

Article

Distinct Bacterial Communities Among *Halimeda* Thalli, Seawater, Sediment, and Sea Cucumber Feces in a *Halimeda*-Dominated Habitat

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Abstract

Halimeda-dominated habitats are ecologically significant tropical benthic ecosystems whose structural complexity supports diverse marine organisms, particularly associated microbes. However, the characteristics and ecological roles of these bacterial communities and their connectivity within *Halimeda*-dominated habitats remain poorly understood. This study examined microbial diversity and composition across four microenvironments of a *Halimeda* meadow, namely, ambient seawater, sediment, *Halimeda* thalli, and *Holothuria atra* feces, using the V3–V4 region of the 16S rRNA gene. A total of 44 phyla, 733 genera, and 827 species were identified, with *Proteobacteria*, *Cyanobacteria*, *Bacteroidota*, *Desulfobacterota*, and *Actinobacteriota* dominating across all samples. Microbial diversity and composition differed significantly among microenvironments, with sediment showing the highest species diversity and seawater showing the lowest. *Halimeda* thalli were dominated by *Alphaproteobacteria*, particularly *Phycisphaerae* and *Parcubacteria*, associated with nutrient cycling on macroalgal surfaces. Sediment was enriched with *Desulfobacteria*, *Actinobacteria*, and *Chloroflexi*. Seawater was characterized by *Synechococcus* CC9902, a primary producer in tropical waters. *H. atra* feces were dominated by *Bacteroidia* and *Bdellovibrionota*, representing a distinct bacterial community shaped by both gut-associated taxa and microorganisms derived from ingested sediments. Only 6.95% of genera were shared across all microenvironments, and feces and sediment shared the highest overlap (47.28%), suggesting that *H. atra* acts as a biogeochemical bridge that repackages benthic organic matter without fundamentally altering its microbial identity. The low overlap between *Halimeda* and seawater, as well as between seawater and both sediment and sea cucumber feces, suggests strong habitat-specific bacterial community assembly, with algal-associated bacterial communities being distinct from pelagic communities and seawater communities differing from benthic and fecal communities.

Keywords: benthic community; *Halimeda*; *Holothuria atra*; microenvironment; microbes



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1. Introduction

Microbial communities are major drivers of nutrient cycling and biogeochemical processes in marine ecosystems, mediating the transformation of carbon, nitrogen, phosphorus, and other essential elements involved in the cycling of organic and inorganic

matter [1,2]. In marine benthic ecosystems, microbial abundance and distribution are often heterogeneous among habitats due to variation in physicochemical conditions, resource availability, and substrate characteristics [3–6]. Consequently, distinct microbial communities have been reported among sediments, seawater, and host-associated microhabitats, reflecting their differing environmental conditions and ecological functions [7,8]. In particular, host-associated bacterial communities can influence host growth, productivity, nutrient acquisition, stress tolerance, and health [9–13]. Despite growing interest in marine host-associated microbiomes, research has primarily focused on ecologically important coastal ecosystems, such as coral reefs, seagrass meadows, and mangrove forests, resulting in substantial advances in our understanding of microbial diversity, ecological functions, and environmental drivers within these systems [14–16], bacterial communities associated with *Halimeda*-dominated habitats remain comparatively understudied.

In addition to primary producers, benthic detritivores are important components of marine ecosystems and contribute substantially to sediment biogeochemical processes. *Holothuria atra* is a deposit-feeding sea cucumber commonly found in tropical coastal habitats, including seagrass meadows, coral reefs, and *Halimeda*-dominated environments [17–19]. Dense *Halimeda* patches trap sediments, accumulate organic matter, and provide habitat complexity that supports a variety of benthic organisms, including sea cucumbers [20,21]. Consequently, *H. atra* is frequently observed within *Halimeda* habitats, where it feeds on sediment-associated organic matter and detritus [19]. Through sediment ingestion and excretion, *H. atra* plays important roles in bioturbation, detritus processing, organic matter recycling, and nutrient redistribution [21–23]. These processes can alter sediment physicochemical properties and influence microbial diversity and community composition [22,23]. Furthermore, the unique physicochemical conditions within the digestive tract of sea cucumbers may selectively enrich, suppress, or transform specific microbial taxa [24]. As a result, bacterial communities in sea cucumber feces may differ substantially from those in surrounding sediments and may contribute to microbial redistribution and nutrient cycling within *Halimeda*-dominated benthic ecosystems.

Halimeda (Bryopsidales, Chlorophyta) is a genus of calcified green macroalgae widely distributed throughout tropical and subtropical marine ecosystems, including coral reefs and coastal intertidal and subtidal habitats [25]. As an important primary producer, *Halimeda* contributes substantially to ecosystem productivity, carbonate sediment formation, and carbon sequestration [20,26]. *Halimeda* has been identified as a major contributor to carbonate production on tropical reefs, with reported production rates of up to $4 \text{ kg CaCO}_3 \text{ m}^{-2} \text{ yr}^{-1}$, and it is increasingly recognized as an important component of blue carbon ecosystems [20]. In addition, the structural complexity of *Halimeda* patches provides habitat for a diverse assemblage of marine organisms, including fishes, sea slugs, sea cucumbers, and other invertebrates [21,27,28]. Beyond providing physical habitat, *Halimeda* thalli offer an extensive surface area for microbial colonization and create unique microenvironments that may support distinct bacterial communities [29,30]. Similar to other marine macrophytes, bacterial communities associated with *Halimeda* may contribute to nutrient cycling, organic matter degradation, host health maintenance, and other ecosystem processes [30–33]. Understanding the composition and distribution of bacterial communities associated with *Halimeda* is therefore important for elucidating host–microbe interactions and assessing the ecological role of microorganisms within *Halimeda*-dominated ecosystems.

