Potential importance of physiologically diverse benthic foraminifera in sedimentary nitrate storage and respiration

Joan M. Bernhard,[1] Karen L. Casciotti,[2,3] Matthew R. McIlvin,[2] David J. Beaudoin,[4] Pieter T. Visscher,[5] and Virginia P. Edgcomb[1]

Received 13 January 2012; revised 8 May 2012; accepted 10 May 2012; published 3 July 2012.

[1] Until recently, the process of denitrification (conversion of nitrate or nitrite to gaseous products) was thought to be performed exclusively by prokaryotes and fungi. The finding that foraminifera perform complete denitrification could impact our understanding of nitrate removal in sediments as well as our understanding of eukaryotic respiration, especially if it is widespread. However, details of this process and the subcellular location of these reactions in foraminifera remain uncertain. For example, prokaryotic endobionts, rather than the foraminifer proper, could perform denitrification, as has been shown recently in an allogromiid foraminifer. Here, intracellular nitrate concentrations and isotope ratios ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) were measured to assess the nitrate dynamics in four benthic foraminiferal species (*Bolivina argentea, Buliminella tenuata, Fursenkoina cornuta, Nonionella stella*) with differing cellular architecture and associations with microbial endobionts, recovered from Santa Barbara Basin, California. Cellular nitrate concentrations were high (12–217 mM) in each species, and intracellular nitrate often had elevated $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values. Experiments including suboxic and anoxic incubations of *B. argentea* revealed a decrease in intracellular nitrate concentration and an increase in $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ over time, indicating nitrate respiration and/or denitrification within the foraminifera. Results illustrate that nitrate reduction occurs in a range of foraminiferal species, including some possessing endobionts (including a chloroplast-sequestering species) and others lacking endobionts, implying that microbial associates may not solely be responsible for this process in foraminifera. Furthermore, we show that benthic foraminifera may represent important reservoirs of nitrate storage in sediments, as well as mediators of its removal.

Citation: Bernhard, J. M., K. L. Casciotti, M. R. McIlvin, D. J. Beaudoin, P. T. Visscher, and V. P. Edgcomb (2012), Potential importance of physiologically diverse benthic foraminifera in sedimentary nitrate storage and respiration, *J. Geophys. Res.*, *117*, G03002, doi:10.1029/2012JG001949.

1. Introduction

[2] Nitrogen can be a limiting nutrient in the marine realm. Bioavailable nitrogen occurs mainly in the forms of nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+), which can be converted to less accessible nitrogen gas (N_2) by the processes of denitrification (NO_3^- to N_2) and anammox (anaerobic ammonium oxidation; NO_2^- and NH_4^+ to N_2). These processes occur in marine sediments and portions of the water column with oxygen levels below ~5 μM. Denitrification was thought to be performed exclusively by prokaryotes (eubacteria and archaea) and a few fungi until a report suggested that denitrification is performed by certain foraminifera [*Risgaard-Petersen et al.*, 2006]. Additional recent reports support this finding for a relatively wide range of foraminifera [i.e., *Høgslund et al.*, 2008; *Koho et al.*, 2011; *Piña-Ochoa et al.*, 2010]. This important observation impacts our understanding of nitrogen cycling in the marine environment as well as fundamental concepts of protistan respiration. Whether foraminifera themselves perform denitrification or host symbionts that perform denitrification, the capacity of foraminifera to store large quantities of nitrate and migrate in sediments to reach the optimal redox potential for denitrification may affect the relationship between

[1]Geology and Geophysics Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, USA.
[2]Marine Chemistry and Geochemistry Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, USA.
[3]Now at Department of Environmental Earth System Science, Stanford University, Stanford, California, USA.
[4]Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, USA.
[5]Department of Marine Sciences, University of Connecticut, Groton, Connecticut, USA.

Corresponding author: J. M. Bernhard, Geology and Geophysics Department, Woods Hole Oceanographic Institution, MS 52, Woods Hole, MA 02543, USA. (jbernhard@whoi.edu)

0148-0227/12/2012JG001949

bottom water conditions and denitrification potential in sediments.

[3] Currently, the details of the denitrification process and the subcellular localization of denitrification subreactions in most foraminifera remain unclear. A recent study found that a gene for nitrite reduction, the second step in the denitrification process, was localized to endobionts in an allogromiid foraminiferal species, indicating that this process is performed by the endobionts, not the eukaryote itself in this case [*Bernhard et al.*, 2012]. Sequence analysis and fluorescent in situ hybridization (FISH) approaches led to the conclusion that the endobionts of this allogromiid foraminifer are likely denitrifying pseudomonads. It should be recognized that there is a wide variety of physiologies, morphologies, and lifestyles among benthic foraminifera [e.g., *Sen Gupta*, 1999]; these attributes may lead to differing physiological strategies for survival in low-oxygen and anoxic sediments.

[4] In an effort to gain more insights into the role of foraminifera in benthic nitrogen cycling, a series of experiments, field analyses, and laboratory analyses were performed on four foraminiferal species that live with or near the allogromiid foraminifer found to have denitrifying endobionts [*Bernhard et al.*, 2012]. The species analyzed in this study, all of which have calcareous tests (shells), were *Nonionella stella*, *Bulliminella tenuata*, *Fursenkoina cornuta*, and *Bolivina argentea.* Among these species, *N. stella* is the only one to have been previously assayed for denitrification [*Risgaard-Petersen et al.*, 2006], although other species of *Bolivina* have been found to denitrify (*B. seminuda, B. plicata, B. subaenariensis*) [*Piña-Ochoa et al.*, 2010]. The abundances of the four species studied here are known to vary in the Santa Barbara Basin (SBB, California, USA) along oxygen-concentration and water-depth gradients (0−∼15 μM O_2; ∼430–595 m, respectively), with *N. stella* tolerant of anoxia, *B. tenuata* and *F. cornuta* occurring in varved sediments with detectable but depleted oxygen (∼2–15 μM O_2 and ∼550–585 m water depth) [*Bernhard et al.*, 1997; *Bernhard et al.*, 2000], and *B. argentea* occurring in very high densities in bioturbated sediments from shallower water depths (430 m; ∼3.5–15.4 μM O_2) [*Bernhard et al.*, 1997]. With respect to cell physiology and ultrastructure, *N. stella* harbors chloroplasts [*Bernhard and Bowser*, 1999; *Grzymski et al.*, 2002], *B. tenuata* in SBB harbor rod-shaped endobionts [*Bernhard et al.*, 2000; *Buck and Bernhard*, 2001], and, at the beginning of this study, we hypothesized that *B. argentea* lacked symbionts given its prevalence in more aerated habitats [*Bernhard et al.*, 1997]. The ultrastructural attributes of *F. cornuta* were unknown at the beginning of this study. Our aim was to study nitrate accumulation and reduction in a variety of foraminiferal physiologies such as symbiont-bearing, non-symbiont bearing, and chloroplast-sequestering taxa in order to assess their potential importance in benthic nitrogen cycling.

2. Materials and Methods

2.1. Sites

[5] Foraminifera were collected from sediments in and near SBB; which is centered at 34°13.5′N, 120°02′W off Southern California (USA). The SBB has restricted water circulation below the depth of a sill (∼475 m) [e.g., *Reimers et al.*, 1990], isolating it from the Southern California Bight; the deepest part of the Basin (∼570–600 m) commonly supports copious growth of the filamentous sulfur-oxidizing bacterium *Beggiatoa* [e.g., *Bernhard et al.*, 2003]. Sediment samples were collected from multiple water depths using either an MC-800 multicorer or a Soutar boxcorer, during five different sampling events between 2007 and 2010 [*Bernhard et al.*, 2012]. Most targeted sites were ≥570 m water depth, where bottom waters are typically greatly depleted in or even lacking dissolved oxygen [*Bernhard et al.*, 1997; *Bernhard et al.*, 2006] and contain sulfide, sometimes in high concentrations [*Bernhard et al.*, 2003; *Kuwabara et al.*, 1999]. A shallower site on the SBB periphery at 430 m was also sampled to obtain foraminifera from a relatively aerated habitat [*Bernhard et al.*, 1997] compared to the deeper sites. Collections from that 430 m-deep site served as the source of *B. argentea.*

2.2. Cellular Ultrastructure

[6] Transmission Electron Microscopy (TEM) was performed on *F. cornuta* and *B. argentea.* Foraminiferal-bearing sediments were fixed in 3% TEM-grade glutaraldehyde buffered with 0.1 M sodium-cacodylate acid (pH 7.2) within ∼30 min of sediment recovery. Samples were kept chilled until aliquots were sieved over a 90-μm screen using chilled (5°C) buffer. Specimens were processed for TEM using our standard methods [e.g., *Bernhard et al.*, 2000] and examined with either a Zeiss 902A or a Zeiss 10CA Transmission Electron Microscope.

2.3. Collection of Samples for Chemical Analyses

[7] To determine in situ intracellular nitrate concentrations and nitrate isotope values of foraminifera, small sediment cores (2.6-cm inner diameter) were obtained from boxcores immediately after the corer was secured on deck. The cores were securely capped on each end and were taken into an environmental van set at 5°C (±3°C), which is similar to bottom water temperature of ∼6°C (www.CalCOFI.org). Within ∼20 min, the overlying water was removed for nitrate analyses (see below) and each core was sectioned into 1-cm intervals to a depth of 3 cm. Pore waters were isolated from each sediment interval by allowing coarse sediments (and foraminifera) to settle for a short period (∼30 s), after which water was carefully pipetted from settled sediments. These pore waters were separated from entrained fine sediments by successive centrifugation (5000 g for 10 min, repeated 2 or 3 times until clear). The settled coarse (noncentrifuged) sediments served as the source for foraminifera for these analyses. Specimens were isolated from the coarse residue after sieving sediments with a 90-μm screen, rinsed twice in 0.2 μm-filtered, nitrate-free seawater, measured for length and diameter or width, and individually air-dried in acid-cleaned tubes. Further procedures are described in Section 2.6.

2.4. Maintenance of Live Specimens

[8] To serve as a source for living specimens for experimental manipulations at a later time, sediments collected from >570 m that were taken to our shore-based laboratory were maintained in tightly closed bottles in a 7°C environmental room. In contrast, sediments from the aerated (430 m) site were placed on a recirculating seawater system in the same 7°C environmental room and fed weekly an algal mixture of *Dunaliella tertiolecta* and *Isochrysis galbana* [e.g., *Filipsson et al.*, 2010], for up to six months.

2.5. DNA Extraction, PCR Amplification, Alignment and Phylogenetic Analysis

[9] DNA was extracted from ~10–20 conspecifics obtained from sediments, after briefly washing with sterile seawater and homogenizing in lysis buffer, using either a standard CTAB extraction protocol [*Winnepenninckx et al.*, 1993] or the Qiagen DNeasy Plant DNA Extraction kit. The same DNA extraction protocol did not work for all organisms, most likely due to unidentified differences between species. For *B. argentea* and *B. tenuata*, 18S rRNA gene fragments were amplified from foraminiferal DNA extracts using the primers S14F3A [*Habura et al.*, 2004] or S14F1 with Rib sB [*Pawlowski*, 2000]. PCR conditions were: 95°C for 2 min, 48°C for 1 min, and 70°C for 2 min followed by 35 cycles of 30 s at 95°C, 1 min at 48°C, and 2 min at 70°C followed by 95°C for 30 s, 48°C for 1 min, and 70°C for 15 min. 16S rRNA genes were amplified from the symbiont-bearing *B. tenuata* using primers 8F [*Lane*, 1991] and 1492R [*Longnecker and Reysenbach*, 2001]. PCR conditions were: 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 45°C for 1 min, and 72°C for 90 s followed by an additional 7-min 72°C incubation. Dissimilatory nitrite reductase genes (*nirK*, *nirS*) were PCR amplified from 3 species by touchdown PCR using extracted DNA and published primers and protocols: Cunir3/Cunir4 [*Casciotti and Ward*, 2001] for *B. argentea*, Cunir4/nirK1F [*Braker et al.*, 1998] for *N. stella*, and FlaCu/R3Cu [*Hallin and Lindgren*, 1999] for *B. tenuata.* Touchdown PCR conditions were: 95°C for 2 min followed by 10 cycles of 94°C for 30 s, 45–50°C for 40 s, and 72°C for 40 s with −0.5°C/cycle followed by 31 cycles of 94°C for 30 s, 43–50°C for 40 s, and 72°C for 40 s followed by 70°C for 7 min.

