

# Survival and life strategy of the foraminiferan *Globobulimina turgida* through nitrate storage and denitrification

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**ABSTRACT:** In a laboratory experiment, we examined the prolonged survival and behaviour of the benthic foraminiferan *Globobulimina turgida* under 3 simulated natural conditions: oxygenated with added nitrate, anoxic with added nitrate, and anoxic. The survival rates, adenosine triphosphate (ATP) reserve and intracellular nitrate pool of *G. turgida* were measured periodically under these conditions. Furthermore, to evaluate the efficiency and energy yield of the respiration system, denitrification rates of individual specimens were quantified using the acetylene inhibition and N<sub>2</sub>O microsensor technique at the start of the experiment. Our results demonstrate that the long-term (56 d) survival rate (64%) and ATP concentrations of *G. turgida* were not significantly different in oxygenated and anoxic, nitrate-containing conditions (Mann-Whitney test,  $p > 0.05$ ). Thus, *G. turgida* can survive prolonged anoxia (3 mo) as long as nitrate is available to sustain its respiration. However, it remains unsure whether growth or reproduction can take place under anoxia. Short-term (21 to 35 d) survival rates were lower in nitrate-free, anoxic conditions (22% recovered alive compared to 62 to 82% in nitrate-oxic or nitrate-anoxic conditions), but foraminifera were observed to survive up to 56 d if respiring from their intra-cellular nitrate pool only. The foraminiferal nitrate pool appears very dynamic, as wide ranges of concentrations were measured in living specimens (0 to 463 mM ind.<sup>-1</sup>). We postulate that the scatter in the nitrate pool measurements highlights the ability of the foraminifera to actively collect and respire on nitrate, depending on individuals' history of exposure to oxygen and nitrate.

**KEY WORDS:** Benthic foraminifera · Denitrification · Intracellular nitrate · ATP · Anoxia

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## INTRODUCTION

Foraminifera are unicellular eukaryotic organisms that are abundantly present in the marine realm. They are amongst the most successful benthic organisms and are found in a wide range of environments from shallow brackish waters to the deepest parts of the ocean (van der Zwaan et al. 1999). Many species thrive below the oxic-anoxic interface (e.g. Jorissen et al. 1995, 2007, Fontanier et al. 2003, Koho et al. 2008a), which implies that they have either a fermentative or a respiratory metabolism based on electron acceptors other than oxygen. Recently, it has been demonstrated

that many benthic foraminiferal species appear to be facultative anaerobes, allowing them to use both oxygen and nitrate for respiration (Risgaard-Petersen et al. 2006, Høglund et al. 2008, Piña-Ochoa et al. 2010). These species seem to respire nitrate through denitrification (i.e. the dissimilatory reduction of nitrate to gaseous N). Foraminiferal denitrification is sustained through an intracellular nitrate pool (Risgaard-Petersen et al. 2006). Thus, foraminifera actively collect and store nitrate in their cell, enabling them to sustain respiratory activities even when the desired electron acceptors (oxygen, nitrate) are absent from the environment. This versatile metabolic capacity of

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these organisms may be one of the reasons for their successful colonisation of diverse marine environments. However, little is known about the metabolic activity of foraminifera in anoxic environments (Bernhard 1993, Bernhard & Alve 1996), and it remains unknown whether energy gained from nitrate respiration is enough to sustain growth, reproduction and completion of their lifecycle. Data from Bernhard & Reimers (1991) and Moodley et al. (1997, 1998) suggest that foraminifera, typically found in oxygen-depleted environments, cannot survive prolonged anoxia. Therefore, questions arise regarding foraminiferal survival mechanisms and how they can gain energy to sustain their metabolic activities under such apparently adverse conditions.

This study was designed to assess the survival and ATP activity of the denitrifying foraminiferan *Globobulimina turgida* and its use and development of the intracellular nitrate pool under various (prolonged) oxygen/nitrate conditions. Furthermore, we also addressed whether *G. turgida* survives anoxia by being dormant versus active. Throughout the experiment, the number of living specimens was monitored, and their internal nitrate pool and cellular energy (adenosine triphosphate, ATP) reserves were measured under such conditions to establish foraminiferal survival and activity. Knowledge on the respiratory strategy of individual foraminiferal species will explain the survival strategy of foraminifera occurring in seasonally or permanently oxygen-depleted environments and also add to our understanding of foraminiferal microhabitats, an important parameter in foraminiferal pollution monitoring (e.g. Irabien et al. 2008, Mojtahid et al. 2008) and in palaeoenvironmental reconstructions based on foraminiferal species distributions (e.g. van der Zwaan et al. 1999, Jorissen et al. 2007).

## MATERIALS AND METHODS

**Study site and sediment processing.** Sediment was collected from the deep basin (Alsäck, depth: 119 m; 58° 19.4' N; 11° 32.7' E) of the Gullmar Fjord, Sweden, in April 2008. The deep fjord has a relatively stable bottom water temperature (6°C) and salinity (~34; Nordberg et al. 2000). The bottom water concentrations of oxygen and nitrate were 160 µM and 15 µM, respectively. Sediment at the study site is organic rich (~3 wt%, Filipsson et al. 2004), muddy with a porosity of 0.75 to 0.85 (v/v) and molar C/N ratio of 10 (Engström et al. 2005). An Olausson-grab designed to collect intact, cubic samples of the soft bottom seafloor was used onboard RV 'Oscar von Sydow' to collect the sediment. The cubic samples were subsequently subsampled for the top 10 to 15 cm of sediment using plas-

tic tubes of various dimensions (on average Ø 6 cm). Intact cores were then transported to Utrecht University in cool boxes. In Utrecht, the cores were placed in an aquarium (10°C) that was filled with *in situ* seawater.

In April 2009, the top 2 cm of the sediment cores were sliced (at 1 cm intervals) and sieved over 150 µm mesh. The sieving was carried out using *in situ* seawater. *Globobulimina* spp. normally occur relatively deep in the sediment (e.g. Jorissen et al. 1995, Kitazato et al. 2000, Fontanier et al. 2003, Koho et al. 2008a); however, in Gullmar Fjord this species is commonly found in the upper 1.5 to 2 cm of the sediment (Risgaard-Petersen et al. 2006), most likely due to the relatively shallow redox zonation in the sediment. The sieved sediment was transported to Aarhus University in a cool box where the experiment was set up.

