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A comparison of free-living and sponge-associated bacterial communities from a remote oceanic island with a focus on calcareous sponges

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## Abstract

Calcareous sponges are an often overlooked element of sponge communities. In contrast to most other sponges, calcareous sponges produce calcium carbonate spicules, as opposed to the siliceous spicules of most sponges. Here, we investigated bacterial communities of 17 sponge species, including type and paratype specimens of recently described calcareous species, sampled off the remote island of Rodrigues, in the Indian Ocean. The main axis of variation in a PCO analysis of all samples separated non-calcareous sponge species including *Axinyssa aplysinoides*, *Cinachyrella* aff. *australiensis*, *Petrosia seychellensis*, *Ircinia* aff. *variabilis*, *Spongia ceylonensis*, *Plakinastrella* aff. *clipptertonensis*, *Agelas* aff. *ceylonensis*, *Agelas* aff. *Mauritiana*, and *Hyrtios erectus* from calcareous sponges, the non-calcareous *Biemna tubulata*, sediment and seawater. Overall, the bacterial communities of calcareous sponges revealed unique prokaryotic profiles with low abundances of several bacterial phyla, and relatively high abundances of other taxa e.g., the phyla Fibrobacterota, Proteobacteria, and SAR324 clade, the class Alphaproteobacteria and orders Cytophagales and Cyanobacteriales, although there was considerable variation among species. Calcareous sponges also had a high dominance of unknown bacterial operational taxonomic units (OTUs). Considering the unique nature of these communities, further studies are needed to better understand the environmental and ecological drivers of calcareous sponge-associated bacterial communities and their relevance as potential sources of novel microbes of biotechnological interest.

Keywords: bacteria, Calcarea, composition, Mauritius, Rodrigues

## Introduction

Sponges come in all shapes and forms from thinly encrusting to massive, and basket-shaped to flabelliform. The colours range from drab brown to hues of red, yellow, purple and a range of other colours. They also include hard, coral-like sponge species known as sclerosponges, which lay a carbonate skeleton, delicate glass sponges, denizens of the deep sea, bath sponges and other soft-bodied species. They can be found in freshwater and marine environments, in the tropics and Antarctic and in rivers and canals (Hooper and van Soest 2002; Bell 2008; Wulff 2001; Erpenbeck et al. 2011; Maldonado et al. 2016). An ancient group, sponges have received considerable attention from microbiologists because of the presumed, ancient (~ 600 million years), relationships they have established with microbial symbionts (Taylor et al. 2007; Thacker and Freeman 2012). These symbionts are believed to have played crucial roles during the evolution of sponges enabling them to adapt to the wide range of environmental conditions in which they are presently found (Wilkinson 1984).

Microbes perform a large number of important functions within their sponge hosts ranging from nutrient translocation and vitamin synthesis to the production of secondary metabolites involved in defense against predators. The resident microbial communities also produce antibiotic compounds, which inhibit colonisation of potentially harmful pathogens (Piel 2009; Fan et al. 2012; Hentschel et al. 2012). The wide array of secondary metabolites found in sponges are often derived from resident microbes. Many of these compounds have considerable biotechnological and pharmaceutical potential (Blunt and Munro 1998; Osinga et al. 1998; van Soest and Braekman 1999; Faulkner et al. 2000; Hentschel et al. 2002; Sipkema et al. 2005; Taylor et al. 2007).

Sponges have been deemed an excellent model for studying host-microbe interactions (Taylor et al. 2007; Hentschel et al. 2012; Cleary et al. 2013, 2015, 2019; Cleary, Polónia, Becking et al. 2018; Thomas et al. 2016; Swierts et al. 2018). A large number of studies have highlighted the importance

of sponge host identity (species) in structuring microbial communities followed by microbial abundance status (Cleary et al. 2015, Cleary, Polónia, and de Voogd 2018; 2020; de Voogd et al. 2015, 2019; Ribes et al. 2015; Thomas et al. 2016; Chaib De Mares et al. 2017). Microbial abundance status refers to the density of microbes inhabiting sponge tissues. A number of early studies highlighted marked differences in microbial densities among different sponge species (Vacelet and Donadey 1977; Reiswig 1981; Hentschel et al. 2002, 2003). These were initially referred to as bacterial and non-symbiont sponges, which was later replaced by high microbial abundance (HMA) and low microbial abundance (LMA) sponges due to the generally much greater microbial densities of HMA species ( $10^8$ - $10^{10}$  microbial cells/g of tissue) compared to LMA species ( $10^5$ - $10^6$  microbial cells/g of tissue) (Hentschel et al. 2006).

Much of the research on sponge microbiomes has focused on various demosponge species. However, not all sponges have received the same degree of scrutiny. Calcareous sponges (class Calcarea), which first appeared during the Cambrian (Botting et al. 2012), are exclusively marine organisms and comprise approximately 8% of total sponge species (van Soest and de Voogd 2018). Most species occur at shallow depths and only few species are known from the deep sea (Voigt et al. 2012). Unlike most other sponges that may have a skeleton made of siliceous spicules, calcareous sponges only produce calcite spicules, which constitute a synapomorphy of the group (Voigt et al. 2021). Most calcareous sponges are inconspicuous, small and white organisms, and tend to grow in cryptic environments, such as underneath coral overhangs and marine caves. Their cryptic nature and lack of specialist taxonomists has led to them being largely overlooked in biodiversity surveys (Manuel et al. 2002; van Soest and de Voogd 2015). Only a few species are common such as the Indo-Pacific *Leucetta chagosensis*, or can even be nuisance, exotic species such as *Paraleucilla magna* along the Mediterranean coast (Longo et al. 2007; van Soest and de Voogd 2018).

As a consequence of the above, the microbes of calcareous sponges have also been largely understudied in comparison to demosponges, with just a few recent studies assessing their microbiomes (Cleary et al. 2020; Kallscheuer et al. 2020; Moreno-Pino et al. 2020; Ribeiro et al. 2022). Thus far, only five calcareous sponge genera have been studied using next-generation sequencing, namely *Clathrina*, *Leucetta*, and *Leucosolenia*, *Sycettusa*, and *Paraleucilla* (Ribeiro et al. 2022). These studies highlighted the predominance of the phyla Proteobacteria, Cyanobacteria, Planctomycetes, Actinobacteria and Bacteroidetes, with Proteobacteria as the most abundant phylum (Cleary et al. 2020; Kallscheuer et al. 2020; Lopes et al. 2019; Moreno-Pino et al. 2020; Ribeiro et al. 2022). Moreover, the relatively low microbial densities and diversities identified suggested LMA status (Cleary et al. 2020). Promising antibacterial and antioxidant compounds, probably involved in the bacterial cross-talk within the sponge, have been isolated from calcareous sponge-associated bacteria (Flemer et al. 2012; Roué et al. 2012; Fromont et al. 2016).

In the present study, we sampled several sponge species belonging to three different sponge classes (Calcarea, Demospongiae and Homoscleromorpha), sediment and seawater in waters surrounding the remote island of Rodrigues in the Indian Ocean. Rodrigues is a small volcanic island in the Indian Ocean surrounded by a wide lagoon with a fringing reef and in the north by channels, reefs and caves. The marine fauna is currently not well-studied (Turner and Klaus 2005; Jahajeeah et al. 2021).

The sponge species sampled included several new to science (including type material), of which the calcareous sponges have already received formal scientific description (van Soest and de Voogd 2018). This is the first study to characterise the prokaryotic communities of five different calcareous sponge species, including genera and species that have never been studied before. The main goals of the present study were to compare the prokaryotic communities of calcareous sponges with communities housed in other sponge species belonging to two other classes, sediment and seawater.

Specific aims of the present study were to (1) compare bacterial diversity, composition and higher taxon relative abundance among sponge host- associated and free-living (sediment and seawater) bacterial communities, and (2) identify abundant OTUs and closely related organisms using the NCBI nucleotide database.

## Material and Methods

### *Sample collection and study sites*

Rodrigues is a small volcanic island in the Indian Ocean, about 620 km northeast of Mauritius and is part of the Mascarene plateau. Rodrigues has a surface area of about 119 km<sup>2</sup> and is enclosed by a fringing reef at the north side, but a large lagoon envelops the reef from the east to the western part of the island and has a surface almost twice that of the island itself (240 km<sup>2</sup>). Several dredged and natural channels are present in the north near the harbour of Port Mathurin and two in the south near Port Sud Est and in the east at St. François. The sea surface temperature varies between 22-28° C winter and summer (Zinke et al. 2016). A map of the study area and sampling locations is shown in Fig. 1.