[10] PCR products from positive amplifications were gel purified using the Qiaquick Gel Extraction Kit (Qiagen) and cloned into the pCR4 vector in the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Selected clones were sequenced using an Applied Biosystems 3730XL capillary sequencer. Sequences were then edited and assembled into contigs using Sequencher (Gene Codes Corporation). Chimeric and low-quality sequences were removed from further analyses by visual inspection and by using the CHECK_CHIMERA program [*Cole et al.*, 2003].

[11] Clone sequences were aligned using the autoaligner function within the software ARB [*Ludwig et al.*, 2004] using the SILVA Reference database, and then the alignment was manually corrected using secondary structure information. Only reliably aligned sites were included in subsequent phylogenetic analyses. Bootstrapping and determination of the best estimate of the ML tree topology were conducted with the Rapid Bootstrapping algorithm of RAxML (1000 bootstrap replicates) version 7.0 under the GTR+I model running on the CIPRES portal [*Stamatakis*, 2006; *Stamatakis et al.*, 2008] (www.phylo.org).

2.6. Nitrate Content and Isotopic Composition

[12] To determine intracellular nitrate concentrations, air-dried individuals (isolated and rinsed twice in nitrate-free seawater on the ship, as noted above) were analyzed as described in *Bernhard et al.* [2012]. Briefly, nitrate content was measured using isotope ratio mass spectrometry (IRMS) and the denitrifier method [*Casciotti et al.*, 2002; *Sigman et al.*, 2001], which relies on conversion of NO_3^- to N_2O and sensitive detection of N_2O via IRMS. The size variation in targeted foraminiferal species was substantial, so while analyses could be conducted on individual foraminifera for three of the species (*B. argentea; F. cornuta; B. tenuata*), a pooled sample of 5–10 specimens was typically required for analyses of *N. stella.* Air-dried specimens were dissolved overnight in 100 μL of 1% acetic acid in distilled deionized water. These extracts were injected into replicate 20 mL headspace vials containing 3 mL of the denitrifier cultures [*McIlvin and Casciotti*, 2011] that had been purged with high purity nitrogen gas for four hours prior to injection of the sample to remove all background nitrate, nitrite, and N_2O from the vials. The resulting N_2O was quantified against low-level (0.1–2.5 nmol) nitrate standards (also injected in 50 μL volumes) using the peak area of the major ion beam (m/z = 44). Typical standard regressions yielded signals of around 1 mV·s per pmol NO_3^-, with an uncertainty on the regression of less than 2%. Major ion peak areas from 100 μL injections of the 1% acetic acid solution were within 18 mV·s of the denitrifier vials with no additions, reflecting a small and consistent blank of less than 20 pmol per 100 μL, or 10 pmol per 50 μL sample. Intracellular nitrate concentrations were determined from the nitrate contents using recorded dimensions and volume calculations for idealized geometric shapes. *B. tenuata* was considered a cone $(1/3\pi[1/2W]^2L$, where L = length and W = width); *B. argentea* considered an flattened elliptical cone $(1/8\pi L[1/2W]^2)$; *F. cornuta* a prolate spheroid $(4/3\pi[1/2L][1/2W]^2)$; *N. stella* an oblate spheroid $(4/3\pi[1/2L]^2[1/2W])$.

[13] When nitrate content was sufficient (>0.5 nmol), the nitrate $\delta^{15}N$ ($\delta^{15}N_{NO3} = (({}^{15}N/{}^{14}N)_{NO3} \div ({}^{15}N/{}^{14}N)_{AIR} - 1)$ * 1000) and $\delta^{18}O$ ($\delta^{18}O_{NO3} = (({}^{18}O/{}^{16}O)_{NO3} \div ({}^{18}O/{}^{16}O)_{VSMOW} - 1)$ * 1000) were determined for the sample. $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ were normalized to the AIR and VSMOW reference scales, respectively, by analysis of nitrate reference materials USGS32 ($\delta^{15}N$ = +180‰ versus AIR, $\delta^{18}O$ = +25.7‰ versus VSMOW), USGS34 ($\delta^{15}N$ = −1.8‰ versus AIR, $\delta^{18}O$ = −27.9‰ versus VSMOW), and USGS35 (used for $\delta^{18}O$ only; $\delta^{18}O$ = +57.5‰ versus VSMOW [*Böhlke et al.*, 2003]) at 0.5–5.0 nmol levels. Triplicate analyses of each nitrate standard were performed at four nmol levels for each batch of samples (0.5, 1.0, 2.5, and 5.0). Standards were made fresh for each run from 200 μM stock nitrate solutions, and standards, samples, and acetic acid blanks were all injected into prepared vials in 50 μL volumes. Isotopic values of the samples were calculated using the isotopic standards that bracketed the amount of nitrate in the sample to minimize blank effects. Some *B. argentea* (n = 21) were also analyzed for the presence of nitrite by treating an aliquot of the acetic acid digest from each of the specimens with sulfamic acid to remove any nitrite [*Granger and Sigman*, 2009]. Both treated and untreated aliquots were then analyzed for nitrate concentration and isotopic composition using the methods described above, and the difference between the two was used to determine nitrite content in each of the 21 specimens.

2.7. Laboratory Experiments With *B. argentea*

[14] To establish whether the concentration and $\delta^{15}N$ value of nitrate contained in foraminifera was altered in response to exposure to environmental redox perturbations,

a manipulative experiment was conducted using *Bolivina argentea* collected from the 430-m site that had been maintained under oxic conditions in the laboratory environmental room. *B. argentea* were picked from these sediments into 50-ml glass tubes containing a 2% agar plug and low nutrient seawater that was either (1) fully aerated, (2) purged with N_2 gas, or (3) purged with N_2 gas in addition to the agar plug being permeated with 100 mM H_2S. For this first experiment, two types of seawater were used: artificial seawater (Instant Ocean®) and natural aged (nitrate free) seawater from the Sargasso Sea. The first experiment lasted 14 days.

[15] In a second experiment, freshly collected *B. argentea* were incubated in He-purged bottom water collected from the site using the same three treatments described above (aerated, N_2-purged and N_2-purged with H_2S added). For this (second) experiment, half of the vials from each treatment also received an antibiotic treatment of mixed chloramphenicol (0.3%), dihydrostreptomycin sulfate (1%), neomycin sulfate (1.5%), K penicillin G (2.5%), and tetracycline HCl (0.4%), which is known not to inhibit growth or reproduction in certain benthic foraminifera [*Pierce*, 1965]. The foraminifera were incubated for 1 (T_1) or 2 weeks (T_2) at 7°C under these conditions, then individually sorted and analyzed for $[NO_3^-]$, $\delta^{15}N_{NO3}$, and $\delta^{18}O_{NO3}$ as described above. For reference, a set of individuals were collected and analyzed as described at each experiments' initiation (T_0).

2.8. Denitrification Rate Measurements

[16] Two separate experiments were carried out to measure denitrification potential (^{15}N-labeled N_2 production from $^{15}NO_3^-$) by *B. argentea* and *F. cornuta.* Individual foraminifera were picked in groups of 5 (February 2009) or 10 (June 2009) into replicate 12 mL Exetainer® vials containing N_2-purged 0.2 μm filtered natural bottom water obtained from the collection site. The vials were sealed without a gas headspace and 12 μL of 50 mM 95-atom % $^{15}NO_3^-$ was added, for a final NO_3^- concentration near 80 μM and atom % $^{15}N - NO_3^-$ of 60%. In the first $^{15}NO_3^-$ experiment (February 2009), vials were killed with $HgCl_2$ after incubating for 0, 6, 12, 24, 36, and 48 h (*B. argentea*) or 48 h (*F. cornuta*) at 7°C. In the second $^{15}NO_3^-$ experiment (June 2009), vial contents were killed with $HgCl_2$ after 0, 26, 51, 75, and 98 h (*B. argentea* only). Controls containing the N_2-purged filtered seawater, but no added foraminifera, were incubated in parallel.

[17] Production of ^{15}N-labeled N_2 (both $^{29}N_2$ and $^{30}N_2$) was determined by membrane inlet mass spectrometry in the Giblin laboratory at the Ecosystems Center of the Marine Biological Laboratory (MBL, Woods Hole, MA). Samples were analyzed for $^{28}N_2$:Ar, $^{29}N_2$:Ar, and $^{30}N_2$:Ar, and were normalized to the same measurements from air-equilibrated seawater held at the same temperature as the samples (technique described by *Kana et al.* [1994]). Denitrification rates (pmol/day) were calculated using the isotope pairing technique [*Nielsen*, 1992], which includes production of both $^{29}N_2$ and $^{30}N_2$; rates were divided by the number of individuals to give a cell-specific rate. Atom % $^{15}NO_3^-$ in the incubations was verified by isotope ratio mass spectrometry at the University of California Davis after conversion to N_2O using the denitrifier method [*Sigman et al.*, 2001]. These samples were analyzed by purging and trapping the N_2O cryogenically using a Finnigan GasBench and PreCon in line with a Finnigan Delta V Plus isotope ratio mass spectrometer. Atom % $^{15}NO_3^-$ values were normalized to aliquots of $^{15}NO_3^-$ (99.5%) analyzed in parallel.

3. Results

3.1. Cellular Ultrastructure

[18] All four species investigated in this study contain large vacuoles (Figures 1b–1c and 1f–1h) that appear to lack particulate material (i.e., not food vacuoles). Prior publications have noted the plethora of large vacuoles in foraminiferal cytoplasm [e.g., *Bernhard and Reimers*, 1991; *Bernhard and Bowser*, 2008], and some have asserted that these large vacuoles contain seawater with high concentrations (70 ± 49 mM) of nitrate [e.g., *Bernhard et al.*, 2012]. *Bolivina argentea* exhibits typical benthic foraminiferal cell ultrastructure in that mitochondria can be concentrated at the cell periphery and at pore plugs (Figure 1a). Also, *B. argentea* cytoplasm occasionally includes chloroplasts, although these can be in a state of significant decay (Figure 1c) or can appear intact and, thus, in a state of potential sequestration (Figure 1c). *B. argentea* appears to be a detritivore (Figure 1b) as does *F. cornuta* (Figure 1f). Some *F. cornuta* vacuoles contained ingested bacteria (Figure 1f), but their sparse abundance and inconsistent presence among specimens is not indicative of symbiosis (compare Figure 1f to Figure 1h). Thus, *F. cornuta* from SBB are considered symbiont-free bacterivores. Our observations are also consistent with *N. stella* harboring plastids (Figure 1g) and *B. tenuata* harboring rod-shaped endobionts (Figure 1h) [*Bernhard et al.*, 2000; *Buck and Bernhard*, 2001]. In sum, these new TEM results and prior knowledge indicate that the species analyzed for this study included a variety of cell physiologies (Table 1), ranging from non-symbiont bearing taxa (*B. argentea*; *F. cornuta*) to a symbiont-bearing species (*B. tenuata*) to a plastid-harboring species (*N. stella*).