**Identification and collection of living *Globobulimina turgida*.** Identification of living foraminiferans was carried out using non-terminal methods. Specimens assumed to be alive in the sieve residue (bearing a good colouration, cytoplasm well-defined, sediment gathered around the aperture) were gently picked out and placed into a Petri dish containing artificial seawater (ASW, Red Sea Salt; salinity ~34 to 35) and a thin layer of fine-grained sediment (<38 µm; sediment sieved from the same experimental cores). ASW was used throughout the experiment, so nitrate concentrations could be controlled. Only the specimens that moved (within ~16 h) and thus produced a burrow or a trace on the sediment surface were considered living and used in the experiment.

**Experimental design and set-up.** Three aquaria (Fig. 1) were set up with distinctive chemical conditions, although all aquaria were kept at constant salinity (35) and temperature (15°C). Aquarium 1 (Oxic+Nitrate\_AQ) contained well oxygenated ASW amended with 50 µM NO<sub>3</sub><sup>-</sup>. High oxygen concentrations were sustained by vigorous bubbling of seawater with compressed air. The oxygen concentrations were stable throughout the experiment, measuring approximately 200 µmol. Aquarium 2 (Anoxic+ Nitrate\_AQ) contained anoxic ASW with added 50 µM NO<sub>3</sub><sup>-</sup>. Aquarium 3 (Anoxic\_AQ) contained anoxic nitrate-free ASW. The low oxygen conditions in Anoxic+ Nitrate\_AQ and Anoxic\_AQ were maintained by a steady introduction of N<sub>2</sub> gas mixed with 0.04 % CO<sub>2</sub> (to keep the pH stable) into the overlying water column. Oxygen concentrations were monitored with permanently installed Clark-type oxygen microelectrodes with a tip diameter of 125 µm (Revsbech 1989). The oxygen concentrations in both anoxic aquariums remained below the detection limit (0.5 µM) of the sensor throughout the incubation period. The sensors were 2-point calibrated in air-saturated ASW (salinity 35,

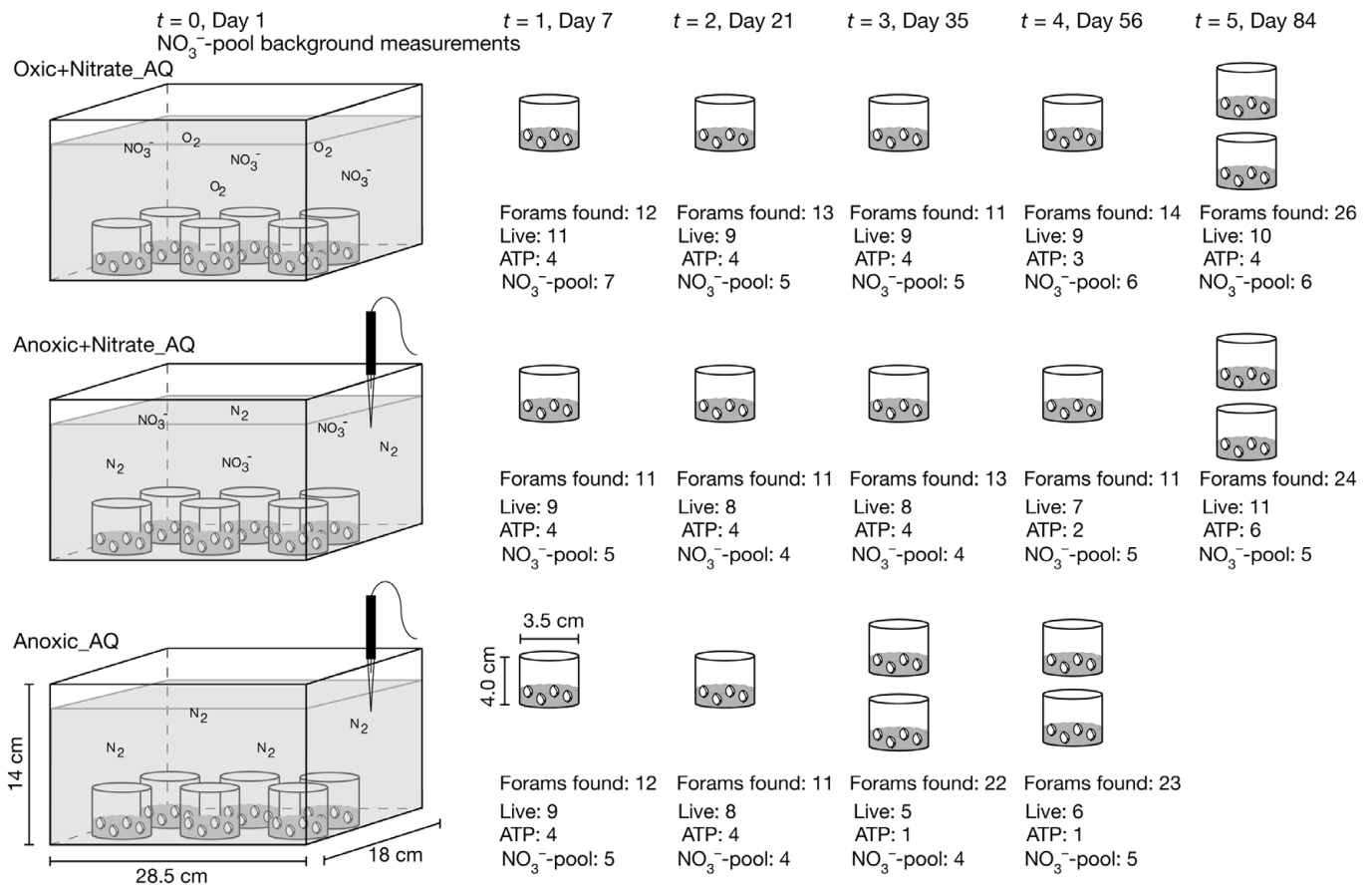


Fig. 1. Experimental set up and time line. Numbers of *Globobulimina turgida* recovered (Forams found) and identified live (Live; based on FDA staining). The number of living specimens measured for ATP and the nitrate pool at each sampling time is indicated. Artificial seawater (ASW) was used to set up all aquaria, allowing the manipulation of nitrate levels in each aquarium. Nitrate was added to the oxic aquarium to create natural conditions; natural nitrification under oxic conditions could not have been excluded from the set up. At  $t = 0$  (Day 1), background measurements of the intracellular nitrate pool were made. In total, 8 specimens were measured

