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ORIGINAL ARTICLE

Comparison of the anaerobic microbiota of deep-water *Geodia* spp. and sandy sediments in the Straits of Florida

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Marine sediments and sponges may show steep variations in redox potential, providing niches for both aerobic and anaerobic microorganisms. *Geodia* spp. and sediment specimens from the Straits of Florida were fixed using paraformaldehyde and 95% ethanol (v/v) for fluorescence *in situ* hybridization (FISH). In addition, homogenates of sponge and sediment samples were incubated anaerobically on various cysteine supplemented agars. FISH analysis showed a prominent similarity of microbiota in sediments and *Geodia* spp. samples. Furthermore, the presence of sulfate-reducing and annamox bacteria as well as other obligate anaerobic microorganisms in both *Geodia* spp. and sediment samples were also confirmed. Anaerobic cultures obtained from the homogenates allowed the isolation of a variety of facultative anaerobes, primarily *Bacillus* spp. and *Vibrio* spp. Obligate anaerobes such as *Desulfovibrio* spp. and *Clostridium* spp. were also found. We also provide the first evidence for a culturable marine member of the *Chloroflexi*, which may enter into symbiotic relationships with deep-water sponges such as *Geodia* spp. Resuspended sediment particles, may provide a source of microorganisms able to associate or form a symbiotic relationship with sponges.

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Introduction

Sponges contribute significantly to the total biomass of the tropical reef fauna and may dominate the benthic community in the Caribbean and other tropical waters (Wilkinson and Cheshire, 1990). As filter feeders, sponges process large quantities of seawater removing significant amounts of suspended particles and mircoorganisms (Duckworth *et al.*, 2006). Overall, microorganisms may constitute up to 60% of the tissue biomass in sponges ('bacteriosponges') (Reiswig, 1981). Sponges and their microbiota have been intensively studied for their biochemical profiles and it has been shown that species-specific microbial communities differ from those in ambient seawater with certain sponges hosting a uniform microbial population in different oceans (Vacelet and Donadey, 1977; Wilkinson, 1978; Schmidt *et al.*, 2000; Webster and Hill, 2001; Hentschel *et al.*, 2002).

Similarly, estuarine and coastal marine sediments and their microbial communities have been studied in detail for a number of years for their biogeochemical significance (Skyring, 1987; Takii and Fukui, 1991). These, frequently muddy, shallow water sediments may contain up to 3×10^9cells ml^{-1} and show a clear seasonal variability in their geochemical and microbial composition (Musat *et al.*, 2006). The microbiota associated with sediments retrieved from deep-water environments have also been studied for some time (Kato *et al.*, 1997;

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Colquhoun *et al*., 1998; Luna *et al*., 2004, 2006; Wang *et al*., 2004). However, it is currently uncertain if these benthic sediments, which do not undergo significant seasonal changes in their geochemical and microbial composition, host specific microbial communities. Although the sediment microbial community has been shown to be dominated by members of the Planctomycetes, the *Cytophaga/Flavobacterium* group, Gammaproteobacteria and bacteria of the *Desulfosarcina/Desulfococcus* group, the role and importance of anaerobes such as sulfate-reducing bacteria (SRB) and methanogenic archaea have been well documented (Purdy *et al*., 2001, 2003; Loy *et al*., 2002; Dalsgaard *et al*., 2005; Musat *et al*., 2006).

Both sponges and marine sediments show steep variations in redox potential. Hoffmann *et al*. (2005) showed that the oxygen concentrations in *Geodia barretti*, one of the most ancestral demosponges with origins in the early Cambrian era, is strongly dependent on pumping activity, leading to anoxia in parts of the tissue and the canal system (Gruber and Reitner, 1991; Hoffmann *et al*., 2005). This anoxia seemed to be a common feature of living *Geodia* specimens and did not influence their survival while providing an environment favorable for the growth of symbiotic anaerobes. Furthermore, it was shown that in actively pumping *Geodia* individuals, the cortex and the subcortical spaces were well oxygenated but that the oxygen levels were depleted 4–6 mm below the sponge surface. In non-pumping individuals, oxygen was depleted directly beneath the cortex and diffusive oxygen consumption could be observed in the overlying water.

It may be assumed that anoxia is responsible for regulating the bacterial biota in sponges and marine sediments and may thus be responsible for seasonal fluctuations in the shallow water sediment microbiota. In turn, anoxia in sponges may benefit the sponge host by providing an environment favorable for chemoautotrophic microbial processes that contribute to sponge nutrition (Taylor *et al*., 2007). Therefore, it would be expected that although sponges and marine sediments may provide similar environments, sponges would harbor bacteria that would specifically aid in nutrient assimilation and cycling.

In this study, we used a combination of anaerobic culture and fluorescence *in situ* hybridization (FISH) with a comprehensive suite of probes to compare the microbiota of marine sediments and *Geodia* spp. collected from the Straits of Florida to detect microbiota important in deep-water nutrient cycling and sponge symbiosis. Particular consideration was given to anaerobic microbiota that are not commonly found in seawater to determine whether marine sediments may act as a reservoir for sponge associated microorganisms.

Materials and methods

All chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA). PCR reactions were performed using IQ Master Mix from BioRad (Hercules, CA, USA). Restriction enzymes were obtained from Promega (Madison, WI, USA). Pre-formulated Nutrient Broth, Marine Agar, Brain Heart Infusion Agar, Sabouraud-Dextrose Agar and Marine Broth were prepared according to the manufacturer's instructions unless stated otherwise and supplemented with cysteine 100 μg ml^{-1} (DIFCO Laboratories, Detroit, MI, USA).

Sample collection and treatment

Collection of sponge specimens and sediment samples (top 10 cm of the seafloor) was performed on two separate research expeditions (April 2005 and August 2005) to the Straits of Florida using the R/V *Seward Johnson* and the *Johnson-Sea-Link* I (JSL I) research submersible. Collection sites and depths are given in Table 1. All sponge specimens and sediments were handled with nitrile examination gloves. Sponges were identified using standard spicule analysis. Approximately 10 g of each sponge specimen or 10 g of each sediment sample was homogenized under sterile conditions for 3 min (25 °C) in 100-ml sterile Artificial Seawater (pH: 7.2) using a commercial Waring blender (Waring Laboratory Science, Torrington, CT, USA). This homogenate was then used to establish bacterial cultures on solid agar plates. Intact specimens of sponge tissue were shock frozen at −80 °C in 50% (v/v) glycerol.

Table 1 Collection sites

Sample No.	*Taxa*	*Lattitude*	*Longitude*	*Location*	*Country*	*Depth (m)*
10-VIII-05-1-202	Sediment	24° 14.0132′N	82° 18.0236′W	Florida, Pourtales Terrace	USA	670
2-VIII-05-1-201	Sediment	26° 57.0494′N	79° 59.2467′W	Florida, Jupiter Reef	USA	233
12-IV-05-1-201	Sediment	26° 31.3488′N	78° 56.7606′W	Grand Bahama Island, Freeport	Bahamas	644
9-VIII-05-1-201	Sediment	24° 21.8081′N	81° 50.7809′W	Florida, Pourtales Terrace	USA	191
6-VIII-05-1-201	Sediment	25° 41.8835′N	79° 51.9809′W	Florida, Miami Terrace Escarpment	USA	342
HBOM 003:01040	Geodiidae	26° 11.7081′N	79° 59.2363′W	Florida, Miami Terrace Escarpment	USA	304
HBOM 003:01041	Geodiidae	25° 41.8835′N	79° 51.9809′W	Florida, Miami Terrace Escarpment	USA	341
HBOM 003:01042	Geodiidae	24° 43.9859′N	80° 25.6972′W	Florida, Pourtales Terrace	USA	197

Fluorescence in situ *hybridization*
FISH with ribosomal RNA-targeted probes was carried out using a variety of probes targeting archaea, common classes of bacteria, and selected anaerobic bacteria (Table 2). Results of microscopic counting were corrected by subtracting fluorescence signals of the probes with that of nonsense probe NONEUB (NON-EUB338), which was previously shown not to hybridize with any prokaryotic cells (Amann *et al.*, 1990; Wallner *et al.*, 1993). All probes were synthesized and monolabelled at the 5′ end with Cy3 (Ex 552 nm, Em 568 nm) by Sigma-Genosys (Dublin, Ireland). Approximately 1 ml of sponge homogenate or 1 ml of sediment homogenate was fixed in 10 ml of either 95% (v/v) ethanol (for Gram-positive bacteria) or 4% (w/v) paraformaldehyde in phosphate-buffered saline (pH 7.2, for Gram-negative bacteria) overnight. Fixed samples in 1-ml aliquots were washed three times in phosphate-buffered saline and resuspended in a mixture of 150 µl phosphate-buffered saline and 150 µl of 96% (v/v) ethanol for storage at −20 °C until further use.