3.2. Sequence Analysis

[19] The 18S rRNA gene sequence obtained from *Bolivina argentea* indicates that it is closely related to *Bolivina subaenariensis*, as indicated by BLAST analysis (94% sequence similarity). Specimens of *Buliminella tenuata*, as identified by morphology [i.e., *Bernhard and Bowser*, 2008; *Bernhard et al.*, 1997; *Bernhard et al.*, 2000; *Cannariato et al.*, 1999; *Stott et al.*, 2002], were sequenced from the shallower (430 m) site as well as deeper sites (>580 m). BLAST analysis indicates that the 18S rRNA sequences obtained for *B. tenuata* from both water depths are nearly identical (2 nucleotide differences out of 1000) and group most closely with *Bulimina* sp. (GenBank ID: FM999863.1, 99% sequence similarity over 84% coverage). In a phylogenetic analysis incorporating the nearest relatives of this sequence, *B. tenuata* groups with *Bulimina* sp. with 80% bootstrap support under maximum likelihood (Figure 2). As noted, the SBB *Nonionella stella* 18S rRNA sequence was obtained previously [*Grzymski et al.*, 2002], and we did not sequence the 18S rRNA gene from *F. cornuta*, so that taxon's identification is based on morphology only. Small subunit rRNA sequences for *B. tenuata* and *B. argentea* have been deposited in GenBank (accession numbers JQ013743–JQ013745).

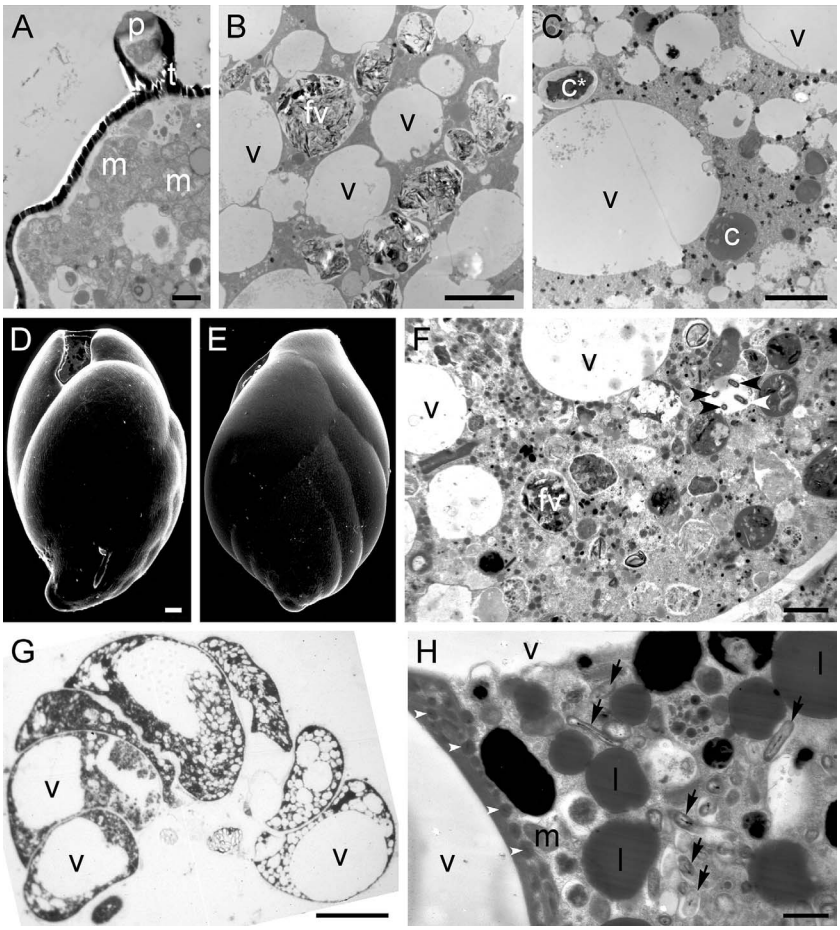

Figure 1. Micrographs of the four species studied. TEM micrographs of *Bolivina argentea*: (a) Image showing intact mitochondria (m) under a pore plug (p), t = test; (b) Image showing food vacuoles (fv) and "empty" vacuoles (v); (c) Image showing chloroplasts (c), one of which is being digested (c*) and one of which appears sequestered. Note lack of endobionts in Figures 1a–1c. *Fursenkoina cornuta*: (d, e) SEM micrographs showing overall morphology from two side views; (f) TEM micrograph showing vacuoles (v) and food vacuoles (fv), sometimes containing bacteria (black arrowheads). (g) Transmitted light micrograph through a section of *Nonionella stella* showing the large vacuoles (v) in most chambers. (h) TEM micrograph of *Buliminella tenuata* showing mitochondria (m), lipid (l), peroxisomes (white arrowheads) complexed with endoplasmic reticulum, vacuoles (v), and abundant rod-shaped endobionts (arrows). Scale bars equal 1 μm in Figures 1a and 1 h; 5 μm in Figures 1b, 1c, and 1f; 20 μm in Figure 1d; and 50 μm in Figure 1g.

[20] The 16S rRNA sequences obtained from pooled specimens of symbiont-bearing *B. tenuata* from the deeper (>580 m, low-oxygen) sites included sequences affiliating with uncultured gammaproteobacteria, sulfate-reducing deltaproteobacteria, and uncultured Fe-oxidizing bacteria. The most common sequence recovered (12/23 high-quality sequences) affiliated with the uncultured gammaproteobacteria, which we presume are the copious rod-shaped

Table 1. General Characteristics, Distributions, Detected Genes, Nitrate Concentrations and Nitrogen Isotope Values of the Four Species Studied[a]

	SBB Depth Range (m)	SBB [O_2] Range (μM)	Symbionts	Ref(s)	Nitrite Reduction Gene	[NO_3^-] (mM) ±SD (n)	$\delta^{15}N_{NO3}$ (‰) ±SD (n)
Bolivina argentea	431–537	2–15.4	none[b]	1	*nirS*	195.1 ± 160.3 (47)	25.6 ± 4.2 (39)
Buliminella tenuata	339–591	1.2–22.6	rod-shaped endobionts	1–3	*nirK*	217.4 ± 150.5 (13)	19.8 ± 0.9 (11)
Fursenkoina cornuta	431–578	1.2–15.4	none[b]	1	No data	125.2 ± 68.9 (18)	27.8 ± 5.8 (14)
Nonionella stella	431–591	0–15.4	sequestered plastids	1–2, 4–5	*nirS*	11.6 ± 15.7 (6[c])	36.4 ± 4.8 (3)

[a]Depth and oxygen concentration ranges and symbiont attributes are from published literature on SBB. References are as follows: 1 = *Bernhard et al.* [1997]; 2 = *Bernhard et al.* [2000]; 3 = *Buck and Bernhard* [2001]; 4 = *Grzymski et al.* [2002]; 5 = *Bernhard et al.* [2006].

[b]Ultrastructural observations presented here.

[c]Due to the small size of *N. stella*, each analysis required pooling of specimens. In this case, 6 analyses were made, each of from 1 to 7 pooled individuals. The mean accounts for this pooling.

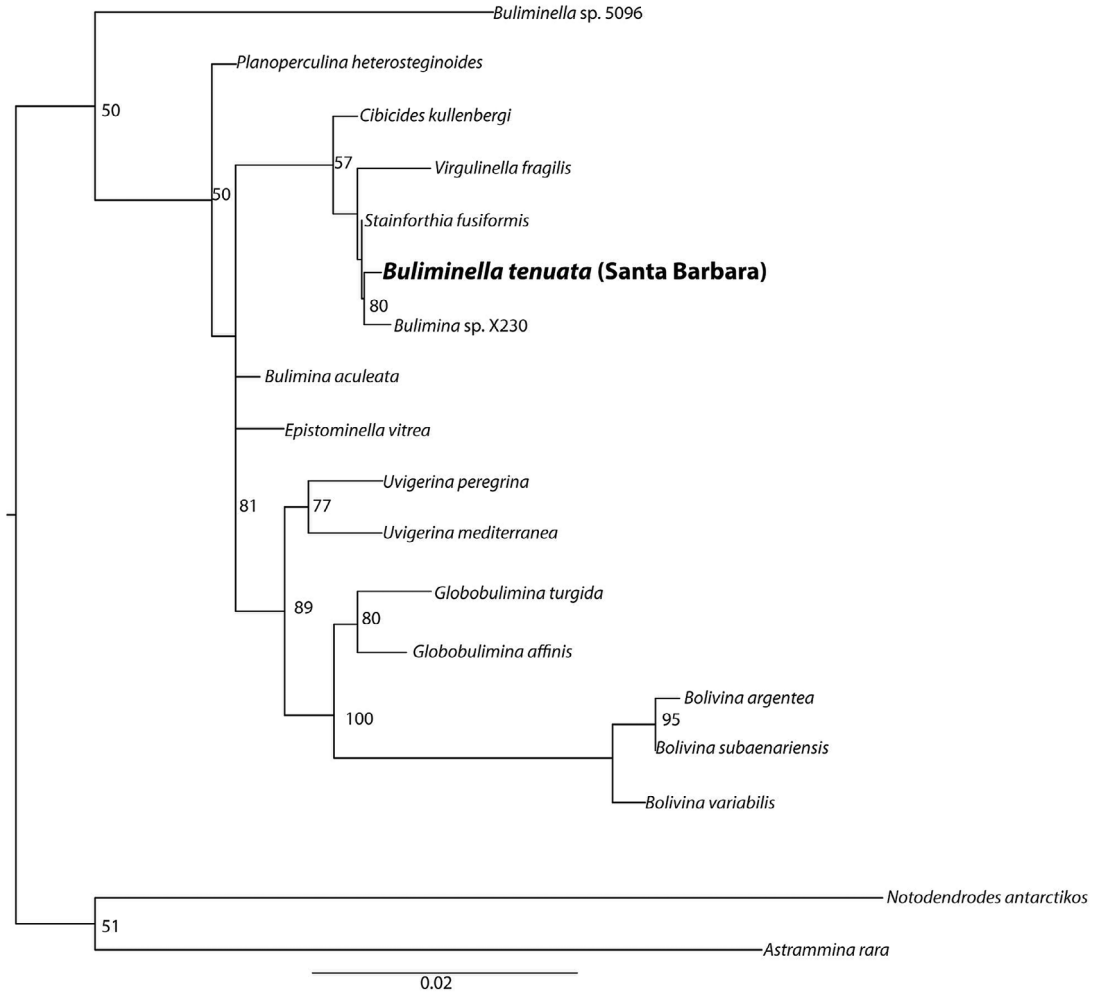

Figure 2. Phylogenetic analysis of the 18S rRNA gene from *Buliminella tenuata* from Santa Barbara Basin (>580 m). Maximum likelihood tree is based on an alignment of 559 nucleotides. Bootstrap support values ≥50% under maximum likelihood are shown at nodes. Scale is given as substitutions per site. See the description of our methods in section 2.5 for details.

endobionts (Figure 1h) [see also *Bernhard et al.* 2000; *Buck and Bernhard* 2001]. In *B. tenuata* specimens collected from the aerated site (430 m), we only recovered one sequence type (n = 18 out of 18 nonchimeric sequences) also affiliating with the gammaproteobacteria. In preliminary phylogenetic analyses within ARB, all the 16S rRNA sequences obtained from *B. tenuata* from the aerated site (430 m) and 2 out of 12 sequences from the low oxygen (>580 m) sediments were 99% similar, and loosely affiliated with a clade of gammaproteobacterial sequences that include members of the Enterobacteriales and sequences of uncultured bacteria, some of which are from marine sediments (data not shown). Ten of twelve gammaproteobacterial sequences recovered from the *B. tenuata* in the low oxygen (>580 m) sediments were 99% similar and affiliated with members of Alteromonadacea and, in particular, the genus *Marinobacter.* Many known sulfide-oxidizers are gammaproteobacteria that also carry out denitrification [*Kelly and Wood*, 2006; *Robertson and Kuenen*, 2006]. The confirmation of this identity will constitute future work in our laboratories using fluorescent in situ hybridization (FISH) studies with fresh specimens. Bacterial small subunit rRNA sequences have been deposited in GenBank (accession numbers JQ846276 and JQ846277).

