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Diversity and abundance of ammonia-oxidizing *Archaea* and *Bacteria* in tropical and cold-water coral reef sponges

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ABSTRACT: We analysed the diversity and abundance of ammonia-oxidizing *Archaea* (AOA) and *Bacteria* (AOB) in the shallow warm-water sponge *Halisarca caerulea* and the deep cold-water sponges *Higginsia thielei* and *Nodastrella nodastrella*. The abundance of AOA and AOB was analysed using catalyzed reporter deposition-fluorescence *in situ* hybridization and (real-time) quantitative PCR (Q-PCR) targeting archaeal and bacterial *amoA* genes. Archaeal abundance was similar between sponge species, while bacterial abundance was higher in *H. caerulea* than in *N. nodastrella* and *H. thielei*. Q-PCR showed that AOA outnumbered AOB by a factor of 2 to 35, suggesting a larger role of AOA than of AOB in ammonia oxidation in sponges. PCR-denaturing gradient gel electrophoresis was performed to analyse the taxonomic affiliation of the microbial community associated with these sponges. Archaeal and bacterial *amoA* genes were found in all 3 sponges. The structure of the phylogenetic trees in relation to temperature and sponge species was analysed using all published *amoA* sequences retrieved from sponges. Temperature was an important factor influencing the distribution of nitrifiers in sponges. Both archaeal and bacterial *amoA* sponge sequences tended to cluster with sequences retrieved from habitats of similar temperature. This is the first time that similarity in AOB diversity is described between distantly related species (*H. thielei* belonging to the class Demospongiae, and *N. nodastrella* to Hexactinellida). The results described here support the idea of a relatively uniform microbial community between distantly related sponges and suggest that temperature (rather than phylogenetic distance) is determining the diversity of AOA and AOB in sponges.

KEY WORDS: *amoA* gene · *Archaea* · *Bacteria* · Temperature · Marine · Sponges

INTRODUCTION

Nitrification is the first step in the nitrogen cycle, involving the oxidation of ammonia (NH_3) to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-). These 2 steps are catalysed by distinct groups of microorganisms: ammonia oxidizers and nitrite oxidizers. It is now well established that some marine sponges (phylum Porifera) live in association with microorganisms which are able to nitrify (Corredor et al. 1988, Diaz & Ward 1997, Diaz et al. 2004, Jiménez & Ribes 2007, Taylor et al. 2007, Southwell et al. 2008, Van Duyl et al. 2008, Hoffmann et al. 2009, Schläppy et al. 2010, Hentschel et al. 2012). Ammonia-oxidizing *Bacteria* (AOB) and nitrite-oxidizing *Bacteria* (NOB) have been observed in sponges (Taylor et al. 2007 and references therein). Ammonia-oxidizing *Archaea* (AOA) have also been receiving increasing attention since

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the discovery of the ammonia-oxidizing thaumarchaeote *Cenarchaeum symbiosum* associated with the sponge *Axinella mexicana* (Preston et al. 1996). Thaumarchaeotes, in particular, appear capable of oxidizing ammonia (Bayer et al. 2008, Turque et al. 2010, Liu et al. 2011, Pester et al. 2011 and references therein). Recently, the presence of AOB and AOA was described for a diverse range of sponges from the Pacific, Caribbean, Mediterranean and North Atlantic (Bayer et al. 2008, Meyer & Kuever 2008, Steger et al. 2008, López-Legentil et al. 2010, Mohamed et al. 2010, Liu et al. 2011, Radax et al. 2012). Nitrification by sponges may well be significant in coral reef ecosystems, considering the high biomass of sponges in such systems (Diaz & Rützler 2001, Reitner & Hoffmann 2003, Van Soest et al. 2007a).

The tropical shallow-water sponge *Halisarca caerulea* (Vacelet & Donadey 1987) (class Demospongiae, order Halisarcida, family Halisarcidae) and the cold deep-water sponges *Higginsia thielei* (Topsent, 1898) (class Demospongiae, order Halichondrida, family Heteroxyidae) and *Nodastrella nodastrella* (until recently known as *Rossella nodastrella* Topsent, 1915; Dohrmann et al. 2012) (class Hexactinellida, order Lyssacinosida, family Rossellidae) are common inhabitants of Atlantic coral reefs. Nitrification has been reported in the latter 2 species, most likely being mediated by sponge-associated microbes (Van Duyl et al. 2008). *H. thielei* and *N. nodastrella* harbor relatively high amounts of *Archaea* and *Bacteria* (ca. 7 to 30% *Archaea* and 36 to 65% *Bacteria* of the total microbial counts with DAPI staining; Van Duyl et al. 2008). Furthermore, evidence for microbial bicarbonate fixation by these sponges in the dark ocean suggests that sponge-associated microorganisms may be involved in ammonia oxidation in these areas. *H. caerulea* also harbours sponge-associated microorganisms (De Goeij et al. 2008). This sponge lives in cryptic habitats in the reef, like crevices, which have shown net release of nitrate (Van Duyl et al. 2006). Since sponges cover up to 50% of the calcareous rock in these crevices (Scheffers et al. 2004) and several tropical reef sponges have already been reported to nitrify (e.g. Corredor et al. 1988, Diaz & Ward 1997, Southwell et al. 2008), it is assumed that the measured nitrate efflux was at least partly coming from cavity sponges including *H. caerulea*. Despite these suggestions, it is still unknown whether microorganisms associated to *H. caerulea*, *H. thielei* and *N. nodastrella* could be directly involved in the N-cycle. 16S rRNA genes and the ammonia-monooxygenase subunit A (*amoA*) gene have been commonly used to detect the presence of ammonia-oxidizing microorganisms in sponges (see Taylor et al. 2007 and references therein, Bayer et al. 2008, Cheng et al. 2008, Meyer & Kuever 2008, Mohamed et al. 2008, 2010, Steger et al. 2008, Hoffmann et al. 2009).

Ammonia-monooxygenase (AMO) is an integral membrane protein occurring in ammonia oxidizers, which is composed of 3 subunits (A, B and C) and various metal centres (Hyman & Arp 1992, McTavish et al. 1993, Klotz et al. 1997). The *amoA* subunit contains the active site of AMO (Hyman & Arp 1992). Despite the fact that fewer studies have used *amoA* when compared to 16S rRNA, the *amoA* gene has the advantage that it encodes a protein involved directly in ammonia oxidation and is, therefore, a functional gene important to the nitrification process (O'Mullan & Ward 2005). In the present study, we analysed the diversity and abundance of the *amoA* functional gene in *Higginsia thielei*, *Nodastrella nodastrella* and *Halisarca caerulea* with the aim of assessing whether (1) these sponge species harbour bacterial and archaeal nitrifiers and (2) the diversity of the ammonia-oxidizing microbial community is mainly sponge related or temperature related. Since this study includes sponges belonging to different taxonomic classes (1 hexactinellid sponge and 2 demosponges), the diversity of AOA and AOB associated with phylogenetically distant host sponges was also analysed.

Here, 'sponge-associated' microorganism refers merely to the presence of a certain microorganism in the sponge, assuming nothing regarding the existence of interaction or dependence.