For hybridization, 1 ml of fixed sample was centrifuged at 2000 × *g* for 2 min to remove large particulate matter. A volume of 100 µl of centrifuged sample was added to Epoxy-printed three well (14-mm diameter) microscope slides (Menzel, Braunschweig, Germany) and dried at room temperature. Slides were dehydrated in ethanol (50, 80 and 96% (v/v) for 3 min each). While slides dried, 1 ml of hybridization buffer containing 0.9 M NaCl, 0.02 M TrisHCl, and 0.01% (w/v) sodium dodecyl sulfate was prepared. The formamide concentration of the hybridization buffer was adapted according to the individual probe's requirements with high performance liquid chromatography grade water being added to bring the final volume to 1 ml. Hybridization buffer 10 µl was added to 1 µl of the appropriate probe (50 ng µl^{-1}) and then added to the sample slide. The remaining buffer solution was poured over a piece of tissue paper that was added to a 50-ml centrifuge tube in which the sample slide was placed. The tube was then placed horizontally with the cap tightly closed in a hybridization oven at 46 °C for 3 h. After hybridization, the buffer was removed from the sample using a small amount of preheated (48 °C) washing buffer containing 0.02 M TrisHCl, 0.01 M ethylene-diaminetetraacetic acid and 5–900 mM NaCl, depending on probe requirements. For total counts, 50 nM SYTO 9 (Invitrogen, Dun Laoghaire, Ireland) was added to the washing buffer. After all liquid was removed from the slide, 500 µl of the washing buffer was added to the sample slide with a further 1 ml of washing buffer being poured over a piece of tissue paper that was added to a new 50-ml centrifuge tube. The sample slide was then placed horizontally into the new tube, which was capped tightly and placed in a hybridization oven at 48 °C for 15 min. After washing, the slide was dipped in ice-cold high performance liquid chromatography grade water for 3 s and air dried. Slides were stored at −20 °C until use. For analysis, one drop of SlowFade-Light Antifade Kit component A (Invitrogen) was added to the slide. Organisms were evaluated using an Olympus IX51 microscope (Olympus UK, London, UK) with epifluorescence attachment (Olympus U-RFL-T) and appropriate filter sets. Image analysis was performed using an Olympus DP70 camera system and Olympus Cell^{F} imaging software. Fifteen random fields with a good distribution of cells (10–100) were counted for each probe and sample.

Anaerobic culture
Aliquots (10 and 100 µl) of each sponge and sediment homogenate were spread onto standard Nutrient Agar, diluted Marine Agar (1/5 strength Marine Agar 2216 diluted with Artificial Seawater and supplemented with agar to a final concentration of 1.5%(w/v)), Brain-Heart Infusion agar, Brain-Heart Infusion agar with Artificial Seawater and Sabouraud Dextrose Agar plates in triplicate. Plates were incubated at ambient temperature in screw top 2.5-l anaerobic jars containing one sachet of anaerobic atmosphere generator (Oxoid, Basingstoke, UK). The jars were further sealed in clear plastic bags filled with N_2. Once back on shore, all samples were immediately transferred to an anaerobic chamber (Coy, Grass Lake, MI, USA). Bacterial growth was monitored for 4 weeks and individual colonies with unique morphotypes were serially streaked on their respective medium until pure cultures were obtained. Pure cultures were then transferred to diluted Marine Agar plates before DNA isolation. Pure cultures on diluted Marine Agar plates were also used to check cultures for aerobic growth by aerobic incubation at ambient temperature for a maximum of 4 weeks. For long-term storage, purified bacterial isolates were grown in 10-ml Marine Broth 2216 and 1-ml aliquots were frozen in 10% (v/v, final concentration) glycerol at −80 °C.

DNA extraction and PCR of cultured bacterial isolates
DNA of bacterial isolates was extracted by touching the colony with a sterile needle, which was then placed in 10 µl sterile 5% (w/v) Chelex (BioRad) solution. Samples were boiled for 10 min before centrifugation at 14 000 × *g*. Supernatant containing extracted DNA was transferred to a new tube and stored at −80 °C before further use. Eubacterial universal primers FC27 (5′-AGAGTTTGATCCTGGC TCAG-3′) and RC1492 (5′-TACGGCTACCTTGTTAC GACTT-3′) (Mincer *et al.*, 2004) were used to amplify the 16S ribosomal DNA in standard PCR reactions containing 10 pmol of each primer, 12.5 µl IQ Supermix (BioRad), and sterile H_2O to a final volume of 24 µl. The PCR mix was added to 1 µl of DNA template and cycled as follows: initial denaturing at 95 °C for 5 min, 35 cycles at 95 °C for

Table 2 Hybridization (FISH) probes (available at http://www.microbial-ecology.net/probebase/; Loy *et al.*, 2002)

Probe	*Sequence (5′- 3′)*[a]	*Positions*[b]	*Target*	*% Formamide*[c]	*NaCl (mm)*[d]	*Label*	*Reference*
ARC 915	5′-GTGCTCCCCCGCCAATTCCT-3′	915–934	Archaea	35	70	Cy3	Stahl and Amann, 1991
EUB338	5′-GCTGCCTCCCGTAGGAGT-3′	338–355	Most bacteria	35	70	Cy3	Amann *et al.*, 1990
EUB338II	5′-GCAGCCACCCGTAGGTGT-3′	338–355	Planctomycetales	50	10	Cy3	Daims *et al.*, 1999
EUB338III	5′-GCTGCCACCCGTAGGTGT-3′	338–355	Verruncomicrobiales	50	10	Cy3	Daims *et al.*, 1999
NONEUB	5′-ACTCCTACGGGAGGCAGC-3′	338–355	Nonsense negative control	35	80	Cy3	Wallner *et al.*, 1993
ALF968	5′-GGTAAGGTTCTGCGCGTT-3′	968–985	α—Proteobacteria	35	70	Cy3	Manz *et al.*, 1992
BET42a	5′-GCCTTCCCACTTCGTTT-3′	1027–1043	β—Proteobacteria	35	70	Cy3	Manz *et al.*, 1992
GAM42a	5′-GCCTTCCCACATCGTTT-3′	1027–1043	γ—Proteobacteria	35	70	Cy3	Manz *et al.*, 1992
DELTA495a,b,c	5′-A**(G/A)**TTAGCCGG**(T/C)**GCTTCCT-3′	495–512	δ—Proteobacteria	35	70	Cy3	Loy *et al.*, 2002
EPSY549	5′-CAGTGATTCCGAGTAACG-3′	549–566	ε—Proteobacteria	55	10	Cy3	Manz *et al.*, 1992
CF319a	5′-TGGTCCGTGTCTCAGTAC-3′	319–336	Cytophaga—Flavobacterium	35	70	Cy3	Manz *et al.*, 1992
LGC354a,b,c	5′- **(T/C)(G/C)**GAAGATTCCCTAC-3′	354–371	Firmicutes	35	70	Cy3	Meier *et al.*, 1999
GNS934	5′-ACCACACGCTCCGCTGCTTGT-3′	934–955	Chloroflexi cluster I	30	100	Cy3	Fieseler *et al.*, 2004
DLP	5′-CCACCATGCGGCAGGAGGTCA-3′	182–202	Actinobacteria	40	40	Cy3	Schuppler *et al.*, 1998
CHIS150	5′-TTATGCGGTATTATTCT**(T/C)**CCTTT-3′	150–172	Clostridium cluster I and II (A)	0	900	Cy3	Franks *et al.*, 1998
SRB385	5′-CGGCGTCGCTGCGTCAGG-3′	385–402	SRB of δ—Proteobacteria (A)	35	70	Cy3	Amann *et al.*, 1990
SRB441	5′-CATGCACTTCTTTCCACTT-3′	441–459	Desulfobulbaceae (A)	5	60	Cy3	Tonolla *et al.*, 2000
DSV698	5′-GTTCCTCCAGATATCTACGG-3′	698–717	Genus Desulfovibrio (A)	40	40	Cy3	Manz *et al.*, 1992
DSV698c	5′-GTTCCTCCAGATATCTACGC-3′	698–717	DSV968 competitor	40	40	None	Manz *et al.*, 1992
NS01225	5′-CGCCATTGTATTACGTGTGA-3′	1224–1243	Nitrifying β—Proteobacteria	35	70	Cy3	Mobarry *et al.*, 1996
AMX368	5′-CCTTTCGGGCATTGCGAA-3′	368–385	All Anammox organisms (A)	15	338	Cy3	Kartal *et al.*, 2007
SHEW227	5′-AGCTAATCCCACCTAGGTI[e]**(G/A/T)(A/T)**CAT-3′	227–251	Genus Shewanella	40	40	Cy3	Huggett *et al.*, 2006
cSHEW227	5′-AGCTAATCCCACCTAGGC**(A/T)**TATC-3′	227–251	SHEW227 competitor	40	40	None	Huggett *et al.*, 2006

Abbreviation: FISH, fluorescence *in situ* hybridization.
[a]The nucleotides in boldface type are degenerate.
[b]*Escherichia coli* numbering.
[c]Concentration (v/v) in hybridization buffer.
[d]Concentration (w/v) in washing buffer.
[e]Inosine.
(A) After probe target denotes strict anaerobe organisms.