15°C) and anoxic alkaline ascorbate. All 3 aquaria were sealed to prevent any evaporation and to maintain constant gas concentration. The nitrate content in all aquaria was monitored periodically and ranged between 30 and 60  $\mu\text{M}$  in Oxic+Nitrate\_AQ and Anoxic+Nitrate\_AQ. During the experiment, nitrate was added to the Oxic+Nitrate\_AQ and Anoxic+Nitrate\_AQ to sustain the desired high nitrate concentrations.

Six small vials (each 30 ml), all containing 14 living *Globobulimina turgida* and a thin layer of fine-grain sieved sediment ( $<38 \mu\text{m}$ ), were placed in each aquarium. Fine-grained sediment slurry (4 ml) was transferred with a syringe into each vial to create more 'natural' conditions for the foraminifera. Following compaction, the sediment layer was  $\sim 1$  ml in volume.

The experiment ran for 3 mo. All aquaria were sampled at 7 d ( $t = 1$ ), 21 d ( $t = 2$ ), 35 d ( $t = 3$ ) and 56 d ( $t = 4$ ). In addition, Oxic+Nitrate\_AQ and Anoxic+Nitrate\_AQ were sampled at 84 d ( $t = 5$ ). Furthermore, back-

ground measurements of the intracellular nitrate pool were carried out on Day 1 ( $t = 0$ ). At each sampling time, a vial (or 2 if only a few living foraminifera were identified at the time: at  $t = 3$  and 4 in Anoxic\_AQ; at  $t = 5$  in Oxic+Nitrate\_AQ and Anoxic+Nitrate\_AQ) was taken from each aquarium. The majority (79 to 100%) of foraminifera were found, checked for viability using the fluorescein diacetate (FDA) technique (see next section) and counted. The living specimens were measured for their intracellular nitrate pool (see section 'Internal nitrate content in foraminifera') or cellular ATP content (see section 'ATP extraction and measurements'). In addition, at sample times  $t = 3$  and 5, 8 dead specimens were also measured for intracellular nitrate content. Following every sampling, some fresh ( $\sim 1$  ml) fine-grained sediment was added to the surface of the sediment in the vials to provide a food source for the foraminifera. In addition, at the start of the experiment ( $t = 0$ ) the denitrification rate of *Globobulimina turgida*

was measured using a microsensors technique (see section 'Denitrification measurements').

**Identification of living individuals during the experiment: FDA staining.** Identification of living foraminifera based on movement is time consuming, which is not ideal while processing several incubation chambers simultaneously. In addition, time-consuming methods may bias the experimental results. Therefore, epifluorescence microscopy was used to distinguish living from dead specimens. The fluorescence technique is a powerful, reliable and quick method to identify living individuals in experimental setups (Bernhard et al. 1995), particularly under anoxic conditions. Incubation in fluorogenic probes causes live specimens to become fluorescent, while dead specimens will not easily be detected. The fluorogenic probe used in this experiment was FDA, which was diluted in a dimethyl sulphoxide solution and ASW in order to obtain a final solution of 100  $\mu\text{M}$  FDA. All samples were incubated in ASW-FDA solution for 3 to 4 h. Foraminifera were then picked out and placed in ASW with no FDA. Each specimen was checked individually for fluorescence using an Axiovert 200M Apotome epifluorescence microscope (Carl Zeiss).

**Reliability of the FDA staining:** To investigate the possible FDA staining of dead foraminifera, 10 living *Globobulimina turgida* (identified by movement) were either heat killed (2 h at 45°C) or placed for a short while in 70 % ethanol. Specimens killed in ethanol were placed back in natural seawater for 2 h to allow any bacterial recolonisation. Both living and dead specimens were then incubated in FDA-ASW for 3 to 4 h. After the incubation, the specimens were placed in fresh ASW (no FDA), and the intensity of fluorescence in specimens was visually examined by epifluorescence microscopy. Live specimens produced a very intense bright glow, the glow being brightest in the outermost chamber of foraminifera. In contrast, the dead foraminifera produced no glow, or a very dull homogenous glow often absent in the outermost chamber of foraminifera. The dull glow in the dead foraminifera was most likely produced by bacteria.

**FDA test on the internal nitrate content in *Globobulimina turgida*:** Prior to the experiment, the possible influence of FDA staining on the internal nitrate pool was measured in 10 living specimens of *G. turgida* (5 stained with FDA and 5 non-stained; the non-stained specimens were identified as alive according to movement within ca. 16 h. The internal nitrate pool measurements in both stained and non-stained foraminifera were comparable and within the values previously reported for *G. turgida* (Risgaard-Petersen et al. 2006), ranging between 2094 and 5153 pmol cell<sup>-1</sup> for non-stained and between 4564 and 9119 pmol cell<sup>-1</sup> for stained specimens. Therefore, FDA probing was applied without further considerations.

**Internal nitrate content in foraminifera.** Foraminifera were gently cleaned of sediment with a brush and rinsed in nitrate-free ASW. The specimens were then transferred to PCR tubes and analysed for nitrate content using the VCl<sub>3</sub> reduction method (Braman & Hendrix 1989) on a chemiluminescence detector (Model CLD 86, Eco Physics AG) as described by Risgaard-Petersen et al. (2006) and Høgslund et al. (2008), with modification by Piña-Ochoa et al. (2010). The analysis was carried out immediately when possible; alternatively, specimens were stored at -20°C until analysis within 1 wk of sampling.

