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Compositional analysis of bacterial communities in seawater, sediment, and sponges in the Misool coral reef system, Indonesia

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Abstract Sponge species have been deemed high microbial abundance (HMA) or low microbial abundance (LMA) based on the composition and abundance of their microbial symbionts. In the present study, we evaluated the richness and composition of bacterial communities associated with one HMA sponge (*Xestospongia testudinaria*; Demospongiae: Haplosclerida: Petrosiidae), one LMA sponge (*Stylissa carteri*; Demospongiae: Scopalinida - Scopalinidae), and one sponge with a hitherto unknown microbial community (*Aaptos suberitoides*; Demospongiae: Suberitida: Suberitidae) inhabiting the Misool coral reef system in the West Papua province of Indonesia. The bacterial communities of these sponge species were also compared with seawater and sediment bacterial communities from the same coastal coral reef habitat. Using a 16S rRNA gene barcoded pyrosequencing

approach, we showed that the most abundant phylum overall was Proteobacteria. The biotope (sponge species, sediment or seawater) explained almost 84% of the variation in bacterial composition with highly significant differences in composition among biotopes and a clear separation between bacterial communities from seawater and *S. carteri*; *X. testudinaria* and *A. suberitoides* and sediment. The Chloroflexi classes SAR202 and Anaerolineae were most abundant in *A. suberitoides* and *X. testudinaria* and both of these species shared several OTUs that were largely absent in the remaining biotopes. This suggests that *A. suberitoides* is a HMA sponge. Although similar, the bacterial communities of *S. carteri* and seawater were compositionally distinct. These results confirm compositional differences between sponge and non-sponge biotopes and between HMA and LMA sponges.

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Introduction

Coral reefs are highly diverse, but also globally threatened ecosystems. Local perturbations including overfishing and pollution combined with global phenomena such as coral bleaching have had a disastrous effect on coral reefs (Bruno and Selig 2007; De'ath et al. 2012; Pandolfi et al. 2003). Coral cover in certain reefs has virtually disappeared leading to the loss of important ecosystem services such as renewable resources (e.g., fisheries), protection against erosion, and nutrient cycling (Moberg and Folke 1999). The loss of coral cover also leads to the loss of dependent species such as numerous fish species and shifts in composition to reefs dominated by non-coral taxa such as algae or sponges (Bellwood et al. 2004).

Sponges (Phylum *Porifera*) are ancient metazoans (van Soest et al. 2012) with a fossil record dating back to the Precambrian (Li et al. 1998). The majority of the 8826 valid living species belong to the class Demospongia (demosponges) (Borchiellini et al. 2001; van Soest et al. 2012). The remaining species represent the classes Hexactinellida (glass sponges), Calcarea (calcareous sponges), and Homoscleromorpha. They have successfully colonised a range of aquatic environments from tropical to polar, shallow to deep water, and marine and freshwater (Rützler 2004). They are also structurally important components of coral reefs where they provide shelter for numerous organisms and contribute to reef regeneration and stabilisation (de Voogd et al. 2006). In addition to the above, they are one of the most important marine sources of promising pharmaceutical compounds (Faulkner 2002; Taylor et al. 2007).

Sponges are known to host large communities of microbial symbionts of known ecological and biotechnological importance (Freeman and Thacker 2011). They are also unique and, depending on the species, highly selective environments for microbes (Cleary et al. 2013, 2015; Freeman and Thacker 2011; Hentschel et al. 2006). The prokaryote metabolism requires nitrogen and carbon; sponges produce both, often in ample quantities, by releasing ammonia, as an end product of their metabolism, and carbohydrates and amino acids, as a consequence of phagocytosis (Hentschel et al. 2006). Microbial symbionts, in turn, provide sponges with important nutrients (Flatt et al. 2005), process metabolic waste, improve host defence and stabilise the host skeleton (Hentschel et al. 2006). Some sponge species also house specific microbial communities that may be similar over large geographical distances (Flatt et al. 2005).

Microbes inhabit the mesophyll-matrix of most desmosponges (Hentschel et al. 2003). The abundance and composition of sponge-associated microorganisms can vary greatly among sponge species, including those inhabiting the same habitat (Cleary et al. 2013, 2015; de Voogd et al. 2015). High microbial abundance (HMA) sponges can contain around 10^{10} bacterial cells g^{-1} wet weight of sponge (2 to 4 orders of magnitude higher than concentrations in sea water) (Hentschel et al. 2006), while low microbial abundance (LMA) sponges contain around 10^6 cells g^{-1} (similar to concentrations in sea water) (Kamke et al. 2010).

HMA sponges also tend to host more diverse bacterial communities including Proteobacteria, Chloroflexi, Acidobacteria, and Actinobacteria (Gloeckner et al. 2014). Previously, the determination of HMA or LMA status was made on the basis of electron microscopy and morphotype with HMA sponges containing more densely packed microbial communities and smaller canals and choanocyte chambers compared to LMA sponges (Schlappy et al. 2010; Vacelet and Donadey 1977). Gloeckner et al. (2014), however, showed that electron microscopy was not always sufficient to determine HMA or LMA status and that sponges existed with intermediate microbial abundances. They, therefore, suggested combining electron microscopy

with 16S rRNA gene sequence data. The latter may in fact be a better determinant given the sometimes ambiguous results obtained by Gloeckner et al. (2014).

In the present study, we compared the richness and composition of bacteria in three sponge species inhabiting open coastal habitat, sediment and seawater from a coral reef system in South East Misool, Raja Ampat, West Papua, Indonesia. Located on the northwestern tip of Papua, eastern Indonesia, the Raja Ampat region consists of nearly 1500 islands and is considered an area with a global priority for conservation (Roberts et al. 2002). It is among the most biodiverse regions on Earth possessing over 75% of the world's coral species and almost a thousand species of reef fish (e.g., Allen 2008; Allen and Erdmann 2009; Mangubhai et al. 2012). The reefs and mangrove systems in SE Misool encompass an area of outstanding marine biological diversity (e.g., Allen 2008; Becking et al. 2014; Mangubhai et al. 2012) and harbour some of the most pristine reefs in Indonesia (Grantham et al. 2013; Mangubhai et al. 2012). As a result, a Marine Protected Area of 343,200 ha was established in SE Misool in 2009 (KKPD Misool Timur-Selatan).

