabolic pathways.

#### 4.4 Variations in foraminiferal densities

Overall densities significantly decreased with time (Table 4, Model 2), but the values after one month are somewhat higher than after nine days. This difference is much clearer in the values from the topmost 0.5 cm than from the whole core. In fact, the temporal density variation does not follow a gradual decrease. In the 0–0.5 cm depth interval, the standing stocks in the “normoxia” and the “1 month” chambers are both significantly higher than those in the “9 days” and “2 months” chambers. The cores of the latter two (which are not significantly different) both have significantly higher standing stocks in the 0–0.5 cm interval than the “10 months” cores (Table 4, Model 3). We hypothesize that these density variations are explained either by spatial variability, the effect of the anoxia or by labile organic matter availability. These three hypotheses are discussed below.

##### 4.4.1 Spatial heterogeneity versus temporal variability

Since the living fauna has been analyzed in two replicate cores, we can to some extent assess the spatial variability of the meiofauna at a decimetric spatial scale. The two replicate cores that differ most are the “normoxia” cores sampled before the beginning of the experiment, with total (0–5 cm)

standing stocks of  $\sim 1980$  and  $\sim 820$  individuals per  $10\text{ cm}^2$ , respectively. This difference is not due to a different vertical distribution, which is fairly similar (Fig. 3): the density differs at all depths. Considerable patchiness at this site is the logical explanation. The substrate here is a poorly sorted silty sand, which is colonized by very patchy macroepibenthic assemblages, defined as multi-species clumps or bioherms (Fedra et al., 1976; Stachowitsch, 1984, 1991). Also the sediment geochemistry shows a nonnegligible variability in the intensity and depth of the major diagenetic reactions (Metzger et al., 2013). We expect that the areas devoid of visible macroepifauna (such as those selected for this experiment) also show important spatial patchiness. Burrowing activities of infaunal macrofauna, for example, may have affected the homogeneity of the sediment column. The presence of burrows generally leads to a deeper oxygen penetration depth (Aller, 1988), which can explain the higher infaunal foraminiferal standing stocks (e.g., Jorissen, 1999; Loubere et al., 2011; Phipps et al., 2012).

For comparison, at a 14.5 m-deep site located in the northern Gulf of Trieste, Hohenegger et al. (1993) compared the faunal composition (rose bengal method) of 16 sediment cores (4.8 cm inner diameter, as in our study) sampled in a single  $1\text{ m}^2$  surface. Foraminiferal standing stocks varied from  $\sim 2450$  to  $\sim 6300$  individuals per  $100\text{ cm}^2$ , with an average of  $\sim 3500$  individuals per  $100\text{ cm}^2$  (Hohenegger et al., 1993). The authors explained this by the influence of burrows and specific food requirements. The ratio of about 2.6 between their richest and poorest cores is very similar to the maximum ratio of 2.4 found for our replicate cores at the beginning of the experiment. Such a difference may be typical for shallow Gulf of Trieste sites. A number of long-term (weeks to months) in situ observations clearly show strong changes in foraminiferal standing stocks in coastal environments (e.g., Murray, 1983; Horton and Murray, 2007). In the northern Adriatic Sea, between 30 and 60 m depth, Barawidjaja et al. (1992) and Duijnsteet et al. (2004) described substantial seasonal variability, probably in response to seasonal primary production changes, organic carbon fluxes to the seafloor, temperature and/or salinity. It is very unlikely that such changes (except perhaps temperature) have had an impact the foraminiferal faunas in our closed benthic chambers. The observed temporal changes are therefore rather due to the effect of the experimental conditions than to natural environmental changes concerning the whole northern Adriatic.

The spatial heterogeneity at our study site precludes definitively interpreting the differences in foraminiferal densities between the different chambers (and different sampling times) as being entirely the consequence of the experimental conditions. Nevertheless, all statistical tests indicate that the intra-chamber effect is less important than the other tested variables (time, sample ID or depth). Accordingly, experimental conditions appear to have a stronger impact on

density differences between pairs of replicate cores than spatial patchiness.

