Sponges from Zanzibar host diverse prokaryotic communities with potential for natural product synthesis

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Abstract

Sponges are one of the most dominant organisms in marine ecosystems. One reason for their success is their association with microorganisms that are besides the host itself responsible for the chemical defence. Sponge abundances have been increasing on coral reefs in the Western Indian Ocean (WIO) and are predicted to increase further with rising anthropogenic impacts on coral reefs. However, there is a paucity of information on chemical ecology of sponges from the WIO and their prokaryotic community composition. We used a combination of Illumina sequencing and a predictive metagenomic analysis to (1) assess the prokaryotic community composition of sponges from Zanzibar, (2) predict the presence of KEGG metabolic pathways responsible for bioactive compound production and (3) relate their presence to the degree of observed chemical defence in their respective sponge host. We found that sponges from Zanzibar host diverse prokaryotic communities that are host species-specific. Sponge-species and respective specimens that showed strong chemical defences in previous studies were also predicted to be highly enriched in various pathways responsible for secondary metabolite production. Hence, the combined sequencing and predictive metagenomic approach proved to be a useful indicator for the metabolic potential of sponge holobionts.

Introduction

Sponges harbour the highest diversity of prokaryotic symbionts in any invertebrate host recorded to date with at least 63 to 72 prokaryotic phyla and candidate phyla described (Webster and Thomas, 2016; Moitinho-Silva *et al.*, 2017). Sponge hosts are assumed to select their specific prokaryotic community and both partners use mechanisms to maintain their association (Hentschel *et al.*, 2012; Webster and Thomas, 2016). The densities of prokaryotic symbionts can approach up to 10^{10} cells per gram of sponge wet weight in high microbial abundance (HMA) sponges (Hentschel *et al.* 2003; Hentschel *et al.* 2006; Gloeckner *et al.*

2014). In contrast, low microbial abundance (LMA) sponges host only 10^5 - 10^6 cells per gram of sponge wet weight, closely resembling the abundances of prokaryotic communities in the surrounding seawater (Hentschel *et al.* 2006; Weisz *et al.* 2007). The sponge microbiome in both, LMA and HMA sponges, consists partially of unique prokaryotes that are rarely found outside their sponge host in free-living communities (Hentschel *et al.*, 2012; Simister *et al.*, 2012; Thomas *et al.*, 2016).

These associations with prokaryotes likely enabled sponges to be one of the dominant benthic components in many ecosystems worldwide since they add to the fitness of the sponge host (Freeman and Thacker, 2011; Hentschel *et al.*, 2012; Pawlik *et al.*, 2016; Pita *et al.*, 2018). The abundances of sponges in coral reefs exposed to increasing anthropogenic stressors are rising in recent years since sponges are able to cope better with global and local anthropogenic stressors than corals (Fabricius *et al.*, 2011; Bell *et al.*, 2013). Their prokaryotic communities are to a certain extent stable across changes in nutrient concentration (Ward-Paige *et al.*, 2005; Luter *et al.*, 2014), temperature (Simister *et al.*, 2012; Pita *et al.*, 2013), low-pH conditions (Ribes *et al.*, 2016) and light exposure (Cárdenas *et al.*, 2014) as well as along geographical (Steinert *et al.*, 2016; Thomas *et al.*, 2016) and temporal (Hardoim and Costa, 2014b; Erwin *et al.*, 2015) gradients. Further, secondary metabolites used in the chemical defence of the sponge host against space competitors and predators in the reef are suspected to be often produced by sponge-associated prokaryotes (Wilson *et al.*, 2014; Graça *et al.*, 2015; Bhushan *et al.*, 2017), and play a key role in the increasing prevalence of sponge communities in the Caribbean (Loh and Pawlik, 2014). However, the link between the chemical ecology of sponges and the prokaryotic ecology remains poorly understood (Hardoim and Costa, 2014a; Marino *et al.*, 2017). There is especially a lack of information on the ecological role of sponges and the functions of their prokaryotic symbionts in the Western Indian Ocean (WIO) region, which remains highly underrepresented in research efforts, even though it was identified a biodiversity hotspot (Fisher *et al.*, 2011; Obura, 2012). In some

locations of the WIO region, such as the Barrier Reef of Toliara in Madagascar and the Quirimba Archipelago in Mozambique, coral reefs shifted already from coral- to sponge-dominance (Barnes, 1999; Barnes and Bell, 2002). On Zanzibar's west coast, sponge cover already increased significantly from < 1 % to 7.5 % and is predicted to increase further in the next years (Muhando, 2009; Lokrantz *et al.*, 2010; Helber *et al.*, 2017). Currently, there is no information on the prokaryotic communities inhabiting sponge species from Zanzibar. Thus, it is important to gain deeper insights into the sponge-prokaryotic symbiosis and how it might give sponges competitive advantages over other benthic organisms, such as reef building corals. Recent efforts in next generation sequencing, such as the sponge microbiome project (Moitinho-Silva, *et al.*, 2017), as well as in predictive functional analysis now enable us to examine the microbial communities of sponges with a previously unmatched accuracy, and to predict specific functions of these microbes based on the detection of marker genes (Langille *et al.*, 2013; de Voogd *et al.*, 2015; Cleary *et al.*, 2017).

The present study uses prokaryotic 16S rRNA gene community data obtained from the sponge-related Earth Microbiome Project (EMP) (Gilbert *et al.*, 2014; Moitinho-Silva *et al.*, 2017; Thompson *et al.*, 2017). A combination of Illumina sequencing derived amplicon sequencing variants, here called sub-operational-taxonomic-units (i.e., sOTUs) (Amir *et al.*, 2017) and predictive metagenomic analysis applying software package Tax4Fun based on the amplicon data (Aßhauer *et al.*, 2015) was used to investigate the prokaryotic communities of the sponge hosts and to predict the presence of potential pathways for the production of bioactive compounds. In addition, we linked the results of previously conducted studies (Helber *et al.*, 2017, 2018) on the antipredatory and antimicrobial activities as well as cytotoxic properties of the same sponge species collected from the same locations to the predictive metagenomic analysis.

The main goals of our study were to (1) compare prokaryotic taxon abundance between the most abundant sponge species (i.e., *Biemna* sp., *Callyspongia* sp., *Paratetilla* sp.,

Haliclona atra, *Haliclona fascigera*, *Scopalina hapalia*, *Tetrapocillon minor*) on the reefs of Bawe and Changuu island on the west coast of Zanzibar and seawater samples, (2) assess if chemically stronger defended sponge species (Helber *et al.*, 2017, 2018) harbour similar microbial communities, (3) identify the prevalence and presence of predicted KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Kanehisa *et al.*, 2014) involved in the production of secondary metabolites and (4) relate their prevalence and presence to the degree of chemical defence of its respective sponge species.