Despite the ecological importance of *Halimeda*-dominated habitats and their associated benthic fauna, little is known about bacterial communities across habitat microenvironments that are ecologically linked through nutrient cycling, organic matter transfer, microbial dispersal, and trophic interactions. In particular, variation in bacterial assemblages among *Halimeda* thalli, surrounding seawater, sediment, and sea cucumber feces

within dense *Halimeda* patches has received limited attention. Previous studies of *Halimeda*-associated bacterial communities have primarily focused on comparisons between algal surfaces and surrounding seawater. For example, Kuba et al. [30] reported distinct bacterial diversity and community composition between *Halimeda* thalli and adjacent seawater. However, studies simultaneously investigating bacterial communities across multiple interconnected microenvironments, including *Halimeda* thalli, seawater, sediment, and sea cucumber feces within *Halimeda*-dominated habitats, remain scarce.

Therefore, this study aimed to characterize and compare bacterial community structures across four interconnected microenvironments—*Halimeda* thalli, seawater, sediment, and sea cucumber feces—using 16S rRNA gene metabarcoding. It was hypothesized that bacterial communities associated with these microenvironments would differ significantly in both diversity and community composition because each habitat provides distinct physicochemical conditions, resource availability, and ecological niches that selectively favor different bacterial assemblages. By examining bacterial communities across multiple habitat compartments, this study provides new insights into microbial niche partitioning and potential ecological linkages within tropical benthic ecosystems. The findings improve our understanding of host–microbe and environment–microbe interactions and contribute to a more comprehensive understanding of microbial processes occurring within *Halimeda*-dominated coastal habitats.

2. Materials and Methods

2.1. Study Site

This study was conducted in the intertidal zone of Lidee Island (6°47'18" N 99°46'17" E), Satun Province, Andaman Sea, Thailand, in December 2020 (Figure 1). The study site was dominated by *Halimeda macroloba* Decaisne, which provides habitat for a variety of marine organisms, particularly the sea cucumber *Holothuria atra*. In addition, three seagrass species were present at this study site: *Halophila ovalis* (R. Brown) J. D. Hooker, *Thalassia hemprichii* (Ehrenberg) Ascherson, and *Cymodocea rotundata* Ascherson & Schweinfurth. Seawater temperature and salinity were approximately 30 °C and 30 ppt, respectively. Nitrate and phosphate concentrations were 7.37 ± 0.02 mg/L and 2.14 ± 0.39 mg/L, respectively. All samples, including seawater, sediment, *H. macroloba* thalli, and sea cucumber feces, were collected randomly within *Halimeda* patches at a depth of 1.5–2.0 m via snorkeling, with three independent replicates collected for each sample type. The distance between sampling points was approximately 5 m. This spacing was selected to obtain independent biological replicates while maintaining similar environmental conditions within the study area and minimizing potential confounding effects arising from small-scale habitat heterogeneity. Seawater samples were collected approximately 0.5 m above the sediment surface using sterilized 2 L bottles. Sediment samples were collected from a depth of approximately 5 cm using a corer with a diameter of 5 cm, and then they were transferred into sterile 50 mL centrifuge tubes. For *H. macroloba* and sea cucumber feces, each replicate was collected directly into sterile 50 mL centrifuge tubes. Immediately after collection, all samples were transferred to sterile sampling containers and maintained in an insulated cooler with ice packs during transport to the laboratory. The samples were transported to the Prince of Songkla University laboratory and subsequently stored at −20 °C until DNA extraction. The seawater samples were thawed and filtered through a sterile 0.2 µm membrane filter. The membrane filters containing the retained microbial biomass were subsequently used for DNA extraction. To ensure that subsequent analyses targeted the tightly associated bacterial community, the *Halimeda* samples were washed with sterile 3.5% artificial seawater prepared using commercial sea salt (Marinium, Bangkok, Thailand) to remove loosely attached epibionts and residual sediment [30].

