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Received: 14 November 2017 | Revised: 29 June 2018 | Accepted: 30 June 2018

DOI: 10.1111/maec.12517

ORIGINAL ARTICLE

WILEY marine ecology

Assessing the bacterial communities of sponges inhabiting the remote western Indian Ocean island of Mayotte

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Funding information
ANR-Netbiome, Grant/Award Number: ANR-11-EBIM-0006; Foundation for Science and Technology, Grant/Award Number: 115304 and 2009

Abstract

Marine sponges are known to host diverse and abundant communities of microbial symbionts. It has been generally assumed that the bacterial communities of low microbial abundance (LMA) sponges are less diverse than those of high microbial abundance (HMA) sponges. In this study, we used next-generation sequencing technology to explore the bacterial communities of several biotopes including sponges, seawater, and sediment from the remote Western Indian Ocean island of Mayotte. The species investigated were the known LMA sponges: *Jaspis splendens*, *Stylissa carteri*, and *Stylissa massa*, and the known HMA sponges: *Hyrtios erectus and Xestospongia testudinaria*. In addition to this, we also investigated the following sponge species: *Ectyoplasia coccinea*, *Paratetilla bacca*, *Liosina paradoxa*, and *Petrosia* aff. *spheroida* of which the exact HMA/LMA status is unknown although we preliminarily classified them as HMA or LMA based on the status of closely related species. Certain HMA sponges shared similar bacterial communities dominated by Actinobacteria and Chloroflexi, whereas another species (*E. coccinea*) had a bacterial community closer to that of LMA sponges. Most LMA sponges housed a bacteriome dominated by Proteobacteria and Cyanobacteria, but the bacteriome of *P. bacca* also included dominant Chloroflexi and actinobacterial OTUs. Together with *S. carteri*, this sponge housed a more diverse bacterial community at the phylum, class, and order levels than HMA sponges. Although certain LMA sponges housed a bacterial community similar to the surrounding environment (seawater), they also included highly abundant, possibly species or genus specific, OTUs. Based on this study and small set of sponges studied, we conclude that a clear dichotomy between HMA and LMA sponges does not appear to exist.

KEYWORDS
bacterial diversity, coral reef, pyrosequencing, sponges

1 | INTRODUCTION

Microbial communities, housed in multicellular hosts, have influenced the evolution of their hosts and are an integral part of plant and animal life (McFall-Ngai et al., 2013). In the recent past, important advances have been made in our understanding of the impact of symbiotic microbial communities on the health and well-being of marine host organisms. In corals, algal symbionts, *Symbiodinium* spp., provide up to 60% of the nutrient requirements of host organisms; loss of the symbionts due to environmental stress often results in host death (Ainsworth et al., 2011; Brown, 1997; Rosenberg, Koren, Reshef, Efrony, & Zilber-Rosenberg, 2007). In addition to corals, sponges are abundant and ecologically important components of coral reef ecosystems (Diaz & Rützler, 2001). In general, bacteria are the most abundant component of the prokaryotic community in sponges (Fan et al., 2012; Hardoim & Costa, 2014;

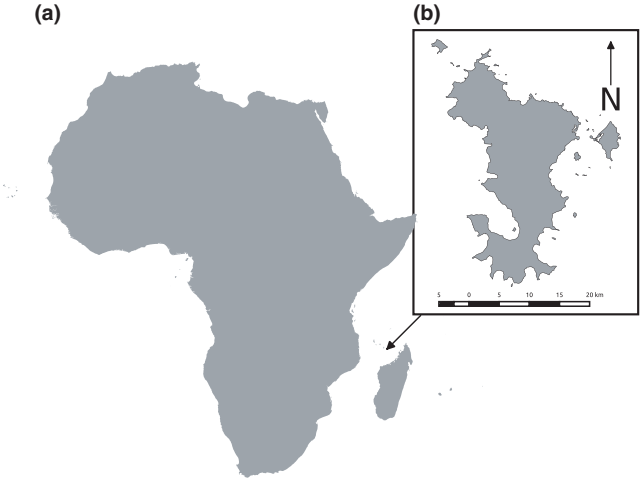

FIGURE 1 (a) Location map with (b) inset showing the island of Mayotte

Lee et al., 2011; Taylor, Radax, Steger, Steger, & Wagner, 2007). In some sponges, nearly 40% of the volume of the organism consists of microbes, of which some contribute significantly to the host metabolism (Hentschel, Usher, & Taylor, 2006; Taylor et al., 2007). Because of this, sponges have long been referred to as the sponge holobiont, thus including the sponge cells plus communities of persistent symbionts (Hentschel, Piel, Degnan, & Taylor, 2012; Reveillaud et al., 2014). The evolutionary and ecological success of sponges may, in part, be related to their intimate relationship with these microbial communities (Sipkema, Franssen, Osinga, Tramper, & Wijffels, 2005). In the late 1970s, certain sponges were first shown to harbor very high densities of bacteria, although other sponges appeared to be largely devoid of such symbionts (Vacelet & Donadey, 1977). This distinction eventually led to the terms high microbial abundance (HMA) sponges and low microbial abundance (LMA) sponges, whereby these two groups differed in bacterial diversity and abundance, in addition to exhibiting major physiological differences. HMA sponges can contain 10^{10} bacterial cells/g wet weight of sponge, that is, 2–4 orders of magnitude higher than surrounding seawater (Hentschel et al., 2002, 2012, 2006). These types of sponges have been shown to host diverse communities of Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, and Poribacteria that provide their hosts with inorganic and organic carbon and play an important role in the nitrogen metabolism (Bayer et al., 2014; Hoffmann et al., 2009; Siegl et al., 2010). Many of these higher taxa are generally rare or absent in LMA sponges, the exception being Proteobacteria (Poppell et al., 2014; Schläppy et al., 2010). HMA sponge species have also been shown to transfer their symbionts horizontally, thus from the surrounding environment, although the latter process has never been demonstrated in situ (Bright & Bulgheresi, 2010; Webster et al., 2010). In general, it is assumed that the microbiota of LMA sponges are horizontally transmitted, as the bacterial communities are similar to those found in the surrounding seawater (Gloeckner et al., 2014; Moitinho-Silva et al., 2014; Thacker & Freeman, 2013). Bacterial symbionts are also transmitted by vertically through sponge reproductive stages (Enticknap, Kelly, Peraud, & Hill, 2006; Maldonado, 2007; Schmitt et al., 2012; Thacker & Freeman, 2013). In comparison to HMA sponges, LMA sponges are in general thought to have higher pumping rates, more extensive aquiferous channels, and higher choanocyte chamber density thus reflecting a more heterotrophic feeding mode (Poppell et al., 2014; Weisz, Lindquist, & Martens, 2008). It is, however, unknown whether the sponges are preconditioned to host microbes or whether the morphology of the sponge interior is a result of hosting the microbes (Gloeckner et al., 2014). Recent work has shown that the HMA/LMA dichotomy is not as strict as was once presumed; in contrast, some prokaryotes are shared widely among different LMA sponge hosts, whereas others are host specific (Cleary, Voogd, Polonia, Freitas, & Gomes, 2015; de Voogd, Cleary, Polonia, & Gomes, 2015; Moitinho-Silva et al., 2014, 2017). Moitinho-Silva et al. (2014) proposed to change the term "sponge specific" to sponge-enriched, because sponge-specific prokaryotes appear to occur in low numbers in the surrounding environment. Although we are able to categorize the HMA/LMA dichotomy to a large degree, it is not yet known what causes it, or the reason for its existence. For instance, Gloeckner et al. (2014) investigated 56 sponges belonging to a subset of different orders (some of which are presently disused) and showed that some sponge orders only consist of HMA sponges, for example, the orders Verongida and Agelasida, although others, for example, the Poecilosclerida, only consist of LMA sponges and that most orders contain a mixture of both types.

We do know that HMA/LMA characteristics are often conserved in closely related species across large geographical scales (Gloeckner et al., 2014; Montalvo & Hill, 2011). Bacterial communities have been shown to be important for the functional ecology of sponges (Bell, 2008; Ribes et al., 2012). It is still unclear, however, whether HMA and LMA sponges provide distinct ecological functions and what role they play in key ecological processes such as carbon and nitrogen cycling. An important first step is to assess the large range of HMA and LMA sponges in order to assess to what extent both groups of sponges house compositionally distinct bacterial communities and whether there is, indeed, a true dichotomy between both groups or whether, in contrast, there is evidence of a continuum in symbiont composition.

In this study, we assessed bacterial communities using 454-pyrosequencing of several biotopes including seawater, sediment, and a number of relatively abundant sponge species of the remote island of Mayotte located in the Western Indian Ocean. Our main goal was to explore the HMA/LMA dichotomy by sampling replicates of HMA/LMA species and also some additional species of which the status is still unknown. We assessed whether these species house distinct bacterial communities. Specific goals were to compare OTU composition among sponge species and surrounding biotopes (sediment and seawater) and to assess how dominant (> 500 sequences) bacterial OTUs were distributed among sponge hosts using a set of tools including ordination, heatmap, and network visualization.

