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Composition and Predictive Functional Analysis of Bacterial Communities in Seawater, Sediment and Sponges in the Spermonde Archipelago, Indonesia

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Abstract In this study, we used a 16S rRNA gene barcoded pyrosequencing approach to sample bacterial communities from six biotopes, namely, seawater, sediment and four sponge species (*Stylissa carteri*, *Stylissa massa*, *Xestospongia testudinaria* and *Hyrtios erectus*) inhabiting coral reefs of the Spermonde Archipelago, South Sulawesi, Indonesia. Samples were collected along a pronounced onshore to offshore environmental gradient. Our goals were to (1) compare higher taxon abundance among biotopes, (2) test to what extent variation in bacterial composition can be explained by the biotope versus environment, (3) identify dominant (>300 sequences) bacterial operational taxonomic units (OTUs) and their closest known relatives and (4) assign putative functions to the sponge bacterial communities using a recently developed predictive metagenomic approach. We observed marked differences in bacterial composition and the relative abundance of the most abundant phyla, classes and orders among sponge species, seawater and sediment. Although all biotopes housed compositionally distinct bacterial communities, there were three prominent clusters. These included (1) both *Stylissa* species and seawater, (2) *X. testudinaria* and *H. erectus* and (3) sediment. Bacterial communities sampled from the same biotope, but different environments (based on proximity to the coast) were much more similar than bacterial communities from different biotopes in the same environment. The biotope thus appears to be a much more important structuring force than the surrounding environment. There were concomitant differences in the predicted counts of KEGG orthologs (KOs) suggesting that bacterial communities housed in different sponge species, sediment and seawater perform distinct functions. In particular, the bacterial communities of both *Stylissa* species were predicted to be enriched for KOs related to chemotaxis, nitrification and denitrification whereas bacterial communities in *X. testudinaria* and *H. erectus* were predicted to be enriched for KOs related to the toxin–antitoxin (TA) system, nutrient starvation and heavy metal export.

Keywords 16S rRNA gene · KEGG orthologs · Makassar · Ordination · Pyrosequencing

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Introduction

Coastal marine ecosystems influence climate, nutrient cycling and primary productivity on a global scale [1]. Despite the acknowledged importance of these ecosystems, they have been severely affected by anthropogenic disturbances. This is particularly the case with coral reef ecosystems that have been adversely affected by a number of disturbances including local perturbations such as overfishing, eutrophication and heavy metal pollution [2–4] and global disturbances related to warming such as coral bleaching [3–6]. The intensity of these disturbances is predicted to increase over the coming decades [7, 8].

Microbes play key roles in the functioning of coral reef ecosystems [9]. Relatively little research has, however, focused on microbial communities in coral reefs when compared to other taxa such as corals or fish. In coral reef ecosystems, microbes can be found in the plankton and sediment but are also important symbionts in higher taxa such as corals and sponges. Here, we studied communities of bacteria in six coral reef biotopes in the Spermonde Archipelago, a coral reef system off the coast of Makassar, Indonesia, and located in an area known as the coral triangle. These included two non-host biotopes namely sediment and seawater and four host biotopes namely the sponge species *Stylissa carteri* and *Stylissa massa* (order Halichondrida: family Dictyonellidae), *Xestospongia testudinaria* (order Haplosclerida: family Petrosiidae) and *Hyrtios erectus* (order Dictyoceratida: family Thorectidae). Sponges are both abundant and ecologically important in coral reef ecosystems [10]. They also harbour very high microbial densities; high microbial abundance (HMA) sponges can contain 10^{10} bacterial cells per gram wet weight of sponge. This is orders of magnitude higher than the surrounding seawater [11–15]. In most cases, bacteria make up the lion's share of prokaryotic diversity [15–17]. There has been a recent surge in studies of bacteria and their functions in a number of biotopes including sponges [18–20]. At present, however, relatively little is known about the functions of sponges and their bacterial symbionts in the reefs of the coral triangle, which contains the most diverse coral reefs in the world [21]. It is important, however, to have some idea of how sponges may affect the coral reef environment given that they are predicted to increase in abundance in the future [22, 23].

Unfortunately, very few bacterial symbionts of sponges have been cultured. It is, therefore, difficult to identify the functions of the majority of sponge-associated symbionts [24]. Recent advances in 'omics' techniques such as metatranscriptomics [18] and proxy techniques including predictive analysis using marker genes, however, now enable predictions of metagenomic functional content. In the present paper, we use a recently developed bioinformatic tool, PICRUSt, that enables us to both predict gene enrichment and identify the taxonomy of bacteria carrying these genes [25].

Few studies have assessed the composition and functions of bacteria in multiple coral reef biotopes, particularly in the coral triangle. Our main goals with this study were to (1) compare higher taxon abundance among biotopes, (2) assess to what extent the biotope and environment (sampling zone) influence composition, (3) assess if different biotopes harbour functionally distinct bacterial communities and (4) assign putative functions to the bacterial communities of different sponge species.

Material and Methods

Study Site

All sampling took place in the Spermonde Archipelago, South Sulawesi, Indonesia, which consists of 160 fringing, barrier and patch reefs [26]. The Spermonde is situated adjacent to the city of Makassar, a city with more than two million inhabitants [27]. Previous studies have shown a pronounced onshore to offshore gradient in environmental conditions related to anthropogenic disturbances and river discharge including sedimentation, agricultural runoff, oil spills, destructive fisheries, tourism and coral mining [28–30].

Sampling

Sediment, seawater and four sponge species were collected from reefs in the Spermonde Archipelago (Lae Lae, Samalona, Kudingkareng Keke, Bone Baku and Langkai) using SCUBA in August 2012 (Fig. 1). The Spermonde is a well-documented carbonate coastal shelf subject to several environmental influences along an onshore to offshore gradient. The environmental influences include sewage and other forms of pollution from the city of Makassar and fluvial discharge and erosion products from the Jene Berang River that transverses Makassar [31]. Previous studies have divided the Spermonde into four zones that run parallel to the coast. These zones were based on geomorphology, reef development, geology, shelf depth and offshore distance [32, 33]. The innermost zone, zone 1, is approximately bounded by the 20-m isobath and mainly consists of cay-crowned reefs. Visibility is limited in zone 1, and salinity is lower and nutrient, silt and sand content higher than the other zones. This zone is most under influence of land-based pollution. The sample site Lae Lae was sampled in zone 1. Nutrient levels in the other zones are comparable and exhibit minor fluctuations throughout the year [34]. Zone 2 begins >4 km offshore, mainly consists of reefs crowned with islets, and maximum depth is approximately 30 m. The sites Samalona and Bone Baku were sampled in zone 2. Zone 3 begins 12.5 km offshore, mainly consists of submarine shoals with few emerging cays, and maximum depth ranges from 30 to 50 m depending on the reef. The site Kudingareng Keke was sampled in zone 3. Zone 4 consists of the outer rim of the reef system and starts approximately 30 km offshore. Maximum depth ranges from 40 to 50 m on the eastern side and beyond 100 m on the westward drop. The reefs of zone 4 form a barrier-type reef crowned by some islets. The site Langkai was sampled in zone 4.

