

Intraspecific Variation in Microbial Symbiont Communities of the Sun Sponge, *Hymeniacidon heliophila*, from Intertidal and Subtidal Habitats

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Sponges host diverse and complex communities of microbial symbionts that display a high degree of host specificity. The microbiomes of conspecific sponges are relatively constant, even across distant locations, yet few studies have directly examined the influence of abiotic factors on intraspecific variation in sponge microbial community structure. The contrast between intertidal and subtidal environments is an ideal system to assess the effect of environmental variation on sponge-microbe symbioses, producing two drastically different environments on a small spatial scale. Here, we characterized the microbial communities of individual intertidal and subtidal *Hymeniacidon heliophila* sponges, ambient seawater, and sediment from a North Carolina oyster reef habitat by partial (Illumina sequencing) and nearly full-length (clone libraries) 16S rRNA gene sequence analyses. Clone library sequences were compared to *H. heliophila* symbiont communities from the Gulf of Mexico and Brazil, revealing strong host specificity of dominant symbiont taxa across expansive geographic distances. Sediment and seawater samples yielded clearly distinct microbial communities from those found in *H. heliophila*. Despite the close proximity of the sponges sampled, significant differences between subtidal and intertidal sponges in the diversity, structure, and composition of their microbial communities were detected. Differences were driven by changes in the relative abundance of a few dominant microbial symbiont taxa, as well as the presence or absence of numerous rare microbial taxa. These findings suggest that extreme abiotic fluctuations, such as periodic air exposure in intertidal habitats, can drive intraspecific differences in complex host-microbe symbioses.

Sponges form ancient symbioses (as described in reference 1) with a great diversity of bacterial, archaeal, and eukaryotic microorganisms (1, 2). Most of these microbial symbionts elude cultivation (3); thus, culture-independent techniques such as next-generation DNA sequencing are necessary to elucidate the immense diversity of microbial communities in marine sponges. For example, the sponge *Rhopaloeides odorabile* hosts nearly 3,000 unique microbial taxa (4). Symbiotic microbes can constitute up to 40% of the sponge mesohyl volume (5) and perform a variety of ecological functions in the host sponge (6). Sponge-associated microbial symbionts may play roles in the nutrient acquisition, chemical defense production, antifouling activity, and disease susceptibility of host sponges (1, 7). Despite the ubiquity of microbial symbionts in sponges, much is still unknown about their structure and ecological function.

Sponges host microbial communities that are distinct from those found in seawater and exhibit a high degree of host specificity (4, 8, 9), even between closely related sponge species (10). Determining what structures these symbiotic communities is a current research priority, because it will provide insight into the maintenance of one of the most ancient metazoan-microbial symbioses. The comparison of microbial assemblages in multiple sponge species has revealed a very small community (<1%) of symbiont taxa found in many species, while a much larger portion of the community (ca. 70%) is host species specific (11). The host specificity of sponge microbial symbionts explains most of the interspecific variation in microbial communities (12); however, relatively few studies have examined the effects of environmental factors on intraspecific variation in microbial community structure. Rare microbial taxa, which can represent a large proportion (>90%) of the diversity of microbes found in marine sponges, exhibit similar levels of host species specificity, as reported for

more dominant symbiont taxa (13). Next-generation sequencing has provided insight into the extraordinary diversity of these low-abundance microbial taxa, often referred to as the “rare biosphere” (14), but further research is necessary to understand how they are shaped by their environment.

Recent studies report spatially and temporally stable microbial communities in multiple sponge species collected across geographic distances of hundreds of kilometers (8, 15, 16), seasonal and annual time scales (17, 18), and depths of 10 to 100 m (19). In some cases, however, local environmental conditions may drive intraspecific differences in sponge microbial community structure. For example, microbial symbionts of *Mycale hentscheli* differed over spatial and temporal scales (20) and symbionts of *Petrosia ficiformis* differed by location within the Mediterranean Sea (21). Biotic factors, including the presence of diseased tissue, have been shown to affect microbial community structure in *Aplysina cauliformis* (22). In addition, abiotic factors such as light, nutrient concentrations, and temperature can drive intraspecific variation in sponge microbial symbiont communities. Individual *Petrosia*

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ficiformis sponges growing in well-illuminated habitats harbor cyanobacterial symbionts, while those growing in shaded areas, including caves, do not (21). A study near a highly polluted estuary in Brazil noted that nutrient concentrations played a role in the structuring of the different archaeal communities of conspecific sponges (23). In another study, experimental warming induced changes in the microbial community structure of *Rhopaloeides odorabile* (24). Notably, these shifts in symbiont communities were elicited by drastic changes in environmental factors (light versus dark, high pollution levels, temperatures exceeding the annual maxima), while symbiont communities in sponges were stable across less intense changes in light exposure (25), eutrophication (26), and temperature (16). Environmental conditions may play a role in the structuring of the microbial community of marine sponges, in particular, when fluctuations are intense, but more research is needed to gain a better understanding of the effects of abiotic factors on sponge-microbe symbioses.

Marine ecologists have extensively studied the intertidal environment because of the unique physical stressors that it places on organisms, including thermal stress and desiccation, strong competition for space, and physical disturbance from waves (27). The intertidal environment is physiologically stressful for sessile filter-feeding organisms, and sponges that live in the intertidal zone experience periodic air exposure and consequential fluctuations in temperature, irradiance, and UV exposure (28). The demosponge *Hymeniacidon heliophila* (Parker 1910), commonly called the sun sponge, is abundant in coastal ecosystems of the Atlantic Ocean spanning a vast latitudinal range from the Gulf of Maine in the United States to Brazil (29). The sun sponge is able to live in the intertidal zone, with exposure to air twice daily at low tide, and in the subtidal zone at depths of 1 to 20 m. The sun sponge has adapted to high UV radiation exposure in the intertidal zone by producing antioxidant compounds such as L-5-hydroxytryptophan that prevent oxidative cell damage (30). The microbial symbiont community structure of *H. heliophila* has been previously examined in Brazil and the Gulf of Mexico (29, 31), although these studies focused exclusively on subtidal sponges.