[21] The dissimilatory nitrite reductase gene *nirK* was detected only in DNA extracts of *B. tenuata* collected from >570-m water depth, while another nitrite reductase gene, *nirS*, was detected in DNA extracts from *N. stella* and *B. argentea* (*F. cornuta* was not screened for either *nir* gene). The *B. tenuata nirK* sequence was most closely related to a *nirK* sequence from a uncultured soil bacterium (GenBank ID:DQ784059) and the closest cultured relative based on BLAST analysis was *Rhodopseudomonas palustris*, a (phototrophic) purple nonsulfur bacterium. The two *nirS* sequences from *B. argentea* were most closely related to the *nirS* of uncultured bacteria from the Hai River, China (JF966833 and JF966815). Four *nirS* sequence types (differing by 1–3 bp over ~850 bp sequence length) were obtained from *N. stella* and all were related to uncultured bacterial *nirS* sequences (Jiaozhou Bay, China, GenBank ID EU048455; San Francisco Bay SF04–BC11–B07, GenBank ID GQ453735; Hai River, China, GenBank ID JF966844; and a sequence found in the *Cupriavidus metallidurans* complete genome, GenBank ID CP000352). *Cupriavidus*

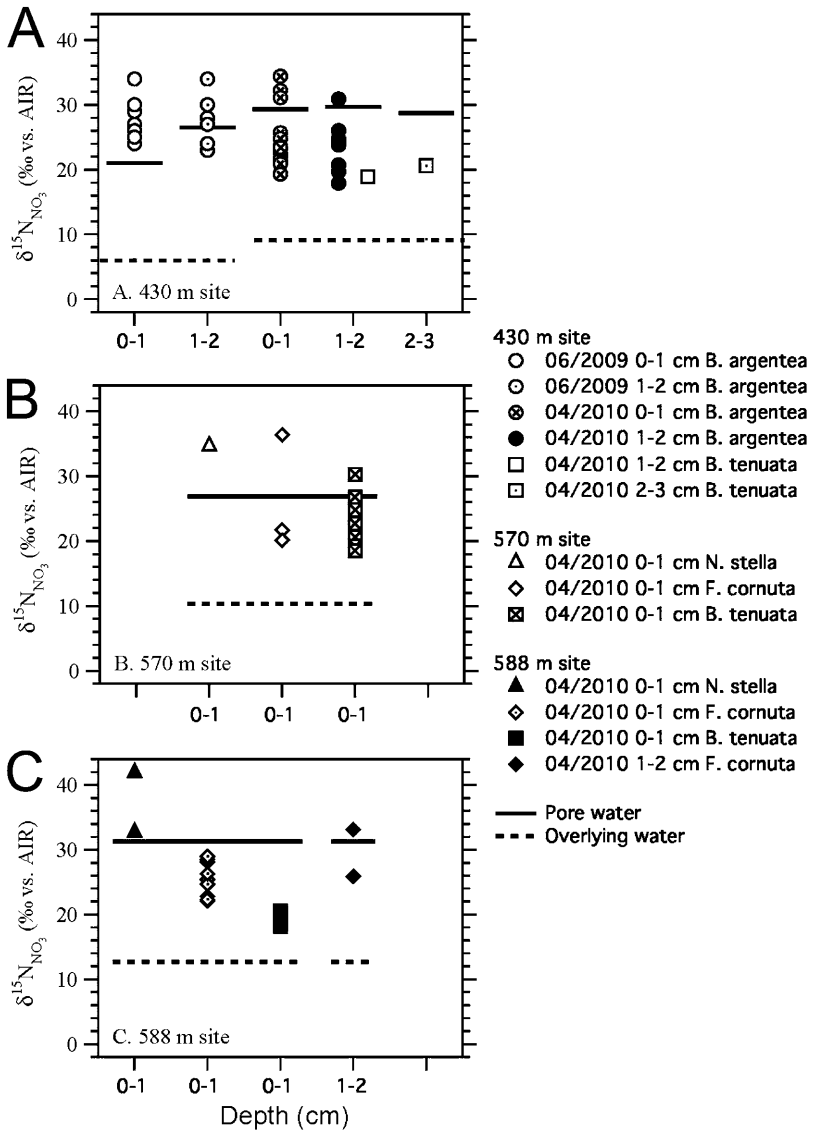

Figure 3. Plots showing $\delta^{15}N_{NO3}$ from SBB foraminifera, presented by species and site, with respect to co-occurring pore waters (solid horizontal lines) and overlying bottom (dashed lines) water. Different symbols reflect sediment depth interval and/or sampling date (see key).

metallidurans is a heavy metal-resistant facultative chemolithoautotrophic betaproteobacterium that appears to use reduced sulfur compounds as electron donors and to be capable of full denitrification by the successive reduction of nitrate to molecular nitrogen based on its possession of a *nirS* gene and other genes associated with denitrification [*Janssen et al.*, 2010]. Sequences for *nirK* and *nirS* obtained in this study have been deposited in GenBank (accession numbers JQ846278–JQ846284).

3.3. Nitrate Concentrations and Isotopic Ratios

[22] In specimens sacrificed at sea, nitrate contents were variable within populations of each species. Mean NO_3^- concentrations for each species were, however, quite high (Table 1) relative to bottom water and pore water nitrate concentrations. In those field-collected specimens with sufficient NO_3^- contents to measure $\delta^{15}N_{NO3}$ (>0.5 nmol) values ranged from 15 to 42‰ (Figure 3). Each species had $\delta^{15}N_{NO3}$ values higher than overlying bottom waters, although the relationship of foraminiferal $\delta^{15}N_{NO3}$ to pore water showed differences among foraminiferal species as well as between individuals of a single species. For example, *N. stella* contained $\delta^{15}N_{NO3}$ values consistently elevated relative to pore water (Figures 3b–3c), while *B. argentea* and *F. cornuta* had individuals with higher and lower $\delta^{15}N_{NO3}$ values (Figures 3a–3c). *B. tenuata* $\delta^{15}N_{NO3}$ values were generally lower than pore water values.

[23] *B. argentea* specimens were collected from the 430-m site in June 2009 and April 2010, and while the pore water $\delta^{15}N_{NO3}$ values showed some variation with depth in the sediment and between dates, *B. argentea* $\delta^{15}N_{NO3}$ values did not show significant differences in a Tukey-Kramer test of means ($p = 0.05$). *N. stella*, which were collected from surface sediments of the 570-m and 588-m sites in April 2010, contained NO_3^- with consistently elevated $\delta^{15}N$ values that did not differ significantly between the two sites (Tukey-

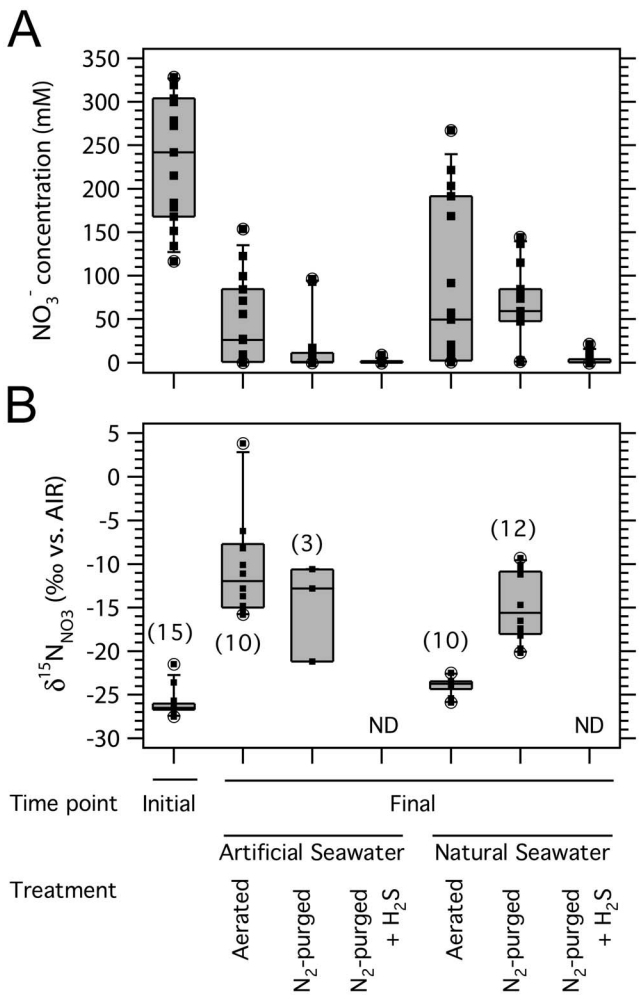

Figure 4. Box and whisker plots showing results from natural abundance Experiment 1, with *B. argentea.* (a) Intracellular nitrate concentrations just before the experiment began (Initial) and after 2 weeks in each treatment (Final). (b) $\delta^{15}N_{NO3}$ values in specimens with sufficient nitrate remaining to obtain a reliable analysis. Each box represents the 25th–75th percentile of individual measurements, while the bar inside each box represents the mean value (50th percentile), and the whiskers represent the full range of data for each treatment. Parenthetic values indicate numbers of specimens included in the analysis. ND = not determined because NO_3^- contents were too low for isotopic analysis. Circled dots indicate outliers.

Kramer test, p = 0.05). *F. cornuta* was also collected from the 570-m and 588-m sites in April 2010. These cells had mid-range $\delta^{15}N$ values: significantly lower (Tukey-Kramer test, p = 0.05) than *N. stella*, but higher than *B. tenuata* for cells collected from the 588-m site. Similar trends were also observed at the 570-m site, although the differences were not significant. *F. cornuta* $\delta^{15}N_{NO3}$ values were often lower than in pore water. *B. tenuata* $\delta^{15}N_{NO3}$ values were generally the lowest among the foraminiferal species, and rarely exceeded pore water $\delta^{15}N_{NO3}$. Regardless of site or depth, *B. tenuata* $\delta^{15}N_{NO3}$ values remained fairly constant at approximately 20‰ (Table 1). *B. argentea* and *F. cornuta* had $\delta^{15}N_{NO3}$ values around 26‰ and 28‰, respectively. *N. stella* had $\delta^{15}N_{NO3}$ around 36‰ (Table 1).