At each site in each zone (Lae Lae, Samalona, Kudingareng Keke), one sample of each biotope (sediment, seawater, *S. carteri*, *S. massa*, *X. testudinaria*, *H. erectus*) was

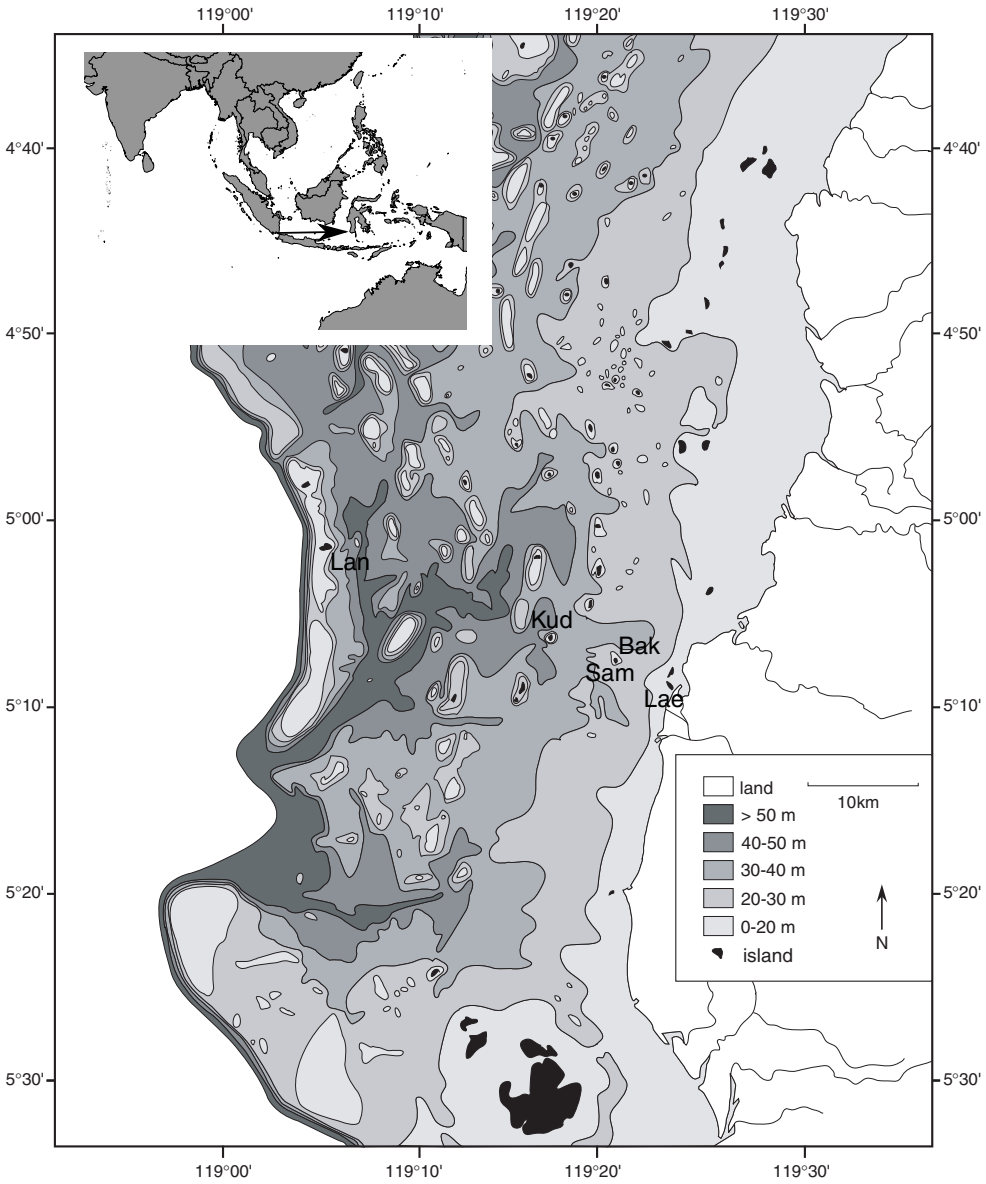

Fig. 1 Bathymetric map of the Spermonde Coral Reef System showing the location of the sample sites: Lae (Lae Lae), Sam (Samalona), Bak (Bone Baku), Kud (Kudingareng Keke) and Lan (Langkai). The *inset in the upper left corner* shows the location of the Spermonde Archipelago in Southeast Asia

taken. Sediment, seawater, *S. massa*, *X. testudinaria* and *H. erectus* were sampled from Lae Lae, Samalona, Kudingkareng Keke and Langkai. Unfortunately, *S. carteri* was not present in two of these sites (Lae Lae and Langkai). We, therefore, sampled additional specimens of *S. massa* from Kudingareng Keke and another site, Bone Baku. Sediment was sampled using the mini core method as previously described [35, 36]. Seawater was sampled by filtering approximately 1 l [37, 38] of seawater (collected between 1 and 3 m depth) through a Millipore® White Isopore Membrane Filter (0.22-μm pore size). Sponges were sampled including fragments of surface and interior following previously described methods [36]. All samples were stored in 96 % EtOH [39, 40] and kept cool (<4 °C) after collection and during transport. In the laboratory, samples were stored at −20 °C until DNA extraction.

DNA Extraction and Pyrosequencing

PCR-ready genomic DNA was isolated from seawater, sediment and sponge samples with FastDNA® SPIN Kit (MP Biomedicals) following the manufacturer's instructions. This is an extraction method frequently used for this purpose [36, 41, 42]. Briefly, 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 °C until use. Prior to pyrosequencing, the amplicons of the bacterial 16S ribosomal RNA (rRNA) gene were obtained using bacterial specific primers 27F and 1494R [43]. After a denaturation step at 94 °C for 5 min, 25 thermal

cycles of 1 min at 94 °C, 1 min at 56 °C and 2 min at 68 °C were carried out followed by an extension step at 68 °C for 10 min. Using 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′ [44] and V4 reverse degenerate primer 5′-TACNVRRGTHTCTAATYC-3′ (Ribosomal Database Project [RDP], Release 10, Update 20, http://rdp.cme.msu.edu/; last checked 06 April 2015). Sequence analysis was performed using previously described methods ([36, 39, 45]; see Online Resource 1 for a detailed description). Briefly, barcoded pyrosequencing libraries were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) software package ([46]; http://www.qiime.org/; last checked 20 Jan 2014) on a computer running the BioLinux 7 operating system (http://nebc.nerc.ac.uk/; checked 02 June 2015). In QIIME, fasta and qual files were used as input for the split_libraries.py script. OTUs were selected using UPARSE with usearch7 [47]. Chimera checking was performed using the UCHIME algorithm, which is the fastest and most sensitive chimera checking algorithm currently available [48]. OTU clustering was performed using the -cluster_otus command (cut-off threshold at 97 %). The DNA sequences generated in this study can be downloaded from the NCBI SRA: SRP047468.