2 | METHODS

2.1 | Sample collection and study area

Mayotte is part of the overseas department of France and is part of the Comores archipelago (Indian ocean). The Comores are located in the Mozambique channel just northwest of Madagascar. Mayotte has a surface area of 374 km^2 and consists of two main islands of volcanic origin, Grande Terre and Petite Terre, and some smaller islands around these main islands. The main island is surrounded by an almost continuous barrier reef and the lagoon is 3–15 km wide, with an area of 1,500 km^2 making it one of the world's largest lagoons (Figure 1). We collected fragments from 27 sponge specimens from nine different sponge species belonging to six different orders (three samples per species) at 12 different sites inside and just outside the lagoon at the western side of Grande Terre (between 12°56.470′S 45°04.305′E and 13°00.375′S 45°08.250′E) using SCUBA diving and snorkeling (depth range: 3–25 m) between May 4 to 11, 2013. The sponges were identified by the first author using classical morphological characters and voucher specimens have been deposited in the sponge collection of Naturalis Biodiversity Center (RMNH POR.#, see Figure 2, Table 1). The species investigated were the known LMA sponges: *Jaspis splendens* (*Js*) (order Tetractinellida), *Stylissa carteri* (*Sc*), and *Stylissa massa* (*Sm*) (order Scopalinida), and

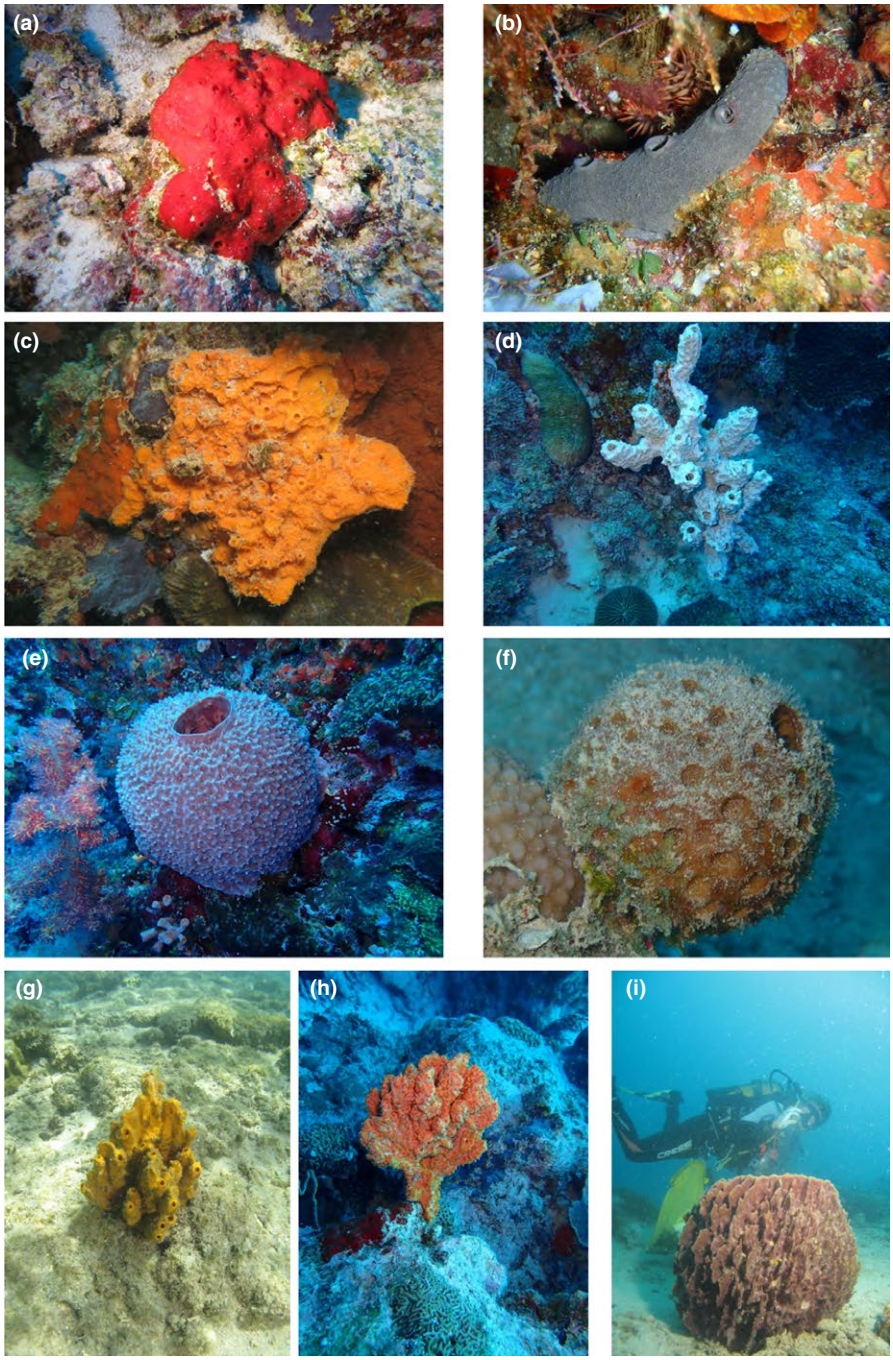

FIGURE 2 Underwater images of the target sponge species, (a) *Ectyoplasia coccinea*, (b) *Hyrtios erectus*, (c) *Jaspis splendens*, (d) *Liosina paradoxa*, (e) *Petrosia* aff. *spheroida*, (f) *Paratetilla bacca*, (g) *Stylissa massa*, (h) *Stylissa carteri*, and (i) *Xestospongia testudinaria*

TABLE 1 Sample list with the sample number, collection voucher number, sponge species, high microbial abundance (HMA) or low microbial abundance (LMA) type, pooled rarefied richness, collection site (location), and GPS coordinates

Sample	Naturalis collection code	Species	Type	Pooled rarefied richness (n = 4,600)	Location	Latitude	Longitude
Ec050	RMNH POR.8350	*Ectyoplasia coccinea*	HMA	115.2 ± 3.7 OTUs	Pointe Sud Mayotte	13°00.375'S	45°08.250'E
Ec057	RMNH POR.8357	*Ectyoplasia coccinea*	HMA		Passe Boueni Sud	12°55.265'S	44°58.126'E
Ec103	RMNH POR.8403	*Ectyoplasia coccinea*	HMA		Passe Sada	12°54.141'S	44°57.862'E
He055	RMNH POR.8355	*Hyrtios erectus*	HMA	112.6 ± 2.6 OTUs	Passe Boueni Sud	12°55.265'S	44°58.126'E
He126	RMNH POR.8426	*Hyrtios erectus*	HMA		Dans lagon, face a la passe Boueni	12°55.163'S	44°59.233'E
He149	RMNH POR.8449	*Hyrtios erectus*	HMA		Grande Passe de l'Ouest	12°48.356'S	44°57.793'E
Js043	RMNH POR.8343	*Jaspis splendens*	LMA	51.6 ± 4.2 OTUs	Pointe Sud Mayotte	13°00.375'S	45°08.250'E
Js077	RMNH POR.8377	*Jaspis splendens*	LMA		Rocchi	12°59.536'S	45°03.183'E
Js128	RMNH POR.8428	*Jaspis splendens*	LMA		Dans lagon, face a la passe Boueni	12°55.163'S	44°59.233'E
Lp025	RMNH POR.8325	*Liosina paradoxa*	LMA	195.0 ± 3.0 OTUs	Ranikiki (recif corallien)	12°56.470'S	45°04.305'E
Lp067	RMNH POR.8367	*Liosina paradoxa*	LMA		Recif de Chira Le Poe	12°58.021'S	45°03.778'E
Lp127	RMNH POR.8427	*Liosina paradoxa*	LMA		Dans lagon, face a la passe Boueni	12°55.163'S	44°59.233'E
Ps100	RMNH POR.8400	*Petrosia* aff. *spheroida*	HMA	139.2 ± 2.1 OTUs	Passe Sada	12°54.141'S	44°57.862'E
Ps160	RMNH POR.8460	*Petrosia* aff. *spheroida*	HMA		Passe Bateau	12°58.653'S	44°58.949'E
Ps164	RMNH POR.8464	*Petrosia* aff. *spheroida*	HMA		Passe Bateau	12°58.653'S	44°58.949'E
Pb033	RMNH POR.8333	*Paratetilla bacca*	LMA	43.3 ± 3.4 OTUs	Ranikiki (recif corallien)	12°56.470'S	45°04.305'E
Pb052	RMNH POR.8352	*Paratetilla bacca*	LMA		Pointe Sud Mayotte	13°00.375'S	45°08.250'E
Pb060	RMNH POR.8360	*Paratetilla bacca*	LMA		Passe Boueni Sud	12°55.265'S	44°58.126'E
Sc023	RMNH POR.8323	*Stylissa carteri*	LMA	174.5 ± 6.3 OTUs	Ranikiki (recif corallien)	12°56.470'S	45°04.305'E
Sc044	RMNH POR.8344	*Stylissa carteri*	LMA		Pointe Sud Mayotte	13°00.375'S	45°08.250'E
Sc061	RMNH POR.8361	*Stylissa carteri*	LMA		Passe Boueni Sud	12°55.265'S	44°58.126'E
Sm145	RMNH POR.8445	*Stylissa massa*	LMA	91.9 ± 4.2 OTUs	N'Gouja	12°57.784'S	45°02.806'E
Sm153	RMNH POR.8453	*Stylissa massa*	LMA		N'Gouja	12°57.784'S	45°02.806'E
Sm155	RMNH POR.8455	*Stylissa massa*	LMA		N'Gouja	12°57.784'S	45°02.806'E
Xt154	RMNH POR.8454	*Xestospongia testudinaria*	HMA	152.9 ± 3.2 OTUs	N'Gouja	12°57.784'S	45°02.806'E
Xt162	RMNH POR.8462	*Xestospongia testudinaria*	HMA		Passe Bateau	12°58.653'S	44°58.949'E
Xt172	RMNH POR.8472	*Xestospongia testudinaria*	HMA		Pointe Kani	12°57.624'S	45°04.697'E

