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Complex nitrogen cycling in the sponge *Geodia barretti*

Friederike Hoffmann,[1*†] Regina Radax,[2] Dagmar Woebken,[1‡] Moritz Holtappels,[1] Gaute Lavik[1], Hans Tore Rapp,[3] Marie-Lise Schläppy,[1] Christa Schleper[2,3] and Marcel M. M. Kuypers[1]

[1]*Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany.*

[2]*Department of Genetics in Ecology, Faculty of Life Sciences, Althanstr. 14, 1090 Vienna, Austria.*

[3]*Centre for Geobiology, Department of Biology, University of Bergen, PO Box 7803, 5020 Bergen, Norway.*

Summary

Marine sponges constitute major parts of coral reefs and deep-water communities. They often harbour high amounts of phylogenetically and physiologically diverse microbes, which are so far poorly characterized. Many of these sponges regulate their internal oxygen concentration by modulating their ventilation behaviour providing a suitable habitat for both aerobic and anaerobic microbes. In the present study, both aerobic (nitrification) and anaerobic (denitrification, anammox) microbial processes of the nitrogen cycle were quantified in the sponge *Geodia barretti* and possible involved microbes were identified by molecular techniques. Nitrification rates of 566 nmol N cm^{-3} sponge day^{-1} were obtained when monitoring the production of nitrite and nitrate. In support of this finding, ammonia-oxidizing *Archaea* (crenarchaeotes) were found by amplification of the *amo*A gene, and nitrite-oxidizing bacteria of the genus *Nitrospira* were detected based on rRNA gene analyses. Incubation experiments with stable isotopes ($^{15}NO_3^-$ and $^{15}NH_4^+$) revealed denitrification and anaerobic ammonium oxidation (anammox) rates of 92 nmol N cm^{-3} sponge-day^{-1} and 3 nmol N cm^{-3} sponge day^{-1} respectively. Accordingly, sequences closely related to '*Candidatus* Scalindua sorokinii' and '*Candidatus* Scalindua brodae' were detected in 16S rRNA gene libraries. The amplification of the *nir*S gene revealed the presence of denitrifiers, likely belonging to the *Betaproteobacteria*. This is the first proof of anammox and denitrification in the same animal host, and the first proof of anammox and denitrification in sponges. The close and complex interactions of aerobic, anaerobic, autotrophic and heterotrophic microbial processes are fuelled by metabolic waste products of the sponge host, and enable efficient utilization and recirculation of nutrients within the sponge–microbe system. Since denitrification and anammox remove inorganic nitrogen from the environment, sponges may function as so far unrecognized nitrogen sinks in the ocean. In certain marine environments with high sponge cover, sponge-mediated nitrogen mineralization processes might even be more important than sediment processes.

Received 3 October, 2008; accepted 30 March, 2009. *For correspondence. E-mail Friederike.Hoffmann@sars.uib.no; Tel. (+47) 55584345; Fax (+47) 55584305. Present address: †Sars International Centre for Marine Molecular Biology, Thormøhlensgate 55, 5008 Bergen, Norway; ‡Departments of Chemical Engineering and of Civil and Environmental Engineering, Stanford University, 318 Campus Drive, Stanford, CA 94305, USA.

Introduction

Phylogenetically complex, yet highly sponge-specific microbial communities live in close association with numerous marine sponge species, sometimes in such high densities that sponges can be viewed as 'microbial fermenters' (Hentschel *et al.*, 2006). While our knowledge about the phylogeny of sponge microbes is increasing rapidly (Hentschel *et al.*, 2002; 2003; Taylor *et al.*, 2007), many open questions remain concerning their metabolic functions and their possible interactions with the host.

The role of associated microbes in nitrogen cycling in sponges has received particular attention. Symbiotic cyanobacteria may contribute to the sponge nitrogen budget via fixation of atmospheric nitrogen (Wilkinson and Fay, 1979). This was observed for shallow-water sponges in oligotrophic waters, for example in coral reefs. Usually, sponges ingest nitrogen with their food and excrete NH_4^+ as a metabolic end-product (Brusca and Brusca, 1990), which can fuel microbial ammonia and nitrite oxidation. Nitrification rates based on the release of nitrite and nitrate have been reported from numerous tropical and temperate sponges (Diaz and Ward, 1997; Bayer *et al.*, 2007; Jimenez and Ribes, 2007; Southwell *et al.*, 2008). Additionally, 16S rRNA gene sequences of several clades of ammonia-oxidizing *Gamma*- and *Betaproteobacteria* and nitrite-oxidizing *Nitrospira* were recovered from

sponges (Hentschel *et al*., 2002; Diaz *et al*., 2004; Bayer *et al*., 2007). Recently, the involvement of Marine Group I *Crenarchaeota* (*Archaea*) in ammonia oxidation has received particular attention, and their stable affiliation with numerous sponge species has been demonstrated (Preston *et al*., 1996; Margot *et al*., 2002; Lee *et al*., 2003; Schleper *et al*., 2005; Hallam *et al*., 2006a; Pape *et al*., 2006; Holmes and Blanch, 2007; Bayer *et al*., 2008; Steger *et al*., 2008). Archaeal symbionts can be transmitted vertically through the larvae (Sharp *et al*., 2007; Steger *et al*., 2008), implying an important role of this host–microbe interaction. A fully sequenced genome of a potential archaeal ammonia-oxidizer from the sponge *Axinella mexicana* is available, and demonstrates the genetic potential for ammonia oxidation (Hallam *et al*., 2006a,b), as predicted earlier through metagenomics (Venter *et al*., 2004; Treusch *et al*., 2005). The isolation of an autotrophic, free-living ammonia-oxidizing marine crenarchaeote gave the final proof for the predicted metabolism (Könneke *et al*., 2005).

Anaerobic processes of the nitrogen cycle have not been investigated in sponges so far. Until recently, sponge metabolism was viewed as being based on aerobic respiration, similar to metazoan respiration in general. Oxygen is usually supplied in excess to the sponge body through the water current created by the choanocytes (flagellated cells) (Reiswig, 1974). The remarkable ability of sponges to pump large amounts of water through their body has led to the assumption that permanent oxygen saturation exists within the sponge body. The application of oxygen-sensitive microelectrodes on different sponge species, however, showed remarkable oxygen deficiencies in the sponge matrix, as a consequence of reduced pumping activity (Gatti *et al*., 2002; Schönberg *et al*., 2004; Hoffmann *et al*., 2005a,b; Hoffmann *et al*., 2007; Schläppy *et al*., 2007). Sponges with a massive growth form that stop pumping become anoxic within 15 min; oxygen is only present in the first millimetre of the sponge surface, due to molecular diffusion (Hoffmann *et al*., 2008). Fluctuating ventilation behaviour, as frequently observed for sponges both in the field and in cultivation (Reiswig, 1971; Vogel, 1977; Gerodette and Flechsig, 1979; Pile *et al*., 1997; Schläppy *et al*., 2007; 2009), thus leads to fluctuating oxygen concentrations in sponges.

Consequently, both aerobic and anaerobic microbial processes can be expected in sponges. However the only proof so far of an anaerobic microbial process in a sponge was the detection of microbial sulfate reduction in the cold-water sponge *Geodia barretti*, in line with anoxia in this species as observed with oxygen-sensitive microelectrodes (Hoffmann *et al*., 2005b). Bacterial denitrification, the anaerobic reduction of nitrate (NO_3^-) to nitrogen (N_2), coupled to the oxidation of organic matter or reduced sulfur species, is a major sink for nitrogen in global nitrogen budgets, and most denitrification takes place in the seafloor (Middelburg *et al*., 1996). Recently, anaerobic ammonium oxidation (anammox), which combines NO_2^- and NH_4^+ to produce N_2, was discovered as an alternative pathway for the loss of inorganic nitrogen (Van de Graaf *et al*., 1995), and has so far been identified in a broad range of natural environments such as marine sediments (Thamdrup and Dalsgaard, 2002), oxygen minimum zones (Kuypers *et al*., 2005), anoxic fjords/basins (Dalsgaard *et al*., 2003; Kuypers *et al*., 2003) and in arctic sea ice (Rysgaard and Glud, 2004). Under certain environmental conditions, anammox can even exceed denitrification as the main N-loss process (Kuypers *et al*., 2005; Hannig *et al*., 2007). Molecular methods and stable isotope approaches allow exploration of both the presence and the activity of denitrifying and anammox bacteria in the environment.

The presence of microbial denitrification and anammox in sponges has been hypothesized (Taylor *et al*., 2007) but neither the processes nor the microbes involved were detected until now.