BLAST, Phylogenetic and Predictive Metagenome Analysis

Closely related organisms to numerically dominant OTUs (>300 sequences) were identified using the NCBI Basic Local Alignment Search Tool (BLAST) command line 'blastn' tool with the -db argument set to nt [49]. We used PICRUSt, a bioinformatics tool that uses marker genes, in this case 16S rRNA, to predict metagenome gene functional content. A detailed description of these methods has been published previously [25, 36] and can be found in the supplementary methods (Online Resource 1). In the present study, we used the KEGG database and focused on a selected set of KEGG orthologs (KOs). In the KEGG database, KOs are sets of homologous sequences, from a large array of organisms, that have been assigned a specific molecular function. KOs are in turn arranged hierarchically and grouped into biological pathways. Note that because of functional overlap, some KOs can be represented in more than one pathway. We used R to generate bar graphs showing the estimated number of genes for selected KOs (K00087, K00575, K00673, K00991, K01076, K01426, K01770, K03409, K03696, K04517, K04561, K05522, K05982, K06200, K07239, K07334, K07658, K07665, K10535 and K12339; the selection of KOs was based on a preliminary analysis of KO variation among biotopes) for each sample and the contribution of selected taxonomic orders; the latter was obtained using the metagenome_contributions.py script in PICRUSt. Note that the PICRUSt results as presented are predictive and thus provide information on potential enrichment and putative function as opposed to measuring actual gene presence/expression and function.

Higher Taxon Abundance

We tested for significant differences in the relative abundance of selected higher taxa (classes and orders) and dominance (the relative abundance of the most abundant OTU in each sample) among biotopes with an analysis of deviance using the glm() function in R [50]. Because the data was proportional, we first applied a glm with the family argument set to binomial. The ratio, however, of residual deviance to residual d.f. in the models substantially exceeded 1, so we set family to 'quasibinomial'. In the quasibinomial family, the dispersion parameter is not fixed at one so that it can model overdispersion. Using the glm model, we tested for significant variation among biotopes 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. 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; accessed 27 Feb 2015).

Composition

Two tables containing (1) the presence and abundance of all OTUs per sample and (2) a table of predicted KO counts were imported into R using the read.Table() function. For the OTU table, OTUs with <20 sequences, not classified as bacteria or classified as chloroplasts and mitochondria, were removed prior to statistical analysis. Both tables were $\log_{10}(x+1)$ transformed (in order to normalise the distribution of data) and distance matrices constructed using the Bray–Curtis index with the vegdist() function in the vegan package [51] in R. The Bray–Curtis index is one of the most frequently applied (dis)similarity indices used in ecology [52, 53]. Variation in OTU and KO composition among biotopes (sediment, seawater, *S. massa*, *S. carteri*, *X. testudinaria* and *H. erectus*) was assessed with principal coordinate analysis (PCO) using the cmdscale() function in R with the Bray–Curtis distance matrix as input. Variations among biotopes and reef zones (pooling samples from the same zone but different biotopes) were tested separately 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 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

and KOs on the first two PCO axes using the wascores() function in the vegan package.

Results

Sequencing yielded 76,510 sequences assigned to 4141 OTUs after quality control, OTU picking and removal of chimera. All OTUs were assigned to 44 phyla, 101 classes and 124 orders. Most (57,409) sequences were assigned to the *Proteobacteria* followed by the *Bacteroidetes* (3318 sequences) and *Nitrospirae* (3115 sequences) (Online Resource 2).

Higher Taxon Abundance

Proteobacteria was the dominant abundant phylum in all biotopes but was particularly abundant in both *Stylissa* species (Online Resource 2). There were highly significant differences in the relative abundance of selected classes and orders among biotopes (Fig. 2). OTUs assiged to the Entotheonellales, for example, were mainly restricted to *X. testudinaria* and H. *erectus* whereas OTUs assiged to the NB1-j were most abundant in both *Stylissa* species. Dominance was most pronounced in both *Stylissa* species, particularly in *S. carteri*, and was least pronounced in sediment. At the phylum level, the main effect was a marked increase in the abundance of *Bacteroidetes* in seawater and *H. erectus* in the inshore site (Lae Lae). This effect, however, was not apparent in sediment or other sponge taxa (Online Resource 2).

OTU Composition

The two, by far, most abundant OTUs (OTUs 1 and 2) were related to organisms previously obtained from *S. carteri* sampled in Saudi Arabia (Table 1). Both of these OTUs (and the alphaproteobacterium OTU-11) were restricted to both *Stylissa* hosts and were absent in all other biotopes. In addition to the above, OTU-12 was restricted to *S. massa*. A large number of OTUs were restricted to *X. testudinaria* and *H. erectus* (e.g., OTUs 4, 8, 9, 13 and 16) including OTUs such as OTU-17 that was restricted to *X. testudinaria* and OTU-14 that was restricted to *H. erectus*. A number of OTUs were more abundant in seawater (e.g., OTUs 3, 15, 51, 58) or sediment (e.g., OTUs 44 and 741), but these organisms were also found in sponges albeit in low abundances. Most of the abundant OTUs were closely related to organisms previously isolated from other sponges (e.g., OTUs 1, 2, 4, 5, 7, 8, 9 and 10; Table 1 and Online Resource 3). Phylogenetic trees of the most numerically dominant OTUs and selected cultured organisms are presented in Online Resources 4 and 5.

There was a highly significant difference in composition among biotopes (all biotopes: $F_{5,18}$=25.64, P<0.001, R^2= 0.877; excluding *S. massa*: $F_{4,15}$=29.97, P<0.001, R^2= 0.889). Variation among biotopes thus explained >87 % of the variation in bacterial composition. In contrast, there was no significant difference among zones when pooling samples according to zone ($F_{3,16}$=0.12, P=0.999, R^2=0.022). A PCO ordination (Fig. 3) of the first two axes revealed three distinct clusters representing samples from the six biotopes. One cluster consisted of samples from seawater and both *Stylissa* hosts, another cluster consisted of samples from sediment, and the last cluster consisted of samples from *X. testudinaria* and *H. erectus*. The first PCO axis separated samples from seawater and both *Stylissa* hosts from samples of *X. testudinaria* and *H. erectus*. The second PCO axis separated sediment samples from all other samples. Most OTUs were restricted to or showed a pronounced preference for specific biotopes as evidenced by the distribution of OTUs in Fig. 3. Including all OTUs (thus, also OTUs <20 sequences), only 1 OTU (OTU-45, family *Rhodobacteraceae*) out of 4141 OTUs was found in all six biotopes and only 6 were found in five biotopes. In contrast, more than 90 % of OTUs (3749) were only found in a single biotope.