30 s, 55 °C for 30 s and 72 °C for 1.5 min. A final extension of 7 min at 72 °C was added. Each PCR product was checked by gel electrophoresis. Expected PCR amplicons of the correct size (~1.5 kbp) were manually excised and gel purified using the Minelute gel extraction kit (Qiagen, Valencia, CA, USA). The PCR products were screened and grouped by restriction fragment length polymorphism analysis using *Hha*I restriction patterns (data not shown). PCR products from each restriction fragment length polymorphism group and those cultures with unique morphotypes were sequenced by Northwoods DNA (Solway, MN, USA). The 16S recombinant DNA sequences were viewed and edited in Chromas (Technelysium, Tewantin, Australia). Sequences were identified using BLAST N at the NCBI database (www.ncbi.nlm.nih.gov, Altschul *et al.*, 1997)

Phylogenetic reconstruction

All phylogenetic reconstruction was performed using ARB (Ludwig *et al.*, 2004). Alignments of 16S small subunit (SSU) ribosomal RNA sequences were made with the ARB fast alignment tool and were checked manually before being added to the ARB data set using the Arb_Parsimony tool to select suitable outgroups. *De novo* trees were constructed using the ARB neighbor-joining distance matrix with Felsenstein correction and termini (parameters: .– = 0) and position variance (parameters: 123456789.– = 0) filters (Felsenstein, 1988). The most appropriate DNA substitution model for distance analyses was determined by MODELTEST (Posada and Crandall, 1998). New sequences generated in this study were submitted to GenBank under the following accession numbers: EF114127–EF114207 and EU203318–EU203332.

Results

Three deep-water sponge specimens used for this study were collected in August 2005 with Harbor Branch Oceanographic Institute's JSL research submersible from the support vessel R/V *Seward Johnson* (Table 1). A taxonomic voucher specimen is deposited for each at the Harbor Branch Oceanographic Museum: catalog numbers 003:01040, 003:01041 and 003:01042.

Specimen 003:01040 was collected (dive number JSL I-4820) in the Straits of Florida, ~13-nm offshore southeastern Florida at a depth of 304 m from a rocky slope of the Miami Terrace escarpment. The specimen is pear-shaped, ~20-cm tall, with a single 5-cm apical osculum. It is light brown in color; the apical tip is lighter with alternating brown and white stripes around the osculum. The striped area of the osculum is hispid with 2- to 3-mm fringing spicules. The surface is hard, smooth and finely pitted, forming a detachable ectosomal cortex 2- to 3-mm thick. The specimen fits the description of the genus *Geodia* Lamarck, 1815 (Phylum- Porifera, Class- Demospongiae, Order- Astrophorida; Hooper and Van Soest, 2002).

Specimen 003:01041 was collected (dive JSL I-4826) from the Miami Terrace escarpment, ~30 nm south of the first specimen and at a depth of 341 m. It also fits the description of the genus *Geodia* but is a different species than the previous. The specimen is oblong to subspherical, ~11-cm maximum diameter, and has a gray to light brown color *in situ*. The hard detachable ectosomal cortex is 2- to 3-mm thick and the surface is dimpled. Three oscula are 4 mm in diameter and slightly raised on 4-mm cones.

Specimen 003:01042 was collected (dive JSL I-4829) further south in the Straits of Florida off the Florida Keys on the Pourtales Terrace at a depth of 197 m. It also fits the description of the genus *Geodia* but is also an unknown species that is different from the other two specimens. It is a flattened sphere ~25 cm in diameter and 15-cm tall. The hard 2-mm ectosomal cortex is hispid, covered with 2- to 5-cm long spicules and highly sedimented.

Analysis of homogenates by FISH showed that a wide variety of microbes, including some putative anaerobes are present in both *Geodia* spp. and sediments (Figure 1). Archaea were present at 1×10^6 cells g^{-1} of sample (wet weight) while the total bacterial population was 1×10^8 cells g^{-1} of sample. *Gammaproteobacteria* and *Firmicutes* gave the most signals in both sediment and *Geodia* spp. samples. *Clostridium* spp. and *Shewanella* spp. constituted only a minor part on the total *Firmicutes* and gammaproteobacterial population, respectively. In contrast, SRB were shown to be a significant part of the deltaproteobacterial population (between 30 and 87%) for both sample types. Anammox organisms made up between 0.65 and 2.4% of the overall population. Betaproteobacterial ammonia-oxidizing bacteria made up between 12 and 57% of the overall betaproteobacterial population. Although *Chloroflexi* were present at 1×10^7 cells g^{-1} of sample in *Geodia* spp., there was a significantly ($P > 0.005$) smaller number of *Chloroflexi* present in sediment samples with 1.5×10^5 cells g^{-1} of sample.

A total of 96 bacterial isolates from sediment and *Geodia* spp. were derived using standard anaerobic bacterial techniques on four different microbial media, which varied in pH, nutrient content and ionic strength (salt content) to mimic different bacterial micro-environments (Tables 3a,b, Figure 2). A majority of the microbial isolates presented here, grew exclusively on a single medium type. All bacterial isolates were analyzed by classical microbiological and genetic methods, including determination of cell morphology, gram staining and 16S ribosomal RNA gene sequence analysis.

Fifty unique organisms from three *Geodia* spp. specimens (Table 3a, Figure 2) were found in