We collected 49 fragments of sponges using SCUBA diving between 16-21<sup>st</sup> October 2016 at four different sites (Fig. 1 and Supplementary data 1) between 3 and 21 m depth (Fig. 1). After collection, the sponges were carefully cleaned of debris and subsequently stored in 96% ethanol and stored at -20 °C. The sponges were identified by NjdV (but see van Soest and de Voogd, 2018) using classical morphological characters and voucher specimens have been deposited in the sponge collection of Naturalis Biodiversity Center (as RMNH POR#) (Supplementary data 1).

The species investigated in the present study were assigned to 3 classes, 8 orders and 17 species. The demosponge species sampled included seven Heteroscleromorph sponges, namely, *Agelas* aff. *ceylonensis* (Fig. 2a), *Agelas* aff. *mauritiana* (Fig. 2b), *Biema tubulata* (Dendy 1905) (Fig. 2c), *Axinyssa aplysinoides* (Dendy 1922), *Cinachyrella* aff. *albatridens*, *Cinachyrella* aff. *australiensis* and *Petrosia seychellensis* Dendy 1922 (Fig. 2d), three keratose sponge species, namely, *Hyrtios erectus* (Keller, 1889), *Ircinia* aff. *variabilis* (Fig. 2f), and *Spongia ceylonensis* (Dendy, 1905) and one Homoscleromorph sponge species, namely, *Plakinastrella* aff. *clipptertonensis* (Fig. 2e). The calcareous sponges sampled included six species all belonging to the subclass Calcinea of which 9 samples are part of the original type material, namely, *Borojevia pirella* Van Soest and de Voogd, 2018 (Fig. 2i, holotype), *Clathrina luteoculcitella* Wörheide and Hooper, 1999, *Clathrina rodriguesensis* van Soest and de Voogd 2018 (Fig. 2g, holotype and 4 paratypes), *Ernstia naturalis* (van Soest and de Voogd, 2015) (Fig. 2h), *Leucetta chagosensis* Dendy 1913 and *Leucetta sulcata* van Soest and de Voogd, 2018 (holotype and 2 paratypes). The number of samples per biotope varied from one (*A. aplysinoides*, *P. seychellensis*, *B. pirella*, and sediment), two (*L. chagosensis*, *C. luteoculcitella*, *Cinachyrella* aff. *albatridens*, and *H. erectus*), three (*L. sulcata*, *Agelas* aff. *ceylonensis*, *Cinachyrella* aff. *australiensis*, *Plakinastrella* aff. *clipptertonensis*, and *I. variabilis*), four (*Agelas* aff. *mauritiana*, *Biema tubulata*, *Spongia ceylonensis*, and water), and five (*C. rodriguesensis*) to six (*E. naturalis*). Some of the investigated species have been studied before such as *A. aplysinoides*, *L. chagosensis*, *H. erectus*, *S. ceylonensis* and *Ircinia* aff. *variabilis* (de Voogd et al. 2015; Cleary et al. 2020); others may be restricted (endemic) to Rodrigues, e.g., *Plakinastrella* aff. *clipptertonensis*, *Cinachyrella* aff. *albatridens*, *L. sulcata*, *B. pirella*, *C. rodriguesensis* or have been observed in other regions, but were not previously studied such as *B. tubulata*, *C. luteoculcitella*, *E. naturalis*, or *P. seychellensis*. Selected sponge species are shown in Fig. 2. In addition to the above, sediment and seawater samples were collected. Sediment was collected by scraping surface sediment into a falcon tube. The bacterioplankton community was sampled by first

collecting 1 l of seawater at  $\approx$  1 m depth and subsequently filtering this through a Millipore® White Isopore Membrane Filter (0.22  $\mu$ m pore size), which was preserved in 96% ethanol.

### *DNA extraction and next-generation sequencing analysis*

PCR-ready genomic DNA was isolated from all samples using the FastDNA® SPIN soil Kit (MPbiomedicals) following manufacturer's instructions. Briefly, the whole membrane filters, for water samples, were cut into small pieces and transferred to Lysing Matrix E tubes containing a mixture of ceramic and silica particles; for the other samples  $\pm$  500 mg of sponge tissue (including parts of the surface and interior) or sediment were added. The microbial cell lysis was performed in the FastPrep® Instrument (Q Biogene) for 80 s at 6.0 ms<sup>-1</sup>. The extracted DNA was eluted into DNase/Pyrogen-Free water to a final volume of 50  $\mu$ l and stored at  $-20$  °C until use. The 16S rRNA gene V3-V4 variable region PCR primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 785R 5'-GACTACHVGGGTATCTAATCC-3' (Klindworth et al. 2013) with barcode on the forward primer were used in a 30 cycle PCR assay using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. After amplification, PCR products were checked on a 2% agarose gel to determine the success of amplification and the relative intensity of bands; the blank control did not yield any bands. PCR product was used to prepare the DNA library following the Illumina TruSeq DNA library preparation protocol. Next-generation, paired-end sequencing was performed at MrDNA (Molecular Research LP; <http://www.mrdnalab.com/>; last checked 18 November 2016) on an Illumina MiSeq device using MiSeq Reagent Kit v3 (Illumina Inc., San Diego, CA, USA) following the manufacturer's guidelines. Sequences from each end were joined following Q25 quality trimming of the ends followed by reorienting any 3'-5' reads back into 5'-3' and removal of short reads (<150 bp). The DNA sequences generated in this study can be downloaded from NCBI BioProject Id: PRJNA715750.

### *16S rRNA Gene Sequencing Analysis*

The 16S rRNA amplicon libraries were analysed using QIIME2 (version 2019.7; Bolyen et al. 2019). Raw data was imported yielding a demultiplexed 'qza' data file (artifact). The DADA2 plugin (Callahan, et al., 2016) in QIIME 2 was subsequently used to trim sequences (final length 400 nt). The DADA2 analysis yielded output archives containing an OTU (also known as amplicon sequence variant or 'ASV') table, denoising stats and a fasta file of representative sequences. The feature-classifier plugin with the extract-reads method was then used with the *i*-sequences argument set to silva-138-99-seqs.qza. This was followed by the feature-classifier plugin with the fit-classifier-naive-bayes method and the *i*-reference-taxonomy method set to silva-138-99-tax.qza. Both silva-138 files can be obtained from <https://docs.qiime2.org/2020.8/data-resources/?highlight=silva>. The feature-classifier plugin was then used with the classify-sklearn method and the *i*-reads argument set to the representative sequences file generated by the DADA2 analysis to produce a table with taxonomic assignment for all OTUs. Finally, mitochondria, chloroplasts, and Eukaryota were filtered out using the qiime taxa plugin with the filter-table method. The OTU and taxonomy tables were later merged in R. Accession numbers of closely related organisms to selected OTUs were obtained using NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990).

### *Statistical Analyses*

Supplementary data 2 contains all OTU counts per sample and taxonomic assignments of all OTUs. Variation in composition among biotopes was visualised with Principal Coordinates Analysis (PCO). For the PCO, a Bray-Curtis distance matrix was first obtained using the Phyloseq package

(McMurdie and Holmes 2013) whereby the count data was rarefied using the `rarefy_even_depth` function with the `sample.size` argument set to the minimum sample size (12206 in the present study) and subsequently  $\log_{10}$  transformed.

We used a machine learning technique, Boruta, to identify significant features (bacterial classes and orders), which distinguished calcareous sponge species from non-calcareous biotopes. Boruta, named after a slavic forest demon, is a random forest wrapper, which is used to evaluate feature importance (Kursa et al. 2010). The first step in the Boruta algorithm is the generation of a data frame consisting of randomly shuffled versions of all original features, which are termed shadow features. This data frame is then attached to the data frame of original features creating a new data frame with double the amount of features. A random forest machine learning algorithm is then fitted to the new data frame consisting of all the original and shadow features in addition to the response variable. The importance of each original feature is then compared with a threshold based on the shadow features. This process is repeated a given number of times (permutations) producing a binomial distribution. Original features at the tails of the distribution are either rejected or accepted while those in the middle are considered tentative. Increasing the number of randomisations can help to resolve tentatively-classified features. In the present study, we generated three data frames consisting of orders, classes, OTUs and a response variable indicating whether a sample was a calcareous sponge or a non-calcareous biotope. Prior to the Boruta analysis, highly correlated features ( $> 0.8$ ) were removed from the data frames. The Boruta function from the Boruta package in R (Kursa and Rudnicki 2010) was then used with phyla, classes, or orders as features (predictive variables). The `doTrace` argument in the Boruta function was set to 2 and the `maxRuns` argument set to 1000; other arguments used default values. The number of significant features was identified and the features (phyla, classes, or orders) with the highest importance presented. Certain features (taxa), however, were similar across taxonomic levels, e.g., the phylum Dadabacteria only consisted of members assigned to the class Dadabacteriia. We, therefore, included different features when this was the case and although both the phylum Dadabacteria and class Dadabacteriia were both significant features, we only included the phylum Dadabacteria in the results.