MATERIALS AND METHODS

Study site, species and sampling

The marine sponge *Halisarca caerulea* is a thin encrusting sponge living in coral cavities and common in shallow waters (2 to 25 m) of the Caribbean Sea (Vacelet & Donadey 1987, Collin et al. 2005, De Goeij et al. 2008). *H. caerulea* were carefully chiselled out of coral cavities under coral slabs or coral rock overhangs on the forereef slope of Curaçao, southern Caribbean. Material was collected between 15 and 17 m depth from the walls of dead end cavities of 50 to 250 l volume at the Carmabi reef (Buoys 0 and 1) in February 2003 and at Blue Bay in April to May 2004. In addition, samples of 2 to 3 l of water surrounding the sponges were collected (for details of collected samples see Table S1 in the supplement at

www.int-res.com/articles/suppl/a068p215_supp.pdf). Water samples were first pre-filtered through a 0.8 µm pore size polycarbonate filter (Poretics) and then filtered through a 0.2 µm pore size polycarbonate filter (Poretics). Filters were wrapped in clean aluminium foil and kept frozen at –80°C until DNA extraction. Total DNA was extracted as described below.

The marine sponges *Higginsia thielei* and *Nodastrella nodastrella* are present in deep-water coral mounds (up to >1000 m depth) off the Azores and in the south-eastern Rockall Bank, in the North East Atlantic (Van Soest et al. 2007b, 2012). *H. thielei* is a small, round and rigid sponge, while *N. nodastrella* is a large, thin-walled, tubular, trumpet-shaped sponge (Van Soest et al. 2007a,b). These sponges were collected on the south-eastern part of Rockall Bank (Logachev mounds) from 24 June to 12 July 2006 with a box corer (stainless steel cylindrical barrel: 50 cm inner diameter, 55 cm high) at a depth between 558 and 578 m (for details on methodology see Van Duyl et al. 2008). Due to the large size of *N. nodastrella*, 2 to 3 samples per box core were taken; these were considered to belong to the same colony. In the case of *H. thielei*, each sample corresponded to a different colony (Table S1 in the supplement).

The species names of sponges were determined on the basis of morphological characteristics, spicula morphology and composition (Van Soest 1978, Kobluk & Van Soest 1989, Hooper & Van Soest 2002).

Microbial abundance in sponges and surrounding seawater

Sponge samples of 6 specimens of *Halisarca caerulea* were fixed with paraformaldehyde (4 g per 100 ml) in phosphate-buffered saline solution (1× PBS) for up to 12 h at 4°C. After washing twice with 1× PBS, samples were stored in a PBS/80% ethanol mixture (1:1) at –20°C. To determine the microbial abundance in the sponge tissue, a small piece of sponge (0.5 cm^2 and 2 mm thick) representing a volume of ca. 100 mm^3 was crushed with a rubber stick in a reaction vial containing 200 µl Lysis T solution (Sigma-Aldrich) to dissociate the sponge tissue and release the microbial cells. Subsequently, several washing steps with artificial seawater (ASW) and centrifuging were conducted to collect microorganisms in the supernatant. Pellets were checked on filters for remaining microorganisms with 4′,6-diamidino-2-phenylindole (DAPI). From the water surrounding *H. caerulea*, 7 samples were taken and fixed with 37% formaldehyde (final concentration: 2%). Microorganisms in the supernatant, as well as samples from the water surrounding the sponge, were collected on GTTP filters (0.2 µm, 25 mm diameter) and stained according to the catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) protocol by Pernthaler et al. (2002). The following probes were applied for targeting *Bacteria*: EUB338 (Amann et al. 1990), EUBmix (mixture of EUB338, EUB338II and EUB338 III; Daims et al. 1999) and NonEUB338 (an oligonucleotide probe which is complementary to the probe EUB338 and serves as a control for non-specific binding; Wallner et al. 1993). For targeting *Archaea* the following probes were used: EURY806 (*Euryarchaea*) and Cren 537 (*Crenarchaea*, now *Thaumarchaea*) (Teira et al. 2004).

In short, the protocol comprises the following steps. (1) Cells were embedded in the filter by dipping them into low-gelling-point 0.1% agarose and drying them upside down in a Petri dish, followed by a dehydration step with 95% ethanol. (2) Cell walls were made permeable by incubation in a solution of lysozyme for *Bacteria* and proteinase K for *Archaea* during 1 h at 37°C. Filters were washed with Milli-Q and incubated in 0.01 M HCl at room temperature for 20 min (Teira et al. 2004), and filters were cut into sections to allow incubations with different solutions and probes. (3) A hybridization buffer was used, containing 55% formamide for EUB and NonEUB probes and 20% formamide for archaeal probes. Hybridization took place in the dark at 35°C for 14 h. (4) Fluorescent dye (tyramide-Alexa488) was added to amplify the signal by incubating the samples for 45 min at 37°C. After amplification, filter pieces were washed in PBS-T (0.05% Triton) in the dark at room temperature for 25 min, followed by washing with Milli-Q and dehydration with 95% ethanol. When filters were dry, they were mounted in a drop of DAPI-mix (DAPI solution in 1× PBS with Vectashield and Citifluor anti-fading reagents) on a glass slide and stored at –20°C. Slides were analysed with an epifluorescent Zeiss Axioplan microscope. For quantification of bacterial cells, 6 filter sections were analysed for *Halisarca caerulea* and 7 for the surrounding water. For quantification of archaeal cells, 4 filter sections were analysed for *H. caerulea* and 2 for the surrounding water. Total counts (DAPI) and specific probe counts were made in 20 randomly selected fields per filter section. Per filter section, >400 microorganisms were counted for DAPI and EUB probes and >70 for EURY and CREN probes (for an example see Fig. S1 in the supplement at www.int-res.com/articles/suppl/a068p215_supp.pdf). To circumvent problems related to auto-fluores-

cence, a double-labelled NonEUB338 probe (Wallner et al. 1993) was applied on a separate filter section and visualized under the same conditions as described above for the CARD-FISH protocol. No signals were detected with the NonEUB338 probe, indicating that all signals detected with the EUB338 probe were *Bacteria*. Microbial abundance in *H. caerulea* and the surrounding water was compared with data from Van Duyl et al. (2008) on microbial abundance in *Nodastrella nodastrella*, *Higginsia thielei* and the surrounding water.

DNA extraction

DNA was extracted from ethanol-preserved sponges (5 samples of *Halisarca caerulea*, 4 samples of *Higginsia thielei* and 8 samples of *Nodastrella nodastrella*) and water sample filters surrounding *H. caerulea* (2 samples) using the UltraClean Soil DNA isolation kit (Mo Bio). About 2 mm^3 of sponge was grinded with a mortar in Lysis solution (UltraClean Soil DNA isolation kit, Mo Bio). Resulting cell suspension was added to the tube with mineral beads, and DNA extraction was done according to the Mobio kit's protocol for maximum yield, involving 10 min of bead beating and binding of the DNA to a silica column. Filters of water samples were cut into small pieces with sterile scissors and processed in a similar way as the sponges.