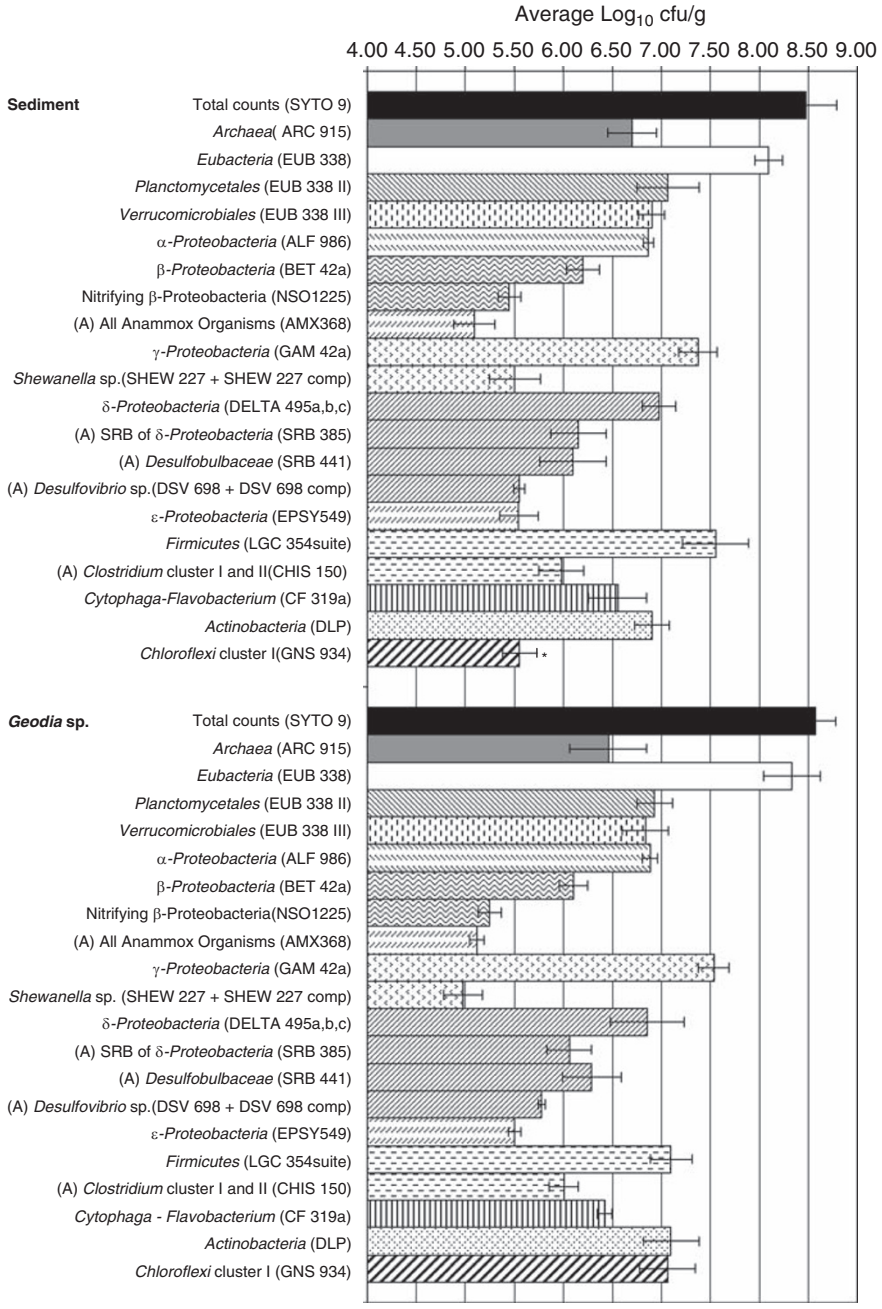

Figure 1 Log_{10} average populations of Eubacteria associated with sediment and *Geodia* spp. samples estimated by means of FISH counting. Error bars are ± one s.d. Log cells g^{-1} is the logarithmic (base 10) count of cells (bacteria) per gram of sample (wet weight). *Indicates a significant difference ($P<0.005$) between *Geodia* spp. and sediment populations. (A) In front of probe target denotes strict anaerobe organisms.

anaerobic culture of which *Firmicutes* (22 isolates, 44%) represented the largest organism cluster. *Gammaproteobacteria* formed the second largest bacterial group in overall microbial diversity, comprising 40% (20 isolates) of all bacterial isolates. Minor culturable components of the microbial isolates consisted of *Chloroflexi* (one isolate, 2%), *Actinobacteria* (three isolates, 6%), *Betaproteobacteria* (three isolates, 6%) and *Bacteroidetes* (one isolate, 2%). With the exception of six obligate anaerobic organisms (WMB24A-E, W060), all bacteria were facultative anaerobes. A total of 46 isolates were 96–100% homologous to GenBank sequences; four were 91–95% homologous. *Bacillus* spp. were the most abundant organisms found in culture comprising 11 isolates (22%). *Vibrio* spp. were represented with 10 isolates (20%), while *Staphylococcus* spp. were represented by 4 isolates (8%).

Surprisingly, the phylogenetic analysis (Figure 3) of the cultured *Chloroflexi* species found in this

Table 3 Microbial isolates from (a) *Geodia* spp. and (b) sediments

Isolate	*GenBank no. of isolate*	*Isolation medium*	*Cell morphology*	*Gram stain*	*Phylogenetic association*	*Closest GenBank match*	*% Similarity*	*GenBank no. of closest relative*
(a)								
W013	EU203320	BHI	Rods, single	+	Actinobacteria	Actinomycetales bacterium GP-5	100	AY145533
W067	EU203325	MA	Rods, single	+/−	Actinobacteria	Mycobacterium marinum cyprinum CC240299	99	AF456239
W072	EU203326	BHI	Rods, single	+	Actinobacteria	uncultured actinobacterium S15A-MN100	96	AJ534681
W076	EU203327	BHI	Pleomorphic rod	−	Bacteroidetes	Formosa sp. 5IX/A01/134	98	AY576730
W023	EF114168	MA	Rods, single	−	Betaproteobacteria	*Variovorax* sp. HAB-27	91	AB051689
W057	EF114178	SB	Rods, single	−	Betaproteobacteria	*Achromobacter xylosoxidans*	93	AJ560626
WMB22	EU203322	BHI	Rods, single	−	Betaproteobacteria	Alcaligenes sp.	98	EF111196
W014	EU203321	BHI	Filamentous rod	−	Chloroflexi	uncultured Chloroflexi bacterium ST01-SN2H	98	AY222298
W060	EU203323	MA	Rods, single, spores	+	Firmicutes	uncultured Clostridiales bacterium RSW02-092	100	AB198553
W077	EF114179	BHI	Rods, single, spores	+	Firmicutes	*Bacillus* sp. VAN35	99	AF286486
WMB10A	EF114185	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	99	AY651924
WMB11A	EF114188	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	98	CP000001
WMB12A	EF114189	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	99	CP000001
WMB13A	EF114190	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	99	CP000001
WMB14	EF114191	BHI	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	99	AY647292
WMB15	EF114186	BHI	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	100	AY651924
WMB16	EF114187	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	98	AJ508706
WMB18	EF114184	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	99	CP000001
WMB1B	EF114197	BHI ASW	Cocci	+	Firmicutes	*Staph. epiderminis*	99	AY167804
WMB21	EF114195	BHI	Rods, single	+	Firmicutes	Clone W4B-B03	99	AY345491
WMB24A	EF114201	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	97	AF018038
WMB24B	EF114202	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	96	AF018038
WMB24C	EF114203	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	98	AF018038
WMB24D	EF114204	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	99	AF018038
WMB24E	EF114205	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	97	AF018038
WMB2B	EF114198	BHI ASW	Cocci	+	Firmicutes	*Staph. epiderminis*	96	AY730712
WMB3B	EF114199	BHI ASW	Cocci	+	Firmicutes	*Staph. epiderminis*	99	AY167864
WMB4B	EF113200	BHI ASW	Cocci	+	Firmicutes	*Staphylococcus sp.*	94	AE016749
WMB6B	EF114192	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus* sp.	99	AB126763
WMB6C	EF114193	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus* sp.	99	AB126763
T580	EF114196	BHI	Rods, single	−	Gammaproteobacteria	*Photobacterium leiognathi*	99	AY292944
W010	EU203318	MA	Rods, single	−	Gammaproteobacteria	Pseudomonas pachastrellae	99	AB125366
W011	EU203319	BHI	Rods, single	−	Gammaproteobacteria	Pseudoalteromonas ruthenica	97	AF316891
W046	EF114170	MA	Rods, single	−	Gammaproteobacteria	*Vibrio* sp. 12G12	99	AY836978
W047	EF114171	MA	Rods, single	−	Gammaproteobacteria	*Vibrio* sp. Ex25	99	AF319769
W049	EF114172	MA	Rods, single	−	Gammaproteobacteria	*Vibrio* sp. 14C06	99	AY837077
W051	EF114173	BHI	Rods, single	−	Gammaproteobacteria	Uncultured *Vibrio* sp. 'Artemia'	99	AF108137
W052	EF114174	MA	Rods, single	−	Gammaproteobacteria	*Pseudomonas putida*	93	DQ229317
W053	EF114175	NA	Rods, single	−	Gammaproteobacteria	*Pseudomonas oryzihabitans*	99	AF501336
W055	EF114176	SB	Rods, single	−	Gammaproteobacteria	*Stenotrophomonas maltophilia*	99	AJ011332
W056	EF114177	SB	Rods, single	−	Gammaproteobacteria	*Stenotrophomonas* sp. 3A3C	99	AF368754
W066	EU203324	MA	Rods, single	−	Gammaproteobacteria	Halomonas marisflavi SW32	97	AF251143
W078	EF114180	BHI	Rods, single	−	Gammaproteobacteria	*Vibrio parahaemolyticus* RIMD 2210	99	BA000032
W082	EF114181	NA	Rods, single	−	Gammaproteobacteria	*Marinobacter alkaliphilus*	99	AB125942
W098	EF114182	MA	Rods, single	−	Gammaproteobacteria	*Vibrio alginolyticus*	99	X74691
W099	EF114183	NA	Rods, single	−	Gammaproteobacteria	*Vibrio* sp. 12G12	99	AY836978
W27A	EF114207	MA	Rods, single	−	Gammaproteobacteria	*Vibrio corallyticus*	98	AJ440004
W28B	EF114169	BHI ASW	Rods, single	−	Gammaproteobacteria	*Vibrio* sp. OC25	98	AB038026

Table 3 (Continued)