**ATP extraction and measurements.** ATP, a basic chemical fuel of living cells, is produced during oxidation of food molecules and powers most of the cell's activities (Alberts et al. 1998) and thus served here as an indication of metabolism and activity of foraminifera in the various experimental conditions. ATP extractions and measurements followed protocols described by Linke (1992) and Witzel (1979), respectively. ATP was extracted from individual specimens of *Globobulimina turgida*. Each foraminiferan was transferred into a test tube and rinsed with 2 ml of boiling 0.02 M Tris buffer (pH 7.8). Tubes were held in an Erlenmeyer flask with boiling water for 30 s. To optimise the extraction efficiency, the tests of the foraminifera were crushed with a glass rod. The sample extracts were stored at -20°C until later analysis. The real dilution of the samples was determined by weighing the test tubes with and without the extractions. The measurements were carried out using a Berthold detection system FB12 Luminometer.

**Denitrification measurements.** The viability of *Globobulimina turgida* used for denitrification measurements was based on movement of specimens (within ca. 16 h), and the measurements were carried out immediately after the harvest of live specimens. Foraminiferal nitrate respiration rates were measured using the acetylene inhibition technique. Acetylene blocks the reduction of N<sub>2</sub>O to N<sub>2</sub>, thus allowing the measurement of N<sub>2</sub>O as the end product of denitrification (Smith et al. 1978). N<sub>2</sub>O production was measured with an N<sub>2</sub>O microsensors (Andersen et al. 2001), as described by Risgaard-Petersen et al. (2006), Høgslund et al. (2008) and Piña-Ochoa et al. (2010).

## RESULTS AND DISCUSSION

### Foraminiferal response to different treatments: survival and cellular ATP reserve

For the first 3 wk, the numbers of living *Globobulimina turgida* were relatively similar in all aquaria, ranging from 70 to 92 % (Fig. 2). However, over the

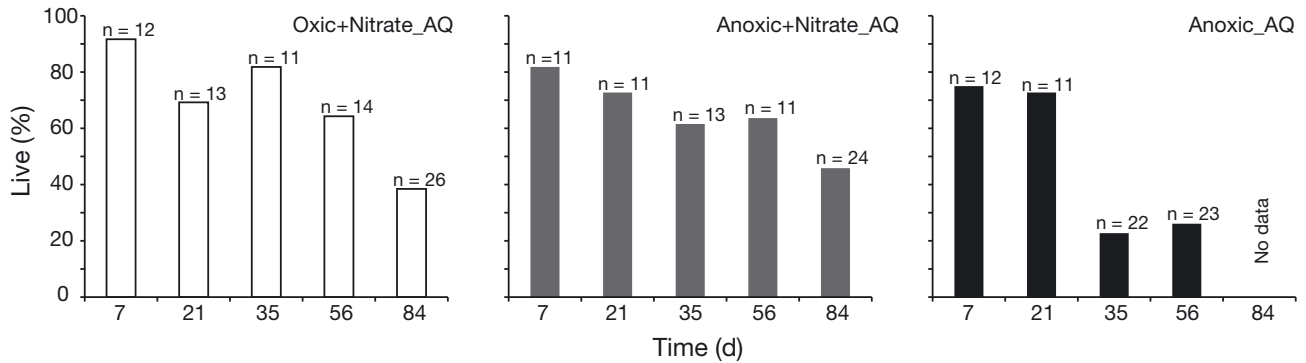


Fig. 2. *Globobulimina turgida*. Fraction of live foraminifera (%) at each sampling time. Note that Anoxic\_AQ was not sampled at 84 d

longer term, the survival rates were higher in the aquaria where either oxygen and nitrate (Oxic+Nitrate\_AQ), or nitrate (Anoxic+Nitrate\_AQ) was readily available in the surrounding environment to sustain foraminiferal respiration. In Anoxic\_AQ, where foraminiferal respiration was restricted to the intracellular nitrate pool, only ~25% of *G. turgida* were found alive after an incubation period of 35 (n = 22) and 56 d (n = 23). Death of specimens in Anoxic\_AQ most likely resulted from declining intracellular nitrate concentrations, which at the end were insufficient to sustain cellular respiration. In contrast, in Oxic+Nitrate\_AQ at 35 d (56 d), >80% (>60%) of foraminifera were still alive. Similarly, in Anoxic+Nitrate\_AQ at both sample times, >60% of foraminifera were still alive. Thus, the long-term survival in Oxic+Nitrate\_AQ and Anoxic+Nitrate\_AQ appeared similar, or even somewhat higher in Anoxic+Nitrate\_AQ. At the end of the experiment, after 84 d, 38% (n = 26) of foraminifera were still alive in Oxic+Nitrate\_AQ and 46% (n = 24) were alive in Anoxic+Nitrate\_AQ. These results indicate that *G. turgida* does not need oxygen to sustain metabolic activities, but can maintain long-term nitrate respiration. However, as no reproduction was observed during the experiment (always  $\leq 14$  specimens found in each vial, Fig. 1), it remains unsure what the optimal conditions are for reproduction to take place.

Large variations were measured in the ATP content within each treatment at various sampling times (Fig. 3, Table 1). The highest ATP concentration ( $243.5 \text{ ng g}^{-1}$ ) was measured in Anoxic+Nitrate\_AQ at  $t = 3$  (after 35 d) followed by  $224.1 \text{ ng g}^{-1}$  in the Oxic+Nitrate\_AQ at  $t = 1$ , and the lowest (below detection limit) in Oxic+Nitrate\_AQ at  $t = 5$  (after 84 d). ATP concentrations varied significantly through time only in Oxic+Nitrate\_AQ (Table 2), and a general decrease was observed with a longer incubation period (Fig. 3). In Anoxic+Nitrate\_AQ and Anoxic\_AQ, no statistically significant variations were observed, and no trend was