## Results

The data in the present study consisted of 53 samples belonging to 19 groups (17 sponge species, sediment and seawater) (Supplementary data 1). After quality control, the dataset consisted of 3542767 sequences and 12429 OTUs. In terms of sequences, the most abundant phyla in the present report were Proteobacteria (2034002 sequences, 4709 OTUs representing 57.4% of sequences and 37.9% of OTUs), Actinobacteriota (274749 sequences, 369 OTUs representing 7.8% of sequences and 3.0% of OTUs), Bacteroidota (229091 sequences, 1981 OTUs representing 3.7% of sequences and 16.0% of OTUs) and Chloroflexi (219324 sequences, 456 OTUs representing 6.2% of sequences and 3.7% of OTUs). In Fig. 3, the mean relative abundances of the eight most abundant phyla are shown. These accounted for 91.69% of all sequences and 66.31% of all OTUs.

Proteobacterial relative abundance was highest in the calcareous sponges (with the exception of *L. chagosensis*), and the non-calcareous sponges *B. tubulata* and *Cinachyrella* aff. *albatridens*. In contrast, the relative abundances of Actinobacteriota, Gemmatimonadota and Acidobacteriota were lower in these groups and seawater than all other groups. Chloroflexi relative abundance, in turn, was particularly high in *Plakinastrella* aff. *clipptertonensis*. The SAR324 clade was most abundant in the calcareous species *C. luteoculcitella*, *C. rodriguesensis* and, particularly, *L. chagosensis*., but much less so in the calcareous species *E. naturalis*, and *L. sulcata*. Calcareous sponges also had very low relative abundances of Nitrospirota.

A PCO ordination showing the variation in composition among samples is presented in Fig. 4. The first axis of variation separated samples from all calcareous sponges, *Cinachyrella* aff. *albatridens*, *B. tubulata*, sediment and seawater from the remaining samples. The second axis separated samples of *E. naturalis*, *B. pirella* and the single sediment sample from samples of the calcareous *C. luteoculcitella*, *C. rodriguesensis*, *L. chagosensis*, *L. sulcata*, and non-calcareous *Cinachyrella* aff. *albatridens* and *B. tubulata*.

The Boruta analysis yielded 16 significant phylum predictors, 21 significant class predictors and 18 significant order predictors ( $P < 0.01$ ). Six of the phyla, classes and orders with the greatest importance values are presented in Figs. 5, 6 and 7. Supplementary Figs. 1 – 3 show variation in the relative abundances of the same predictors at the biotope level. Of the significant predictor phyla, the Fibrobacterota, Proteobacteria, and SAR324 clade were more abundant in calcareous sponges although there was considerable variation. The Nitrospirota, Dadabacteria and Actinobacteriota phyla were relatively abundant in non-calcareous sponges, albeit highly variable in sponges of the subclass Heteroscleromorpha. All of these phyla had relatively low abundances in seawater and sediment samples. The class Alphaproteobacteria was relatively more abundant in calcareous sponge samples whereas the remaining classes were more abundant in other sponge samples. The classes bacteriap25 and Vicinamibacteria reached greatest abundance in the Homoscleromorpha and Keratosa and the Dehalococcoidia class was particularly abundant in the Homoscleromorpha, which was represented by a single species, *Plakinastrella* aff. *clipptertonensis*. At the order level, the Cytophagales and Cyanobacteriales were more abundant in calcareous sponge samples. The orders AT-s3-44, Defluviococcales, and PAUC34f were most abundant in the non-calcareous sponges. The BD2-11 terrestrial group was most abundant in sponges of the Heteroscleromorpha and Keratosa subclasses.

The 50 most abundant OTUs are presented in Fig. 8 and supplementary data 3. *Biemna tubulata* and the calcareous species were dominated by just a few OTUs assigned to the Proteobacteria, Bacteroidota and the SAR324 clade. OTUs 39, 60, 104 and 105, assigned to the BD2-7 genus (Gammaproteobacteria), OM182 clade (Gammaproteobacteria), and Rhodobacteraceae (OTUs 104 and 105), respectively dominated the bacterial community of *B. tubulata*. These OTUs had 94.25 to 98.5% sequence similarities to organisms in the NCBI nucleotide database (NCBI accession numbers [Acc]: JQ197137, KT974745, MF102057 and MK588888). OTU-72, assigned to the Alphaproteobacteria, had 93.25% sequence similarity to an organism obtained from an ascidian identified as *Ciona intestinalis*, dominated the bacterial community of *B. pirella* (Acc: KF798911).

Four OTUs (33, 49, 87 and 192), assigned to the Gammaproteobacteria, Alphaproteobacteria, SAR324 clade and pltb-vmat-80 order (Gammaproteobacteria) dominated the bacterial community of *C. luteoculcitella* and had sequence similarities ranging from 92.7 to 97% to organisms in the NCBI nucleotide database (Supplementary data 5). Six OTUs, assigned to the Alphaproteobacteria (49, 148), Gammaproteobacteria (118, 126), pltb-vmat-80 order (159) and SAR324 clade (183) dominated the bacterial community of *C. rodriguesensis*. These organisms had 92.56 to 96.75% sequence similarities to organisms in the NCBI nucleotide database (Supplementary data 5). Three OTUs, all assigned to the Alphaproteobacteria, dominated the bacterial community of *E. naturalis*. These OTUs had 91.79 to 93% sequence similarities to organisms in the NCBI nucleotide database. A single OTU (OTU-110), assigned to the SAR324 clade, and with 87.56% sequence similarity to an organism obtained from a white microbial mat in Norway, accounted for 57.6% of all sequences in the bacterial community of *L. chagosensis* (Acc: FR875936). The two next most abundant OTUs in *L. chagosensis*, OTUs 15 and 63, assigned to the Bacteroidia and Gammaproteobacteria classes, respectively, accounted for less than 2% of sequences and both were more abundant in water. Three OTUs (11, 42 and 47), assigned to the SAR116 clade (Alphaproteobacteria; OTUs 11 and 47) and the species *Aureibacter tunicatorum* (Bacteroidia: Cytophagales; OTU 42) dominated the bacterial



community of *L. sulcata*. These OTUs had sequence similarities ranging from 92 to 96.5% to organisms in the NCBI nucleotide database (Supplementary data 3).

## Discussion

Calcareous sponges play potentially important roles in marine ecosystems, particularly cave and other cryptic habitats, where they provide habitat for a variety of other marine organisms, have been shown to produce a diverse array of bioactive natural products, and are possibly involved in nutrient cycling. They, however, remain understudied compared to demosponges (Roué et al. 2012). Understanding the ecology of these organisms can help us to better understand the functioning of marine ecosystems and the interactions between different species. The results of our study show that calcareous sponges host bacterial communities distinct from those of other biotopes although there was considerable variation among different calcareous sponge species. Certain calcareous sponge species, for example, hosted bacterial communities with greater similarities to other non-calcareous sponges than to other calcareous sponge species.