Geodia barretti, which is common in the North Atlantic shelf and slope area, is a sponge with high microbial abundance, hosting $> 10^{10}$ microbes cm^{-3} (Hoffmann *et al*., 2006). The detection of both anaerobic zones (Hoffmann *et al*., 2005a,b) and sulfate reduction (Hoffmann *et al*., 2005b), as well as the possibility to grow explants of this species in the lab (Hoffmann *et al*., 2003), make it a suitable candidate to explore the anaerobic nitrogen cycle in sponges. The aim of the present study is to quantify the aerobic (nitrification) and anaerobic (denitrification, anammox) processes of the microbial nitrogen cycle in *G. barretti*, and to identify the microbes that are potentially involved.

Results

Nitrification, denitrification and anammox rates

For nitrification measurements, small sponge explants were incubated in ammonium-amended natural sea water under aerobic conditions and ammonium, nitrite and nitrate concentrations were monitored over a time-course of 48 h. Figure 1A shows the production of NO_3^- and NO_2^- per cm^3 sponge as an average of three replicate experiments. The amount of NO_3^- and NO_2^- production in a control incubation (without sponge) has been deducted from these values. Ammonium consumption exceeded production in the first 24 h, while after that, net production of ammonium was observed (Fig. 1B). Ammonium depletion was also observed in the control incubation without sponge, which indicates algal growth during the experiment. Although we do not know to which extent the bottle

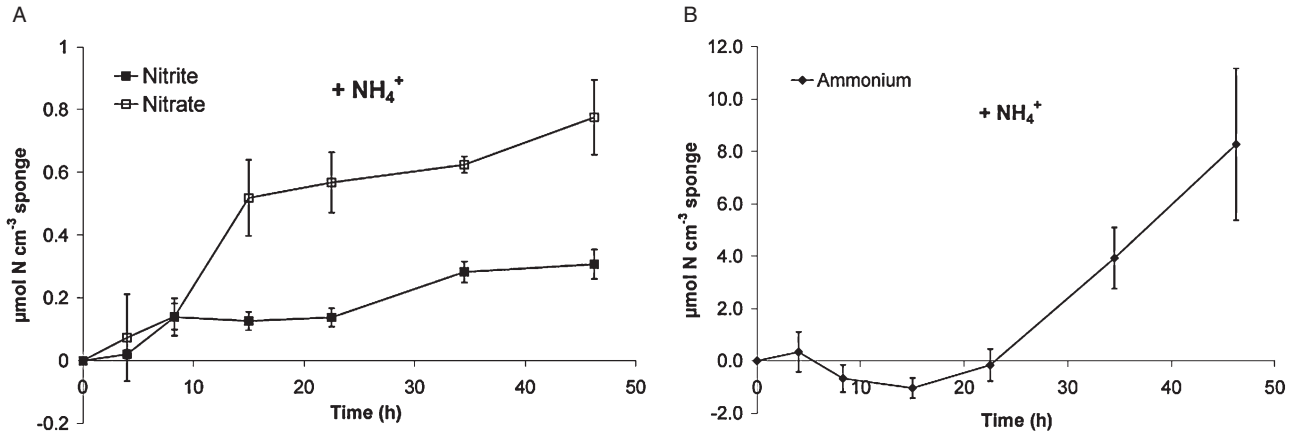

Fig. 1. Incubation of *G. barretti* explants with 12 µM NH_4^+: production/consumption of ammonium, nitrite and nitrate per cm^3 sponge as average water concentrations in three replicate incubations minus concentration in control incubation at a given time point. Nitrite and nitrate were produced during the experiment. A net nitrification rate of 566 nmol N cm^{-3} sponge day^{-1} was revealed by the linear slope of increase (A). Ammonium consumption exceeded production in the first 24 h of the experiments; thereafter, a net production of ammonium was observed (B).

effect of unwanted algal growth may have masked some of the turnover of inorganic nitrogen, it is obvious that (i) the sponge–microbe system was always supplied with NH_4^+ in excess and (ii) both nitrite and nitrate production was observed in all sponge treatments. A total net nitrification rate of 566 nmol N cm^{-3} sponge day^{-1} was calculated from the linear slopes of increase of NO_3^- and NO_2^- production as presented in Fig. 1A.

Production of N_2 as an end-product of denitrification and anmmox was examined by incubating sponge pieces in gas-tight glass vials filled with natural sea water. Sea water was amended with 10 µM $^{15}NO_3^-$ ($^{14}NO_3^-$ background: 8.2 µM) for the denitrification experiment and 10 µM $^{15}NH_4^+ + 2$ µM $^{14}NO_2^-$ (background: 0.4 µM NH_4^+, 0.3 µM NO_2^-) for the anammox experiment. Linear production of both $^{14}N^{15}N$ and $^{15}N^{15}N$ was observed in the $^{15}NO_3^-$ incubation (Fig. 2A). We assume that denitrifiers were not nitrate limited: The small diameter of the sponge explant and the high natural $^{14}NO_3^-$ concentration of 8 µmol l^{-1} allowed a fast diffusive transport that exceeds the denitrification rate. Therefore, the rates of $^{28}N_2$, $^{29}N_2$ (corrected for the $^{29}N_2$ rate of the anammox experiment, see below) and $^{30}N_2$ production were used to calculate a total denitrification rate of 92 nmol N cm^{-3} sponge day^{-1}.

The source of $^{14}NO_3^-$ used for denitrification is either the ambient water (8 µmol per litre of $^{14}NO_3^-$) or $^{14}NO_3^-$ produced via nitrification in the sponge. The sources can be distinguished by comparing the ratio of labelled and unlabelled NO_3^- in the ambient water with the ratio calculated from the $^{29}N_2$ and $^{30}N_2$ production (Nielsen, 1992).

Of 49 nmol $^{14}NO_3^-$ cm^{-3} sponge day^{-1} used for denitrification only 26% can be attributed to coupled nitrification–denitrification whereas 74% derived from $^{14}NO_3^-$ in the ambient water.

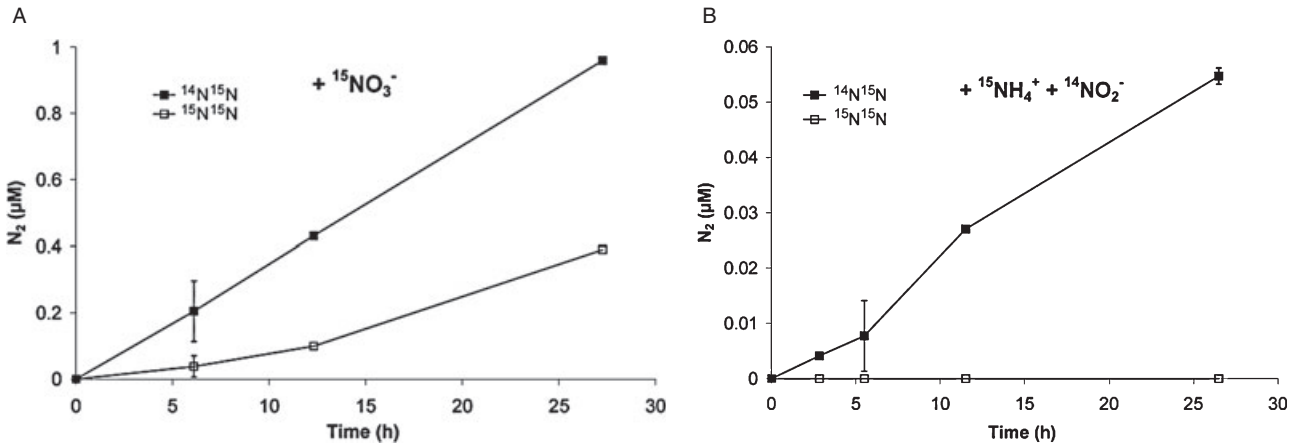

Fig. 2. Incubation experiments with linear production of $^{14}N^{15}N$ and $^{15}N^{15}N$ from $^{15}NO_3^-$ indicating denitrification (A), and $^{14}N^{15}N$ from $^{15}NH_4^+ + {}^{14}NO_2^-$ indicating anammox (B).

In $^{15}NH_4^+ + {}^{14}NO_2^-$ incubations, a linear production of $^{14}N^{15}N$, but no production of $^{15}N^{15}N$ was observed (Fig. 2B). This indicates the process of anammox and an anammox rate of 3.0 nmol N cm^{-3} sponge day^{-1} was calculated. Since no lag phase in the production of $^{14}N^{15}N$ could be observed, coupled nitrification–denitrification of $^{15}NH_4^+$ can be neglected, and the observed $^{14}N^{15}N$ production must be due to anammox.