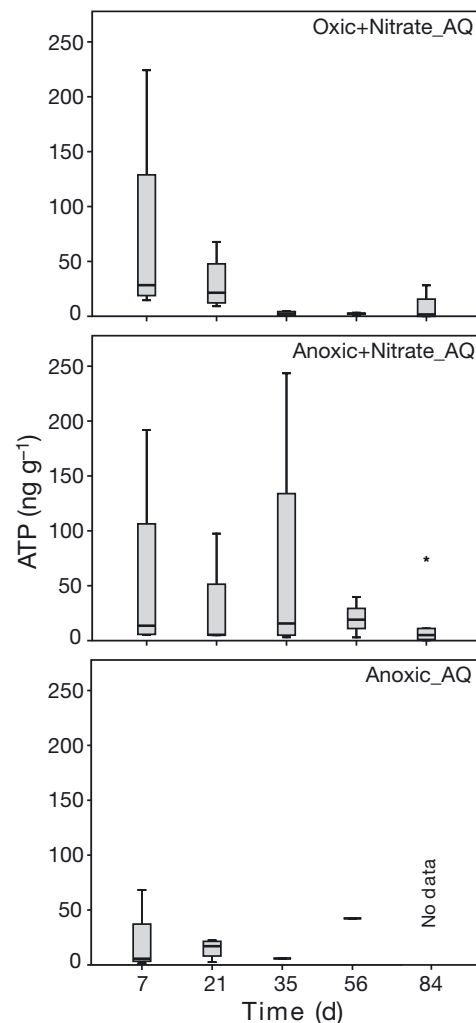


Fig. 3. *Globobulimina turgida*. Median ATP content of foraminiferans versus time for each aquarium. No ATP was measured at  $t = 0$ . The box limits are set at the 25th and 75th percentiles. The whiskers extend to 1.5 times the height of the box, thus representing approximately a 95% range. If no case/row has a value in the whisker range, the whiskers are defined by the minimum or maximum values. An outlier is shown with an asterisk

observed in the median ATP content through time (Fig. 3), implying that foraminifera were metabolically equally active in both setups. Further, the Anoxic+

Table 1. *Globobulimina turgida*. Summary of the foraminiferal ATP data for each sampling time and aquarium; minimum (min.), maximum (max.), median (mdn), average (ave.) and number of individuals measured (n). Note no data for Anoxic\_AQ at  $t = 5$ ; bd: below detection limit; na: not applicable

Treatment/ time	n	ATP (ng g <sup>-1</sup> )			
		Min.	Max.	Mdn	Ave.
<b>Oxic+Nitrate_AQ</b>					
1 (7 d)	4	14.6	224.1	28.4	68.8
2 (21 d)	4	9.1	67.6	21.5	30.0
3 (35 d)	4	0.6	4.5	2.5	2.5
4 (56 d)	3	1.3	3.3	2.4	2.3
5 (84 d)	4	0 (bd)	28.2	1.7	7.8
<b>Anoxic+Nitrate_AQ</b>					
1 (7 d)	4	5.1	191.8	13.6	56.0
2 (21 d)	3	4.8	97.5	5.3	35.9
3 (35 d)	4	3.0	243.5	15.6	69.4
4 (56 d)	3	2.9	39.6	19.1	20.5
5 (84 d)	6	0.2	74.3	5.0	11.7
<b>Anoxic_AQ</b>					
1 (7 d)	4	1.5	68.2	5.6	20.2
2 (21 d)	4	2.5	22.5	17.1	14.8
3 (35 d)	1	na	na	5.9	5.9
4 (56 d)	1	na	na	42.4	42.4

Table 2. Outcome of the Kruskal-Wallis test based on the foraminiferal ATP data from each aquarium

Treatment	H (df)	p
Oxic+Nitrate_AQ	10.47 (4)	0.033
Anoxic+Nitrate_AQ	2.30 (4)	0.682
Anoxic_AQ	1.90 (3)	0.603

Table 3. Outcome of the Mann-Whitney test (2-tailed p), comparing the foraminiferal ATP data from anoxic, N<sub>2</sub>-flushed aquaria (Anoxic+Nitrate\_AQ, Anoxic\_AQ) to the aerated control aquarium (Oxic+Nitrate\_AQ)

Mann-Whitney test	Oxic+Nitrate_AQ				
	(7 d)	(21 d)	(35 d)	(56 d)	(84 d)
Anoxic+Nitrate_AQ;	0.248;				
Anoxic_AQ (7 d)	0.149				
Anoxic+Nitrate_AQ;		0.480;			
Anoxic_AQ (21 d)		0.386			
Anoxic+Nitrate_AQ;			0.083;		
Anoxic_AQ (35 d)			0.157		
Anoxic+Nitrate_AQ;				0.127;	
Anoxic_AQ (56 d)				0.180	
Anoxic+Nitrate_AQ					0.394

Nitrate\_AQ and Anoxic\_AQ were statistically not different from the aerated control aquarium (Oxic+Nitrate\_AQ) at any sample moment (Table 3, Mann-Whitney test, p always > 0.05). Therefore, it seems that the ATP content does not differ between denitrifying and aerobically respiring *Globobulimina turgida*, and that *G. turgida* is 'equally' active in both environments and does not display potential dormancy under anoxic conditions.

In previous studies, the ATP content of foraminifera experiencing prolonged anoxia (N<sub>2</sub>-purged) versus aerobic conditions has been reported as similar (Bernhard 1993) or lower in the former (Bernhard & Alve 1996). The contradictory evidence may be related to interspecies differences, and differences in their ultrastructure (Bernhard & Alve 1996). A decrease in ATP content in foraminifera has also been previously related to low food supply (Graf & Linke 1992, Linke 1992), as the ATP concentrations in deep-sea benthic foraminifera were observed to increase drastically following phytoplankton deposition. In our experiment, food supply may have played a role at least in the Oxic+Nitrate\_AQ where the median ATP concentrations were seen to decline with time (Fig. 3). As the Oxic+Nitrate\_AQ was continuously aerated, sedimentary organic matter in it would have been more rapidly oxidised (due to the higher energy yield of oxygen than nitrate, e.g. Bender & Heggie 1984) than in the anoxic aquaria, thus leading to lower food supply. The low food content in the Oxic+Nitrate\_AQ could also explain the somewhat higher long-term survival rates in the Anoxic+Nitrate\_AQ (Fig. 2). This observation is also supported by laboratory observations of Koho et al. (2008b), who reported declining foraminiferal standing stocks in non-fed control cores (especially the 63 to 150 µm fraction) after a 56 d incubation period. Further, our ATP measurements of Anoxic+Nitrate\_AQ and Anoxic\_AQ may have been compromised, as the isolation of foraminifera for the ATP measurements was performed under oxygenated conditions that could have led to a sudden increase in metabolic activity. However, this appears unlikely, as following re-aeration of a previously anoxic experimental setting, the ATP concentration in foraminifera remained unchanged (J. Bernhard & E. Alve unpubl. data, as cited by Bernhard & Alve 1996).