We mainly focused our analysis on bacterial communities of the LMA sponge *Stylissa carteri* (Dendy, 1889) and the HMA giant barrel sponge *Xestospongia testudinaria* (Lamarck, 1815) in addition to the sponge *Aaptosuberitoides* (Brøndsted, 1934). *Stylissa carteri* (Fig. 1) is a common Indo-Pacific bright orange flabelliform sponge that occurs from the Red Sea to Taiwan (de Voogd and Cleary 2008; Giles et al. 2015). Numerous bromopyrrole alkaloids with promising antiviral, antibacterial, and anti-cancer properties have been isolated from species belonging to the genus *Stylissa* (Ebada et al. 2015; Rohde et al. 2012).

Xestospongia testudinaria (Fig. 2) is one of the largest known sponges. It usually has an erect and barrel-shaped

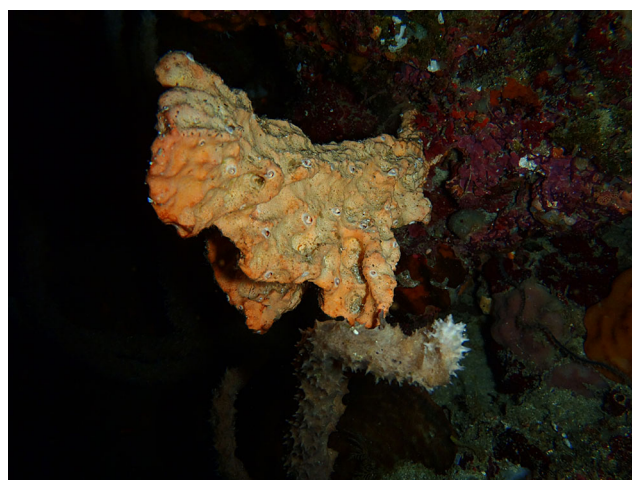


Fig. 1 Photograph of *Stylissa carteri* (Dendy, 1889) taken by LE Becking in Misool

structure that can measure up to 2.4 m in height and width. The surface texture varies from smooth to highly digitate or lamellate (Swierts et al. 2013). It also has an incredible life span that may exceed 2000 years (McMurray et al. 2008). It can be found from the Red Sea to the Great Barrier Reef (de Voogd et al. 2006; Moitinho-Silva et al. 2014; Pham et al. 1999) and can be locally abundant in coral reefs, usually at depths greater than 10 m, but can also occur at shallower depths in certain reefs (de Voogd and Cleary 2009; Huang et al. 2016; Swierts et al. 2013). *Xestospongia* species are among the richest sources of pharmacologically active chemical compounds isolated from marine organisms. *Xestospongia testudinaria* has been shown to produce compounds with potential applications in the treatment of obesity, diabetes mellitus, arteriosclerosis, and Alzheimer's disease (Akiyama et al. 2013; Liang et al. 2014).

Aptos suberitoides (Fig. 3) occurs in shallow coral reefs in the coral triangle (de Voogd and Cleary 2008). It forms thick irregular lobate masses that can occupy large parts of the reef. Its exterior is dark brown, but the interior is canary yellow and stains dark brown after preservation. It has been shown to produce compounds with antitumor, antimicrobial, and antiviral activity (Aoki et al. 2006; Jin et al. 2011; Larghi et al. 2008; Liu et al. 2012; Pham et al. 2013; Tsukamoto et al. 2010).

The main objectives of this study were to identify the most abundant higher bacterial taxa, compare bacterial richness and composition among sponge hosts and non-sponge biotopes (sediment and seawater), and identify dominant bacterial OTUs and their closest known relatives.



Fig. 2 Photograph of *Xestospongia testudinaria* (Lamarck, 1815) taken by LE Becking in Misool

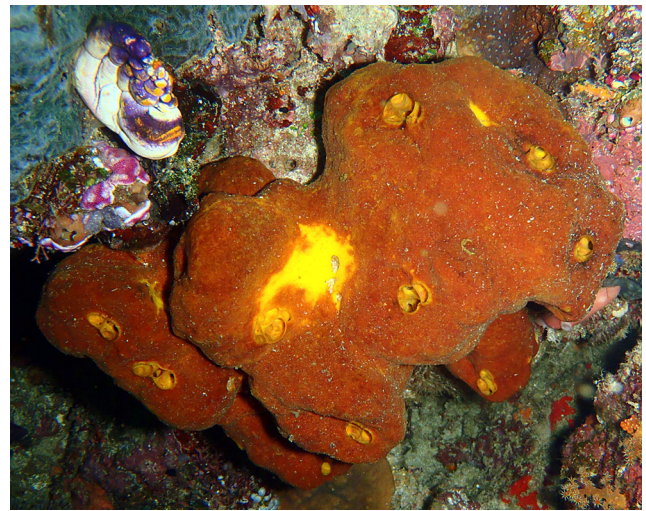


Fig. 3 Photograph of *Aptos suberitoides* (Brøndsted, 1934) taken by LE Becking in Misool

