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Research Article

RESEARCH ARTICLE

Transmission studies and the composition of prokaryotic communities associated with healthy and diseased *Aplysina cauliformis* sponges suggest that *Aplysina* Red Band Syndrome is a prokaryotic polymicrobial disease

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One sentence summary: A combination of transmission studies where healthy and diseased sponges were separated by filters of varying pore size followed by sequencing of partial 16S rRNA genes suggested that ARBS is a prokaryotic yet polymicrobial disease.

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ABSTRACT

Aplysina cauliformis, the Caribbean purple rope sponge, is commonly affected by *Aplysina* Red Band Syndrome (ARBS). This transmissible disease manifests as circular lesions with red margins and results in bare spongin fibers. *Leptolyngbya* spp. appear to be responsible for the characteristic red coloration but transmission studies with a sponge-derived isolate failed to establish disease, leaving the etiology of ARBS unknown. To investigate the cause of ARBS, contact transmission experiments were performed between healthy and diseased sponges separated by filters with varying pore sizes. Transmission occurred when sponges were separated by filters with pore sizes $\geq$ 2.5 μm, suggesting a prokaryotic pathogen(s) but not completely eliminating eukaryotic pathogen(s). Using 16S rRNA gene sequencing methods, 38 prokaryotic taxa were significantly enriched in diseased sponges, including *Leptolyngbya,* whereas seven taxa were only found in some, but not all, of the ARBS-affected sponges. These results do not implicate a single taxon, but rather a suite of taxa that changed in relative abundance with disease, suggesting a polymicrobial etiology as well as dysbiosis. As a better

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understanding of dysbiosis is gained, changes in the composition of associated prokaryotic communities may have increasing importance for evaluating and maintaining the health of individuals and imperiled coral reef ecosystems.

Keywords: sponge-associated bacteria; sponge disease; ARBS; dysbiosis; transmission; *Leptolyngbya*

INTRODUCTION

Due to a combination of global climate change and human activities, coral reefs have experienced dramatic losses in abundance and biodiversity worldwide (Pandolfi *et al.* 2003; Hughes *et al.* 2017; Bellwood *et al.* 2019; Williams *et al.* 2019). Infectious diseases of marine organisms appear to be one of the major causes for population declines (e.g. Harvell *et al.* 2004; Weil 2004; Richardson 2005; Weil and Rogers 2011; Peters 2015). Although diseases affecting scleractinian corals have dominated the recent scientific literature, diseases that affect a wide range of other marine taxa including fish, sponges, shellfish, crustose coralline algae, sea stars and seagrasses have also been reported (e.g. Harvell *et al.* 1999, 2002, 2009; Lafferty, Porter and Ford 2004; Bally and Garrabou 2007; Haapkylä *et al.* 2007; Miner *et al.* 2018). Diseases impacting sponges have been reported from across the globe (reviewed by Webster 2007; Luter and Webster 2017), including observations in freshwater sponge populations (Denikina *et al.* 2016). Sponge diseases can severely affect the fitness and survival of sponges (e.g. Easson *et al.* 2013), and have the potential to directly or indirectly modify the composition of the entire benthic community (Brandt *et al.* 2019), and thus warrant additional investigation.

Sponges are a fundamental structural component of coral reef ecosystems, and perform numerous important ecological processes, such as transferring pelagic nutrients (e.g. carbon, nitrogen and sulfur) into benthic food webs via water filtration, consolidating reef substrata and serving as habitat and food for other reef taxa (Wulff 1995; Diaz and Rützler 2001; Lesser 2006; Rützler 2012; de Goeij *et al.* 2013; Bell *et al.* 2018). Many of these processes are aided by the presence of an associated microbial community within the sponge mesohyl (e.g. Hentschel *et al.* 2012; Radax *et al.* 2012; de Voogd *et al.* 2015; Lurgi *et al.* 2019), although there is much variation in the abundance and diversity of microbial populations between species of sponges. Despite the ecological and commercial importance of sponges, our understanding of sponge diseases remains limited, as information on the etiologic agents, vectors, reservoirs, pathologies and transmission mechanisms of these syndromes is sparse (reviewed by Webster 2007 and Luter and Webster 2017; Gochfeld *et al.* 2019; Olson, Easson and Gochfeld 2021).

Aplysina Red Band Syndrome (ARBS) affects rope sponges in the genus *Aplysina* (Verongida and Demospongiae), and most commonly, *Aplysina cauliformis*, which is widely distributed in the Caribbean basin (Gochfeld, Schlöder and Thacker 2007; Thacker *et al.* 2010; Loh and Pawlik 2014). Reported for the first time in 2004 in the Bahamas, ARBS has since been documented on reefs throughout the broader Caribbean basin, including the Florida Keys, Belize, Cayman Islands, Panama, Curaçao, Dominica, St. Kitts and the US Virgin Islands (Olson, Gochfeld and Slattery 2006; Gochfeld, Schlöder and Thacker 2007; Olson, Thacker and Gochfeld 2014; pers. obs.). Macroscopically, ARBS is easily recognized in *A. cauliformis* due to the presence of red-rimmed lesions that advance toward healthy sponge tissue, leaving behind necrotic tissue that becomes colonized by epibionts (Olson, Gochfeld and Slattery 2006). Similar to Black Band Disease (BBD) in scleractinian corals, ARBS seems to be associated with the presence of a consortium of microorganisms, although the filamentous cyanobacteria *Leptolyngbya* spp. appear to be responsible for the distinctive red pigmentation. To date, the specific role of *Leptolyngbya* spp. in the etiology of ARBS remains unclear, and although it has been isolated from lesions of infected sponges, experiments placing healthy individuals in contact with actively growing cyanobacterial isolates failed to recreate the disease (Olson, Thacker and Gochfeld 2014). In spite of this, both field and laboratory experiments have demonstrated that the primary mode of ARBS transmission is through direct contact between sponge branches, while waterborne transmission is also possible but occurs less frequently (Olson, Gochfeld and Slattery 2006; Easson *et al.* 2013). Infection of *A. cauliformis* with ARBS leads to changes in physiology, biochemistry and immune function, reduced growth and biomass, alterations in the production of secondary metabolites, as well as general physical weakening of the sponge skeleton and enhanced probability of sponge breakage, particularly when exposed to increased mechanical stress, such as during storm events (Gochfeld *et al.* 2019, 2012a; Easson *et al.* 2013; Olson, Easson and Gochfeld 2021).

Aplysina cauliformis hosts an extremely diverse microbial community whose cell density is several orders of magnitude higher than that of the surrounding seawater (Easson and Thacker 2014; Thomas *et al.* 2016). The cyanobacterial photosymbiont *Synechococcus spongiarum* provides the sponge with inorganic carbon and nitrogen, accounting for approximately 75% of its nutritional requirements and energy budget (Freeman and Thacker 2011; Freeman *et al.* 2015). In sponges affected by ARBS, there is a notable reduction in the abundance of *S. spongiarum* in visibly healthy tissue and an increase in the presence of *Leptolyngbya* spp. in discolored tissue associated with the lesions (Gochfeld *et al.* 2012b; Olson, Thacker and Gochfeld 2014; Olson, Easson and Gochfeld 2021). Nevertheless, the etiology of this disease remains elusive and further work is needed to address this knowledge gap. The current study aimed to (1) use field-based, size-exclusion transmission experiments to determine whether ARBS infection via direct contact was facilitated by viral, prokaryotic or eukaryotic organisms, and (2) leverage next-generation sequencing techniques to more fully characterize the component(s) of the microbial community implicated in aim (1), specifically, the prokaryotic community, which included both Bacteria and Archaea.