[24] For *B. argentea* (n = 17), the nitrate concentrations of aliquots from which nitrite was removed via sulfamic acid treatment were not significantly lower than those from paired aliquots without nitrite removal (Wilcoxon signed ranks, p > 0.05). The mean $\delta^{15}N_{NO3}$ values of these two populations also did not differ significantly (Paired t-test, p > 0.05). Although in almost all paired analyses, the concentration of nitrate was slightly less in samples that had been treated with sulfamic acid, the lack of statistical difference leads us to infer that the amount of NO_2^- was small, and not sufficient to affect the measured $\delta^{15}N_{NO3}$ values.

3.4. Laboratory Experiments

[25] At T_0 for Experiment 1, the *B. argentea* had high nitrate concentrations (Figure 4a) and very low $\delta^{15}N_{NO3}$ values (Figure 4b). We believe these patterns are due to their pre-incubation in our laboratory's recirculating seawater system, which contains artificial seawater (Instant Ocean®) with (reagent) nitrate containing an anomalously low $\delta^{15}N$ value (−27‰).

[26] At the end of Experiment 1 (t = 2 weeks), mean intracellular nitrate concentrations had decreased significantly in all treatments (Figure 4a), and mean $\delta^{15}N_{NO3}$ values had increased significantly, compared to T_0 (Tukey-Kramer test, p = 0.05) in all treatments except in aerated natural seawater (Figure 4b). Among the final NO_3^- concentration and $\delta^{15}N_{NO3}$ measurements, there were no significant differences between treatments in artificial seawater; however, in natural seawater addition of H_2S to the agar plug resulted in significantly lower nitrate concentrations than in N_2-purged seawater alone (Figure 4a; Tukey-Kramer test, p = 0.05). It should be noted that in many specimens incubated with N_2-purged artificial seawater (ASW), N_2 purged ASW + H_2S, and N_2-purged natural seawater (NSW) + H_2S, specimens contained less than 500 picomoles of nitrate, and thus, could not be reliably analyzed for $\delta^{15}N_{NO3}$. This could explain, for example, why mean $[NO_3^-]$ and $\delta^{15}N_{NO3}$ comparisons sometimes give contrasting results (e.g., aerated natural seawater).

[27] In general, for Experiment 1, high $\delta^{15}N_{NO3}$ values were associated with low NO_3^- concentrations, although low concentrations were not always associated with high $\delta^{15}N_{NO3}$ values (Figure 5a). This may represent a non-fractionating loss of NO_3^- (such as leakage) occurring in some of the individuals. Leakage could arise from cell lysis if a given specimen died during the experiment. However, it should be noted that the range in NO_3^- concentration among individuals at T_0 was quite high, although the ranges in $\delta^{15}N_{NO3}$ (Figure 4) and $\delta^{18}O_{NO3}$ (Figure 5b) were not. The trends in Figure 5a therefore may represent a population of foraminifera with different starting NO_3^- concentrations but similar $\delta^{15}N_{NO3}$ values undergoing a fractionating NO_3^- consumption process along different trajectories. This interpretation is partially supported by the proportional increase in both $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ (Figure 5b), which shows that at least 85% of the increase in $\delta^{15}N$ is paralleled by an increase in $\delta^{18}O_{NO3}$. Individual treatments did not yield significantly different $\delta^{18}O_{NO3}$ versus $\delta^{15}N_{NO3}$ slopes when regressed separately (95% CI overlapped). This suggests that initial $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ were relatively uniform among

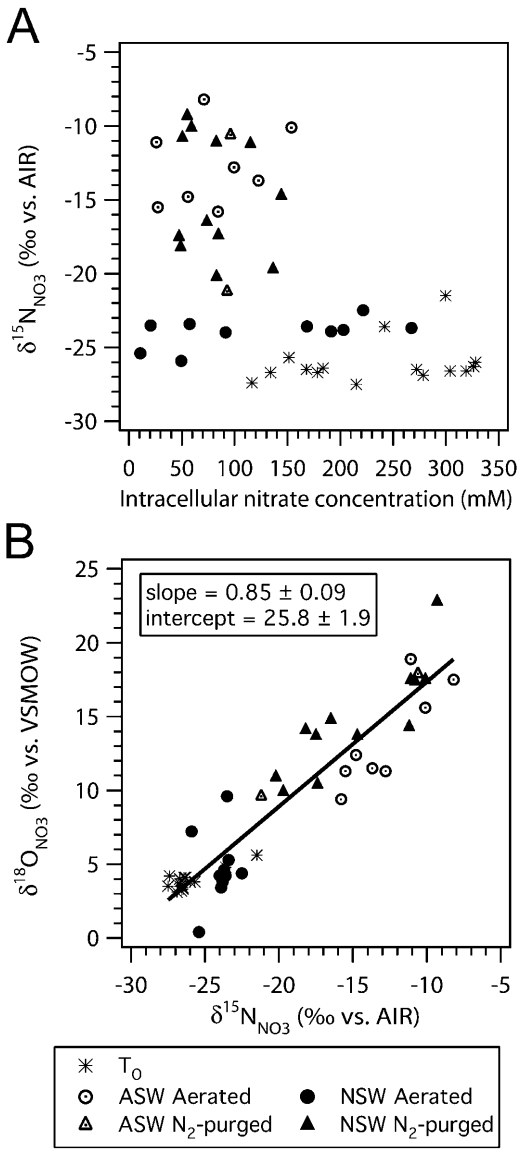

Figure 5. (a) Plot of $\delta^{15}N_{NO3}$ value versus foraminiferal nitrate concentration for Experiment 1. (b) Plot of $\delta^{18}O_{NO3}$ versus $\delta^{15}N_{NO3}$ values for Experiment 1. Results from different treatments are shown in unique symbols, although data from all treatments are included in the regression.

individuals, and that the same fractionating consumption process occurred in most individuals.

[28] In Experiment 2, which included antibiotic treatments, the mean nitrate concentration of freshly collected *B. argentea* (that had not been installed on the recirculating artificial-seawater system) also decreased compared to T_0 in all treatments (Figure 6a). Nitrate removal was observed in incubations with and without antibiotic, and the differences in mean nitrate concentration between +/− antibiotic treatments were not statistically significant (Figure 6; Tukey-Kramer test, p = 0.05). Due to the low levels of nitrate in many specimens, fewer measurements were made for $\delta^{15}N_{NO3}$. In two cases (aerated + antibiotics T_2, N_2-purged – antibiotics T_1), the decrease in nitrate concentration was associated with increases in nitrate $\delta^{15}N_{NO3}$ (Figure 6b) and $\delta^{18}O_{NO3}$ (not shown) values relative to T_0, while in one case (aerated + antibiotics T_1), nitrate disappearance was associated with a decrease in both $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values. In the remaining cases, there was no significant difference (Tukey-Kramer test, p = 0.05) in mean $\delta^{15}N_{NO3}$ or $\delta^{18}O_{NO3}$ values relative to T_0, despite lowered nitrate concentrations. It should be noted, however, that similar to Experiment 1 (Figures 4 and 5), there are real differences in $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values among individuals, with lowered concentrations generally associated with higher $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values, and a correlation between $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values (slope = 0.83, r^2 = 0.89) (not shown), suggestive of intracellular nitrate consumption rather than leakage.

3.5. Foraminiferal Denitrification

[29] *B. argentea*, *F. cornuta*, and *N. stella* were assayed for denitrification (production of $^{29}N_2$ and $^{30}N_2$ from $^{15}NO_3^-$) over a 48-h period (^{15}N Experiment 1), and *B. argentea* was assayed over a 98-h period (^{15}N Experiment 2). In ^{15}N

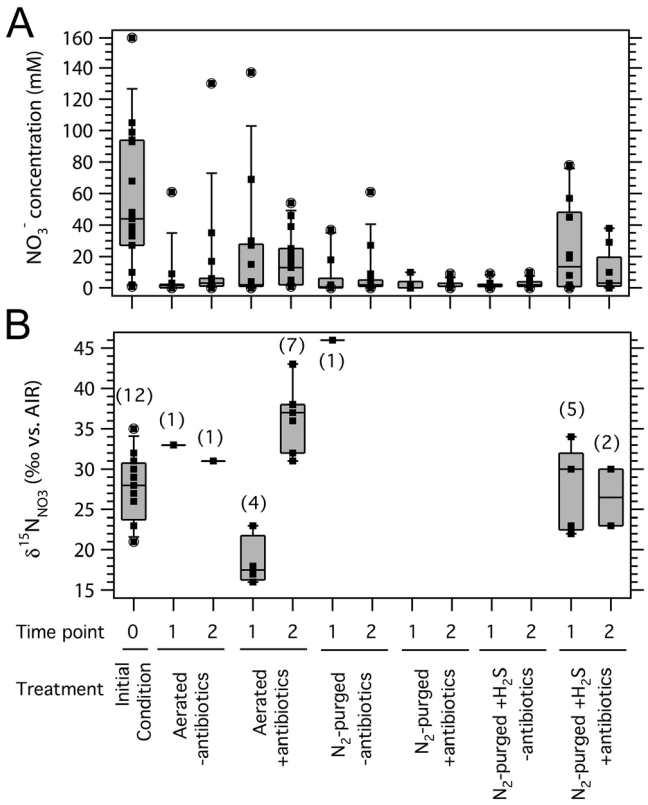

Figure 6. Box and whisker plots showing results from Experiment 2, with *B. argentea.* (a) Nitrate concentration of freshly collected *B. argentea* (time point 0), after 7 days (time point 1) and after 14 days (time point 2) of incubation. (b) $\delta^{15}N_{NO3}$ values from freshly collected *B. argentea* (T_0), after 7 (T_1) and 14 days (T_2). Note that in many treatments, nitrate contents were too low to obtain reliable isotopic measurements. Each box represents the 25th–75th percentile of individual measurements. The bar inside each box represents the mean value (50th percentile), and the whiskers represent the full range of data for each treatment. Parenthetic values indicate numbers of specimens included in the analysis. Circled dots indicate outliers.

Table 2. Denitrification Rates Measured for the Two Species Analyzed[a]

	Frequency of Positive Results	Denitrification Rate (pmol N/cell/day)	Intracellular NO_3^- (pmol N/cell)
Bolivina argentea	53% (8 of 15)	1976 ± 1103	1285 ± 696
Fursenkoina cornuta	67% (2 of 3)	1386 ± 320	796 ± 809

[a]Values presented are means (and standard deviations) of the positive results.

Experiment 1, where incubations contained 5 individuals per replicate vial, denitrification was only detected in *F. cornuta*, not in the vials containing *B. argentea* or *N. stella.* In the second ^{15}N experiment, we focused on *B. argentea* (this was the only species for which enough specimens were available), and we included 10 individuals per tube. Here, we detected production of $^{29}N_2$ and $^{30}N_2$ over 98 h, in ratios consistent with random isotope pairing of NO_3^- during denitrification. Denitrification was not observed in controls, containing N_2-sparged filtered seawater spiked with $^{15}NO_3^-$ with no added foraminifera, from either experiment. The calculated denitrification rate obtained for *F. cornuta* in the first experiment and for *B. argentea* in the second experiment were 1386 ± 320 pmol N/cell/day and 1976 ± 1103 pmol N/cell/day, respectively (Table 2). Together with the average intracellular NO_3^- contents (Table 2), these rates suggest that nitrate should be turning over 1.5–2 times per day.