Microbial community patterns in freshly sampled and cultivated sponges

ARISA (automated rRNA intergenic spacer analysis) and statistical analyses were used to compare microbial communities in samples of sponge explants (used for the incubation experiments) with those of freshly sampled *G. barretti* sponges (used for the molecular studies). Nmds (non-metric multidimensional scaling) – plots and ANOSIM (non-parametric analysis of similarities) of ARISA results showed that sponge explants and fresh sponges had overlapping community patterns. An *R*-value of 0.29 ($P < 0.001$) was obtained by *R*-test statistic measures. *R*-values > 0.75 are commonly interpreted as well separated, $R > 0.5$ as separated, but overlapping, and $R < 0.25$ as barely separable (Ramette, 2007). In contrast, microbial communities of explants used for the experiments were well separated from dead explants ($R = 0.75$, $P < 0.001$). It can thus be concluded that explants which were used for the experiments were alive and healthy, and contained microbial communities that were similar to those in freshly sampled *G. barretti.*

Identification of microbes involved in nitrogen cycling

Ammonium monooxygenase of archaea. The presence and diversity of archaea potentially involved in ammonia oxidation was analysed by targeting the *amo*A gene in PCR-based studies. *amo*A encodes an archaeal homologue of subunit A of the ammonia monooxygenase enzyme. The homologous enzyme in bacteria has been shown to catalyse the transformation from ammonia to hydroxylamine, the first step in ammonia oxidation. The deduced amino acid sequences of 38 cloned PCR products of archaea obtained from *G. barretti* formed a specific, monophyletic cluster, which was stable with four treeing methods (neighbour joining, maximum parsimony, maximum likelihood, Fitch). Although the phylogenetic classification of other marine AmoA sequences cannot be unambiguously resolved (see Fig. 3) the *G. barretti* cluster was clearly affiliated to the crenarchaeotal marine group and together with these was separated from the second major group of archaeal AmoA sequences mostly derived from soils (100% bootstrap support). Similarities of *G. barretti*-derived amino acid sequences to those of '*Candidatus* Cenarchaeum symbiosum' ranged from 93.3% to 94.4% and to those of '*Candidatus* Nitrosopumilus maritimus' from 92% to 93.2% (both on amino acid level). The closest related sponge-derived sequence was from *Chondrosia reniformis* with a similarity of 95.6%. Intracluster similarities, i.e. differences between *G. barretti*-derived sequences only, were 99.38–100%. PCR amplifications of bacteria-derived *amo*A genes were often at the detection limit, indicating far lower amounts of bacterial ammonia oxidizers (not shown). These results were confirmed by metatranscriptomic studies and quantitative PCR data in which *amo*A genes of archaea outnumbered by three to six orders of magnitude *amo*A genes of bacteria in different sponge individuals, both on cDNA and on DNA level (R. Radax, A. Lanzen, F. Hoffmann, T. Urich, and S. Schleper, unpublished). Therefore, a gene library of bacterial *amo*A genes was not constructed.

16S rRNA genes of nitrite-oxidizing bacteria (NOB). To assess the presence and diversity of potential nitrite oxidizers in *G. barretti*, a 16S rRNA gene library with primers specific for the phylum *Nitrospira* was constructed. The produced amplicons were approximately 1100 nucleotides in length. Phylogenetic analysis of 20 genes suggested the presence of sponge-specific nitrifiers from the phylum *Nitrospira* (Fig. 4) with 87.3–91.4% identity to the *Nitrospira marina* 16S rRNA gene and 84.2–87.5% identity to the *Nitrospira moscoviensis* 16S rRNA gene (Fig. 4). The nearest full-length sequence derived from a sponge (AJ347039) had an identity of 98.6–98.9% to the *G. barretti*-derived sequences. *Geodia barretti*-derived clones formed an internal monophyletic group within this cluster with parsimony-bootstrap support of 95%. The 16S rRNA similarity range within the *G. barretti* group was 95.3–99.7% and within the sponge cluster it was 92.8–99.7%.

16S rRNA genes of anaerobic ammonium-oxidizing (anammox) bacteria. Based on the process measurements we concluded that another group of microbes involved in the nitrogen cycling in *G. barretti* should be the anaerobic ammonium-oxidizing (anammox) bacteria. Four different primer combinations were used to amplify the 16S rRNA genes of putative anammox bacteria: PLA46F/BS820R, PLA46F/Amx820R, Amx368F/1392R and PLA46F/1392R. Only the primer combination PLA46F and BS820R was successful in retrieving six gene sequences related to anammox bacteria; the rest of the sequences of this clone library were related to the candidate phylum *Poribacteria. Poribacteria*-related sequences were also retrieved with the primer pairs PLA46F/Amx820R and PLA46F/1392R. The clone library with primers Amx368F and 1392R produced sequences

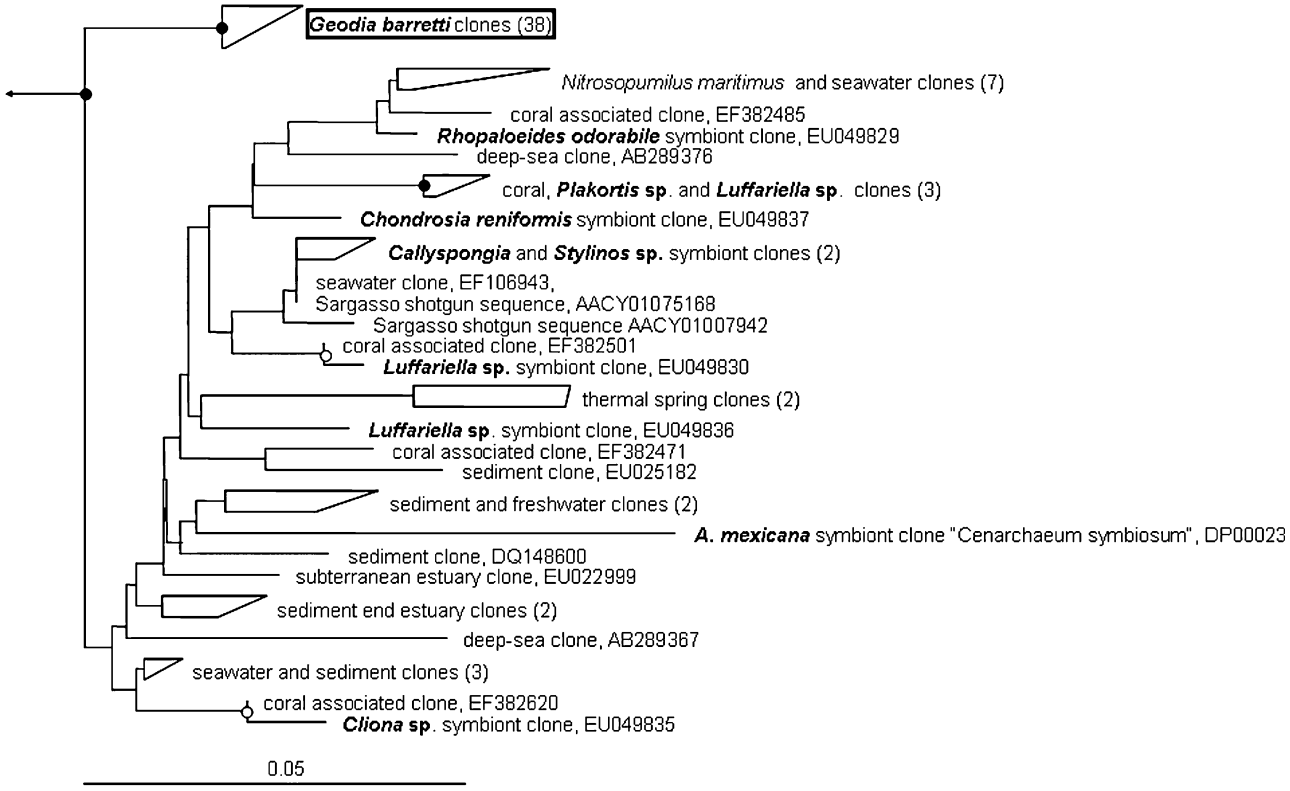

Fig. 3. Phylogenetic tree based on archaeal AmoA sequences of ammonia-oxidizing archaea showing the placement of AOA associated with the marine sponge *Geodia barretti* (bold and framed) within the crenarchaeal group of mostly marine sequences. In this group are also numerous other sponge-derived sequences (bold) and the AmoA sequence from '*Candidatus* Nitrosopumilus maritimus', a known ammonia-oxidizing archaeon. Two hundred amino acids were used for tree construction and parsimony-based bootstraps values are given. Filled circles indicate ≥ 90% bootstrap support and blank circles indicate ≥ 70% bootstrap support. The scale bar indicates 5% sequence divergence. Tree is rooted with a second group of AmoA sequences of *Crenarchaeota* that mostly stem from soils and sediments. The tree was constructed from a distance matrix using the Fitch–Margoliash algorithm.