MATERIALS AND METHODS

Study location and transmission experiment

This research was conducted on shallow reefs near Lee Stocking Island, Exuma Cays, Bahamas (Fig. 1), in August 2013 and July 2014. The Exuma Cays are relatively pristine by most Caribbean standards, with the study sites located over 40 km from the nearest major town. Samples of ARBS-affected *A. cauliformis* (~15 cm) were collected from North Norman's reef (23° 47.388′ N, 76° 08.273′ W) at a depth of 5 m, where ARBS is relatively common, and placed into resealable plastic bags. These were placed into a cooler and transported by boat 0.3 km to Big Point (23° 47.301′ N, 76° 08.118′ W) where the reef is at the same depth.

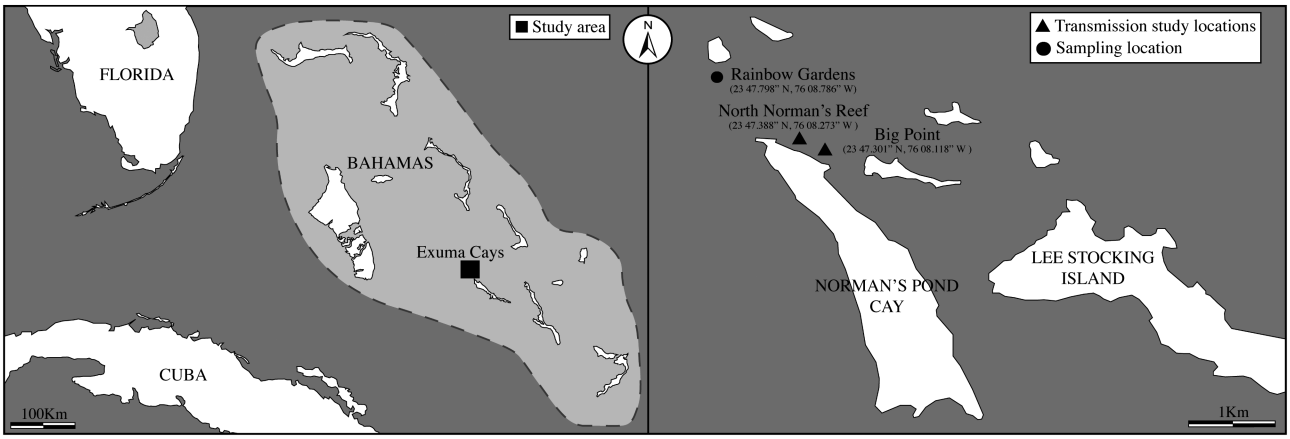


Figure 1. Map of the study area and sampling locations.

To test whether ARBS pathogenesis was likely driven by viral, prokaryotic or eukaryotic agents, healthy and diseased *A. cauliformis* were used in direct contact transmission experiments, as described previously by Olson *et al.* (2006, 2021). Briefly, the red bands of ARBS lesions on collected ARBS-affected sponges were placed in contact with *in situ* healthy conspecifics and cable tied together. Treatments consisted of 47-mm cellulose acetate filter membranes (Millipore, Burlington, Massachusetts, USA) with varying pore sizes placed between the healthy and ARBS-affected sponges prior to attaching the cable tie. These filter membranes (detailed below) selectively exclude potential etiologic agents based on cell size. At the end of each experiment, sponges were visually evaluated for the presence of the characteristic ARBS lesion. Previous studies (Olson, Gochfeld and Slattery 2006; Olson, Easson and Gochfeld 2021) have found these observations to be reliable in assessing ARBS infection.

In 2013, 30 visibly healthy *A. cauliformis* were labeled with flagging tape. The first treatment ($n = 10$) consisted of placing a 0.2 μm filter (excludes the vast majority of prokaryotes) between the healthy and ARBS-affected sponges prior to cable tying the sponges together (Fig. 2). The second treatment ($n = 10$) used 0.45 μm filters (excludes most prokaryotes and all eukaryotes), while the third treatment served as a control for transmission of ARBS, in which the lesion on the diseased sponge was attached directly to a healthy sponge ($n = 10$). After 10 days of contact, a period adequate to cause disease transmission in previous experiments (Olson, Gochfeld and Slattery 2006; Olson, Easson and Gochfeld 2021), all sponge pairs were collected, placed into individual resealable plastic bags for transfer to the laboratory and examined for evidence of disease transmission to the healthy sponge.

A follow-up study was conducted in 2014 on the same reefs using the methodology detailed above to test additional filter pore sizes. A total of 40 healthy sponges were marked with flagging tape and four treatments ($n = 8$ of each) consisted of wrapping filters with pore sizes of 1.2, 2.5, 6 and 15 μm around the healthy sponge prior to attaching a diseased sponge with a cable tie. A no-filter treatment served as the transmission control. After 10 days of contact, the sponges were removed from the reef and examined for evidence of disease transmission.

Sample collection and preparation for DNA sequencing

To evaluate the composition of the associated prokaryotic communities and identity putative etiologic agent(s) based on the transmission experiment results, visibly healthy ($n = 5$) and ARBS-affected ($n = 5$) *A. cauliformis* (thin lilac branching morph) were selected haphazardly from Rainbow Gardens in 2014 (23° 47.798′ N, 76° 08.786′ W) at a depth of 5 m. Approximately 10 cm portions of a branch were removed from healthy individuals using scissors and placed into re-sealable plastic bags. For diseased sponges, affected branches were cut approximately 5 cm below the lesion. Samples were kept in individual bags in seawater until processing (within 3 h). Small wedges (~1 mm thick) that included both the outer pinacoderm and interior mesohyl were aseptically cut from each healthy branch or from the healthy tissue adjacent to the margin of the ARBS lesion using a razor blade and placed into sterile cryovials with 1.8 mL RNAlater® (Life Technologies, Carlsbad, California, USA) for molecular analysis.

DNA was extracted from the small pieces of *A. cauliformis* (five healthy and five ARBS-affected) using the Qiagen DNeasy Blood (Germantown, Maryland, USA) and Tissue kit. The manufacturer's protocol was followed, except that samples were incubated at 55°C overnight in the lysis buffer and proteinase K solution prior to completing the protocol. The quality and quantity of DNA were evaluated by electrophoresis on 1% agarose gels and spectrophotometrically using a Nanodrop ND2000 (Thermo Fisher, Waltham, Massachusetts, USA), respectively. DNA was shipped to HudsonAlpha Genomics Services Lab (now HudsonAlpha Discovery) for 250 bp paired-end reads on an Illumina (San Diego, California, USA) MiSeq following amplification of the V3-4 region of the prokaryotic 16S rRNA gene using the primer pair 341F/785R (Klindworth *et al.* 2013; Table S1, Supporting Information). Sequence data have been deposited at the NCBI Sequence Read Archive under Bioproject accession number PRJNA741542.