#### 4.4.2 Effect of anoxia and hydrogen sulfides on the foraminiferal faunas

The foraminiferal standing stocks exhibit a significant exponential decrease with the log-transformed experiment time (Fig. 2). In detail, the densities do not decrease gradually with time, the highest densities occur in the initial conditions and after one month of anoxia, and the values are lower in the “9 days” and “2 months” cores, with the lowest densities in the “10 months” anoxia samples. If the observed density changes in the 0–0.5 cm interval are not entirely due to spatial patchiness, standing stocks could be negatively affected by the development of anoxia in the first week (Fig. 1). However, in an earlier experiment using the same protocol (in situ incubation, CTG probe), performed in 2009 at the same site, no significant difference in foraminiferal density was observed between a reference core (normoxic conditions) and a core sampled after seven days of anoxia (Geslin, unpublished data). Also, a previous experiment based on rose-bengal-stained organisms using Adriatic Sea faunas did not show a significant impact of up to two months of anoxia on the hard-shelled foraminiferal density (Moodley et al., 1997). Conversely, in a study on the temporal variability of densities in the northern Adriatic Sea (close to the Po Delta), total foraminiferal standing stocks tended to decrease by roughly 20 % during periods of bottom water hypoxia in late summer (Duijnsteet et al., 2004). A previous laboratory study conducted in the northern Adriatic Sea (also using rose bengal staining) found lower standing stocks (roughly 30 % fewer individuals) in cores incubated for 1.5 months in anoxic conditions (Ernst et al., 2005). Furthermore, considering that many foraminifera (of all common species) survived more than 10 months of anoxia, the decrease in density in the first week can hardly reflect decreasing oxygen concentration, especially since the sediment surface wasoxic most of the time. This observation and the literature suggest that short-term anoxia (0–1 month) cannot explain the density drop; the observed differences reflect spatial patchiness. Nevertheless, the overall density decrease suggests that long-term anoxia (> 2 months) negatively impact on density.

In the present study, the vertical density profiles (Fig. 3) are very similar for all experimental cores and are not significantly different from the “normoxia” cores. In some other studies (Alve and Bernhard, 1995; Duijnsteet et al., 2003; Ernst et al., 2005; Pucci et al., 2009), densities between 2 and 4 cm decreased rapidly after the onset of hypoxia/anoxia. This was ascribed to upward migration. In our study, apparently no major vertical migration took place. The standing stocks in the whole cores (0–5 cm) do not differ significantly over time, unlike the standing stock in the first depth interval (0–0.5 cm). This difference may reflect biogeochemical changes (Metzger et al., 2013): they were much more im-

portant in the uppermost sediment interval (which became anoxic, where macrofaunal mortality and degradation resulted in hydrogen sulfides release) than in deeper sediment intervals, which were already anoxic at the start of the experiment (oxygen penetration about 0.5 cm).

Benthic foraminifera were still alive in large numbers in the “1 month” cores, for which the estimated hydrogen sulfide concentration in the water overlying the sediment exceeded 100  $\mu\text{M}$  (Metzger et al., 2013). The toxic nature of hydrogen sulfides for most metazoans could help explain the density decrease between the “1 month” and “2 months” cores (average values of  $\sim 930$  and  $\sim 380$  individuals per  $10\text{ cm}^3$  in the topmost 0–0.5 cm depth interval, respectively). In an earlier laboratory experiment using northern Adriatic Sea sediments, Moodley et al. (1998) found that a 6–2  $\mu\text{M}$  concentration of dissolved sulfides led to a significant decrease of the rose-bengal-stained foraminiferal densities, from about 700 to 200 individuals per  $10\text{ cm}^3$  after 30 days of incubation, corresponding to about 4 % per day. In our study the observed average decrease between 29 and 58 days from  $\sim 940$  to  $\sim 760$  individuals per  $10\text{ cm}^3$  in the 0–5 cm interval, or from 460 to 200 individuals per  $10\text{ cm}^3$  in the 0–0.5 cm layer, corresponds to a loss of 0.75 and 1.3 % per day, respectively. These values are in the same order of magnitude as those obtained by Moodley et al. (1998). Thus, despite of the high presence of toxic, toxic hydrogen sulfides concentrations (Giere, 1993; Fenchel and Finlay, 1995), a substantial part of the foraminifera survived up to 308 days of anoxic and sulfidic experimental conditions. The higher mortalities in other studies (Moodley et al., 1988) may be due to the presence of additional stress factors in their experimental setups.