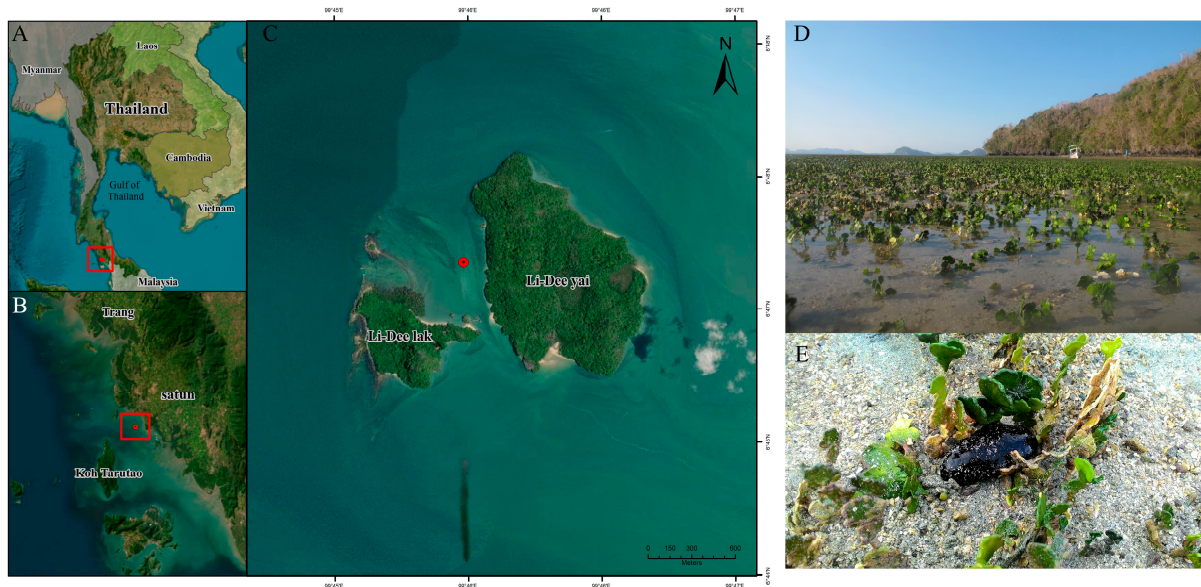


Figure 1. Maps of Thailand (A), Satun Province (B), Lidee Island (C), a *Halimeda* patch in the intertidal zone of Lidee Island (D), and *Holothuria atra* inhabiting *Halimeda* patches in the natural environment (E). The red square with red dot in panel (A,B) and the red dot in panel (C) indicate the sample collection site. The sampling site was located at 6°47′03″ N, 99°46′01″ E.

2.2. DNA Extraction and 16S rRNA Sequencing

Microbial DNA was extracted from a total of 12 samples representing four sample types, with three biological replicates per sample type, using a DNeasy® PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA quantity and purity were assessed with a DS-11 Series spectrophotometer (DeNovix Inc., Wilmington, DE, USA). The purified DNA was then sent to GENEWIZ Biological Technology (Suzhou, China) for library preparation and sequencing. The V3–V4 hypervariable region of the 16S rRNA gene was selected because it is widely used for Illumina-based microbial community profiling and provides a balance between broad taxonomic coverage and sufficient resolution for community-level comparisons. This region was amplified using degenerate primers recommended by GENEWIZ/Azenta: forward 5'-CCTACGRRBGCASCAGKVRVGAAT-3' and reverse 5'-GGACTACNVGGGTWTCTAATCC-3'. These primers target the V3–V4 region of the prokaryotic 16S rRNA gene and are designed to amplify diverse bacterial taxa from complex environmental samples. Related Pro341F/Pro805R-type V3–V4 primers have been reported to provide a broad coverage of both Bacteria and Archaea [34]; however, as with all amplicon-based approaches, primer-associated bias may remain. Therefore, because the present study focused mainly on the bacterial community composition based on the downstream taxonomic classification, the results are interpreted primarily as bacterial community profiles. PCR amplification was performed as a single amplicon PCR with two annealing temperature phases rather than as a two-stage Illumina indexing PCR. Briefly, amplification began with an initial denaturation at 95 °C for 5 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 45 s, and extension at 72 °C for 1 min. This was followed by 15 additional cycles using the same denaturation and extension conditions but with the annealing temperature reduced to 47 °C. A final extension was performed at 72 °C for 10 min. Each 25 µL reaction contained 2.5 µL TransStart Buffer (TransGen, Beijing, China), 2 µL dNTPs, 1 µL of each primer, and 20–30 ng template DNA. The primer sequences reported above represent the locus-specific V3–V4 primer regions. Illumina adaptor and index sequences were incorporated during library preparation by GENEWIZ/Azenta according to the provider's standard amplicon sequencing workflow. The

indexed amplicon libraries were quantified, purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA), and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a 2×300 bp paired-end configuration. Dedicated PCR-negative controls, extraction blanks, mock community controls, or positive control samples were not included in this study. Therefore, potential low-level background contamination from reagents or laboratory handling could not be directly assessed using control samples.