Bioinformatics and statistical analyses

The raw sequences were processed and analysed using the microbiome analysis package QIIME 2–2019.10 (Bolyen *et al.* 2019) run remotely on the Dense Memory Cluster of the Alabama Supercomputer Authority (Huntsville, AL). The forward and reverse end demultiplexed sequence reads were imported into QIIME 2 and primers trimmed using the Cutadapt program (Martin 2011). DADA2 microbiome pipelines were then implemented to describe the prokaryotic communities of the sampled sponges using amplicon sequence variants (ASVs) rather than clustering sequences into operational taxonomic units (OTUs; Callahan *et al.* 2016; Callahan, McMurdie and Holmes 2017). ASV

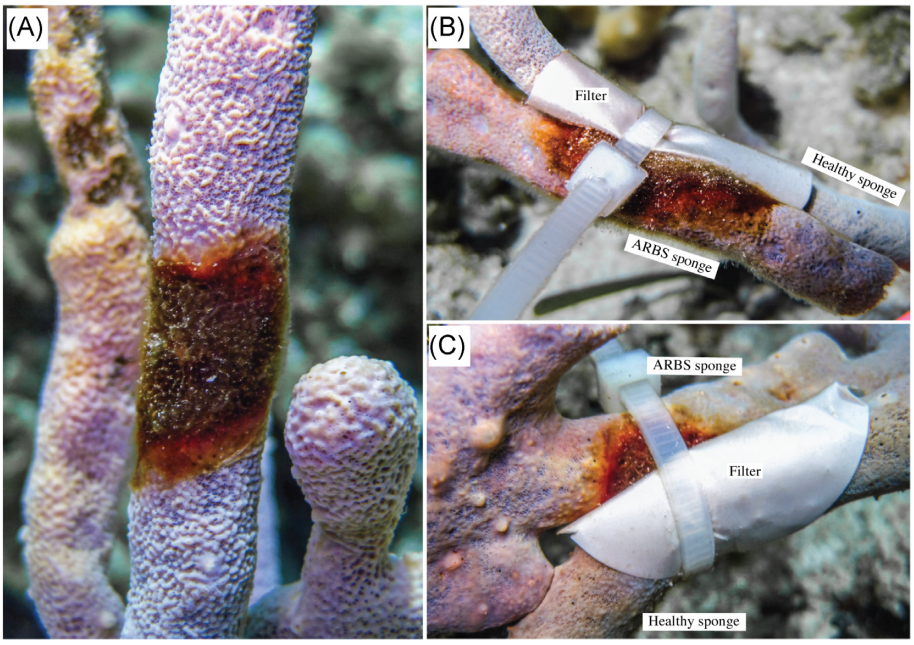


Figure 2. **(A)** *Aplysina cauliformis* affected by ARBS; **(B)** and **(C)** Direct contact transmission experiments where ARBS lesions on collected sponges were cable-tied to *in situ* healthy conspecifics with filters of varying pore sizes in between the diseased and healthy sponges.

methods have demonstrated sensitivity and specificity that is as good, or better than, OTU methods (Eren *et al.* 2015; Callahan, McMurdie and Holmes 2017; Needham, Sachdeva and Fuhrman 2017), and were therefore employed in this study. Briefly, DADA2 pipelines were used to quality filter, merge paired ends, dereplicate and remove chimeras. Taxonomy was assigned to ASVs at 99% sequence identity cutoff by applying the QIIME 2 feature-classifier classify-sklearn against the Naïve Bayes classifier trained on the SILVA-132–99 full-length sequences database (Quast *et al.* 2013). Reads identified as belonging to mitochondria and/or chloroplasts were removed.

Diversity analyses in QIIME 2 were conducted using the 'q2-diversity' plugin, which computes alpha and beta diversity metrics after generating an alpha rarefaction curve to normalize the read counts between samples. Data were checked for normality using a Shapiro–Wilk test with a significance level of 0.05. To evaluate richness, Shannon diversity, evenness, Chao I and Faith's phylogenetic diversity within the ASVs detected in healthy and ARBS-affected sponges, Wilcoxon–Mann–Whitney tests were performed in R (v. 3.4.3; R Core Team 2013) with a significance level of 0.05.

Beta diversity distance matrices calculated in QIIME 2 were imported into R for further analyses using the R package 'qiimer' (Bittinger 2015). After verifying that there were not significant differences in homogeneity of variances using the function 'betadisper' (Anderson 2006; Anderson, Ellingsen and McArdle 2006) in the R package 'vegan' (Oksanen 2013), the Weighted UniFrac distance matrix was used for analysis of similarity (ANOSIM) to test for differences in the prokaryotic communities between healthy and diseased sponges and visualized using Principal Coordinates Analysis (PCoA).

At the genus level, relative abundances for each microorganism were retrieved from QIIME 2 and the resulting relative abundance matrix was analyzed using linear discriminant analysis (LDA) effect size (LEfSe; Segata *et al.* 2011) to compare the prokaryotic communities associated with healthy and diseased sponges using the website https://huttenhower.sph.harvard.edu/galaxy/. LDA was performed to estimate the effect size of each differentially abundant prokaryotic taxon between sponge health conditions. Sample communities were considered significantly different at $P \leq 0.05$ and a LDA score (log10) > 3, one order of magnitude greater than the default value of the LEfSe methodology (Segata *et al.* 2011).

RESULTS

Filter transmission experiment

In 2013, none of the healthy sponges separated from an ARBS-affected sponge by an intact filter developed ARBS lesions after 10 days of contact. One of the 0.45 μm pore size filters was excluded because it had a tear in the filter in the area of contact and the healthy individual developed a lesion. Lesions were observed in 9 of the 10 sponges (90%) in direct contact after 10 days.

In 2014, none of the eight healthy sponges separated from an ARBS-affected sponge by an intact 1.2 μm pore size filter showed evidence of ARBS transmission after 10 days. One filter had a rip in the area of contact and was excluded. For the 2.5 μm pore size filters, no ARBS transmission was observed in six of the eight healthy sponges, with the remaining two (25%) showing small ARBS lesions. In total, two ARBS lesions (25%) were also observed on the healthy sponges separated by 6 μm pore size filters while seven lesions (87.5%) resulted from the eight sponge pairs separated by filters with a pore size of 15 μm. ARBS lesions were observed in all eight (100%) of the healthy sponges in direct contact with an ARBS-affected sponge.

Prokaryotic community analysis

Given the results of the transmission experiment, which indicated that the etiologic agent(s) is most likely $\geq$ 2.5 μm (i.e. prokaryotic), next-generation amplicon sequencing was used to compare the prokaryotic communities of healthy and ARBS-affected *A. cauliformis*. After removing 79 ASVs (6135 reads)

identified as mitochondria and/or chloroplasts, a total of 4446 prokaryotic ASVs were obtained from the 10 sponge samples. As the ASV accumulation curve reached a plateau for all individuals analyzed, the rarefaction applied to avoid artifacts of sequence depth did not result in any loss of samples (Figure S1, Supporting Information).

The five healthy *A. cauliformis* individuals hosted 744–835 prokaryotic ASVs, compared to the 731–1497 ASVs detected within the five ARBS-affected *A. cauliformis* sponges. No significant difference in ASV number (Wilcoxon test, W = 20, n = 10 and P = 0.151) was observed between healthy and ARBS-affected sponges (Table 1). Chao 1 richness estimates indicated that sequencing efforts captured most of the diversity within the sampled prokaryotic communities (Table 1). The Shannon diversity index reported a significantly higher prokaryotic diversity within the ARBS-affected sponges than in healthy individuals (Wilcoxon test, W = 20, n = 10 and P = 0.016; Table 1). Diseased sponges also supported a slightly higher average evenness than healthy individuals, although the difference was not significant (Wilcoxon test, W = 20, n = 10 and P = 0.38; Table 1). These results were reinforced by the Faith's Phylogenetic Diversity index values, which were on average higher in ARBS-affected *A. cauliformis* than in healthy sponges (Table 1). The prokaryotic communities within healthy and diseased *A. cauliformis* sponges were significantly different (ANOSIM, P = 0.019 and R = 0.488). Similarly, weighted Unifrac PCoA showed that sponge individuals separated into two distinct clusters based on health condition (Fig. 3).