Materials and Methods

Sample collection and study area

Seven sponge species and seawater samples for the microbial community analyses were collected by SCUBA between March to May 2014 from Bawe (06° 09′8.1" S, 39° 08′3.839" E) and Changuu Island reef (06° 07′7.356" S, 39° 10′10.919" E). Seawater samples have been collected at 10 m depth close to the reef before sponge samples were taken. Bawe and Changuu Island are located along the Western Coast of the main island Ugunja (Zanzibar) in the Zanzibar channel, about 7 km and 6 km, respectively, from the capital Stonetown (Muhando *et al.*, 2002). Both reefs are heavily influenced by fishing and tourism activities as well as untreated sewage discharge from Stonetown harbour (Muhando, 2009; Lokrantz *et al.*, 2010; Moynihan *et al.*, 2012; Limbu and Kyewalyanga, 2015). The samples for the determination of the LMA or HMA status were collected in November 2015 at Bawe Island reef (06° 09′25.56" S, 39° 08′0.96" E) at 10 m depth and at least 20 m apart to avoid collection of clones. Sponge samples were transferred into zip block bags filled with seawater and were immediately transferred to the laboratory facilities at the Institute of Marine Sciences (IMS, Stonetown). Sponge vouchers used for species identification have been stored at the Naturalis Biodiversity Center in Leiden, Netherlands. Details on taxonomy and pictures have been published by Helber and colleagues (2017).

The sponge samples (6 replicates per species) for the microbial community analysis were transferred in 99.9 % of ethanol (3 replicates) or RNA later (3 replicates). Seawater samples (10 ml; 3 replicates per month) were filtered through a Whatman Cyclopore PC Polycarbonate Membrane Filter (47 mm diameter, 0.22 μm pore size). Both, sponge and seawater samples, were stored at -20°C until further processing at the Institute for Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Germany. Sponge samples (3 – 4 replicates per species) for the analysis of their LMA or HMA status were cut into few cubic millimetre sized pieces and immediately fixed in 2.5 % glutaraldehyde/phosphate- buffered saline and stored at 4°C until further processing.

Transmission Electron Microscopy (TEM)

Following 5 x washing of the samples with 50 mM cacodylate buffer (pH 7.2) and 90 min post-fixation at 4°C with 2 % osmium tetroxide in the buffer, the samples were rinsed with Milli-Q water and stained overnight in 0.5 % uranyl acetate. Samples were then rinsed with Milli-Q water and dehydrated through a series of ethanol and propylene oxide. Following overnight infiltration in a propylene oxide-Epon 812 mix (1:1), samples were rinsed in Epon 812 resin twice for 2 h and transferred into fresh resin for 1 h. Subsequently, the sponge samples were embedded in this resin for at least 48 h. Embedded specimen blocks were trimmed, cut into ultrathin (70 nm) sections with an ultramicrotome (Leica EM UC7, Austria), and then deposited on pioloform coated grids, double contrasted with 2.5 % uranyl acetate and Reynold´s lead citrate. Imaging was performed with a Tecnai G2 Spirit BioTwin transmission electron microscope (80 kV, FEI, USA) at the Central Microscopy of University of Kiel (Germany). For each sponge species, two biological replicates and two technical replicates were investigated. For each sample, the entire ultrathin-section was inspected at TEM and at least two fields-of-views per ultrathin-section were imaged.

Sequence data processing and 16S rRNA gene community analyses

The present data subset was obtained from the final release of the sponge-related Earth Microbiome Project (for further details see http://www.spongeemp.com/ and http://gigadb.org/dataset/100332) (Moitinho-Silva *et al.*, 2017). Sample processing, sequencing, and core amplicon data analysis were performed following the Earth Microbiome Project protocols by the sponge microbiome project collaborators, and all amplicon sequence data and metadata have been made public through the EMP data portal (qiita.microbio.me/emp and http://gigadb.org/dataset/100332). In brief, DNA extraction, Illumina MiSeq sequencing, quality-control, and de-noising using Deblur was carried out by the sponge microbiome project collaborators (Moitinho-Silva *et al.*, 2017). The extracted subset created using R v.3.3.3 (R Core Team, 2017), consisted of 41 samples and 3970 sOTUs. The full sponge microbiome dataset can be downloaded from the GigaScience Database: http://gigadb.org/dataset/100332). The taxonomy of the sponge microbiome sOTUs was updated using mothur v.1.39.5 (Schloss *et al.*, 2009) and the SILVA128 reference files (https://www.mothur.org/wiki/ Silva_reference_files).

The alpha- and beta-diversity analyses (e.g., sOTU richness, Pielou' s evenness, Shannon and Inverse Simpson index) were performed in R using the vegan v.2.4-2 package if not stated otherwise (Oksanen *et al.*, 2013). Prior to the analyses the abundance table was standardized using *decostand* (method = 'hellinger') and pairwise Bray-Curtis dissimilarities were calculated using *vegdist* (method = 'bray'). Univariate relationships between alpha-diversity indices (i.e., OTU richness, Shannon index, Simpson's inverse index and evenness) and differences between the samples were analysed with the Kruskal-Wallis rank sum test using *kruskal.test* (R stats package v.3.3.3) followed by Dunn's Kruskal-Wallis multiple comparisons test using *dunnTest* (FSA package v.0.8.21) and the Benjamini-Hochberg *p*-value correction for multiple testing (Benjamini and Hochberg, 1995; Ogle, 2018). For multivariate analyses (i.e., *betadisper* / *permanova* & *adonis*) all samples were grouped by source type (i.e., host-identity or seawater). Multivariate analyses based on the host-identity

and non-metric multidimensional scaling (nMDS) were performed using the *permutest*, *betadisper*, *adonis* and *metaMDS* functions of the vegan package. Hierarchical cluster analysis was performed using *hclust* (method = 'average'). All heatmaps were created using JColorGrid (Joachimiak *et al.* 2006). Finally, we used the software package Statistical Analysis of Metagenomic Profiles (STAMP) v.2.1.3, to identify significantly different distributed prokaryotic phyla among the sample groups (Parks *et al.*, 2014).

Functional predictive analysis

Functional predictive profiling using PICRUSt (Langille *et al.*, 2013) has been successfully applied in several recent sponge-microbiota studies (de Voogd *et al.*, 2015; Cleary *et al.*, 2017; Weigel and Erwin, 2017). Here, we used the R package Tax4fun (Aßhauer *et al.*, 2015), which was successfully applied for bacterioplankton communities and provided valid correlations of functional profiles to prokaryotic and environmental parameters. In comparison to PICRUSt, Tax4Fun displays higher correlation of functional predictions with the metagenome profile (Aßhauer *et al.*, 2015). When these two software packages were compared, Tax4Fun predicted almost 15 % more KEGG Orthologs than PICRUSt (Koo *et al.*, 2017).

The artificial metagenomic functional profile was calculated by utilizing the ultrafast protein classification (UProC) option on the normalized 16S OTU table (Tax4Fun parameters: refProfile = "UProC", shortReadMode = FALSE, normCopyNo = TRUE). The resulting KEGG abundance profile was analysed using STAMP to identify significant pathways for secondary metabolite production among the sample groups. In the present study, we used the KEGG database and selected a set of KEGG pathways based on their potential for secondary metabolite synthesis (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2016, 2017). The comparison of differences in single pathways for secondary metabolite production and microbial phyla composition between the groups was made by an ANOVA followed by the Tukey–Kramer post hoc test and a Bonferroni multiple test correction. Differences were considered

significant at corrected $p < 0.05$. Additionally, a pathway map was created (https://www.genome.jp/kegg/tool/map_pathway2.html) and the related BRITE KEGG Orthology was collected (https://www.genome.jp/kegg/tool/map_brite2.html) to show significantly enriched KEGG Orthologs (KOs) involved in the biosynthesis of polyketides. To complement the functional prediction based on 16S rRNA profiles, we included the results of previously conducted studies (Helber *et al.*, 2017, 2018) on the biological activities of the investigated sponge species to interpret the predictive metagenomic analysis.