### Nitrate storage in foraminifera

Nine out of 9 dead *Globobulimina turgida* specimens measured for intracellular nitrate did not contain any nitrate. However, extraction of relatively high amounts (>250 mM) of intracellular nitrate from living *G. turgida* was possible in all 3 treatments (Fig. 4). Nitrate

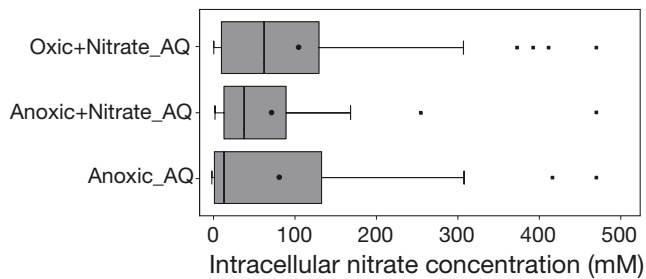


Fig. 4. *Globobulimina turgida*. Intracellular nitrate concentration in live specimens sampled in 3 different aquaria. Boxes include 25 to 75% of overall data, medians are indicated by the centre line of the boxes, black circles are mean values and whiskers comprise the whole range of data. Outliers are shown as small black squares

was measured in 28 out of 29, 24 out of 24 and 19 out of 21 living specimens from Oxidic+Nitrate\_AQ, Anoxic+Nitrate\_AQ and Anoxic\_AQ, respectively. Nitrate concentration among *G. turgida* individuals ranged from 0 to 463 mM, and these concentrations corresponded to an average concentration in all treatments of about 93 mM— $104 \pm 126$  (SD),  $72 \pm 93$ ,  $97 \pm 128$  in Oxidic+Nitrate\_AQ, Anoxic+Nitrate\_AQ and Anoxic\_AQ, respectively. The measured intracellular nitrate concentrations are well within the range of values reported for *Globobulimina* by Risgaard-Petersen et al. (2006) and Glud et al. (2009). Similar intracellular nitrate concentrations (up to 500 mM) have also been observed in white sulphur bacteria belonging to the family *Beggiatoaceae* (*Beggiatoa*, *Thioploca* and *Thiomargarita*; Fossing et al. 1995, McHatton et al. 1996, Schulz et al. 1999, Sayama 2001), corresponding to 4000-fold higher concentration levels than in the ambient environment. These findings suggest that nitrate accumulation is a universal property of vacuolated, filamentous sulphur bacteria, as well as Foraminifera and Gromiida (Piña-Ochoa et al. 2010).

High variability was measured in the intracellular nitrate pool within each treatment (Figs. 4 & 5); the coefficient of variation was 120, 129 and 132% for Oxidic+Nitrate\_AQ, Anoxic+Nitrate\_AQ and Anoxic\_AQ, respectively. To explain some of the data scatter, a size-dependent relationship was suggested by Glud et al. (2009) for the intracellular nitrate store in *Globobulimina affinis*. In our study (data not shown), the variability in the size of intracellular nitrate store was not related to the size of the foraminifera, and no relationship was observed between these parameters. In fact, some of the highest intracellular nitrate pools were measured in relatively small specimens. Instead, we postulate that the scatter in the measurements highlights the active nature of the foraminifera to collect and respire on nitrate, depending on individuals' his-

tory of exposure to oxygen and nitrate. For example, it appears that under 'optimal' conditions in Oxidic+Nitrate\_AQ, where both oxygen and nitrate are present to sustain respiratory activity, the intracellular nitrate pool throughout the 84 d incubation period was not directly affected, as the measurements were not statistically different ( $p > 0.05$ , Kruskal-Wallis test) at any point in time. However, it can be argued that the nitrate concentrations in Oxidic+Nitrate\_AQ did show some decline with time, as only very low concentrations were found at the end of the experiment (Fig. 5). These low nitrate values may be related to the low ATP, or energy, levels (Fig. 3) and potentially low food availability in this treatment. The maintenance of nitrate storage, against the strong environmental gradient, costs the cell energy, and the minimum energy required to concentrate a solute  $x$ -fold up from ambi-

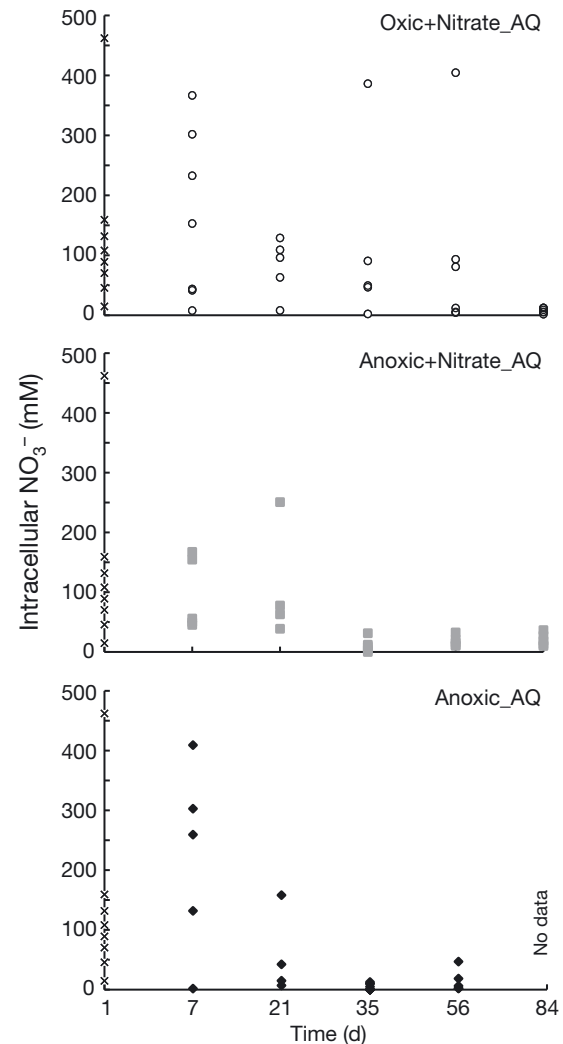


Fig. 5. *Globobulimina turgida*. Intracellular nitrate pool of foraminiferans versus time for each aquarium. Background measurements of  $t = 0$  (Day 1) are shown on the y-axis