The reads generated from the 10 samples represented 36 recognized phyla (33 within domain Bacteria and three within domain Archaea), with seven phyla exclusively found in one or more of the ARBS-affected *A. cauliformis* individuals and 21 phyla with a relative abundance of less than 1% in either healthy or diseased sponges (Fig. 4 and Table S2, Supporting Information). None of the seven phyla found only in diseased sponges had a relative abundance greater than 0.10% and each was detected in some, but not all, of the ARBS-affected sponges. The phylum Chloroflexi dominated the prokaryotic communities of all 10 individuals, followed by the Proteobacteria, Acidobacteria and Actinobacteria (Fig. 4). The LEfSE analysis identified five bacterial taxa that were significantly enriched in healthy sponges (Fig. 5). Among these, Chloroflexi (LDA score = 4.44 and P = 0.047) and Acidobacteria (LDA score = 4.18 and P = 0.047) differed in relative abundance from 45.25±1.25% and 13.27±1.17% in healthy sponges to 40.33±1.66% and 9.82±0.83% (mean±SE) in diseased individuals, respectively. Conversely, LEfSE analysis identified 38 taxa that were significantly enriched in ARBS-affected sponges, with the clade SAR202 (Chloroflexi) having the highest LDA score (LDA score = 4.17 and P = 0.016) and increasing in relative abundance from 15.14±1.10% to 19.41±1.98%. A total of six other taxa also surpassed LDA scores of four within this group and consist of members of the Gammaproteobacteria and Actinobacteria, including three within the order Microtrichales (Fig. 5). Within the phylum Cyanobacteria, the genus *Leptolyngbya* was significantly enriched in ARBS-affected sponges (LDA score = 3.71 and P = 0.008), while the average abundance of *S. spongiarum*, although reduced in diseased individuals compared to healthy sponges, did not differ significantly (Table 2 and Fig. 5). The Gammaproteobacteria had the highest number of taxa significantly enriched in diseased samples, including members of the KI89A clade (LDA score = 3.59 and P = 0.028), the JTB23 clade (LDA score = 3.37 and P = 0.028), the order Oceanospirillales (LDA score = 3.40 and P = 0.028), the UBA10353 marine group (LDA score = 3.20 and P = 0.047), the family Endozoicomonadaceae (LDA score = 3.11 and P = 0.028,) and the genus *Pseudohongiella* (LDA score = 3.10 and P = 0.028).

DISCUSSION

Reports of diseases affecting marine sponges have been increasing over the past two decades (reviewed by Webster 2007 and Luter and Webster 2017; Deignan, Pawlik and Erwin 2018; Greco *et al.* 2019) yet our understanding of the causes of these diseases remains limited. Only a handful of etiologic agents for diseases of sponges have been identified and include the cyanobacterial endosymbiont *Aphanocapsa feldmanni,* which caused disease in *Geodia papyracea* sponges in Belize (Rützler 1988), the spongin-boring alphaproteobacterium *Pseudoalteromonas agarivorans* strain NW4327 responsible for necrosis of the Great Barrier Reef sponge *Rhopaloeides odorabile* (Webster *et al.* 2002), and the fungus *Aspergillus tubingensis* which results in a fatal infection in the Mediterranean sponge *Chondrosia reniformis* (Greco *et al.* 2019). Additionally, Sweet *et al.* (2015) identified a polymicrobial consortium consisting of a fungus and a bacterium that caused necrosis in *Callyspongia (Euplacella)* aff *biru* in the Indian Ocean, fulfilling Koch's postulates. Whereas evidence to date suggests a role for the cyanobacteria *Leptolyngbya* spp. in the etiology of ARBS (Olson, Gochfeld and Slattery 2006; Gochfeld *et al.* 2019), contact experiments with *Leptolyngbya* isolates from ARBS lesions failed to recreate ARBS in healthy *A. cauliformis* (Olson, Thacker and Gochfeld 2014). To address the question of where to focus our efforts towards identifying an etiologic agent(s), transmission experiments utilizing filters with varying pore sizes were conducted, followed by high-throughput sequencing approaches targeting the prokaryotic 16S rRNA gene, with the results suggesting a polymicrobial but largely prokaryotic etiology.

Although recent metagenomic analyses of purified viral fractions demonstrated that reef sponges harbor a diverse community of viruses (Laffy *et al.* 2016, 2018; Pascelli *et al.* 2018), no studies to date have directly implicated viruses in sponge diseases (Claverie *et al.* 2009; Luter, Whalan and Webster 2010; Webster and Taylor 2012; Butina *et al.* 2019). Only one viral infection, affecting a single sponge individual, was suggested to cause localized tissue lesions (Vacelet and Gallissian 1978). The absence of ARBS lesion formation with filters that excluded most small particles (0.2 and 0.45 μm pore sizes) suggests that this disease is not caused by a virus alone. Ultramicrobacteria, which were potentially able to pass through filters with small pore sizes (e.g. 0.2 and 0.45 μm), are also not likely to be the etiologic agent(s) of ARBS due to the lack of observed disease transmission and absence of these organisms in TEM images of healthy and diseased tissues (Gochfeld *et al.* 2019).

Similarly, although eukaryotic organisms have been associated with a variety of coral diseases (e.g. Cróquer, Bastidas and Lipscomb 2006; Raghukumar and Ravindran 2012; Sweet and Séré 2016), their role in sponge disease is less well known (Sweet *et al.* 2015; Greco *et al.* 2019). In addition to *A. tubingensis* being reported as the etiologic agent of a sponge disease (Greco *et al.* 2019), another *Aspergillus* sp., *A. sydowii*, the causative agent of Aspergillosis in gorgonian corals (Geiser *et al.* 1998; Alker, Smith and Kim 2001), has been found within healthy individuals of *Spongia obscura* (Ein-Gil *et al.* 2009), suggesting that sponges may serve as environmental reservoirs for coral pathogens without suffering negative effects themselves (Negandhi *et al.* 2010). Filamentous fungi can be isolated from visibly healthy sponge tissues (e.g. Höller *et al.* 2000; Li and Wang 2009; Passarini *et al.* 2013; Henríquez *et al.* 2014), yet have also been implicated in

Table 1. Alpha diversity indexes for each *A. cauliformis* sponge. # ASVs = total number of ASVs; Shannon = Shannon diversity index; Faith's PD = Faith's phylogenetic diversity; H = healthy individuals and D = ARBS-affected individuals. *P* values less than 0.05 are in bold for comparisons between healthy and diseased sponges.