4. Discussion

4.1. Contributions of Benthic Foraminifera to Sedimentary Nitrate Storage

[30] Nitrate concentrations (12–217 mM) in all four foraminiferal taxa analyzed here were elevated relative to bottom water, although not all specimens of any species had high nitrate concentrations and the data for all species varied considerably. This latter observation is consistent with earlier publications regarding nitrate content and its variability in foraminifera [*Høgslund et al.*, 2008; *Koho et al.*, 2011; *Piña-Ochoa et al.*, 2010; *Risgaard-Petersen et al.*, 2006]. Intracellular concentrations of nitrate in our foraminifera were comparable to those reported by *Risgaard-Petersen et al.* [2006] (35–180 mM), even though the species analyzed were different, aside from *N. stella.* Reports of nitrate concentrations in other *Bolivina* species average 153 ± 49 mM [*Piña-Ochoa et al.*, 2010], which are slightly lower than nitrate concentrations in our *B. argentea* (195 ± 160 mM). Although *Buliminella* was not analyzed by other authors, species of the closely related genus *Bulimina* is documented to have nitrate concentrations of 116 ± 43 mM [*Piña-Ochoa et al.*, 2010], compared to our *Buliminella tenuata*, which had 217 ± 150 mM nitrate. The *B. tenuata* concentrations were the highest we obtained, but these were lower than the highest values reported by *Piña-Ochoa et al.* [2010] for a species from the same superfamily (*Globobulimina* cf. *ovula*, 375 ± 174 mM).

[31] The SBB sediments are known to have high subsurface nitrate + nitrite peaks [*Bernhard and Reimers*, 1991; *Reimers et al.*, 1996] but, at the time of those reports, the source of this peak was unclear. In the past, it was thought that the peak could be due to the large populations of the sulfur-oxidizing denitrifying bacterium *Beggiatoa*, which can contain high concentrations of nitrate [*McHatton et al.*, 1996] that could have been released during pore water sampling. Our present data suggest that nitrate storage in foraminiferal vacuoles may have also contributed to the peak in SBB sediment nitrate + nitrite reported by *Bernhard and Reimers* [1991]. However, a more quantitative assessment of foraminiferal abundances and distributions, with respect to sediment porosity, are required to answer this question completely.

4.2. Bacterial Associations With Benthic Foraminifera

[32] High intracellular nitrate contents suggest that these SBB foraminifera are either intentionally transporting nitrate inside their cells or producing it intracellularly. Because these organisms inhabit oxygen-deficient sediments, and nitrate production by nitrification requires oxygen, we discount intracellular production in our populations. Furthermore, our data from *B. tenuata* did not include any known nitrifying genera among the 16S rRNA genes obtained. While future studies may more directly address the question of nitrifying symbionts through functional gene (ammonia monooxygenase) analysis, it would be difficult to sustain high rates of nitrification in sulfidic sediments, and we find the most plausible explanation to be active transport of NO_3^- into the foraminifer from surrounding pore waters. Molecular characterization and/or localization of nitrate transport proteins in these foraminifera may help shed light on this issue.

[33] Most of the small subunit rRNA sequences obtained from endobionts of *B. tenuata* from oxygen-depleted sites most closely affiliate with relatives of *Marinobacter*, which is a denitrifying rod-shaped bacterial genus within the gammaproteobacteria found commonly in marine sediments [e.g., *Nakano et al.*, 2010]. Thus, these sequencing results are consistent with morphologic results obtained with TEM (i.e., rod-shaped bacteria; Figure 1h) [see also *Bernhard et al.*, 2000; *Buck and Bernhard*, 2001]. There are many representatives of denitrifying bacteria within the gammaproteobacteria, and most denitrifiers are facultative, and can grow by aerobic respiration in the presence of oxygen [*Kuenen et al.*, 1991]. Some sulfide-oxidizing bacteria will also denitrify [*Kuenen et al.*, 1991], an ability that may serve an additional role in detoxification for foraminiferal hosts in anoxic sulfidic sediments. A *nirK* gene was detected in DNA extracts from *B. tenuata* from oxygen-depleted sediments, but it has not yet been localized to the endobionts, attached bacteria, or the foraminifer host. The bacteria associated with *B. tenuata* from more aerated sediments most closely affiliate with a clade of gammaproteobacterial sequences that include 16S rRNA gene sequences from uncultured bacteria and some Enterobactriales. The localization and identity of the gammaproteobacterial associates of the *B. tenuata* from different habitats needs to be confirmed by positive FISH using probes specific to each sequence type, but symbiosis plasticity is not too surprising given some *B. tenuata* from other habitats lack endobionts altogether [*Bernhard et al.*, 2001]. In this species, the potential for and mediator of denitrification remain equivocal.

[34] The other species investigated in this study contained no endobionts, although *N. stella* is known to sequester plastids that contain nitrate reductase [*Grzymski et al.*, 2002]. The fact that a gene encoding dissimilatory nitrite reductase was obtained from each of the foraminiferal species analyzed indicates that these eukaryotes and/or their external bacterial community can perform a critical step of the denitrification process. We did not survey for genes involved in the remainder of the denitrification process (i.e., *norB* and *norQ* for nitric oxide reduction and *nosZ* for nitrous oxide reduction); those analyses remain for future investigations.

4.3. Contributions of Benthic Foraminifera to Sedimentary Denitrification

[35] Interestingly, the negligible amounts of nitrite present in our foraminifera, as evidenced by differences between paired sulfamic and non-sulfamic-treated samples, suggests that the turnover of nitrite in the SBB foraminifera must be tightly coupled between nitrate reduction and nitrite reduction. Furthermore, our measured denitrification rates (1976 ± 1103 pmol/*B. argentea*/day; 1386 ± 320 pmol/*F. cornuta*/day), while containing a high degree of uncertainty, are higher than those reported for other species (88 pmol/*N. stella*/day and 565 pmol/*G. pseudospinescens*/day; [*Høgslund et al.*, 2008; *Risgaard-Petersen et al.*, 2006]). While nitrate contents were not determined for the incubated specimens, average nitrate contents for these species indicate nitrate turnover times of 0.5 to 0.7 days, which is similar to the estimate of 2.5 days for *N. stella* [*Høgslund et al.*, 2008] and much faster than the estimate of over a month for *G. pseudospinescens* [*Risgaard-Petersen et al.*, 2006]. Our data indicate that, when these SBB foraminiferal populations are actively denitrifying, they need to replace their cellular quotient of nitrate at least once every day. These rates are supported by the observation that intracellular nitrate contents in *B. argentea* cells dropped dramatically over a 7-day incubation under anoxic conditions (Figure 6a).

[36] SBB sedimentary denitrification rates have been estimated to be quite high (~4.5 mM N m^{-2} day^{-1}) relative to surrounding basins, although the cause of such high rates is unclear [*Sigman et al.*, 2003]. A typical density of living (as determined by adenosine triphosphate analysis) benthic foraminifera in SBB surface sediments is ~200 specimens/cm^3 [*Bernhard and Reimers*, 1991]. Using our denitrification rates for *B. argentea* and *F. cornuta* (~1500 pmol/cell/day), foraminifera could account for ~3 mM N m^{-2} day^{-1}, or 67% of the estimated sedimentary denitrification [*Sigman et al.*, 2003]. Other cases where benthic foraminifera account for two thirds of sedimentary denitrification are known [*Piña-Ochoa et al.*, 2010], while considerably lower proportions have also been reported [*Glud et al.*, 2009].

[37] The $\delta^{15}N$ of the foraminiferal nitrate provides additional insights about nitrogen cycling in these protists. To begin with, when sufficient nitrate was present for analysis, the observed $\delta^{15}N_{NO3}$ values for fresh specimens were generally elevated, as were those reported for another SBB foraminifer (~18‰) [*Bernhard et al.*, 2012]. Intracellular $\delta^{15}N_{NO3}$ values in endobiont-free *B. argentea* and *N. stella* were often higher than the $\delta^{15}N_{NO3}$ values of pore waters bathing the foraminifera, suggesting that the foraminifera may have actively consumed nitrate in their natural environment. *N. stella* has previously been shown to denitrify [*Risgaard-Petersen et al.*, 2006], while *B. argentea* had not. Foraminiferal $\delta^{15}N_{NO3}$ values that lie between pore water and overlying-water $\delta^{15}N_{NO3}$ (*F. cornuta, B. tenuata*, and some *B. argentea*) suggest that these specimens may have been exposed to overlying water in the surface sediments, that our pore water sampling scale was too coarse to resolve $\delta^{15}N_{NO3}$ gradients at microhabitat scales relevant to these microbes, and/or that these species or individuals had lower nitrate reducing activity.

[38] In our experiments with *B. argentea*, the magnitude of decrease in nitrate concentrations in individuals from the aerated experimental treatments was slightly less than the decrease in nitrate concentrations from specimens from either of the oxygen-depleted treatments. Although the difference in the mean nitrate concentrations was not significant, there were more individuals with measurable nitrate concentrations in aerated experiments, and the range of nitrate concentrations was greater in cells from oxygenated incubations. These results suggest that the presence of oxygen may have partially inhibited or delayed the onset of nitrate respiration. This species inhabits a more oxygenated habitat and is thought to be less tolerant of low oxygen than other species occurring in the vicinity of SBB [*Bernhard et al.*, 1997; *Stott et al.*, 2002], but elevated $\delta^{15}N_{NO3}$ values in pore waters clearly indicate that these sediments support denitrification. Prior research has shown that benthic foraminifera appear to be highly plastic with regards to metabolism, apparently having the ability to switch from aerobic respiration to denitrification as necessary [e.g., *Piña-Ochoa et al.*, 2010]. Indeed, certain benthic foraminifera have been known to survive considerable periods of anoxia [e.g., *Bernhard*, 1993; *Bernhard and Reimers*, 1991; *Moodley and Hess*, 1992; *Moodley et al.*, 1998] and some must have an as-yet-unknown alternative oxidative pathway, as evidenced by experiments done with electron transport inhibitors [*Travis and Bowser*, 1986].

[39] As observed for matched field specimens and pore water data, the disappearance of nitrate associated with increases in $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ in our experiments provides evidence for reductive nitrate removal, rather than nitrate leakage from the foraminifera. In cases where nitrate disappearance was associated with no change, or even a decrease, in $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$, the fate of intracellular nitrate is uncertain. Specimens may have died yet not fully lysed, thereby continuing to have concentrated nitrate in their cytoplasm. Dormancy may also have occurred [e.g., *Alve and Goldstein*, 2010; *Bernhard and Alve*, 1996]. The ratio of increases in $\delta^{18}O_{NO3}$ to $\delta^{15}N_{NO3}$ (0.83–0.85) was lower than expected for most bacterial denitrifiers (slope of 1) [*Granger et al.*, 2008]. This may be indicative of activity from a periplasmic nitrate reductase, rather than a more typical respiratory nitrate reductase. This possibility will have to be pursued in future studies.