Material and methods

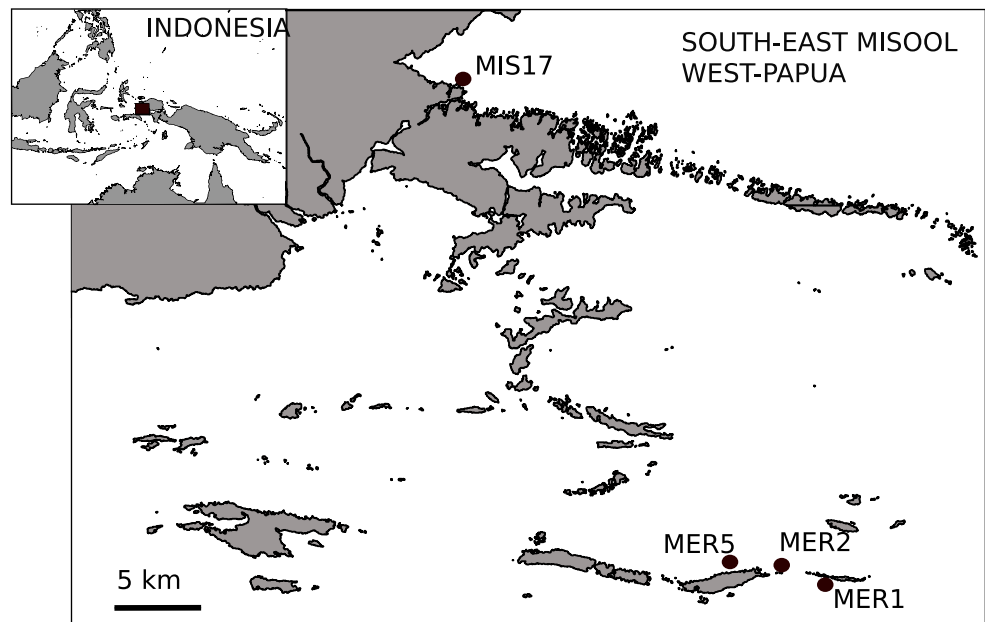
Study site

Samples of *S. carteri*, *A. suberitoides*, and *X. testudinaria* were collected by snorkelling and SCUBA diving from the 13th to the 18th of September 2013 in Southeast Misool, Raja Ampat region, West Papua province in Indonesia (Fig. 4). SE Misool is part of a marine protected area of 343,200 ha established in 2009 (KKPD Misool Timur-Selatan). The equatorial location of Misool means that the main seasonal influence is driven by monsoons (Prentice and Hope 2007). Misool is most influenced by the southeast monsoon from May to October, which is characterised by cooler sea surface temperatures (SSTs), persistent winds and strong ocean swell. The annual rainfall in Papua averages 2500–4500 mm with inter-annual variability in rainfall due to the El Niño Southern Oscillation (ENSO; Prentice and Hope 2007). There are seasonal differences in SSTs with an average SST of 29.0 °C, ranging from 19.3 to 36.0 °C (Mangubhai et al. 2012).

Data collection

In the study area, small fragments of the interior and exterior parts of the sponges *S. carteri* (Demospongiae: Scopalinida - Scopalinidae), *A. suberitoides* (Demospongiae: Suberitida: Suberitidae), and *X. testudinaria* (Demospongiae: Haplosclerida: Petrosiidae) were sampled in order to sample, as much as possible, the whole bacterial community. Specimens were collected from shallow water reefs (depth range: 7–17 m) and identified in the field. Voucher specimens of sponges have been deposited in the sponge collection of Naturalis Biodiversity Center, the Netherlands (RMNH Porifera). Sediment samples were taken using mini cores; this consisted of sampling the top 5 cm of sediment with a plastic disposable syringe from which

Fig. 4 Map of study area showing the location of the study sites



the end had been cut in order to facilitate sampling (Capone et al. 1992). Seawater samples were collected by filtering 1 L of seawater through a Millipore® White Isopore Membrane Filter (GTTP04700, 47 mm diameter, 0.22 μm pore size). Samples were stored in 96% EtOH. After sampling, tubes containing the samples were frozen or carried in ice during travel between field-work lodging and the Netherlands and Portugal, where the samples were stored at -80 . $^{\circ}\text{C}$ until processing. For the present study, three samples each of the sponges *S. carteri*, *A. suberitoides*, and *X. testudinaria*, sediment and seawater were assessed for bacterial community analysis.

DNA extraction and pyrosequencing

Total community-DNA extraction and 16S rRNA gene barcoded-pyrosequencing

We isolated PCR-ready total community DNA (TC-DNA) from sediment, seawater, and sponge samples using the FastDNA® SPIN Kit (MP Biomedicals) following the manufacturer's instructions. Briefly, we prepared sediment samples by centrifuging each one for 30 min at 4400 rpm and 4 $^{\circ}\text{C}$; the membrane filter (seawater sample) and sponge samples were each cut into small pieces. The whole membrane filter and 500 mg of sediment or sponge were transferred to Lysing Matrix E tubes containing a mixture of ceramic and silica particles. The microbial cell lysis was performed in the FastPrep® Instrument (Q Biogene) for 80 s at speed 6.0. Extracted DNA was eluted into DNase/Pyrogen-Free Water to a final volume of 50 μL and stored at -20 $^{\circ}\text{C}$ until use. Prior to pyrosequencing, the amplicons of the bacterial 16S rRNA gene were obtained using bacterial specific primers 27F and 1494R (Gomes et al. 2001). After a denaturation step at 94 $^{\circ}\text{C}$ for 5 min, 25 thermal cycles of 45 s at 94 $^{\circ}\text{C}$, 45 s at

56 $^{\circ}\text{C}$, and 1:30 min at 72 $^{\circ}\text{C}$ were carried out, followed by an extension step at 72 $^{\circ}\text{C}$ for 10 min. With the amplicons of the bacterial 16S rRNA gene as template, the V3V4 region was amplified using barcoded fusion primers with the Roche-454 A Titanium sequencing adapters, a six-base barcode sequence, forward V3 primer 5'-ACTCCTACGGGAGGCAG-3' (Yu et al. 2005), and V4 reverse degenerate primer 5'-TACNRRGTHCTAATYC-3' (Vaz-Moreira et al. 2011).

Sequence analyses were performed using previously described methods (Cleary et al. 2015; de Voogd et al. 2015). Briefly, in QIIME, fasta and qual files were used as input for the split_libraries.py script. OTUs were selected using UPARSE with usearch7 (Edgar 2013). Chimera checking was performed using the UCHIME algorithm, which is the fastest and most sensitive chimera checking algorithm currently available (Edgar et al. 2011). OTU clustering was performed using the cluster_otus command (cut-off threshold at 97%). (see Online Resource 1 for a detailed description). Closely related organisms of numerically abundant OTUs (≥ 200 sequences) were identified using the NCBI Basic Local Alignment Search Tool (BLAST) command line "blastn" tool with the -db argument set to nt (Zhang et al. 2000). The DNA sequences generated in this study can be downloaded from the NCBI SRA: SRP069346.

Phylogenetic tree

Selected 16S rRNA gene sequences of the most dominant OTUs and representative cultured and uncultured closest relatives in GenBank [<http://www.ncbi.nlm.nih.gov/>] were aligned using ClustalW and a phylogenetic analysis conducted using MEGA 6 software (<http://www.megasoftware.net/>; last checked 2014 07 09) (Tamura et al. 2013). A phylogenetic tree was constructed using the

neighbour-joining method and evolutionary distances were computed using the maximum composite likelihood method with a discrete Gamma distribution. In the results, we present a bootstrap consensus tree based on 1000 replicates. The bootstrap value is shown next to each branch when this exceeds 49%. This value represents the percentage of replicate trees in which the associated taxa clustered together.

Statistical analysis

A table containing the presence and abundance of all OTUs per sample was imported into R using the read.table() function. Plant organelles, mitochondria and sequences not classified as Bacteria (e.g., Archaea) were removed prior to statistical analysis.