(Continues)

TABLE 1 (Continued)

Sample	Naturalis collection code Species	Type	Pooled rarefied richness (n = 4,600)	Location	Latitude	Longitude
Sd004	Sediment		243.3 ± 2.4 OTUs	Pointe Sud Mayotte	13°00.375'S	45°08.250'E
Sd005	Sediment			Passe Boueni Sud	12°55.265'S	44°58.126'E
Sd017	Sediment			Passe Bateau	12°58.653'S	44°58.949'E
Wt004	Seawater		104.3 ± 0.8 OTUs	Pointe Sud Mayotte	13°00.375'S	45°08.250'E
Wt005	Seawater			Passe Boueni Sud	12°55.265'S	44°58.126'E
Wt017	Seawater			Passe Bateau	12°58.653'S	44°58.949'E

the known HMA sponges: *Hyrtios erectus* (*He*) (family Thorectidae, order Dictyoceratida) and *Xestospongia testudinaria* (*Xt*) (family Petrosiidae, order Haplosclerida). All of these species are widespread species and have been observed from the Western Indian Ocean to the Pacific Ocean (Cleary et al., 2015; Coelho et al., 2018; Swierts et al., 2017). In addition, four species were investigated of which the HMA/LMA status was unknown: *Ectyoplasia coccinea* (*Ec*) (family Raspailiidae, order Axinellida), *Liosina paradoxa* (*Lp*) (family Dictyonellidae, order Bubarida), *Paratetilla bacca* (*Pb*) (family Tetillidae, order Tetractinellida), and *Petrosia* aff. *spheroida* (*Ps*) (family Petrosiidae order Haplosclerida). For the purposes of this study we preliminarily assigned them HMA or LMA status based on the status of their closest known relative using Gloeckner et al. (2014) and Moitinho-Silva et al. (2017). *Ectyoplasia coccinea* (new combination) was described from the Red Sea as *Reniera coccinea* and later transferred to *Dragmacidon* (as *Dragmacidon coccineum* also family Axinellidae). Examination of the type specimen revealed clavulate acanthostyles characteristic for the genus *Ectyoplasia*. The morphological identification was later confirmed with molecular techniques by Erpenbeck et al. (2016) as OTU030. This species has been observed from the Red Sea, Mauritius, and western Thailand by the first author. The sponge species *Petrosia. spheroida* has been observed in the Saudi Arabia's Red Sea, Mayotte, and Madagascar (Vacelet, Vasseur, & Lévi, 1976 and N.J. de Voogd pers. obs.). We conclude that the characters of this species are different from the original description by Tanita (1967) from Japan and, therefore, name this species *P.* aff. *spheroida*. The sponge species *P. bacca* and *L. paradoxa* are common and also widespread species in the Indo-Pacific region. The sponge samples included the surface and interior in order to sample as much of the bacterial community as possible. In addition to this, three sediment samples were taken from three different sites using mini cores; this consisted of sampling the top 5 cm of sediment with a plastic disposable syringe from which the end had been cut to facilitate sampling (Capone, Dunham, Horrigan, & Duguay, 1992). Also, three 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). All samples were kept in absolute alcohol and in a cooling box. After landing, tubes containing the samples were stored in a refrigerator at a temperature of about −7°C. In Portugal, the samples were stored at −80°C.

2.2 | 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. In brief, we prepared sediment samples by centrifuging each one for 30 min at 4,400 rpm and 4°C (to remove the absolute alcohol); the membrane filter (seawater sample) and sponge samples were each cut into small pieces. Where difficulties in extraction occurred a lysozyme pretreatment was performed (sediment and sponge samples). 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 the speed of 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. To generate highly replicable results and obtain a higher genetic diversity in pyrosequencing libraries (Berry, Mahfoudh, Wagner, & Loy, 2011; Vissers, Bodelier, Muyzer, & Laanbroek, 2009), a nested approach was used. Prior to pyrosequencing, the amplicons of the bacterial 16S rRNA gene were obtained using bacterial-specific primers 27F and 1494R (Gomes et al., 2010). 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′ (Yu, Lee, Kim, & Hwang, 2005 and V4 reverse degenerate primer 5′-TACNVRRGTHTCTAATYC-3′ (Vaz-Moreira, Egas, Nunes, & Manaia, 2011).

Following previous studies (Cleary et al., 2015; de Voogd et al., 2015), barcoded pyrosequencing libraries were analyzed using the QIIME (Quantitative Insights Into Microbial Ecology software package (Caporaso et al., 2010; https://www.qiime.org/; last checked 2014–01–20). In QIIME, separate fasta and qual files were used as input for the split_libraries.py script. Default arguments were used except for the minimum sequence length, which was set at 218 bps after removal of forward primers and barcodes; backward primers were removed using the "truncate only" argument and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script.

The minimum average qual score allowed in a read was the default value of 25. In addition to user-defined cutoffs, the split_libraries.py script performs several quality filtering steps (https://qiime.org/scripts/split_libraries.html). OTUs were selected using UPARSE with usearch7 (Edgar, 2013). The UPARSE sequence analysis tool (Edgar, 2013) provides clustering, chimera checking, and quality filtering on de-multiplexed sequences. Chimera checking was performed using the UCHIME algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011). The quality filtering as implemented in usearch7 filters noisy reads, and preliminary results suggest it gives results comparable to other denoisers such as AmpliconNoise but is much less computationally expensive (https://drive5.com/usearch/features.html; last checked 2014–01–20). First, reads were filtered with the -fastq_filter command and the following arguments -fastq_trunclen 250 -fastq_maxee 0.5 -fastq_truncqual 15. Sequences were then dereplicated and sorted using the -derep_fulllength and -sortbysize commands. This initial quality control produced a file with 241,019 sequences with a mean sequence length of 412.5 ± 35.6 bp and minimum and maximum sequence lengths of 250 and 493 bps, respectively. After quality control, OTU clustering was performed using the -cluster_otus command. Singletons were maintained in the analysis. AWK scripts were then used to convert the OTU files to QIIME format. In QIIME, representative sequences were selected using the pick_rep_set.py script in QIIME using the "most_abundant" method. Taxonomy was assigned to reference sequences of OTUs using default arguments in the assign_taxonomy.py script in QIIME with the rdp method (Wang, Garrity, Tiedje, & Cole, 2007). In the assign_taxonomy.py function, we used a fasta file containing reference sequences from the Greengenes 13_8 release and the rdp classifier method. We used a modified version of the taxonomy file supplied with the Greengenes 13_8 release to map sequences to the assigned taxonomy. All OTUs were assigned to the Bacteria domain and only 206 OTUs remained unassigned at the phylum level. Finally, we used the make_otu_table.py script in QIIME to generate a square matrix of OTUs x samples. This was subsequently used as input for further analyses using the R package (R Core Team, 2013).

2.3 | Higher taxon abundance

We tested for significant differences in the relative abundance of selected higher taxon groups (the most abundant classes and orders) among biotopes with an analysis of deviance using the generalized linear model glm() function in R. Because the data were proportional, we first applied a glm with the family argument set to binomial. The ratio, however, of residual deviance to residual *df* 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 over-dispersion. Using the glm model, we tested for significant variation among biotopes using the ANOVA() function in R (R Core Team, 2013) with the *F* test, which is most appropriate when the dispersion is estimated by moments as is the case with quasibinomial fits.

2.4 | Statistical analysis

A square matrix containing the presence and abundance of all OTUs per sample was imported into R using the read.table() function. Sequences classified as chloroplasts or mitochondria were removed prior to all statistical analysis. The OTU abundance matrix was $\log_e(x + 1)$ transformed, and a distance matrix constructed using the Bray–Curtis index with the vegdist() function in the vegan package in R (Oksanen et al., 2009). The Bray–Curtis index is one of the most frequently applied (dis)similarity indices used in ecology (Cleary, 2003). Variation in OTU composition among biotopes (sponge species, sediment, and seawater) was assessed with principal coordinates analysis (PCO) using the cmdscale() function in R with the Bray–Curtis distance matrix as input. We tested for significant variation in composition among biotopes using the adonis() permutational 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 on the first two PCO axes using the wascores() function in the vegan package. We used a self-written function in R (Gomes et al., 2010) to estimate rarefied OTU richness for each biotope (pooling the replicates per biotope).

2.5 | BLAST and phylogenetic analysis

We used the NCBI Basic Local Alignment Search Tool (BLAST) command line "blastn" tool with the -db argument set to nt to identify the most closely related organisms to numerically dominant OTUs (≥500 sequences) based on sequence similarity scores and bit scores (Zhang, Schwartz, Wagner, & Miller, 2000). See https://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html#head3 (last checked 2017 06 24) for detailed descriptions of sequence similarity and bit scores. A maximum-likelihood phylogenetic tree including all dominant OTUs (≥500 sequences) and selected cultured organisms was constructed using the Mega5 program (https://www.megasoftware.net/; last checked 02–07–2014; Tamura et al., 2011) with the Nearest-Neighbor-Interchange and Generalised Time-Reversible model (Tavaré, 1986) with Gamma distributed and invariant sites. Prior to this analysis, representative sequences of the dominant OTUs were aligned using the ClustalW algorithm in Mega5 (Higgins et al., 1994). In the results, we present a bootstrap consensus tree based on 100 replicates (Felsenstein, 1985). In addition to the phylogenetic tree, we also used the heatmap.2() function from the "gplots" library in R to create a heatmap of all dominant OTUs and their distribution across biotopes (pooling the replicates). Finally, we used the make_otu_network.py script in QIIME to generate network edge and node tables that were subsequently uploaded to Cytoscape version 3.2.1 (Shannon et al., 2003). In Cytoscape, we used the "organic layout" under the yfiles section (https://www.yworks.com/; last checked 11–11–2015). The "Analyze network" function, part of the Network Analyzer plugin, was used to map node size to edge count and edge size to edge weight. The size of the node is, thus,

proportional to the number of connections. A small OTU node, thus, indicates that the OTU in question is only found in a limited number of hosts. The edge size is proportional to the weight, which is a proxy for the abundance of an OTU. A thick edge connecting a biotope and an OTU indicates that the OTU in question was relatively abundant in that particular biotope. Network analysis can help to visualize relationships that may not be apparent using other techniques such as ordination and provide an efficient means of presenting complex information.