2.3. Bioinformatic and Statistical Analyses

All sequence data were processed using QIIME 2 [35]. Demultiplexed reads were initially inspected using the QIIME 2 demux summary to evaluate read quality profiles. Amplicon sequence variants (ASVs) were inferred using the DADA2 plugin [36]. Because the final ASV table was generated from single-end reads, denoising was performed using the QIIME 2 dada2 denoise-single command. Reads were trimmed by removing the first 10 bases from the 5' end and truncated at 430 bp. Reads with ambiguous bases were discarded, and reads with expected errors greater than 2.0 were removed. Quality truncation was performed at the first base with a quality score of 2 or lower. ASVs were inferred using the independent pooling method, and chimeric sequences were removed using the consensus method. The minimum fold-parent-over-abundance parameter for chimera detection was set to 1.0, and 1,000,000 reads were used for error model learning. Taxonomic assignment was performed using the QIIME 2 feature-classifier classify-sklearn method with a naïve Bayes classifier trained on the SILVA 138 SSURF NR99 reference database [37]. The classifier was generated using RESCRIPt and q2-feature-classifier, and taxonomy was assigned with a confidence threshold of 0.7. After taxonomic classification, the ASVs were screened for non-target assignments, including Archaea, chloroplasts, and mitochondria. No ASVs were assigned to the domain Archaea, and no mitochondrial ASVs were detected. Chloroplast-assigned ASVs were detected and removed before downstream analyses. Non-bacterial and unassigned ASVs were also excluded. The final filtered bacterial ASV table was used for taxonomic composition, alpha-diversity, beta-diversity, Venn diagram, and LEfSe analyses. To normalize the sequencing depth prior to diversity analysis, the filtered bacterial ASV table was rarefied to an even depth of 24,000 sequences per sample. This rarefaction depth was selected based on the distribution of the final bacterial read counts after chloroplast removal, which ranged from 24,589 to 35,680 reads per sample, allowing all samples to be retained while minimizing the loss of sequences. The rarefied ASV table was used for alpha- and beta-diversity analyses, whereas taxonomic composition was summarized as relative abundance using the filtered bacterial ASV table. The bacterial community composition was expressed as relative abundance for each replicate across treatments. Alpha diversity was assessed using Simpson's diversity index, observed features (observed ASV richness), Faith's phylogenetic diversity, the Shannon diversity index, and Pielou's evenness. Differences in alpha diversity among treatments were tested with the non-parametric Kruskal–Wallis test. The unweighted pair group method with arithmetic mean (UPGMA) based on Bray–Curtis dissimilarity was used to cluster the bacterial community at the phylum level. Beta diversity was evaluated using principal coordinate analysis (PCoA) based on unweighted UniFrac distances. Differences in community composition among treatments were determined using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, after confirming the homogeneity of dispersions with PERMDISP. Differentially abundant taxa were identified using linear discriminant analysis effect size (LEfSe) [38], applying an LDA threshold of 4.5 and a significance level of 0.05. The overlap of core microbiota between treatments was visualized with a Venn diagram generated using the online tool jvenn (<https://www.bioinformatics.com.cn/static/others/jvenn/example.html>, accessed 20 July

2024). Differences in the relative abundance of microbial taxa at the phylum level among samples were tested using one-way analysis of variance (ANOVA), followed by Tukey's HSD test for pairwise comparisons.

3. Results

A total of 382,400 high-quality bacterial reads were retained after demultiplexing, quality filtering, denoising, chimera removal, and removal of chloroplast-assigned ASVs. The sequencing depth ranged from 24,589 to 35,680 reads per sample (mean: 31,867; median: 32,871 reads per sample). Taxonomic classification in QIIME 2 assigned all recovered ASVs to the domain Bacteria, and no ASVs were assigned to Archaea. No mitochondrial ASVs were detected. Chloroplast-assigned ASVs accounted for 18,271 reads, representing 4.56% of the post-denoising dataset, and were removed before downstream bacterial community analyses. After filtering, the final bacterial dataset comprised 44 phyla, 104 classes, 222 orders, 363 families, 733 genera, and 827 species-level annotations. Sample-wise sequencing and DADA2 processing statistics are provided in Supplementary Table S1.

For bacterial alpha diversity, the sediment samples exhibited the highest richness and diversity, with an average of 1528.52 ± 73.62 observed features and a Shannon index of 9.77 ± 0.12 , followed by the sea cucumber feces (870.26 ± 195.38 observed features; Shannon index = 8.33 ± 0.47) and *Halimeda* samples (426.87 ± 25.48 observed features; Shannon index = 5.69 ± 0.10). Ambient seawater showed the lowest alpha diversity, with an average of 363.67 ± 14.50 observed features and a Shannon index of 5.54 ± 0.02 (Figure 2A and C). Species evenness showed a similar pattern, with sediment (Pielou's evenness = 0.924 ± 0.005), sea cucumber feces (0.859 ± 0.021), and *Halimeda* (0.653 ± 0.018) exhibiting higher evenness than ambient seawater, which showed the lowest value (0.652 ± 0.001) (Figure 2D). Overall, microbial richness, diversity, and evenness differed significantly among sample types ($p < 0.05$, Kruskal–Wallis). However, no significant differences were detected between the *Halimeda* and seawater samples for either diversity or evenness ($p > 0.05$, Dunn's Test) (Figure 2).