ent concentrations is:  $\log_{10}(x) \times 5.7 \text{ kJ mol}^{-1}$  (Harold 1986). Thus, the average 6000-fold-higher intracellular nitrate concentration observed throughout the experiment in *G. turgida* relative to the ambient levels would cost the cells a minimum of 21.56 KJ of free energy  $\text{mol}^{-1}$  of nitrate taken up. If we assume a free energy yield of  $-566 \text{ kJ mol}^{-1}$  nitrate from denitrification (recalculated after Jørgensen 2000) and subtract 66% lost in ATP formation (recalculated after Jørgensen 2000 and Spanning et al. 2007), then only about 10% of the energy gained from respiration is used for nitrate storage. Therefore, it can be speculated that at the end of the experiment due to low food supply, in the Oxid+Nitrate\_AQ, foraminifera were either not able to sustain the high cellular nitrate concentrations due to lack of energy reserves, or they simply did not have to because they were respiring on oxygen.

In the Anoxic+Nitrate\_AQ and Anoxic\_AQ, where foraminifera depended on nitrate respiration, we found statistically significant differences ( $p = 0.001$ ,  $p = 0.003$ ; Kruskal-Wallis test) between medians of intracellular nitrate concentration in live *Globobulimina turgida* in both nitrogen-flushed aquaria (Anoxic+Nitrate\_AQ and Anoxic\_AQ) and at different sampling times (1, 7, 21, 35, 56 and 84 d), especially after 35, 56 and 84 d in the Anoxic+Nitrate\_AQ and 21, 35 and 56 d in Anoxic\_AQ. Furthermore, the intracellular nitrate concentrations were observed to decrease fastest in the Anoxic\_AQ where under anoxic and nitrate-free conditions, *G. turgida* was 'forced' to use its intracellular nitrate without being able to replenish it over time.

#### Nitrate respiration and intracellular nitrate turnover

A complete denitrification to  $\text{N}_2$  gas has been shown to be a mutual ability among the nitrate-storing benthic foraminifera (Risgaard-Petersen et al. 2006, Høgslund et al. 2008, Piña-Ochoa et al. 2010). Our results also confirmed the ability of *Globobulimina turgida* to respire nitrate to  $\text{N}_2$ , thus validating that the intracellular nitrate pool was used for respiration. Denitrification rates ranged between  $263 \pm 8$  ( $\pm$  SE) and  $453 \pm 30 \text{ pmol N ind.}^{-1} \text{ d}^{-1}$ , as derived from Fig. 6, similar to activity measured previously for *G. turgida* ( $565 \text{ pmol N ind.}^{-1} \text{ d}^{-1}$ ), using  $^{15}\text{N}$ -techniques (Risgaard-Petersen et al. 2006). Production of nitrous oxide by *G. turgida* was also measured in the absence of acetylene, similar to *Bolivina plicata*, *Valvulineria cf. laevigata* and *Stainforthia* sp. from the Peruvian Oxygen Minimum Zone (OMZ, Piña-Ochoa et al. 2010). Nevertheless, the  $\text{N}_2\text{O}$  production in *G. turgida* without acetylene was only 27 to 46% of the rates measured with acetylene, while

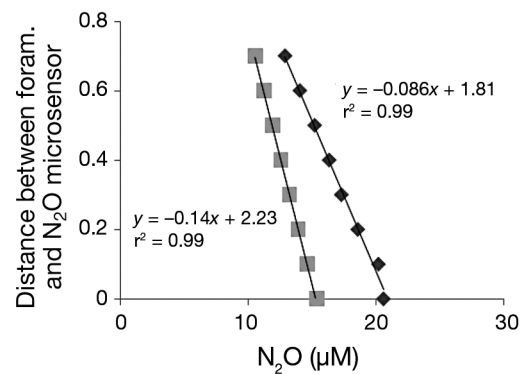


Fig. 6. *Globobulimina turgida*. Profile of nitrous oxide production in 2 replicate batches of foraminiferans (foram.). Profiles measured in anoxic microtubes using 3 individuals (dark gray symbols) or 2 individuals (light gray symbols)

$\text{N}_2\text{O}$  production in the OMZ species was 91 to 95%. Thus, the role of foraminifera as a greenhouse gas source still needs to be assessed.

The intracellular nitrate pool turnover time for *Globobulimina turgida* was estimated by Risgaard-Petersen et al. (2006) to be on the order of 1 mo. Based on our denitrification rate of  $453 \pm 30 \text{ pmol N ind.}^{-1} \text{ d}^{-1}$  and the maximum cell bound nitrate content ( $42480 \text{ pmol cell}^{-1}$ ) found in Anoxic\_AQ at  $t = 1$ , our results suggest that foraminifera can sustain their respiratory activity for up to 3 mo if using their intracellular nitrate pool only (Fig. 7), or for about 5.5 mo when considering the lower turnover rate of  $263 \pm 8 \text{ pmol N ind.}^{-1} \text{ d}^{-1}$ . However, the maximum cell bound nitrate content is nearly 10 times higher than the average nitrate pool measured in all treatments at all sampling times. Therefore, it seems more likely that on average the nitrate turnover time is on the order of 20 d (Fig. 7), which is within the estimates of Risgaard-Petersen et al. (2006). Furthermore, this observation is in agreement with our intracellular nitrate pool data for Anoxic\_AQ, where the median cell-bound nitrate started clearly declining after the 21 d incubation period (Fig. 5), and by 35 d the nitrate pool had become very limited, leading to increased mortality among foraminifera (Fig. 2).