ID	Health	# ASVs	Chao1	Shannon	Evenness	Faith's PD
1	H	787	787.05	7.12	0.74	100.50
2	H	792	792.05	6.84	0.71	88.20
3	H	769	770.04	6.92	0.72	92.43
4	H	744	745.00	6.78	0.71	104.34
5	H	835	836.04	7.30	0.75	112.34
6	D	1497	1500.04	7.58	0.72	135.85
7	D	1128	1135.03	7.59	0.75	104.71
8	D	1115	1115.23	7.46	0.74	109.10
9	D	1016	1017.10	7.43	0.74	100.87
10	D	731	731.10	7.24	0.76	75.98
P-value (H-D)		0.151	0.150	**0.016**	0.380	0.548

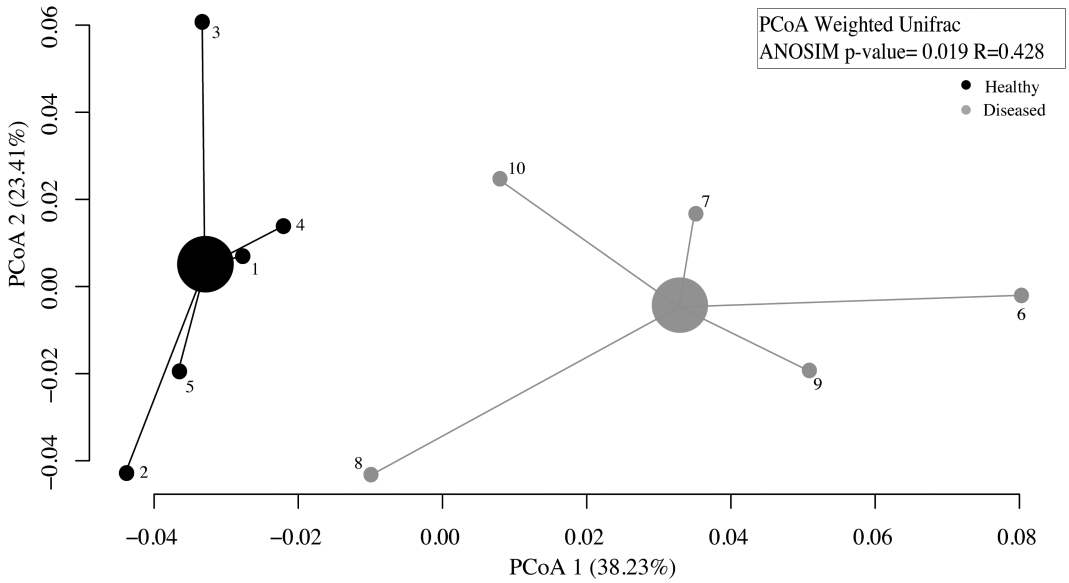
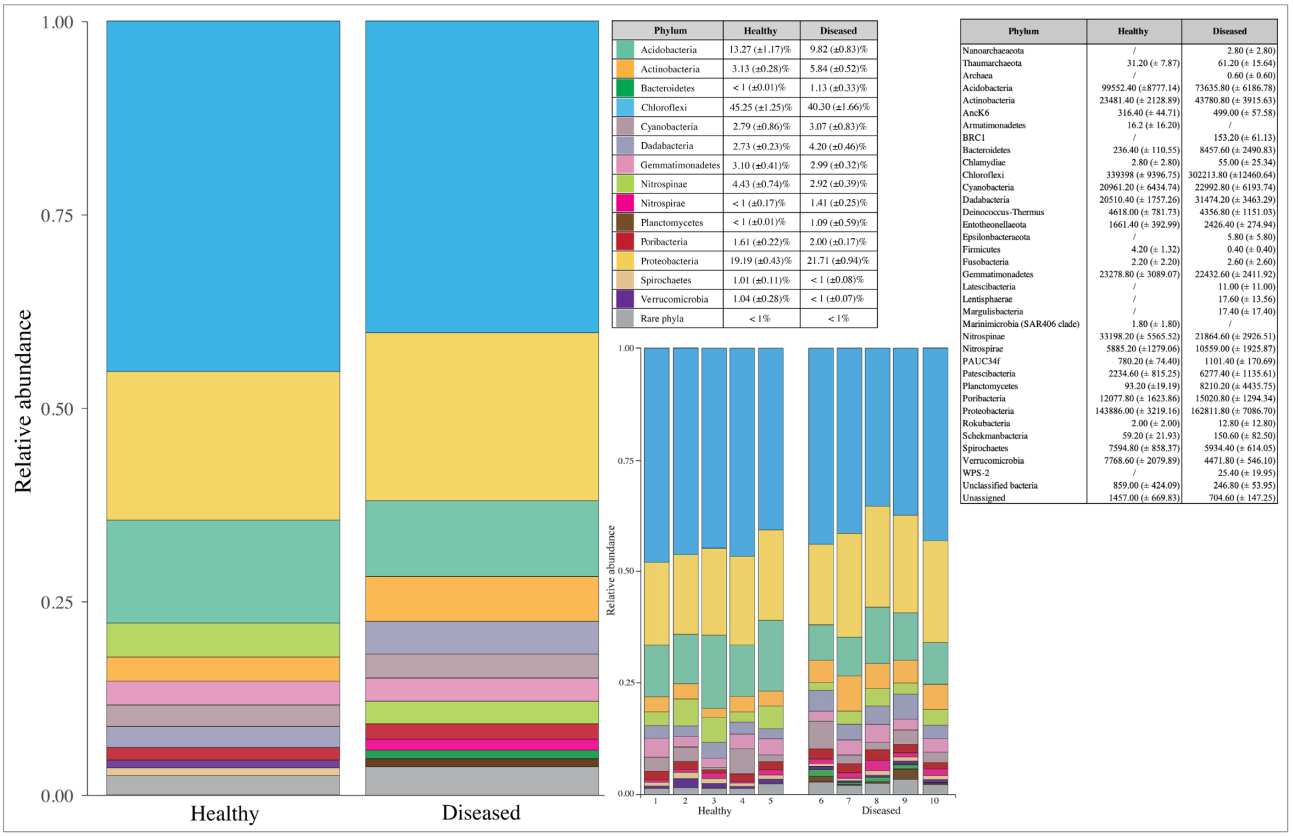


Figure 3. PCoA based on the Weighted UniFrac distance matrix of the *A. cauliformis*-associated prokaryotic community showing that the sponges separated into two distinct clusters based on health condition. Small circles reflect communities within individual sponges while large circles represent the centroids of the groups.

disease development in sponges (Smith 1941; Galstoff 1942). In a histological examination of healthy and ARBS-affected *A. cauliformis* tissue, Gochfeld *et al.* (2019) reported the presence of unidentified, round eukaryotic cells of ~2 μm diameter that were only found in ARBS lesions. In the current study, transmission of ARBS was observed in two of the eight (25%) sponges separated by filters with a pore size of 2.5 μm, suggesting, but not completely eliminating, the possibility that the etiologic agent(s) of ARBS is not eukaryotic. In total, two ARBS lesions (25%) were also observed on the healthy sponges separated by 6 μm pore size filters while seven lesions (87.5%) resulted from the eight sponge pairs separated by filters with the largest pore size (15 μm). Some fouling was present on the filters after the transmission experiments, but it was present on all of the filters. Although biofilm constituents were not characterized, if these biofilms were important for ARBS causation, lesion formation would have been expected to occur under filters regardless of pore size. As this was not the case, it suggests that the biofilms were not associated with disease onset.