The contrast between intertidal and subtidal environments, which produces large environmental variation over a small spatial scale, is an ideal system to study the ecological factors that drive intraspecific variation in the sponge microbiome. We hypothesized that tidal exposure would result in intraspecific variation in the microbial symbiont community of intertidal and subtidal *H. heliophila* sponges. To address this question, we compared the microbial communities of intertidal and subtidal *H. heliophila* sponges, along with ambient seawater and sediment samples, by next-generation sequencing of the bacterial/archaeal 16S rRNA genes. To achieve greater taxonomic resolution and to compare North Carolina *H. heliophila* microbial communities to those previously described from the Gulf of Mexico, we also generated a clone library of nearly full-length bacterial 16S rRNA gene sequences.

MATERIALS AND METHODS

Sample collection and DNA extraction. In July 2014, individual marine *Hymeniacidon heliophila* (Parker 1910) sponges were collected at low tide from Loosin Creek (34.1722N, -77.8328W) in Wilmington, NC. The benthic habitat in Loosin Creek includes soft mud, sand, and patches of oyster reef that sponges frequently use as substrates. Sponges were identified as *H. heliophila* in the field on the basis of gross morphology and

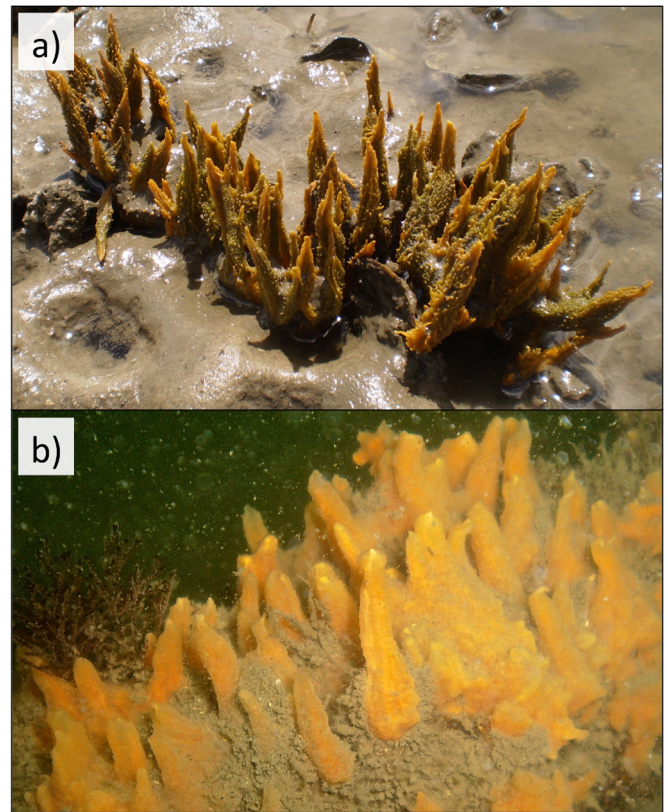


FIG 1 Photographs of intertidal *H. heliophila* (a) exposed during low tide in Loosin Creek and subtidal *H. heliophila* (b) at an approximately 3.5-m depth in Banks Channel, Wilmington, NC.

confirmed by viewing style spicules typical of *H. heliophila* (32) by light microscopy. Air and seawater temperature data for July 2014 (recorded at 15-min intervals) were acquired from nearby (<3 km) meteorological and water quality monitoring stations (Loosin Creek Station [34.1722N, -77.8328W] and Research Creek Station [34.1555N, -77.8509W]) maintained by the North Carolina National Estuarine Research Reserve. Six *H. heliophila* sponges were sampled from the intertidal zone; sponges were considered intertidal if they were fully exposed to the air at low tide (Fig. 1a). Six *H. heliophila* sponges were sampled from the subtidal zone in the same tidal creek at 2 to 3 m below the low-tide water depth (Fig. 1b). All sponges were collected at least 5 m apart, rinsed with 0.2- μ m-filtered seawater, and placed immediately in 95% ethanol. Triplicate ambient seawater samples were collected in 1-liter bottles, 300-ml volumes were concentrated onto 0.2- μ m filters, and the filters were frozen at -80°C until analysis. In close proximity to the sponges (<5 m), triplicate samples were collected from the top 10 cm of sediment and preserved in 95% ethanol. DNA was extracted from sponge, sediment, and seawater samples with the DNeasy blood and tissue kit (Qiagen). DNA extracts were used for PCR amplification of 16S rRNA gene sequences for both clone library construction (ca. 1,500-bp sequences) and Illumina next-generation sequencing (ca. 300-bp sequences).

Sponge nuclear DNA amplification and sequencing. To investigate intraspecific variation in the genotypes of intertidal and subtidal *H. heliophila* sponges, we amplified a segment of the second internal transcribed spacer region (ITS-2) and the 5' end of the 28S ribosomal subunit with sponge-specific primers SP58bF and SP28cR (33). PCR amplifications were conducted with a total volume of 25 μ l including 5 pmol of each primer, 2 \times MyTaq HS Red Mix (Biolone), and 1 μ l (ca. 10 ng) of template DNA. Negative controls were included to test for contamination in all reaction mixtures (none detected). The thermocycler program consisted

of an initial denaturation at 95°C for 2 min; 35 cycles of 95°C for 15 s, 50°C for 15 s, and 72°C for 20 s; and a final extension at 72°C for 2 min. Positive amplicons were prepared for DNA sequencing with BigDye Terminator v. 3.1 (Applied Biosystems) and the same forward and reverse primers used in the initial amplifications. PCR products were purified by isopropanol precipitation and sequenced on an ABI 3130xl genetic analyzer (Applied Biosystems) at the University of North Carolina Wilmington (UNCW) Center for Marine Science. Sequence reads were processed in Geneious (version 8.02) (34) by aligning forward and reverse reads to create consensus sequences of 602 bp. All reads were identical; thus, a single representative sequence was deposited in GenBank.