PCR for DGGE-sequencing

Amplification of bacterial and archaeal *amoA* genes was performed using 2-step amplification protocols. For *Bacteria*, the PCR protocol and the primers were used as described by Hornek et al. (2006), with modifications: in 20 µl reactions we used 10 pmol of each degenerate primer amoA-1F (5′-GGG GHT TYT ACT GGT GGT-3′; H = not G, Y = T or C) and amoAr NEW (5′-CCC CTC BGS AAA VCC TTC TTC-3′; S = G or C, V = G or A or C, B = C or G or T), 1 U Picomaxx enzyme and 1× Picomaxx buffer (Stratagen), 5 nM of each dNTP and 8 µg of bovine serum albumin (BSA) for the first step amplification. The annealing temperature was increased to 60°C for maximum specificity, and a total of 33 cycles were run to generate template for the second reaction. The second step was performed with a GC-clamp on the forward primer amoA-1F and with the inosine primer amoAr-i (5′- CCC CTC iGi AAA iCC TTC TTC-3′; i = inosin) in order to reduce complex band patterns in the denaturing gradient gel electrophoresis (DGGE). For this second reaction, we added 1 U of Genescript *taq* polymerase, $MgCl_2$ to a final concentration of 2.0 mM and 5 pmol of each primer. We ran 20 cycles with an annealing temperature of 60°C and added a final 30 min extension step.

For *Archaea*, the PCR protocol and the primers were described by Wuchter et al. (2006). For the first PCR step, we used 4 pmol of each primer Arch-amoA-for (5′-CTG AYT GGG CYT GGA CAT C-3′) and Arch-amoA-rev (5′-TTC TTC TTT GTT GCC CAG TA -3′), 1 U Picomaxx enzyme, 5 nM of each dNTP and 8 µg BSA. The annealing temperature was 57°C. The second step was performed with a GC-clamp on the reverse primer (Arch-amoA-rev) and with a newly developed inosine variant of the Arch-amoA-for primer, amoAf-i-BA (5′-CTG AiT GGG CiT GGA CAT C-3′; present paper). This was done to reduce the complexity of the DGGE banding pattern (Hornek et al. 2006). Conditions were as described for the second step of bacterial *amoA* amplification, except for an optimized annealing temperature of 51.8°C.

DGGE

DGGE for *Bacteria* was performed as described by Hornek et al. (2006), by using approximately 100 ng of the product from the second step PCR on a 20 to 80% urea-formamide (UF) denaturing gradient gel. For *Archaea*, DGGE was performed as described by Wuchter et al. (2006) by using around 100 ng of the product from the second step PCR on a 10 to 50% UF denaturing gradient gel. Electrophoresis was performed using a D-Code system (Bio-Rad) with 1× Tris-acetate-EDTA (TAE) buffer (pH 8.3) at a constant temperature of 55°C and a voltage of 10 V for 10 min plus 200 V for 5 h for *Bacteria*, and at a constant temperature of 60°C and a voltage of 10 V for 15 min plus 200 V for 3 h for *Archaea*. Gels were stained with a solution of 2× SYBR gold in 1× TAE to visualize banding patterns. All clear bands in each sample were excised from the gel. Excised bands were soaked in 50 µl sterile 10 mM TRIS-buffer (pH 8.0) for a minimum of 48 h at 4°C. Of this 50 µl volume, 0.4 µl was used in a re-amplification reaction according to the protocols described for the second-step PCR, but without GC-clamps.

For cycle-sequencing reactions, we used the Big Dye Terminator solution V1.1 (Applied Biosystems). Products were analyzed on the ABI prism 310 genetic analyzer.

Sequence analyses

Electropherograms were inspected manually for ambiguities. If the height of a second peak was at least 50% of the highest peak then the ambiguity was uncorrected. In addition, when sequences with double peaks were seen, these were always due to a combination of 2 sequences (without double peaks) present lower in the gel; therefore, double peak sequences were discarded. Consensus sequences (assembled forward and reverse sequences) were aligned using the BLAST algorithm (Altschul et al. 1997) and, together with their close relatives, imported into the program ARB (Ludwig et al. 2004). Other relevant marine *amoA* sequences present in GenBank were also imported. A multiple alignment was made of the nucleotide sequences. Nucleotide sequences were translated into amino-acid sequences which could be easily aligned. No gaps were found in the alignment of amino-acid sequences; therefore, the nucleotide sequences were aligned accordingly. Different nucleotide and protein sequences were considered when >1 substitution was found compared to another sequence in the database.

Non-redundant bacterial *amoA* nucleotide sequences from this study (n = 17) were compared with all non-redundant bacterial nucleotide sequences from marine sponges, sequences from water and sediment, and from cultivated *Nitrosomonas* spp. and *Nitrosospira* spp., available in GenBank (date of September 2012). In total, 115 nucleotide sequences were considered. The backbone was constructed using 105 sequences with 387 informative positions and was analysed with Rapid Maximum-Likelihood (RaxML-VI Version 2.2.1; Stamatakis 2006) and neighbour-joining (NJ) algorithms implemented in ARB. Trees were calculated to visualize the affiliation of the derived sequences. Other (shorter) sequences were added to the reference tree using ARB parsimony, starting with the longest sequence and ending with the shortest (377 to 387 bp, from *Dysidea avara*; Ribes et al. 2012), using the respective sequence as a filter. Bootstrap analyses (1000 runs for NJ and 100 runs for RaxML) were used to estimate the support of the affiliations.

Non-redundant archaeal *amoA* nucleotide sequences from this study (n = 14) were compared with non-redundant sequences present in Genbank (date of September 2012) of other marine sponge–associated *Archaea*, including '*Candidatus* Cenarchaeum symbiosum' (Hallam et al. 2006), of water, sediment and corals, and of *Nitrosopumilus maritimus* (Könneke et al. 2005). In total, 158 nucleotide sequences were considered. The backbone, consisting of 130 sequences of 550 bp, was used to construct the tree topology using RaxML and NJ algorithms with bootstrap analyses (100 and 1000 replicates, respectively). Then, 28 shorter sequences (161 to 217 bp, including our own sequences) were inserted in the reference tree via ARB Parsimony, one by one, starting with the longest sequence.

Sequence data have been submitted to the GenBank database under Accession Numbers GQ353375 to GQ353399 and GQ353427 for *Bacteria* (n = 26), and GQ353400 to GQ353426 for *Archaea* (n = 27).

Q-PCR analysis

Quantification of archaeal and bacterial *amoA* gene copies in samples from *Halisarca caerulea* (5 samples) and the surrounding water (2 samples), *Higginsia thielei* (4 samples) and *Nodastrella nodastrella* (7 samples) were performed by (real-time) quantitative PCR (Q-PCR) analysis using primers described by Wuchter et al. (2006) for *Archaea* and by Hornek et al. (2006) for *Bacteria* (see previous subsections). Cycling conditions were the ones described above for PCR reactions. The reactions were performed in a CFX96 system (Bio-Rad, Hercules). Calibration curves were prepared from the nearly complete *amoA* gene of *Nitrosopumillus maritimus* (940 bp; dilution series: 1 to 1×10^7 copies per microlitre) and from a partial fosmid of the *amoA* gene of *Nitrosomonas eutropha* (490 bp; dilution series: 1 to 1×10^7 copies per microlitre). Efficiency was 88% ($r^2 = 0.99$, linear standard curve over 7 decades) for the archaeal *amoA* gene and 67% ($r^2 = 0.98$, linear standard curve over 6 decades) for the bacterial *amoA* gene. All DNA extracts were analysed in triplicate. No samples were excluded from the analysis.