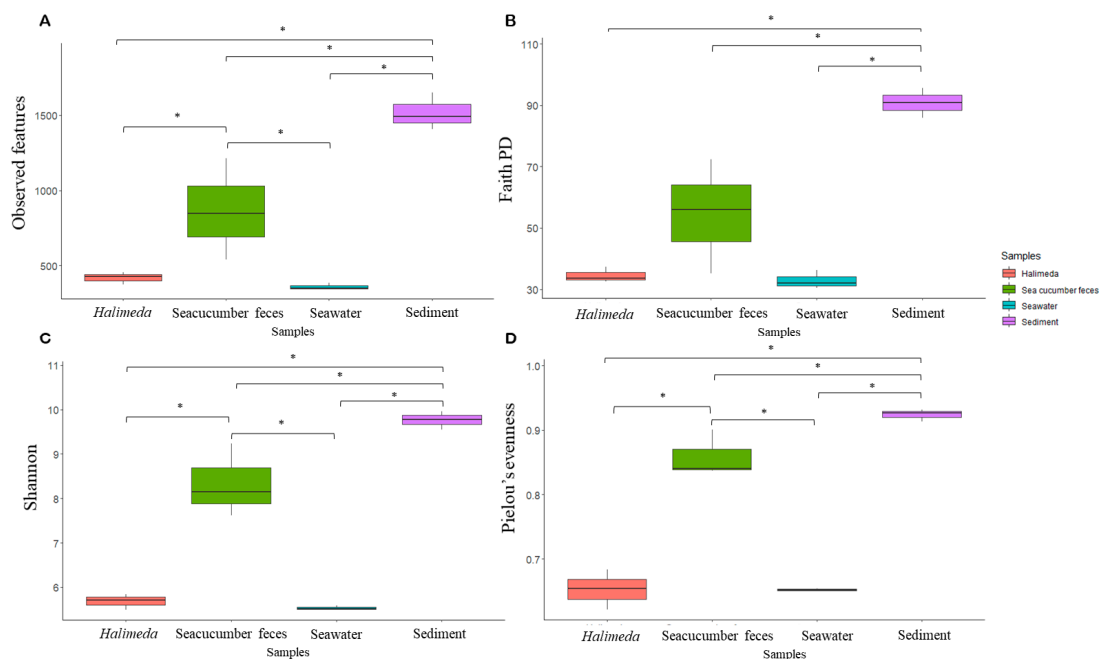


Figure 2. Boxplots showing bacterial alpha diversity across the four microenvironments (*Halimeda* thalli, seawater, sediment, and sea cucumber feces), based on (A) observed features (richness), (B) Faith's phylogenetic diversity (Faith's PD), (C) Shannon diversity index, and (D) Pielou's evenness index. Asterisks (*) indicate statistically significant differences between groups ($p < 0.05$).

The bacterial community composition differed markedly among seawater, sediment, *Halimeda* thalli, and sea cucumber feces, indicating strong compartment-specific structuring of the bacterial communities ($p < 0.05$, PERMANOVA, Supplementary Table S2). At the phylum level, *Proteobacteria*, *Cyanobacteria*, *Bacteroidota*, *Desulfobacterota*, and *Actinobacteriota* were dominant across all sample types, comprising 32.32–89.39%, 0.96–45.07%, 6.98–19.65%, 0.02–12.50%, and 0.11–11.41% of the total community, respectively (Figure 3). At the class level, *Alphaproteobacteria*, *Gammaproteobacteria*, *Cyanobacteriia*, *Bacteroidia*, and *Acidimicrobiia* were the most abundant groups, accounting for 7.71–74.80%, 11.40–36.80%, 0.95–45.07%, 6.95–19.65%, and 0.09–10.54%, respectively (Figure 4). At lower taxonomic levels, several taxa showed marked variation among sample types. For example, the *Synechococcus* CC9902 lineage, *Agarilytica rhodophyticola*, and *Lutibacter* sp. reads were among the most abundant assignments, with relative abundances ranging from 0.05 to 28.64%, 0.00 to 10.42%, and 0.00 to 9.04%, respectively.

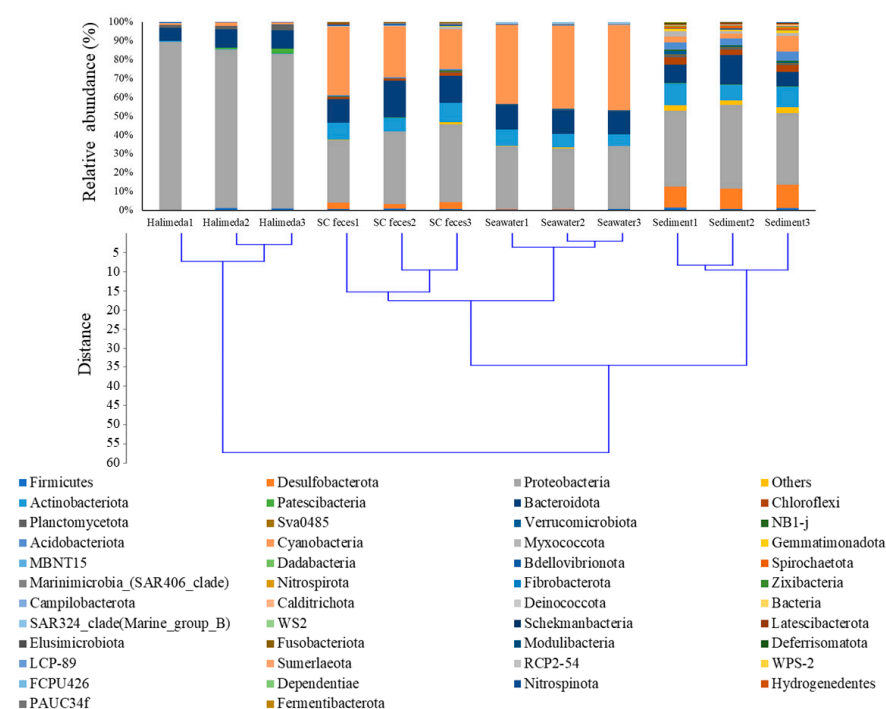


Figure 3. UPGMA dendrogram based on the relative abundance of bacterial phyla across the four microenvironments (*Halimeda* thalli, seawater, sediment, and sea cucumber feces). The dendrogram illustrates the similarity in bacterial community composition among samples based on phylum-level relative abundance. Each colored bar represents a bacterial phylum, and the length of each colored segment corresponds to its relative abundance within each sample.