#### Nitrate respiration strategies: prokaryotes versus eukaryotes

Traditionally, nitrate respiration is considered mainly a prokaryotic process, and only a few eukaryotic organisms, such as ciliates (Finlay et al. 1983) and fungi (Kobayashi et al. 1996, Takaya et al. 1999, Tielens et al. 2002), have been described as reducing nitrate. However, the discovery of denitrification com-



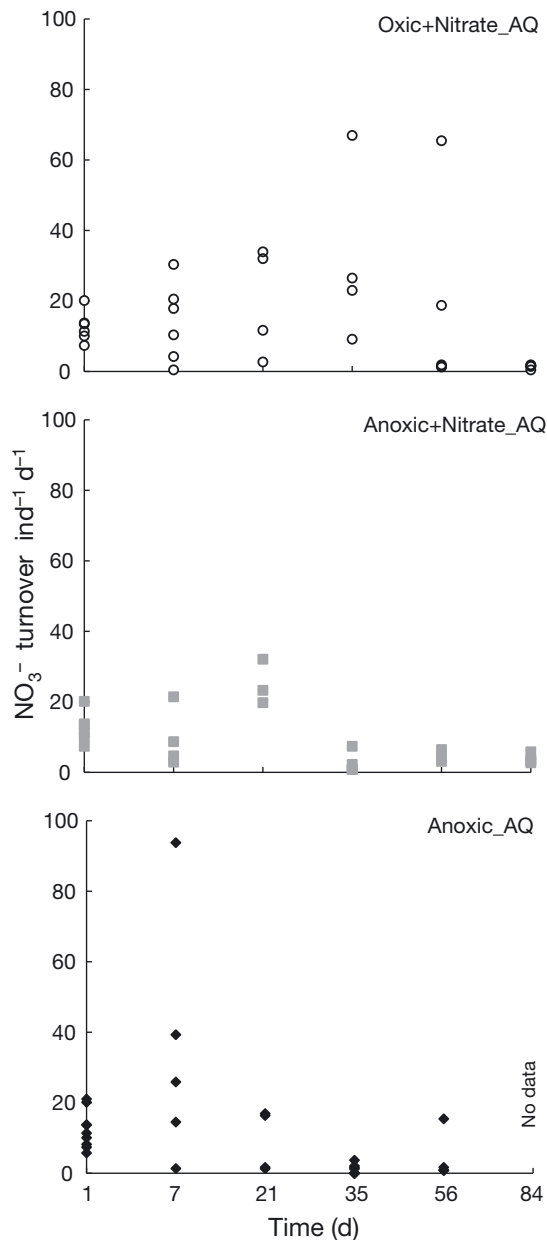


Fig. 7. *Globobulimina turgida*. Nitrate turnover rates in Oxidic+Nitrate\_AQ, Anoxic+Nitrate\_AQ and Anoxic\_AQ based on the denitrification rate of  $453 \pm 30$  pmol N ind.<sup>-1</sup> d<sup>-1</sup>

monly occurring among Foraminifera has challenged our understanding of the role of eukaryotes in the nitrogen cycle (Piña-Ochoa et al. 2010). Large differences, however, exist between the nitrate respiration in prokaryotes, ciliates, fungi and foraminifera. Firstly, where ciliates (*Loxodes* spp., Finlay et al. 1983) and fungi (e.g. *Fusarium oxysporum* and *Cylindrocarpum tonkinense*, Tielens et al. 2002) are capable of reducing nitrate only to nitrite, foraminifera can perform complete denitrification with N<sub>2</sub> as the end product of res-

piration. Further, foraminifera are able to reduce nitrate in the absence of oxygen/nitrate and move to areas where there is no measurable oxygen/nitrate. In contrast, sessile fungi only switch to nitrate reduction when oxygen concentrations become limiting (Takaya et al. 1999).

It seems that the nitrate-accumulating and -respiring foraminifera share some life strategies with other nitrate-storing prokaryotes, such as 3 genera of sulphur bacteria (*Beggiatoa*, *Thioploca* and *Thiomargarita*) that frequently dominate nitrate rich and oxygen-depleted sediments of the seafloor (Fossing et al. 1995, McHatton et al. 1996, Schulz et al. 1999). For instance, large sulphur bacteria, such as *Thioploca* and *Beggiatoa*, are able to store nitrate in vacuoles and carry it deep into oxygen- and nitrate-free environments by migration, which, similar to foraminifera, enables the organisms to sustain nitrate respiration in a nitrate-free environment (Høgslund et al. 2009). The migration behaviour between redox zones is also commonly observed among foraminifera (e.g. Geslin et al. 2004, Pucci et al. 2009), and thus it is very likely that this behaviour is closely linked to the nitrate uptake, or nitrate refuelling, and respiration of stored nitrate. A major difference between foraminifera and nitrate-storing sulphur bacteria, however, is the end product of nitrate respiration. While the preferred respiratory pathway of bacteria is dissimilatory reduction of nitrate to ammonium (Otte et al. 1999), foraminifera reduce intracellular nitrate to dinitrogen gas.

## CONCLUSIONS

In our laboratory experiments, we observed that the benthic foraminiferan *Globobulimina turgida* is capable of long-term survival under simulated anoxic conditions if nitrate is readily available to sustain cellular respiration. In the absence of desired electron acceptors (oxygen and nitrate), the survival rates of *G. turgida* are reduced. Nevertheless, *G. turgida* can survive up to 56 d if respiring the intracellular nitrate pool only. Therefore, the capacity of *G. turgida* to respire on oxygen and nitrate, and its relatively long-term survival through use of the intracellular nitrate pool, allows the foraminiferan to reside in a wide range of sediment depths and to inhabit various sediment redox zones. Our results also showed similar ATP concentrations in specimens incubated under different oxygen/nitrate conditions, indicating that in the long term, the metabolic activity of *G. turgida* remained the same in both environments.

The capacity of *Globobulimina turgida* to denitrify nitrate to N<sub>2</sub> gas was confirmed. The rates varied between  $263 \pm 8$  ( $\pm$ SE) and  $453 \pm 30$  pmol N ind.<sup>-1</sup> d<sup>-1</sup>.

The production of  $N_2O$  was also measured in the absence of acetylene; however, the production was only 27 to 46% of the rates measured with acetylene. Clearly, further observational and experimental data are required to clarify the relevance of foraminifera as a potential greenhouse gas source and their role in the N cycle.

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