Clone library construction and analysis. To construct clone libraries, 16S rRNA gene sequences were amplified from DNA extracts with universal bacterial forward primer 8F (35) and reverse primer 1509R (36). The PCR amplifications and thermocycler conditions were the same those as described above, except that 0.5 μ l of template DNA was used. PCR products were gel extracted and purified with the GeneJET Gel Extraction and DNA Cleanup Micro kit (Thermo Scientific). All amplifications were conducted in triplicate and combined following gel extraction and purification. Purified PCR products were ligated into plasmids and transformed into One Shot Chemically Competent *Escherichia coli* cells with the TOPO TA cloning kit for sequencing (Invitrogen). For each sponge, sediment, or seawater sample, 17 transformants were selected and PCR screened with vector primers T3 and T7. Positive transformants were prepared for DNA sequencing with BigDye Terminator v. 3.1 (Applied Biosystems) with forward and reverse vector primers in separate reaction mixtures. PCR products were purified by isopropanol precipitation and sequenced on an ABI 3130xl genetic analyzer (Applied Biosystems) at the UNCW Center for Marine Science. Sequence reads were processed in Geneious (version 8.02) (34) by aligning forward and reverse reads to create near full-length 16S rRNA consensus sequences (ca. 1,500 bp). Consensus sequences were converted to FASTA format, imported into the mothur software package (37), and aligned with the Greengenes database (gg_13_5_99). Sequences were checked for chimeras by self-reference searching with UChime (38) and classified by using a naive Bayesian classifier and bootstrap algorithm for confidence scoring (39) based on the improved Greengenes taxonomy template (40) as implemented in mothur. Nontarget taxa (chloroplasts, mitochondria, and eukarya) were removed from the data set. High-quality sequences ($n = 243$) were assigned to operational taxonomic units (OTUs) in mothur by using 97% sequence identity and the average neighbor clustering algorithm, and the taxonomic classification of each OTU was constructed by majority consensus (41).

Next-generation DNA sequencing and processing. DNA extracts were sent to Molecular Research LP (Shallowater, TX) for amplification, library construction, and multiplexed (see Table S1 in the supplemental material) sequencing of partial (V4) 16S rRNA gene sequences with the universal bacterial/archaeal forward primer 515f and reverse primer 806r (42) on an Illumina MiSeq platform. Raw sequences were processed with a modified version of the Illumina MiSeq SOP pipeline (43) in mothur (http://www.mothur.org/wiki/MiSeq_SOP). Briefly, raw sequence reads were filtered for ambiguous base calls (maxambig = 0), amplicon size (maxlength = 300, minlength = 200), barcode mismatches (bdiffs = 0), primer mismatches (pdiffs = 2), and homopolymers (maxhomop = 8). The resulting sequences were aligned with the Greengenes reference database (gg_13_5_99) and trimmed to the V4 region. To further reduce sequencing errors in the data set, the precluster (44) and UChime algorithms were run, as implemented in mothur, and putative chimeras were removed. Sequences were then classified, nontarget reads were removed, and 97% OTUs were constructed as described above. Because the sampling depths (i.e., number of sequence reads) varied among replicates, each data set was subsampled to the lowest read count ($n = 3,514$) from the final shared file. All subsequent data analyses were based on the final subsampled data sets. To define the rare and abundant microbial taxa, a 1% threshold was employed (45), resulting in a cutoff value of 35 se-

quences (i.e., abundant OTUs were present in the data set >35 times, and rare OTUs were present ≤ 35 times).

Statistical methods. (i) Microbial community diversity. To compare the microbial community diversities of the four sources (intertidal and subtidal *H. heliophila*, sediment, and seawater), the diversity indices OTU richness (S), the Shannon-Weaver diversity index (H'), and inverse Simpson index (D) were calculated with the R package vegan (46) and code developed by Easson and Thacker (12). Because of the greater depth of sampling from the Illumina MiSeq platform (3,514 sequences/sample) than from clone libraries (17 sequences/sample), microbial diversity indices were calculated only for next-generation Illumina sequence data. Analyses of variance (ANOVA) were used to statistically compare the diversity indices of the four sources, and Tukey's honest significant difference (HSD) tests were conducted for multiple pairwise *post hoc* comparisons of means.

(ii) Microbial community structures. To compare the microbial community structures of sampling sources, Bray-Curtis similarity (BCS) matrices were created by using next-generation OTU abundances in PRIMER (version 6.1.11). BCS matrices were visualized by using cluster dendrograms. Permutational multivariate ANOVA (PERMANOVA) were used to test for significant differences in microbial community structure among the different sampling sources, with subsequent pairwise comparisons for significant PERMANOVA results. Multiple pairwise comparisons were corrected on the basis of the Benjamini-Yekutieli (B-Y) false-discovery rate control and an experiment-wise error rate of $\alpha = 0.05$. To ensure that significant PERMANOVA results were due to structural differences and not unequal dispersion of variability among groups, permutational multivariate analyses of dispersion (PERMDISP) were conducted for all significant PERMANOVA outcomes. Next-generation sequences were divided into abundant and rare-OTU partitions, and BCS matrices were recreated for each partition to test for differences in microbial community structures of abundant and rare taxa.