A functional predictive analysis in a recent study (Weigel and Erwin, 2017) found similar KO counts and abundances in genes responsible for nitrogen cycling when compared to metagenomic sequencing, but still showed noticeable deviations. Thus, functional predictive approaches cannot replace whole metagenomic profiling. Results obtained from predictive functional analyses using 16S rRNA gene amplicon sequencing can deviate from metagenomic profiling and functional gene annotation, as not all predicted genes may indeed be present or functional in the respective prokaryotic OTU (Aßhauer *et al.*, 2015; Weigel and Erwin, 2017). In addition, because of functional overlap, some KOs can be assigned to more than one pathway. Despite these limitations, functional predictive approaches provide a cost-effective first estimate of metagenomic profiles.

Results

Transmission Electron Microscopy (TEM)

Of the seven sponge species examined (each species with two biological and two technical replicates), five (*Callyspongia* sp., *Paratetilla* sp., *H. atra*, *H. fascigera*, *S. hapalia*) were identified as low microbial abundance (LMA) and one (*Biemna* sp.) was identified as high microbial abundance (HMA) sponge. Unfortunately, one (*T. minor*) has completely degenerated during embedding process. *T. minor* was suggested to be a LMA host based on prediction results by machine learning (Moitinho-Silva *et al.*, 2017). An LMA status of

T. minor would indeed be consistent with our amplicon results. *Biemna* sp. showed HMA characteristics based on morphological diversity (Vacelet and Donadey, 1977; Friedrich *et al.*, 1999) and moderate abundance of extracellular bacteria (Fig. 1). Bacteria in *Biemna* sp. displayed different morphologies, ranging from (1) ovoid to spherical cyanobacteria (up to 2.5 μm in length; Fig. 1F, b1) that exhibited thylakoids, (2) small bacteria (0.5 - 0.6 μm by 0.3 - 0.5 μm in size; Fig. 1F, b2) with compact DNA in the middle, (3) bacteria with nuclear area and dense deposits (0.7 - 1.3 by 0.6 -1.0 μm in size; Fig. 1F, b3), (4) thread-like bacteria (1.1 - 1.5 μm by 0.2 - 0.5 μm in size; Fig. 1F, b4), and other types of bacteria (Fig. 1F, b5). Compared to the HMA sponge *Biemna* sp., the mesohyl tissues of the six investigated LMA sponges are noticeably devoid of microorganisms, and prokaryotic cells can only be occasionally observed (Fig. 1). Overall, the HMA-LMA status was very clear in that the mesohyl of the LMA species was nearly devoid of microorganisms while the mesohyl of the HMA species, *Biemna* sp., contained a variety of different bacterial morphotypes.

Prokaryotic community composition

The prokaryotic communities of the following sponge species were analysed within the framework of the final EMP "Global Sponge Microbiome Dataset" (Moitinho-Silva, *et al.*, 2017): *Callyspongia* sp. (n = 4), *Biemna* sp. (n = 5), *H. atra* (n = 5), *H. fascigera* (n = 6), *Paratetilla* sp. (n = 5), *S. hapalia* (n = 5), *T. minor* (n = 6) and seawater samples (n = 5). Sequencing yielded 4.278.491 sequences assigned to 3.970 sOTUs after quality control. Altogether, sOTUs of 32 bacterial and three archaeal phyla and 91 classes were recovered from the sponge and seawater samples of which the phylum Proteobacteria (1.320 sOTUs, accounting for approximately 33 % of all sOTUs) was the most abundant-(Fig. 2). Within the Proteobacteria phylum, Alphaproteobacteria exhibited the highest sOTU richness (n = 419 sOTUs), followed by Gammaproteobacteria (372), unclassified Proteobacteria (290), Deltaproteobacteria (177), Epsiloproteobacteria (40) and Betaproteobacteria (19). Other sOTU rich phyla, next to a high number of unclassified Bacteria (n = 929 sOTUs), included

Bacteroidetes (373), Firmicutes (336), Planctomycetes (213), Actinobacteria (140), Cyanobacteria (100), Chloroflexi (95), Verrucomicrobia (87) and Acidobacteria (78). Seawater samples were dominated by Alphaproteobacteria, whereas most sponges were dominated by Gammaproteobacteria, except for *H. atra*, which was the only sponge species that harboured unclassified Proteobacteria as their main prokaryotic component (Fig. 2). From the 35 prokaryotic phyla, 14 contributed significantly to differences between the sample groups (Supporting Information Fig. 1). Out of those phyla, *Biemna* sp. hosted most sequences assigned to Acidobacteria, PAUC34f, Gemmatimonadetes and Chloroflexi; all phyla known as HMA indicator phyla (Fig. 2; Moitinho-Silva, *et al.*, 2017). The microbial community of the LMA sponges was mainly composed of LMA indicator phyla or a mix of other and LMA indicator phyla (Fig. 2). The LMA sponges *H. fascigera*, *S. hapalia*, *Paratetilla* sp., *Callyspongia* sp. and *H. atra* had the highest phylum level diversities with 24 to 21 prokaryotic phyla discovered. The HMA sponge *Biemna* sp. and the LMA sponge *T. minor* possessed the lowest phylum diversity of all sponge samples hosting on average only 18 and 17 prokaryotic phyla. Seawater samples had the lowest phylum level diversity with 12 bacterial and three archaeal phyla discovered. These results have to be interpreted with caution because of the small volume of seawater used in this study (10ml per sample) that might have resulted in lower diversity and richness values for prokaryotic communities in seawater samples. However, our results detecting significant differences between sponge and seawater samples as well as similarities between LMA sponges and seawater are in line with previously published studies that used larger volumes of water (1-2l; e.g. Steinert *et al.*, 2016; Thomas *et al.*, 2016).

The mean sOTU richness of sponge species ranged from 136 for *Biemna* sp. to 507 in *H. fascigera*. The HMA sponge *Biemna* sp. had the most diverse (Shannon Index: 3.62 ± 0.04; Inverse Simpson Index: 25.70 ± 5.60) and evenly distributed (Evenness: 0.74 ± 0.04) prokaryotic community (Table 1) and differences in those alpha diversity indices

compared to the other sponge and seawater samples were significant in pairwise comparisons (Supporting Information Table S1).