The filter transmission experiments indicate that ARBS likely results from a prokaryotic agent(s), due to absence of ARBS infections when these organisms were excluded with the use of filters $\leq$ 1.2 μm pore size and the appearance of ARBS when filters of $\geq$ 2.5 μm pore size were employed. Several other studies have also implicated bacterial pathogens for sponge diseases (e.g. Webster *et al.* 2002; Stabili *et al.* 2012). Olson *et al.* (2014), using terminal restriction fragment length polymorphism (T-RFLP) analyses of *A. cauliformis* prokaryotic communities, suggested that ARBS was caused by a prokaryotic polymicrobial infection. Similarly, our current characterization of the prokaryotic communities using high-throughput sequencing approaches also does not implicate a single taxon, but rather a suite of taxa that both increase and decrease in relative abundance in association with ARBS infection. This dysbiosis, or perturbation to the composition of resident commensal microbial communities relative to the community found within healthy individuals (Petersen and Round 2014), is not surprising, as diseases in nature often involve a complex network of causative agents and host responses, creating difficulties for the identification of etio-



Phylum	Healthy	Diseased
Acidobacteria	13.27 (±1.17)%	9.82 (±0.83)%
Actinobacteria	3.13 (±0.28)%	5.84 (±0.52)%
Bacteroidetes	< 1 (±0.01)%	1.13 (±0.33)%
Chloroflexi	45.25 (±1.25)%	40.30 (±1.66)%
Cyanobacteria	2.79 (±0.86)%	3.07 (±0.83)%
Dadabacteria	2.73 (±0.23)%	4.20 (±0.46)%
Gemmatimonadetes	3.10 (±0.41)%	2.99 (±0.32)%
Nitrospinae	4.43 (±0.74)%	2.92 (±0.39)%
Nitrospirae	< 1 (±0.17)%	1.41 (±0.25)%
Planctomycetes	< 1 (±0.01)%	1.09 (±0.59)%
Poribacteria	1.61 (±0.22)%	2.00 (±0.17)%
Proteobacteria	19.19 (±0.43)%	21.71 (±0.94)%
Spirochaetes	1.01 (±0.11)%	< 1 (±0.08)%
Verrucomicrobia	1.04 (±0.28)%	< 1 (±0.07)%
Rare phyla	< 1%	< 1%

Phylum	Healthy	Diseased
Nanoarchaeaota	/	2.80 (± 2.80)
Thaumarchaeota	31.20 (± 7.87)	61.20 (± 15.64)
Archaea	/	0.60 (± 0.60)
Acidobacteria	99552.40 (±8777.14)	73635.80 (± 6186.78)
Actinobacteria	23481.40 (± 2128.89)	43780.80 (± 3915.63)
AncK6	316.40 (± 44.71)	499.00 (± 57.58)
Armatimonadetes	16.2 (± 16.20)	/
BRC1	/	153.20 (± 61.13)
Bacteroidetes	236.40 (± 110.55)	8457.60 (± 2490.83)
Chlamydiae	2.80 (± 2.80)	55.00 (± 25.34)
Chloroflexi	339398 (± 9396.75)	302213.80 (±12460.64)
Cyanobacteria	20961.20 (± 6434.74)	22992.80 (± 6193.74)
Dadabacteria	20510.40 (± 1757.26)	31474.20 (± 3463.29)
Deinococcus-Thermus	4618.00 (± 781.73)	4356.80 (± 1151.03)
Entotheonellaeota	1661.40 (± 392.99)	2426.40 (± 274.94)
Epsilonbacteraeota	/	5.80 (± 5.80)
Firmicutes	4.20 (± 1.32)	0.40 (± 0.40)
Fusobacteria	2.20 (± 2.20)	2.60 (± 2.60)
Gemmatimonadetes	23278.80 (± 3089.07)	22432.60 (± 2411.92)
Latescibacteria	/	11.00 (± 11.00)
Lentisphaerae	/	17.60 (± 13.56)
Margulisbacteria	/	17.40 (± 17.40)
Marinimicrobia (SAR406 clade)	1.80 (± 1.80)	/
Nitrospinae	33198.20 (± 5565.52)	21864.60 (± 2926.51)
Nitrospirae	5885.20 (±1279.06)	10559.00 (± 1925.87)
PAUC34f	780.20 (± 74.40)	1101.40 (± 170.69)
Patescibacteria	2234.60 (± 815.25)	6277.40 (± 1135.61)
Planctomycetes	93.20 (±19.19)	8210.20 (± 4435.75)
Poribacteria	12077.80 (± 1623.86)	15020.80 (± 1294.34)
Proteobacteria	143886.00 (± 3219.16)	162811.80 (± 7086.70)
Rokubacteria	2.00 (± 2.00)	12.80 (± 12.80)
Schekmanbacteria	59.20 (± 21.93)	150.60 (± 82.50)
Spirochaetes	7594.80 (± 858.37)	5934.40 (± 614.05)
Verrucomicrobia	7768.60 (± 2079.89)	4471.80 (± 546.10)
WPS-2	/	25.40 (± 19.95)
Unclassified bacteria	859.00 (± 424.09)	246.80 (± 53.95)
Unassigned	1457.00 (± 669.83)	704.60 (± 147.25)

Figure 4. Relative abundances of the 16S rRNA gene reads generated from the 10 *A. cauliformis* samples. The large graph and table show means of the prokaryotic phyla associated with the healthy ($n = 5$) and ARBS-affected ($n = 5$) sponges while the small graph indicates the relative abundance of the phyla within each individual.

logical agents and polymicrobial infections have been suggested to be responsible for some coral diseases (e.g. Miller and Richardson 2011; Sato *et al.* 2016).

The prokaryotic community profiles of healthy *A. cauliformis* reported here are consistent with previous descriptions of these communities and are characterized by a complex assemblage dominated by Chloroflexi, Proteobacteria, Acidobacteria, Actinobacteria, Gemmatimonadetes and Cyanobacteria (Easson and Thacker 2014; Thomas *et al.* 2016; Freeman *et al.* 2020). Based on relative read abundances, these phyla accounted for more than 80% of the sponge prokaryotic associates, confirming that healthy *A. cauliformis* host a relatively stable 'core' community consisting of a few bacterial taxa with a diversity of transient microorganisms at much lower abundances. However, some variation exists among the current and previous studies, likely due to a combination of factors, including different molecular techniques (e.g. clone-library approaches, T-RFLP profiling, high throughput sequencing with the amplification of different hypervariable regions of the 16S rRNA gene using diverse primers) and potential temporal and geographic variation in sampling.

Recently, research on diseases of marine invertebrates has pivoted towards understanding the importance of microbial community shifts rather than the identification of single pathogens (Luter, Whalan and Webster 2010; Angermeier *et al.* 2011; Zaneveld, McMinds and Thurber 2017; MacKnight *et al.* 2021). In accordance with previous work by Olson *et al.* (2014), the current study detected a significant change in the *A. cauliformis*-associated prokaryotic community between healthy and ARBS-affected individuals, reflecting dysbiosis. Although some researchers have suggested that dysbiosis may be a hallmark of disease in both corals (e.g. Shore and Caldwell 2019; MacKnight *et al.* 2021) and sponges (Olson, Thacker and Gochfeld 2014; Olson, Easson and Gochfeld 2021; Taylor *et al.* 2021), dysbiosis in sponges has also been reported with exposure to various environmental stressors that did not necessarily result in disease or mortality (e.g. temperature, pH and contact with conspecifics; e.g. Lesser *et al.* 2016; Kandler *et al.* 2018; Ramsby *et al.* 2018; Vargas, Leiva and Wörheide 2021). During dysbiosis, the critical functions provided by the sponge-associated microbial communities may be negatively impacted, but it remains to be seen whether or how these communities stabilize or recover.