(iii) OTU level analysis of microbial community differences. To investigate the contribution of each OTU to the observed dissimilarity between intertidal and subtidal *H. heliophila* samples, a one-way similarity percentages species contributions (SIMPER) analysis was conducted by using microbial OTU relative abundance matrices in PRIMER. We used a cutoff percentage of 0.50 to identify the OTUs that contributed to half of the intertidal-subtidal dissimilarity in the overall, abundant, and rare microbial community data sets. In addition, Venn diagrams were constructed in mothur to identify the numbers of OTUs overlapping between sampling sources.

Nucleotide sequence accession numbers. The nucleotide sequence determined in this study has been submitted to GenBank and assigned accession numbers KT880231 to KT880468. Raw sequence data were deposited as FASTQ files in the Sequence Read Archive of the National Center for Biotechnology Information under accession no. SRP065064.

RESULTS

Host genotype and temperature regimes in intertidal and subtidal habitats. No intraspecific variation in *H. heliophila* genotypes was detected between intertidal and subtidal environments, with identical ITS-2 and partial 28S rRNA gene sequences recovered in all 12 sponges. Furthermore, the recovered sequences were 99.9% identical to *H. heliophila* ITS-2/28S rRNA gene sequences from the Gulf of Mexico (31). Air and seawater temperatures exhibited similar average values during the collection months ($26.3^{\circ}\text{C} \pm 2.6$ and $27.7^{\circ}\text{C} \pm 1.2$, respectively); however, air temperatures exhibited greater daily fluctuations (see Fig. S1 in the supplemental material), averaging a daily range more than twice that of seawater ($7.0^{\circ}\text{C} \pm 1.9$ and $3.2^{\circ}\text{C} \pm 1.1$, respectively) and reaching peak values (maximum, 34.0°C ; minimum, 20.5°C) exceeding those recorded in seawater (maximum, 32.5°C ; minimum, 24.3°C).

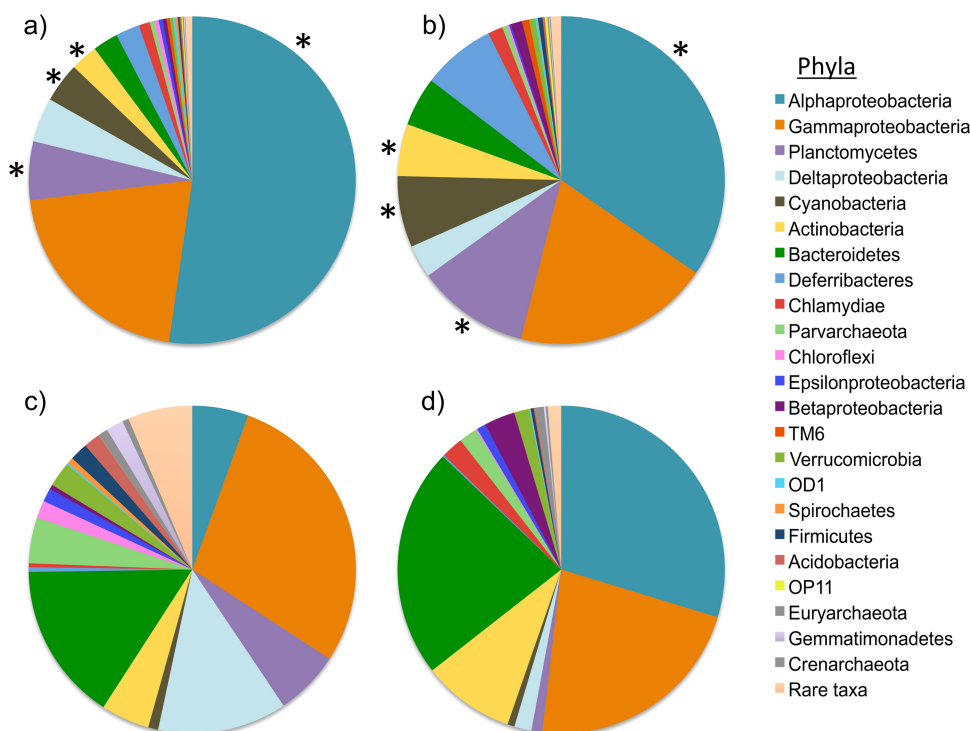


FIG 2 Compositions of microbial communities associated with intertidal *H. heliophila* (a), subtidal *H. heliophila* (b), sediment (c), and seawater (d). Pie charts depict the relative abundances of archaeal and bacterial phyla, with *Proteobacteria* divided into five major classes. Asterisks denote taxa that exhibit significant differences between intertidal and subtidal *H. heliophila* sponges (see Fig. S3 in the supplemental material).

Comparative analysis of microbial community diversity and composition. In total, 7,646 OTUs were recovered from sponge ($n = 4,224$), sediment ($n = 2,999$), and seawater ($n = 1,451$) samples, representing 59 bacterial and 3 archaeal phyla (*Euryarchaeota*, *Crenarchaeota*, and *Parvarchaeota*). All archaeal phyla were detected in *H. heliophila* and ambient sediment and seawater samples, while the presence and relative abundance of bacterial phyla differed across sources (Fig. 2). The sediment and seawater communities included 55 and 46 bacterial phyla and were dominated by the bacterial phyla *Proteobacteria* (49 and 58%, respectively) and *Bacteroidetes* (16 and 23%; Fig. 2). The *H. heliophila* community included 48 bacterial phyla, but in contrast, these sponge samples had a much lower abundance of *Bacteroidetes* (4%) and the top three phyla were *Proteobacteria* (67%), *Planctomycetes* (8%), and *Cyanobacteria* (5%; Fig. 2). *Proteobacteria* in *H. heliophila* displayed a significantly greater relative abundance in intertidal (76%) than in subtidal (58%) sponges (t test, $P < 0.01$). Within the *Proteobacteria*, there was a significantly greater relative abundance of *Alphaproteobacteria* in intertidal (52%) than in subtidal sponges (35%, t test, $P < 0.01$). Subtidal *H. heliophila* had a greater proportion of *Planctomycetes*, *Cyanobacteria*, and *Actinobacteria* than intertidal *H. heliophila* did (Fig. 2; see Fig. S2 in the supplemental material; t test, $P < 0.05$).