Isolate	*GenBank no. of isolate*	*Isolation medium*	*Cell morphology*	*Gram stain*	*Phylogenetic association*	*Closest GenBank match*	*% Similarity*	*GenBank no. of closest relative*
WMB20	EF114194	BHI ASW	Rods, single	–	Gammaproteobacteria	Clone GLB-2	98	AY345576
WMB26	EF114206	NA	Rods, single	–	Gammaproteobacteria	*Vibrio chagasii*	98	AJ490158
(b)								
W007	EU203330	MA	Short rods, single	+	Actinobacteria	Actinomyces gerencseriae	98	X80414
W030	EF114132	BHI	Rods, single	–	Alphaproteobacteria	*Erythrobacter* sp. MB-16	98	AF325446
W069	EU203331	NA	Pleomorphic rod	–	Bacteroidetes	Salegentibacter flavus FG69	96	AY682200
W002	EU203328	MA	Rods, single	–	Deltaproteobacteria	Desulfovibrio sp. DR1	99	Y17758
W003	EU203329	MA	Rods, single, spores	+	Firmicutes	Clostridium beijerinckii	98	X68180
W029	EF114131	NA	Rods, single, spores	+	Firmicutes	*Bacillus* sp. KMM 3737	99	AY228462
W031	EF114133	BHI	Rods, single, spores	+	Firmicutes	*Bacillus baekryungensis*	97	AF541965
W033	EF114135	BHI	Rods, single, spores	+	Firmicutes	*Bacillus* sp. CSS-8	92	DQ084469
W034	EF114136	BHI	Rods, single, spores	+	Firmicutes	*Bacillus* sp. KR4	95	AB126757
W035	EF114137	NA	Rods, single, spores	+	Firmicutes	*Bacillus thuringiensis*	99	AY795567
W036	EF114138	MA	Rods, single, spores	+	Firmicutes	*Bacillus* sp. VAN35	99	AF286486
W037	EF114139	MA	Rods, single, spores	+	Firmicutes	*Bacillus* sp. R-6782	98	AY422984
W038	EF114140	MA	Rods, single, spores	+	Firmicutes	*Bacillus cereus* strain ATCC 25621	99	AY795568
W040	EF114142	MA	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	99	DQ207729
W041	EF114143	MA	Rods, single, spores	+	Firmicutes	*Bacillus cereus* isolate CECRIbio	98	DQ207562
W042	EF114144	MA	Rods, single	+	Firmicutes	*Halobacillus trueperi* strain GSP38	99	AY505522
W044	EF114146	MA	Rods, single, spores	+	Firmicutes	*Bacillus* sp. P01	100	AY964602
W079	EF114148	BHI	Rods, single, Spores	+	Firmicutes	*Bacillus* sp. 9B_1	99	AY689061
W080	EF114149	NA	Rods, single, spores	+	Firmicutes	*Bacillus pumilus*	99	AY741720
W081	EF114150	NA	Cocci	+	Firmicutes	*Staphylococcus* sp. R-23212	100	AJ969171
W108	EU203332	MA	Rods, single	+	Firmicutes	Paenibacillus sp. 2	99	DQ105970
WMB1A	EF114153	BHI ASW	Cocci	+	Firmicutes	*Staph. epiderminis*	99	AY030342
WMB24F	EF114158	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	97	AF018038
WMB24G	EF114159	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	96	AF018038
WMB24H	EF114160	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	98	AF018038
WMB24I	EF114161	BHI ASW	Rods, single, spores	+	Firmicutes	Uncultured bacterium 08SE	98	AF018038
WMB2A	EF114154	BHI ASW	Cocci	+	Firmicutes	*Staph. epiderminis*	99	AY030342
WMB3A	EF114155	BHI ASW	Cocci	+	Firmicutes	*Staph. epiderminis*	99	AY167804
WMB4A	EF114156	BHI ASW	Cocci	+	Firmicutes	*Staphylococcus sp.*	99	AY437631
WMB5	EF114157	BHI ASW	Cocci	+	Firmicutes	*Staphylococcus sp.*	98	AY057450
WMB6A	EF114167	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus* sp.	99	AB126763
WMB7A	EF114165	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	98	CP000001
WMB9A	EF114166	BHI ASW	Rods, single, spores	+	Firmicutes	*Bacillus cereus*	98	CP000001
W024	EF114127	MA	Rods, single	–	Gammaproteobacteria	*Halomonas* sp. wp4	98	AJ551091
W026	EF114128	NA	Rods, single	–	Gammaproteobacteria	*Vibrio* sp. 14D09	98	AY837090
W027	EF114129	NA	Rods, single	–	Gammaproteobacteria	Marine bacterium Tw-6	99	AY028201
W028B	EF114130	NA	Rods, single	–	Gammaproteobacteria	*Vibrio* sp. 12D10	98	AY028201
W032	EF114134	MA	Rods, single	–	Gammaproteobacteria	*Photobacterium* sp. 2220	99	DQ219367
W039	EF114141	MA	Rods, single	–	Gammaproteobacteria	Uncultured Aeromonadaceae	96	AY711717
W043	EF114145	MA	Rods, single	–	Gammaproteobacteria	*Ferrimonas marina*	97	AB193750
W045	EF114147	NA	Rods, single	–	Gammaproteobacteria	*Vibrio vulnificus* strain MP-4	100	AY911393
W083	EF114151	BHI	Rods, single	–	Gammaproteobacteria	*Pseudomonas* sp. 7325	99	AM111063
W115	EF114152	BHI	Rods, single	–	Gammaproteobacteria	*Vibrio harveyi* strain S35	99	AY750578
WMB27B	EF114162	MA	Rods, single	–	Gammaproteobacteria	*Vibrio corallyticus*	98	AJ440004
WMB27C	EF114163	MA	Rods, single	–	Gammaproteobacteria	*Vibrio corallyticus*	98	AJ440004
WMB27D	EF114164	MA	Rods, single	–	Gammaproteobacteria	*Vibrio corallyticus*	96	AJ440004

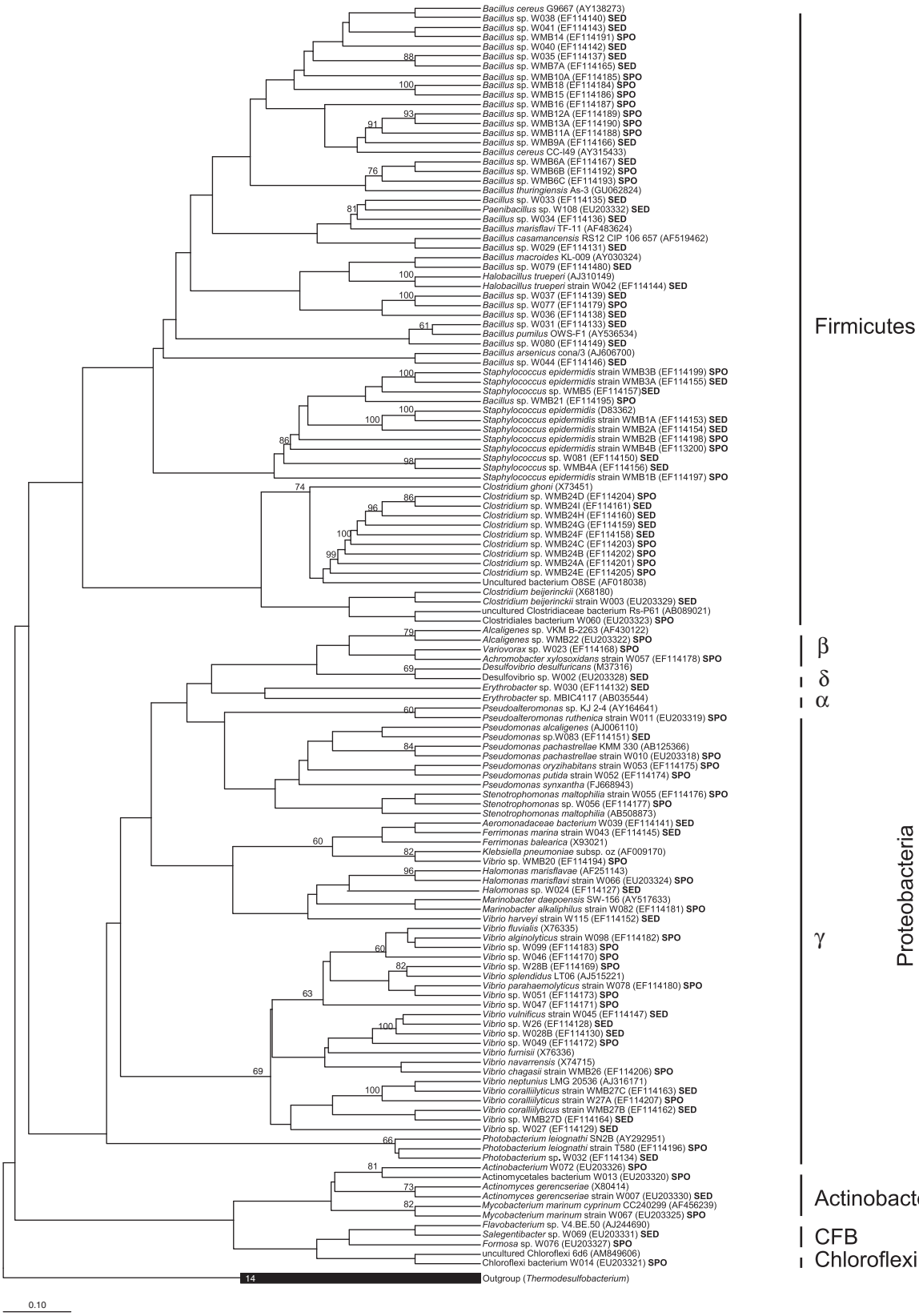

Figure 2 Distance-based neighbor-joining phylogeny of 16S ribosomal RNA (rRNA) gene sequences obtained from anaerobic isolates from sediment and sponge tissue. Numbers at nodes are percentages indicating levels of bootstrap support, based on neighbor-joining analysis of 1000 re-sampled data sets. Only values $\geqslant 60\%$ are shown. Scale bar represents 0.1 substitution per nucleotide position. SPO = Isolate from Geodia spp., SED = Isolate from sediment.