related to the phylum *Acidobacteria*. Of the six anammox bacterial sequences retrieved with primers PLA46F and BS820R (Fig. 5), five sequences (clones 2G8_1, 2G8_6, 2G8_7, 2G8_8 and 2G8_12) were almost identical to each other (sequence identity of 99.8–100%). Based on calculations with full-length anammox bacterial sequences they were most closely related to a sequence from Barrow Canyon sediment (Alaska, DQ869384, 97.5% sequence identity), whereas the sequence identity with '*Candidatus* Scalindua sorokinii' and '*Candidatus* Scalindua brodae' (Kuypers *et al*., 2003; Schmid *et al*., 2003; Schubert *et al*., 2006) was only ~95%. One sequence (clone 2G8_47) showed only 96% sequence identity to the former cluster of anammox bacterial sequences from *G. barretti* and was more closely related to '*Candidatus* Scalindua sorokinii' and '*Candidatus* Scalindua brodae' (~98% sequence identity). The highest sequence identity was observed with amplified gene fragments from the Peruvian upwelling system (Woebken *et al*., 2008) (98.3%, e.g. clones Peru_23, Peru_54 and Peru_88 of the Peruvian OMZ sea water cluster I).

Cytochrome cd1 nitrite reductase (nirS) of bacterial denitrifiers. The denitrification rates measured in *G. barretti* implied the presence of denitrifying organisms. Since this metabolic capacity is spread between species of different bacterial phyla, we attempted to amplify genes encoding the key metabolic enzyme of this process, i.e. the dissimilatory nitrite reductase. Two unrelated forms of this enzyme exist, the copper-dependent enzyme, NirK, and the cytochrome *cd*1 nitrite reductase, NirS. Several attempts to amplify *nir*K genes from *G. barretti* failed, as only PCR products of unexpected size were obtained. Nevertheless, 20 potential candidate genes obtained with nirK1F and nirK5R (Braker *et al*., 1998) under conditions of low stringency were sequenced, but as expected these were no *nir*K genes. Similarly, the use of the same reverse primer in combination with the forward primer nirK583F (Santoro *et al*., 2006) resulted in no products. In contrast, we were able to amplify bacterial *nir*S genes encoding the cytochrome *cd*1 nitrite reductases of bacterial nitrifiers. Thirteen out of 19 NirS sequences were related to NirS of *Thauera mechernensis* with 99.2–100% amino acid

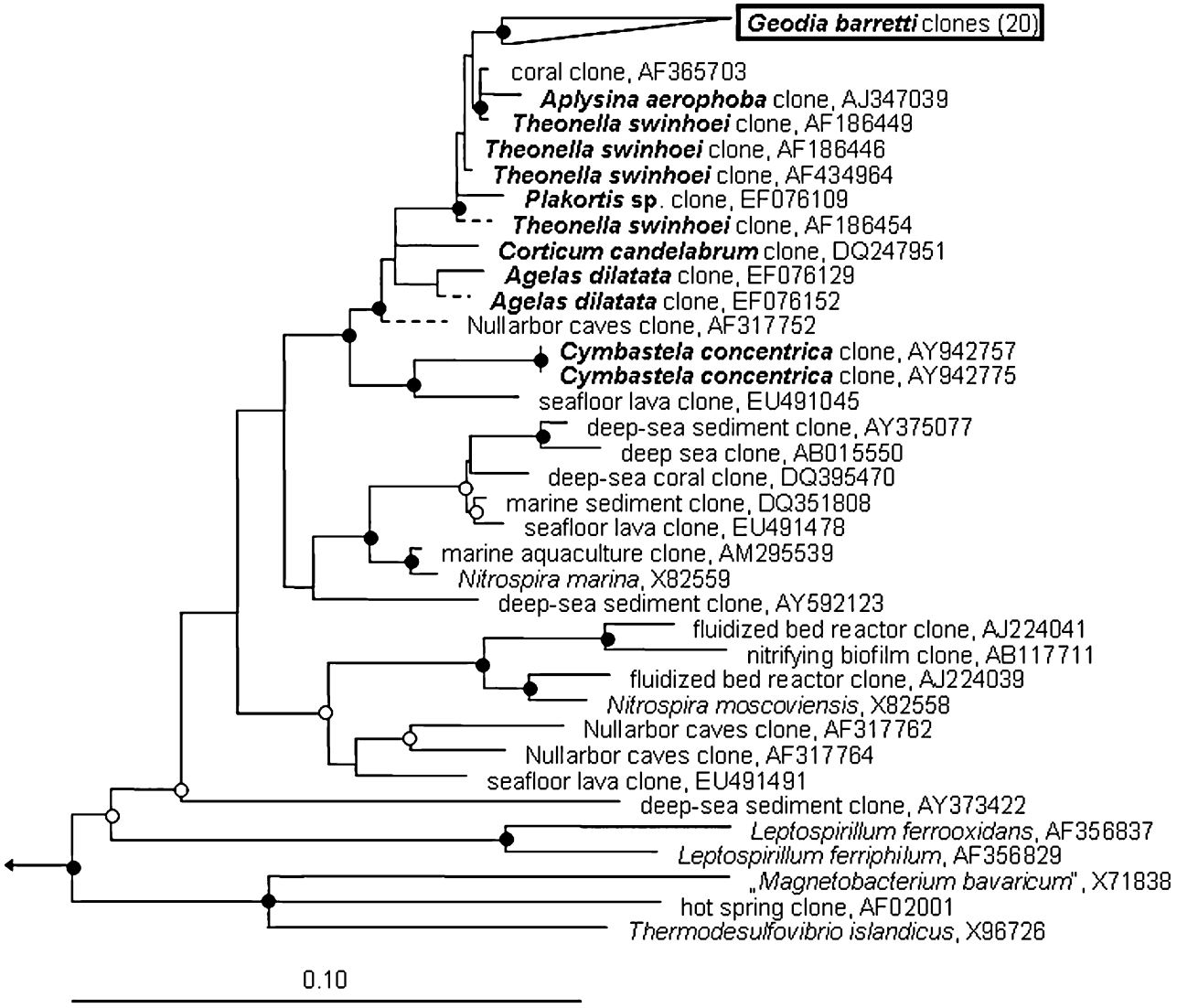

Fig. 4. Consensus tree based on 16S rRNA gene sequences affiliated to the phylum *Nitrospira*, recovered from the marine sponge *Geodia barretti*. Sponge-derived sequences are highlighted in bold and sequences from this study have an additional frame. The tree shown is based on neighbour joining, maximum likelihood and maximum parsimony treeing methods. Branches not unambiguously resolved by different treeing methods are indicated by polytomies. Short sequences were added using the parsimony insertion tool from ARB after tree calculation and are indicated with a dashed line. Filled circles indicate ≥ 90% bootstrap support and open circles indicate ≥ 75% parsimony-bootstrap support. The scale bar indicates 10% 16S rRNA sequence divergence. The out-group (not shown) consisted of 16S rRNA sequences of several other bacterial phyla.

sequence identity and the second group with six sequences had 89–90.6% sequence similarity to NirS of *Pseudomonas stutzeri* and 91.3–92.9% sequence identity to NirS of *Alcaligenes faecalis* on the protein level (Fig. 6).