With respect to composition, the first axis of the PCO ordination separated non-calcareous sponge species including *H. erectus*, *Ircinia* aff. *variabilis*, *S. ceylonensis* and *A. aplysinoides* from the remaining samples consisting of the calcareous sponges, sediment, seawater and the non-calcareous sponges *B. tubulata* and *Cinachyrella* aff. *albatridens*. The second axis of variation in the PCO analysis, separated taxa with greater similarity to water samples and other taxa, namely, the calcareous species *B. pirella* and *E. naturalis* with greater similarity to the single sediment sample. In addition to *B. pirella* and *E. naturalis*, de Voogd et al. (2015) previously showed that the sponge *Liosina paradoxa* (order Tethyida), sampled in Mayotte, also housed a highly diverse bacterial community similar to that found in sediment samples; de Voogd et al. (2015) attributed this to incorporation of sediment within the sponge skeleton. Most of the sampled calcareous species grew on the walls of (semi)-dark caves, and sediment entered through cracks and fissures in the cave ceiling. Trapped sediment was observed in between the anastomosing tubes of both *B. pirella* and *E. naturalis* (see Figs. 12a, and 31a-d in de Voogd and van Soest, 2018). Although we cleaned the sponges of excess sediment grains, sediment particles can be seen in a cross section of the skeletons of *B. pirella* and *E. naturalis* (see Figs. 13a, and 32a-b in de Voogd and van Soest, 2018). However, the incorporation of sediment grains of these two species is not similar to that observed in *L. paradoxa* where incorporated sand grains are part of the sponge skeleton. Unfortunately, the present study only included a single sediment sample. Future studies of calcareous sponges should include more sediment samples in order to better assess the potential importance of sediment in structuring the bacterial communities of calcareous sponges.

The Boruta analysis identified several phyla, classes and orders, which distinguished calcareous sponges from other biotopes. The phyla Fibrobacterota, Proteobacteria, and SAR324 clade, class Alphaproteobacteria and orders Cytophagales and Cyanobacteriales were more abundant in calcareous sponges than other biotopes although there was considerable variation in abundance among calcareous species. Relatively little is known about the Fibrobacterota phylum as pertains to ecology and physiology despite it being a diverse group (Rahman et al. 2016). Previous studies, however, have shown that different *Fibrobacter* strains are capable of lignocellulose degradation with potential applications for biofuel production (Bera-Maillet et al. 2004; Ransom-Jones et al. 2012, Raut et al. 2019). Members of this class have been previously identified in landfill sites and freshwater lakes (Ransom-Jones et al. 2012). The candidate phylum SAR324, formerly assigned to the Deltaproteobacteria, is widespread across a range of environments and can be abundant in various marine ecosystems including hydrothermal vents, the deep sea and as was a dominant member of a sponge species in the present study. Metagenome-assembled genomes (MAGs) of the SAR324 clade were shown to be highly versatile and included hetero- and autotrophic pathways

and pathways for oxic and anoxic conditions (Malfertheiner et al. 2022). In the present study, the calcareous sponge *L. chagosensis* was dominated by a single OTU (OTU-110) assigned to the SAR324 clade. In a previous study of sponges sampled from the Red Sea region of Saudi Arabia (Cleary et al. 2020), we showed that the bacterial community of *L. chagosensis* was also dominated by OTUs assigned to the SAR324 clade. This indicates that the association between SAR324 and *L. chagosensis* may be of a more stable nature and that its bacterial community is not simply a reflection of the bacterioplankton community. OTU-110, furthermore, only accounted for 0.01% of sequences in seawater.

Alphaproteobacterial members have been reported as one of the most widespread, dominant and diverse groups of sponge-associated bacteria (Hardoim et al. 2014; Thomas et al. 2016; Cleary et al. 2019; Karimi et al. 2019), and have been previously shown to be abundant in calcareous sponges (Lopes et al. 2019). Alphaproteobacteria are, furthermore, a metabolically extremely diverse group of microorganisms with important biological functions (Williams et al. 2007). Cytophagales are chemoorganotrophs known for the production of flexirubin-type pigments and starch hydrolysis (Reichenbach 2006). They have been shown to produce a range of hydrolytic enzymes, which can degrade various high molecular weight macromolecules including proteins, chitin, pectin, agar, starch, or cellulose (Kirchman 2002). As such, they may play an important role in the turnover of dissolved organic matter. In sponges, Cytophagales members were found to be abundant in Antarctic sponge species with the Cytophagales order representing around 48% of the bacterial community of the sponge *Hymeniacidon torquata* (Cristi et al. 2022). In the present study Cytophagales were particularly abundant in the species *L. sulcata*. Perea-Blázquez et al. (2009) studied the diet of two calcareous sponges (*Leucosolenia echinata* and *Leucetta* sp.) in New Zealand and used flow cytometry to quantify food particles obtained via filter feeding. They showed that the diet consisted of heterotrophic and phototrophic (*Prochlorococcus* spp. and *Synechococcus* spp.) bacteria with the latter removed with the highest efficiency by both species. The selection of Cyanobacteriales as a significant indicator of calcareous sponges suggests a similar role in the present study although this requires further confirmation.

The calcareous sponges and *Cinachyrella* aff. *albatridens* had very low levels of Nitrospirota, Dadabacteria and Actinobacteriota. The Nitrospirota phylum includes members, which oxidise nitrite to nitrate (the last step in nitrification) in addition to commamox (complete ammonia oxidation), which oxidises ammonia to nitrate (Norton et al. 2008; Daims et al. 2016). The prevalence of Nitrospirota members in non-calcareous sponges in the present study suggests that these sponge species may be an important link in marine nitrification. Their rarity in calcareous sponges is interesting and worthy of additional research. Dadabacteria are relatively understudied, but the available genomic research suggests that members are involved in the degradation of microbially-derived dissolved organic matter, such as peptidoglycans and phospholipids (Graham et al. 2021). This would help to explain their prevalence in selected sponge species. Actinobacteriota are a diverse group of gram-positive bacteria, commonly found in terrestrial and aquatic environments, and known for their ability to produce a wide variety of secondary metabolites, including antibiotics with considerable biotechnological potential (Manivasagan et al. 2014).

At the class level, the classes Dehalococcoidia, bacteriap25, Vicinamibacteria, and TK17 were under-represented in calcareous sponges. Dehalococcoidia and TK17 are both taxa within the Chloroflexi phylum, an important HMA indicator taxon in addition to the phylum Actinobacteriota (Hentschel et al. 2002; Moitinho-Silva et al. 2017). The bacteriap25 is a class of the Myxococcota, a phylum consisting of gram-negative, facultatively anaerobic bacteria. They are known for their ability to form multicellular fruiting bodies, which are structures that contain spores and are used for reproduction (Velicer and Stredwick 2002). The class Vicinamibacteria was recently assigned to the Acidobacteriota (Dedysh and Yilmaz 2018).

The orders Defluviococcales, AT-s3-44, BD2-11 terrestrial group, and PAUC34f were all under-represented in calcareous sponges. In the the southeastern Mediterranean Sea, a number of taxa including the Defluviococcales, SAR202, bacteriap25 and Gemmatimonadales were significantly associated with increasing sediment depth (Rubin-Blum et al. 2021). Wang et al. (2020), furthermore, described a Defluviococcales-dominated community, which alternately accumulated glycogen and polyhydroxyalkanoate, the latter of which was used for growth coupled with nitrite oxidation to N<sub>2</sub>O. The order AT-s3-44 was assigned to the Alphaproteobacteria class and, in contrast to the general trend among alphaproteobacterial orders, was more abundant in non-calcareous sponges. The BD2-11 terrestrial group was assigned to the Gemmatimonadota phylum. Sequences assigned to the BD2-11 terrestrial group have been recovered from marine sediments, sponges, and soils (Hanada and Sekiguchi 2014). Note that the Acidobacteriota, PAUC34f, and Gemmatimonadota are all HMA indicator taxa (Moitinho-Silva et al. 2017).

Although the random forest machine-learning method used in Boruta resulted in taxa, which significantly differentiated between calcareous sponges and other biotopes, it is important to note that most of the variation was attributable to differences among species as opposed to between calcareous sponges and other biotopes. Future studies should also try to include greater numbers of known LMA species and sediment samples in addition to a range of calcareous sponge species in order to better understand how their bacterial communities compare to those of other sponges.

In a comparison of two calcareous sponges, Ribeiro et al. (2022) showed that the calcareous sponge *Sycettusa hastifera*, had a spatially stable bacterial community. This was not, however, the case for *Paraleucilla magna*. It was unclear, however, whether this was related to spatial or local environmental processes. The bacterial communities of both species mainly consisted of Proteobacteria, Cyanobacteria and Bacteroidetes. At the class level, *S. hastifera*, was dominated by OTUs assigned to the Betaproteobacteria whereas *P. magna* was dominated by OTUs assigned to the Alphaproteobacteria. Proteobacteria, Cyanobacteria and Bacteroidetes are often the dominant taxa in LMA species thus indicating LMA status for both species (Moitinho-Silva et al. 2017). In the case of *P. magna*, the most abundant OTU was assigned to the alphaproteobacterial species *Mycoplana ramosa*, which has the potential to reduce nitrate (Urakami et al. 1990). In contrast to *P. magna* and the calcareous sponges in our study, *S. hastifera* housed an abundant OTU assigned to the genus *Nitrosospira*.