3 | RESULTS

In this study, sequencing yielded 216,364 sequences, assigned to 4,001 OTUs after quality control, OTU picking, and removal of chloroplasts and mitochondria. Most sequences belonged to OTUs assigned to Proteobacteria (123,983) followed by Cyanobacteria (39,490), Chloroflexi (21,722), Actinobacteria (15,031), Acidobacteria (3,395), and Gemmatimonadetes (2,846; Figure 3). There was a large degree of variation in the percentage

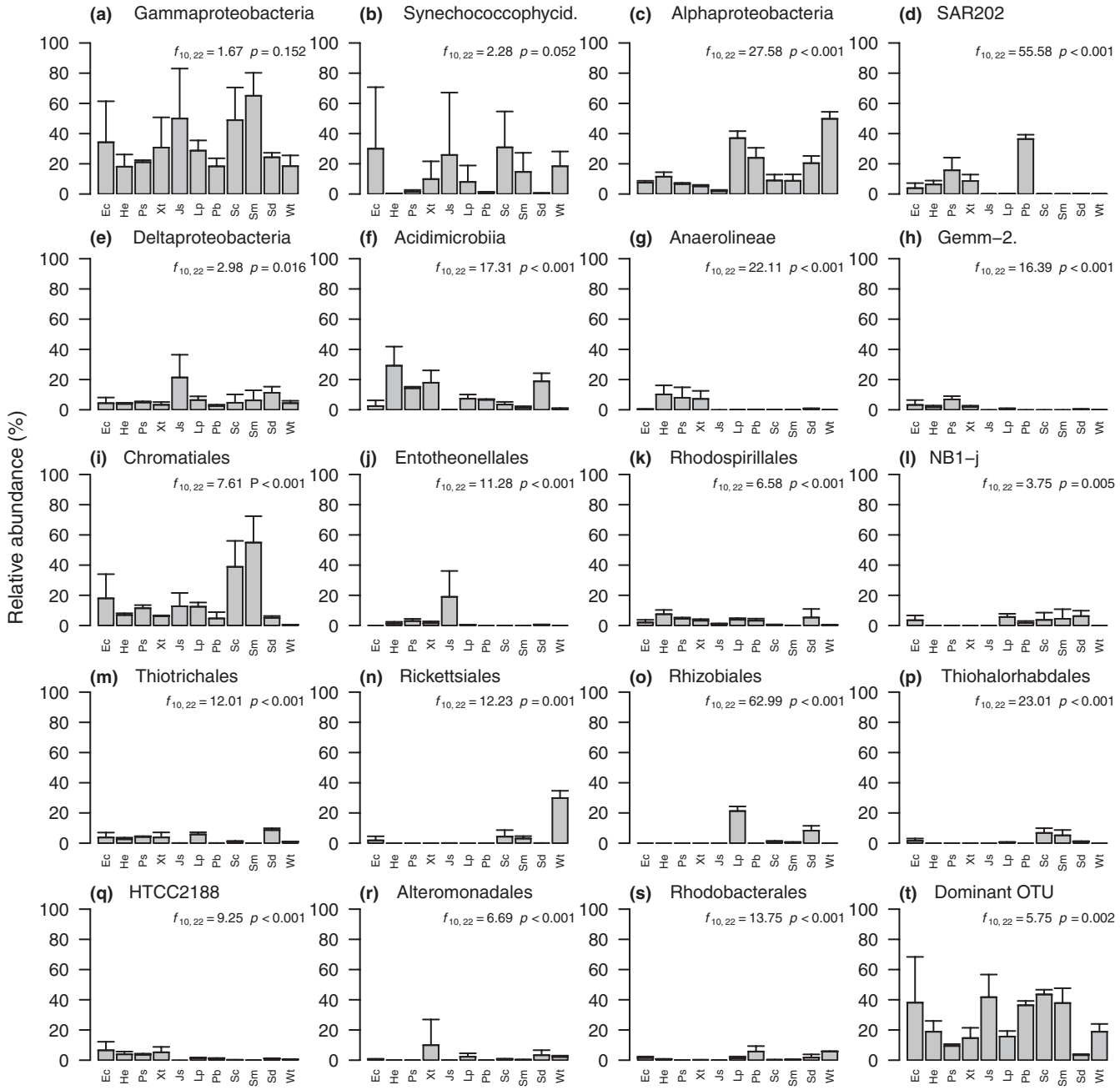

FIGURE 3 Mean relative abundance of the most abundant bacterial classes (a–h), orders (i–s) and the relative abundance of the most abundant OTU (t) from *Ectyoplasia coccinea* (Ec), *H. erectus* (He), *Petrosia* aff. *spheroida* (Ps), *Xestospongia testudinaria* (Xt),*Jaspis splendens* (Js), *Liosina paradoxa* (Lp), *Paratetilla bacca* (Pb), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), 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 the GLM are shown in the top-right corner of each subfigure

of sequences assigned to various phyla among biotopes. The percentage of Proteobacteria, for example, varied from 32.6% ± 1.5% in *Petrosia* aff. *spheroida* (Ps) to 80.3% ± 14.0% in *S. massa* (Sm). The percentage of Chloroflexi, in turn, varied from 0.1% ± 0.0% in *J. splendens* (Js) to 36.5% ± 2.9% in *P. bacca* (Pb). The total number of phyla recorded per biotope also varied considerably from 14 in *L. paradoxa* (Lp) to 33 in sediment. The number of classes varied from 23 in *H. erectus* to 82 in sediment and the number of orders varied from 29 in *H. erectus* (He) to 99 in sediment. At all three levels of taxonomic resolution, at least some LMA sponges housed more phyla (HMA: *H. erectus*: 17, *P.* aff. *spheroida*: 17, *X. testudinaria*: 18; LMA: Pb: 20, *S. carteri*:29), classes (HMA: *H. erectus*: 23, *P.* aff. *spheroida*: 25, *X. testudinaria*: 29; LMA: *P. bacca*: 38, *S. carteri*: 65) and orders (HMA: He: 29, Ps: 33, Xt: 40; LMA: Pb: 59, Sc:85) than HMA sponges. In all instances, sediment was the most diverse biotope with water housing more diverse bacterial communities (phyla: 16, classes: 32, orders: 50) than HMA sponges, but less diverse than several LMA sponges. The relative abundance of all higher taxa differed significantly among biotopes with the exception of the class Gammaproteobacteria and subclass Synechococcophycideae. For example, OTUs assigned to Entotheonellales were most abundant in *J. splendens* (Figure 3j), whereas OTUs assigned to the Chromatiales (Figure 3i) were most abundant in both *Stylissa* species. Certain taxa, notably Gemm−2, Thiotrichales, HTCC2188 (Figure 3h,m,q), were most abundant in HMA sponges and sediment and largely absent from LMA sponges and seawater. OTUs assigned to the Chloroflexi class SAR202 (Figure 3d) were absent in the LMA sponges *J. splendens*, *L. paradoxa*, both *Stylissa* species, sediment and seawater, but relatively abundant in all HMA sponges and the LMA sponge *P. bacca*. OTUs assigned to the Chloroflexi class Anaerolineae (Figure 3g) were largely restricted to the sponges *H. erectus*, *P.* aff. *spheroida*, and *X. testudinaria* and formed a small component of *E. coccinea*, *P. bacca*, and sediment. The relative abundance of the most abundant OTU (Figure 3t) in each sample was higher in LMA sponges

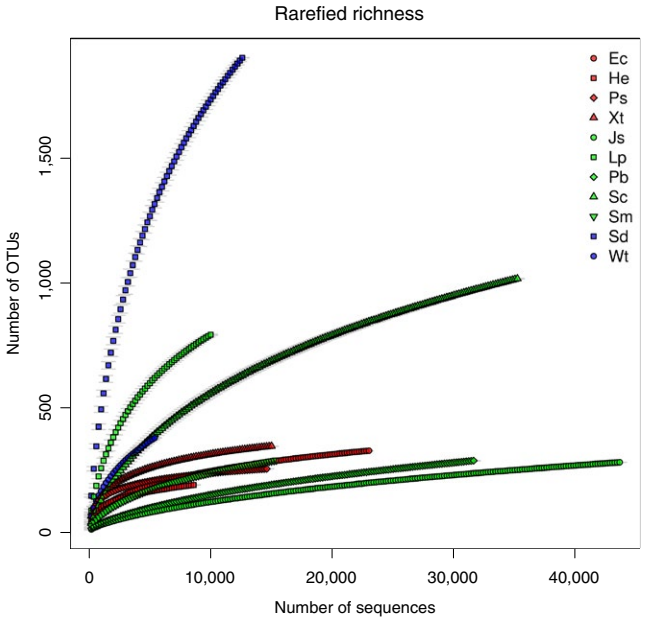

FIGURE 4 Rarefaction plot of OTU diversity for each biotope. *Ectyoplasia coccinea* (Ec), *Hyrtios erectus* (He), *Petrosia* aff. *spheroida* (Ps), *Xestospongia testudinaria* (Xt), *Jaspis splendens* (Js), *Liosina paradoxa* (Lp), *Paratetilla bacca* (Pb), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), *sediment* (Sd), *and seawater* (Wt)

(*J. splendens*: 41.6% ± 15.0%, *S. carteri*: 43.5% ± 3.1%), with the exception of *L. paradoxa* (15.6% ± 3.7%), than HMA sponges (*H. erectus*: 18.8% ± 7.2%, *X. testudinaria*: 14.6% ± 6.8%), with the exception of *E. coccinea* (38.1% ± 30.3%).