As seen in other studies of diseased sponges (Webster *et al.* 2008; Blanquer *et al.* 2016; Deignan, Pawlik and Erwin 2018), ARBS-affected sponges also supported a significantly higher diversity of prokaryotes than healthy conspecifics, which may facilitate the establishment and proliferation of opportunistic microorganisms, disrupting the commensal community assemblage found in healthy sponges (Simister *et al.* 2012) and resulting in unstable microbial community states. The wider variation in prokaryotic community composition in ARBS-affected sponges than in healthy sponges can be readily visualized in Fig. 3. Samples from the ARBS-affected sponges analysed in this study were taken from visibly healthy tissue immediately adjacent to the disease lesions in order to capture changes within the sponge-associated prokaryotic community yet avoid including opportunistic and/or epibiotic microorganisms residing in necrotic tissue. While these tissues from diseased sponges appeared healthy, bacterial richness was found to be almost 2-fold higher than in samples from healthy sponges. These results

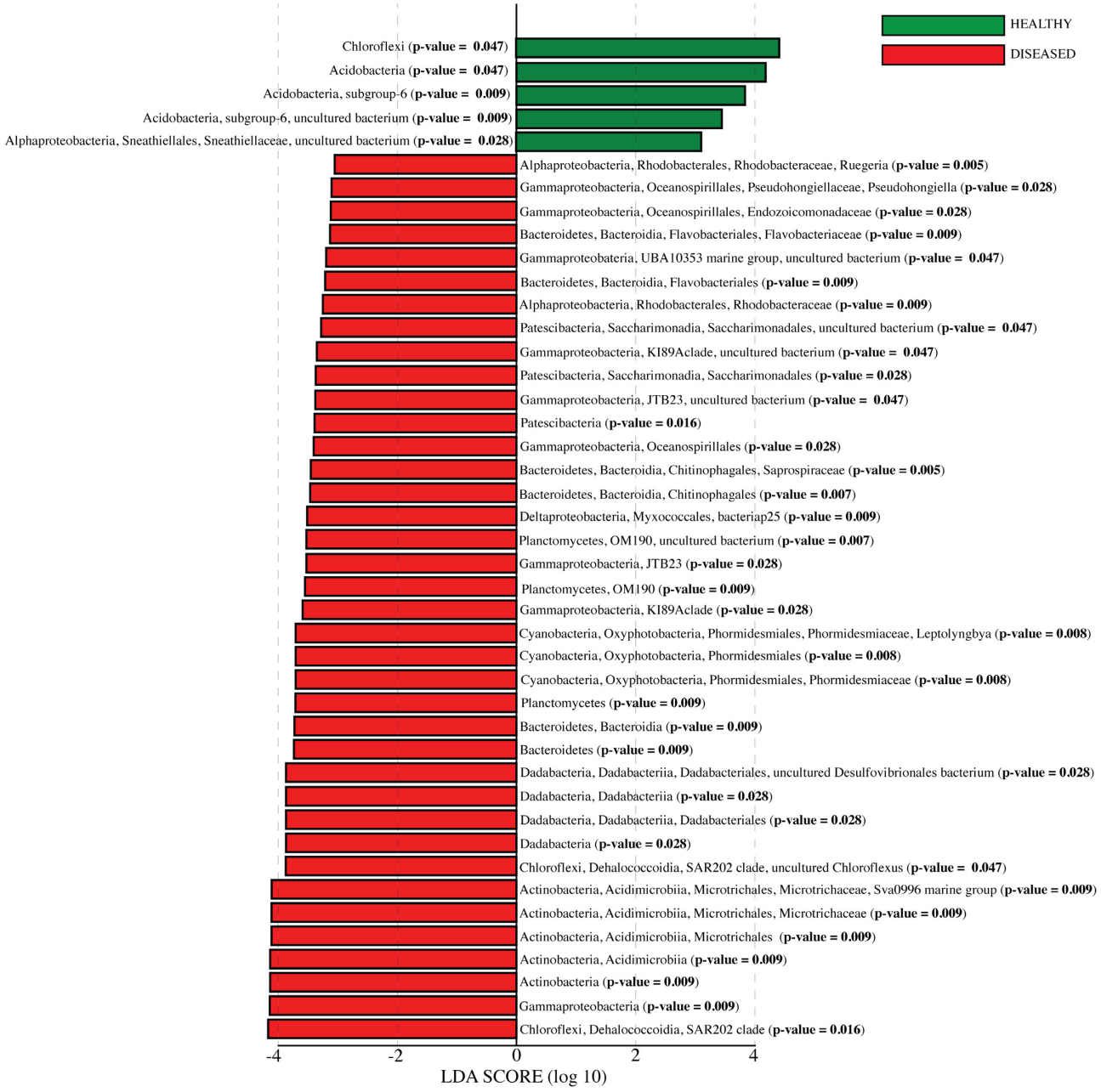


Figure 5. LEfSe analysis comparing the prokaryotic communities associated with healthy and ARBS-affected sponges. Sample communities were considered significantly different at $P \leq 0.05$ and a LDA score (log10) > 3.

appear to be in contrast with other studies, which observed a disruption to the host microbial community that was confined exclusively to the disease lesions, such as in the sponges *Aplysina aerophoba* (Webster *et al.* 2008) and *Agelas tubulata* (Deignan, Pawlik and Erwin 2018).

Other changes in healthy tissue from ARBS-affected sponges, including concentrations of heat shock protein 70, soluble protein and secondary metabolites, also provide evidence that ARBS elicits a systemic, rather than localized, response in *A. cauliformis* holobionts (Gochfeld *et al.* 2012a, b; Olson *et al.* 2021). Interestingly, Maldonado *et al.* (2010) found that sponges within the genus *Ircinia* have the ability to isolate damaged tissue from healthy biomass by building barriers of collagen and concentrating phagocytic cells (e.g. archaeocytes) at these margins, limiting disease advancement. Although transmission electron microscopy (TEM) of *Amphimedon compressa* with sponge white patch disease did not display these barriers, a distinct demarcation was observed between healthy and diseased tissues (Angermeier *et al.* 2012). In diseased *Xestospongia muta* and *A. cauliformis*, TEM images showed abundant cyanobacteria within sponge archaeocytes, but no collagen barriers (Angermeier *et al.* 2011; Gochfeld *et al.* 2019). All of these changes suggest that sponges use a variety of innate immune responses to counter or limit damage resulting from disease, but that there is not a uniform response within all Porifera.

This study reported a shift in the composition of the prokaryotic communities between healthy and ARBS-affected *A. cauliformis* individuals and identified the taxa that changed

Table 2. Mean reads from the 16S rRNA gene of cyanobacterial taxa found in healthy and diseased *A. cauliformis* individuals. H = Healthy sponges ($n = 5$) and D = ARBS affected sponges ($n = 5$).