OTU richness (S), the Shannon-Weaver diversity index (H'), and the inverse Simpson index (D) were significantly different among the four sampling sources (ANOVA, $P < 0.01$; Table 1). Seawater samples exhibited a diversity similar to that of subtidal *H. heliophila* for both S and H' (Table 1), while the sediment community displayed significantly higher microbial diversity than sponge and seawater samples for all three diversity indices (Table 1).

Comparison of subtidal and intertidal *H. heliophila* communities revealed that diversity was significantly higher in the subtidal than in the intertidal symbiont communities for two diversity indices (S and H' ; *post hoc* Tukey's HSD test, $P < 0.05$), while there was no difference for the third (D; *post hoc* Tukey's HSD test, $P = 0.85$).

Comparative analysis of microbial community structure. In addition to the differences in the diversity of microbial communities from each source, the community structure of *H. heliophila*-associated microbial symbionts differed from those of the microbial communities in ambient seawater and sediment samples, with samples clustering according to source (Fig. 3). Accordingly, BCS matrices revealed significant differences in microbial community structure among *H. heliophila*, sediment, and seawater samples (PERMANOVA, $P < 0.01$), with 77% of the variability in microbial community structure explained by the source. Pairwise com-

TABLE 1 Diversity metrics for microbial communities associated with intertidal and subtidal *H. heliophila*, sediment, and seawater^a

Source	S ^b	H' ^c	D ^d
<i>H. heliophila</i>			
Intertidal	466 ± 35*	3.51 ± 0.11*	11.36 ± 1.07†
Subtidal	600 ± 24†	4.06 ± 0.09†	17.63 ± 1.68†
Seawater	619 ± 22†*	4.44 ± 0.05†	24.42 ± 1.07†
Sediment	1,334 ± 74‡	6.54 ± 0.06‡	293.37 ± 20.49‡

^a Values are means ± 1 standard error, and different symbols represent significant pairwise differences between the values (Tukey's HSD test).

^b S, OTU richness.

^c H' , Shannon-Weaver diversity index.

^d D, inverse Simpson index.

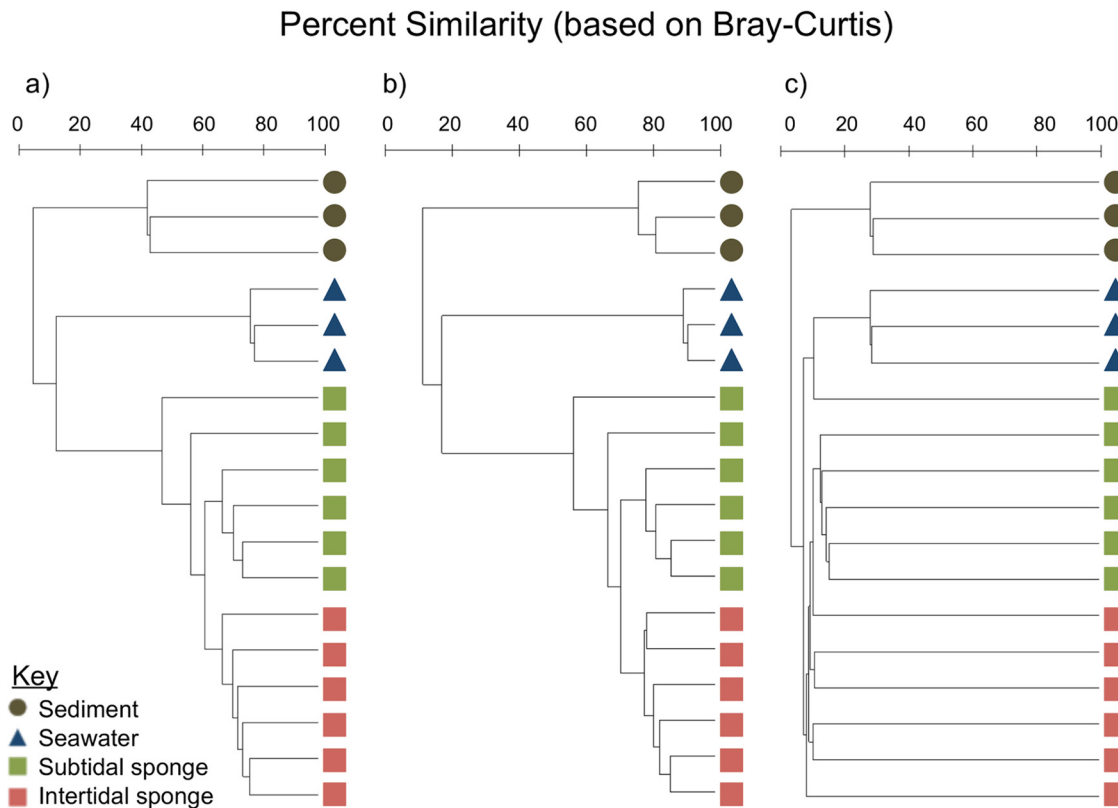


FIG 3 Cluster dendrogram based on Bray-Curtis dissimilarity matrices of the overall (a), abundant (b), and rare (c) microbial community partitions from sediment (●), seawater (▲), and subtidal (green ■) and intertidal (red ■) *H. heliophila*.

parisons revealed significant differences in community structure among all of the sources (PERMANOVA pairwise test; Table 2), including intertidal and subtidal communities of *H. heliophila*, with the exception of the sediment-seawater pairwise comparison ($P = 0.094$). Subtidal sponge symbionts displayed greater variability in microbial community structure (average similarity = 60.5%) than intertidal sponges (69.9%), which formed a distinct cluster within the *H. heliophila* samples (Fig. 3). While the overall permutational multivariate analysis of dispersion was significant (PERMDISP, $P < 0.01$), indicating unequal variances among some of the sources, the only significant pairwise comparison was between the intertidal *H. heliophila* and sediment microbial communities (Table 2).