OTU richness followed this general pattern with some exceptions (Figure 4). Most biotopes, with the exception of sediment, seawater, *S. carteri*, and *L. paradoxa*, appeared to be approaching a richness asymptote. LMA sponges contained the least rich (*J. splendens* and *P. bacca*) and richest sponge bacterial communities (*S. carteri*). *Liosina paradoxa* (Lp) was interesting in having the richest bacterial community in terms of OTU richness but the poorest in terms of phylum

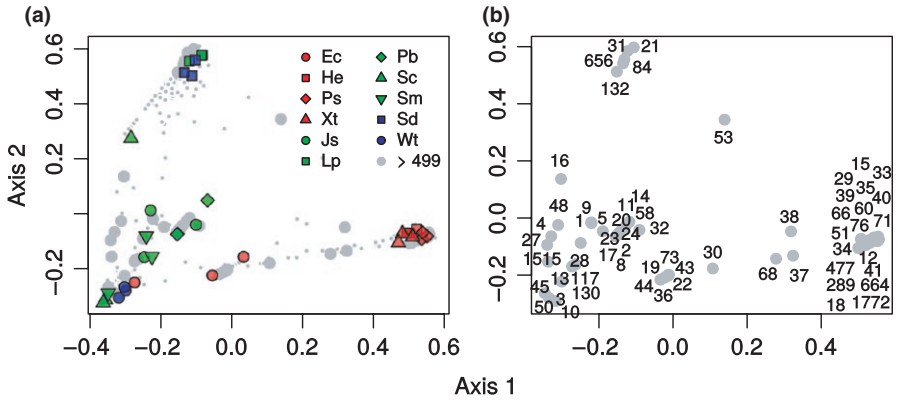

FIGURE 5 Ordination showing the first two axes of the PCO analysis. (a) Symbols represent samples from *Ectyoplasia coccinea* (Ec), *Hyrtios erectus* (He), *Petrosia* aff. *spheroida* (Ps), *Xestospongia testudinaria* (Xt), *Jaspis splendens* (Js), *paradoxa paradoxa* (Lp), *Paratetilla bacca* (Pb), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), sediment (Sd), and seawater (Wt). Very small light gray circles represent OTUs < 100 sequence reads; large light gray circles represents OTUs with ≥ 500 sequence reads; (b) numbers represent abundant (≥100 sequence reads) OTUs referred to in Table 2

richness. There was pronounced variation in the composition among individuals of certain biotopes. This was particularly evident in *E. coccinea* and *J. splendens* where the percentage of Cyanobacteria among individuals varied from 2.6% to 76.8% in *E. coccinea* and from 1.5% to 73.5% in *J. splendens*. In contrast, individuals of *P. bacca* harbored Proteobacteria, Chloroflexi, and Actinobacteria in similar relative abundances (Supporting Information Figure S1).

There was a highly significant difference in composition among biotopes (adonis: $F_{10,22} = 11.05$, $p < 0.001$, $R^2 = 0.834$). Variation among biotopes thus explained >83% of the variation in composition. The first PCO axis separated the *H. erectus*, *X. testudinaria*, and *P.* aff. *spheroida* from all other samples, and the second axis separated sediment and *L. paradoxa* samples from remaining samples (Figure 5). A number of abundant OTUs were found predominantly or exclusively in *H. erectus*, *P.* aff. *spheroida*, and *X. testudinaria* (Figure 6). These included OTUs 12, 15, and 1772 assigned to the Actinobacteria, OTU-33 assigned to the Acidobacteria, OTUs 35, 40, 60, 66, and 664 assigned to the Chloroflexi, OTUs 76 and 289 assigned to the Proteobacteria, OTU-39 assigned to SBR1093 and OTU-41 that was unclassified at the phylum level. All of these OTUs were closely related (sequence similarity >98%) to organisms previously found in other sponges including *X. testudinaria* from Indonesia (Table 2). Actinobacterial OTUs in HMA sponges and *P. bacca* also formed a well-supported cluster distinct from the only abundant actinobacterial OTU (OTU-45) in the other LMA sponges. The Actinobacteria in HMA sponges and *P. bacca* clustered together with two cultured organisms, *Ferrimicrobium acidiphilum* and *Acidimicrobium ferrooxidans*. OTUs associated with sediment and *L. paradoxa* samples included OTUs 21, 84, and 656 assigned to the Alphaproteobacteria, OTU-132 assigned to the gammaproteobacterial order Thiotrichales and OTU-31 assigned to the genus *Synechococcus*. OTUs 21 and 656 were both assigned to the family Phyllobacteriaceae and were closely related (sequence similarity >98%) to organisms found in the sponges *Corticium candelabrum* and *Haliclona* (*Gellius*) sp. OTU-21 was also strongly enriched in *L. paradoxa* compared to sediment (1,403 sequences in *L. paradoxa* vs. five sequences in sediment).

The third PCO axis separated samples of *P. bacca* from all other samples and the fourth PCO axis separated *J. splendens* and *S. massa* from the remaining samples (Supporting Information Figure S2). *Paratetilla bacca* housed a number of abundant OTUs that were predominantly or exclusively found there. The fifth PCO axis separated samples of *S. massa* from samples of *J. splendens* (Supporting

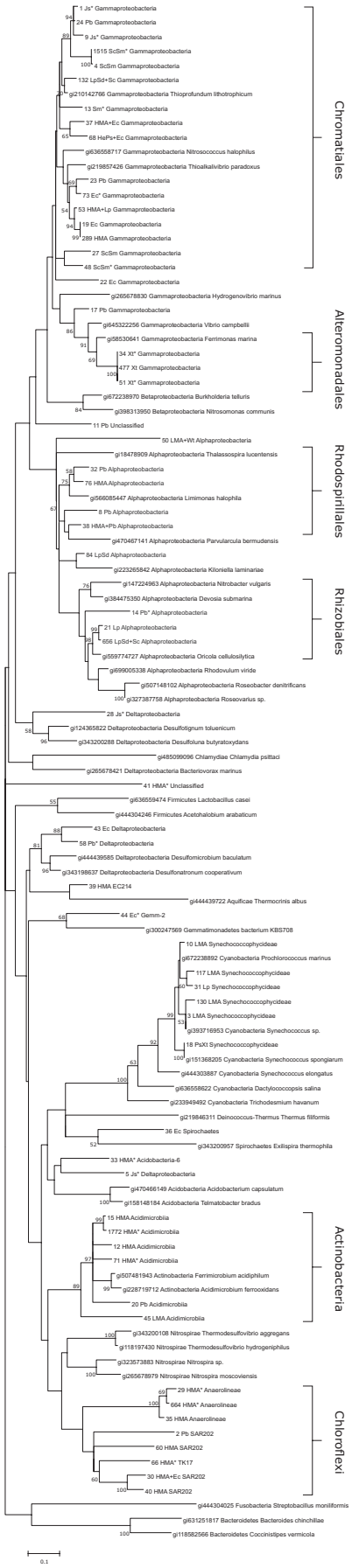

FIGURE 6 Phylogenetic tree of the bacterial 16S rRNA gene sequences recovered from sponges, *(Ectyoplasia coccinea*, *Hyrtios erectus*, *Petrosia aff. spheroida*, *Xestospongia testudinaria*, *Jaspis splendens*, *Liosina paradoxa*, *Paratetilla bacca*, *Stylissa carteri*, *Stylissa massa)* seawater (Wt), and sediment (Sd); bootstrap values lower than 50% were omitted. The number of each OTU is indicated as are GenBank GenInfo sequence identifiers of cultured bacterial sequences. Phyla and orders of Bacteria are indicated. OTUs are assigned to the following clusters HMA (Ps), (Xt), (Ec) and (He), LMA (Pb), (Js), (Lp), (Sc) and (Sm), Seawater (Wt), and Sediment (Sd)

TABLE 2 List of most abundant OTUs (≥500 sequences) including OTU number (OTU), total sequences (Sum), biotope or biotopes where OTU was (mainly) found (Group), taxonomic affiliation of OTU, GenBank Geninfo sequence identifiers (GI) of closely related organisms identified using BLAST, sequence identity (Seq) of those organisms with our representative OTU sequences, isolation source of closely related organisms identified using BLAST. In the 'Group' category, OTUs restricted to a given biotope or biotopes are indicated by an asterisk (*). Ectyoplasia coccinea (Ec), H. erectus (He), Petrosia aff. spheroida (Ps), Xestospongia testudinaria (Xt), Jaspis splendens (Js), paradoxa paradoxa (Lp), Paratetilla bacca (Pb), Stylissa carteri (Sc), Stylissa massa (Sm), , sediment (Sd), and seawater (Wt), high microbial abundance sponges (HMA), low microbial abundance sponges (LMA)