The top 30 sOTUs in all samples included members of the Cyanobacteria, Proteobacteria (Alpha-, Gamma- and unclassified), Actinobacteria, Thaumarchaeota, Bacteroidetes, Nitrospirae and Acidobacteria. Interestingly, 24 of the top 30 sOTUs were quite novel and they had low sequence similarity, sometimes only 84 %, when compared against known prokaryotes of the 16S NCBI (i.e., rRNA_typestrains/prokaryotic_16S_ribosomal_RNA) reference taxonomy (Supporting Information Table S2). However, when 16 of the unknown sequences were BLAST searched against the nucleotide (nt) database, they showed high similarity (> 98 % identity over approximately 100 bp) to previously discovered sponge-associated prokaryotes from the Great Barrier Reef, Japan, Florida, the Red Sea, the South China Sea and the Mediterranean (Supporting Information Figure S2; Supporting Information Table S2). Nine of those sOTUs were exclusively found in one sponge species in the present study or only discovered in very low abundances (< 1 %) in others. The most abundant sOTU (sOTU 592), accounting for 7.65 % of all sOTUs, belonged to the phylum Cyanobacteria, genus *Synechococcus*, and it was present in all samples (Supporting Information Figure S2). The second (sOTU 16635) and third (sOTU 1359) most abundant sOTUs were similar to (1) an uncultured bacterium clone and (2) an uncultured *Candidatus Branchiomonas* sp. clone retrieved previously from a sponge host when BLAST searched against the nucleotide (nt) database (Supporting Information Figure S2; Supporting Information Table S2). The most dominant sOTU (sOTU 3073) in the seawater samples, was an Alphaproteobacterium assigned to the order of Caulobacterales (genus *Brevundimonas*), closely related to an organism isolated from seawater and it was absent from almost all sponge samples.

The prokaryotic communities in the different sponge hosts are highly host specific and they differ significantly between each of the sponge species and seawater (adonis: $df = 7$,

$F = 6.4296$, $R^2 = 0.577$, $p < 0.001$). Additionally, the non-metric multidimensional scaling plots also demonstrated that the LMA sponges, the HMA sponge and seawater formed clear clusters and the LMA sponges displayed greater similarity with the seawater samples (Fig. 3). Pairwise comparisons of mean group dispersions demonstrated that *H. fascigera*, *T. minor*, *Callyspongia* sp. and seawater samples contributed significantly to the differences between the groups even though the overall group dispersion was not significant (betadisper: $df = 7$, $F = 1.3414$, $p = 0.271$; Supporting Information Table S3).

Core and species-specific microbiome

A small core community (100 % presence among all sponge samples – excluding seawater) could be found in our investigated sponge species, consisting of 3 sOTUs (0.88%) belonging to two bacterial phyla, Cyanobacteria (genus *Synechococcus*) and Planctomycetes. One of those, sOTU592 (Cyanobacteria; *Synechococcus*), belongs to a formerly described sponge-specific sequence cluster, namely SC51 (see Simister *et al.*, 2012). However, when we used the core community definition of Thomas *et al.* (2016), that is 'presence in 85 % of sponges', we could find 29 sOTUs making up on average 8.6% of the total community composition. Only two of those 29 sOTUs belonged to sponge-specific clusters (SCs) and sponge-coral-specific clusters (SCCs) (sOTU592 – SC51 and sOTU222 - SC3/SCC4 see Simister *et al.*, 2012). The majority of core sOTUs were affiliated with either Proteobacteria, Bacteroidetes, unclassified bacteria or Planctomycetes.

The number of species-specific prokaryotes varied greatly among the different sponge species. *T. minor* had the smallest species-specific community, which consisted of ca. 24 % of the total community whereas species-specific prokaryotes accounted up to 86 % for *Paratetilla* sp. and 85 % for *H. fascigera*. The species-specific community of the other four sponge species (i.e., *S. hapalia*, *H. atra*, *Callyspongia* sp. and *Biemna* sp.) consisted of approximately 42 – 58 % of the total community. sOTUs belonging to sponge-specific SCs (Simister *et al.*, 2012) or later on termed as "sponge-enriched" sequence clusters (Moitinho-

Silva *et al.*, 2014) made up only a smaller portion (5 – 23 %) of the species-specific communities of the individual host sponges. "Sponge-enriched" SCs accounted only for 7 – 17 % of the overall microbial communities of the different host sponges, but the numbers of their reads made up 8 – 30 % of the overall sOTU reads of their microbial communities. Approximately 70 % of all "sponge-enriched" SCs were exclusively found in sponges, while only 5 % were exclusively discovered in seawater samples.

Functional Predictive analysis

We focused on predicted KEGG pathways that are involved in the production of bioactive secondary metabolites (Supporting Information Table S4; Fig. 4) and also examined which microbial phyla in the associated communities of the hosts explained the main differences between the samples (Supporting Information Figure S1). To support the quality of our approach we included the FTU values, which represent the fraction of the unmapped Tax4Fun OTUs (Table 1). A low FTU value suggests that the majority of OTUs were included in the functional predictive analysis and therefore the results might be more similar to actual metagenomic data. However, two sponge species in our study, *H. atra* (FTU= 0.51) and *H. fascigera* (FTU= 0.65), had FTU values >0.5. It was shown that FTU values of up to 0.5 still resulted in a correlation coefficient of 0.65 between the predictive analysis with Tax4Fun and the functional profile obtained by whole metagenome sequencing (Aßhauer *et al.*, 2015).

Pathways involved in the production of secondary metabolites that explained the main proportion of variance between the samples include (KO01057) Biosynthesis of type II polyketide products, (KO00521) streptomycin biosynthesis and (KO00100) steroid biosynthesis (Fig. 4). Monoterpenoid biosynthesis (KO00902) and indole alkaloid biosynthesis (KO00901) were predicted to be particularly enriched in *Callyspongia* sp., while *H. atra* was more enriched in various antibiotic pathways, such as streptomycin (KO00521), vancomycin (KO01055) and tetracycline (KO00253) biosynthesis as well as terpenoid backbone (KO00900), and ubiquinone and other terpenoid-quinone biosynthesis (KO00130).

The HMA sponge *Biemna* sp. was predicted to be significantly enriched in pathways responsible for steroid biosynthesis (KO00100), indole alkaloid biosynthesis (KO00901), sesquiterpenoid and triterpenoid biosynthesis (KO00909), biosynthesis of type II polyketide backbone (KO01056) and nonribosomal peptide structures (KO01504). *T. minor* was only enriched in the biosynthesis of type II polyketide products (KO01057) and had otherwise low predicted gene counts for pathways associated with secondary metabolite production.

Both, HMA and LMA sponges, were predicted to be enriched in various genes and proteins involved in the biosynthesis of type I and II polyketide synthases (Supporting Information Table S5; Supporting Information Fig. S3). However, there was no significant difference in enrichment of KOs involved in the production of type I and II polyketide synthases between HMA and LMA sponges (Supporting Information Table S6). The functional predictive approach proved to be a highly useful tool to get first insights into the potential functional capabilities of the symbiotic prokaryotes in our investigated sponges. However, it cannot substitute whole metagenome sequencing because its quality depends on the amount of prokaryotic reference profiles in the KEEG database and the presence as well as the functionality of predicted gene clusters is not verified.

