LETTERS

Evidence for complete denitrification in a benthic foraminifer

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Benthic foraminifera are unicellular eukaryotes found abundantly in many types of marine sediments. Many species survive and possibly reproduce in anoxic habitats[1], but sustainable anaerobic metabolism has not been previously described. Here we demonstrate that the foraminifer *Globobulimina pseudospinescens* accumulates intracellular nitrate stores and that these can be respired to dinitrogen gas. The amounts of nitrate detected are estimated to be sufficient to support respiration for over a month. In a Swedish fjord sediment where *G. pseudospinescens* is the dominant foraminifer, the intracellular nitrate pool in this species accounted for 20% of the large, cell-bound, nitrate pool present in an oxygen-free zone. Similarly high nitrate concentrations were also detected in foraminifera *Nonionella* cf. *stella* and a *Stainforthia* species, the two dominant benthic taxa occurring within the oxygen minimum zone of the continental shelf off Chile. Given the high abundance of foraminifera in anoxic marine environments[1–3], these new findings suggest that foraminifera may play an important role in global nitrogen cycling and indicate that our understanding of the complexity of the marine nitrogen cycle is far from complete.

In 2005 we investigated foraminifera in the oxygen-free zone of sediment from the Gullmar Fjord, Sweden. By promoting cell lysis we identified a large nitrate pool beneath the oxygenated zone at a depth where the sediment pore water is devoid of nitrate (Fig. 1). This cell-bound nitrate pool positively correlated with foraminiferal abundance (Pearson correlation, $r = 0.98$; $P = 0.0001$, $n = 7$). One of the dominant foraminifera in this sediment was *G. pseudospinescens* (Fig. 2a), which accounted for 37% of the standing stock in the >150 μm fraction. As is typical for this organism[4], it mainly occurred in sediment layers where the pore water lacked oxygen and nitrate (Fig. 1). The average nitrate content of individual *G. pseudospinescens* was 18 nmol (range 0–72 nmol; Table 1), corresponding to an average intracellular concentration well above 10 mM, or more than 500-fold higher than the maximum concentration in the sediment pore water. The nitrate in *G. pseudospinescens* accounted for about 20% of the total cell-bound nitrate pool in the sediment, thus leaving open the possibility that other foraminifera also accumulate nitrate in this sediment. Possible candidates include *Nonionella turgida* and *Stainforthia fusiformis*, which also occurred in anoxic layers of the sediment but were not analysed for nitrate.

That accumulation of nitrate in foraminifera might be a widespread phenomenon was confirmed through a study of the epibenthic community within the oxygen-minimum zone of the continental shelf off Chile in January 2006. The intracellular nitrate concentration in the two dominant taxa—*Nonionella* cf. *stella* (Fig. 2b) and an undescribed *Stainforthia* species (Fig. 2c)—was even higher than in *G. pseudospinescens* and up to 15,000-fold higher than the maximum porewater concentration (Table 1).

To accumulate nitrate against these large electrochemical gradients requires energy[5] and hence is only sustainable if the foraminifera benefit from their intracellular nitrate pool. It has been previously proposed that species preferring anoxic environments can respire nitrate[6]. We therefore tested whether the intracellular nitrate pool in *G. pseudospinescens* is denitrified (that is, respired).

When individual *G. pseudospinescens* were incubated in oxygen-free water containing $^{15}NO_3^-$, ^{15}N-labelled dinitrogen gas was formed, thus indicating complete denitrification (that is, full reduction of nitrate to dinitrogen gas (one-tailed *t*-test, $P = 0.0005$, $n = 8$, $\alpha = 0.05$; Fig. 3a). The following observations

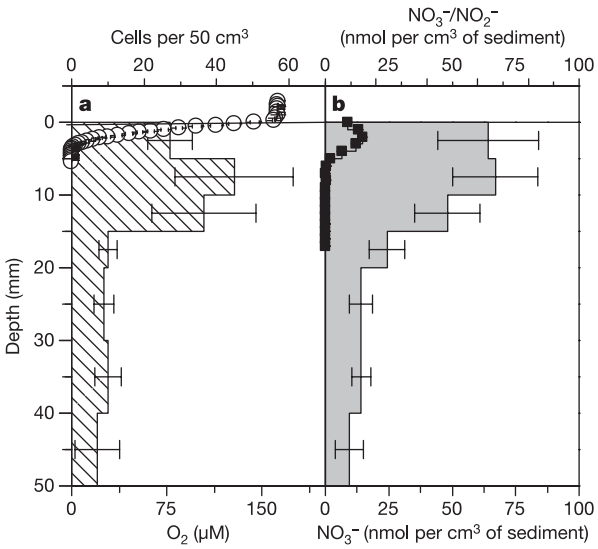

Figure 1 | Foraminifera, oxygen and nitrate in sediment. a, Depth distribution of Rose-Bengal stained *G. pseudospinescens* (hatched area) and oxygen (open circles). **b**, Porewater nitrate/nitrite (squares) and cell-bound nitrate (grey area) in sediment cores from the Gullmar Fjord, Sweden, August 2005. The error bars indicate the s.e.m ($n = 3$).