OTU	Sum	Group	Phylum	Class	Order	Family
1	14,810	Js*	Proteobacteria	Gammaproteobacteria	Unclassified	Unclassified
2	11,617	Pb	Chloroflexi	SAR202	Unclassified	Unclassified
3	19,296	LMA	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae
4	19,031	ScSm	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified
5	5,790	Js*	Proteobacteria	Deltaproteobacteria	[Entotheonellales]	[Entotheonellaceae]
8	4,274	Pb	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified
9	5,266	Js*	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified
10	6,718	LMA	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae
11	2,855	Pb	Proteobacteria	Unclassified	Unclassified	Unclassified
12	2,852	HMA	Actinobacteria	Acidimicrobiia	Acidimicrobiales	TK06
13	2,223	Sm*	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified
14	1667	Pb*	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae
15	2,338	HMA	Actinobacteria	Acidimicrobiia	Acidimicrobiales	wb1_P06
16	1899	ScSm*	Proteobacteria	Deltaproteobacteria	NB1-j	NB1-i
17	2,364	Pb	Proteobacteria	Gammaproteobacteria	Unclassified	Unclassified
18	1790	PsXt	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae
19	2,894	Ec	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae
20	2067	Pb	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Unclassified
21	1,410	Lp	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae
22	1,378	Ec	Proteobacteria	Gammaproteobacteria	HTCC2188	HTCC2089
23	1,408	Pb	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae
24	1814	Pb	Proteobacteria	Gammaproteobacteria	Unclassified	Unclassified
27	3,252	ScSm	Proteobacteria	Gammaproteobacteria	Thiohalorhabdales	Unclassified
28	1,275	Js*	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae
29	1,322	HMA*	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae
30	806	HMA + Ec	Chloroflexi	SAR202	Unclassified	Unclassified
31	1,038	Lp	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae
32	745	Pb	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
33	939	HMA*	Acidobacteria	Acidobacteria–6	BPC015	Unclassified
34	661	Xt*	Proteobacteria	Gammaproteobacteria	Alteromonadales	Unclassified
35	770	HMA	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae

Genus	OTU	GI	Seq	Source	Location
Unclassified	1	295,639,186	95.97	Sponge: *Aplysina fulva*	Bahamas: Sweetings Cay, Mangrove
Unclassified	2	400,269,182	99.05	Sponge: *Cinachyra* sp.	
Synechococcus	3	786,319,984	99.76	Sea water from G−9 station(depth = 0 m)	
Unclassified	4	407,913,000	100	Sponge: *Stylissa carteri*	
Unclassified	5	334,303,082	95.08	Medea hypersaline basin, Mediterranean Sea	
Unclassified	8	400,269,153	98.82	Sponge: *Cinachyra* sp.	
Unclassified	9	400,269,037	95.02	Sponge: *Cymbastella coralliophila*	
Prochlorococcus	10	672,374,773	99.76	Seawater	West Pacific
Unclassified	11	441,084,656	90.87	Sponge: *Dysidea avara*	Mediterranean Sea: Medas Islands
Unclassified	12	768,028,613	100	Coral: *Porites lutea*	
Unclassified	13	597,437,727	99.78	Sponge: *Axinella* sp.	
Unclassified	14	400,269,113	94.77	Sponge: *Coelocarteria singaporensis*	
Unclassified	15	768,028,476	99.76	Coral: *Porites lutea*	
Unclassified	16	407,912,992	100	Sponge: *Stylissa carteri*	
Unclassified	17	400,269,041	95.3	Sponge: *Cymbastella coralliophila*	
Synechococcus	18	308,217,458	99.76	Sponge: *Xestospongia muta*	
Unclassified	19	678,605,864	98.43	Sponge: *Astrosclera willeyana*	
Unclassified	20	384,161,909	99.53	Sponge: *Cinachyra* sp.	
Unclassified	21	82,470,213	98.58	Sponge: *Corticium candelabrum*	
Unclassified	22	110,265,023	98.66	Sponge: larva marine sponge	
Unclassified	23	745,791,420	96.66	Sponge: Plakortis halichondrioides	
Unclassified	24	295,639,186	95.02	Sponge: Aplysina fulva	
Unclassified	27	407,912,993	98.34	Sponge: *Stylissa carteri*	
Bdellovibrio	28	350,627,483	96.71	Sponge: *Xestospongia muta*	
Unclassified	29	526,299,835	98.82	Sponge: taxon: 166,587	
Unclassified	30	295,639,177	98.82	Sponge: *Aplysina fulva*	
Synechococcus	31	82,470,805	99.29	?	?
Unclassified	32	195,945,265	97.16	Sponge: *Aplysina fulva*	
Unclassified	33	400,269,348	100	Sponge: *Xestospongia testudinaria*	
Unclassified	34	283,831,330	98.21	Sponge	
Unclassified	35	350,627,534	100	Sponge: *Xestospongia testudinaria*	

(Continues)

TABLE 2 (Continued)

OTU	Sum	Group	Phylum	Class	Order	Family
36	934	Ec	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae
37	1502	HMA + Ec	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae
38	836	HMA + Pb	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
39	776	HMA	SBR1093	EC214	Unclassified	Unclassified
40	617	HMA	Chloroflexi	SAR202	Unclassified	Unclassified
41	517	HMA*	Unclassified	Unclassified	Unclassified	Unclassified
43	722	Ec	Proteobacteria	Deltaproteobacteria	NB1-j	MND4
44	737	Ec*	Gemmatimonadetes	Gemm−2	Unclassified	Unclassified
45	1,011	LMA	Actinobacteria	Acidimicrobiia	Acidimicrobiales	OCS155
48	786	ScSm*	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified
50	2,338	LMA + Wt	Proteobacteria	Alphaproteobacteria	Rickettsiales	Pelagibacteraceae
51	516	Xt*	Proteobacteria	Gammaproteobacteria	Alteromonadales	Unclassified
53	830	HMA + Lp	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae
58	574	Pb*	Proteobacteria	Deltaproteobacteria	NB1-j	Unclassified
60	732	HMA	Chloroflexi	SAR202	Unclassified	Unclassified
66	866	HMA*	Chloroflexi	TK17	TK18	Unclassified
68	519	HePs + Ec	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae
71	559	HMA*	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Unclassified
73	543	Ec*	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified
76	684	HMA	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
84	746	LpSd	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified
117	5,558	LMA	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae
130	3,997	LMA	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae
132	549	LpSd + Sc	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae
289	780	HMA	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae
477	683	Xt	Proteobacteria	Gammaproteobacteria	Alteromonadales	Unclassified
656	533	LpSd + Sc	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae
664	791	HMA*	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae
1515	932	ScSm*	Proteobacteria	Gammaproteobacteria	Chromatiales	Unclassified
1772	634	HMA*	Actinobacteria	Acidimicrobiia	Acidimicrobiales	wb1_P06

Information Figure S3). This was primarily related to the presence of OTUs only found in those hosts, and thus possibly species specific (*S. massa*: OTU-13; *J. splendens*: OTUs 1, 5, 9, 28). The four OTUs restricted to *J. splendens* also only had sequence similarities varying from 95.02 to 96.71 (Table 2) and thus may represent novel taxa. In addition to the above, there were a number of other abundant OTUs restricted to certain species or genera. These included OTUs 16, 786, and 1,515 restricted to both *Stylissa* species; OTUs 34, 51, and 477 restricted to *X. testudinaria* and OTU-44 restricted to *E. coccinea* (Figure 6). OTUs 16, 786, and 1,515 were assigned to the Chromatiales and NB1-j orders and were similar (sequences similarity >99%) to organisms obtained from the sponges *S. carteri* in the Red Sea and *Axinella* spp. from the Caribbean and China. The second most abundant OTU overall, OTU-4, was largely restricted to both *Stylissa* species (19,028 sequences in both *Stylissa* species vs. three sequences in *J. splendens*) and assigned to the order Chromatiales. It is closely related (sequence similarity = 100%) to an organism found in *S. carteri* from the Red Sea (Table 2). LMA

Genus	OTU	GI	Seq	Source	Location
Unclassified	36	678,605,894	98.44	Sponge: *Astrosclera willeyana*	
Unclassified	37	678,605,908	99.33	Sponge: *Astrosclera willeyana*	
Unclassified	38	559,767,691	98.82	Sponge: *Holoxea* sp.	
Unclassified	39	559,767,717	100	Sponge: *Holoxea* sp.	
Unclassified	40	678,605,861	99.76	Sponge: *Astrosclera willeyana*	
Unclassified	41	134,290,601	98.58	Sponge: *Xestospongia muta*	
Unclassified	43	338,186,100	93.72	Paddy rice soil	
Unclassified	44	134,290,488	95.1	Sponge: *Ectyoplasia ferox*	
Unclassified	45	672,374,888	99.52	Seawater	West Pacific
Unclassified	48	209,364,851	99.55	Sponge: *Axinella corrugata* (sponge 4)	
Unclassified	50	827,025,978	100	Seawater surface	Red Sea
Unclassified	51	646,280,565	97.55	Sponge: *Arenosclera brasiliensis*	
Unclassified	53	379,771,393	98.21	Sponge: *Geodia barretti*	
Unclassified	58	400,269,180	99.29	Sponge: *Cinachyra* sp.	
Unclassified	60	345,330,237	99.29	Sponge: *Rhopaloeides odorabile*	
Unclassified	66	526,299,944	98.82	Sponge: *Aplysina cauliformis*	
Unclassified	68	511,630,187	99.78	Sponge: *Vaceletia crypta*	
Unclassified	71	768,028,817	99.53	Coral: *Porites lutea*	
Unclassified	73	379,771,373	98.22	Sponge: *Geodia barretti*	
Unclassified	76	678,605,876	99.76	Sponge: *Astrosclera willeyana*	
Unclassified	84	333,799,055	99.53	Permeable coral reef sands	
Synechococcus	117	700,288,759	99.76	Saline lake water	Croatia
Synechococcus	130	597,437,734	99.53	Sponge: *Axinella* sp.	
Unclassified	132	571,134,685	99.55	Marine coastal sediment	
Unclassified	289	451,353,954	100	Sponge: Ircinia strobilina	
Unclassified	477	646,280,563	98.88	Sponge: *Arenosclera brasiliensis*	
Unclassified	656	334,847,231	99.76	Coral: *Siderastrea stellata*	
Unclassified	664	350,627,590	99.76	Sponge: *Xestospongia testudinaria*	
Unclassified	1515	597,437,717	100	Sponge: *Axinella* sp.	
Unclassified	1772	350,627,490	99.76	Sponge: *Xestospongia muta*	

and HMA sponges housed a phylogenetically diverse community of Chromatiales including a well-supported cluster of three OTUs found in *J. splendens* and *P. bacca* (OTUs 1, 9, and 24), and a cluster of two OTUs of which OTU-19 was found mainly in *E. coccinea* and OTU-289 mainly in the three HMA sponges (Figure 7).