Discussion

Nitrification, denitrification and anammox rates in sponges

Both anammox and denitrification are important processes for nitrogen cycling in virtually all habitats on Earth, as they remove nitrogen from the environment and recycle it back to the atmosphere. While denitrification in other invertebrates was described recently (Stief *et al.*, 2009), our study represents the first indication for anammox to occur in an animal and shows that nitrification, denitrification and anammox can occur simultaneously in one sponge individual. Rates for nitrification, denitrification and anammox were quantified and putative involved microbes identified. Under suboxic and microoxic conditions as in *G. barretti* explants, these aerobic and anaerobic processes can apparently happen

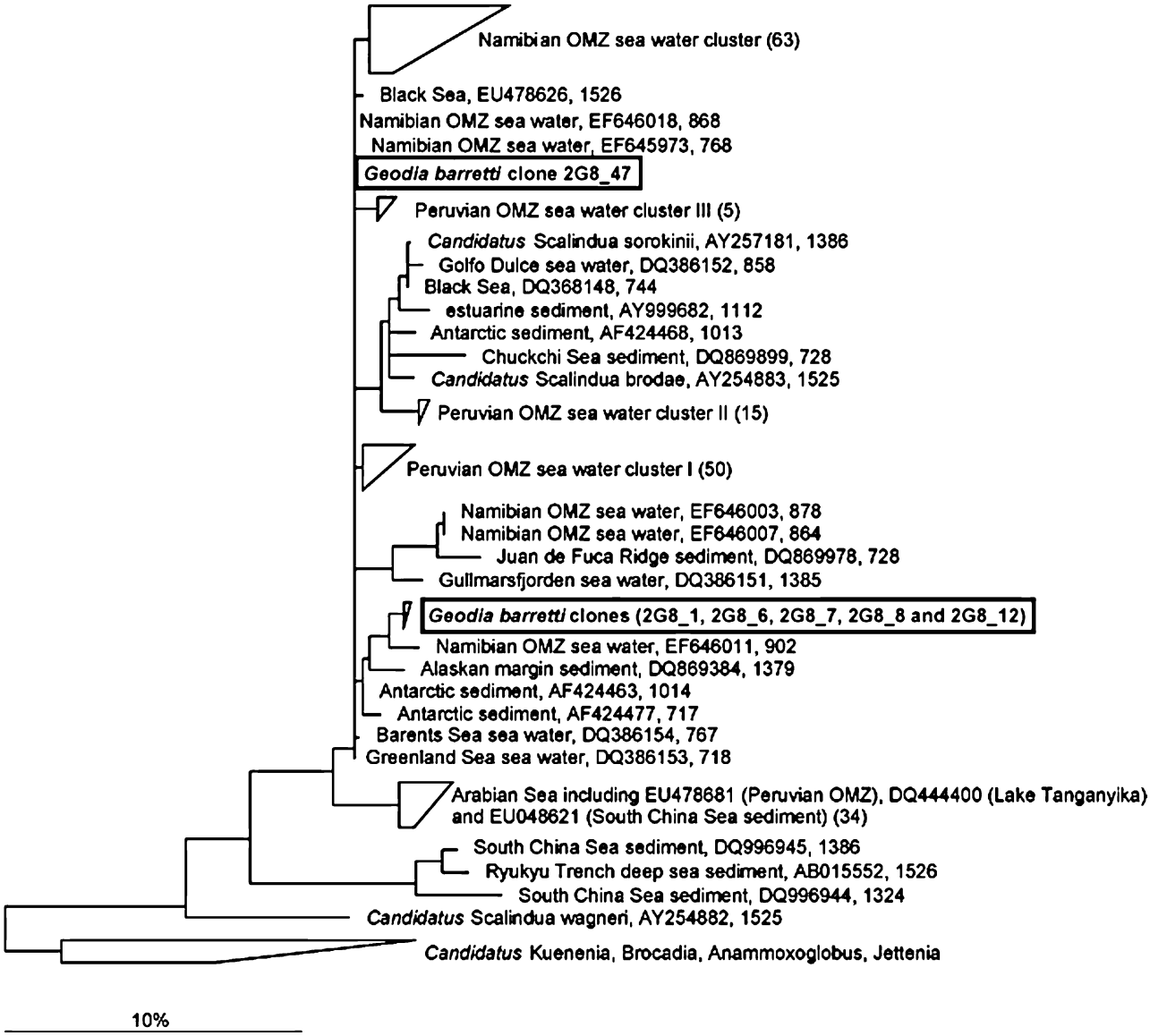

Fig. 5. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic affiliation of the anammox bacterial sequences retrieved from *G. barretti* (bold, framed). The consensus tree was constructed based on maximum likelihood, neighbour joining and maximum parsimony trees, calculated without and with 50% position variability filters using other cultured and uncultured planctomycete sequences as out-group (not shown in tree). The bar represents 10% estimated sequence divergence. Numbers following accession numbers refer to sequence lengths (6p).

at the same time, as recently described for the suboxic zone of Black Sea sediments (Lam *et al.*, 2007). Our bulk nitrification rate (566 nmol N cm^{-3} day^{-1}) must thus be considered as a net rate, since part of the products are used to fuel denitrification and anammox. As pointed out in *Results*, 26% of the nitrate used for denitrification (denitrification rate: 92 nmol N cm^{-3} day^{-1}) stems directly from nitrification. Adding this amount to the net nitrification rate reveals a total nitrification rate of 582 nmol N cm^{-3} day^{-1}. Similarly, the calculated anammox rate is a conservative estimate, since alternatively to the $^{15}NH_4^+$ added, the microbes could use unlabelled NH_4^+ as a substrate which is continuously excreted by the sponge. This would result in the production of unlabelled N_2, which would not be detected with our analysis method and thus is not attributed to the calculated anammox rate.

We also observed nitrification and denitrification in a parallel study with two Mediterranean sponges (M.-L. Schläppy, S. I. Schöttner, G. Lavik, M. Kuypers, D. de Beer, and F. Hoffmann, Submitted). In *Dysidea avara*, net nitrification was 218 nmol N cm^{-3} day^{-1} while denitrification was 242 nmol N cm^{-3} day^{-1}; in *Chondrosia reniformis*, nitrification was 319 and denitrification 360 nmol N cm^{-3} day^{-1}. Compared with the present study, nitrifica-