Discussion

In the present study we demonstrated that sponge-associated prokaryotic communities of seven different reef sponge taxa from Zanzibar's west coast are largely sponge host species-specific. Thus, host identity is a significant factor in explaining the microbiome of the sponges as shown before (e.g., Easson and Thacker, 2014; Reveillaud *et al.*, 2014; Steinert *et al.*, 2016, 2017). Very low intraspecific variability could be detected in samples of the HMA sponge *Biemna* sp. and of the three LMA sponges *T. minor*, *Paratetilla* sp. and *H. fascigera*. This indicates that prokaryotic communities in both, HMA and LMA sponges, are not randomly taken up, but strongly selected for (Blanquer *et al.*, 2013; Moitinho-Silva *et al.*,

2014). Previously identified HMA indicator phyla (Moitinho-Silva, *et al.*, 2017) were especially enriched in the HMA sponge *Biemna* sp. whereas the prokaryotic communities of the LMA sponges and seawater samples were mainly composed of LMA indicator phyla. The different LMA sponges did not form strict host-specific clusters when they were grouped at prokaryotic phylum level, which is not unusual (Steinert *et al.*, 2017). Even though the phylogenetic composition of the prokaryotic communities of LMA sponge species resembled the ones in seawater, they also displayed strong sponge-host specificity consistent with previous studies (Easson and Thacker, 2014; Moitinho-Silva *et al.*, 2014; Erwin *et al.*, 2015). In accordance with previous studies (Schmitt *et al.*, 2012; Reveillaud *et al.*, 2014; Astudillo-García *et al.*, 2017), we discovered a small core community consisting of 0.88 % (100 % presence) or 8.6 % (i.e., present in 85 %of samples) of the overall prokaryotic community. The core microbiome (85 % presence) was dominated by Proteobacteria and Bacteroidetes and did not mainly consist of "sponge-enriched" SCs as reported before for various sponge species (Schmitt *et al.*, 2012; Thomas *et al.*, 2016; Weigel and Erwin, 2017). The "sponge-enriched" SCs in our investigated sponge species accounted in general only for 7 – 17 % of the overall prokaryotic communities of the different host species. However, previous studies (Simister *et al.*, 2012; Schmitt *et al.*, 2012; Thomas *et al.*, 2016) reported that "sponge-enriched" SCs constituted between 27 – 65 % of the overall prokaryotic community in various host sponges. In fact, sponge species from Zanzibar harboured a large number of unclassified bacteria, several of them being related to sponge-associated bacteria recovered from Great Barrier Reef sponges which also possessed less sequences belonging to "sponge-enriched" SCs (Webster *et al.*, 2013).

Approximately 30 % of all "sponge-enriched" SCs were also found in seawater. It is still contentious whether symbiotic prokaryotes are metabolically active in seawater or whether they could be only detected in seawater due to leakage from sponges (e.g., wounding by storm or predation, collapse from disease or spawning events) (Angermeier *et al.*, 2011;

Gloeckner *et al.*, 2013; Moitinho-Silva *et al.*, 2014). Recent deep sequencing approaches discovered an increasing number of so-called "sponge-specific" prokaryotes in seawater or sediments, which might serve as seed bank for sponges (Taylor *et al.*, 2013; Sipkema *et al.*, 2015; Thomas *et al.*, 2016). These findings are consistent with our study in which we discovered sponge-specific candidate phyla, such as Tectomicrobia, SBR1093 and Poribacteria in very low abundances in seawater samples. Several bacteria from the surrounding seawater, including sponge-specific representatives of the phyla Actinobacteria and Gemmatimonadetes, are assumed to be actively finding their sponge hosts via chemotactic migration (Tout *et al.*, 2017). Our findings that prokaryotic communities in both, LMA and HMA sponges, differ considerably from seawater and that HMA sponges harbour a more diverse community are in line with previous studies. These findings indicate that both horizontal and vertical transmission potentially occur in sponges, but that horizontal transmission is likely more prevalent in LMA species (Reveillaud *et al.*, 2014; Ribes *et al.*, 2015).

Functional prediction of secondary metabolite biosynthesis pathways

Functional prediction of secondary metabolite biosynthesis pathways revealed distinct differences between sponge species. *Callyspongia* sp. harboured a considerable number of bacteria belonging to the candidate phylum Tectomicrobia (Wilson *et al.*, 2014). Tectomicrobia have large genomes containing many gene clusters dedicated to bioactive compound production, similar to members of the phyla Actino- and Cyanobacteria (Omura *et al.*, 2001; Flores and Herrero, 2010; Lackner *et al.*, 2017). Next to Tectomicrobia, this sponge species harboured the most sOTUs that belonged to yet unclassified bacteria. The strong reported cytotoxic activities of *Callyspongia* sp. could be ascribed to the production of alkylpiperidine alkaloids (Fusetani, 2008; Helber *et al.*, 2018) as it was enriched in various pathways for alkaloid biosynthesis (KO00901 and KO00960). Thus, *Callyspongia* sp. would be interesting for further exploration of its prokaryotic communities since it was previously

reported the chemically strongest defended sponge of the species investigated in the present study (Helber *et al.*, 2018).

Sponges of the genus *Biemna* are known for their broad spectrum of bioactive metabolites, particularly steroids (e.g. Ehrenasterol and Biemnasterol) and alkaloids (e.g. Biemnadin, Hydroxyascididemin and Netamines), often with cytotoxic properties especially against human carcinoma cells (Zeng *et al.*, 1993; Govinden- Soulange *et al.*, 2014; Youssef *et al.*, 2015). Consistent with these previous studies, the sponge *Biemna* sp. at our site was enriched in pathways for steroid (KO00100) and indole alkaloid biosynthesis (KO00901). Moreover, the high abundance of type II polyketide backbone (KO01056) and nonribosomal peptide structures (KO01054) could be related to the large presence of the phyla PAUC34f, Chloroflexi as well as Actinobacteria. Sequences for polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) were detected in more than half of the isolated Actinobacteria from various sponge species (Jiang *et al.*, 2007, 2008). Additionally, Chloroflexi and PAUC34f are also known to contain PKS and NRPS (Fieseler *et al.*, 2007; Siegl and Hentschel, 2010).

Predicted functional profiles of the sponge species *H. atra* revealed the potential presence of several pathways involved in the biosynthesis of antimicrobial products. These findings are in line with previous studies, in which sponges of the genus *Haliclona* (Ely *et al.* 2004; Aishwarya *et al.* 2013; Skariyachan *et al.* 2014) and specifically *H. atra* (Helber *et al.*, 2018) demonstrated high antimicrobial activities against a range of bacteria and pathogens.

The sponge *H. fascigera* had similar predicted pathways for the production of antimicrobial compounds as *H. atra*. *H. fascigera* displayed intermediate antimicrobial activities in disc diffusion assays, while *H. atra* demonstrated activity against more than twice as much bacteria (Helber *et al.*, 2018). *H. fascigera* was predicted to harbour genes associated with Sphingolipid mechanism (KO00600), which might explain its potent cytotoxic activities (Helber *et al.*, 2018). Diverse sphingolipid metabolites have been isolated from a range of

sponge species that displayed cytotoxic and antitumor activities (Muralidhar *et al.*, 2003; Ando *et al.*, 2010).