Predictive Metagenome Analysis

Mean (and standard deviation) Nearest Sequenced Taxon Index (NSTI) values for the biotopes sampled in Makassar were 0.220 (0.025) for Sc, 0.195 (0.012) for Sm, 0.197 (0.025) for Xt, 0.198 (0.020) for He, 0.145 (0.004) for Sd and 0.145 (0.010) for Wt. There was a significant difference among biotopes in KO composition ($F_{5,18}$=18.23, P<0.001, R^2=0.835). Variation among biotopes thus explained almost 84 % of the variation in KO composition. The first axis was primarily related to variation between samples from seawater versus samples from *X. testudinaria* and *H. erectus* with samples from sediment and both *Stylissa* species intermediate. The second axis was primarily related to variation between sediment samples and samples from both *Stylissa* species (Fig. 4).

KOs predicted to be enriched in both *Stylissa* species and *X. testudinaria* and *H. erectus* are indicated by their KO identifiers (Fig. 4 and Table 2). KOs predicted to be enriched in both *Stylissa* species included K00087 (benzoate and aminobenzoate degradation), K00575 (chemotaxis protein methyltransferase CheR), K00673 (arginine and proline metabolism), K01076 (limonene and pinene degradation), K03409 (chemotaxis protein CheX), K04561 (denitrification, nitrate => nitrogen) and K10535 (nitrification, ammonia => nitrite). KOs predicted to be enriched in *X. testudinaria* and *H. erectus* included K00991 (terpenoid backbone biosynthesis), K01426 (styrene and aminobenzoate degradation; arginine and proline metabolism, phenylalanine metabolism, and tryptophan metabolism), K01770 (terpenoid backbone biosynthesis), K03696 (heat shock protein), K04517 (phenylalanine, tyrosine and tryptophan biosynthesis; novobiocin biosynthesis), K05522 (replication and repair), K05982 (DNA

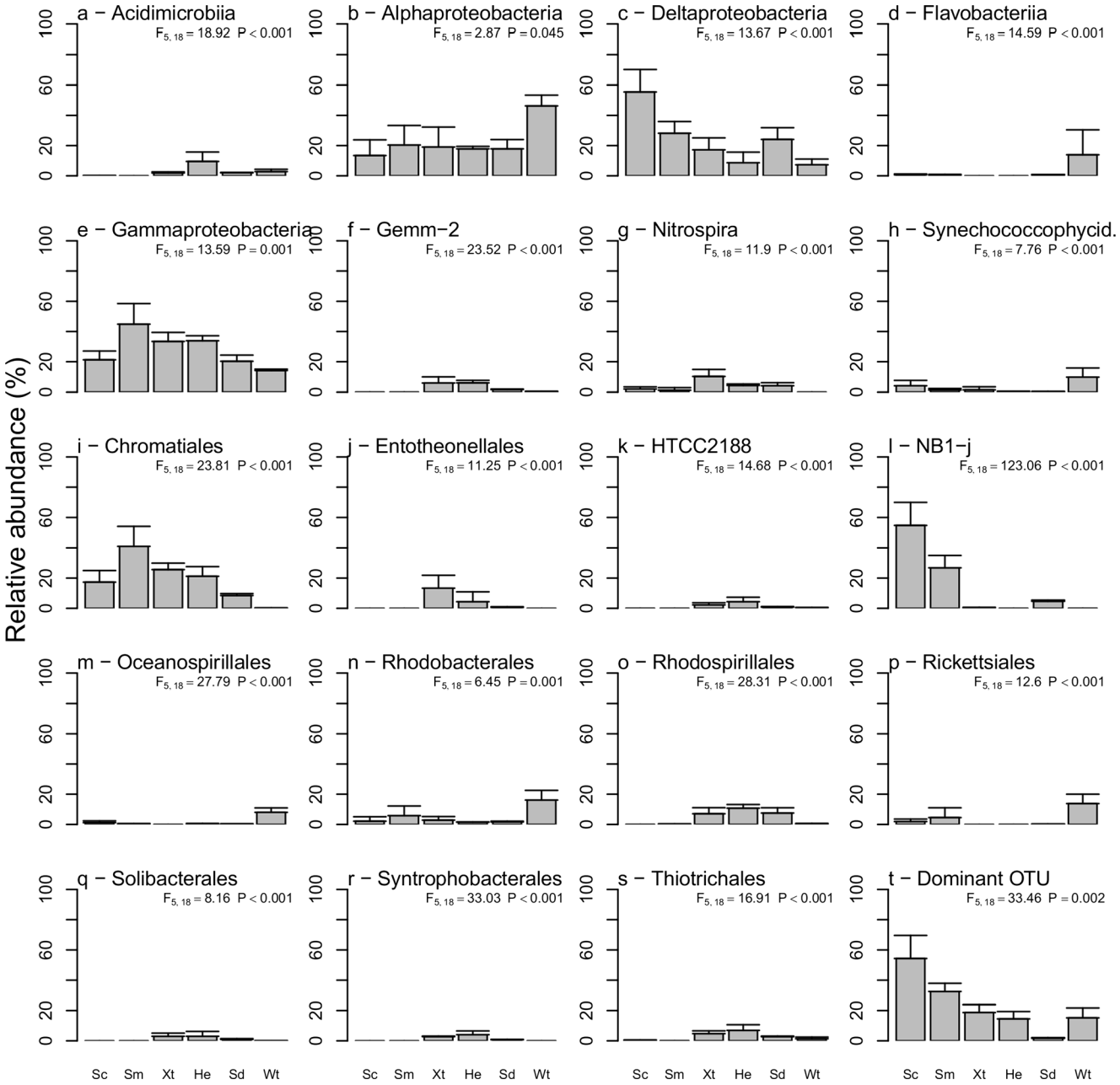

Fig. 2 Mean relative abundance of the most abundant bacterial classes, orders and the dominant OTU for samples from *S. carteri* (Sc), *S. massa* (Sm), *X. testudinaria* (Xt), *H. erectus* (He), sediment (Sd) and seawater (Wt). *Error bars* represent a single standard deviation. The dominant OTU represents the mean abundance for the single most abundant OTU in each sample, thus not necessarily the same OTU. Results of glm are shown in the *top-right corner* of each graph

repair and recombination protein), K06200 (carbon starvation protein), K07239 (heavy metal exporter), K07334 (proteic killer suppression protein), K07658 (PhoR-PhoB phosphate starvation response two-component regulatory system), K07665 (copper resistance phosphate regulon response regulator CusR two-component regulatory system) and K12339 (sulphur metabolism; cysteine and methionine metabolism).

The contributions of selected orders to KO enrichment are presented in Online Resources 6 and 7. In both *Stylissa* species, OTUs (primarily OTU-1) belonging to the *Deltaproteobacteria* class contributed strongly to enrichment of K00087, K03409, K04561 and K10535. In *X. testudinaria* and *H. erectus*, *Solibacteres* contributed strongly to enrichment of K05522, K07239, K07334 and K07665. Other important classes that contributed to enrichment were *Acidimicrobiia* and *Gammaproteobacteria* (primarily OTU-4) for K05522, *Gammaproteobacteria* and *Nitrospira* for K07239, *Gammaproteobacteria* and *Nitrospira* for K07334 and *Solibacteres* and *Nitrospira* and 'other' for K07665.