Taxonomic composition and bacterial community patterns differed among the four sample types, as shown in Figure 3. Hierarchical clustering using the unweighted pair group method with arithmetic mean (UPGMA) at the phylum level separated the samples into two major clusters. The first cluster comprised sediment, seawater, and sea cucumber feces, whereas the second cluster consisted exclusively of the *Halimeda* samples (Figure 3). *Halimeda* thalli were characterized by a high relative abundance of *Proteobacteria* and *Planctomycetota*, accounting for $85.17 \pm 2.18\%$ and $2.08 \pm 0.47\%$ of the community, respectively. The relative abundance of *Proteobacteria* in *Halimeda* was higher than that in ambient seawater, sediment, and sea cucumber feces ($p < 0.05$). Likewise, *Planctomycetota* were significantly more abundant in *Halimeda* than in seawater and sea cucumber feces ($p < 0.05$, Kruskal–Wallis). The seawater samples were characterized by high relative abundances of *Cyanobacteria*, the SAR324 clade (Marine Group B), and *Marinimicrobia* (SAR406 clade),

accounting for $43.63 \pm 0.97\%$, $0.93 \pm 0.04\%$, and $0.42 \pm 0.04\%$, respectively. Cyanobacteria was significantly more abundant in seawater than in *Halimeda*, sea cucumber feces, and sediment ($p < 0.05$). Additionally, the SAR324 clade (Marine Group B) and *Marinimicrobia* (SAR406 clade) were significantly more abundant in seawater than in *Halimeda* and sea cucumber feces ($p < 0.05$). The sediment samples showed elevated abundances of *Desulfobacteria*, *Actinobacteria*, *Chloroflexi*, *Myxococcota*, *Acidobacteriota*, and *Firmicutes*, with relative abundances of $11.38 \pm 0.57\%$, $10.11 \pm 0.95\%$, $3.46 \pm 0.34\%$, $1.92 \pm 0.41\%$, $4.06 \pm 0.40\%$, and $1.13 \pm 0.20\%$, respectively. *Actinobacteria* and *Chloroflexi* were significantly more abundant in sediment than in seawater and *Halimeda* ($p < 0.05$). Additionally, *Acidobacteriota*, *Myxococcota*, and *Desulfobacterota* were significantly more abundant in sediment than in *Halimeda* and seawater ($p > 0.05$). *Firmicutes* were also relatively abundant in sediment, although no significant differences were detected among sample types ($p > 0.05$). Sea cucumber feces were enriched in *Bacteroidota*, *Fusobacteriota*, and *Bdellovibrionota*, with relative abundances of $15.43 \pm 2.17\%$, $0.87 \pm 0.04\%$, and $0.71 \pm 0.05\%$, respectively. *Fusobacteriota* were significantly more abundant in sea cucumber feces than in *Halimeda* and seawater ($p < 0.05$). Similarly, the abundance of *Bdellovibrionota* was higher in feces than in seawater ($p < 0.05$). In contrast, the abundance of *Bacteroidota* did not differ significantly among the sample types (Figure 3). The distribution of the 30 most abundant bacterial classes is shown in a heatmap in Figure 4, where the color gradient from dark blue to dark red indicates increasing relative abundance across samples. The *Halimeda* samples were enriched in *Alphaproteobacteria*, *Phycisphaerae*, *Parcubacteria*, *Phycisphaerae*, *Bacilli*, and *Gracilibacteria*, with relative abundances of $69.06 \pm 3.39\%$, $1.96 \pm 0.45\%$, $1.05 \pm 0.73\%$, $0.74 \pm 0.38\%$, and $0.16 \pm 0.09\%$, respectively. In sea cucumber feces, *Bacteroidia* and *Bdellovibrionia* were more abundant, accounting for $15.37 \pm 2.19\%$ and 0.86 ± 0.04 , respectively. The seawater samples were dominated by *Cyanobacteriia* and the SAR324 clade (Marine group B), with significantly higher relative abundances ($46.63 \pm 0.97\%$ and $0.93 \pm 0.04\%$, respectively) than in the other sample types. In sediment, 19 bacterial classes were relatively abundant; among these, *Gammaproteobacteria*, *Acidimicrobiia*, *Desulfobacteria*, *Anaerolineae*, and *Polyangia* were the most dominant, with relative abundances of $30.88 \pm 2.96\%$, $9.40 \pm 0.82\%$, $4.23 \pm 0.43\%$, $2.42 \pm 0.29\%$, and $1.77 \pm 0.38\%$, respectively (Figure 4). At the genus level, the three most dominant genera in seawater were *Synechococcus* CC9902, *Cyanobium* PCC-6307, and *Candidatus Actinomarina*, with relative abundances of $30.90 \pm 0.35\%$, $7.56 \pm 0.72\%$, and $5.69 \pm 0.93\%$, respectively. In the sediment samples, *Woeseia* and *Ilumatobacter* were the dominant genera, accounting for $5.99 \pm 0.25\%$ and $1.80 \pm 0.04\%$, respectively. Sea cucumber feces were dominated by *Pleurocapsa* PCC-7319, *Lutibacter*, and *Vibrio*, with relative abundances of $7.11 \pm 1.89\%$, $4.69 \pm 2.28\%$, and $3.15 \pm 0.45\%$, respectively. In contrast, *Halimeda* thalli were primarily associated with *Parvularcula*, *Kordiimonas*, *Agarilytica*, *Aestuariibacter*, and *Nisaea*, accounting for $5.70 \pm 1.09\%$, $5.45 \pm 0.44\%$, $5.00 \pm 2.09\%$, $4.68 \pm 0.78\%$, and $3.24 \pm 0.47\%$ of the community, respectively (Supplementary Table S3).