Statistical analysis

To analyse the structure of the phylogenetic trees in relation to temperature (warm, cold, temperate) and sponge species, distance matrixes were exported from ARB into the PRIMER 6.1.7 software package (Primer-E Ltd). Within PRIMER, non-parametric permutation tests (ANOSIM, analysis of similarity) were done according to Clarke (1993). Two-way nested ANOSIMs were performed to test the null hypotheses that there is no structure in, respectively, AOA and AOB communities between different temperatures, considering differences in AOA and AOB community structure related to different sponge species. Because

the interpretation of ANOSIM results has limitations (e.g. it is based on ranks) 2-way nested PERMANOVA tests (permutational multivariate analysis of variance) were conducted as well. For that, PERMANOVA+ add-on package was used in PRIMER (Anderson et al. 2008). Monte Carlo (MC) sampling was used to stress the problem of limited possible permutations. Because of the unbalanced design, tests were done using Type III sums of squares. Additional PERMANOVA tests were conducted to test whether the distribution of AO microorganisms was related to their substrate (sponge, coral, water, sediment).

RESULTS

Abundance of AOA and AOB

Total microbial abundance (DAPI) in *Halisarca caerulea* was on average $12.3 \times 10^8 \pm 7.1 \times 10^8$ (SD) cm^{-3} of sponge (Table 1). For comparison, cell abundance data for *Higginsia thielei* and *Nodastrella nodastrella* collected in 2005 at the same locations as in the present study (Van Duyl et al. 2008) are also presented in Table 1. Total microbial abundance was higher in *H. caerulea* than in *H. thielei* ($2.0 \times 10^8 \pm 1.4 \times 10^8$) and *N. nodastrella* ($2.5 \times 10^8 \pm 1.1 \times 10^8$). CARD-FISH results revealed that the abundance of sponge-associated *Bacteria* was higher in *H. caerulea* ($9.7 \times 10^8 \pm 6.3 \times 10^8$) than in the 2 cold-water species (1.0×10^8 to 1.4×10^8), while the abundance of sponge-associated *Archaea* (*Euryarchaea* + *Thaumarchaea*, i.e. total *Archaea*) was comparable between *H. caerulea* ($0.2 \times 10^8 \pm 0.1 \times 10^8$) and the 2 other species (0.3×10^8 to 0.4×10^8 total *Archaea* cm^{-3} of sponge) (Table 1). Numbers of *Thaumarchaea* were slightly lower in *H. caerulea* ($0.06 \times 10^8 \pm 0.05 \times 10^8$) than in *N. nodastrella* and *H. thielei* (~0.2×10^8). About 25% of the DAPI counts of sponge-associated microorganisms could not be identified with the bacterial and archaeal CARD-FISH probes applied. The concentration of microorganisms was (1000×) higher in the sponge tissues than in the surrounding water. *Thaumarchaeota* were (10×) more abundant in water surrounding *N. nodastrella* and *H. thielei* than in water around *H. caerulea* (Table 1).

Q-PCR results showed that there were always more (2 to 35) archaeal *amoA* copies than bacterial *amoA* copies in the tested sponges (Table S2 in the supplement at www.int-res.com/articles/suppl/a068p215_supp.pdf), suggesting a larger role of AOA than of AOB in ammonia oxidation in sponges. On average, AOA/AOB ratios in the tropical sponge *Halisarca caerulea* (mean ratio = 5; individual ratios: 2 to 10 ± 1 to 3 SD) were lower than in the cold-water sponges *Higginsia thielei* (mean ratio = 16; individual ratios: 4 to 25 ± 1 to 5 SD) and *Nodastrella nodastrella* (mean ratio = 11; individual ratios: 4 to 35 ± 1 to 6 SD). Bacterial *amoA* copies in the water surrounding *H. caerulea* were below the detection limit in 1 of the 2 samples. In the water sample where bacterial *amoA* was detected, archaeal *amoA* copies were 81 ± 9 (SD) times higher than bacterial *amoA* copies (Table S2).

Diversity of sponge-associated AOA

The tropical sponge *Halisarca caerulea* hosted a higher diversity of archaeal *amoA* (8 different nucleotide sequences) than the cold-water sponges

Table 1. Calculated number (n) of *Bacteria*, total *Archaea* (*Euryarchaea* + *Thaumarchaea*) and *Thaumarchaea* related to total microbial counts (DAPI) in *Halisarca caerulea*, *Higginsia thielei* and *Nodastrella nodastrella* (n cm^{-3}), and in the water (n ml^{-1}). Standard deviations for *H. caerulea* data are in parentheses

Type	Temperature	Microbial counts	*Bacteria*	Total *Archaea*	*Thaumarchaea*
Sponge					
H. caerulea	Warm	12.28×10^8 (7.14×10^8)	9.71×10^8 (6.34×10^8)	0.15×10^8 (0.11×10^8)	0.06×10^8 (0.05×10^8)
H. thielei[a]	Cold	2.00×10^8	0.96×10^8	0.36×10^8	0.24×10^8
N. nodastrella[a]	Cold	2.53×10^8	1.39×10^8	0.28×10^8	0.23×10^8
Ambient water					
H. caerulea	Warm	11.01×10^5 (1.59×10^5)	5.77×10^5 (1.80×10^5)	0.14×10^5 (0.11×10^5)	0.004×10^5 (0.004×10^5)
H. thielei[a]	Cold	3.14×10^5	0.88×10^5	0.06×10^5	0.06×10^5
N. nodastrella[a]	Cold	4.83×10^5	1.55×10^5	0.11×10^5	0.09×10^5

[a]Data from Van Duyl et al. (2008)

Nodastrella nodastrella (5 nucleotide sequences) and *Higginsia thielei* (1 nucleotide sequence) (Fig. 1, Table 2). Different methods (NJ and RaxML) resulted in similar tree topologies (no significant difference between them in 90% of 10000 permutations) for both nucleotides and amino acids. Therefore, only the results of the RaxML trees will be described in the following paragraphs.

Sequences of sponge-associated AOA retrieved from excised DGGE bands (Figs. S2b & S3b in the supplement at www.int-res.com/articles/suppl/a068p215_supp.pdf) grouped with sequences retrieved from other cold- and warm-water sponges, corals, sediment and water (Fig. 1). All archaeal *amoA* sequences obtained corresponded to the *Thaumarchaeota* group. Phylogenetic analysis revealed 6 well-supported clusters (bootstrap value ≥70%), some of them including the sequences obtained in this study. Cluster 1 includes sequences from the cold-water sponge *Nodastrella nodastrella* and sequences from water surrounding *Halisarca caerulea*, but also sequences from other cold- and warm-water marine sponges and sequences from corals and water of many different origins. We found 99% identity between a nucleotide sequence present in water surrounding *H. caerulea* (Water1_B27) and a sequence retrieved from the marine sponge *Siphonochalina* sp. collected in the Coral Sea (Australia). Another sequence found in water (Water1_B27) was identical (100% similarity) to a sequence retrieved from a warm-water coral (*Fungia granulosa*). Cluster 2 includes *amoA* sequences retrieved from the Mediterranean sponge *Agelas oroides* and sequences from a warm-water coral (*Porites astreoides*, Caribbean) and a warm-water sponge (*Luffariella* sp., Coral Sea). Cluster 3 includes *amoA* sequences retrieved from *H. caerulea* and the surrounding water and sequences retrieved from *N. nodastrella* and *Higginsia thielei*. In this cluster, there are also sequences retrieved from the cultivated AOA *Nitrosopumilus maritimus*. Also archaeal *amoA* sequences retrieved from a warm-water coral (*Diploria strigosa*, Caribbean), a warm-water sponge (*Hymeniacidon heliophila*, Atlantic), a cold-water sponge (*Phakelia ventilabrum*, Atlantic) and sequences retrieved from water and sediment are part of this cluster. Clusters 4, 5 and 6 do not include any *amoA* sequences retrieved from *H. caerulea*, *H. thielei*, or *N. nodastrella*. These clusters include mainly sequences from other warm-water sponges and corals. Archaeal *amoA* gene sequences from *H. caerulea*, *H. thielei* and *N. nodastrella* showed 82 to 94% sequence identity (90 to 96% on an amino-acid level) to the *amoA* sequence of *N. maritimus* and 74 to 79% identity (92 to 93% on an amino-acid level) to the *amoA* sequence of '*Candidatus* Cenarchaeum symbiosum'. The presence of silent mutations in different nucleotide sequences resulted in the reduced amino-acid diversity of 6 different amino-acid sequences in *H. caerulea*, 4 in *N. nodastrella* and 1 in *H. thielei* (Table 2, Fig. S4 in the supplement at www.int-res.com/articles/suppl/a068p215_supp.pdf).