Most abundant OTUs in the present study were found across a range of host species in addition to sediment and seawater in line with a previous study of ours (Cleary et al. 2019). The bacterial communities of the calcareous sponges were, however, dominated by one to five highly abundant OTUs, again in line with previous studies (Cleary et al. 2020, 2021). In a comparison with the NCBI nucleotide database, we also found relatively low sequence similarities to sequences in the database. This indicates that the abundant OTUs associated with the calcareous sponges in the present study represent hitherto undiscovered bacterial taxa. In addition to this, none of the database sequences were from sponge hosts, in contrast to previous studies and the non-calcareous sponges in the present study (Cleary et al. 2020, 2021; Supplementary data 5). It is possible that the remote, and isolated, nature of Rodrigues plays a role in the potential novelty of the OTUs in the present study and the lack of research in that particular part of the globe, namely the southern Indian Ocean. The hosts/isolation sources with the highest total scores using BLAST search to query sequences from calcareous sponges included organisms collected from sediment, seawater, a mud volcano, microbial mat, corals, and ascidians.

## *Conclusion*

Calcareous sponge species in the present study harboured very low relative abundances of several phyla including HMA indicator taxa such as Chloroflexi, Gemmatimonadota and Acidobacteriota in addition to other taxa such as Nitrospirota and Dadabacteria suggesting a distinct role of their communities in nutrient dynamics. Notable in this study, Fibrobacterota was a phylum-level predictor of calcareous sponges. Members of this phylum are often described in the literature as fiber-degrading organisms with potential applications in the process of lignocellulose degradation for biofuel production. Interestingly, all of the dominant OTUs associated with these sponges belonged to unknown bacterial taxa. In addition to this, none of these OTUs were closely related to organisms previously detected in other sponge hosts. The uniqueness of the calcareous sponge microbiome indicates that these sponges may be a hitherto untapped source of novel microbes of biotechnological interest. Future research should strive to include calcareous sponges in surveys in order to assess to what extent they consistently house bacterial communities with distinct sets of abundant OTUs in comparison to other sponge taxa.

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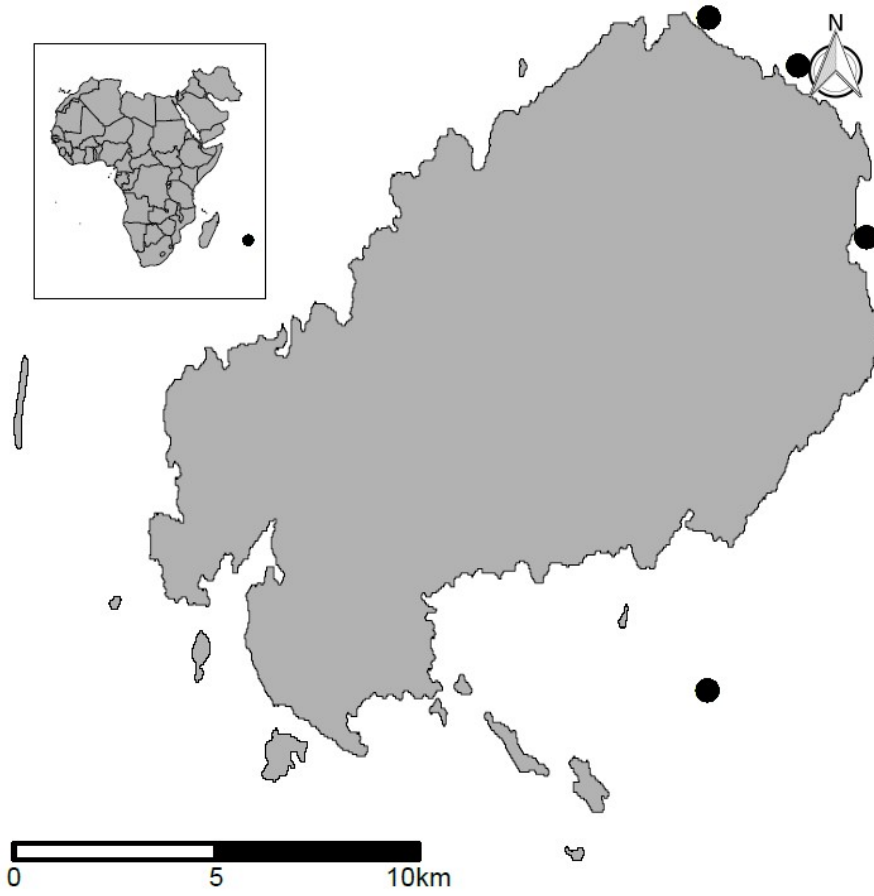


Fig. 1. Map of study area showing location of sample sites (black circles).

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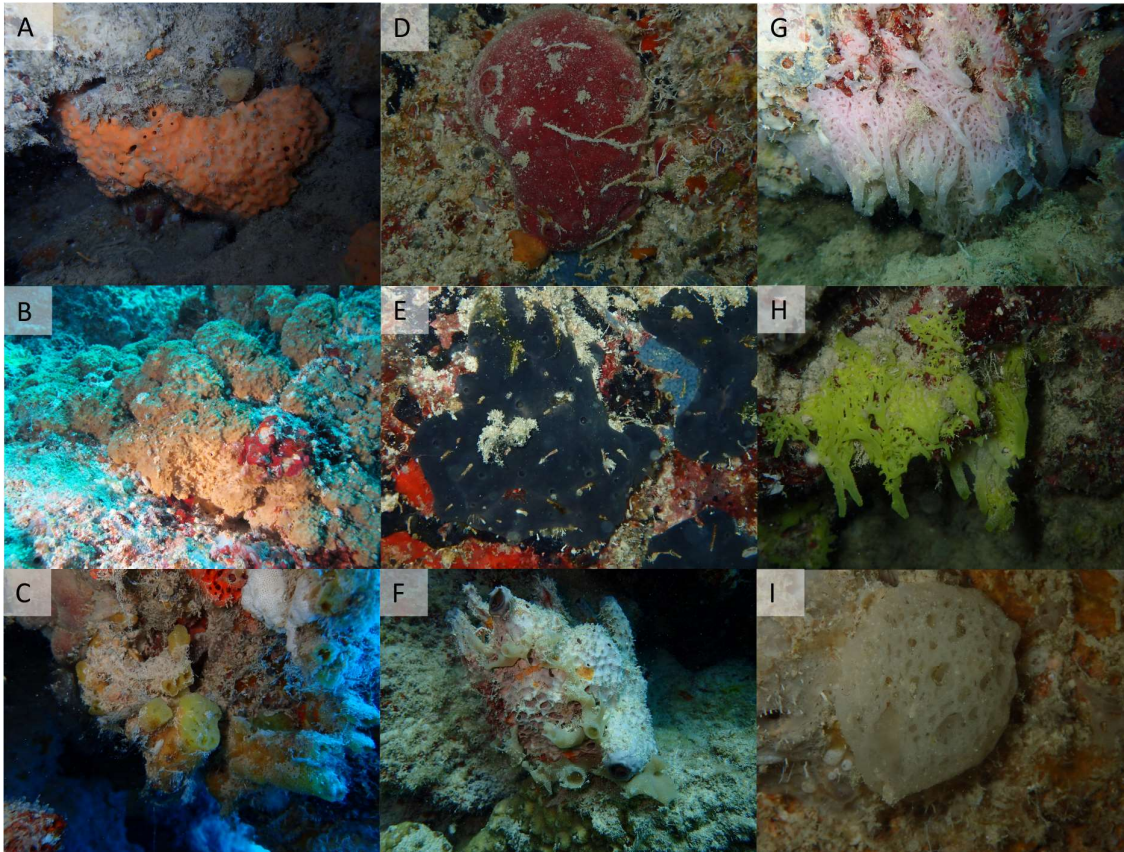


Fig. 2. Photos of selected sponge species. a. *Agelas* aff. *ceylonensis*, b. *Agelas* aff. *mauritiana*, c. *Biema tubulata*, d. *Petrosia seychellensis*, e. *Plakinastrella* aff. *Clipptertonensis*, f. *Ircinia variabilis*, g. *Clathrina rodriguesensis*, h. *Ernsta naturalis*, i. *Borojevia pirella* (photos a, c-i by NJ de Voogd, and b, by S. Fichou).