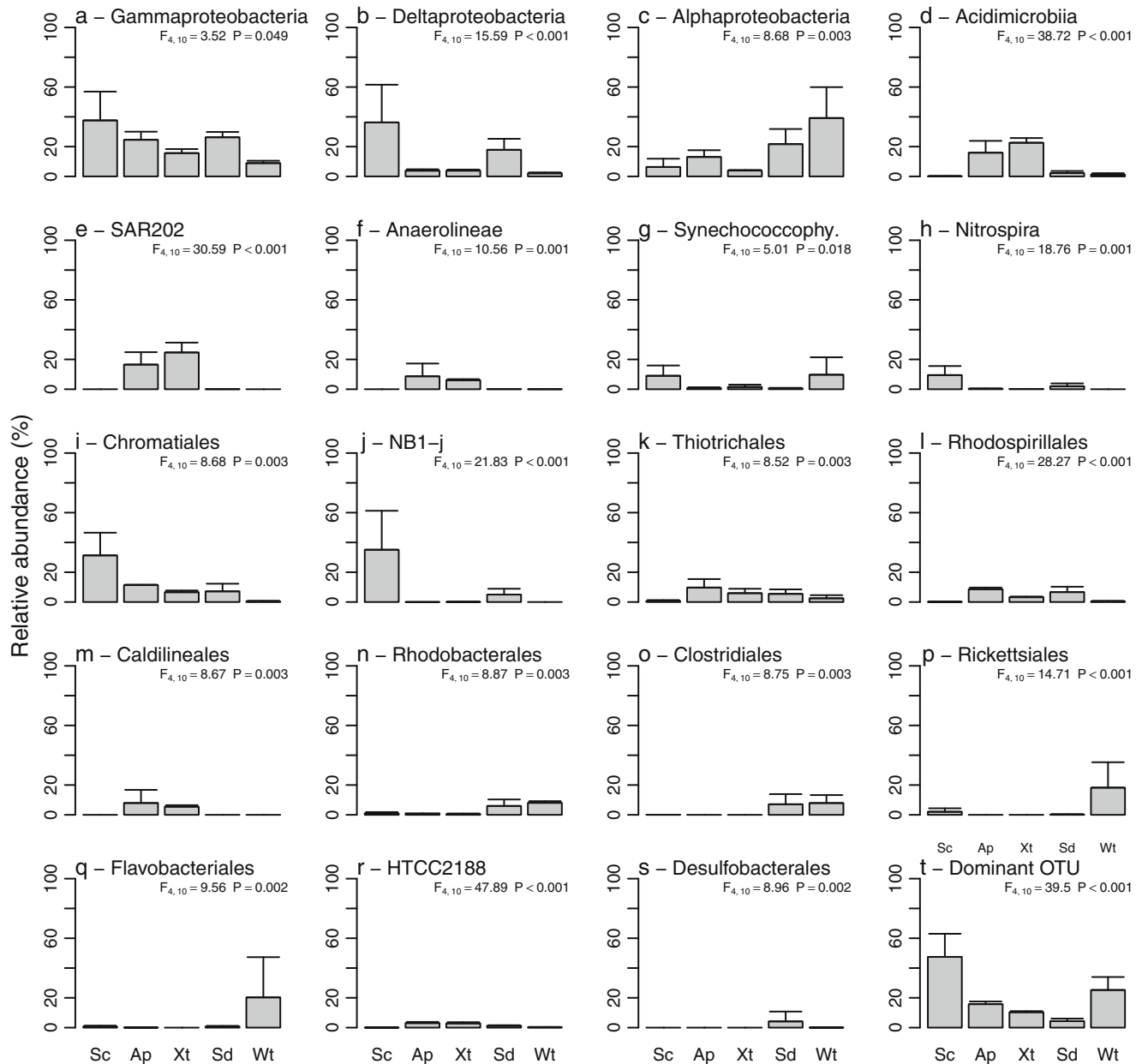


Fig. 5 Mean (error bars represent a single standard deviation) relative abundance of the most abundant bacterial classes and orders and the most abundant OTU (dominant OTU) for samples from *S. carteri* (Sc), *A. suberitoides* (Ap), *X. testudinaria* (Xt), sediment (Sd), and seawater (Wt). Note that the abundance of the dominant OTU refers to the abundance of the most abundant OTU per sample and thus not the most abundant OTU overall. a Gammaproteobacteria, b Deltaproteobacteria, c

Alphaproteobacteria, d Acidimicrobiia, e SAR202, f Anaerolineae, g Synechococrophy, h Nitrospira, i Chromatiales, j NB1-j, k Thiotrichales, l Rhodospirillales, m Caldilineales, n Rhodobacterales, o Clostridiales, p Rickettsiales, q Flavobacteriales, r HTCC2188, s Desulfobacteriales, and t the dominant OTU. Results of the GLM analyses for each taxon are presented in the top right of each subfigure

Richness and higher taxon abundance

We used a self-written function in R (Gomes et al. 2010) to estimate rarefied OTU richness for each sample. Care, however, should be taken in the interpretation of richness estimates based on sequence data given the prevalence of sequencing errors (Edgar 2013). We tested for significant differences in the relative abundance of selected higher taxon groups (classes and orders) among habitats with an analysis of deviance using the `glm()` function in R. As data were proportional, we first applied a GLM with the family argument set to binomial. However, the ratio of residual deviance to residual degrees of freedom in the models substantially exceeded one so we set family to “quasibinomial”. In the “quasibinomial” family, the dispersion parameter is not fixed at one so that it can model over-dispersion. Using the GLM model, we tested for significant variation among habitats using the `anova()` function in R with the F test, which is most appropriate when dispersion is estimated by moments as is the case with quasibinomial fits.

Composition

The OTU abundance matrix was $\log_e(x + 1)$ transformed (in order to normalise the distribution of the data), and a distance matrix was constructed using the Bray-Curtis index with the `vegdist()` function in the VEGAN package (Oksanen et al. 2009) in R. The Bray-Curtis index is one of the most frequently applied (dis)similarity indices used in ecology (Cleary 2003; Legendre and Gallagher 2001; Polónia et al. 2014). Variation in OTU composition among biotopes (*S. carteri*, *A. suberitoides*, *X. testudinaria*, sediment, and seawater) was assessed with principal coordinates analysis (PCO) using the `cmdscale()` function in R with the Bray-Curtis distance matrix as input. Variation among biotopes was tested for significance using the `adonis()` function in VEGAN. In the `adonis` analysis, the Bray-Curtis distance matrix of species composition was the response variable with the biotope as independent variable. The number of permutations was set at 999; all other arguments used the

default values set in the function. Weighted averages scores were computed for OTUs on the first four PCO axes using the `wascors()` function in the `vegan` package. Detailed descriptions of the functions used here can be found in R (e.g., `?cmdscale`) and online in reference manuals (<http://cran.r-project.org/web/packages/vegan/index.html>; 2015/05/29).