Table 1 List of most abundant OTUs (>300 sequences) including OTU-numbers; total sequences (Sum), taxonomic affiliation of OTU, GenBank GenInfo sequence identifiers (GI) of closely related organisms identified using BLAST; sequence identity (Seq) of these organisms with our representative OTU sequences; isolation source (Source) of closely related organisms identified using BLAST; location where the isolation source was sampled (Location)

OTU	Sum	Phylum	Class	Order	Family	GI	Seq	Source	Location
1	10859	Proteobacteria	Deltaproteobacteria	NB1-j	NB1-i	407912992	99.5	Sponge: *Stylissa carteri*	Saudi Arabia
2	5337	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified	407913000	99.5	Sponge: *Stylissa carteri*	Saudi Arabia
4	3613	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	451353954	100.0	Sponge: *Ircinia strobilina*	Bahamas:Exumas
5	1744	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified	127692586	99.8	Sponge: *Axinella corrugata*	?
6	1505	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	516559154	100.0	Seawater	Northern Adriatic Sea: Gulf of Trieste
7	1640	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	451353960	100.0	Sponge: *Ircinia strobilina*	Bahamas: Exumas
8	1213	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	158342489	99.0	Sponge: *Rhopaloeides odorabile*	Australia
9	952	Actinobacteria	Acidimicrobiia	Acidimicrobiales	TK06	526299843	100.0	Sponge: *Aplysina cauliformis*	Belize: Carrie Bow Cay
10	817	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	451354068	99.5	Sponge: *Ircinia felix*	Bahamas: Exumas
11	1683	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	340764414	99.8	Sponge: *Phakellia fusca*	China: South China Sea
13	1379	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified	315020304	100.0	Sponge: *Xestospongia testudinaria*	Indonesia: Manado
14	695	Bacteroidetes	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	262528650	100.0	Coral: *Montastraea faveolata*	Panamá: Bocas del Toro
16	1356	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	315020278	99.8	Sponge: *Xestospongia muta*	USA: Key Largo
17	1195	Proteobacteria	Deltaproteobacteria	[Entotheonellales]	[Entotheonellaceae]	315020294	100.0	Sponge: *Xestospongia muta*	USA: Key Largo
18	636	Acidobacteria	Solibacteres	Solibacterales	PAUC26f	407728807	100.0	Sponge: *Ircinia oros*	Spain: Catalunya
19	787	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	134290593	99.8	Sponge: *Xestospongia muta*	USA: Key Largo
20	498	Proteobacteria	Deltaproteobacteria	[Entotheonellales]	[Entotheonellaceae]	295638981	98.9	Sponge: *Haliclona hogarthi*	Bahamas: Sweetings Cay, Mangrove
21	552	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified	209364851	100.0	Sponge: *Axinella corrugata*	?
22	624	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	388955550	100.0	Seawater	USA: San Diego, CA
23	508	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	451354037	100.0	Sponge: *Ircinia felix*	Bahamas:Exumas
24	447	Gemmatimonadetes	Gemm-2	Unclassified	Unclassified	511630180	97.7	Sponge: *Vaceletia crypta*	Australia: Great Barrier Reef
25	421	Proteobacteria	Deltaproteobacteria	Sva0853	Unclassified	429510884	100.0	Seawater	West Pacific Ocean
26	609	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	451963772	99.0	Seawater	Taiwan
27	395	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	526299871	100.0	Sponge: *Aplysina cauliformis*	Bahamas: Lee Stocking Island
28	408	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	451775225	100.0	Seawater	Pacific Ocean
29	454	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	530540136	100.0	Sponge: *Haliclona* sp.	India: Gulf of Mannar
30	348	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	154125226	100.0	Seawater	Singapore
31	650	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	158342459	100.0	Sponge: *Rhopaloeides odorabile*	Australia
32	312	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	511630187	100.0	Sponge: *Vaceletia crypta*	Australia: Great Barrier Reef
33	347	Proteobacteria	Unclassified	Unclassified	Unclassified	441084663	90.1	Sponge: *Dysidea avara*	Mediterranean Sea: Medas Islands
34	348	Gemmatimonadetes	Gemm-4	Unclassified	Unclassified	290575663	100.0	Sponge	?

Table 1 (continued)

OTU	Sum	Phylum	Class	Order	Family	GI	Seq	Source	Location
35	409	Proteobacteria	Deltaproteobacteria	[Entotheonellales]	Unclassified	563402270	99.8	Sponge: *Smenospongia aurea*	Bahamas
37	418	Gemmatimonadetes	Gemm-2	Unclassified	Unclassified	482678041	100.0	Coral	?
41	555	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified	350627613	99.8	Sponge: *Xestospongia testudinaria*	Indonesia: Manado
42	470	Proteobacteria	Gammaproteobacteria	Thiohalorhabdales	Unclassified	340764412	100.0	Sponge: *Phakellia fusca*	China: South China Sea
43	429	SBR1093	EC214	Unclassified	Unclassified	315020327	100.0	Sponge: *Xestospongia testudinaria*	Indonesia: Manado
45	429	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	451775465	100.0	Sponge: *Mycale laxissima*	USA
48	342	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	451354044	100.0	Sponge: *Ircinia felix*	Bahamas: Exumas
49	312	Actinobacteria	Acidimicrobiia	Acidimicrobiales	wb1_P06	288730901	100.0	Sponge: *Holoxea* sp.	China:South China Sea
50	310	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	511630242	99.8	Sponge: *Vaceletia crypta*	Australia: Great Barrier Reef
51	797	Proteobacteria	Alphaproteobacteria	Rickettsiales	Pelagibacteraceae	545346901	100.0	Seawater	Gulf of Gdansk, Baltic Sea
53	423	Gemmatimonadetes	Gemm-2	Unclassified	Unclassified	400269057	98.4	Sponge: *Luffariella variabilis*	Australia: Great Barrier Reef
60	404	Acidobacteria	Acidobacteria-6	BPC015	Unclassified	451354030	99.1	Sponge: *Ircinia strobilina*	Bahamas: Exumas
1156	806	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	451353960	99.8	Sponge: *Ircinia strobilina*	Bahamas:Exumas
3248	305	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	511630227	99.8	Sponge: *Vaceletia crypta*	Australia: Great Barrier Reef