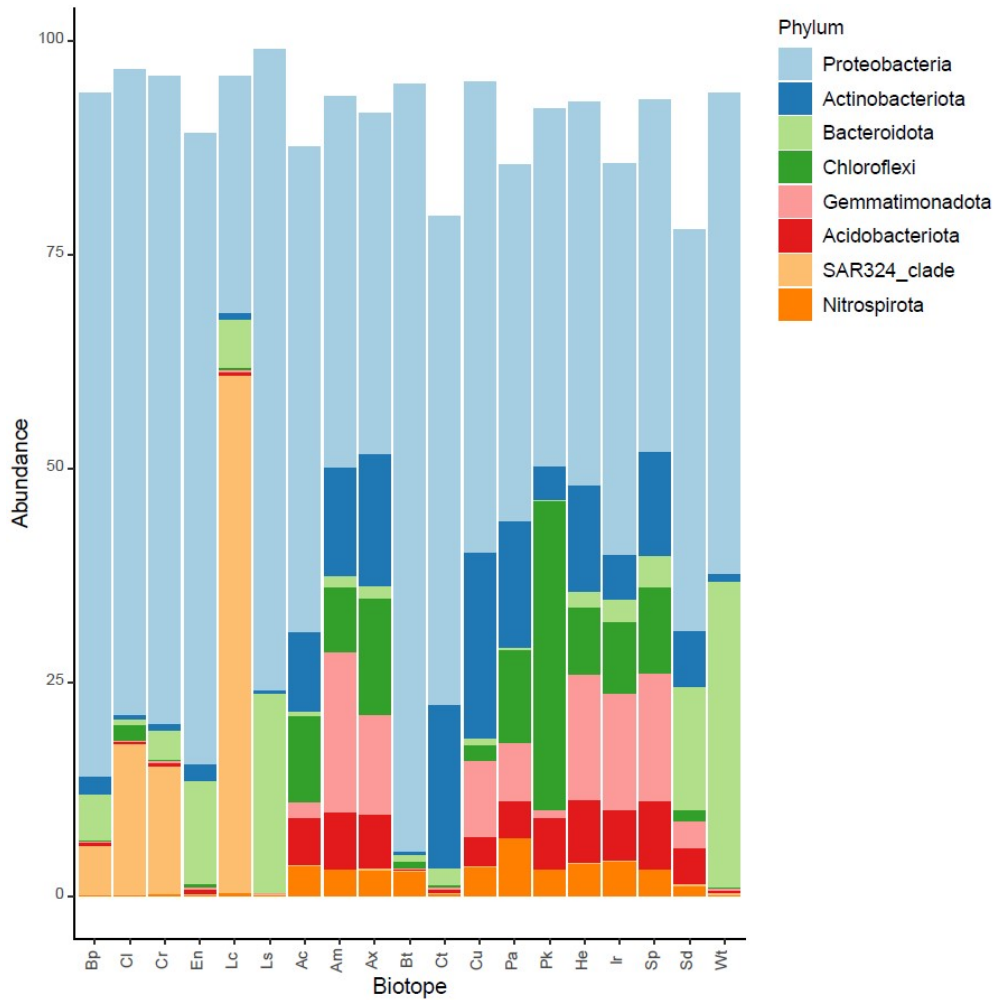


Fig. 3. Stacked barplots of the mean relative abundances of the eight most abundant phyla for the following groups: Ac: *Agelas* aff. *ceylonensis*, Am: *Agelas* aff. *mauritiana*, Bt: *Biema tubulata*, Ax: *Axinyssa aphysinoides*, Ct: *Cinachyrella* aff. *albatridens*, Cu: *Cinachyrella* aff. *australiensis*, Pa: *Petrosia seychellensis*, He: *Hyrtios erectus*, Ir: *Ircinia* aff. *variabilis*, Sp: *Spongia ceylonensis*, Pk: *Plakinastrella* aff. *clipptertonensis*, Bp: *Borojevia pirella*, Cl: *Clathrina luteoculcitella*, Cr: *Clathrina rodriguesensis*, En: *Ernstia naturalis*, Lc: *Leucetta chagosensis*, Ls: *Leucetta sulcata*, Sd: sediment, and Wt: water. The bars are colour-coded according to the phylum as indicated by the legend in the topright corner.



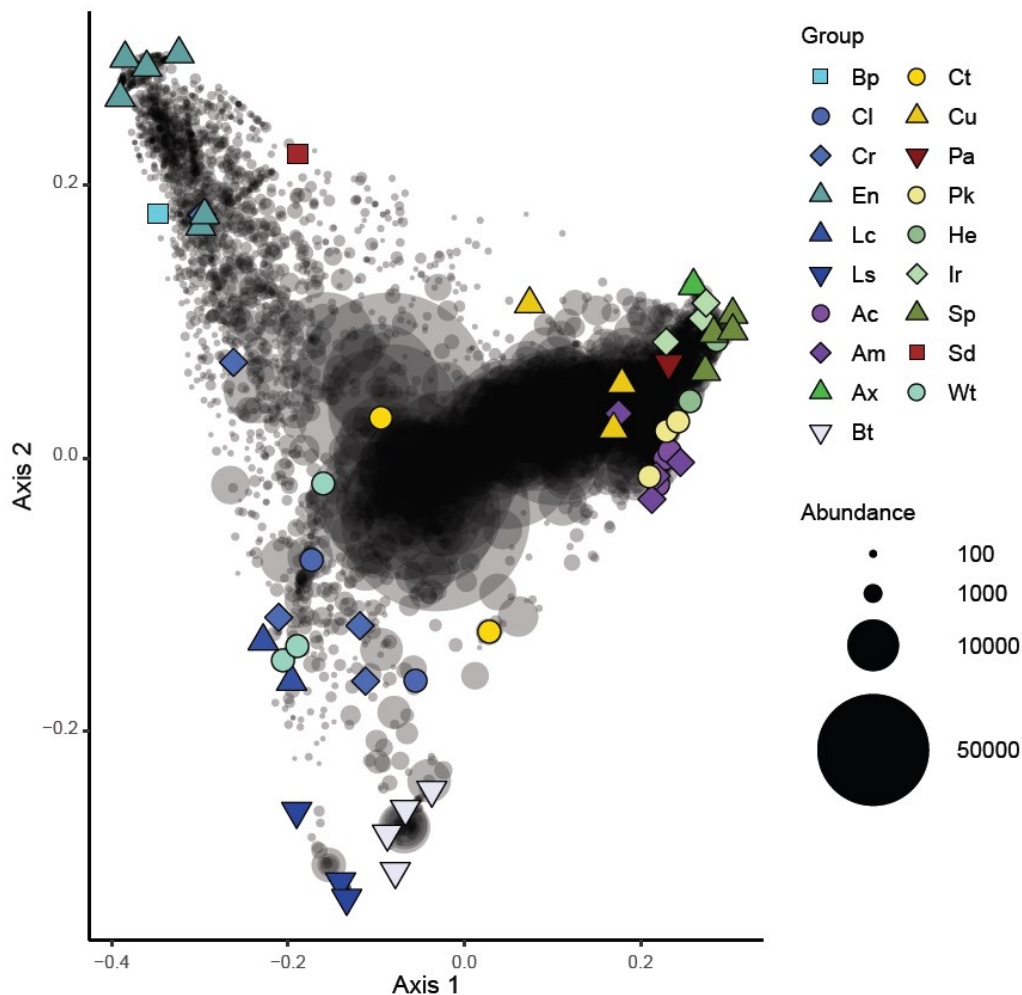


Fig. 4. Ordination showing the first two axes of the principal coordinates analysis (PCO) of OTU composition. Symbols are colour coded and represent samples from different groups as shown in the legend on the right side of the figure. Grey symbols represent weighted averages scores for OTUs. The symbol size is proportional to group abundance (number of sequence reads). The legend symbols represent the following groups: Ac: *Agelas* aff. *ceylonensis*, Am: *Agelas* aff. *mauritiana*, Bt: *Biema tubulata*, Ax: *Axinyssa aphysinoides*, Ct: *Cinachyrella* aff. *albatridens*, Cu: *Cinachyrella* aff. *australiensis*, Pa: *Petrosia seychellensis*, He: *Hyrtios erectus*, Ir: *Ircinia* aff. *variabilis*, Sp: *Spongia ceylonensis*, Pk: *Plakinastrella* aff. *clipptertonensis*, Bp: *Borojevia pirella*, Cl: *Clathrina luteoculcitella*, Cr: *Clathrina rodriguesensis*, En: *Ernsta naturalis*, Lc: *Leucetta chagosensis*, Ls: *Leucetta sulcata*, Sd: sediment, and Wt: water.