S. hapalia was predicted to be enriched in similar pathways as *H. atra* as they shared the highest similarity in their microbial communities. Though, in contrast to *H. atra, S. hapalia* displayed only moderate antibacterial activities and was the sponge species with the second highest activity in the cytotoxicity assay (Helber *et al.*, 2018), in line with previous studies investigating species of the genus *Scopalina* (Biegelmeyer *et al.*, 2015; Vicente *et al.*, 2015). Cytotoxic properties might be attributed to the high abundance of Cyanobacteria which are known to produce a range of toxic compounds (Faulkner *et al.*, 2000; Matthew *et al.*, 2008, 2010). No previous information on secondary metabolites for *S. hapalia, Paratetilla* sp. and *T. minor* have been published so far, highlighting a knowledge gap that needs to be filled.

Sponges from Zanzibar harboured prokaryotes that produce type I and II polyketide synthases, but contrary to previous studies (Fieseler *et al.*, 2007; Siegl and Hentschel, 2010) PKS genes and proteins were also predicted to be enriched in LMA sponges. Compared to the LMA sponges, the HMA sponge *Biemna* sp. was not predicted to be significantly enriched in genes involved in PKS production. Second to polyketides, terpenoids are also assumed to play a role in host defence (Keyzers *et al.*, 2006; Karimi *et al.*, 2017, 2018). The high number of different microbial terpenoid pathways found in *Biemna* sp., *Callyspongia* sp. and *H. atra*, is noteworthy because terpenoids were until recently suspected to be of plant or fungal origin (Yamada *et al.*, 2015). This finding combined with recent studies detecting that terpenoid biosynthesis genes are widespread across uncultured sponge-associated bacteria (Karimi *et al.*, 2017) could indicate that terpenoids play a more important role in sponge chemical defence than previously assumed.

The strong chemical defences of the investigated sponge species (Helber *et al.*, 2017, 2018) in combination with continuing ocean warming, destructive fishing practices and damage to the reef through tourism activities (Jiddawi and Öhman, 2002; Muhando *et al.*,

2002; Lokrantz *et al.*, 2010) might explain the increasing abundances of sponges on the reef over the last years. Based on our results on the biological activities of the sponges and the functional predictive analyses, the sponges *H. atra*, *Biemna* sp. and *Callyspongia* sp. would be the most interesting species for further investigations of secondary metabolism and biotechnological potential.

Our results obtained by a functional predictive approach based on 16S rRNA amplicon sequencing cannot replace whole genome sequencing. Thus, the results of our study need to be confirmed by (meta)genomic and/ or (meta)transcriptomic work to demonstrate that the predicted pathways in the prokaryotic communities of each sponge host are actually present and functional.

Conclusions

The present study is, to our knowledge, the first to investigate the prokaryotic community composition of sponges from the WIO. We have demonstrated that the highly diverse prokaryotic communities in sponges from Zanzibar are host species-specific, and that LMA sponges, despite being associated with low abundances of prokaryotes, harbour a diverse microbiome. The 16S rRNA gene sequencing combined with the functional predictive analysis can be a helpful tool to assess the potential capacity to synthesize natural products in sponge species and to prioritize sponge species for different follow-up chemistry approaches. The high number of unclassified bacteria in all sponge samples was striking, with 24 of the 30 most abundant sOTUs showing low sequence similarities to known sponge-associated prokaryote sequences. These findings render the sponges of Zanzibar interesting for future studies, especially with regards to their natural product repertoire.

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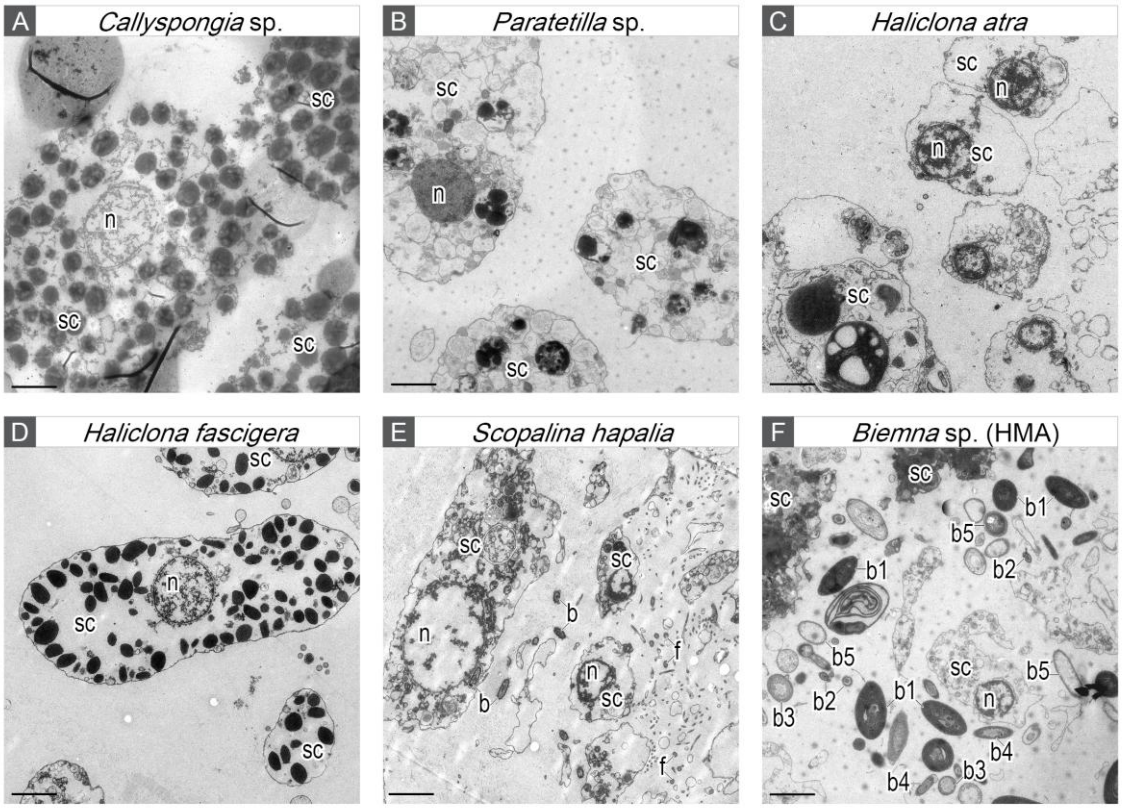

Fig. 1: Transmission electron microscopy showing the HMA or LMA status in six sponge species. LMA: (A) *Callyspongia* sp., (B) *Paratetilla* sp., (C) *Haliclona atra*, (D) *Haliclona fascigera*, and (E) *Scopalina hapalia*. HMA: (F) *Biemna* sp. with (b1) cyanobacteria, (b2) small bacteria with compact DNA in the middle, (b3) bacteria with nuclear area and dense deposits, (b4) thread-like bacteria, and (b5) other types of bacteria. Scar bar = 2 μm; b, bacteria; f, choanocyte flagella; n, nucleus; sc, sponge cell. Note that there are background artefacts in Figs 1B, 1E and 1F probably due to preparation, cutting, or overexposure.