	Mean reads count (±SE)	
Cyanobacteria	H	D
Melainabacteria, Caenarcaniphilales, uncultured bacterium	0.00 (±0.00)	3.60 (±2.91)
Melainabacteria, Caenarcaniphilales, uncultured bacterium	0.00 (±0.00)	3.80 (±3.80)
Cyanobacteria, Melainabacteria, Caenarcaniphilales	0.00 (±0.00)	25.60 (±26.60)
Melainabacteria, Vampirovibrionales, uncultured bacterium	0.00 (±0.00)	9.40 (±8.44)
Oxyphotobacteria, Limnotrichales, Limnotrichaceae, *Limnothrix*	0.00 (±0.00)	22.00 (±16.12)
Oxyphotobacteria, Nostocales, Cyanobacteriaceae, Cyanobacterium CLg1	0.00 (±0.00)	10.80 (±10.80)
Oxyphotobacteria, Nostocales, Cyanobacteriaceae, uncultured bacterium	0.00 (±0.00)	1.60 (±1.60)
Oxyphotobacteria, Nostocales, Oscillatoriaceae, *Planktothricoides* SR001	0.00 (±0.00)	360.60 (±122.34)
Oxyphotobacteria, Nostocales, Paraspirulinaceae, *Spirulina* DRTO-55.2	0.00 (±0.00)	53.40 (±51.67)
Oxyphotobacteria, Nostocales, Xenococcaceae, *Xenococcus* PCC-7305	0.00 (±0.00)	4.40 (±3.49)
Oxyphotobacteria, Nostocales, Xenococcaceae	1.60 (±1.60)	3.40 (±2.36)
Oxyphotobacteria, Nostocales	0.00 (±0.00)	3.20 (±3.20)
Oxyphotobacteria, Phormidesmiales, Phormidesmiaceae, *Acrophormium* PCC-7375	0.00 (±0.00)	16.00 (±8.50)
Oxyphotobacteria, Phormidesmiales, Phormidesmiaceae, *Phormidium* MBIC10003	0.00 (±0.00)	5.60 (±4.46)
Oxyphotobacteria, Phormidesmiales, Phormidesmiaceae, uncultured *Leptolyngbya*	1.00 (±0.63)	8487.80 (±5601.10)
Oxyphotobacteria, Synechococcales, Cyanobiaceae, *Cyanobium* PCC-6307	2.60 (±2.60)	0.00 (±0.00)
Oxyphotobacteria, Synechococcales, Cyanobiaceae, *Synechococcus* CC9902	49.40 (±20.10)	4.40 (±2.98)
Oxyphotobacteria, Synechococcales, Cyanobiaceae, *Synechococcus spongiarum* group	20890.60 (±6412.42)	13993.40 (±2113.40)
Oxyphotobacteria, Synechococcales, Cyanobiaceae	0.00 (±0.00)	2.40 (±2.40)
Oxyphotobacteria	0.00 (±0.00)	62.80 (±35.01)
Sericytochromatia	0.00 (±0.00)	2.60 (±2.60)

in relative abundance with health condition (Figs 4 and 5). Within the cyanobacterial assemblage, *S. spongiarum*, the photosymbiont that dominates healthy individuals, was replaced in diseased sponges with a more heterogeneous cyanobacterial assemblage dominated by members of the genus *Leptolyngbya* (Table 2). This result is consistent with that observed in previous studies on ARBS (Olson, Gochfeld and Slattery 2006; Olson, Thacker and Gochfeld 2014) and for *X. muta* tissue affected by sponge orange band disease (Angermeier *et al.* 2011). Although cultivated *Leptolyngbya* isolates failed to recreate disease in ARBS transmission studies (Olson, Thacker and Gochfeld 2014), our data suggest that *Leptolyngbya* spp. are likely involved in ARBS pathogenesis but are not the sole etiological agent of this disease.

Supporting results reported by Blanquer *et al.* (2016) in diseased *Ircinia fasciculata*, but contrary to results from diseased *A. aerophoba* (Webster *et al.* 2008), the class Gammaproteobacteria was significantly enriched in ARBS-affected individuals. Within this class, Endozoicomonadaceae are commonly found in association with marine invertebrates (e.g. Kurahashi and Yokota 2007; Nishijima *et al.* 2013; Bourne, Morrow and Webster 2016; Neave *et al.* 2017; Robbins *et al.* 2019; Jensen *et al.* 2021), with putative roles in nutrient acquisition and cycling, regulation of host health and microbiome structuring (Morrow *et al.* 2012; Rua *et al.* 2014; Neave *et al.* 2016; Jensen *et al.* 2021). Although disease can reduce the abundance of *Endozoicomonas* spp. in corals (Vezzulli *et al.* 2013; Meyer *et al.* 2014), our limited understanding of the functional roles of these bacteria in sponges restricts our ability to exclude members of the Endozoicomonadaceae as potential etiologic agents of ARBS.

Some of the significantly enriched bacterial taxa in ARBS-affected sponges included the phyla Bacteroidetes, Actinobacteria and Dadabacteria and the orders Microtrichales, Rhodobacterales and Flavobacteriales, all known to increase in abundance in stressed and/or diseased marine invertebrates (Webster *et al.* 2008; Webster, Cobb and Negri 2008; Reis *et al.* 2009; Sunagawa *et al.* 2009; Mouchka, Hewson and Harvell 2010; Bourne, Morrow and Webster 2016; Luter *et al.* 2017, 2020; Kandler *et al.* 2018; Rosales *et al.* 2020; MacKnight *et al.* 2021). Several of these taxa were recently described as habitat generalists, capable of becoming dominant in disturbed ecosystems, which was hypothesized to be due to their metabolic flexibility (Chen,

Boyaci and Campbell 2021) and which would support their increased abundance in diseased sponges where tissue damage could affect nutrients required for growth. Within the order Rhodobacterales, the genus *Ruegeria* was significantly enriched in the ARBS-affected sponges. Rubio-Portillo *et al.* (2021) also detected an increase in *Ruegeria* sp. in necrotic tissues of the Mediterranean corals *Oculina patagonica* and *Leptogorgia sarmentosa* and identified members of this genus as putative microbial indicators of marine invertebrate diseases, but not necessarily as pathogens. Although intriguing, the polyphyly of the genus *Ruegeria* suggests that its potential as an indicator genus needs to be interpreted with caution (Liang *et al.* 2021). However, members of the Flavobacteriales warrant further investigation as potential etiologic agent(s) of ARBS as this order has been associated with various coral diseases and found in stressed scleractinian corals (Frias-Lopez *et al.* 2002; Sekar, Kaczmarky and Richardson 2008; Thurber *et al.* 2009; Gignoux-Wolfsohn and Vollmer 2015). Members of the Planctomycetes, which also occurred in greater relative abundance in ARBS-affected sponges, should undergo further investigation as they have recently been reported to be potential opportunistic human pathogens (Kaboré *et al.* 2020) and have been widely reported within sponge-associated communities (Pimentel-Elardo *et al.* 2003; Kallscheuer *et al.* 2020; Kohn *et al.* 2020; Wiegand *et al.* 2020).

Of the seven taxa detected solely in ARBS-affected individuals, all were found at very low relative abundance and were only present in some, but not all, of the ARBS-affected sponges. As a result, these taxa are not likely to represent the etiologic agent of ARBS. Within the seven taxa, members of the Saprospiraceae are generally associated with the degradation of complex organic materials (Flint and Duncan 2014; McIlroy and Nielsen 2014) and, as such, are not unexpected as ARBS causes necrosis of the affected tissue. Similarly, although archaeal sequences were detected, no pathogenic archaea have been reported, with members instead being important in nutrient cycling (reviewed by Turon and Uriz 2020). Taken collectively, the changes in relative abundance of prokaryotic taxa detected in the current study are similar to patterns of community alterations detected in various stressed and/or diseased marine invertebrates and rather than providing insight into putative etiologic agent(s), they confirm that dysbiosis occurs in ARBS-affected sponges.

Although much is known about microbial communities associated with healthy and ARBS-affected *A. cauliformis*, there is still no definitive etiologic agent(s) of this highly transmissible disease. With additional information on the functions of the sponge-associated microorganisms, it may be possible to tease apart their putative roles. Thus, future research should utilize a metagenomic and/or transcriptomic approach to assess the functional changes in microbial community composition within ARBS-affected sponges over space and time in order to determine which microbial constituents contribute to homeostasis and defense of the sponge holobiont and which may be pathogenic or opportunistic. With the increase in emerging diseases reported from sponges worldwide, improving our understanding of the functionality and stability of sponge-associated microbial communities is essential for recognizing environmental conditions and organisms that result in tissue damage or mortality.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflicts of Interest. None declared.

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