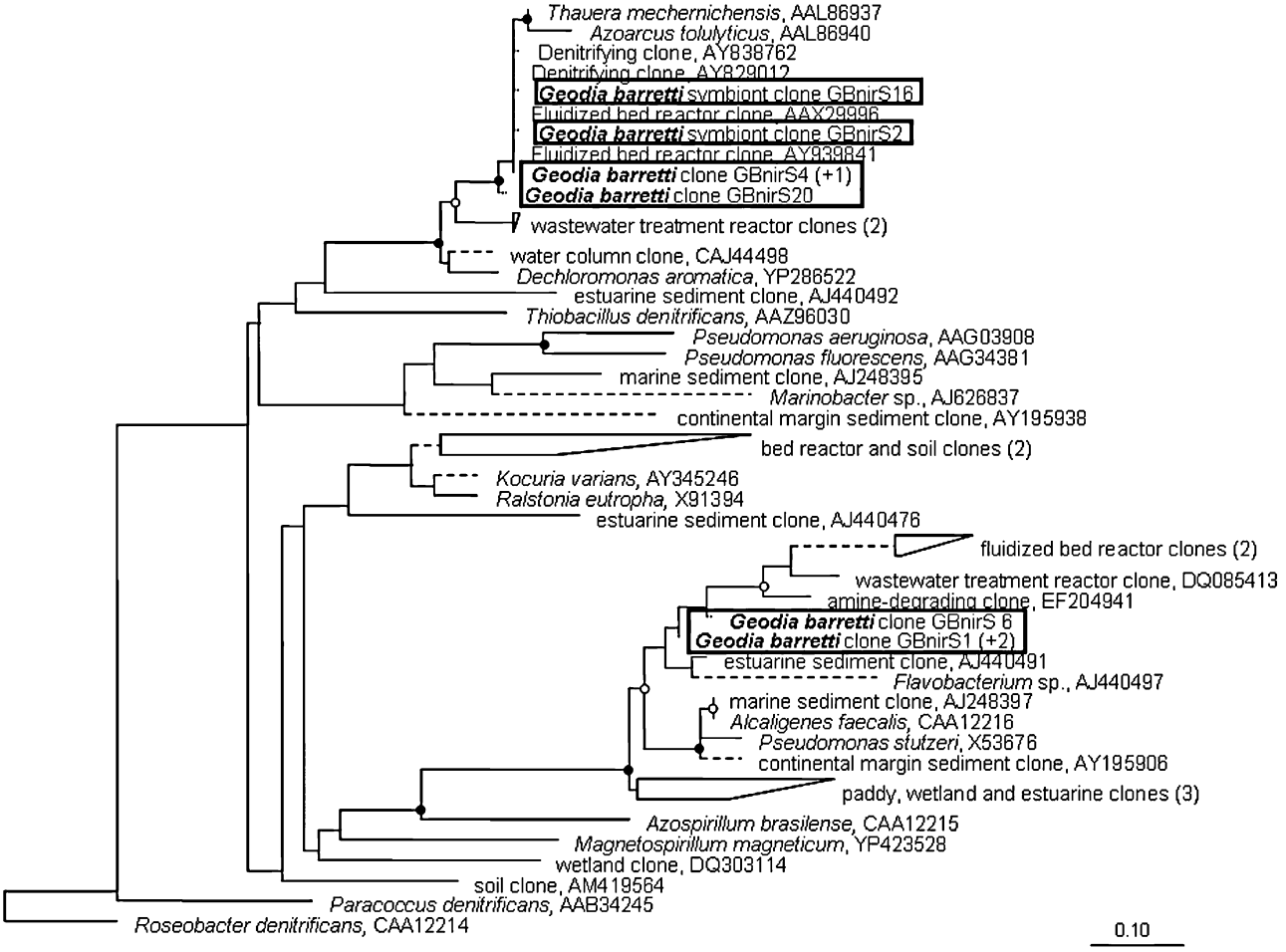

Fig. 6. Phylogenetic Fitch tree based on bacterial NirS protein sequences showing the distribution of denitrifying organisms associated with *Geodia barretti* (bold and framed). The tree was calculated based on sequences with 192 amino acids, whereas short sequences (139 amino acids) were added via the parsimony interactive tool and are indicated by a dashed line. Filled circles indicate ≥ 90% and blank circles indicate ≥ 70% bootstrap support. The scale bar indicates 10% sequence divergence. The tree is rooted with *Roseobacter denitrificans*.

tion rates were lower while denitrification rates were higher in the Mediterranean sponges. Anammox was under the detection limit (M.-L. Schläppy, S. I. Schöttner, G. Lavik, M. Kuypers, D. de Beer, and F. Hoffmann, Submitted). These results show that complex nitrogen-cycling processes as we observed in *G. barretti* may be common in sponges, but rates may be variable in different sponge species. It will be important to investigate the activity in further species to obtain a broader view on the significance of these processes in these and other animals.

Besides our studies, there are no other reports on denitrification or anammox rates in sponges, whereas nitrification has been reported. The net nitrification rate we observed in *G. barretti* (566 nmol cm^{-3} day^{-1}; approx. 196 nmol g^{-1} dry wt h^{-1}, assuming a sponge water content of 90%) is within the range of nitrification rates reported for Mediterranean sponges [89–1325 nmol g^{-1} dry wt h^{-1} (Bayer *et al.*, 2008); 180–780 nmol g^{-1} dry wt h^{-1} (Jimenez and Ribes, 2007) as well as tropical sponges (30–2650 nmol g^{-1} dry wt h^{-1} (Diaz and Ward, 1997)]. It is also within the range of nitrification rates reported from other benthic animals (3–1020 nmol g^{-1} dry wt h^{-1}) and it is orders of magnitude above nitrification rates of estuarine sediments [0.02–7.7 nmol g^{-1} dry wt h^{-1} (Bernhard *et al.*, 2007)].

Rates of anammox have not been reported from animal hosts so far. The rate we found in the sponge *G. barretti* (3 nmol N cm^{-3} sponge day^{-1}) is at least one order of magnitude higher than pelagic anammox rates [0.02–0.38 nmol ml^{-1} day^{-1} (Kuypers *et al.*, 2003; 2005; Hannig *et al.*, 2007)].

Denitrification rates of microbes dwelling in the guts of benthic invertebrates (insects, mollucs and shrimps) range between 7 and 38 nmol N per individual per day, which equals 29–365 nmol cm^{-3} day^{-1} (Stief *et al.*, 2009), and are thus comparable to denitrification rates we found in *G. barretti*.

Microbial community patterns in freshly sampled and cultivated sponges

ARISA analysis showed that healthy explants kept in the laboratory contained similar communities of microbes as wild *G. barretti*. This is consistent with a previous FISH (fluorescence *in situ* hybridization) study targeting the main groups of the microbial community of *G. barretti* during 8 months of cultivation. Hoffmann and colleagues (2006) showed that the respective microbial groups were stable throughout several months of cultivation; while aberrant microbes occurred during the first days.

Microbial processes observed in healthy explants as monitored in this study can thus also be expected in complete sponges; and vice versa, the microbial sequences that we recovered from freshly sampled sponges should also be present in the explants. A more extensive study on the microbial community patterns of *G. barretti* investigating specimens from different sampling sites and seasons using ARISA and DGGE (denaturing gradient gel electrophoresis) revealed a species-specific microbial community in *G. barretti* (F. Hoffmann and S. Fortunato, unpublished). Moreover, these studies showed that the microbial community of *G. barretti* was very close to that of other sponge species of the family of Geodiidae, indicating that the complex nitrogen-cycling processes described here may be found in many or even all sponges of the Geodiidae family.

Phylogeny of microbes involved in Ncycling

We have used PCR-based studies to screen specifically for microorganisms potentially responsible for nitrification, denitrification and anammox processes in the sponge *G. barretti*. The *Nitrospira* found in *G. barretti* and other sponge species formed a monophyletic sequence cluster, mainly consisting of sponge – derived 16S rRNA sequences, within the *N. marina*-related group (Hentschel *et al*., 2002; Taylor *et al*., 2007). The ammonia-oxidizing archaea found in *G. barretti* were closely related to other sponge-derived sequences within the group of *Crenarchaeota*, mainly consisting of marine sequences. A prevalence of archaeal versus bacterial *amoA* genes as obtained by quantitative PCR as well as whole genome transcription patterns of the microbial communities in *G. barretti* (R. Radax, A. Lanzen, F. Hoffmann, T. Urich, and S. Schleper, unpublished) indicates that the ammonia oxidation we observed in *G. barretti* is most likely driven by the activity of archaea. In total, the phylogenetic analyses imply that sequences of *G. barretti*-associated nitrifiers (ammonia-oxidizing archaea and *Nitrospira*) form monophyletic sequence clusters. This indicates a long-term stable association and/or adaptation of the microbes to their host sponge, which lives in environmental conditions notably different from that of shallow water sponges in tropical and temperate areas, i.e. cold water, darkness and high pressure. Anammox bacteria sequences derived from *G. barretti* were most closely related to the '*Candidatus* Scalindua' branch, like most other marine anammox bacteria sequences. The recovery of two groups of genes potentially encoding cytochrome *cd*1-type nitrite reductases (NirS) suggests that the denitrification activity, as measured in the sponge, is most probably performed by more than one group of bacteria. Based on sequence distributions the most likely denitrifying population to be specifically associated with *Geodia* could be related to the *Thauera* group (see Fig. 6). Clear phylogenetic assignments based on the *nir*S gene are not possible as horizontal gene transfer (HGT) occurs frequently and can lead to wrongly assigned taxa (Braker *et al*., 1998). From cultivation studies it is known that organisms harbouring the *nirS* gene are most prevalent in the *Betaproteobacteria* (Heylen *et al*., 2006). In our study we have found two clusters, one related to sequences from *Betaproteobacteria*, and another cluster related to sequences of *Beta*- and *Gammaproteobacteria*, as well as a short *Bacteroidetes*-related sequence. Since this study provides the first phylogenetic analysis for denitrifying and anammox bacteria from a marine sponge, we cannot assign any group as being sponge-specific. It is worth noting in this context that a common alphaproteobacterial associate of marine sponges (Hentschel *et al*., 2001; Webster and Hill, 2001; Enticknap *et al*., 2006) was found to be very closely related to the marine denitrifier *Pseudovibrio denitrificans*, and that at least some of these sponge-derived strains had tested positive for denitrification (Enticknap *et al*., 2006).

Function and effects of complex nitrogen cycling in sponges

It seems obvious that autotrophic partners such as nitrifying microbes may provide an additional source of carbon for the sponge. The exact nature of metabolic exchange between the respective partners is not well understood yet; however, direct feeding of sponge cells on associated microbes is well described (Wilkinson and Garrone, 1980; Ilan and Abelson, 1995; Vacelet *et al*., 1996) and we made similar observations by transmission electron microscope investigation of *G. barretti* (F. Hoffmann, not shown). The ratio of carbon fixation to nitrification by bacterial ammonia and nitrite oxidizers is approximately 1 mol of CO_2 for every 9 mol of NH_4^+ oxidized to NO_3^- (Feliatra and Bianchi, 1993). Thus, nitrification rates of *G. barretti* (582 nmol cm^{-3} day^{-1}, regarding nitrate lost by denitrification) allow the nitrifying community in sponges to fix up to 64 nmol CO_2 cm^{-3} sponge day^{-1}. Differences in carbon fixation mechanisms of

archaeal ammonia-oxidizers (Hallam *et al*., 2006b) could alter this ratio. In any case, the possible carbon fixation rates are small compared with respiration rates of *G. barretti* explants [9 μmol O_2 cm^{-3} day^{-1} (Hoffmann *et al*., 2005a)]. Assuming that 1 μmol O_2 consumption per cm^3 sponge per day is due to aerobic oxidation of ammonium to nitrate and nitrite, and the remainder is due to carbon oxidation, microbial nitrification would provide less than 1% of the carbon demand of *G. barretti*. Due to the even lower rate, carbon fixation by anammox bacteria can be neglected.