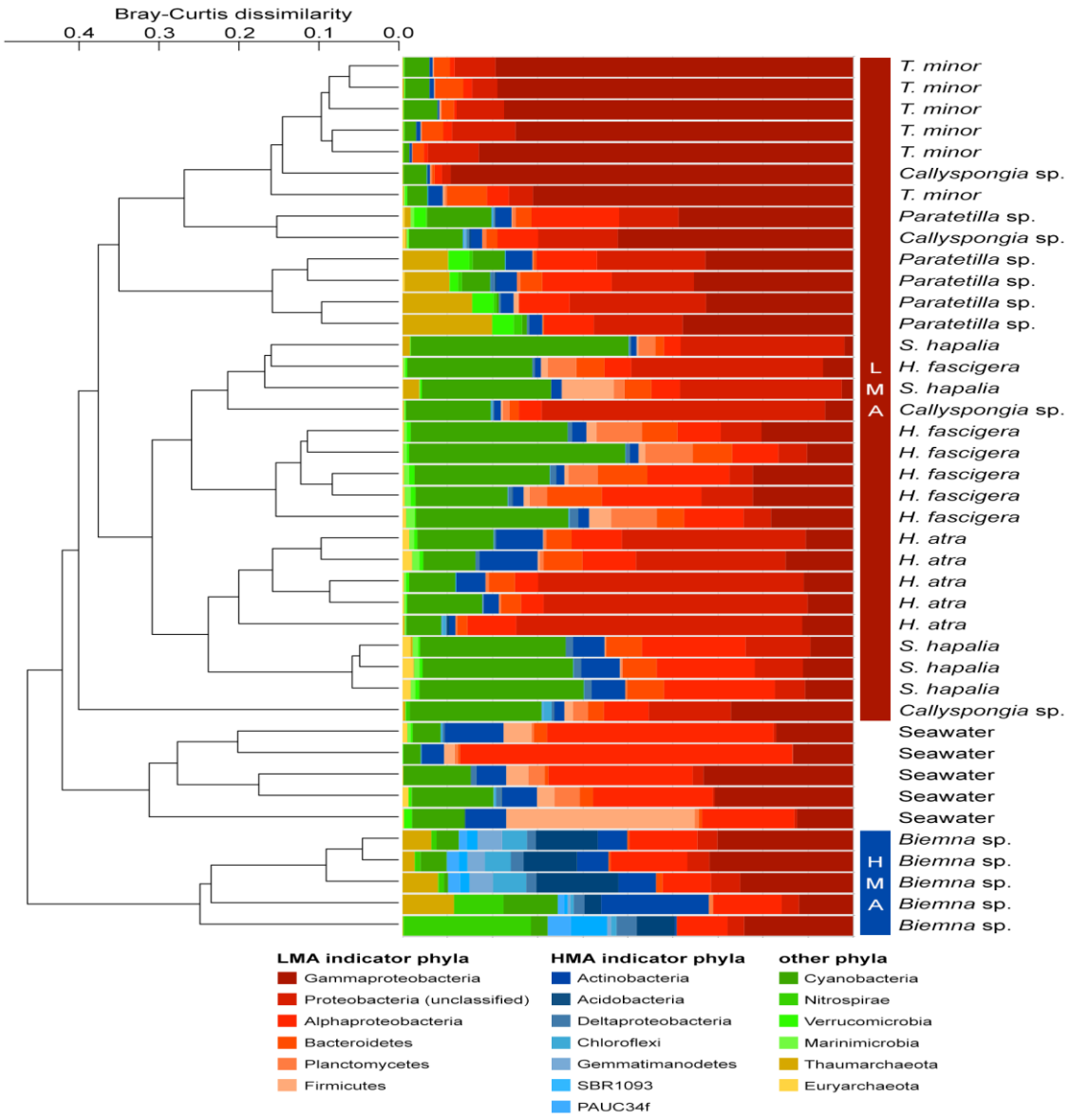

Fig. 2: Relative 16S rRNA gene sequence abundance of microbial phyla (Proteobacteria split to classes) present in each host sponge species and seawater sample. Samples were arranged by Bray-Curtis dissimilarity as shown by the dendrogram on top. Microbial indicator phyla for LMA sponges are displayed in red colors, indicator phyla for HMA sponges in blue colors. The color scheme (i.e., blue or red) and the classification of HMA and LMA microbial indicator phyla are adapted from Moitinho-Silva and colleagues (2017).