Table 2. Number of nucleotide (nuc.) and amino-acid (a.a.) sequences determined by PCR-DGGE-sequencing in the warm-water sponge *Halisarca caerulea* and the cold-water sponges *Higginsia thielei* and *Nodastrella nodastrella*. AOA: ammonia-oxidizing *Archaea*; AOB: ammonia-oxidizing *Bacteria*

Sample	AOA nuc.	AOA a.a.	AOB nuc.	AOB a.a.
H. caerulea	8	6	3	2
H. thielei	1	1	4	4
N. nodastrella	5	4	10	8

Diversity of sponge-associated AOB

Higher bacterial diversity was found in *Nodastrella nodastrella* (10 different nucleotide sequences) than in *Higginsia thielei* (4 nucleotide sequences) and *Halisarca caerulea* (3 nucleotide sequences) (Fig. 2, Table 2). Similarly, as for *Archaea*, description of diversity of sponge-associated AOB is only described for RaxML trees.

Sequences of sponge-associated AOB retrieved from excised DGGE bands (Figs. S2a & S3a in the supplement) were grouped in 10 well-supported clusters (bootstrap value ≥70%; Fig. 2). *AmoA* sequences retrieved from *Halisarca caerulea* fell into 2 clusters (Clusters 5 and 9) which were closely related to sequences derived from the tropical sponges *Mycale laxissima* and *Ircinia strobilina*. In both clusters, sequences retrieved from *H. caerulea* (Hal2_B24 and Hal3_B2) were highly similar to sequences from *Mycale laxissima* (99% identity). In Cluster 9, 99% identity was also found between nucleotide sequences of *H. caerulea* (Hal2_B24) and the surrounding water (Water2_B5). *AmoA* sequences retrieved from *Nodastrella nodastrella* and *Higginsia thielei* fell into 4 clusters (Clusters 1, 3, 6 and 8) and were closely related to published sequences of the sponges *Polymastia* cf. *corticata* (Cluster 1) and *Dysidea avara* (Cluster 3). In Clusters 6 and 8, identical sequences (100% identity) were found in *H. thielei* and *N. nodastrella*. In Cluster 6, these were also identical to a sequence found in cold water from the Pacific. Clusters 2, 4, 7, and 10 did

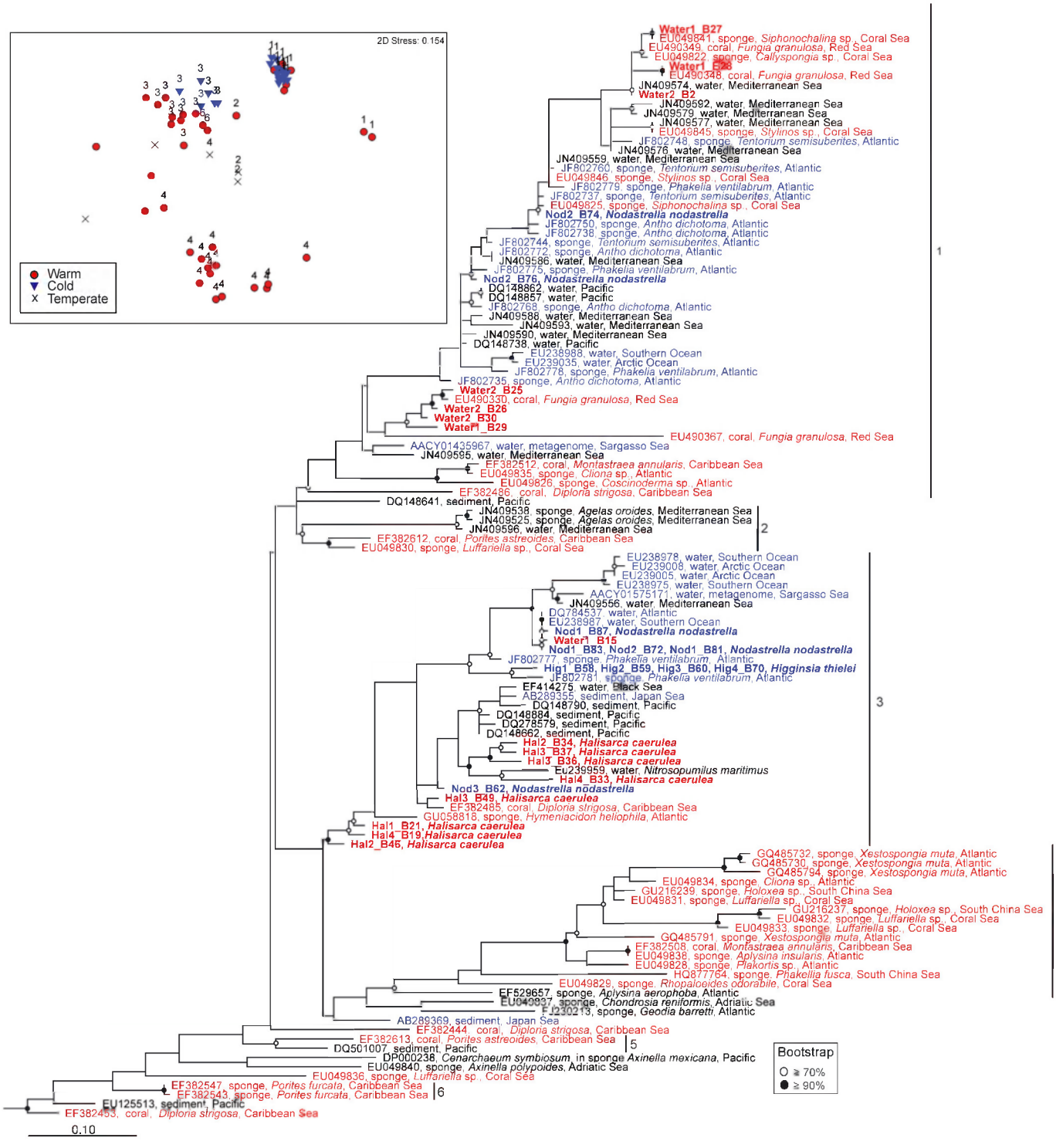

Fig. 1. Maximum-likelihood tree based on nucleotide sequences of *amoA* genes of *Thaumarchaeota* retrieved from sponges, corals, sediment and water. Sequences derived from the present study are shown in bold. Red-coloured sequences originate from habitats with warm-water temperatures (winter temperatures >18°C) and blue-coloured sequences originate from habitats with cold-water temperatures (summer temperatures <12°C); sequences which do not fit in these categories (temperate) are in black. Open circles: bootstrap values ≥70 %; filled circles: bootstrap values ≥90 %. Scale bar indicates 10 % sequence divergence. The out-group (not shown) contained *amoA* sequences of *Thaumarchaeota* isolated from warm-water sediments (DQ5010xx), cold-water sediments (EU885xxx) and the corals *Porites astreoides* and *Colpophyllia natans* (EF382xxx). Inset: a multidimensional scaling plot of the distance matrix underlying the tree (only sponges), with numbers referring to the identified clusters