Partitioning of sponge-, seawater-, and sediment-associated

TABLE 2 Pairwise statistical comparisons of *H. heliophila*, seawater, and sediment microbial community structure (PERMANOVA) and dispersion (PERMDISP)^a

Pairwise comparison	PERMANOVA		PERMDISP	
	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
Intertidal-subtidal <i>H. heliophila</i>	2.05	0.005 ^a	2.17	0.120
Intertidal <i>H. heliophila</i> -sediment	4.39	0.016 ^a	8.77	0.012 ^a
Intertidal <i>H. heliophila</i> -seawater	6.15	0.010 ^a	3.51	0.047
Subtidal <i>H. heliophila</i> -sediment	3.73	0.010 ^a	2.14	0.233
Subtidal <i>H. heliophila</i> -seawater	4.36	0.017 ^a	3.12	0.067
Sediment-seawater	3.34	0.094	35.48	0.101

^a Significant difference revealed by pairwise comparisons following B-Y correction.

microbial communities into abundant and rare components revealed 128 abundant OTUs that accounted for 75.2% of the sequences and 7,518 rare OTUs that accounted for 24.8% of the sequences. The *H. heliophila* microbial community included 123 abundant OTUs (85.6% of *H. heliophila* sequences) and 4,101 rare OTUs (14.4%). The microbial community structure based on the abundant-OTU data partition was significantly different among intertidal and subtidal *H. heliophila*, sediment, and seawater samples (PERMANOVA, $P < 0.01$; Fig. 3), with the source explaining 89.8% of the variability in community structure. At the abundant-OTU level, intertidal and subtidal *H. heliophila* communities displayed significantly different microbial community structures (PERMANOVA pairwise test, $P < 0.01$; Fig. 3). The same analyses of the rare-OTU partition yielded the same result; microbial community structure differed among source environments (PERMANOVA, $P < 0.01$), and intertidal and subtidal *H. heliophila* had significantly different microbial community structures (PERMANOVA pairwise test, $P < 0.01$). However, for the rare-OTU partition, the source explained less of the variation (19%) in community structure and greater dissimilarity was observed between replicates within each source (Fig. 3).

OTU level differences in symbiont communities of intertidal and subtidal sponges. SIMPER analyses yielded the top OTUs that contributed to 50% of the observed dissimilarity between subtidal and intertidal *H. heliophila* for the overall community, the abundant-OTU partition, and the rare-OTU partition. For the overall microbial community, there were 12 OTUs that contributed to 50% of the difference between intertidal and subtidal sym-

TABLE 3 Taxonomic classification and relative abundances of OTUs differentiating microbial communities in intertidal and subtidal *H. heliophila* sponges^a

OTU	Phylum	Lowest taxonomic classification	Contribution to dissimilarity (%)	Avg relative abundance	
				Intertidal	Subtidal
1 ^b	Proteobacteria	<i>Novosphingobium resinovorum</i>	9.90	19.65	13.87
3 ^b	Proteobacteria	<i>Thalassobaculum litoreum</i>	8.90	13.24	5.59
2 ^b	Proteobacteria	Order Kiloniellales	5.62	12.43	8.41
9 ^b	Deferribacteres	Order Deferribacterales	5.24	2.03	6.42
7 ^b	Planctomycetes	Family Pirellulaceae	5.23	4.03	8.58
5	Proteobacteria	Order RCP1-48 (<i>Gammaproteobacteria</i>)	4.09	7.52	4.46
6	Proteobacteria	Order Oceanospirillales	2.98	5.98	4.15
10	Actinobacteria	Order Actinomycetales	2.24	1.88	3.82
14	Spirochaetes	<i>Leptonema</i> sp.	1.88	2.25	1.26
18	Cyanobacteria	<i>Synechococcus</i> sp.	1.62	1.13	2.49
31	Proteobacteria	Order Oceanospirillales	1.37	0.16	1.29
8	Proteobacteria	Order Oceanospirillales	1.27	0.33	1.42

^a Results based on SIMPER analysis and OTUs contributing to 50% of the observed dissimilarity are shown.

^b OTU also detected by SIMPER analysis of the abundant (>1% relative abundance) microbial community partition.

biont communities, with 7 of these OTUs classified as *Proteobacteria* (Table 3). Analysis of the abundant-OTU partition revealed five OTUs that contributed to half of the dissimilarity between intertidal and subtidal sponges. Notably, these were the same top five OTUs that resulted from the overall community level analysis, indicating that the dominant taxa played a large role in shaping intraspecific differences in *H. heliophila*. For the rare-OTU partition, there were 1,259 OTUs that contributed to half of the observed intertidal-subtidal difference in microbial community structure. Thus, the difference between the rare microbial communities of intertidal and subtidal *H. heliophila* was driven by the presence or absence of a great number of microbial taxa. Further, evidence of the influence of the rare microbial OTUs in differentiating subtidal and intertidal symbiont communities was seen in the surprisingly small overlap between the microbial taxa of intertidal and subtidal *H. heliophila*. Among all of the sponge replicates, intertidal *H. heliophila* had 1,574 unique OTUs that accounted for 4.3% of the *H. heliophila* sequences, while subtidal *H. heliophila* had 2,159 unique OTUs (5.9% of the sequences). Notably, most (79.3%) of these unique intertidal and subtidal OTUs were not detected in ambient seawater or sediment communities (see Fig. S3 in the supplemental material). Only 491 OTUs were common to subtidal and intertidal sponges; however, these common OTUs accounted for 89.9% of the *H. heliophila* sequences.