Results

The sequencing effort yielded 50,223 sequences, which were assigned to 3797 OTUs after quality control, OTU picking and removal of chimeras, chloroplasts, mitochondria, and sequences not assigned to the Bacteria domain. OTU richness was by far highest in the sediment biotope and lowest in *S. carteri* (Online Resource 2). The most abundant phylum overall was *Proteobacteria* where mean relative abundance ranged from $23.6 \pm 2.9\%$ in *X. testudinaria* to $80.3 \pm 1.8\%$ in *S. carteri* (Online Resource 3). Chloroflexi were most abundant in *A. suberitoides* ($30.5 \pm 10.6\%$) and *X. testudinaria* ($33.0 \pm 6.0\%$), but represented less than 1% of sequences in *S. carteri*, sediment, and seawater. The same held for Actinobacteria, which represented 16.0 ± 7.8 of *A. suberitoides* sequences and $22.7 \pm 3.2\%$ of *X. testudinaria* sequences. The mean abundance of Actinobacteria in the other biotopes ranged from 0.1 ± 0.1 in *S. carteri* to 2.4 ± 1.5 in sediment. Bacteroidetes were most abundant in sediment (8.2 ± 2.4) and seawater (20.8 ± 26.6) but represented less than 1% of sequences in all sponge biotopes. Although highly variable within biotope samples, Cyanobacteria abundance was highest in *S. carteri* (9.0 ± 6.9) and seawater (9.9 ± 11.9) and much lower in sediment (1.5 ± 0.1), *A. suberitoides* (0.7 ± 0.6), and *X. testudinaria* (1.5 ± 1.5).

Higher taxon abundance

In line with the phylum-level results, there were marked differences in the abundance of selected classes and orders

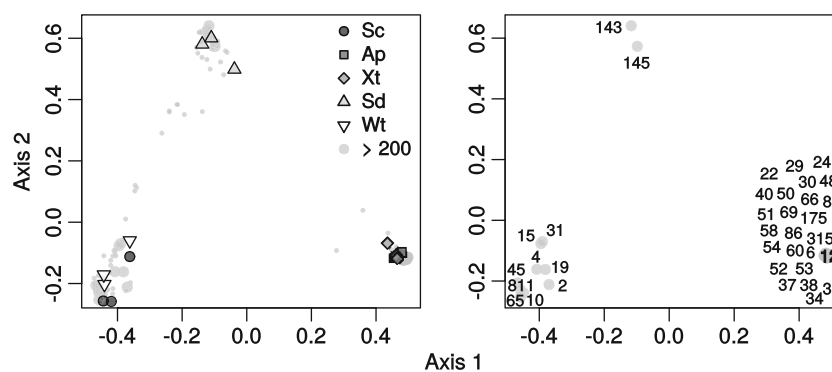


Fig. 6 Ordination showing the first two axes of the PCO analysis. a. Symbols represent samples from *S. carteri* (Sc), *A. suberitoides* (Ap), *X. testudinaria* (Xt), sediment (Sd), and seawater (Wt). b. Ordination

showing only the most abundant OTUs. Numbers represent dominant (≥ 200 sequence reads) OTUs referred to in Table 1. Small circles represent OTUs < 200 sequence reads

Table 1 List of abundant (≥ 200 sequence reads) OTUs and closely related organisms identified using BLAST search. **OTU**: OTU number; **Sum**: number of sequence reads; **Group**: biotope or biotopes where the OTUs were found; **GI**: GenInfo sequence identifiers of closely related organisms identified using BLAST; **Seq**: sequence similarity of these organisms with our representative OTU sequences; **Source**: isolation source of organisms identified using BLAST; **Location**: sampling location of organisms identified using BLAST

OTU	Sum	Group	Phylum	Class	Order	Family	Genus	GI	Seq	Source	Location
2	430	Gen	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	Synechococcus	28,557,443	99.76	Oligotrophic oceanic water	?
4	4110	Sc ^a	Proteobacteria	Deltaproteobacteria	NB1-j	NB1-i	Unassigned	407,912,992	99.54	Sponge: Stylissa carteri	Saudi Arabia
10	2742	Sc ^a	Proteobacteria	Gammaproteobacteria	Chromatiales	Unassigned	Unassigned	407,913,000	100	Sponge: Stylissa carteri	Saudi Arabia
12	2979	ApXt ^a	Actinobacteria	Acidimicrobia	Acidimicrobiales	wb1_P06	Unassigned	768,028,476	99.77	Coral: Porites lutea	China: Hainan
15	287	Wt	Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae	Unassigned	347,439,967	100	Seawater	China: Fujian
19	1145	Sc	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Unassigned	597,437,724	100	Sponge: Aximella sp	China
22	1497	ApXt	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Unassigned	511,630,206	99.55	Sponge: Vaceletia crypta	Australia:Great Barrier Reef
24	871	ApXt ^a	Actinobacteria	Acidimicrobia	Acidimicrobiales	TK06	Unassigned	768,028,613	99.53	Coral: Porites lutea	China: Hainan
29	1447	ApXt	Chloroflexi	SAR202	Unassigned	Unassigned	Unassigned	350,627,603	99.29	Sponge: Xestospongia testudinaria	Indonesia: Manado
30	642	Ap	Proteobacteria	Alphaproteobacteria	Unassigned	Unassigned	Unassigned	345,330,451	99.76	Sponge: Rhopaloeides odorabile	Australia: Great Barrier Reef
31	439	Wt	Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae	Unassigned	586,599,152	100	Shrimp pond	China: Fujian Province
34	488	ApXt ^a	Chloroflexi	TK17	mle1-48	Unassigned	Unassigned	526,300,040	98.82	Sponge: Aplysina cauliformis	Belize: Carrie Bow Cay
35	501	ApXt ^a	Chloroflexi	SAR202	Unassigned	Unassigned	Unassigned	678,605,861	99.76	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
37	616	ApXt ^a	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae	Unassigned	526,299,835	99.76	Sponge: Aplysina cauliformis	Belize: Carrie Bow Cay
38	504	ApXt	Acidobacteria	Acidobacteria-6	BPC015	Unassigned	Unassigned	451,354,030	99.33	Sponge: Ircinia strobilina	Bahamas:Sweeting's Cay
40	430	Xt ^a	Gemmatimonadetes	Gemm-4	Unassigned	Unassigned	Unassigned	400,269,352	99.55	Sponge: Xestospongia testudinaria	Australia: Great Barrier Reef
45	829	ScWt	Proteobacteria	Alphaproteobacteria	Rickettsiales	Pelagibacteraceae	Unassigned	732,664,604	100	Bottom seawater	Atlantic sedimentary abyssal plain
48	284	Xt ^a	Proteobacteria	Gammaproteobacteria	Thioirichales	Piscirickettsiaceae	Unassigned	350,627,620	99.78	Sponge: Xestospongia testudinaria	Indonesia: Manado
50	609	ApXt	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Unassigned	451,353,954	99.78	Sponge: Ircinia strobilina	Bahamas:Exumas
51	286	ApXt ^a	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Unassigned	451,354,037	99.76	Sponge: Ircinia felix	Bahamas:Exumas
52	484	ApXt	Proteobacteria	Gammaproteobacteria	Chromatiales	Unassigned	Unassigned	678,605,848	99.33	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
53	432	ApXt ^a	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Unassigned	678,605,876	99.53	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
54	288	Ap ^a	Gemmatimonadetes	Gemm-4	Unassigned	Unassigned	Unassigned	288,730,915	98.66	Sponge: Theonella swinhoi	China:South China Sea
58	512	ApXt ^a	Actinobacteria	Acidimicrobia	Acidimicrobiales	Unassigned	Unassigned	350,627,578	99.29	Sponge: Xestospongia testudinaria	Indonesia: Manado
60	317	ApXt	SBR1093	EC214	Unassigned	Unassigned	Unassigned	315,020,327	99.54	Sponge: Xestospongia testudinaria	Indonesia: Manado
65	273	Sc ^a	Proteobacteria	Gammaproteobacteria	Thiohalorhabdabales	Unassigned	Unassigned	407,912,993	97.91	Sponge: Stylissa carteri	Saudi Arabia
66	532	ApXt	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Unassigned	678,605,851	99.76	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
69	267	ApXt ^a	Chloroflexi	SAR202	Unassigned	Unassigned	Unassigned	350,627,635	99.53	Sponge: Xestospongia testudinaria	Indonesia: Manado