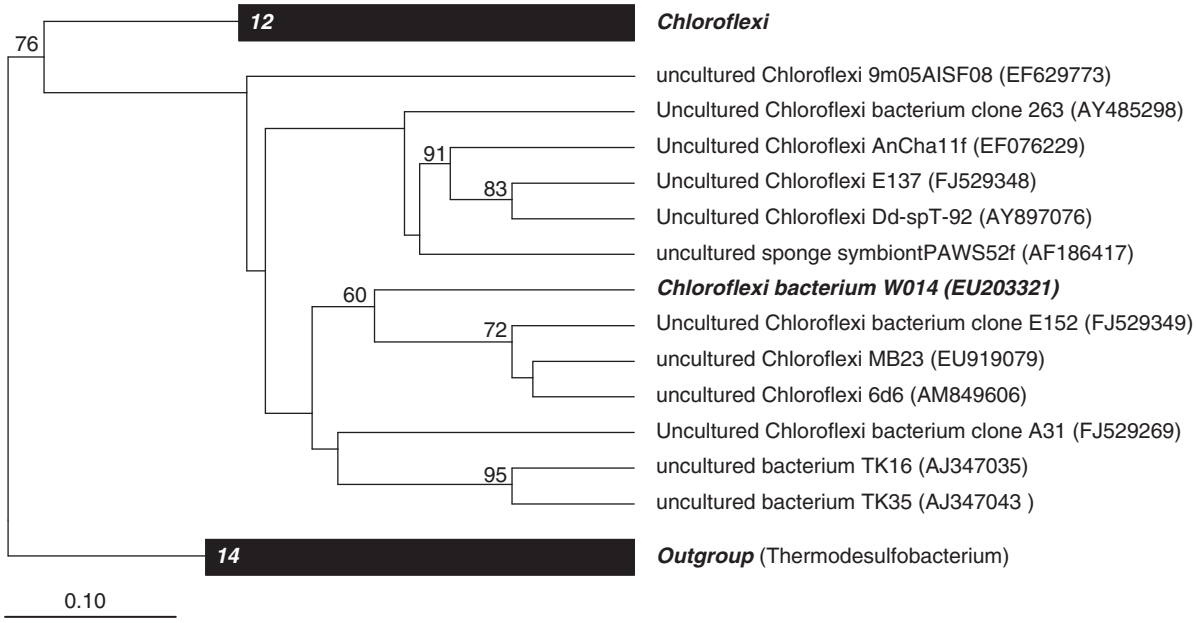

Figure 3 *De novo* neighbor-joining phylogeny comparing the Chloroflexi isolated from *Geodia* spp. in this study (EU203321) with other sponge-origin reference sequences. Numbers at nodes are percentages indicating levels of bootstrap support, based on neighbor-joining analysis of 1000 re-sampled data sets. Only values $\geqslant$60% are shown. Scale bar represents 0.1 substitution per nucleotide position. Reference sequences derived from previous studies and GenBank entries are described in the text or are written with their corresponding accession numbers.

study indicated a close relationship to a cluster of unculturable marine-derived *Chloroflexi*, which have been suggested to form stable symbiotic relationships with sponges (Taylor *et al.*, 2007). In contrast, the cultured *Chloroflexi* species only showed a distant relationship with other *Chloroflexi* 16S RNA sequences found in the NCBI database. This current data infers that the organism isolated in this study forms a symbiotic relationship with deep-water sponges of the genus *Geodia* spp. To our knowledge this is the first evidence of a culturable member of the *Chloroflexi* isolated from a deep-water sponge.

Sediment samples used in this study had a grayish-green to black appearance and a fine sand grain structure. Anaerobic culture of sediment samples (Tab. 3b, Figure 2) again showed a wide array of organisms able to grow anaerobically, with a total of 46 isolates. Again, *Firmicutes* represented the largest group (63%, 29 organisms), while *Gammaproteobacteria* were the second largest group (28%, 13 organisms). One species (2%) each of *Actinobacteria*, *Alphaproteobacteria*, *Bacteroidetes* and *Deltaproteobacteria* was also present. With the exception of six obligate anaerobic organisms (WMB24F-I, W002, W003), all bacteria were facultative anaerobes. Forty-four isolates were 96–100% homologous to GenBank sequences. One isolate (*Bacillus* spp.) showed 92% homology to GenBank sequences while another was 95% (*Bacillus* spp.) homologous, which may indicate that these organisms are novel variants of the species *Bacillus* spp., which represented the major bacterial cluster found in sediment cultures amounting to 16 specimens (34%). *Vibrio* spp. was represented by seven isolates (15%). *Staphylococcus* spp. was represented with four isolates (9%).

In both the sponge and sediment samples, most sequences obtained from obligate anaerobes were most closely associated with uncultured bacterium 08SE (accession number AF018038) from the gut of the Tyrolean iceman (Cano *et al.*, 2000). BLAST analysis further revealed the sequence to be a member of the *Clostridium* spp. Although these isolates are related to the same GenBank database entry, they differed in a combination of other parameters linked to their analysis set. Each culture was either isolated from a different growth medium and/or showed marked differences in gross colony morphology and color. As different bacterial strains can have a divergent biochemical and genetic make-up, these are included in the data presented here. Extensive morphological studies identified the organisms as obligate anaerobic, Gram positive, spore forming (sub-terminal to terminal) single rods, indicating the presence of a *Clostridium* spp.

Discussion

Although it is assumed that sponge-bacterium symbioses have existed for millions of years, the mechanisms by which this association is established are not well understood (Taylor *et al.*, 2007). It may be assumed that microorganisms capable of association with sponges are present in the surrounding seawater and environment, but at abundances below

the detection limit of currently available methods (Müller and Müller, 2003). Therefore, as anaerobic bacteria are not major components of the bacterial communities of seawater, resuspended sediment particles are a likely source for these and other microbiota. To enable the *in situ* assessment of microbial consortia in the deep-water sponge *Geodia* spp. and in surrounding sediment samples, we have utilized a combination of FISH with a diverse oligonucelotide probe set and direct microbial culture, which offered the possibility of characterizing microbes using traditional microbiological techniques. These methodologies have been proven to give a reliable overview of *in situ* microbial associations in various marine organisms, including sponges (Hoffmann *et al.*, 2006; Brück *et al.*, 2007). Our assessment of specimens of *Geodia* spp. and sediment showed that a significant proportion of microbiota is shared between the two. In fact, only *Chloroflexi* were present in significantly larger numbers in *Geodia* spp. in comparison with sediment. The major bacterial constituents *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes* as well as anaerobic microbiota such as SRB and *Clostridium* spp. were present in similar numbers in *Geodia* spp. and sediment. Similar results were observed by Hoffmann *et al.* (2006) who showed that *G. barretti* is dominated by *Alphaproteobacteria* and *Gammaproteobacteria*.

Studies with the Adriatic sponge *Suberites domuncula* have suggested that oxygen levels within the sponge tissue are responsible for regulating the resident microbiota (Müller *et al.*, 2004). *Bacillus* strains represent approximately 20% of the total heterotrophic microbiota in seawater whereas they constitute up to 80% of the total number of the culturable heterotrophic bacteria in marine sediments (Harwood, 1989). Similarly, *Bacillus subtilis* and *Bacillus pumilus* were the most abundant species among those associated with marine sponges (Ivanova *et al.*, 1992, 1999). Spores of *Bacillus* and *Clostridium* species are metabolically dormant and extremely resistant to acute environmental stresses such as low nutrient availability and varying oxygen concentrations (James *et al.*, 2000). Therefore, it is possible that spores of *Bacillus* and *Clostridium* species can survive for many years in marine sediments and sponge tissues until conditions are right for germination and formation of a metabolically active cell. In our study, *Bacillus* spp. constituted 40% of the total culturable sediment microbiota and 22% of culturable sponge associated microorganisms. FISH probing resulted in the detection of significant numbers of *Clostridium* spp. and other *Firmicutes*. Numbers in FISH did not vary significantly between sponge tissue and sediment. This suggests that these organisms were indeed viable within the examined environments.