A network showing relationships between OTUs and biotopes is presented in Figure 8 whereby the size of the biotope or OTU symbol indicates the number of connections between biotopes and OTUs. OTUs with larger symbols were thus found in more biotopes. The thickness of the lines connecting biotopes and OTUs, in turn, is a function of the number of sequences for a particular OTU in a particular biotope. OTUs in the center of the network were thus found in a large number of biotopes. This includes the most abundant OTU overall, OTU-3 assigned to the genus *Synechococcus* and most abundant in *E. coccinea* (6,579 sequences), *J. splendens* (5,155 sequences), *S. carteri* (4,485 sequences), *S. massa* (2,307 sequences), and seawater (538 sequences). Most of the OTUs present in numerous biotopes were assigned to Cyanobacteria and Proteobacteria.

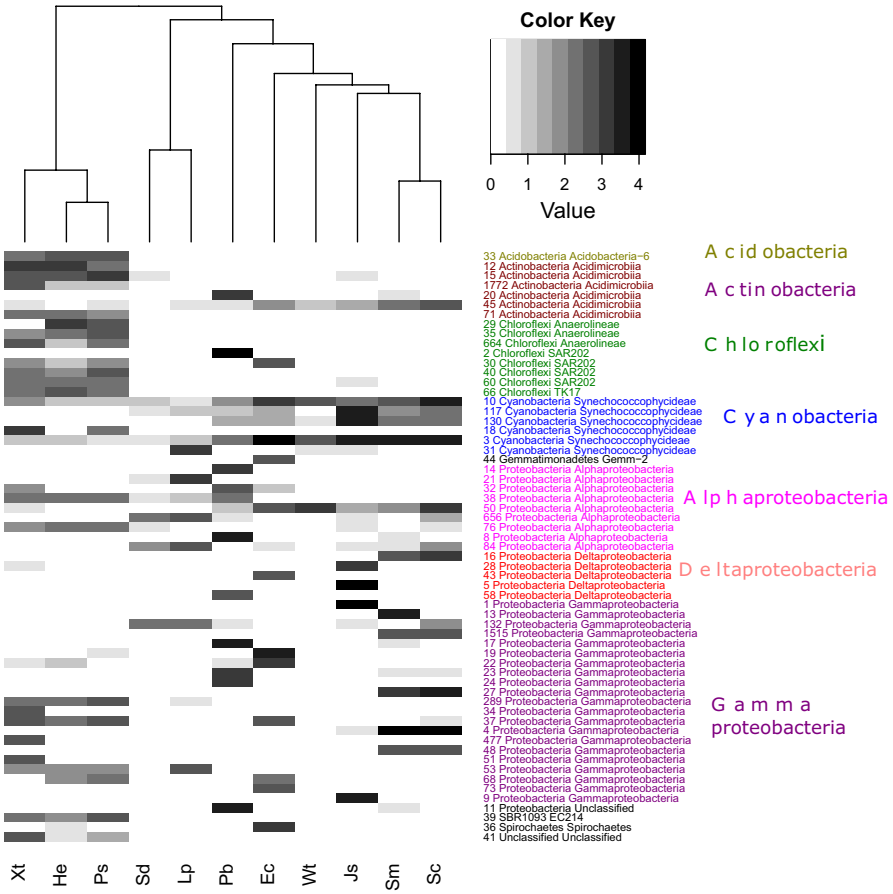

FIGURE 7 Heatmap of the most abundant (≥500 sequences) OTUs (rows) in each biotope (column). The number of sequences of a given OTU in each biotope is indicated by a color key using a logarithmic scale. The OTU number and assigned phylum and orders are given. Biotopes were clustered based on OTU similarity using the Bray–Curtis distance. Xestospongia testudinaria (Xt), H. erectus (He), Petrosia aff. spheroida (Ps), sediment (Sd), Liosina paradoxa (Lp), Paratetilla bacca (Pb), Ectyoplasia coccinea (Ec), seawater (Wt), Jaspis splendens (Js), Stylissa massa (Sm), Stylissa carteri (Sc)

The cyanobacterial OTUs, assigned to the genera *Synechococcus* and *Prochlorococcus*, were found predominantly in LMA sponges and seawater. Interestingly, the main cyanobacterial symbiont in *X. testudinaria* and *P.* aff. *spheroida* (OTU-18) formed a well-supported cluster with *Synechococcus spongiarum*. The network reflects the ordination results with the three HMA species sharing a large number of OTUs. Likewise, the LMA sponges shared a large number of OTUs with one another and with seawater. In the ordination, *E. coccinea*, although presumably a HMA species, clustered with the LMA sponges. In the network, it is apparent that *E. coccinea* houses a more distinct bacterial community sharing a subset of OTUs with HMA species. These included OTUs 19, 43, and 68 assigned to the Gamma- and Deltaproteobacteria, OTUs 30 and 35 assigned to SAR202 and Anaerolineae, and OTU-36 assigned to the Spirochaetes. All of these OTUs were closely related (sequence similarity >98%) to organisms previously found in sponges, including the species *Astrosclera willeyana*, *Geodia barrretti*, and *Ectyoplasia ferox*, with the exception of OTU-43.

4 | DISCUSSION

With the emergence of deep sequencing, it has become possible to obtain a more comprehensive picture of the microbial diversity associated with sponges. Here, we used 454-pyrosequencing to explore the bacterial communities of several biotopes including sponges, seawater, and sediment, in a coral reef system located in the understudied Western Indian Ocean. Proteobacteria were, by far, the most abundant taxa in terms of both sequences and OTUs, although some samples were dominated by Cyanobacteria, Chloroflexi, or Actinobacteria. A number of potentially novel taxa were identified with relatively low sequence similarity to organisms in GenBank. It is generally assumed that LMA sponges are characterized by a low phylum-level diversity with dominant phyla belonging to Proteobacteria and Cyanobacteria (Giles et al., 2013; Hentschel et al., 2006; Moitinho-Silva et al., 2014; Poppell et al., 2014). However, in the present study, this was complemented by Chloroflexi and Actinobacteria in *P. bacca*. Moreover, this sponge together with *S. carteri* housed a higher bacterial diversity at the phylum, class, and order level than the sponges *H. erectus*, *P.* aff. *spheroida*, and *X. testudinaria*. The Chloroflexi clade SAR202 (mainly OTU-2) was particularly abundant in *P. bacca* with 11,617 sequences, and OTU-2 had a sequence similarity of 99.05% to an organism previously found in *Cinachyra* from Australia. These sponges, together with *Cinachyrella*, are all closely related. Sponges belonging to these genera are difficult to identify in the field, because a lack of diagnostic features hampers identification using traditional morphological characters (Chambers, Padovan, Alvarez, & Gibb, 2013; Cuvelier et al., 2014). In the recent past, it was shown that these sponges could be identified based on their distinct bacterial community even over a wide geographic range (Chambers et al., 2013). In our study, we were able to assign our samples to a single morphospecies; interestingly, the different individuals of *P. bacca*