Table 1 (continued)

OTU	Sum	Group	Phylum	Class	Order	Family	Genus	GI	Seq	Source	Location
80	259	ApXt	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Unassigned	451,353,961	99.55	Sponge: <i>Ircinia felix</i>	Bahamas: Exumas
86	209	ApXt	Proteobacteria	Deltaproteobacteria	[Entotheonellales]	Unassigned	Unassigned	451,354,009	98.44	Sponge: <i>Ircinia strobilina</i>	Bahamas: Sweeting's Cay
143	248	Sd ^a	Proteobacteria	Gammaaproteobacteria	Thiotrichales	Thiotrichaceae	Unassigned	270,282,857	99.77	Marine sediments	Philippines: Bolinao
145	244	Sd	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unassigned	576,235,568	100	Sponge: <i>Rhabdastrella globostellata</i>	Guam
175	289	ApXt ^a	Chloroflexi	SAR202	Unassigned	Unassigned	Unassigned	345,330,250	99.53	Sponge: Rhopaloeides odorabile	Australia: Great Barrier Reef
658	376	Xt ^a	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae	Unassigned	350,627,590	99.76	Sponge: <i>Xestospongia testudinaria</i>	Indonesia: Manado
811	748	ScWt	Cyanobacteria	Synechococophycideae	Synechococales	Synechococcaceae	Synechococcus	700,288,666	100	Saline lake water	Croatia
3153	250	ApXt	Actinobacteria	Acidimicrobia	Acidimicrobiales	wb1_P06	Unassigned	226,425,311	99.53	Sponge: <i>Xestospongia muta</i>	USA: Key Largo, Florida

^a restricted to group

(Fig. 5). Sequences assigned to the Chloroflexi classes SAR202 and Anaerolineae and the order Caldilineales were largely restricted to *A. suberitoides* and *X. testudinaria*. The abundance of Gammaproteobacteria was greatest in *S. carteri*, largely due to the prevalence of OTUs assigned to the order Chromatiales. The class Deltaproteobacteria was most abundant in *S. carteri* and sediment, largely due to the order NB1 – j in both biotopes and Desulfobacterales in sediment. Dominance was by far greatest in *S. carteri* with a mean of $47.4\% \pm 15.5$ of sequences assigned to the most abundant OTU. In contrast, dominance was lowest in sediment with a mean of only $4.4\% \pm 1.7$ of sequences assigned to the most abundant OTU.

Importance of biotopes in structuring composition

There was a highly significant difference in bacterial composition among biotopes ($F_{3,8} = 13.07$, $P < 0.001$, $R^2 = 0.839$). Variation among biotopes thus explained almost 84% of the variation in composition. In the PCO ordination of the first two axes (Fig. 6), there are three distinct clusters, namely: a cluster of samples from *A. suberitoides* and *X. testudinaria*, a cluster of samples from sediment and a cluster of samples from *S. carteri* and seawater. The main axis (axis 1) separated samples of *A. suberitoides* and *X. testudinaria* from samples of *S. carteri* and seawater. The second axis (axis 2) separated all these samples from samples of sediment. For the purposes of this study, a total of 36 OTUs were considered abundant (≥ 200 sequences). Only two abundant OTUs were associated with sediment. OTU-143 was restricted to sediment samples and closely related ($>99\%$ sequence similarity) to an organism obtained from marine sediment in the Philippines (Table 1). OTU-145 was mainly found in sediment in this study, but had 100% sequence similarity with an organism previously obtained from the sponge *Rhabdastrella globostellata* in Guam. A number of OTUs were mainly found in seawater and *S. carteri*. This included OTU-45 that was related to an organism previously found in bottom seawater of the Atlantic abyss and OTU-811 that was related to an organism previously found in Croatian marine lake water. Most of the abundant OTUs were associated with *A. suberitoides* and *X. testudinaria* and were often absent in other biotopes. This mainly included OTUs previously isolated from sponges and corals (e.g., OTUs 12 and 24). Most of these OTUs were shared between both sponge species although one (OTU-54) was restricted to *A. suberitoides* and three to *X. testudinaria* (OTUs 45, 48 and 658). OTUs 40 (restricted to *X. testudinaria*) and 54 (restricted to *A. suberitoides*) were both assigned to the phylum Gemmatimonadetes.