The facultative nature of the majority of microbes isolated from sediment and *Geodia* spp. suggests that an anaerobic metabolism may not be a necessary survival strategy for most of the microbiota in sponges and marine sediments, however, it may provide a mechanism for coping with periods of anaerobiosis. The efficient utilization and recycling of nutrients, as previously found in *Geodia* spp., may rely on anoxic cycles within sponge tissue making symbiotic microorganisms capable of anaerobic metabolism an important factor in sponge survival (Schumann-Kindel *et al.*, 1997). Furthermore, anoxic zones in *Geodia* spp. may have developed as an effective buffer system that prevents sulfide toxification and overgrowth of SRB (Hoffmann *et al.*, 2006). Members of the *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria* and *Bacteroidetes* have also been shown to have a critical role in extending the anaerobic oxidation of ammonium and nitrite to nutrient-depleted suboxic water layers by creating anoxic, nutrient-enriched microniches (Woebken *et al.*, 2007). This niche-formation may also occur in sponges such as *Geodia* spp. and hence may have an important role in the removal and bioconversion of dissolved nitrogenous waste products such as nitrite, ammonia and organic detritus (marine snow; Hoffmann *et al.*, 2005). Therefore, *Geodia* spp. may rely on external organic nitrogen sources in addition nitrogen-fixing symbionts for growth and tissue remodeling. As nitrogen is the limiting growth factor for biomass formation in nutrient poor environments, sponges rely on prokaryotic symbionts with specific nitrogen recycling strategies to prevent enduring nutrient stress (Meier *et al.*, 1994; Zehr and Ward, 2002; Lenton and Klausmeier, 2006). The annamox bacterial group is capable of converting nitrite and ammonia to nitrogen, which can be fixed to organic intermediates by subsequent microbial processes (Taylor *et al.*, 2007). Furthermore, a consolidated formation of organic nitrogen intermediates by prokaryotic ammonia and nitrite oxidizers may lead to intermediates that can be assimilated by the sponge host leading to complete nitrogen cycling within a sponge (Taylor *et al.*, 2007). In this study, annamox organisms were found in *Geodia* spp. and sediment samples suggesting that bioconversion and nutrient cycling of nitrogenous compounds may occur in these samples.

It has been suggested that the nutrient exchange between sponge host and microbial symbiont may also be driven by the sponge restricting the symbiont's access to essential nutrients and thus supplying the host with an excess of organic carbon (Hinde, 1988; Wilkinson, 1992). In shallow water sponges, cyanobacteria have been identified as the dominant symbionts responsible transfer of photosynthetically derived organic carbon compounds mainly in form of glycerol (C3) for metabolism in the sponge host (Wilkinson, 1980, 1983). In addition to cyanobacteria, a specific cluster of the bacterial class *Chloroflexi* has been identified as sponge-specific symbionts, which is also supported by our data (Figures 1 and 3, Hentschel *et al.*, 2002; Taylor *et al.*, 2007). However, a possible interaction of these

microbes with the sponge host has so far not been suggested. Much like cyanobacteria, *Chloroflexi* are filamentous microorganisms, which are capable of permanently integrating into sponge tissue. In light suffused shallow water environments *Chloroflexi* are also capable photosynthetic fixation of atmospheric CO_2. On the basis of the similar metabolic capacities between cyanobacteria and chloroflexi, these bacteria may also provide carbonaceous photosynthates, such as glycerol, to the sponge host in shallow water environments.

Although symbiotic cyanobacteria cannot thrive in light-deprived environments (Thacker, 2005), *Chloroflexi* can metabolically adjust to dark, nutrient poor environments such as the deep ocean. FISH data sets reported in this study showed significantly higher counts of *Chloroflexi* in the tissue of *Geodia* spp. when compared with marine sediment samples. This data indicating that deep-water *Geodia* tissue is enriched with *Chloroflexi* points to a potential symbiotic relationship. Previous studies of *Chloroflexi* sponge interactions were complicated by the fact that none of the *Chloroflexi* species could be recovered in culture for more detailed microbiological studies. To our knowledge, this study presents the first account of a culturable marine bacterium of the class *Chloroflexi* isolated from sponge tissue. Comparative phylogenetic analysis of this isolate with database DNA sequences of unculturable *Chloroflexi* suggested that this organism may actually form a true symbiotic relationship with deep-water sponge hosts (Hentschel *et al.*, 2002). *Chloroflexi* can convert inorganic into organic carbon by way of the 3- hydroxyproprionate pathway (Brock *et al.*, 1984). The resulting organic intermediates produced by this microbial symbiont could subsequently be transferred to the host sponge and metabolized, which would impart a clear synergistic survival advantage to the host in nutrient poor environments such as the deep ocean floor. The *Chloroflexi*-catalyzed conversion of inorganic CO_2 to metabolizable organic intermediates certainly contributes to microbial biomass formation in deep-water environments. The inferred symbiotic relationship of *Chloroflexi* may also contribute to additional biomass formation in deep-water *Geodia* species.

In conclusion, as marine microorganisms survive under harsh environmental conditions, they can be expected to be a source of novel biogeochemical and biochemical processes (Barlet *et al.*, 1995; Turley, 2000). Through this study, it has become evident that sponge and sediment microbiota share a striking overall similarity and that facultative and obligate anaerobic microorganisms thrive in these environments, in which they may aid in nutrient recycling and bioconversion or some other, to date unknown, function. Resuspended sediment particles may further form a realistic source, in addition to vertical transfer, of microorganisms able to associate or form a symbiotic relationship with sponges. Such microbes represent another facet of the cultivable microbiota that remains to be examined for its biotechnological potential.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W *et al.* (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl Acid Res* **25**: 3389–3402.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Barlet DH, Kato C, Horikoshi K. (1995). High pressure influences on gene and protein expression. *Res Microbiol* **146**: 697–706.

Brock TD, Madigen MT, Martinko JM, Parker J. (1984). *Biology of Microbiology*. Prentice Hall, International (UK): London.

Brück TB, Brück WM, Santiago-Vázquez LZ, McCarthy PJ, Kerr RG. (2007). Diversity of the bacterial communities associated with the azooxanthellate deep water octocorals *Leptogorgia minimata*, *Iciligorgia schrammi*, and *Swiftia exertia*. *Mar Biotech* **9**: 561–576.

Cano RJ, Tiefenbrunner F, Ubaldi M, Del Cueto C, Luciani S, Cox T *et al.* (2000). Sequence analysis of bacterial DNA in the colon and stomach of the Tyrolean Iceman. *Am J Phys Anthropol* **112**: 297–309.

Colquhoun JA, Heald SC, Li L, Tamaoka J, Kato C, Horikoshi K *et al.* (1998). Taxonomy and biotransformation activities of some deep-sea actinomycetes. *Extremophiles* **2**: 269–277.

Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

Dalsgaard T, Thamdrup B, Canfield DE. (2005). Anaerobic ammonium oxidation (anammox) in the marine environment. *Res Microbiol* **156**: 457–464.

Duckworth AR, Bruck WM, Janda KE, Pitts TP, McCarthy PJ. (2006). Retention efficiencies of the coral reef sponges Aplysina lacunosa, Callyspongia vaginalis and Niphates digitalis determined by Coulter counter and plate culture analysis. *Mar Biol Res* **2**: 243–248.

Felsenstein J. (1988). Phylogenies from molecular sequences: inference and reliability. *Ann Rev Gen* **22**: 521–565.

Fieseler L, Horn M, Wagner M, Hentschel U. (2004). Discovery of the novel candidate phylum 'Poribacteria' in marine sponges. *Appl Environ Microbiol* **70**: 3724–3732.

Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F, Welling GW. (1998). Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* **64**: 3336–3345.

Gruber G, Reitner J. (1991). Isolated poriferan micro- and megascleres from the lower Campanian of Höver (northern Germany) and remarks on the phylogeny of the taxon Geodiidae (Demospongiae). *Berliner Geowissenschaftliche Abhandlungen (A)* **134**: 107–117.

Harwood CR. (1989). *Biotechnology Handbooks 2: Bacillus*. Plenum Press: New York.

Hentschel U, Hopke J, Horn M, Friedrich AB, Wagner M, Hacker J *et al.* (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol* **68**: 4431–4440.

Hinde R. (1988). Symbiotic nutrition and nutrient limitation. *Proc 6th Int Coral Reef Symp* **1**: 199–204.