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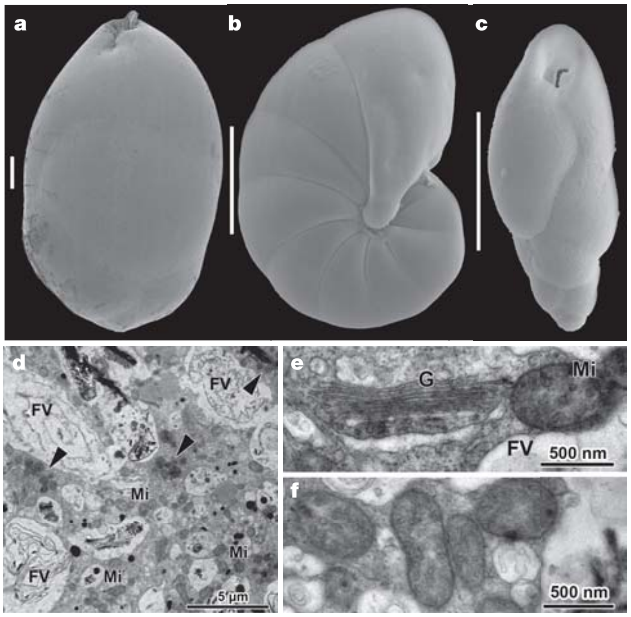

Figure 2 | SEM and TEM micrographs of the investigated foraminifer species. **a**–**c**, SEM micrographs of single cells of *G. pseudospinescens* (**a**), *Nonionella* cf. *stella* (**b**) and *Stainforthia* sp. (**c**). Scale bar, 100 µm. **d**, TEM micrograph showing cellular organization of *G. pseudospinescens* containing the intact cytoplasm with multiple food vacuoles (FV) and clusters of mitochondria (Mi) and peroxisomes (arrows). **e**, Detailed TEM micrograph showing a mitochondrion (Mi) and Golgi apparatus (G). **f**, Detailed TEM micrograph of a cluster of mitochondria.

show that this process took place intracellularly using the intracellular nitrate ($^{14}NO_3^-$) as the substrate: if denitrification had only taken place outside the foraminifera, less than 2% of the recovered ^{15}N would have appeared as mixed-labelled $^{29}N_2$ because the ^{15}N-labelling of nitrate in the medium exceeded 98 atom% throughout the incubation. The appearance of recovered ^{15}N in the $^{29}N_2$ pool was significantly different from (two-tailed *t*-test, $P = 0.0037$, $n = 8$, $\alpha = 0.05$) and generally much higher than this hypothetical fraction. In 6 out of 8 samples, more than 40% of the recovered ^{15}N appeared as the mixed-labelled $^{29}N_2$. As parallel experiments with $^{15}NH_4^+$ and $^{14}NO_3^-$ excluded the possibility of anaerobic ammonium oxidation (that is, the anammox process[7]), $^{29}N_2$ production must have been due solely to denitrification. The foraminifera must therefore have taken up $^{15}NO_3^-$ from the medium, mixed this with their intracellular pool of $^{14}NO_3^-$ and denitrified the mixed $^{14}NO_3^-/^{15}NO_3^-$ pool intracellularly.

In a second series of experiments, the denitrification rate was measured in individual *G. pseudospinescens* containing $^{14}NO_3^-$ and $^{15}NO_3^-$. The specimens were prepared by incubating intact sediment cores with 50 µM $^{15}NO_3^-$ in the overlying water. Foraminifera harvested from the cores after 8 weeks exhibited an average (±s.e.m.) of 40 ± 4 ^{15}N atom% enrichment of their nitrate pool. Nineteen of the twenty-two cells analysed contained measurable $^{15}NO_3^-$. Denitrification was measured on foraminifera harvested after 9 weeks and incubated in nitrate-free and oxygen-free media. In 10 out of 12 cases, $^{29}N_2$ and $^{30}N_2$ production was detected during a 24–72-h incubation period (Fig. 3b). Linear regression analysis revealed an average dinitrogen production rate for a foraminifer cell of 565 pmol N d^{-1} with a 95% confidence interval between 226–902 pmol N d^{-1}.