Clone library analysis and next-generation community comparison. Clone library analysis yielded 174 *H. heliophila* sequences (intertidal $n = 91$, subtidal $n = 83$), 40 seawater sequences, and 31 sediment sequences. *H. heliophila* displayed the highest OTU level microbial species richness ($n = 65$), followed by sediment ($n = 30$) and seawater ($n = 19$); however, sediment and seawater samples had fewer replicates than *H. heliophila*. The clone library microbial community structures of *H. heliophila*, sediment, and seawater sources differed (PERMANOVA, $P < 0.01$), and 41.7% of the variability in community structure was explained by the source. All pairwise comparisons revealed significant differences in microbial communities, except between sediment and seawater ($P = 0.089$) and between intertidal and subtidal *H. heliophila* ($P = 0.082$). However, the clone library analysis had a much lower sampling depth per sponge ($n = 12$ to 16) than next-generation sequencing ($n = 3,514$).

A comparison of next-generation sequences and clone library sequences revealed a large proportion of matching OTUs. There were 45 sequences that matched $\geq 97\%$ with next-generation sequences, accounting for 75.3% of the clone library sequences and 75.9% of the next-generation sequences. Clone library analysis of *H. heliophila* bacterial communities revealed a symbiont composition similar to that reported for the next-generation data set, dominated by *Proteobacteria* (55.7%), *Cyanobacteria* (20.1%), and *Planctomycetes* (8%).

Biogeographic comparison of *H. heliophila* microbial communities. Nearly full-length 16S rRNA gene sequences from North Carolina *H. heliophila* samples were compared to those from previous studies in the Gulf of Mexico (31) and Brazil (29). Clone library analysis revealed greater microbial OTU richness in *H. heliophila* ($n = 65$) from North Carolina than in conspecific hosts from the Gulf of Mexico ($n = 37$) and Brazil ($n = 50$). While only 6.3% of the North Carolina *H. heliophila* microbial OTUs ($n = 11$) displayed $\geq 97\%$ similarity to 16S rRNA sequences from Gulf of Mexico *H. heliophila*, these shared OTUs accounted for 52.3% of the sequences from the present study ($n = 91$) and 71.9% of the sequences from the Gulf of Mexico ($n = 97$). The matching microbial OTUs included *Alphaproteobacteria* ($n = 5$), *Cyanobacteria* ($n = 2$), *Gammaproteobacteria* ($n = 2$), *Bacteroidetes* ($n = 1$), and *Spirochaetes* ($n = 1$).

DISCUSSION

The structure and composition of microbial communities in the sponge *H. heliophila* differed significantly from those of seawater communities, consistent with previous studies reporting that sponge-specific bacterial communities are distinct from free-living microbial taxa in ambient seawater (4, 8, 13). We further demonstrated that *H. heliophila* has a microbial community distinct from the highly diverse sediment microbial community, despite its close association with sediment in tidal creek habitats. While intertidal and subtidal sponges exhibited identical genotypes, they differed in the diversity, structure, and composition of their microbial symbiont communities. In fact, intraspecific variation in microbial community structure between intertidal and subtidal sponges was evident in both the abundant (>1%) and rare ($\leq 1\%$)

members, suggesting a broad impact of tidal exposure on the diverse symbiont communities in *H. heliophila*.

Overall, *H. heliophila* hosted an extremely diverse microbial community of bacteria and archaea. The microbial community was dominated by the bacterial phylum *Proteobacteria*, specifically, the class *Alphaproteobacteria*. Previous studies have categorized *H. heliophila* as a low-microbial-abundance (LMA) sponge on the basis of transmission electronmicroscopic observations and microbial species composition (29, 31). The latter is consistent with our results, as bacterial communities associated with LMA sponges tend to be dominated by *Proteobacteria* or *Cyanobacteria* (47, 48). In addition, the phyla *Planctomycetes*, *Cyanobacteria*, and *Deferribacteres* were common members of the *H. heliophila* microbiome. A recent investigation of bacterial communities in 12 intertidal sponges also reported *Proteobacteria*, *Planctomycetes*, and *Cyanobacteria* to be the most abundant phyla (49). While *Proteobacteria* is commonly the most abundant sponge-associated microbial phylum (11, 12), it is worth noting that *Planctomycetes* and *Cyanobacteria* seem to be particularly abundant in intertidal sponges. With sponge microbiological studies focused almost exclusively on subtidal sponges, additional analyses of intertidal sponges are necessary to further investigate structural patterns among and between intertidal and subtidal sponge species.