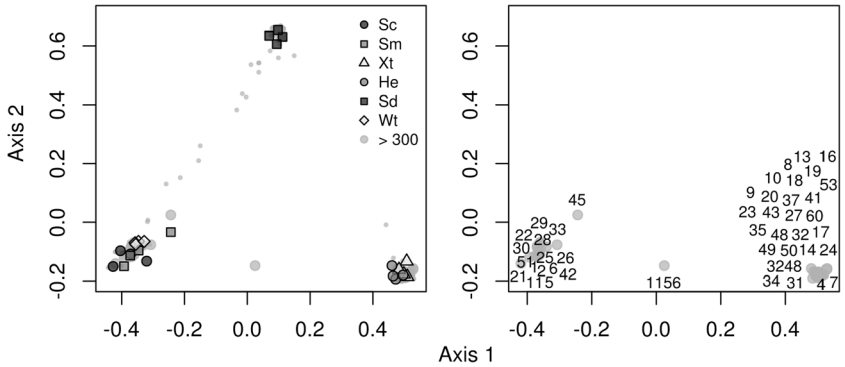

Fig. 3 Ordination showing the first two axes of the PCO analysis for OTU composition. *Symbols* represent samples from *S. carteri* (Sc), *S. massa* (Sm), *X. testudinaria* (Xt), *H. erectus* (He), sediment (Sd) and seawater (Wt). *Numbers* refer to OTU numbers in Table 1. The *small light grey circles* represent OTUs with <300 sequences while the *larger light grey circles* represent OTUs ≥ 300 sequences

Discussion

Higher Taxon Abundance

We recorded highly significant differences in the relative abundances of a number of classes and orders. The *Rhodospirillales* order and Gemm-2 class, for example, were largely restricted to sediment, *H. erectus* and *X. testudinaria*, whereas the *Rickettsiales* were largely restricted to seawater and both *Stylissa* species. Entotheonellales and HTCC2188 also were most abundant in *H. erectus* and *X. testudinaria*. At the phylum level, the relative abundance of *Proteobacteria* was most pronounced in the bacterial communities of both *Stylissa* species. Bacterial communities belonging to the other biotopes, while hosting a majority of OTUs assigned to *Proteobacteria*, had more phylum-level diversity. The relative abundance of the most dominant OTU in each sample was highest in both *Stylissa* species and lowest in sediment. This result reflects similar findings of these biotopes in coral reefs of Jakarta, Indonesia [54].

Bacterial Composition: Biotope Versus Environment

Biotope proved a significant predictor of variation in composition as opposed to the sampling zone. This suggests that much of the variation in bacterial composition in coral reef habitat is due to differences among distinct biotopes, i.e., seawater, sediment, host organisms and possibly other microhabitats such as crevices and biofilms, the latter of which were not investigated in the present study. It remains to be investigated how bacterial communities from different biotopes respond to environmental gradients. It is probable that bacterioplankton and possibly sediment bacteria respond more strongly than bacteria residing in host organisms such as sponges. A number of previous studies, for example, have found that sponge bacterial communities remain remarkably stable across pronounced geographic and environmental gradients [11, 17, 55, 56]. Reveillaud et al. [56] sampled *Hexadella* species over a very large bathymetric gradient (15–960 m) and observed 'remarkably specific and stable sponge–bacteria associations'. Likewise, Lee et al. [17] suggested that sponge microbial communities appear to resist environmental change. This apparent resistance to environmental change of host-related microbes extends beyond sponges. Hawlena et al. [57] collected bacterial communities of fleas and ticks over a range of environmental conditions and sites but found that none of those conditions significantly affected bacterial community composition. Composition was, however, strongly related to the type of host. Bacterioplankton communities while probably more sensitive to changes in environmental conditions are also subject to living in a highly dynamic environment. Bacterioplankton composition though has been shown to vary

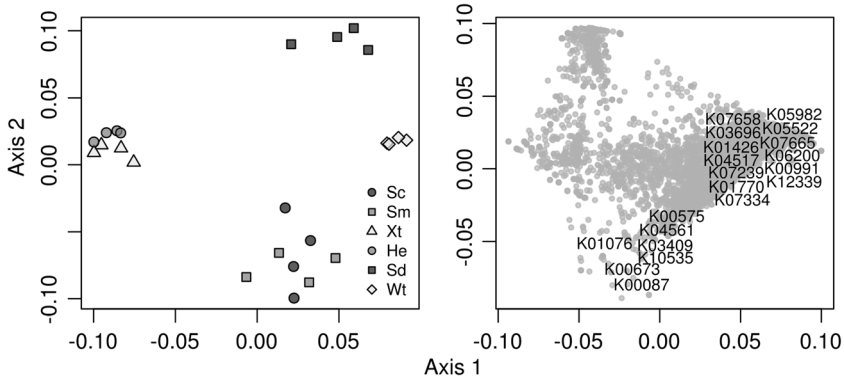

Fig. 4 Ordination showing the first two axes of the PCO analysis for KO composition. *Symbols* represent samples from *S. carteri* (Sc), *S. massa* (Sm), *X. testudinaria* (Xt), *H. erectus* (He), sediment (Sd) and seawater (Wt). *Codes* refer to KO codes in Table 2

Table 2 Selected KOs enriched (Enriched) in both *Stylissa* species (*Sc, Sm*) or *X. testudinaria* and *H. erectus* (Xt, He)

KO	Sum	Enriched	Description	Function
K00087	6485	*Sc, Sm*	Xanthine dehydrogenase molybdenum-binding subunit [EC:1.17.1.4]	Aminobenzoate degradation; benzoate degradation
K00575	59,633	*Sc, Sm*	Chemotaxis protein methyltransferase CheR [EC:2.1.1.80]	Chemotaxis signaling
K00673	8664	*Sc, Sm*	Arginine N-succinyltransferase [EC:2.3.1.109]	Arginine and proline metabolism
K00991	17,668	Xt, He	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase [EC:2.7.7.60]	Terpenoid backbone biosynthesis
K01076	8108	*Sc, Sm*	[E3.1.2.-]	Limonene and pinene degradation; biosynthesis of unsaturated fatty acids
K01426	18,995	Xt, He	Amidase [EC:3.5.1.4]	Styrene degradation; aminobenzoate degradation; arginine and proline metabolism; phenylalanine metabolism; tryptophan metabolism
K01770	16,465	Xt, He	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase [EC:4.6.1.12]	Terpenoid backbone biosynthesis
K03409	16,221	*Sc, Sm*	Chemotaxis protein CheX	Bacterial chemotaxis
K03696	10,676	Xt, He	ATP-dependent Clp protease ATP-binding subunit ClpC	Heat shock proteins
K04517	17,230	Xt, He	Prephenate dehydrogenase [EC:1.3.1.12]	Phenylalanine, tyrosine and tryptophan biosynthesis; novobiocin biosynthesis
K04561	11,308	*Sc, Sm*	Cytochrome b-containing subunit I [EC:1.7.2.5]; nitric-oxide reductase	Denitrification: nitrate => nitrogen
K05522	10,040	Xt, He	Endonuclease VIII [EC:3.2.2.- 4.2.99.18]	Base excision repair
K05982	5872	Xt, He	Deoxyribonuclease V [EC:3.1.21.7]	DNA repair and recombination proteins
K06200	9224	Xt, He	Carbon starvation protein	Carbon starvation response
K07239	21,515	Xt, He	Heavy-metal exporter	Heavy metal export
K07334	28,888	Xt, He	Proteic killer suppression protein	Toxin antitoxin system
K07658	9044	Xt, He	Two-component system OmpR family alkaline phosphatase synthesis response regulator PhoP	Phosphate starvation response
K07665	11,965	Xt, He	Two-component system OmpR family copper resistance phosphate regulon response regulator CusR copR silR	Copper resistance
K10535	7879	*Sc, Sm*	Hydroxylamine oxidase [EC:1.7.3.4]	Nitrification: ammonia => nitrite
K12339	14,927	Xt, He	Cysteine synthase B [EC:2.5.1.47]	Sulphur metabolism; cysteine and methionine metabolism