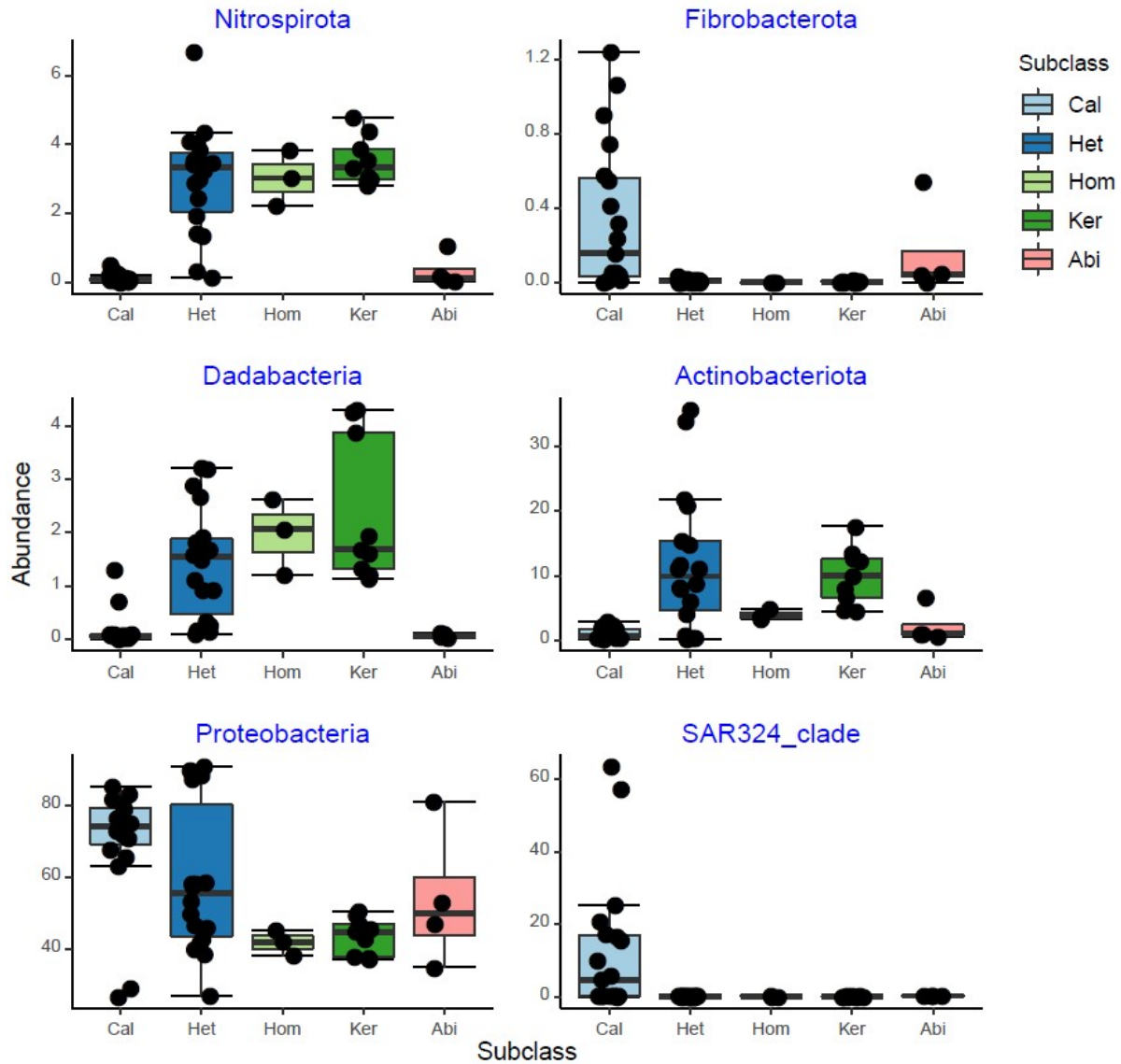


Fig. 5. Relative abundances of bacterial phyla, which differentiated between calcareous sponges and other biotopes: a. Nitrospirota, b. Fibrobacterota, c. Dadabacteria, d. Actinobacteriota, e. Proteobacteria, and f. SAR324 clade. Boxplots are shown with samples grouped according to sponge subclass: Cal (Calcinea), Het (Heteroscleromorpha), Hom (Homoscleromorpha), and Ker (Keratosa), in addition to Abi: abiotic biotopes (sediment and water).

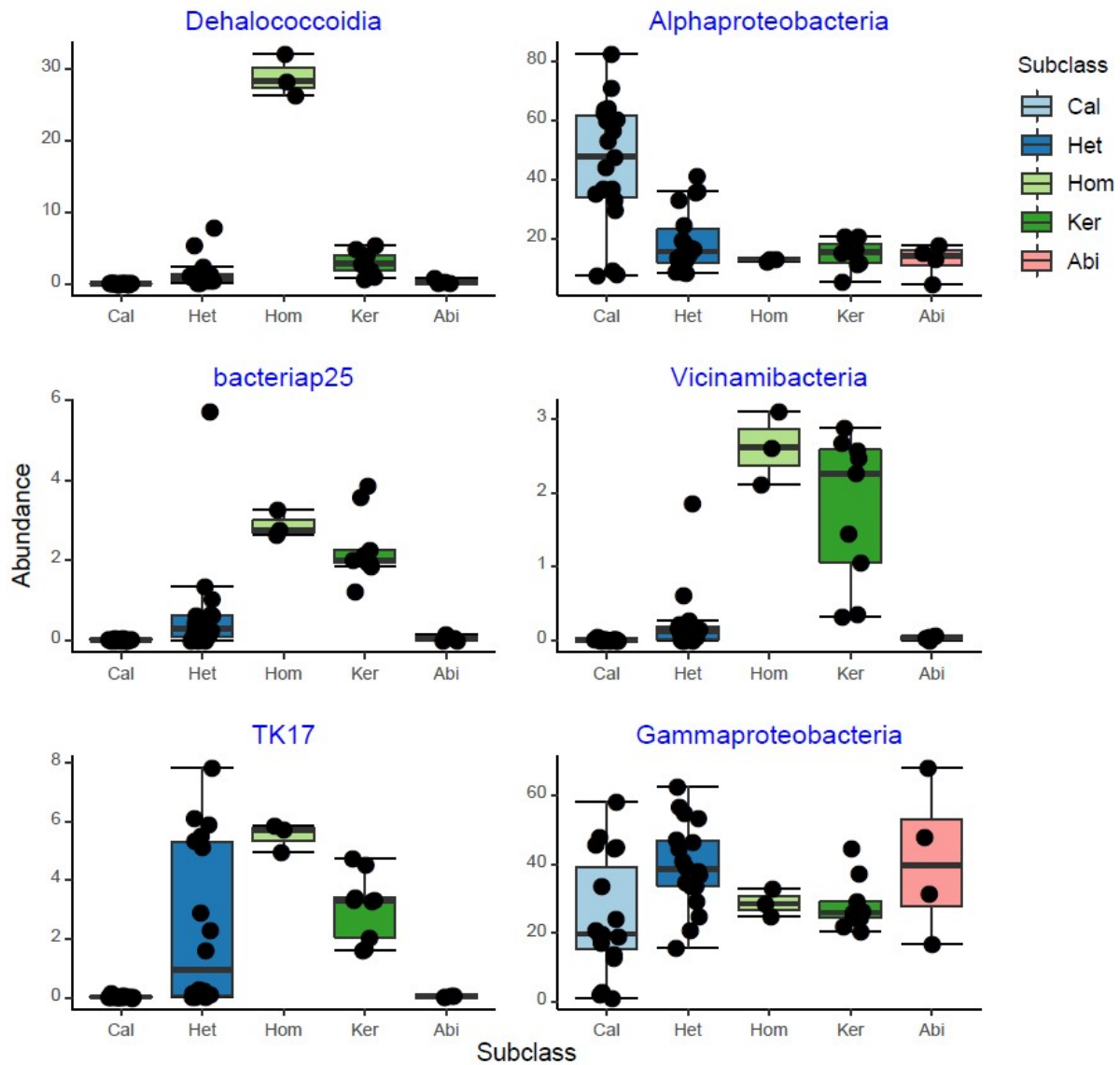


Fig. 6. Relative abundances of bacterial classes, which differentiated between calcareous sponges and other biotopes: a. Dehalococcoidia, b. Alphaproteobacteria, c. bacteriap25, d. Vicinamibacteria, e. TK17, and f. Gammaproteobacteria. Boxplots are shown with samples grouped according to sponge subclass: Cal (Calcinea), Het (Heteroscleromorpha), Hom (Homoscleromorpha), and Ker (Keratosa), in addition to Abi: abiotic biotopes (sediment and water).