In subsequent experiments, evidence for complete denitrification of intracellular nitrate was also obtained for *Nonionella* cf. *stella*. Specimens were incubated in microtubes containing nitrate-free and oxygen-free water in the presence or absence of acetylene, which inhibits the biological reduction of nitrous oxide to dinitrogen. Denitrification was quantified from the accumulation of nitrous oxide (see Supplementary Information for details). Nitrous oxide only accumulated in the presence of acetylene, corresponding to a denitrification rate for a single cell of 84 ± 33 pmol N d^{-1} (±s.e.m.).

Using two different methodologies we have thus shown that the intracellular nitrate in the foraminifera is denitrified. Little is know about the physiology of foraminifera, however, and the denitrification rates *in situ* may well be significantly higher or lower than the rates reported here based on laboratory incubations.

There are two possible mediators of the observed denitrification inside foraminifera: endosymbiotic nitrate-reducing bacteria or denitrifying enzymes in the foraminifera's own organelles.

As an efficient denitrifying prokaryote has a specific denitrification capacity of about 40 fmol N $cell^{-1}$ d^{-1} (see Supplementary Information)[8], the denitrification rate measured in *G. pseudospinescens* would necessitate the presence of between 5,650 and 22,550 endosymbiotic denitrifying bacteria in each cell. To quantify bacteria that might be involved in nitrate respiration we analysed *G. pseudospinescens* by fluorescence *in situ* hybridization (FISH) and scanning electron microscopy (SEM). In addition, we examined its cellular ultrastructure by transmission electron microscopy (TEM) of thin sections. Analysis of individual *G. pseudospinescens* with 4′,6-diamidino-2-phenylindole (DAPI) and FISH probes for members of Archaea or Bacteria revealed less than 100 prokaryotic cells on the surface of each specimen. Crushing the foraminifera did not release more prokaryotic cells. Sediment samples from the same location used as controls for the FISH analyses contained 2.7 ± 0.4 × 10^9 bacterial cells per ml of sediment. Examination of the foraminifera by SEM and TEM supported the FISH results: there were very few prokaryotic cells attached to their surface and no endosymbionts present. The TEM micrographs of fully sectioned cells revealed an intact cytoplasm with mitochondria and other organelles. No bacterial infestation was visible other than occasional occurrences within food vacuoles. These findings therefore strongly suggest that denitrification should be ascribed to the foraminifera's own organelles, for instance, their mitochondria.

Non-symbiotic nitrate respiration is known to occur in three eukaryotes—the anaerobic freshwater protozoa *Loxodes* spp., which only reduces nitrate as far as nitrite[9], and the fungi *Fusarium*

Table 1 | Intracellular nitrate content and concentration in foraminifera

Location	Maximum NO_3^- concentration in pore water (µM)	Species	Intracellular NO_3^- content (pmol per cell)		Intracellular NO_3^- concentration (mM)
			Mean	Range	
Gullmar Fjord, Sweden	17	*G. pseudospinescens*	18,000 (5,000, $n = 20$)	0–72,000	10 (2.3)
Station 18, Chile	12	*Nonionella* cf. *stella*	186 (25, $n = 43$)	8–794	35
Station 18, Chile	12	*Stainforthia* sp.	60 (9, $n = 26$)	0–172	180

The maximum ambient porewater nitrate concentration is shown for comparison. The s.e.m. and number of replicates are given in parentheses. The nitrate content of approximately 2 mm^3 control samples of sediment particles from the sites was less than 20 pmol in the Swedish sediment (that is, close to the detection limit for the method used at this site) and 4.6 ± 1.5 pmol in the Chilean sediment. Note that the intracellular nitrate concentrations should be considered minimum values as the estimates are based on shell volume, rather than cell volume or the volume of possible storage vacuoles. Shell volumes were as follows: *G. pseudospinescens*, 0.5–3.6 mm^3; *Nonionella* cf. *stella*, 5.2 × 10^{-3} ± 7.1 × 10^{-4} mm^3; *Stainforthia* sp., 3.3 × 10^{-4} ± 2.06 × 10^{-5} mm^3.