The third PCO axis mainly separated *S. carteri* samples from seawater samples. OTUs associated with and restricted to *S. carteri* included OTUs 4, 10, and 65 all of which were related to organisms previously obtained from *S. carteri* in the

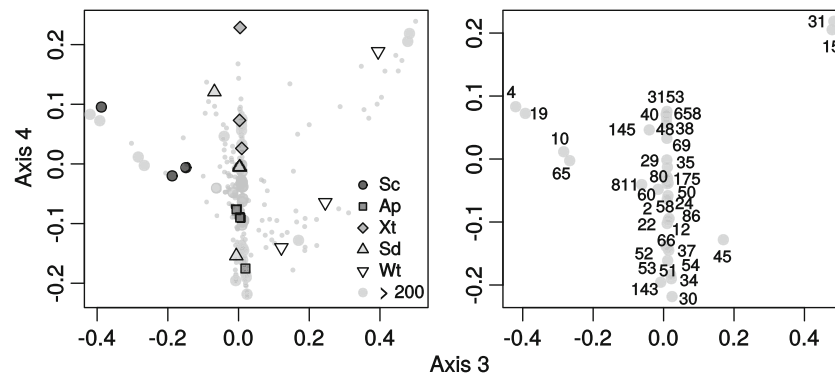


Fig. 7 Ordination showing the third and fourth axes of the PCO analysis. a. Symbols represent samples from *S. carteri* (Sc), *A. suberitoides* (Ap), *X. testudinaria* (Xt), sediment (Sd), and seawater (Wt). b. Ordination

showing only the most abundant OTUs. Numbers represent dominant (≥ 200 sequence reads) OTUs referred to in Table 1. Small circles represent OTUs <200 sequence reads

Red Sea (Fig. 7 and Table 1). OTUs 15 and 31, which were associated with seawater samples, were related to organisms previously obtained from shrimp pond and seawater samples in China. The fourth axis mainly separated samples of *A. suberitoides* and *X. testudinaria*.

In general, most of the sponge OTUs analysed here were either detected in more than one sponge biotope or were closely related to bacterial phylotypes associated with different sponge host species (Table 1 and Fig. 8). For example, *A. suberitoides* and *X. testudinaria* shared dominant bacterial symbionts (ApXt in Table 1) closely related to bacterial phylotypes previously identified in a range of sponge hosts (*Xestospongia muta*, *Aplysina cauliformis*, *Ircinia* spp., *Rhopaloeides odorabile*, *Astrosclera willeyana*, and *Vaceletia crypta*) in different geographical locations. Most abundant sponge OTUs were closely related to sponge bacterial symbionts from other studies from different sponge species. However, a few abundant OTUs, selectively enriched in *X. testudinaria* or *S. carteri* (OTUs 4, 10, 48, 65, and 658), may represent sponge species specific associations.

Discussion

In line with previous studies (Cleary et al. 2015; de Voogd et al. 2015), the sediment biotope proved to be the richest. OTUs in sediment were mainly assigned to the Proteobacteria, but there were substantial numbers of OTUs assigned to other taxa including Actinobacteria, Bacteroidetes, and Acidobacteria, among others. OTUs assigned to the proteobacterial order Desulfobacterales were also restricted to sediment. The Desulfobacterales order consists of anaerobic bacteria known to use sulphate as terminal electron acceptor in order to oxidate H_2 and a wide range of organic compounds. They also have been shown to play an important role in degrading organic contaminants and appear to be sensitive to predicted shifts in ocean pH (Coelho et al. 2015; Muyzer and Stams 2008; Zhou et al. 2011).

Proteobacteria also proved to be the most abundant phylum in *S. carteri* and in two of the three seawater and *A. suberitoides* samples. In contrast, Chloroflexi and Actinobacteria were more prevalent in samples of *X. testudinaria*. The dominance of Proteobacteria in *S. carteri* is in line with previous studies of this species in a different region within Indonesia (Cleary et al. 2015) and of the closely related *S. massa* in the Jakarta Bay-Thousand Islands reef complex (de Voogd et al. 2015). LMA sponges, such as *S. carteri*, have been shown to host bacterial communities with limited phylum-level diversity when compared to HMA species. They are also known to filter large volumes of water, which can explain the presence of large numbers of OTUs found in water and the higher similarity between seawater and *S. carteri* bacterial communities (Giles et al. 2013; Weisz et al. 2008).

The prevalence of Chloroflexi in *X. testudinaria* in the present study agrees with previous studies of the species in other regions including the Red Sea, Celebes Sea, and Great Barrier Reef where Acidobacteria, Actinobacteria and particularly Chloroflexi were the most abundant phyla in terms of OTUs and sequences (Lee et al. 2011; Montalvo et al. 2014; Montalvo and Hill 2011). It, however, contrasts with two of our previous studies (Cleary et al. 2015; de Voogd et al. 2015) where Proteobacteria were more abundant in terms of OTUs and sequence reads although both of these studies also contained a large number of OTUs assigned to the Chloroflexi. Previous studies have also identified Chloroflexi as a consistent component of the bacterial communities of HMA sponges (Gloeckner et al. 2014; Schmitt et al. 2011).

In the present study, OTUs assigned to the Chloroflexi were mainly assigned to the orders SAR202 and Anaerolineae. Members of the SAR202 have been found to be relatively abundant in bathypelagic waters (Varela et al. 2008). Anaerolineae members have been found in a wide range of habitats from arctic permafrost to tropical marine sediment and the mammalian gastrointestinal tract (Campbell et al. 2014; Hug et al. 2013). Campbell et al. (2014) proposed that Anaerolineae in humans occupy an ecological niche where they