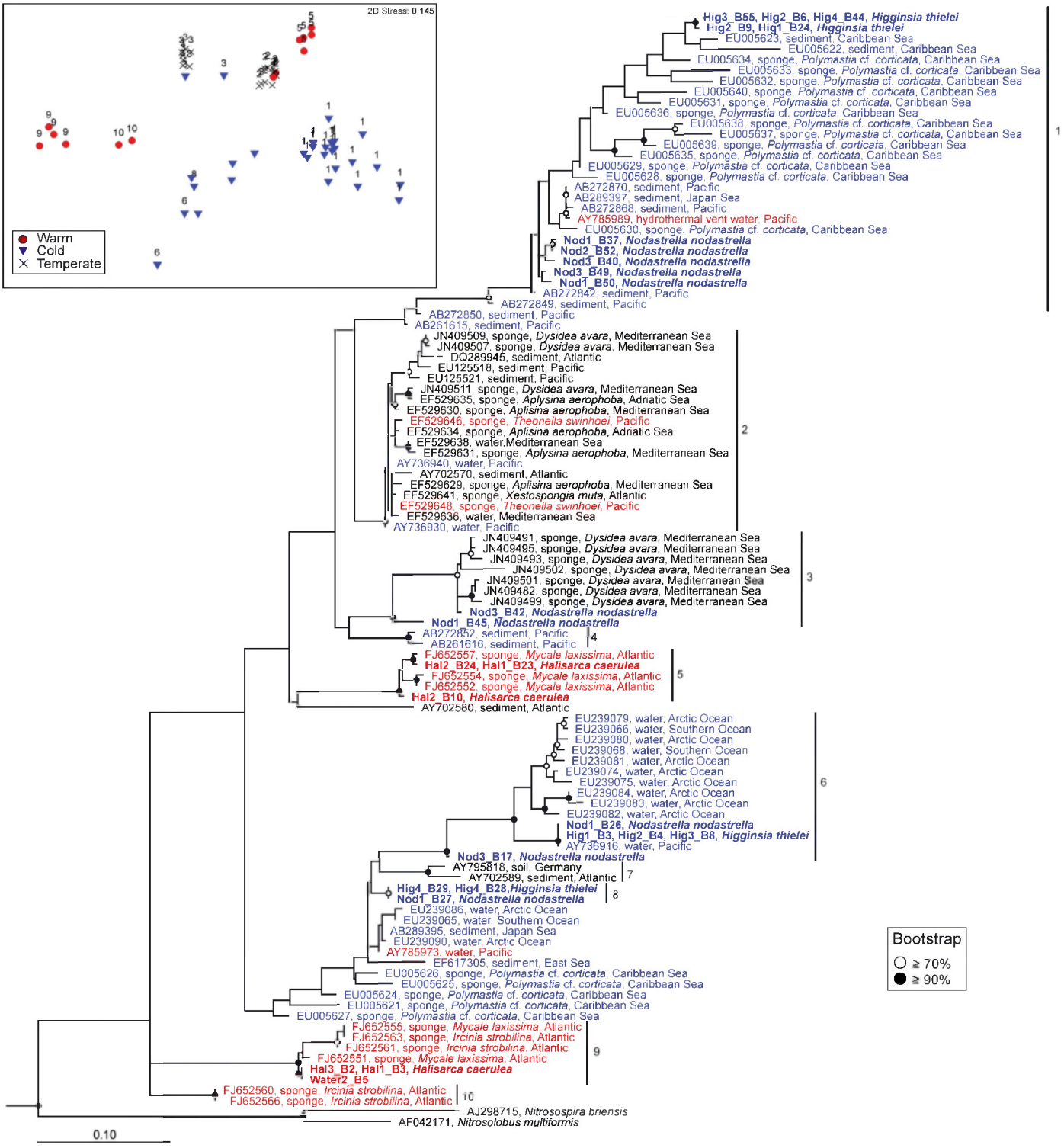

Fig. 2. Maximum-likelihood tree based on nucleotide sequences of *amoA* genes of *Betaproteobacteria* retrieved from sponges, sediment and water. Sequences derived from this study are shown in bold. Red-coloured sequences originate from habitats with warm-water temperatures (winter temperatures >18°C) and blue-coloured sequences originate from habitats with cold-water temperatures (summer temperatures <12°C); sequences which do not fit in these categories (temperate) are in black. Open circles: bootstrap values ≥70%; filled circles: bootstrap values ≥90%. Scale bar indicates 10% sequence divergence. The out-group (not shown) contained *amoA* sequences of cultivated *Nitrosomonas* spp. and *Nitrosospira* spp. Inset: a multidimensional scaling plot of the distance matrix underlying the tree (sponge species only), with numbers referring to the identified clusters

not contain any of our own sequences, but 2 of these clusters (2 and 10) did contain sequences retrieved from sponges. Bacterial *amoA* gene sequences retrieved from *H. caerulea*, *H. thielei* and *N. nodastrella* showed 73 to 77% sequence identity (88 to 91% on an amino-acid level) to the *amoA* sequence of the cultured AOB *Nitrosospira briensis*, and 75 to 77% sequence identity (87 to 93% on an amino-acid level) to the *amoA* sequence of *Nitrosolobus multiformis*. For *H. caerulea* and *N. nodastrella*, amino-acid trees revealed lower diversity than nucleotide trees, showing 8 different amino-acid sequences in *N. nodastrella* and 2 in *H. caerulea* (Table 2, Fig. S5 in the supplement at www.int-res.com/articles/suppl/a068p215_supp.pdf). In *H. thielei* each different nucleotide sequence was also a different amino-acid sequence (4 different amino-acid sequences were retrieved). Overall, similar clustering was observed between nucleotide and amino-acid trees.

Table 3. PERMANOVA and 2-way nested ANOSIM results (p-value) for testing the effects of sponge species and temperature on the existence of structure in the community composition of sponge-associated ammonia-oxidizing *Archaea* (AOA) and ammonia-oxidizing *Bacteria* (AOB) in nucleotide and amino-acid trees. All: all sponge species in the trees; >2×: only species that occur more than twice; nuc.: nucleotide sequence; a.a.: amino-acid sequence. NS: not significant; **bold**: based only on 35 permutations

	Factor	All nuc.	>2× nuc.	All a.a.	>2× a.a.
AOA					
PERMANOVA (MC)	Temperature	0.0070	0.0080	0.0370	0.0540
	Species(Temp.)	0.0001	0.0001	0.0001	0.0001
ANOSIM	Temperature	**0.0290**	**0.0290**	**0.0290**	**0.0700**
	Species(Temp.)	0.0100	0.0010	0.0001	0.0001
AOB					
PERMANOVA (MC)	Temperature	0.0020	0.0002	<0.0001	0.0002
	Species(Temp.)	0.0004	0.0020	0.0500	0.0900
ANOSIM	Temperature	0.0080	0.0040	0.0010	0.0040
	Species(Temp.)	0.0270	0.0090	NS	NS

Effect of host species and temperature on AOA and AOB diversity

Two-way nested PERMANOVA and ANOSIM tests were performed using all non-redundant *amoA* sequences retrieved from sponges and available in GenBank up to September 2012. For *Archaea*, both tests showed a significant effect of sponge species on the structure of archaeal *amoA* trees ($p \leq 0.01$ for nucleotides and amino acids; Table 3). Taking into account the effect of species, temperature also significantly influenced the distribution of sponge archaeal *amoA* sequences in the nucleotide trees ($p = 0.0001$, PERMANOVA test), although the effect of temperature was not very strong (only slightly significant in cases when all sponge sequences were considered) in the amino-acid tree ($0.037 < p < 0.054$; Table 3). The ANOSIM test was not very strong due to the low number of permutations. For *Bacteria*, sponge species significantly affected the structure of *amoA* nucleotide trees ($p < 0.05$), but not amino-acid trees ($p > 0.05$) (Table 3). Temperature significantly affected the distribution of sponge bacterial *amoA* in nucleotide and amino-acid trees ($p \leq 0.001$).