Hoffmann F, Larsen O, Rapp HT, Osinga R. (2005). Oxygen dynamics in choanosomal sponge explants. *Mar Biol Res* **1**: 160–163.

Hoffmann F, Rapp HT, Reitner J. (2006). Monitoring microbial community composition by fluorescence *in situ* hybridization during cultivation of the marine cold-water sponge *Geodia barretti*. *Mar Biotechnol (NY)* **8**: 373–379.

Hooper JNA, Van Soest RWM. (2002). *Systema Porifera, a Guide to the Classification of Sponges* Vol. 1 Kluwer Academic: New York. p 1101.

Huggett MJ, Williamson JE, de Nys R, Kjelleberg S, Steinberg PD. (2006). Larval settlement of the common Australian sea urchin *Heliocidaris erythrogramma* in response to bacteria from the surface of coralline algae. *Oecologia* **149**: 604–619.

Ivanova EP, Mikhailov VV, Andreev LA. (1992). Marine bacilli and some approaches to their identification. *Microbiol Zhurnal* **54**: 27–33.

Ivanova EP, Vysotskii MV, Svetashev VI, Gorshkova NM, Mikhailov VV, Yumoto N *et al.* (1999). Characterisation of *Bacillus* strains of marine origin. *Intern Microbiol* **2**: 267–271.

James AL, Chilvers KF, Perry JD, Armstrong L, Gould FK. (2000). Evaluation of p-naphtholbenzene-β-D-galactoside as a substrate for bacterial β-galactosidase. *Appl Environ Microbiol* **66**: 5521–5523.

Kartal B, Rattray J, van Niftrik LA, van de Vossenberg J, Schmid MC, Webb RI *et al.* (2007). *Candidatus* 'Anammoxoglobus propionicus' a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Syst Appl Microbiol* **30**: 39–49.

Kato C, Li L, Tamaoka J, Horikoshi K. (1997). Molecular analysis of the sediment of the 11000 m deep Mariana Trench. *Extromophiles* **1**: 117–123.

Lenton TM, Klausmeier CA. (2006). Co-evolution of phytoplankton C.N:P stochiometry and the deep ocean N: P ratio. *Biogeosci Disc* **3**: 1023–1047.

Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J *et al.* (2002). Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl Environ Microbiol* **68**: 5064–5081.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al.* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

Luna GM, Dell'Anno A, Danovaro R. (2006). DNA extraction procedure: a critical issue for bacterial diversity assessment in marine sediments. *Envir Microbiol* **8**: 308–320.

Luna GM, Dell'Anno A, Guiliano L, Danovaro R. (2004). Bacterial diversity in deep Mediterranean sediments: relationship with the active bacterial fraction and substrate availability. *Envir Microbiol* **6**: 745–753.

Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

Meier D, Zuniga-Partida V, Ramirez-Cano F, Hahn N-C, Faix O. (1994). Conversion of technical lignin into slow release nitrogenous fertilizers by ammoxidation in the liquid phase. *Bioresource Technol* **49**: 121–128.

Meier H, Amann R, Ludwig W, Schleifer K-H. (1999). Specific oligonucleotide probes for *in situ* detection of a major group of gram-positive bacteria with low DNA G+C content. *Syst Appl Microbiol* **22**: 186–196.

Mincer TJ, Spyere A, Jensen PR, Fenical W. (2004). Phylogenetic analysis and diterpenoid production by marine bacteria of the genus Saprospira. *Curr Microbiol* **49**: 300–307.

Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl Environ Microbiol* **62**: 2156–2162.

Müller WE, Grebenjuk VA, Le Pennec G, Schroder H, Brummer F, Hentschel U *et al.* (2004). Sustainable production of bioactive compounds by sponges—cell culture and gene cluster approach: a review. *Mar Biotechnol* **6**: 105–117.

Müller WEG, Müller IM. (2003). Origin of the metazoan immune system: identification of the moleculaes and their function in sponges. *Integr Comp Biol* **43**: 281–292.

Musat N, Werner U, Knittel K, Kolb S, Dodenhof T, van Beusekom JE *et al.* (2006). Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea. *Syst Appl Microbiol* **29**: 333–348.

Posada D, Crandall KA. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Purdy KJ, Nedwell DB, Embley TM, Takii S. (2001). Use of 16S rRNA-targeted oligonucleotide probes to investigate the distribution of sulphate-reducing bacteria in estuarine sediments. *FEMS Microbiol Ecol* **36**: 165–168.

Purdy KJ, Munson MA, Cresswell-Maynard T, Nedwell DB, Embley TM. (2003). Use of 16S rRNA-targeted oligonucleotide probes to investigate function and phylogeny of sulphate-reducing bacteria and methanogenic archaea in a UK estuary. *FEMS Microbiol Ecol* **44**: 361–371.

Reiswig HM. (1981). Partial carbon and energy budgets of the bacteriosponge *Verongia fistularis* (Porifera: Demospongiae) in Barbados. *Mar Ecol Prog Ser* **2**: 273–293.

Schmidt EW, Obraztsova AY, Davidson SK, Faulkner DJ, Haygood MG. (2000). Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel delta-Proteobacterium, 'Candidatus *Entotheonella palauensis*'. *Mar Biol* **136**: 969–977.

Schumann-Kindel G, Bergbauer M, Manz W, Szewyk U, Reitner J. (1997). Aerobic and anaerobic microorganisms in modern sponges: a possible relationship to fossilization-processes. *Facies* **36**: 268–272.

Schuppler M, Wagner M, Schön G, Göbel U. (1998). *In situ* identification of nocardioform actinomycetes in activated sludge using fluorescent rRNA-targeted oligonucleotide probes. *Microbiology* **144**: 249–259.

Skyring GW. (1987). Sulfate reduction in coastal ecosystems. *Geomicrobiol J* **5**: 295–374.

Stahl DA, Amann RI. (1991). Development and application of nucleic acid probes. In: Stackebrandt E and Goodfellow M (eds). *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons Ltd: Chichester, England. pp 205–248.

Takii S, Fukui M. (1991). Relative importance of methanogenesis, sulfate reduction and dendritification in sediments of the lower Tama river. *Bul Jpn Soc Micro Ecol* **6**: 1–8.

Taylor MW, Radax R, Steger D, Wagner M. (2007). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol Mol Biol Rev* **71**: 295–347.

Thacker RW. (2005). Impacts of shading on sponge-cyanobacteria symbiosis: acomparision between host-sepcific and generalist associations. *Intregr Comp Biol* **45**: 369–376.

Tonolla M, Demarta A, Peduzzi S, Hahn D, Peduzzi R. (2000). *In situ* analysis of sulfate-reducing bacteria related to *Desulfocapsa thiozymogenes* in the chemocline of meromictic Lake Cadagno (Switzerland). *Appl Environ Microbiol* **66**: 820–824.

Turley C. (2000). Bacteria in the cold deep sea benthic boundary layer and sediment-water interface of the NE Atlantic. *FEMS Microbiol Ecol* **33**: 89–99.

Vacelet J, Donadey C. (1977). Electron microscope study of the association between some sponges and bacteria. *J Exp Mar Biol Ecol* **30**: 301–314.

Wallner G, Amann R, Beisker W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.

Wang F, Wang P, Chen M, Xiao X. (2004). Isolation of extremophiles with the detection and retival of *Shewanella* strains in deep-sea sediments from the West Pacific. *Extremophiles* **8**: 156–168.

Webster NS, Hill RT. (2001). The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. *Mar Biol* **138**: 843–851.

Wilkinson CR. (1978). Microbial association in sponges. II. Numerical analysis of sponge and water bacterial populations. *Mar Biol* **49**: 169–176.

Wilkinson CR. (1980). Nutrient translocation from green algal symbionts to the freshwater sponge Ephydatia fluviatilis. *Hydrobiologia* **75**: 241–250.

Wilkinson CR. (1983). Net primary productivity in coral reef sponges. *Science* **219**: 410–412.

Wilkinson CR. (1992). Symbiotic interactions between marine sponges and algae. In: Reisser W (ed). *Algae and Symbioses: Plants, Animals, Fungi, Viruses, Interactions Explored*. Biopress Limited: Bristol, UK. pp 111–151. 478.

Wilkinson CR, Cheshire AC. (1990). Comparisons of sponge populations across the Barrier Reefs of Australia and Belize: evidence for higher productivity in the Caribbean. *Mar Ecol Prog Ser* **67**: 285–294.

Woebken D, Fuchs BM, Kuypers MM, Amann R. (2007). Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system. *Appl Environ Microbiol* **73**: 4648–4657.

Zehr JP, Ward BB. (2002). Nitrogen cycling in the ocean: New perspectives on processes and paradigms. *Appl Environ Microbiol* **68**: 1015–1024.