Heterotrophic microbes consume simple compounds (dissolved organic carbon, DOC), while sponge cells prefer small particles (particulate organic carbon, POC) like pelagic bacteria or phytoplankton (Willenz, 1980; Pile *et al*., 1996; Witte *et al*., 1997; Ribes *et al*., 1999). Sponges with high amounts of associated microbes however have been identified as important DOC sinks (Yahel *et al*., 2003; De Goeij *et al*., 2008a). This leads to the conclusion that DOC uptake in sponges is mediated by sponge microbes. In fact, a recent investigation by De Goeij and colleagues (2008b) showed that assimilation of ^{13}C-labelled DOC was both direct and bacteria mediated, as tracer carbon was recovered both in bacteria-specific and in non-bacteria fatty acids within the sponge. Considering an anoxic situation in the sponge matrix, DOC may also be excreted as a result of fermentation processes in sponge cells, and could thus feed an anaerobic heterotrophic microbial community, such as sulfate-reducing prokaryotes (Hoffmann *et al*., 2005b) and denitrifiers (this study). The importance of these processes for sponge nutrition remains to be proven.

In conclusion, the physiologically complex community of microorganisms involved in sponge nitrogen cycling has the potential to make additional carbon sources available to the host sponge, although its impact on sponge nutrition may be rather small.

We assume therefore that the main benefit of the complex nitrogen cycling in sponges lies within the efficient removal of waste products. Ammonium removal by ammonium oxidizers (aerobic and anaerobic) may even exceed sponge ammonium excretion, as seen in the first 24 h of the nitrification experiment (Fig. 1B). Similar observations were made with the Mediterranean sponge *Aplysina aerophoba*, which functions as an 'ammonium sink' during the cold season (Bayer *et al*., 2008). Accumulation of detrimental nitrite and nitrate is counteracted by nitrite oxidation and nitrate reduction respectively. The energy gain and possible carbon transfer from sponge microbes to sponge cells are a positive, but minor side-effect of this efficient waste product treatment. Nitrogen fixation by symbiotic cyanobacteria has been described for shallow-water sponges (Wilkinson and Fay, 1979; Mohamed *et al*., 2008). We do not expect this process in *G. barretti* which lives in deep water where inorganic nitrogen is always available in excess as NO_3^-, and where cyanobacterial symbionts are not present due to the absence of light.

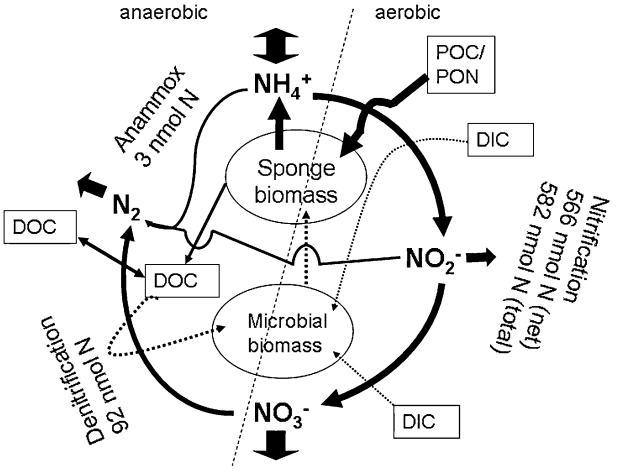

Fig. 7. Complex nutrient cycling in the sponge *G. barretti* showing the interaction of heterotrophic, autotrophic, aerobic and anaerobic processes within the sponge–microbe system and between sponge and environment. Rates of microbial processes of the sponge nitrogen cycle are given in nmol N cm^{-3} sponge day^{-1}. Proposed processes of carbon transfer between sponge and microbes are indicated with dashed arrows. Thickness of the arrows reflect the quantitative importance of this process. DIC, dissolved inorganic carbon; DOC, dissolved organic carbon; POC, particulate organic carbon; PON, particulate organic nitrogen.

Complex nitrogen cycling within *G. barretti* and nitrogen fluxes between the sponge and the marine environment are summarized in Fig. 7.

Impact of sponge nitrogen cycling on marine ecosystems

In sponge mass occurrences at the North Atlantic slope at about 200–500 m water depth (Klitgaard *et al*., 1997; Klitgaard and Tendal, 2004; H.T. Rapp, unpubl. data), sponges of the family Geodiidae can be found in densities of up to 30 kg m^{-2}. Assuming similar rates *in situ* to those we obtained in this study, sponge-mediated nitrification would transform up to 16 mmol N m^{-2} day^{-1}, while sponge-mediated denitrification would remove 2.7 mmol N m^{-2} day^{-1} as N_2. Nitrogen removal by these sponge mass occurrences can thus be 2–10 times higher than by continental shelf sediments at similar water depths, where denitrification rates are 0.1–1 mmol N m^{-2} day^{-1} (Middelburg *et al*., 1996; Seitzinger and Giblin, 1996).

Sponge mass occurrences, which can cover several km^2 at certain areas in the North Atlantic (Klitgaard *et al*., 1997; Klitgaard and Tendal, 2004), can therefore be more important for nitrogen mineralization processes than marine sediments at these depths.

The importance of sponge-mediated nitrification and the resulting fluxes of dissolved inorganic nitrogen in areas with high sponge cover, e.g. tropical coral reefs, has already been pointed out (Southwell *et al*., 2008, and references therein). Denitrification and anammox, in contrast, remove these nutrients from the environment. If denitrification and anammox rates as we observed them in *G. barretti* turn out to be common processes in sponges all over the world, then marine areas with high sponge cover, e.g. sponge mass occurrences of the North Atlantic as well as tropical and cold-water coral reefs, may function as so far unrecognized sinks for inorganic nitrogen. In order to evaluate the impact of sponges as nitrogen sinks in the ocean it will be important to measure such processes in other marine environments and in more sponge species.

Experimental procedures

Sponge sampling and explant culture

Sponges were sampled near the city of Bergen on the west coast of Norway, between 100 and 200 m depth on a hard bottom slope in Korsfjord (60°09′12″N, 05°08′52″E) using a triangular dredge operated from the research vessel R/V Hans Brattstrøm. Since it is not possible to maintain whole specimens of *G. barretti* in the lab, explant cultures of sponge tissue were established: cube- or cuboid-shaped fragments of 0.3–0.4 cm^3 were cut from the choanosomal part of freshly retrieved *G. barretti* and kept in half-open cultivation systems with unfiltered seawater as previously described (Hoffmann *et al*., 2003): sponge explants were placed on fine mesh plastic grids (3 mm mesh) with no artificial connection, allowing the entire surface to be in contact with the ambient water. Explants can be maintained up to 1 year (Hoffmann *et al*., 2003); tissue and skeleton regeneration, growth and even the production of egg cells were observed during cultivation. Within the first months of cultivation, explants of *G. barretti* have no canal system and cannot pump water through their body. Most of the animal is anoxic, except a surface layer of 1 mm where oxygen enters the sponge by molecular diffusion (Hoffmann *et al*., 2005a). Thus, explants of *G. barretti* reflect conditions of both pumping (oxic) and non-pumping (anoxic) sponges and are useful model organisms to investigate aerobic and anaerobic processes in sponges.

Incubation experiments

NH_4^+ incubation – nitrification. Explants, which had been cultivated for 5 months and showed a visually healthy appearance, were used for the experiments. For the nitrification experiment, two explants of 0.3–0.4 cm^3 (total sponge volume: 0.7–0.8 cm^3) were placed in 500 ml of natural, unfiltered sea water from 200 m depth amended with 12 μM NH_4^+. Three parallel incubations and one control (sea water without sponge) were used. Beakers with air bubblers and magnetic stirrers were placed in a temperature-controlled room at 15°C (same as used for explant cultures) in the dark. Water samples were taken over a time-course of 48 h and immediately frozen at –80°C until nutrient analyses were carried out. Ammonium and phosphate concentrations were determined with a Scalar Continuous-Flow-Autoanalyser using the chemistry described by Grasshoff (1983). Nitrite and nitrate were analysed with a chemoluminescence NOx analyser (Thermo Environmental Instruments, USA).