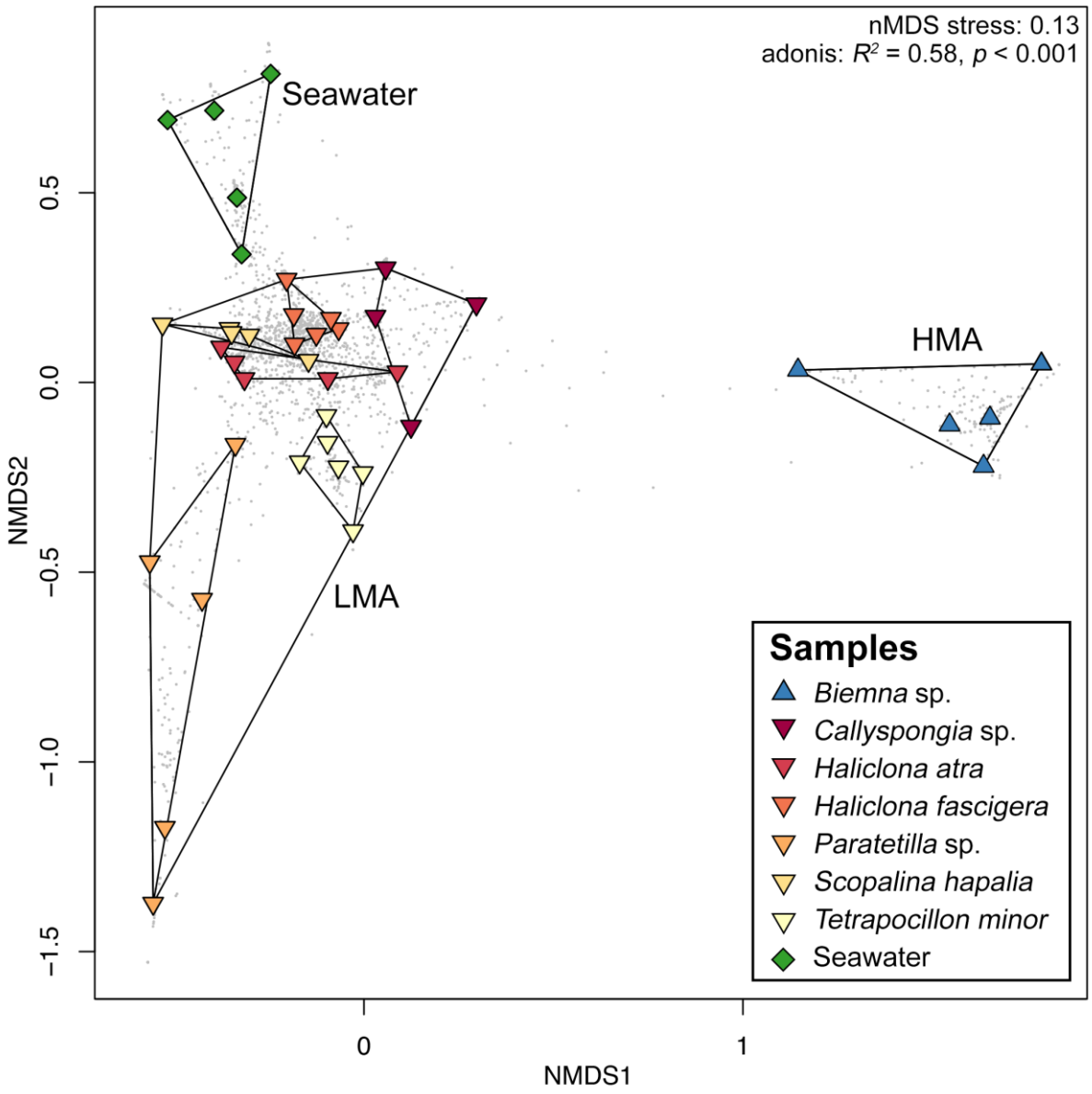

Fig. 3: Non-metric multidimensional scaling (nMDS) plot based on Bray-Curtis dissimilarities for the microbial communities in the different host sponges and seawater samples. The LMA and HMA sponge species and the seawater samples form three distinct clusters.

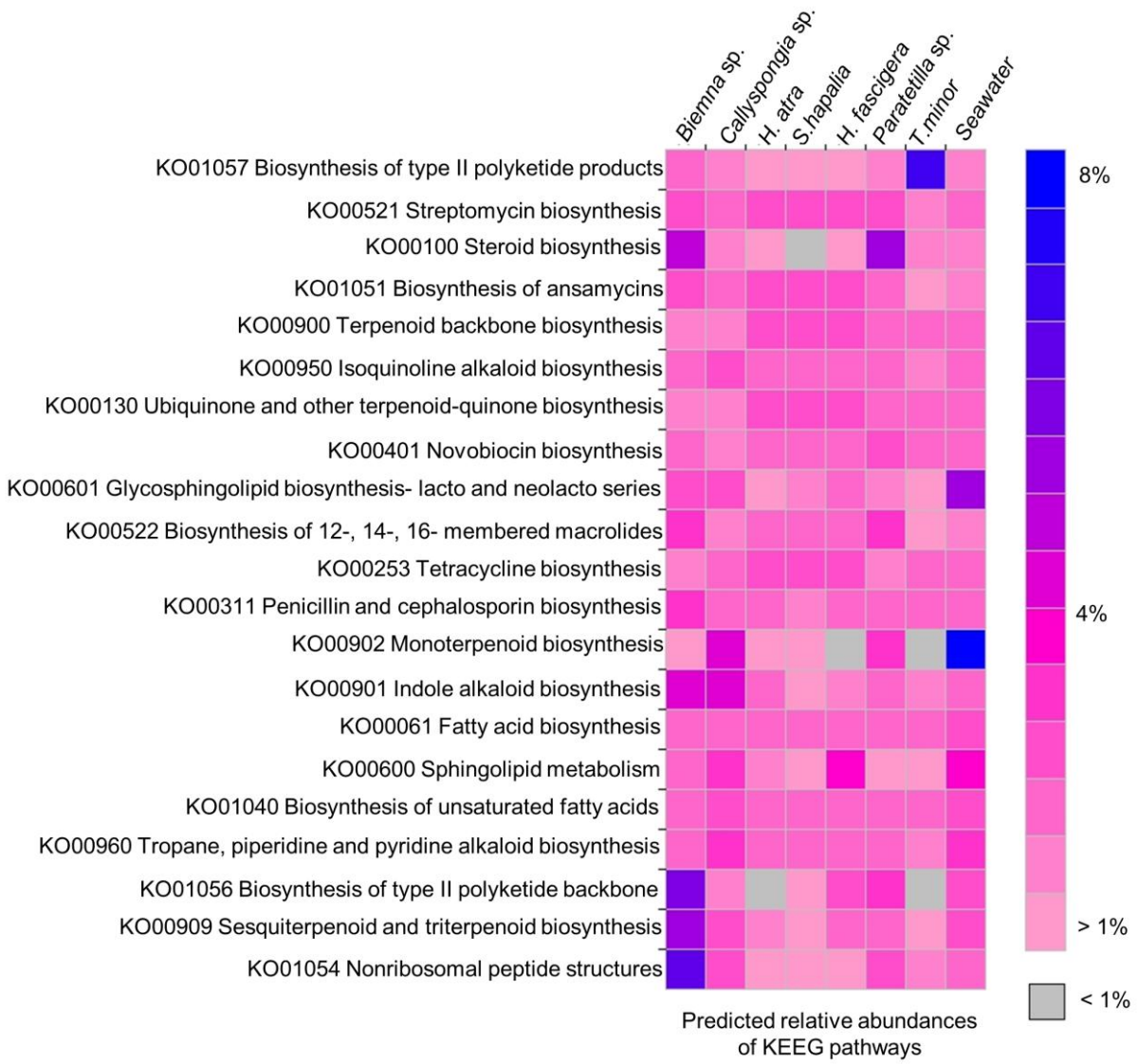

Fig. 4: Heatmap displaying the average, estimated gene count contributions to KEGG pathways involved in secondary metabolite production that have significantly contributed to differences between the samples (determined by STAMP).

Table 1: Alpha microbiome diversity comparisons between the sponge and seawater samples and benthic coverage at 10 m water depth of the most abundant sponge species at Bawe Island, Zanzibar. The average values (± standard error) showing the Shannon diversity index, inverse Simpson index, sOTU richness and evenness as well as fraction the of unmapped tax4fun OTUs (FTU) are displayed for each sponge species and the seawater samples.

Sample	Shannon Index	Inverse Simpson Index	Evenness	sOTU richness	Benthic cover at 10 m depth [%]	n	FTU
***Biemna* sp.**	3.62 (±0.21)	25.70 (±5.60)	0.74 (±0.04)	136 (±12.43)	2.88 (±4.20)	5	0.39 (±0.03)
***Callyspongia* sp.**	2.08 (±0.39)	4.53 (±1.87)	0.38 (±0.08)	266 (±43.66)	0.13 (±0.48)	4	0.15 (±0.12)
H. atra	2.61 (±0.22)	4.76 (±1.01)	0.44 (±0.04)	370 (±38.14)	2.33 (±4.70)	5	0.65 (±0.07)
H. fascigera	3.46 (±0.15)	11.61 (±2.20)	0.56 (±0.02)	507 (±50.57)	0.17 (±0.90)	6	0.51 (±0.14)
S. hapalia	3.45 (±0.17)	9.43 (±0.60)	0.57 (±0.03)	424 (±46.53)	0.04 (±0.14)	5	0.26 (±0.09)
***Paratetilla* sp.**	3.21 (±0.17)	13.86 (±1.75)	0.58 (± 0.01)	304 (±81.96)	0.01 (±0.04)	5	0.26 (±0.03)
T. minor	2.24 (±0.10)	3.64 (±0.27)	0.40 (±0.01)	257 (±22.33)	0.11 (±0.24)	6	0.05 (±0.03)
Seawater	3.33 (±0.23)	14.57 (±2.18)	0.68 (±0.04)	171 (±60.24)	-	5	0.07 (±0.05)