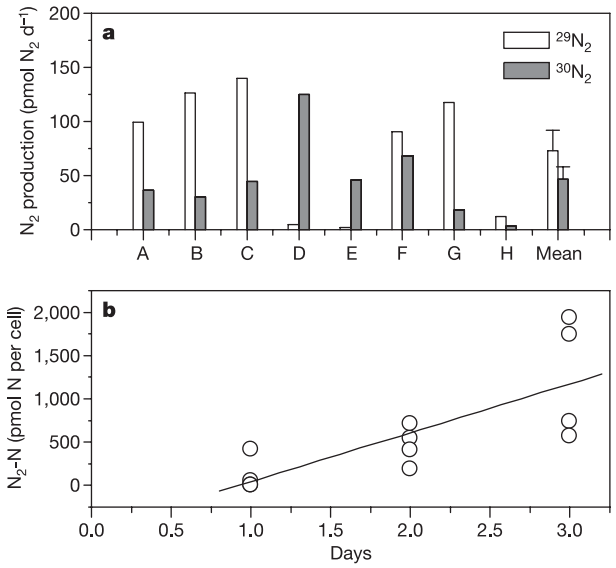

Figure 3 | N_2 production in *G. pseudospinescens*. **a**, $^{29}N_2$ and $^{30}N_2$ production in samples each containing two *G. pseudospinescens* cells and $^{15}NO_3^-$ (98.0 ± 0.05 ^{15}N atom%). Letters A–H, individual samples. Mean, values averaged across all samples. The error bars indicate the s.e.m. ($n = 8$). **b**, N_2-N (where N_2-N is dinitrogen expressed in units of N atoms) produced by individual *G. pseudospinescens* containing a ^{15}N-labelled intracellular nitrate pool. Circles represent discrete samples each containing two cells of *G. pseudospinescens*. The amount of N_2-N produced was calculated from the measured production of $^{29}N_2$ and $^{30}N_2$ during the incubations using the isotope pairing technique[26]. The regression line is shown ($R^2 = 0.58$, $P < 0.01$, $n = 12$).

oxysporum and *Cylindrocarpon tonkinense*[10], which reduce nitrate to nitrous oxide. Complete denitrification such as observed here for foraminifera has not been previously reported for eukaryotic cells.

A specific advantage of using nitrate for respiration is that it is possible to accumulate this non-toxic ion to high concentrations in the cell, thereby enabling respiration to be sustained during periods when nitrate is absent from the ambient environment. Assuming that the denitrification rate estimated for single *G. pseudospinescens* cells (that is, 565 pmol N d^{-1}) is representative for the organisms in their natural habitat, they would thus be able to survive in the absence of external nitrate sources for over a month using their intracellular nitrate pool of about 18 nmol NO_3^- (Table 1). This would explain their ability to remain in sediment layers lacking ambient oxygen and nitrate[4,11]. As *Globobulimina* is known to be motile and can also be found at the surface of sediments[4,11], the organism could replenish its nitrate pool there before migrating to deeper anoxic strata where competition and predation pressure are minimal. Temporary nitrate accumulation by foraminifera may also be beneficial in the oxygen-minimum zone of the continental shelf off Chile, where nitrate uptake by filamentous sulphur bacteria restricts the occurrence of nitrate to the sediment surface[12]. Given their nitrate storage and denitrification capabilities, *Nonionella* cf. *stella* could make short foraging trips into organic-matter-rich, nitrate-free sediment strata.

The present finding of combined nitrate accumulation and complete denitrification within eukaryotic cells indicates that the present conceptual view of marine denitrification as a bacteria-mediated process is incomplete. Denitrification of intracellular nitrate in motile foraminifera represents a hitherto unknown pathway for removal of nitrogen from the marine environment. Future studies addressing the generality among members of the Foraminifera of the traits discussed here will provide valuable information about the evolutionary origin of the trait and the quantitative importance of this pathway for global nitrogen cycling.

METHODS

Sampling was performed in Gullmar Fjord, Sweden (58° 19.4′ N; 11° 32.7′ E; Station S3 in ref.13) in August 2005 and at Station 18 off Concepcion Bay, Chile (36° 32.1′ S; 73° 7.1′ W)[14] in January 2006. The bottom water concentrations of oxygen and nitrate were 160 μM and 15 μM, respectively, in Gullmar Fjord (temperature 6 °C) and <1 μM and 12 μM, respectively, off Concepcion Bay (temperature 11 °C).