Consistent with previous studies examining biodiversity within and below the intertidal zone, we report greater diversity in the subtidal than in the intertidal *H. heliophila* microbiome. Higher diversity of subtidal than intertidal communities has been reported for soft-bottom benthic meiofauna (50) and hard-bottom benthic macrofauna (51). Our results suggest that the physiological stressors that place limits on biodiversity in intertidal environments, including oxidative stress, desiccation, and thermal stress, may also affect symbiotic microbial communities. In response to such stressors, free-living bacteria in the harsh intertidal habitat commonly grow in biofilms, thereby forming a protective shelter from physical stressors (52). Further, intertidal biofilms display signs of adaptation to tidal disturbance, including enriched metal ion and oxidative stress genes, unlike subtidal biofilms (53). An additional stressor resulting from tidal exposure for sponge-associated microbial communities is anoxia, as the cessation of host pumping (filter feeding) turns sponge tissue anoxic within 15 min (54). In the present study, greater ranges and extremes were observed in air temperatures than in seawater temperatures, indicating greater thermal stress acting on periodically exposed intertidal sponges. While further experimentation is necessary to demonstrate physiological differences within host sponges from tidal habitats, we hypothesize that intertidal *H. heliophila* microbial symbiont communities are less diverse than subtidal communities because of the constraints associated with living in the intertidal zone, including air exposure and periodic extreme temperature fluctuations.

In addition to differences in microbial community diversity, tidal exposure affected the structure of both abundant and rare microbial symbionts in *H. heliophila*. Shifts in the relative abundance of the dominant microbial taxa, namely, *Alphaproteobacteria*, were responsible for driving much of the difference between intertidal and subtidal microbial community structures. The identification of these few differentially abundant symbiont OTUs within a complex microbial consortium provides promising and specific targets for subsequent research on symbiont function. For example, the second most abundant OTU that differentiated in-

tertidal and subtidal *H. heliophila* was classified as the alphaproteobacterium *Thalassobaculum litoreum*, which has been shown to possess nitrate reductase enzymes (55). While nitrate reductase activity has yet to be demonstrated in this sponge holobiont, cytoplasmic and periplasmic nitrate reductase genes (*narG* and *napA*) have been detected in multiple sponge microbiomes (56) and other nitrogen cycling pathways have been reported in the *H. heliophila* microbiome (*amoA* genes from ammonia-oxidizing archaea) (15). Further research is necessary to demonstrate the functional implications of the observed microbial community shifts, as different microbial communities can be functionally equivalent (56) and a rich biodiversity of rare symbionts exists within *H. heliophila*.

The advent of next-generation sequencing has provided insight into the factors that structure the extraordinarily diverse rare biosphere. The host specificity of the rare microbiome in sponges was only recently discovered (13), and our study demonstrates that the rare microbiome within one host sponge species can be significantly affected by environmental factors. The rare microbial community of *H. heliophila* accounted for most (93.5%) of the microbial species richness, which is in agreement with the highly diverse rare microbiome in other sponge species (13). Interestingly, the rare microbial communities displayed the same significant clustering by environment as the abundant microbial communities, suggesting that similar ecological constraints act on abundant and rare microbial community members. Consistent with this hypothesis, a study of prokaryotic microbes in the Arctic Ocean found that both the abundant and rare phylotypes exhibited biogeographic trends (57). Within *H. heliophila*, we found that the rare microbial community was composed of unique microbial taxa in intertidal and subtidal hosts and that these taxa were not present in ambient seawater or sediment communities. The vast genetic diversity within the rare component of sponge-associated communities may also contribute to holobiont functionality, as rare microbial taxa can play an important and sometimes disproportionate functional role in nutrient cycling (58, 59) and may increase in abundance in response to environmental stressors (60). While we demonstrated intraspecific differences in the rare microbial symbionts of *H. heliophila*, many questions remain concerning the differences in habitat provisioning that affected the persistence of such a large number of microbial taxa in symbiotic and free-living communities.

Comparison of the symbiont community characterized herein to previous studies of the *H. heliophila* microbiome revealed the stability of dominant symbiont OTUs across a broad geographic range. The microbial symbiont community of *H. heliophila* was previously characterized in the Gulf of Mexico (31) and in Brazil (29), revealing a comparable community structure dominated by *Alphaproteobacteria* and including symbionts affiliated with *Beta*- and *Gammaproteobacteria*, *Cyanobacteria*, *Firmicutes*, and *Actinobacteria*. While a relatively small proportion of North Carolina *H. heliophila* OTUs matched Gulf of Mexico OTUs, the matching OTUs comprised dominant taxa that constituted over half of the total clone library sequences. Similarly, a low number of symbiont clades were common to *H. heliophila* from the Gulf of Mexico and Brazil, yet they accounted for half of the total symbiont communities (25). The maintenance of a species-specific microbial community across broad geographic distances indicates intimate host-symbiont associations that may be maintained by some degree of vertical symbiont transmission, as reported for other sponge spe-

cies (e.g., see reference 61), although further experimentation is needed to document the passage of microbes to *H. heliophila* sponge embryos.

Our results demonstrate that abiotic factors associated with tidal exposure can drive intraspecific differences in sponge microbial symbiont community structure. To date, this is the first study to compare the microbial symbiont communities of intertidal and subtidal conspecific sponges. We found that intertidal and subtidal *H. heliophila* sponges hosted microbial communities with different levels of diversity, relative abundances of dominant taxa, and compositions of rare taxa. The extension of intraspecific differences in structure and composition to the rare microbial community is particularly intriguing, since rare symbionts were only recently discovered to display host specificity (13). These results indicate that rare symbiotic microbial taxa in marine sponges may be structured by ecological mechanisms similar to those of their abundant counterparts. The present study focused on differences in the structure of intertidal and subtidal microbial symbiont communities, yet changes in symbiont composition may beget differences in symbiont functionality with important implications for nutrient cycling in coastal oyster reef ecosystems. The extreme nature of the intertidal zone and the close proximity of intertidal and subtidal sponges provide an ideal environment to study intraspecific variation in microbial symbiont communities. Future studies describing sponge microbial symbiont communities from different environments will help to elucidate the role of abiotic factors in shaping the structure and function of diverse microbiomes associated with marine sponges, as well as the cascading effects of symbiont metabolism on host ecology and coastal nutrient cycles.

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