For beta diversity, principal coordinate analysis (PCoA) based on unweighted UniFrac distances showed clear separation of bacterial communities among the four sample types (Figure 5). In addition, PERMANOVA confirmed that the bacterial community composition differed significantly among sample types ($p < 0.05$, Supplementary Table S1). The Venn diagram revealed that 51 bacterial genera (6.95%) were shared across all sample types. Each microenvironment also contained unique bacterial taxa, including 119 genera in sediment (16.26%), 11 genera in *Halimeda* (1.49%), 35 genera in sea cucumber feces (4.77%), and 22 genera in ambient seawater (2.99%). Pairwise overlap in microbial communities also varied among sample types. Ambient seawater and sediment shared 138 genera (18.80%), whereas *Halimeda* and sediment shared 205 genera (27.92%). *Halimeda* and ambient seawater shared 77 genera (10.49%), while *Halimeda* and sea cucumber feces shared 163 genera

(22.20%). Sea cucumber feces and ambient seawater shared 138 genera (18.80%). The greatest overlap was observed between sea cucumber feces and sediment, which shared 347 genera (47.28%) (Figure 6).

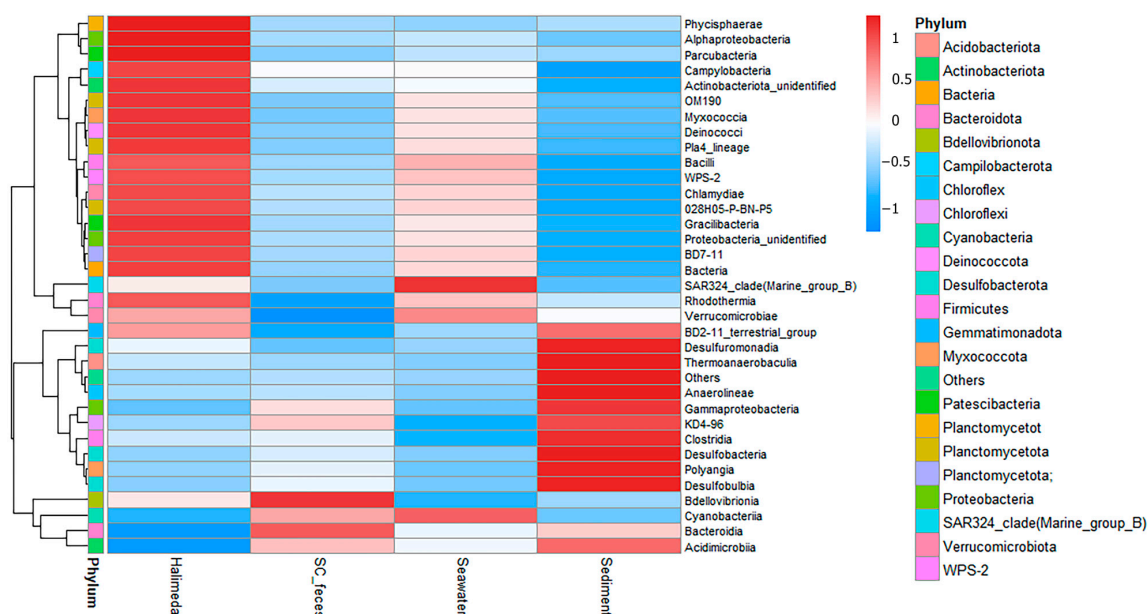


Figure 4. A hierarchically clustered heatmap of the microbial community composition. The heatmap illustrates the relative abundance of various bacterial taxa across all samples, namely, *Halimeda*, sea cucumber feces, seawater, and sediment. The rows represent individual microbial taxa, and the columns correspond to each of the samples. Data were row-scaled into Z-scores for visualization; for each specific taxon across all conditions, red indicates a higher-than-average relative abundance, while blue indicates a lower-than-average abundance. The dendrogram on the left displays the hierarchical clustering of taxa based on the Euclidean distance, grouping microbes that exhibit similar abundance profiles. The adjacent vertical color bar annotates the broader phylum classification for each taxon.

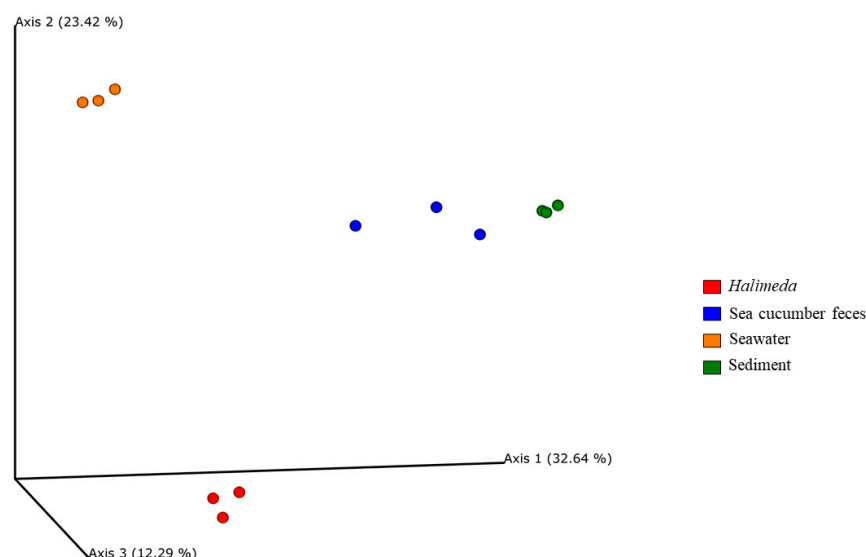


Figure 5. PCoA of bacterial community of all samples was based on an unweighted UniFrac distance matrix. Red, *Halimeda*; blue, sea cucumber feces; orange, seawater; and green, sediment. The bacterial community composition differed significantly among the four microenvironments according to PERMANOVA analysis ($p < 0.05$; Supplementary Table S2).