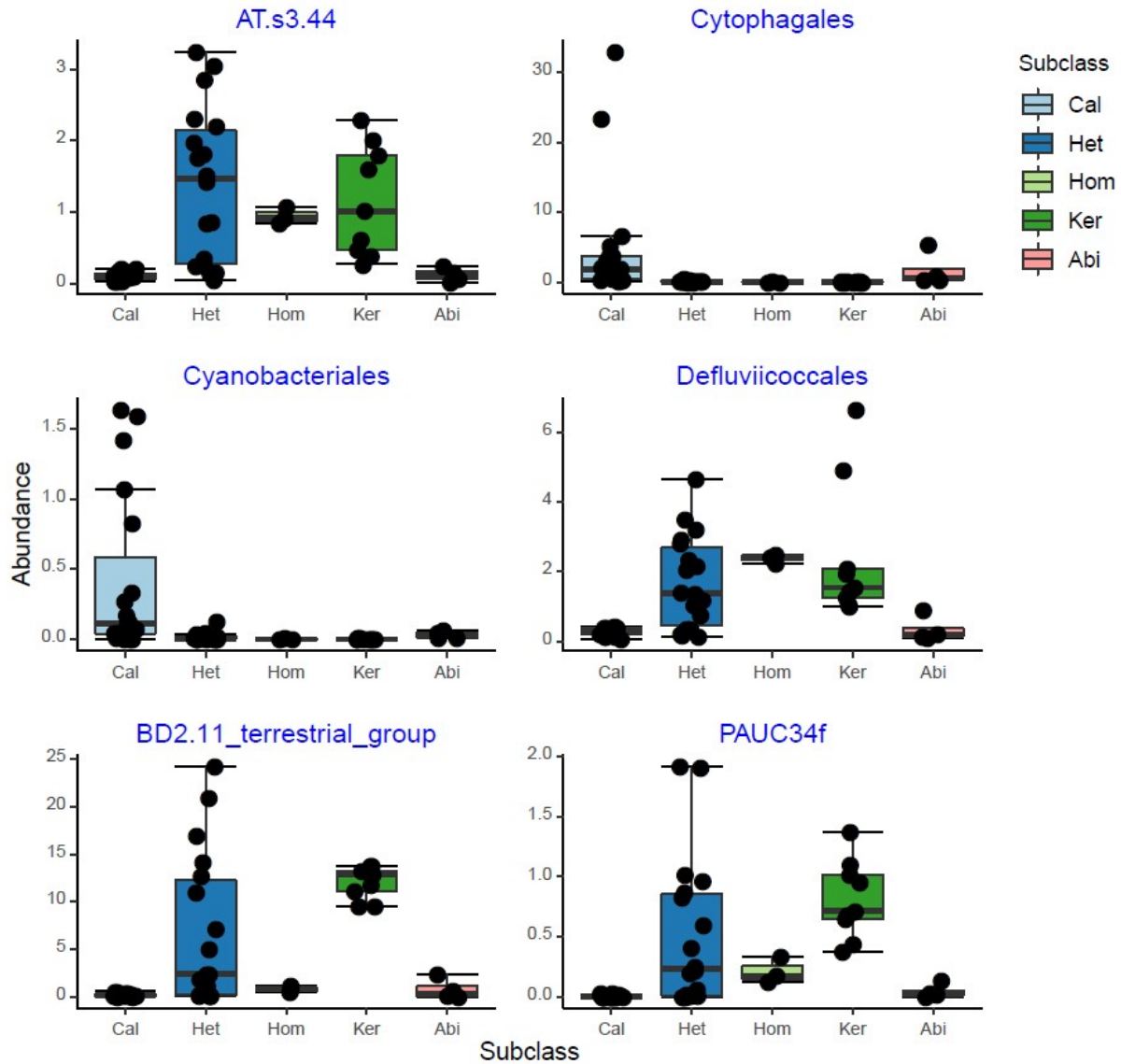


Fig. 7. Relative abundances of bacterial orders, which differentiated between calcareous sponges and other biotopes: a. AT-s3-44, b. Cytophagales, c. Cyanobacteriales, d. Defluviococcales, e. BD2-11 terrestrial group, and f. PAUC34f. Boxplots are shown with samples grouped according to sponge subclass: Cal (Calcinea), Het (Heteroscleromorpha), Hom (Homoscleromorpha), and Ker (Keratosa), in addition to Abi: abiotic biotopes (sediment and water).

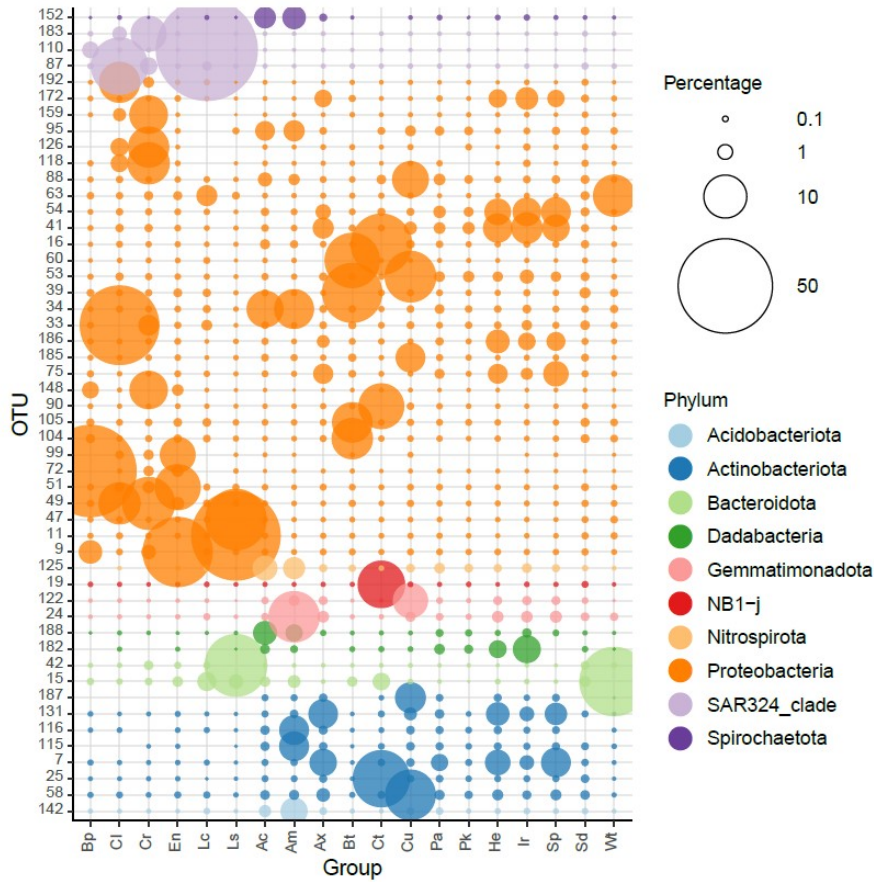


Fig. 8. Relative abundance of the most abundant OTUs colour-coded according to prokaryotic phylum for: Ac: *Agelas aff. ceylonensis*, Am: *Agelas aff. mauritiana*, Bt: *Biema tubulata*, Ax: *Axinyssa aplysinoides*, Ct: *Cinachyrella aff. albatridens*, Cu: *Cinachyrella aff. australiensis*, Pa: *Petrosia seychellensis*, He: *Hyrtios erectus*, Ir: *Ircinia aff. variabilis*, Sp: *Spongia ceylonensis*, Pk: *Plakinastrella aff. clipptertonensis*, Bp: *Borojovia pirella*, Cl: *Clathrina luteoculcitella*, Cr: *Clathrina rodriguesensis*, En: *Ernsta naturalis*, Lc: *Leucetta chagosensis*, Ls: *Leucetta sulcata*, Sd: Sediment, and Wt: Water. The circle size of the OTU is proportional to the mean percentage of sequences per biotope as indicated by the symbol legend in the upper right corner of the figure below the phylum assignment legend.