PERMANOVA tests done on the distribution of archaeal and bacterial *amoA* genes in relation to habitat type (sponge, coral, sediment and water) revealed that for both *Archaea* and *Bacteria*, habitat and temperature have a highly significant effect on the distribution of AOA and AOB ($p < 0.0001$; Table S3 in the supplement at www.int-res.com/articles/suppl/a068p215_supp.pdf). Further pair-wise tests suggested that the distribution of bacterial *amoA* did not differ significantly between sponge and sediment, while the distribution of archaeal *amoA* did not differ significantly between sponge and water (not shown). However, these tests are not statistically strong due to an unbalanced dataset and should, therefore, only be used as an indication.

DISCUSSION

Our results reinforce the notion that sponges harbour microbial organisms with metabolisms that are important to the N-cycle in tropical and cold-water coral reef communities (see reviews by Taylor et al. 2007 and Hentschel et al. 2012). Both bacterial and archaeal *amoA* genes were found in *Halisarca caerulea*, *Higginsia thielei* and *Nodastrella nodastrella*, showing that AOB, as well as AOA, reside in these sponges. In terms of abundance, numbers of AOA were higher than those of AOB in all 3 studied sponge species (on average 5- to 16-fold more AOA than AOB). In 3 other cold-water sponge species, the numbers of AOA per gram of sponge were about 150 times to 4×10^5 times higher than those of AOB (Radax et al. 2012). AOA were also found to be the main ammonia-oxidizing microbes in the warm-

water sponge *Phakelia fusca* (Han et al. 2012). Our results suggest that AOA may be responsible for a major part of the ammonia oxidation, not only in cold-water sponges, as previously suggested by Radax et al. (2012), but also in tropical sponges. Archaeal *amoA* genes in the water surrounding *H. caerulea* were about 2 orders of magnitude higher than bacterial *amoA* genes. Previous studies describe archaeal *amoA* copy numbers in Atlantic and Mediterranean waters as being 1 to 3 orders of magnitude higher than those of bacterial *amoA* (Wuchter et al. 2006, De Corte et al. 2009), and up to 4 orders of magnitude higher in the Pacific (Mincer et al. 2007). Our data support the idea that AOA play a major role in nitrification, not only in the ocean, but also in sponges.

Microbial abundance

In terms of total microbial abundance, *Bacteria* were seen to dominate the microbial community in *Halisarca caerulea*, showing much higher densities than *Archaea*. The same has been observed in *Higginsia thielei* and *Nodastrella nodastrella* (Van Duyl et al. 2008). *Bacteria* were also seen to dominate the microbial community in *Agelas oroides* and *Chondrosia reniformis* (Ribes et al. 2012) and in *Phakellia fusca* (Han et al. 2012). However, in other sponges, *Archaea* were more dominant than *Bacteria* (Margot et al. 2002, Pape et al. 2006). The use of different probes in different studies may be one of the reasons for the differences in abundance of *Archaea* versus *Bacteria* in sponges. In addition, due to the lack of data on microbial abundance of many sponge species, as recently emphasized by Simister et al. (2012), a general trend is difficult to find.

Bacterial and total archaeal abundance varied considerably between specimens of *Halisarca caerulea* (as shown by the large standard deviation observed). Variability in bacterial abundance was larger than described for *Higginsia thielei* and *Nodastrella nodastrella* (Van Duyl et al. 2008). Differences in microbial abundance between specimens could be due to sampling of different tissues within a sponge. In the sponge *Polymastia* cf. *corticata*, high variability in bacterial and archaeal communities was associated with different tissue sections (Meyer & Kuever 2008).

In *Halisarca caerulea*, about 1.3% of FISH-positive microorganisms were total *Archaea* (*Euryarchaea* + *Thaumarchaea*), from which less than half were *Thaumarchaea*. This value is much lower than for *Nodastrella nodastrella* and *Higginsia thielei*. In these 2 cold-water sponges 11 to 18% of the DAPI counts were total *Archaea*, with a dominance of *Thaumarchaea* (Van Duyl et al. 2008). The presence of *Euryarchaea* in marine sponges has been reported in a few studies. *Euryarchaea* were detected in *Rhopaloeides odorabile* (Webster et al. 2001) and 3 other sponges (Holmes & Blanch 2007) from Australia. Also *Agelas oroides* (Ribes et al. 2012) hosted *Euryarchaea*. Apparently, *Euryarchaea* also form a substantial fraction of the total archaeal community in *H. caerulea*.

Diversity of sponge-associated AOA and AOB

Archaeal and bacterial *amoA* sequences retrieved from *Halisarca caerulea*, *Nodastrella nodastrella* and *Higginsia thielei* were compared with sequences retrieved from other sponges, corals and environmental samples (sediment, water). Archaeal *amoA* sequences were distinct from those found in other sponges (Bayer et al. 2008, Meyer & Kuever 2008, Steger et al. 2008, Hoffmann et al. 2009, López-Legentil et al. 2010, Turque et al. 2010, Liu et al. 2011, Han et al. 2012, Radax et al. 2012, Ribes et al. 2012). The closest relative to sequences retrieved from the tropical sponge *H. caerulea* was from the coral *Diploria strigosa* (Beman et al. 2007), while *amoA* sequences from water surrounding the sponge were similar to sequences retrieved from the warm-water sponge *Siphonochalina* sp. (Steger et al. 2008) and the warm-water coral *Fungia granulosa* (Siboni et al. 2008). AOA in the cold-water sponge *H. thielei* were closest to AOA found in the cold-water sponge *Phakelia ventilabrum* (Radax et al. 2012), and the closest relative of archaeal sequences retrieved from *N. nodastrella* were found in water from the Antarctic (Kalanetra et al. 2009). There was no clear biogeographic effect, with many of the sponge-derived sequences forming large clusters comprising sequences from many different locations, such as the Red Sea, Atlantic, Caribbean, Mediterranean, China Sea and more. A widespread distribution of archaeal sequences retrieved from sponges has also been observed in earlier studies (Steger et al. 2008, Ribes et al. 2012). The 6 well-supported clusters contained, not only *amoA* sequences retrieved from sponges, but also sequences from corals, sediments and/or water. No clear sponge-specific clusters were observed either. Cluster 4 included, however, mainly sequences retrieved from sponges, excluding 1 sequence from a coral. It seems, therefore, that most archaeal communities in sponges may be acquired,

above all, horizontally via sediment or water. Nevertheless, *Archaea* have also been found in sponge larvae and gametes, indicating that some AOA may be vertically transmitted (see review by Webster & Taylor 2012 and references therein). The high similarity between archaeal 16S rRNA sequences retrieved from the sponge *Polymastia* cf. *corticata* and sequences from other sponge species (Meyer & Kuever 2008) supports the existence of sponge-specific clusters and vertically acquired *Archaea* within sponges. The existence of sponge-specific associated *Archaea* has also been suggested for other sponge species (Preston et al. 1996, Holmes & Blanch 2007, Bayer et al. 2008, Hoffmann et al. 2009, Turque et al. 2010, Radax et al. 2012).