#### 4.4.3 Potential response to labile organic matter availability

In the present experimental setup, the availability of labile organic matter is another factor that could limit survival: the sealed benthic chamber blocks the sediment organic matter input by the pelagic system. This should considerably reduce labile organic matter availability and potentially starve the benthic faunas. Unfortunately no complete (and conclusive) organic carbon data set is available to confirm such a trend. Nevertheless, the geochemical analyses indirectly show that organic matter availability varied during the experiment. A large input of hydrogen sulfides in the overlying water took place after one month of anoxia. Usually sulfides are produced deep in the sediment and diffuse upwards. Conversely, after one month of anoxia, the source of the sulfide pool was positioned at the sediment–water interface (Metzger et al., 2013). This shallow production of sulfides has been interpreted as due to the death of macrofaunal organisms (the already present macroinfaunal organisms as well as the two introduced brittle stars) in the early stages of the experiment and their subsequent anaerobic degradation.

Unexpectedly, the geochemical data suggest that the organic matter content at the sediment–water interface did not decrease with time (due to the sealing of the chamber), but rather increased, peaking after one month, due to macrofaunal decay. The coincidence between the periods with maximum foraminiferal standing stocks and maximum estimated organic matter availability (at “normoxia” and “1 month”) suggests a causal relationship between the two parameters. Numerous previous studies show a clear foraminiferal response to organic matter input (e.g. Heinz et al., 2001; Ernst and van der Zwaan, 2004; Duijnsteet et al., 2005; Ernst et al., 2005; Nomaki et al., 2005; Pucci et al., 2009). Accordingly, increased labile organic matter (macrofaunal remains) could promote foraminiferal densities in the topmost sediment layer, despite the anoxic conditions. Equally, the considerable standing stock of benthic foraminifera in the “1 month” samples could be the result of a reproduction event. This possibility is discussed in more detail for individual species in Langlet et al. (2013). Consequently, organic matter availability would have a stronger effect on foraminiferal density than both anoxia and hydrogen sulfides.

## 5 Conclusions

We performed an in situ experiment at a 24 m-deep site in the Gulf of Trieste. Benthic chambers enclosing abundant benthic foraminiferal faunas were installed on the sediment surface, which rapidly turned anoxic. Assuming the two independent methods employed here (CTG labeling and ribosomal RNA analysis) are accurate, cores sampled in chambers opened after 1 week, 1 month, 2 months and 308 days all contained abundant foraminiferal assemblages down to 5 cm depth in the sediment. Benthic foraminifera in the Gulf of Trieste are therefore capable of surviving anoxia with co-occurring sulfides for at least 10 months. Large differences between some of the replicate cores point to considerable spatial patchiness, which may be related to the irregular distribution of macroinfaunal burrowing activity, somewhat hampering the interpretation of the temporal trends in density. Nonetheless, our data show an exponential decrease in densities over time. Closer examination suggests slightly increased densities in the topmost 0.5 cm after one month, which we tentatively interpret as a response to increased labile organic matter availability due to macrofaunal mortality at the beginning of the experiment.

**Supplementary material related to this article is available online at <http://www.biogeosciences.net/10/7463/2013/bg-10-7463-2013-supplement.zip>.**

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