[40] The lack of a significant difference in nitrate removal in the presence and absence of the antibiotic cocktail during our second experiment with *B. argentea* is suggestive of nitrate reduction by foraminifera themselves. It is also possible that a subset of bacteria was not affected, or was incompletely inhibited, by the cocktail composed of five antibiotics, and thus, could continue to denitrify throughout the experiment. For example, chloramphenicol inhibits RNA transcription [*Jardetzky*, 1963; *Wolfe and Hahn*, 1965] but

would not inactivate existing enzymes. However, the combination of chloramphenicol, streptomycin, neomycin, tetracycline and penicillin is expected to inhibit most if not all prokaryotic activity. Given that the foraminiferal species used in both experiments (*B. argentea*) lacks symbionts, if bacterial denitrification was occurring, the bacteria would have to be contaminants either on the test exterior or as parasites in foraminiferal endoplasm. Bacterial parasites have recently been documented in foraminifera from deep-sea sediments in and adjacent to hydrocarbon seeps [*Bernhard et al.*, 2010]. However, it is estimated that 6,000–23,000 denitrifying bacteria per foraminifera would be required to account for denitrification rates reported for foraminifera in *Risgaard-Petersen et al.* [2006]; this magnitude of cytoplasmic parasites have never been observed in our electron microscopic imagery of *B. argentea* although serial sections including entire specimens have not received dedicated analysis in this context. To resolve this issue of whether bacteria or the foraminifer itself is responsible for denitrification, methods such as geneFISH [*Moraru et al.*, 2010], which localizes the sites of denitrification genes to the eukaryote, symbionts and/or contaminants, will need to be employed, as has been done for an allogromiid foraminifer [*Bernhard et al.*, 2012].

4.4. Synthesis and Evolutionary Implications

[41] Results of this study and *Bernhard et al.* [2012] suggest that foraminifera have evolved at least two ways to perform the process of denitrification: (1) with symbionts and (2) by the eukaryote. The allogromiid foraminifer studied by *Bernhard et al.* [2012] appears to host denitrifying symbionts. *B. tenuata* also contains symbionts, and although their identity and physiological capacity remain to be determined, initial data do not exclude denitrifiers. If the *nirK* gene obtained from *B. tenuata* DNA extracts can be localized to the endobionts, then this system may function similarly to the allogromiid. The other foraminifera studied here do not contain endobionts (*N. stella* husbands plastids), although they may have external bacterial associates. *B. argentea* was shown to produce N_2 from NO_3^- and rapidly consume intracellular nitrate during both oxic and anoxic incubations. Isotopic fractionation during nitrate consumption indicates that it is being reduced intracellularly, rather than simply leaking out of the cell or being consumed by extracellular contaminants. A gene for nitrite reduction (*nirS*) was also detected in DNA extracts from *B. argentea* although it has not yet been localized. Incubations of *F. cornuta* were also found to produce N_2 from NO_3^-. Further investigation is needed to determine whether this foraminifer is catalyzing the reaction itself, but its intracellular $\delta^{15}N_{NO3}$ values were occasionally elevated relative to pore water nitrate, and no endobionts are known to exist in this species. *N. stella* has previously been shown to sequester chloroplasts containing nitrate reductase [*Grzymski et al.*, 2002] and has previously been shown to denitrify [*Piña-Ochoa et al.*, 2010; *Risgaard-Petersen et al.*, 2006], presumably without endobionts. *N. stella*'s elevated $\delta^{15}N_{NO3}$ values suggest that this organism does participate in nitrate reduction in the field.

[42] It will be fascinating to determine if additional protists such as testate amoebae, ciliates, and/or flagellates can perform complete denitrification and, if they can, determine the mechanisms employed to do so. From a paleontologic perspective, both ciliates and flagellates likely evolved approximately 1.9 Billion years ago [*Hedges et al.*, 2004; *Wright and Lynn*, 1997], a time in the Paleoproterozoic when a "whiff" of oxygen entered the atmosphere [e.g., *Poulton et al.*, 2004]. However, at that time, atmospheric and oceanic oxygenation likely did not last long [*Frei et al.*, 2009], so if these early evolving protists were to survive, an anaerobic metabolic pathway(s) was required. Indeed, it is argued that much of the Proterozoic, during and after the origin of ciliates and flagellates, was in a "nitrogen crisis" where oceanic oxygen concentrations remained very low to undetectable while denitrification was prevalent [*Falkowski and Godfrey*, 2008; *Fennel et al.*, 2005]. Although nitrate may have been limited at this time [*Falkowski and Godfrey*, 2008], the ability to denitrify could have imparted a major ecological advantage to allow success of early evolving eukaryotic lineages. In cases where fossilizable protistan body parts exist, our understanding of protistan physiology with respect to the nitrogen cycle will help interpret the paleontologic record in terms of paleoecology.

5. Conclusions

[43] While the involvement of benthic foraminifera in denitrification has been documented by other workers [e.g., *Piña-Ochoa et al.*, 2010; *Risgaard-Petersen et al.*, 2006], it is difficult to exclude bacteria when assaying foraminifera for denitrification activity. Our approach was to use a combination of antibiotics and changes in intracellular nitrate content and isotopic composition in foraminifera with varying associations with bacteria. We have shown that while bacterial symbionts and/or contaminants may perform a portion of denitrification detected in foraminifera, it remains possible that the foraminifera proper also perform complete denitrification. Our data support the notion that foraminifera and/or their symbionts may play a major role in the marine nitrogen cycle through nitrate storage and consumption, and further define a significant role for protists in benthic marine habitats.

[44] **Acknowledgments.** We dedicate this manuscript to the memory of pioneering evolutionary theorist Lynn Margulis, who would have appreciated the physiologic plasticity of this protist taxon. We thank the captain and crew of the RV *Robert Gordon Sproul*, all science parties who helped to collect samples, Sam Bowser for enlightening us about Pierce's antibiotic cocktail, Edward Leadbetter for helpful comments on the manuscript, and two anonymous reviewers for their comments on an earlier draft. This research was supported by NSF grant EF-0702491 to JMB, KLC, and VPE.

References

Alve, E., and S. T. Goldstein (2010), Dispersal, survival and delayed growth of benthic foraminiferal propagules, *J. Sea Res.*, *63*(1), 36–51, doi:10.1016/j.seares.2009.09.003.

Bernhard, J. M. (1993), Experimental and field evidence of Antarctic foraminiferal tolerance to anoxia and hydrogen sulfide, *Mar. Micropaleontol.*, *20*(3–4), 203–213, doi:10.1016/0377-8398(93)90033-T.

Bernhard, J. M., and E. Alve (1996), Survival, ATP pool, and ultrastructural characterization of benthic foraminifera from Drammensfjord (Norway): Response to anoxia, *Mar. Micropaleontol.*, *28*(1), 5–17, doi:10.1016/0377-8398(95)00036-4.

Bernhard, J. M., and S. S. Bowser (1999), Benthic foraminifera of dysoxic sediments: Chloroplast sequestration and functional morphology, *Earth Sci. Rev.*, *46*(1–4), 149–165, doi:10.1016/S0012-8252(99)00017-3.

Bernhard, J. M., and S. S. Bowser (2008), Peroxisome proliferation in foraminifera inhabiting the chemocline: An adaptation to reactive oxygen species exposure? *J. Eukaryot. Microbiol.*, *55*(3), 135–144, doi:10.1111/j.1550-7408.2008.00318.x.

Bernhard, J. M., and C. E. Reimers (1991), Benthic foraminiferal population fluctuations related to anoxia- Santa Barbara Basin, *Biogeochemistry*, *15*(2), 127–149, doi:10.1007/BF00003221.

Bernhard, J. M., B. K. Sen Gupta, and P. F. Borne (1997), Benthic foraminiferal proxy to estimate dysoxic bottom-water oxygen concentrations: Santa Barbara Basin, US Pacific continental margin, *J. Foraminiferal Res.*, *27*(4), 301–310, doi:10.2113/gsjfr.27.4.301.

Bernhard, J. M., K. R. Buck, M. A. Farmer, and S. S. Bowser (2000), The Santa Barbara Basin is a symbiosis oasis, *Nature*, *403*(6765), 77–80, doi:10.1038/47476.

Bernhard, J. M., K. R. Buck, and J. P. Barry (2001), Monterey Bay cold-seep biota: Assemblages, abundance, and ultrastructure of living foraminifera, *Deep Sea Res., Part I*, *48*(10), 2233–2249, doi:10.1016/S0967-0637(01)00017-6.

Bernhard, J. M., P. T. Visscher, and S. S. Bowser (2003), Submillimeter life positions of bacteria, protists, and metazoans in laminated sediments of the Santa Barbara Basin, *Limnol. Oceanogr.*, *48*(2), 813–828, doi:10.4319/lo.2003.48.2.0813.

Bernhard, J. M., A. Habura, and S. S. Bowser (2006), An endobiont-bearing allogromiid from the Santa Barbara Basin: Implications for the early diversification of foraminifera, *J. Geophys. Res.*, *111*, G03002, doi:10.1029/2005JG000158.

Bernhard, J. M., J. B. Martin, and A. E. Rathburn (2010), Combined carbonate carbon isotopic and cellular ultrastructural studies of individual benthic foraminifera: 2. Toward an understanding of apparent disequilibrium in hydrocarbon seeps, *Paleoceanography*, *25*, PA4206, doi:10.1029/2010PA001930.

Bernhard, J. M., V. P. Edgcomb, K. L. Casciotti, M. M. McIlvin, and D. J. Beaudoin (2012), Denitrification likely catalyzed by endobionts in an allogromiid foraminifer, *ISME J.*, *6*, 951–960, doi:10.1038/ismej.2011.171.

Böhlke, J. K., S. J. Mroczkowski, and T. B. Coplen (2003), Oxygen isotopes in nitrate: New reference materials for O-18: O-17: O-16 measurements and observations on nitrate-water equilibration, *Rapid Commun. Mass Spectrom.*, *17*(16), 1835–1846, doi:10.1002/rcm.1123.

Braker, G., A. Fesefeldt, and K. P. Witzel (1998), Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples, *Appl. Environ. Microbiol.*, *64*(10), 3769–3775.

Buck, K. R., and J. M. Bernhard (2001), Protistan-Prokaryotic symbioses in deep-sea sulfidic sediments, in *Symbiosis*, edited by J. Seckbach, pp. 507–517, Kluwer Acad., Dordrecht, Netherlands.

Cannariato, K. G., J. P. Kennett, and R. J. Behl (1999), Biotic response to late Quaternary rapid climate switches in Santa Barbara Basin: Ecological and evolutionary implications, *Geology*, *27*(1), 63–66, doi:10.1130/0091-7613(1999)027<0063:BRTLQR>2.3.CO;2.

Casciotti, K. L., and B. B. Ward (2001), Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria, *Appl. Environ. Microbiol.*, *67*(5), 2213–2221, doi:10.1128/AEM.67.5.2213-2221.2001.

Casciotti, K. L., D. M. Sigman, M. G. Hastings, J. K. Bohlke, and A. Hilkert (2002), Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method, *Anal. Chem.*, *74*(19), 4905–4912, doi:10.1021/ac020113w.

Cole, J. R., et al. (2003), The Ribosomal Database Project (RDP-II): Previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy, *Nucleic Acids Res.*, *31*(1), 442–443, doi:10.1093/nar/gkg039.

Falkowski, P. G., and L. V. Godfrey (2008), Electrons, life and the evolution of Earth's oxygen cycle, *Philos. Trans. R. Soc. B*, *363*(1504), 2705–2716, doi:10.1098/rstb.2008.0054.

Fennel, K., M. Follows, and P. G. Falkowski (2005), The co-evolution of the nitrogen, carbon and oxygen cycles in the Proterozoic ocean, *Am. J. Sci.*, *305*(6–8), 526–545, doi:10.2475/ajs.305.6-8.526.

Filipsson, H. L., J. M. Bernhard, S. A. Lincoln, and D. C. McCorkle (2010), A culture-based calibration of benthic foraminiferal paleotemperature proxies: Delta O-18 and Mg/Ca results, *Biogeosciences*, *7*(4), 1335–1347, doi:10.5194/bg-7-1335-2010.