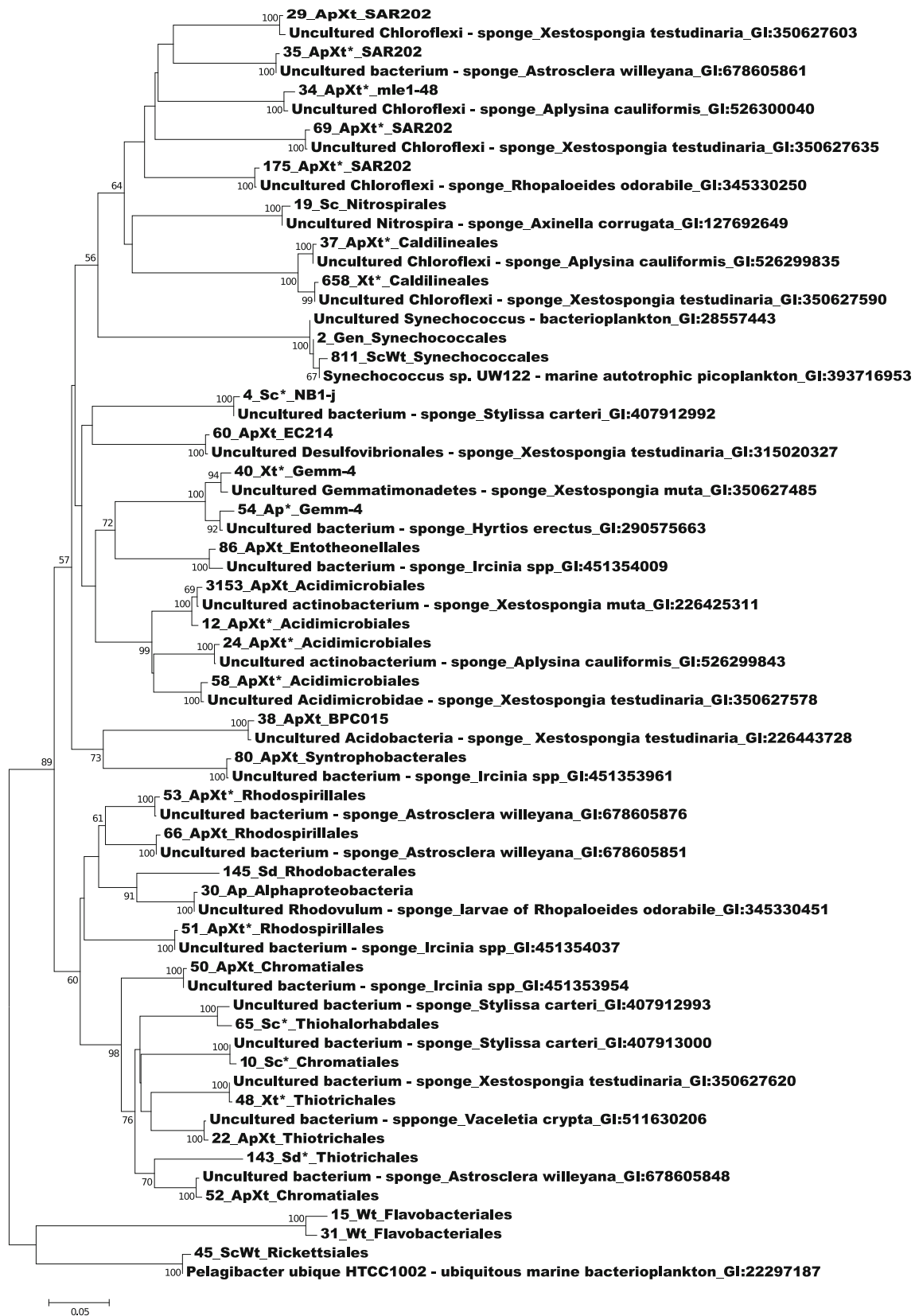


Fig. 8 Phylogenetic tree of the bacterial 16S rRNA gene sequences recovered from *S. carteri*, *A. suberitoides*, *X. testudinaria*, sediment, and seawater from the Misool coral reef system. Bootstrap values lower

than 50% were omitted. The number of each OTU is indicated as are GenInfo sequence identifiers of sequences obtained using BLAST

scavenge material from lysed bacterial cells and human tissue. In sponges, they may occupy a similar niche whereby they exploit the high loss of sponge biomass due to rapid sponge cell turnover (de Goeij et al. 2013).

Seawater samples mainly consisted of OTUs assigned to Proteobacteria, Bacteroidetes, and Cyanobacteria. Alphaproteobacteria were most abundant in seawater samples and mainly consisted of OTUs assigned to the orders Rickettsiales and Rhodobacterales. The order Flavobacteriales was also mainly found in seawater samples. This result follows numerous studies that have found a prevalence of Proteobacteria (mainly Alphaproteobacteria) and Bacteroidetes in the bacterioplankton (Glöckner et al. 1999).

S. carteri and seawater samples clustered together in the PCO ordination as did *A. suberitoides* and *X. testudinaria* samples. With respect to *S. carteri* and seawater, this confirms previous studies highlighting the similarity of LMA sponges and seawater (Giles et al. 2013; Weisz et al. 2008). However, it should be noted that, although similar, samples of *S. carteri* were still distinct from seawater samples as seen in the ordination of the third and fourth axes. *Stylyssa carteri* also contained highly abundant OTUs that were either much more abundant in or restricted to *S. carteri*. These OTUs were also closely related to organisms previously obtained from *S. carteri* samples in Saudi Arabia or *Axinella*, which were previously placed in the same order (Halichondrida).

The similarity in bacterial symbiont composition between *A. suberitoides* and *X. testudinaria* is interesting. These species are otherwise very different, e.g., their outer morphology, skeletal architecture and phylogenetic relationship. For instance, *A. suberitoides* has a massive to lobate growth form, whereas *X. testudinaria* is barrel shaped. However, the skeleton of both sponge species, is composed of high densities of silicious spicules, albeit of different size dimensions and morphologies. The similarity in the composition of their bacterial symbionts is thus in line with the theory that the internal sponge morphology is an important determinant whether a sponge hosts a HMA- or LMA-type bacterial community (Glöckner et al. 2014; Vacelet and Donadey 1977; Weisz et al. 2008).

This is, to the best of our knowledge, the first study of the bacterial community of *A. suberitoides*, a relatively abundant, widespread and easily recognisable sponge species. Recently, the higher classification of sponges has been altered and some sponge orders have been abandoned (Morrow and Cárdenas 2015). Previously, the genus *Aptos* (order Suberitida, family Suberitidae) was placed within the order Hadromerida, which contained HMA sponges, such as *Sphaciospongia vesparium* (currently order Clionaida; family Clionaidae) and LMA sponges such as *Suberites domuncula* (currently order Suberitida, family Suberitidae) and *Suberites diversicolor* (Cleary et al. 2013). Glöckner et al. (2014) previously showed that although certain sponge taxa such as the orders

Agelasida and Verongida only consisted of HMA species and the Poecilosclerida of LMA species, other taxa such as the Haplosclerida, Homoscleromorpha, and Dictyoceratida included both LMA and HMA species.

In conclusion, the present study confirms that sponges harbour microbial communities that are distinct from communities in sediment and the surrounding seawater. This finding extends to LMA sponges as demonstrated by *S. carteri* in this study. Although the bacterial communities of *S. carteri* and seawater were similar, they were still distinct with *S. carteri* hosting a small number of highly abundant OTUs including OTUs restricted to this sponge. Our study also showed that the hitherto unknown bacterial community of *A. suberitoides* is very similar to the known HMA sponge *X. testudinaria* providing evidence that *A. suberitoides* might also be a HMA sponge. Although both *A. suberitoides* and *X. testudinaria* shared numerous OTUs, they also hosted what may be species-specific OTUs.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

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