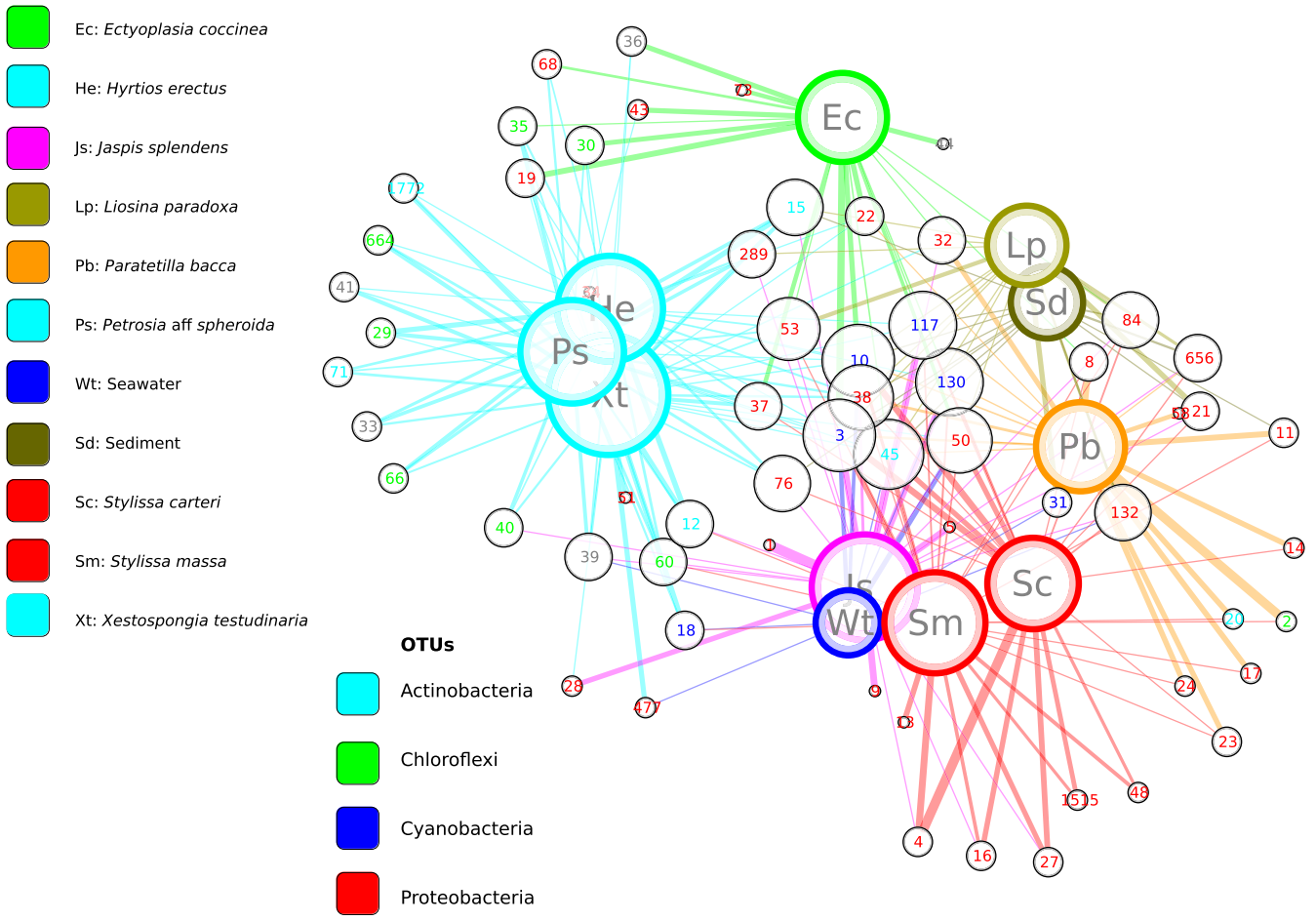

FIGURE 8 Network of biotopes (letters) and OTUs (numbers) constructed using cytoscape based on an OTU table of the most abundant (≥500 sequences) OTUs. The size of the biotope symbol indicates the total number of sequences; the size of the OTU symbol indicates the number of connections to separate biotopes. The thickness of the line connecting a biotope and OTU indicates the number of sequences of a give OTU in a given biotope. Finally, the color of the lines indicates the biotope to which a given OTU is connected. Just three sponge species (Xt, Sc, and Ps) harbored more than 84% of all dominant OTUs

harbored Proteobacteria, Chloroflexi, and Actinobacteria in almost identical relative abundances, suggesting that the bacterial community is well conserved in this species and comparison with samples of this species from a wider geographic range would be interesting to check whether the species indeed has a specific microbial signature. The HMA sponges *X. testudinaria*, *H. erectus*, and presumed HMA sponge *P.* aff. *spheroida* were dominated by OTUs assigned to the phyla Actinobacteria and Chloroflexi, Acidobacteria, Proteobacteria, and the candidate phylum SBR1093, as found previously in other studies (Kamke, Taylor, & Schmitt, 2010; Schmitt et al., 2012).

Sponge morphology has been proposed to be an important determinant of the HMA/LMA dichotomy. HMA sponges are large, massive, and have a firm touch and fleshy consistency, whereas LMA sponges are generally smaller and feel fragile, soft and brittle (U. Hentschel pers. obs in Gloeckner et al., 2014). Indeed, both *X. testudinaria* and *P.* aff. *spheroida* have very similar morphologies; both are large and massive. *Hyrtios erectus*, another HMA sponge, however, forms small firm digits and is embedded in the sediment. The sponge *J. splendens* and *E. coccinea* are very similar in morphology forming irregular lumpy encrustations with elevated oscules and are very soft and brittle. *Jaspis splendens* forms a clear cluster with seawater, *S. massa* and *S. carteri*. However, *E. coccinea* is clearly different, sharing a bacterial community with HMA sponges, but also with LMA sponges. In addition to this, it has two abundant OTUs confined to this species, namely OTU-44 and 73. OTU-44 belongs to the class Gemm-2, and a related OTU was previously isolated from the Caribbean *Ectyoplasia ferox* (Schmitt, Angermeier, Schiller, Lindquist, & Schmitt, 2008). Although *Ectyoplasia* is considered to be a HMA sponge by Gloeckner et al. (2014) and Schmitt et al. (2008), it clearly falls outside the HMA cluster. In the recent past, Easson and Thacker (2014) also showed that the Caribbean sponge species *Ecyoplasia ferox* contains a unique and diverse microbial community with several dominant Proteobacteria OTUs. The LMA sponges *S. massa*, *P. bacca*, and *J. splendens* housed abundant, possibly species-specific taxa. Both *Stylissa* spp., although soft, are not fragile, brittle, or small in appearance and are in our opinion true LMA sponges.

Ambient seawater is often assessed for microbial communities in order to detect seed banks for the colonization and acquisition of

symbionts specifically for LMA sponges; however, the benthic substrate is often overlooked. In the present study, most sponge species sampled were embedded in the reef substrate, and we therefore also sampled the reef sediment to assess its bacterial community. Although the sediment bacterial community was characterized by a higher phylum diversity, its community structure was highly similar to that of the sponge *L. paradoxa*. This species had a low phylum-level richness, but a very high OTU richness and cannot be categorized as either a LMA or HMA sponge. This sponge species forms clusters of large tubes with a very peculiar sandy–muddy surface and incorporates extraneous material in its skeleton. OTU-21 and OTU-656 were observed in both sediment and *L. paradoxa* and are assigned to the order Rhizobiales (family Phyllobacteriaceae). OTU-21 is closely related to the novel taxon *Oricola cellulosilytica*, which was very recently described from surface seashore seawater in Taiwan (Hameed et al., 2015). However, the ecological relevance of many Phyllobacteriaceae representatives remains largely unknown. It is also unclear whether these OTUs are transversal or whether they actually are part of the sponge bacteriome. Interestingly, the skeleton of *H. erectus* is, like *L. paradoxa*, composed of a crust of exogenous material and consists of sponge fibers filled with extraneous detritus, sediment grains, and foreign spicules. However, although *H. erectus* is embedded in the reef sediment, its bacterial community is highly distinct from the sediment bacterial community.

Based on the present study, we conclude that a clear dichotomy between HMA and LMA sponges does not appear to exist. However, certain HMA sponges (*X. testudinaria*, *P.* aff. *spheroida*, and *H. erectus*) are clearly distinguished by sharing very similar bacterial communities dominated by OTUs assigned to the Actinobacteria and Chloroflexi among others. Certain LMA sponges housed a bacterial community that was similar to the surrounding environment (seawater) but also included highly abundant OTUs that may be species or genus-specific. These OTUs mostly belonged to the Proteobacteria and Cyanobacteria, and relative abundance varied considerably among individuals.

Many microbial taxa found in sponges also occur at very low abundances in seawater, which might serve as a seed bank for sponges (Taylor et al., 2013). Microbes might also be leached into the sea by physical damage or by the expulsion of the reproductive material during spawning (Gloeckner, Lindquist, Schmitt, & Hentschel, 2013). In conclusion, the marine bacterial community seems to consist of a complex network of bacterial taxa with the host (e.g., sponge species) and non-host (e.g., sediment and seawater) biotopes harboring partially overlapping bacterial members.

ACKNOWLEDGEMENTS

This study was financed through the ANR-Netbiome under grant No ANR-11-EBIM-0006 and by European Funds through COMPETE (FCOMP-01-0124-FEDER-008657) and by National Funds through the Portuguese Science Foundation (FCT) within the LESS CORAL project [PTDC/AAC-AMB/115304/2009]. Thanks are due for the financial support to CESAM (UID/AMB/50017 - POCI-01-0145-FEDER-007638), to FCT/MCTES through national funds (PIDDAC), and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. Ana R.M. Polónia was supported by a postdoctoral scholarship (SFRH/BPD/117563/2016) funded by FCT, Portugal within the Human Capital Operational Programme (HCOP), subsidized by the European Social Fund (ESF) and National funds MCTES. Research permits were issued via Terres Australes en Antartiques françaises (TAAF). We thank Cécile Debitus, Bruno Fichou, Stephan Aubert, Philippe Prost, and Jean-Pierre Bellanger for their support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

N.J.d.V. and D.F.R.C. designed the study; N.J.d.V. and A.G.B. collected the samples; A.R.M.P. performed the laboratory work; D.F.R.C. and A.R.M.P. performed the data analysis; N.J.d.V, A.G.B., A.R.M.P., and D.F.R.C. wrote the manuscript.

DATA ACCESSIBILITY

The DNA sequences generated in this study can be downloaded from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA): Accession no. SRP071901.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: de Voogd NJ, Gauvin-Bialecki A, Polónia ARM, Cleary DFR. Assessing the bacterial communities of sponges inhabiting the remote western Indian Ocean island of Mayotte. *Mar Ecol*. 2018;39:e12517. https://doi.org/10.1111/maec.12517