Frei, R., C. Gaucher, S. W. Poulton, and D. E. Canfield (2009), Fluctuations in Precambrian atmospheric oxygenation recorded by chromium isotopes, *Nature*, *461*(7261), 250–253, doi:10.1038/nature08266.

Glud, R. N., B. Thamdrup, H. Stahl, F. Wenzhoefer, A. Glud, H. Nomaki, K. Oguri, N. P. Revsbech, and H. Kitazato (2009), Nitrogen cycling in a deep ocean margin sediment (Sagami Bay, Japan), *Limnol. Oceanogr.*, *54*(3), 723–734, doi:10.4319/lo.2009.54.3.0723.

Granger, J., and D. M. Sigman (2009), Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method, *Rapid Commun. Mass Spectrom.*, *23*(23), 3753–3762, doi:10.1002/rcm.4307.

Granger, J., D. M. Sigman, M. F. Lehmann, and P. D. Tortell (2008), Nitrogen and oxygen isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria, *Limnol. Oceanogr.*, *53*(6), 2533–2545, doi:10.4319/lo.2008.53.6.2533.

Grzymski, J., O. M. Schofield, P. G. Falkowski, and J. M. Bernhard (2002), The function of plastids in the deep-sea benthic foraminifer, *Nonionella stella*, *Limnol. Oceanogr.*, *47*(6), 1569–1580, doi:10.4319/lo.2002.47.6.1569.

Habura, A., J. Pawlowski, S. D. Hanes, and S. S. Bowser (2004), Unexpected foraminiferal diversity revealed by small-subunit rDNA analysis of Antarctic sediment, *J. Eukaryot. Microbiol.*, *51*(2), 173–179, doi:10.1111/j.1550-7408.2004.tb00542.x.

Hallin, S., and P. E. Lindgren (1999), PCR detection of genes encoding nitrile reductase in denitrifying bacteria, *Appl. Environ. Microbiol.*, *65*(4), 1652–1657.

Hedges, S. B., J. E. Blair, M. L. Venturi, and J. L. Shoe (2004), A molecular timescale of eukaryote evolution and the rise of complex multicellular life, *BMC Evol. Biol.*, *4*, 2, doi:10.1186/1471-2148-4-2.

Høgslund, S., N. P. Revsbech, T. Cedhagen, L. P. Nielsen, and V. A. Gallardo (2008), Denitrification, nitrate turnover, and aerobic respiration by benthic foraminiferans in the oxygen minimum zone off Chile, *J. Exp. Mar. Biol. Ecol.*, *359*(2), 85–91, doi:10.1016/j.jembe.2008.02.015.

Janssen, P. J., et al. (2010), The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments, *PLoS ONE*, *5*(5), e10433, doi:10.1371/journal.pone.0010433.

Jardetzky, O. (1963), Studies on the mechanism of action of chloramphenicol, *J. Biol. Chem.*, *238*(7), 2498–2508.

Kana, T. M., C. Darkangelo, M. D. Hunt, J. B. Oldham, G. E. Bennett, and J. C. Cornwell (1994), Membrane inlet mass spectrometer for rapid high precision determination of N_2, O_2 and Ar in environmental water samples, *Anal. Chem.*, *66*(23), 4166–4170, doi:10.1021/ac00095a009.

Kelly, D. P., and A. P. Wood (2006), The chemolithotrophic bacteria, in *The Prokaryotes: A Handbook on the Biology of Bacteria*, edited by M. Dworkin et al., pp. 441–456, Springer, Berlin.

Koho, K. A., E. Piña-Ochoa, E. Geslin, and N. Risgaard-Petersen (2011), Vertical migration, nitrate uptake and denitrification: Survival mechanisms of foraminifers (*Globobulimina turgida*) under low oxygen conditions, *FEMS Microbiol. Ecol.*, *75*(2), 273–283, doi:10.1111/j.1574-6941.2010.01010.x.

Kuenen, J. G., L. A. Robertson, and O. H. Tuovinen (1991), The genera *Thiobacillus, Thiomicrospira*, and *Thiosphaera*, in *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, vol. 3, edited by A. Balows et al., pp. 2638–2657, Springer, New York.

Kuwabara, J. S., A. van Geen, D. C. McCorkle, and J. M. Bernhard (1999), Dissolved sulfide distributions in the water column and sediment pore waters of the Santa Barbara Basin, *Geochim. Cosmochim. Acta*, *63*(15), 2199–2209, doi:10.1016/S0016-7037(99)00084-8.

Lane, D. (1991), 16S/23S rRNA sequencing, in *Nucleic Acid Techniques in Bacterial Systematics*, edited by E. Stackebrandt and M. Goodfellow, pp. 115–175, Wiley, New York.

Longnecker, K., and A. L. Reysenbach (2001), Expansion of the geographic distribution of a novel lineage of epsilon-Proteobacteria to a hydrothermal vent site on the Southern East Pacific Rise, *FEMS Microbiol. Ecol.*, *35*(3), 287–293.

Ludwig, W., et al. (2004), ARB: A software environment for sequence data, *Nucleic Acids Res.*, *32*(4), 1363–1371, doi:10.1093/nar/gkh293.

McHatton, S. C., J. P. Barry, H. W. Jannasch, and D. C. Nelson (1996), High nitrate concentrations in vacuolate, autotrophic marine *Beggiatoa* spp, *Appl. Environ. Microbiol.*, *62*(3), 954–958.

McIlvin, M. R., and K. L. Casciotti (2011), Technical updates to the bacterial method for nitrate isotopic analyses, *Anal. Chem.*, *83*(5), 1850–1856, doi:10.1021/ac1028984.

Moodley, L., and C. Hess (1992), Tolerance of infaunal benthic foraminifera for low and high oxygen concentrations, *Biol. Bull.*, *183*(1), 94–98, doi:10.2307/1542410.

Moodley, L., G. J. van der Zwaan, G. M. W. Rutten, R. C. E. Boom, and A. J. Kempers (1998), Subsurface activity of benthic foraminifera in relation to porewater oxygen content: Laboratory experiments, *Mar. Micropaleontol.*, *34*(1–2), 91–106, doi:10.1016/S0377-8398(97)00044-3.

Moraru, C., P. Lam, B. M. Fuchs, M. M. M. Kuypers, and R. Amann (2010), GeneFISH—An *in situ* technique for linking gene presence and cell identity in environmental microorganisms, *Environ. Microbiol.*, *12*(11), 3057–3073, doi:10.1111/j.1462-2920.2010.02281.x.

Nakano, M., T. Inagaki, S. Okunishi, R. Tanaka, and H. Maeda (2010), Effect of salinity on denitrification under limited single carbon source by *Marinobacter* sp. isolated from marine sediment, *J. Basic Microbiol.*, *50*(3), 285–289, doi:10.1002/jobm.200900250.

Nielsen, L. P. (1992), Denitrification in sediment determined from nitrogen isotope pairing, *FEMS Microbiol. Ecol.*, *86*(4), 357–362, doi:10.1111/j.1574-6968.1992.tb04828.x.

Pawlowski, J. (2000), Introduction to the molecular systematics of foraminifera, *Micropaleontology*, *46*, 1–12.

Pierce, S. (1965), A comparative study of two members of the family *Allogromiidae* (Protozoa, Foraminferida), Ph.D. diss., 212 pp., New York Univ., New York.

Piña-Ochoa, E., S. Hogslund, E. Geslin, T. Cedhagen, N. P. Revsbech, L. P. Nielsen, M. Schweizer, F. Jorissen, S. Rysgaard, and N. Risgaard-Petersen (2010), Widespread occurrence of nitrate storage and denitrification among Foraminifera and *Gromiida*, *Proc. Natl. Acad. Sci. U. S. A.*, *107*(3), 1148–1153, doi:10.1073/pnas.0908440107.

Poulton, S. W., P. W. Fralick, and D. E. Canfield (2004), The transition to a sulphidic ocean similar to 1.84 billion years ago, *Nature*, *431*(7005), 173–177, doi:10.1038/nature02912.

Reimers, C. E., C. B. Lange, M. Tabak, and J. M. Bernhard (1990), Seasonal spillover and varve formation in the Santa Barbara Basin, California, *Limnol. Oceanogr.*, *35*(7), 1577–1585, doi:10.4319/lo.1990.35.7.1577.

Reimers, C. E., K. C. Ruttenberg, D. E. Canfield, M. B. Christiansen, and J. B. Martin (1996), Porewater pH and authigenic phases formed in the uppermost sediments of the Santa Barbara Basin, *Geochim. Cosmochim. Acta*, *60*(21), 4037–4057, doi:10.1016/S0016-7037(96)00231-1.

Risgaard-Petersen, N., et al. (2006), Evidence for complete denitrification in a benthic foraminifer, *Nature*, *443*(7107), 93–96, doi:10.1038/nature05070.

Robertson, L. A., and J. G. Kuenen (2006), The colorless sulfur bacteria, in *The Prokaryotes: A Handbook on the Biology of Bacteria*, edited by M. Dworkin et al., pp. 985–1011, Springer, New York.

Sen Gupta, B. K. (1999), *Modern Foraminifera*, Kluwer Acad., Dordrecht, Netherlands.

Sigman, D. M., K. L. Casciotti, M. Andreani, C. Barford, M. Galanter, and J. K. Bohlke (2001), A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater, *Anal. Chem.*, *73*(17), 4145–4153, doi:10.1021/ac010088e.

Sigman, D. M., R. Robinson, A. N. Knapp, A. van Geen, D. C. McCorkle, J. A. Brandes, and R. C. Thunell (2003), Distinguishing between water column and sedimentary denitrification in the Santa Barbara Basin using the stable isotopes of nitrate, *Geochem. Geophys. Geosyst.*, *4*(5), 1040, doi:10.1029/2002GC000384.

Stamatakis, A. (2006), RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models, *Bioinformatics*, *22*(21), 2688–2690, doi:10.1093/bioinformatics/btl446.

Stamatakis, A., P. Hoover, and J. Rougemont (2008), A rapid bootstrap algorithm for the RAxML web servers, *Syst. Biol.*, *57*(5), 758–771, doi:10.1080/10635150802429642.

Stott, L. D., T. Bunn, M. Prokopenko, C. Mahn, J. Gieskes, and J. M. Bernhard (2002), Does the oxidation of methane leave an isotopic fingerprint in the geologic record?, *Geochem. Geophys. Geosyst.*, *3*(2), 1012, doi:10.1029/2001GC000196.

Travis, J. L., and S. S. Bowser (1986), Microtubule-dependent reticulopodial motility: Is there a role for actin?, *Cell Motil. Cytoskeleton*, *6*(2), 146–152, doi:10.1002/cm.970060212.

Winnepenninckx, B., T. Backeljau, and R. De Wachter (1993), Extraction of high molecular weight DNA from molluscs, *Trends Genet.*, *9*(12), 407, doi:10.1016/0168-9525(93)90102-N.

Wolfe, A. D., and F. E. Hahn (1965), Mode of action of chloramphenicol. IX. Effects of chloramphenicol upon a ribosomal amino acid polymerization system and its binding to bacterial ribosome, *Biochim. Biophys. Acta*, *95*, 146–155, doi:10.1016/0005-2787(65)90219-4.

Wright, A. D. G., and D. H. Lynn (1997), Maximum ages of ciliate lineages estimated using a small subunit rRNA molecular clock: Crown eukaryotes date back to the Paleoproterozoic, *Arch. Protistenkd.*, *148*(4), 329–341, doi:10.1016/S0003-9365(97)80013-9.