KO KEGG ortholog identifier, *Sum* sum of gene counts, *Description* description of KO, *Function* function of pathways to which KO belongs

along pronounced environmental gradients of carbon, temperature and salinity [58, 59].

There is a debate about the degree to which sponges host sponge-specific or sponge species-specific microbial communities [11, 15, 60]. In the present study, three of the most abundant OTUs were only found in *Stylissa* species (OTUs 1, 2 and 11). These three OTUs were closely related (with a similarity ≥99 %) to organisms found in *S. carteri* sampled in Saudi Arabia ([61]; OTU-1: GI: 407912992, OTU-2: 407913000; OTU-11: 407913009), *Stylissa* sp. sampled in Australia ([62] OTU-1: GI:400269236), *Axinella* sp. sampled in China (Liu unpublished; OTU-1: GI: 597437720; OTU-2: 597437717; OTU-11: GI: 597437738), *Axinella corrugata* sampled in the Caribbean (Lopez et al. unpublished, Holmes and Blanch unpublished; OTU-1: GI: 209364706; OTU-2: GI: 127692655; OTU-11: GI: 127692617 and GI: 209364724), *Axinella verrucosa* sampled in the Mediterranean (Steffens unpublished; OTU-2: GI: 34368515) and *Phakellia fusca* sampled in China ([63]; OTU-11: GI: 340764414). Interestingly, all these sponge hosts (including the *Stylissa* species) belong to the same taxonomic order (Halichondrida). These results confirm previous findings of Polónia et al. [64] where they found that both *Stylissa* spp. hosted a single very abundant crenarchaeote assigned to the species *Cenarchaeum symbiosum*. This crenarchaeote was also found in other sponges including *Axinella* and *Phakellia* leading Polónia et al. [64] to suggest the presence of a possibly order-specific symbiosis between Halichondrida and *C. symbiosum. C. symbiosum* was itself originally isolated from the sponge *Dragmacidon mexicanum* (previously known as *Axinella mexicana*) off the California coast [65]. We expand on this and suggest the existence of a small core community of possibly sponge order-specific microbes including one crenarchaeote and three bacteria belonging to the orders NB1-j, *Chromatiales* and an unclassified alphaproteobacterium. As with *C. symbiosum*, organisms closely related to these bacterial OTUs were isolated from other halichondrid sponges across a very large geographical range including the Indo-Pacific, Caribbean and Mediterranean. As noted by Polónia et al. [64], this would appear to suggest that this core group is spatially stable and possibly vertically

transmitted, i.e., from parent to offspring. This result contrasts with Schmitt et al. [66] who found that bacterial communities of sponges in the same order were not more similar to one another than bacterial communities of sponges in different orders.

The bacterial communities of the HMA sponges *X. testudinaria* (order: Haplosclerida) and *H. erectus* (order: Dictyoceratida) were compositionally similar and shared a large number of OTUs. In contrast to the low microbial abundance (LMA) *Stylissa* species, both HMA sponges were enriched with OTUs closely related to *Nitrospira marina* (GI: 530902; Online Resource. 3), a well-known lithoautotrophic nitrite-oxidising bacteria previously found in other marine sponges [11]. Very few OTUs found in *H. erectus* were shared with sediment, seawater and both *Stylissa* species despite the fact that phylogenetically, the Haplosclerida is more closely related to the Halichondrida than to the Dictyoceratida [67].

Sponge host phylogeny has been shown to have a weak effect on microbial composition [68], but the structure of the sponge tissue matrix may play a more important role in structuring the sponge bacterial community. *H. erectus* is a small black digitate sponge that lives embedded in sediment and sand. The skeletons of *Hyrtios* species lack silicious spicules and have a crust of exogenous material, and the choanosome consists of dense spongin fibres, extraneous detritus, sediment grains, foreign sponge spicules and broken shells. *X. testudinaria*, in turn, is a very long-lived and slow-growing species, the skeleton of which consists of a dense network of silicious spicules [69]. In contrast to the previous species, *Stylissa* spp. are probably fast growers with a loose collagen-rich skeleton containing relatively large spicules [70]. Like a bath sponge, the loose skeletal structure of *Stylissa* spp. has the capacity to retain much higher amounts of water in their tissue.

In addition to the above, *X. testudinaria* is a confirmed high microbial abundance (HMA) sponge while *H. erectus* is a presumed HMA sponge [71]. Our results would appear to confirm *H. erectus* as a HMA sponge given the similarity of its bacterial community with *X. testudinaria*. *Stylissa* spp., in contrast, are confirmed low microbial abundance (LMA) sponges [72]. LMA sponges typically have limited phylum-level diversity dominated by *Proteobacteria* and are known to filter larger water volumes than HMA sponges, thereby increasing similarity with bacterioplankton communities [14, 61, 72–75]. This fits well with our results and results from archaeal communities inhabiting *Stylissa* spp. in Makassar, Indonesia [64], but not Jakarta [36]. In addition to the above, the sponge metabolism is believed to be only influenced by microbes in HMA sponges, which has led to a focus on HMA sponges [76]. Importantly, our data confirms that both LMA *Stylissa* species maintain a bacterial community that is similar to, but still distinct from, the surrounding seawater and includes highly abundant OTUs that were absent in all other biotopes including seawater. This result is in line with de Voogd et al. [54] who found the same for *S. massa* in Jakarta and Moitinho-Silva et al. [72] who found the same for *S. carteri* in the Red Sea.