^{15}N incubations – denitrification and anammox. The denitrification process combines two NO_3^- ions to form one molecule of N_2. When $^{14}NO_3^-$ is present in addition to the added $^{15}NO_3^-$, denitrification produces $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ through random isotope pairing. The anammox process, in contrast, combines one NO_2^- and one NH_4^+ ion, which result in the production of $^{14}N^{14}N$ or $^{14}N^{15}N$ in incubation experiments with $^{15}NO_3^-$, but no production of $^{15}N^{15}N$ (Thamdrup and Dalsgaard, 2002).

For ^{15}N incubations we slightly modified a previously published method (Dalsgaard *et al*., 2003; Hannig *et al*., 2007). Sponge explants of 0.3–0.4 cm^3 (average 0.35 cm^3) were incubated in gas-tight 12.5 ml glass vials (Labco exetainer, Labco Limited, UK) completely (bubble-free) filled with natural sea water. Sea water was amended with 10 μM $^{15}NO_3^-$ ($^{14}NO_3^-$ background: 8.2 μM) for the denitrification experiment and 10 μM $^{15}NH_4^+ + 2$ μM $^{14}NO_2^-$ (background: 0.4 μM NH_4^+, 0.3 μM NO_2^-) for the anammox experiment. Respiration rates of sponge explants (9 μmol O_2 cm^{-3} sponge day^{-1}) (Hoffmann *et al*., 2005a) depleted 90% of the initial oxygen concentration (280 μM) in the exetainers during the 24 h of the experiment. Biological activity was stopped at 0, 3, 6, 12 and 24 h by adding 150 μl of saturated $HgCl_2$ solution. A head space of 2 ml of He gas was added to trap the produced N_2. One to two replicates and two controls (sea water without sponge) were sampled per time point. To avoid any leakage of gas, samples were stored upside-down at room temperature until analyses. The isotope ratio ($^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$) of the head space nitrogen was determined by gas chromatography-isotopic ratio mass spectrometry by direct injections from the head space according to Kuypers and colleagues (2005). The concentrations of the produced $^{14}N^{15}N$ and $^{15}N^{15}N$ were assessed as excess relative to air and the N_2 production rates were calculated from the slope of increase (Nielsen, 1992; Thamdrup and Dalsgaard, 2002; Risgaard-Petersen *et al*., 2003).

Molecular and phylogenetic analyses of sponge microbes

DNA extraction. For anammox bacterial 16S rRNA gene libraries, as well as for microbial community patterns, samples of freshly collected *G. barretti* were immediately fixed in 99% ethanol, washed once in 99% ethanol, and frozen at –20°C. Prior to DNA extraction, the sponge material was ground with mortar and pestle, and left at room temperature for several minutes to evaporate the ethanol. DNA was extracted using the Fast DNA spin kit for soil (BIO 101) according to the manufacturer's instructions. For construction of gene libraries containing 16S rRNA genes of *Nitrospira*, *amoA* genes of archaea and *nirS* genes of bacteria, samples of freshly harvested *G. barretti* were fixed in liquid nitrogen

directly on the boat and frozen at –80°C until DNA extraction with phenol-chloroform was performed as described before (Leininger *et al*., 2006).

Microbial community patterns obtained by ARISA. Since DNA for the molecular studies was obtained from freshly sampled *G. barretti*, while cultivated sponge explants were used for the physiological experiments, it was necessary to check if explants and wild sponges contain similar microbial communities. The high-resolution molecular fingerprinting technique ARISA was used for this comparison. DNA of three freshly sampled *G. barretti*, of two explants that had been used for the nitrification experiments (two independent DNA extractions per explant), and of five explants that died during cultivation and showed obvious signs of decay (black colour, bad smell), was extracted as described above. Comparison with the microbial community of dead explants was performed to provide an additional proof that experimental explants were viable and healthy; this is not always obvious in explant cultures which lack an aquiferous system and thus can not pump (Hoffmann *et al*., 2005a). Universal bacterial ARISA was performed in triplicate using the primers ITSF and the HEX-labelled ITSReub (Cardinale *et al*., 2004). ARISA-PCR, fragment analysis and processing of ARISA profiles were performed as described elsewhere (Böer *et al*., 2009). Non-metric multidimensional scaling (nmds, Chord distance) was performed with the PAST data analysis package. Testing for significant differences between distinct sample groups (field sponges, experimental explants, dead explants) was performed by ANOSIM in PAST.

Construction of clone libraries. In order to obtain 16S rRNA gene sequences from putative anammox bacteria, we constructed four clone libraries using primers with different specificities. The first clone library was constructed with the planctomycete-specific primer PLA46F (Neef *et al*., 1998) and with BS820R targeting the anammox bacteria *Candidatus* Scalindua wagneri and *Candidatus* Scalindua sorokinii (Kuypers *et al*., 2003). For the second clone library we used PLA46F in combination with Amx820R (Schmid *et al*., 2000), which targets the anammox bacterial genera *Candidatus* Brocadia and *Candidatus* Kuenenia. The third clone library was constructed with the primer Amx368F that should target all anammox bacteria (Schmid *et al*., 2003) and the universal primer 1392R (Stahl *et al*., 1988). For the fourth clone library we used the planctomycete-specific primer PLA46F and the universal primer 1392R in order to target all planctomycetes. Thirty-four PCR cycles were used for amplification with the first and the third primer set, 33 cycles for the second primer set and 26 cycles for the fourth primer set. All PCR reactions were conducted in 12 replicates, the replicates were pooled and the pooled PCR products were cloned. Preparative gels ensured cloning of PCR products of the correct size.

PCR reactions were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), prior to cloning with TOPO TA Cloning kits for sequencing with vector pCR4 (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Clones were screened by PCR for inserts of correct size and these were sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

16S rRNA genes of *Nitrospira* were amplified with the universal primer 616V (Juretschko *et al*., 1998) and the *Nitrospira*-specific primer 1158R (Maixner *et al*., 2006). Amplification of archaeal *amoA* genes was performed with primer set 19F (Leininger *et al*., 2006) and 643R (Treusch *et al*., 2005) and amplification of *nirS* genes was achieved with primers nirScd3AF (Michotey *et al*., 2000) and nirSR3cd (Throbäck *et al*., 2004), using touchdown PCR conditions (Braker *et al*., 1998) in combination with GoTaq Polymerase (Promega Corporation, Madison, WI, USA). The respective amplicons of three sponge individuals were cut out from a low-melting agarose gel and were pooled for cloning with the TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. Clones were screened for inserts of correct size and these were sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

Phylogenetic analysis. 16S rRNA sequences of anammox bacterial clone libraries were edited with the Sequencing Analysis Software (Applied Biosystems) and analysed for their closest relatives using the ARB 16S rRNA gene database (Ludwig *et al*., 2004) and BLAST (Altschul *et al*., 1997). Sequences of about 800 bp length were assembled from individual reads with the Sequencher v4.6 software (Gene Codes Corporation, Ann Arbor, USA).

The 16S rRNA gene sequences were checked for the presence of chimeric sequences by using the CHIMERA_CHECK program from RDP II (Cole *et al*., 2003) and imported into the ARB 16S rRNA gene database. Phylogenetic analyses of the 16S rRNA sequences were made using distance matrix, maximum parsimony and maximum likelihood algorithms in ARB with and without 50% variability filters. A consensus tree was constructed thereafter.

16S rRNA sequences related to *Nitrospira* were edited and analysed using the ARB program package (Ludwig *et al*., 2004) after proofreading and were combined with the sponge symbiont database (Taylor *et al*., 2007). Sequences with high similarity to *G. barretti*-derived sequences were identified by BLAST searches and were also imported into ARB. A consensus tree, based on three different treeing methods (neighbour joining, maximum likelihood and maximum parsimony), was constructed including relevant reference sequences and other available sponge-derived 16S rRNA sequences.

Amino acid sequences of ammonia-monooxygenase (AmoA) and dissimilatory nitrite reductase (NirS) were also edited and analysed in ARB. Databases for both genes were generated by importing sequences from NCBI after BLAST searches using *G. barretti*-derived sequences were performed. Fitch trees (Fitch and Margoliash, 1967) were calculated in PHYLIP and imported into ARB for further formatting and the addition of shorter NirS sequences.

Sequence identities were calculated using the similarity function of the distance matrix algorithm in ARB. Anammox bacteria-related 16S rRNA gene sequences, 16S rRNA gene sequences for *Nitrospira*, AmoA and NirS sequences are available in GenBank under Accession No. FJ230205–FJ230287.

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