Nitrate/nitrite, and oxygen depth profiles ($n = 3$–5) were measured in sediment cores from Gullmar Fjord using a biosensor[15] and a Clark-type oxygen microsensor[16] as previously described[17]. The measurements were performed at 10 °C, which is the lower limit of the biosensor's operational range. The cell-bound nitrate pool was quantified in three cores using a described approach[18], except that cell lysis was promoted by placing sediment slices in centrifuge tubes and immersing them in boiling water for 10 min.

Foraminifera for nitrate measurements were collected from the 0.5–2-cm core depth interval (Swedish sediment) or from the surface of the cores (Chilean sediment). They were gently cleaned with a brush and kept overnight in Petri dishes containing sea water. Only specimens showing renewed collection of material at the aperture were used for the measurements.

The nitrate content of individual *G. pseudospinescens* was measured using the VCl_3 reduction method[19] on a chemiluminescence detector (Model 42c; Thermo Environmental Instruments). Before nitrate determination each foraminifer was measured for estimation of shell volume, rinsed in nitrate-free artificial sea water, dried, decalcified for 1 h at 4 °C in 100 μl of IUPAC buffer solution (pH 4; Radiometer analytical) and then crushed. Nitrate was measured in 12–25-μl subsamples. The specimens of *Nonionella* cf. *stella* and *Stainforthia* sp. were inserted directly into 0.6 ml of 2 M HCl solution with VCl_3 held at 80 °C in a reduction chamber connected to the detector (Model CLD 86; Eco Physics AG). Their shell volume was estimated from the linear dimensions of a separate batch of specimens. The nitrate content of sediment samples (approximately 2 mm^3; $n = 11$ from each site) was determined following the same protocol as for the foraminifera. Intracellular nitrate concentration was estimated from the measured nitrate content and the estimated shell volume.

Quantitative FISH with the probe SD-Arch-0915-aA-20 specific for Archaea, the EUBmix (SD-Bact-0338-aA-18, S-*-BactP-0338-aA-18, S-*-BactV-0338-aA-18) specific for Bacteria (Thermo Electron Corporation), and the general DNA stain (DAPI) was performed on 12 *G. pseudospinescens*; all appropriate controls being included[20]. Control hybridization studies of sieved sediment from the same sampling confirmed that bacteria could be identified and quantified by FISH if present. SEM and TEM were performed on six *G. pseudospinescens* as previously described[21]. One complete cell was sectioned at 0.5–1-μm intervals and examined by light microscopy. From these sections, around 100 randomly selected ultra-thin sections (100 nm) were used for TEM of the cytoplasm.

Denitrification in *G. pseudospinescens* was measured using ^{15}N methods in two experimental rounds. Round one: two series of 6-ml glass vials (Exetainer; Labco) each containing two cells in filter-sterilized (0.2 μm; Sartorius) oxygen-free, He-flushed medium (based on artificial sea water) with either 500 μM $^{15}NO_3^-$ (98 atom% Cambridge Isotope Laboratories), 500 μM acetate and 500 μM $^{14}NH_4Cl$ (denitrification medium; $n = 8$) or 500 μM $^{15}NH_4^+$ and 500 μM $^{14}NO_3^-$ (anammox medium; $n = 7$) was incubated for 24–48 h at 10 °C. Thereafter 4 ml of medium was removed from the vials[22] for determination of $^{15}NO_3^-$ atom%[23] and 100 μl of He-flushed $ZnCl_2$ was injected through the rubber septum of the vials to halt N_2 production[7,22]. $^{29}N_2$ and $^{30}N_2$ were determined as described previously[22].

Round two: *G. pseudospinescens* containing ^{15}N-labelled intracellular nitrate pools were prepared through incubation of intact cores in 30-l tanks for 8 or 9 weeks at 10 °C with 50 μM $^{15}NO_3^-$ in the overlying water. The 8-week incubations were used for determining the ^{15}N atom% of intracellular nitrate using a standard method[23] modified for small volumes. The 9-week incubations were used for measuring denitrification. Twelve Exetainers were prepared with 2 ml of sterile nitrate-free and oxygen-free medium based on sediment pore water[24] with autoclaved algal cells (*Dunaliella* sp.). Two *G. pseudospinescens* were added to each vial, the vials capped and the headspace flushed with He for 4 min. Incubation was halted at 24-h intervals within a 72-h incubation period as described above. Control experiments based on fluorescein diacetate staining[25] revealed 100% survival of *G. pseudospinescens* after incubation for more than 72 h under these conditions.

Denitrification in *Nonionella* cf. *stella* was measured using N_2O microelectrodes and acetylene inhibition (see Supplementary Information for details).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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