Predictive Functional Analysis

As mentioned previously, PICRUSt provides a prediction of microbiome function but not an actual measurement of such function. There are, however, methods of quality control that test the reliability of PICRUSt predictions including the weighted NSTI scores. NSTI, which was developed to evaluate the predictive accuracy of PICRUSt, calculates dissimilarity between reference genomes and the metagenome under study. In poorly characterised environments, there are relatively few reference genome sequences available; thus, the PICRUSt predictions of these genomes tend to be less accurate than for well-known microbial environments. In the present study, NSTI scores were relatively high, most notably for sponges, a reflection of the relative novelty of the bacterial communities of the coral reef sponges studied here. Mean scores for three of the four sponge species were below 0.20, but the highest value was obtained for *S. carteri* at 0.220. Langille et al. [25] showed that the accuracy of PICRUSt decreased with increasing NSTI scores but still produced reliable results for a dataset of soil samples with a mean NSTI score of 0.17. Accuracy was, however, lower for a dataset from the Guerrero Negro microbial mat with a mean NSTI score of 0.23. Langille et al. [25] noted, however, that this was also related to shallow sequencing at a depth that was insufficient to fully sample the community's genomic composition. The relatively high NSTI scores obtained here indicate that the PICRUSt predictions must be treated with caution. The results, however, still provide some interesting insights into potential bacterial community functioning that, in the future, should be tested with studies that measure actual gene presence or expression.

One notable difference between OTU and KO composition was the similarity in bacterial composition between seawater and *Stylissa* samples, but the distinct difference in KO composition. Despite the abundance of symbionts shared between seawater and both *Stylissa* species and the lower number of sponge-specific symbionts found, sponge-specific symbionts exhibited the most pronounced dominance in both *Stylissa* species and contributed strongly to certain predicted metabolic functions. In particular OTU-1, assigned to the *Deltaproteobacteria*, was largely responsible for the pronounced enrichment of both *Stylissa* species for K00087 (Benzoate and Aminobenzoate degradation), K03409 (chemotaxis protein CheX), K04561 (denitrification, nitrate => nitrogen) and K10535 (nitrification, ammonia => nitrite). Moitinho-Silva et al. [18] found that *S. carteri* from the Red

Sea exhibited high expression of functions related to stress response and membrane transporters. In both *Stylissa* species, we observed predicted enrichment of KOs related to bacterial chemotaxis (K00575 and K03409) and xenobiotics degradation (K00087, K01076).

The predicted contribution of *Deltaproteobacteria* to both nitrification and denitrification is in line with similar findings for *S. massa* in Jakarta [54] and highlights the potential importance of this class and OTU-1 in particular to nitrogen cycling with *Stylissa* species. In other marine environments, *Deltaproteobacteria* have also been shown to play a key role in the nitrogen cycle. In the Eastern South Pacific, for example, *Nitrospina*-like bacteria (order *Desulfobacterales*) were identified as the main drivers of nitrite oxidation in a seasonal upwelling area [77].

The contrast in predicted metabolic enrichment of both *Stylissa* species with *X. testudinaria* and *H. erectus* is interesting. KOs enriched in *X. testudinaria* and *H. erectus* included KOs involved in terpenoid backbone biosynthesis (K00991 and K01770), DNA repair (K05522, K05982), heavy metal efflux (K07239), copper resistance (K07665), carbon starvation (K06200) and proteic killer suppression (K07334) proteins. Two KOs (K07658, K07665) involved in copper resistance and phosphate starvation enriched in *X. testudinaria* and *H. erectus* are part of the signal transduction system known as the two-component regulatory system [78]. Signal transductors belonging to the two-component regulatory system enable bacteria to respond to a very wide range of nutrients, stressors (including antibiotics) and environmental conditions [79].

In addition to the previously mentioned KOs related to stress management (nutrient starvation, heat proteins and heavy metal exporters), *X. testudinaria* and *H. erectus* were also enriched for the proteic killer suppression protein higA. The higA (host inhibition of growth) protein is required for cloning of the killer protein HigB, part of the toxin–antitoxin (TA) system. TA systems consist of sets of two or more genes that include a toxin (e.g., higB) and anti-toxin (e.g., higA) and are believed to confer an advantage on the fitness of plasmids that carry them [80]. They are key regulators of cellular processes that influence survival under stressful conditions, are involved in essential cellular processes like replication, gene expression and cell wall synthesis and play a role in persistence, biofilm formation, antibiotic resistance and bacterial virulence [81, 82]. Interestingly, in a survey of TA loci, Pandey and Gerdes [83] found that TA loci were highly abundant in free living prokaryotes but absent from obligate intracellular organisms. They suggested that is a reflection of the beneficial role that TA loci play for free living prokaryotes in coping with stress.

The type of predicted functional enrichment displayed by the bacterial communities of *X. testudinaria* and *H. erectus* would appear to suggest adaptations to surviving and indeed persisting (in the case of *X. testudinaria* for very long periods of time) in stressful environments. Many sponges including *X. testudinaria* and sponge symbionts are known to produce antibacterial compounds, so host symbionts need to have mechanisms such as TA loci to cope with these compounds [84, 85]. *X. testudinaria* is also often found in highly perturbed environments and can even be extremely abundant in such environments [86–88]. The specific bacterial community of *X. testudinaria* may play a role in enabling the sponge to persist and survive in stressful environments.

In addition to the above, KOs related to the phenylalanine metabolism (K01426) and phenylalanine tyrosine and tryptophan biosynthesis (K04517) were predicted to be enriched in *X. testudinaria* and *H. erectus*. Phenylalanine is an essential amino acid, which is converted to tyrosine and is produced for a variety of medicinal and nutritional applications. Tyrosine is an amino acid that occurs in proteins belonging to signal transduction processes, plays a role in photosynthesis and is a precursor to alkaloids and phenols [89, 90]. Enrichment in the tyrosine and phenylalanine metabolic pathways and the importance of these pathways for the biosynthesis of alkaloids and phenols are in line with the numerous bioactive compounds that have been isolated from *X. testudinaria* and *H. erectus* [91–95]. Numerous bioactive compounds have also been isolated from *Stylissa* species including dimeric alkaloids (e.g., dibromophakellin and sceptrin), brominated pyrrole alkaloids and other brominated alkaloids. These compounds are of particular interest due, among other things, to their ability to inhibit protein kinases. Both *Stylissa* species, *X. testudinaria* and *H. erectus*, also produce a large range of highly selective antibiotic compounds [96–99].

Here, we have provided a detailed analysis of the bacterial communities inhabiting distinct coral reef biotopes. More than 87 % of the variation in the composition of these communities could be attributed to differences among biotopes. Despite sampling along a pronounced environmental gradient, the sampling zone proved a poor predictor of bacterial composition. Future research should focus on how bacterial communities from different biotopes respond to environmental variation. Bacterioplankton, for example, may show more of a response than bacterial communities housed within host organisms such as sponges. Although LMA sponges belonging to the genus *Stylissa* contained communities that were similar to seawater, they also contained highly abundant OTUs that were absent in all other biotopes. One of these OTUs, assigned to the class *Deltaproteobacteria*, contributed substantially to the predicted enrichment of genes related to chemotaxis, denitrification and nitrification in both *Stylissa* species. *X. testudinaria* and *H. erectus* displayed diverse microbial communities that differed strongly from seawater. The bacterial communities of *X. testudinaria* and *H. erectus* were predicted to be enriched for genes related to the toxin–antitoxin (TA) system and genes that convey tolerance to heavy metal

pollution and nutrient starvation suggesting adaptation to stressful environmental conditions.

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