Bacterial *amoA* sequences retrieved from *Halisarca caerulea* showed high similarity to sequences retrieved from the tropical sponges *Mycale laxissima* and *Ircinia strobilina* (Mohamed et al. 2010), while sequences retrieved from *Higginsia thielei* and *Nodastrella nodastrella* were similar to sequences obtained from the deep-water sponge *Polymastia corticata* (Meyer & Kuever 2008). The closest relatives of sequences retrieved from the tropical sponge *H. caerulea* were found in the sponge *M. laxissima* (Mohamed et al. 2010) and in the water surrounding *H. caerulea*. Identical AOB sequences were found in *H. thielei*, *N. nodastrella* and in cold water from the Pacific (O'Mullan & Ward 2005). Similar to the archaeal *amoA* tree, no clear biogeographic effect is seen in the AOB tree. Sequences retrieved from sponges fall in clusters containing sequences from different locations. Cluster 2 includes sequences from sponges collected in the Mediterranean, Adriatic, Atlantic and Pacific. And AOB sequences retrieved from *N. nodastrella* in Cluster 3 group with sequences from *Dysidea avara* from the Mediterranean. Such diverse distribution of bacterial communities has also been reported in earlier studies, with geographically distant sponges showing similar sponge-associated *Bacteria* (Montalvo & Hill 2011, Yang et al. 2011, Ribes et al. 2012). Two of the well-supported clusters (Clusters 1 and 2) contained mainly sequences from sponges and sediment, while Cluster 6 contained only sequences from sponges and water. High similarity in bacterial composition between sediment and sponges has also been previously reported (Turque et al. 2008). The distribution of bacterial *amoA* does not seem to differ significantly between sponge and sediment, as suggested by the PERMANOVA test. This fact supports the idea that bacterial communities may be horizontally acquired via the sediment. The fact that 1 cluster contains only sequences from *H. thielei*, *N. nodastrella* and water, suggests the acquisition of AOB via water as well. In addition, several clusters contain only sequences retrieved from sponges, suggesting the existence of sponge-specific AOB. The grouping of sequences retrieved from *H. thielei* and *N. nodastrella* in 1 cluster suggests the existence of cold-water, sponge-specific AOB. Our results corroborate earlier reports that AOB may also be vertically transmitted in sponges (Turque et al. 2008, review by Webster & Taylor 2012 and references therein). In 3 Great Barrier reef sponges, many previously called 'sponge-specific' bacterial clusters were detected in seawater, suggesting that both vertical and horizontal transmission might operate together (Webster et al. 2010).

In general, considering the 3 species, the bacterial community was more diverse than the archaeal community. Seventeen different bacterial nucleotide sequences in contrast to 14 archaeal nucleotide sequences were retrieved from the studied sponges. However, it should be taken into account that some *Bacteria* may present multiple copies of the *amoA* gene (Norton et al. 2002), which will influence the real number of different nucleotide sequences found. It should also be kept in mind that the sequences presented here were obtained by DGGE analysis of PCR products, and our diversity assessment is therefore based on very short fragments. In addition, primer-introduced amplification bias cannot be excluded as one of the reasons for the observed differences in diversity of AOA and AOB in relation to other studies. The primers used may also have influenced the formation of the distinct sequence clusters.

Effect of temperature on AOA and AOB diversity

Temperature significantly affected the distribution of sponge sequences in both archaeal and bacterial *amoA* trees. The effect of temperature on the composition of bacterial and archaeal assemblages has been mentioned in several studies. In the Mediterranean sponge *Aplysina aerophoba*, temperature partially explained the increase in ammonium excretion rates from spring to the end of summer (Bayer et al. 2008), suggesting that seasonal differences in community composition of sponge-associated microorganisms may be responsible for the observed variations. In fact, water temperature was the environmental variable that best explained spring, summer and winter archaeal assemblage structure in freshwater lakes (Auguet et al. 2011). Also in sulphurous

lakes (Casamayor et al. 2001, Llirós et al. 2008), North Sea waters (Wuchter et al. 2006, Herfort et al. 2007) and estuarine sediments (Sahan & Muyzer 2008) temperature was seen to control the diversity of *Bacteria* and *Archaea* (including AOB and AOA). Nevertheless, in Mediterranean Sea waters (De Corte et al. 2009) and in soil (Tourna et al. 2008), no effect of temperature on bacterial or archaeal *amoA* diversity was observed, indicating that other environmental factors also affect the presence of nitrifying microorganisms.

In bacterial *amoA* trees (nucleotide and amino-acid trees) clear clustering could be seen between sponge sequences retrieved from habitats with similar temperatures (cold, warm, or temperate). Phylogenetically similar sponge-associated bacterial communities originating from similar habitats have been reported in earlier studies. The bacterial communities associated with the geographically distant warm-water sponges *Xestospongia muta* and *X. testudinaria* were seen to be similar (Montalvo & Hill 2011). On the other hand, sponge-associated *Bacteria* from the shallow Caribbean Sea were found to be significantly distinct from *Bacteria* retrieved from sponges from deep-water environments of the Caribbean Sea. Therefore, our results suggest the existence of temperature-related, sponge-specific associated *Bacteria*. In archaeal *amoA* trees the effect of temperature in structuring the distribution of AOA was also significant, although in terms of the amino-acid tree the temperature effect was not as strong as for the bacterial tree. Archaeal *amoA* sequences from habitats with similar temperature conditions tended to group together, but in smaller sub-clusters. In earlier studies, archaeal phylotypes retrieved from cold-water sponges were found to be related to sequences from deeper and colder waters (Radax et al. 2012). Also, distinct archaeal communities retrieved from sponges from the same area suggested that environmental conditions have an effect on sponge-associated microbial communities (Turque et al. 2010). Overall, our results suggest the existence of sponge-associated archaeal and bacterial communities adapted to different temperatures.

The relatively high similarity between the microbial community of *Nodastrella nodastrella* and *Higginsia thielei* (often grouping in the same cluster) is quite interesting considering the fact that they are phylogenetically distant species. *H. thielei* belongs to the class Demospongiae, while *N. nodastrella* belongs to the class Hexactinellida. Similarities in microbial communities have often been described in closely related sponges (review by Taylor et al. 2007). Nevertheless, phylogenetically distant sponges (although both Demospongiae) such as *Aplysina aerophoba* and *Theonella swinhoei* were also seen to contain similar microbial communities (Hentschel et al. 2002). To the best of our knowledge, the present study describes for the first time the diversity of AOA and AOB in a hexactinellid sponge. Our results support the idea of a relatively uniform microbial community between distantly related sponges and suggest that temperature (rather than phylogenetic distance) determines the diversity of AOA and AOB communities in sponges.

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