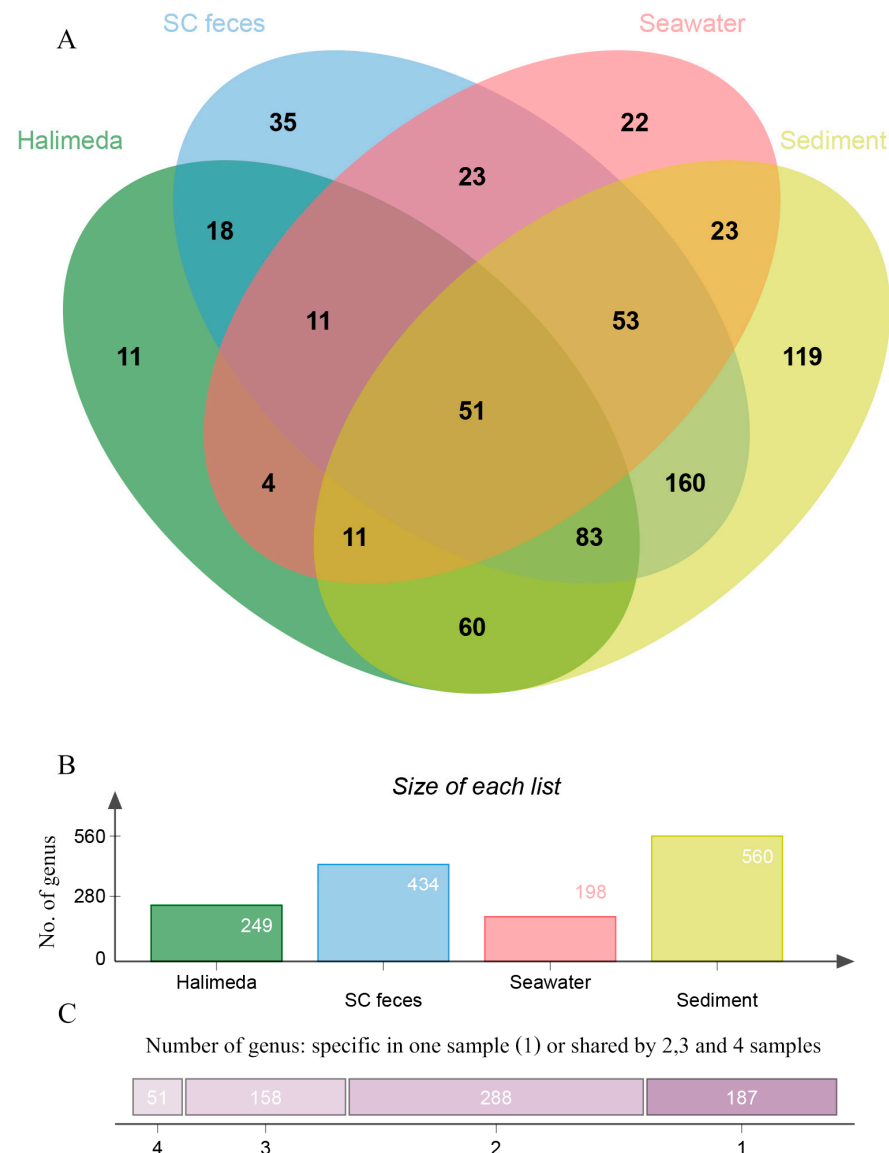


Figure 6. (A) A Venn diagram illustrating the distributions of shared and unique microbial genera across four distinct marine microenvironments: the macroalga *Halimeda*, sea cucumber feces, seawater, and sediment. (B) A bar chart displaying the total number of genera within each individual microenvironment. (C) A summary of the degree to which genera are specific to a single environment or shared across multiple microenvironments. The visualization was generated using the jvenn interactive tool (<https://www.bioinformatics.com.cn/static/others/jvenn/example.html>; accessed on 10 June 2026).

Linear discriminant analysis effect size (LEfSe) was used to identify bacterial taxa that were significantly enriched in each sample type. In sediment, the class *Acidimicrobiia* (phylum *Actinobacteriota*), together with the class *Gammaproteobacteria* (phylum *Proteobacteria*) and the phylum *Desulfobacteriota*, was significantly enriched. The seawater samples were characterized by the enrichment of the family *Cyanobiaceae* (phylum *Cyanobacteria*). In the *Halimeda* samples, several taxa were significantly enriched, including the order *Caulobacteriales* and the families *Rhodobacteraceae* (within the class *Alphaproteobacteria* of the phylum *Proteobacteria*) and *Alteromonadaceae* (within the class *Gammaproteobacteria* of the phylum *Proteobacteria*). In contrast, sea cucumber feces were characterized by the enrichment of *Xenococcaceae* (family within the order *Cyanobacteriales*) and the order *Microtrichales* (class *Actinobacteriota*) (Figure 7).

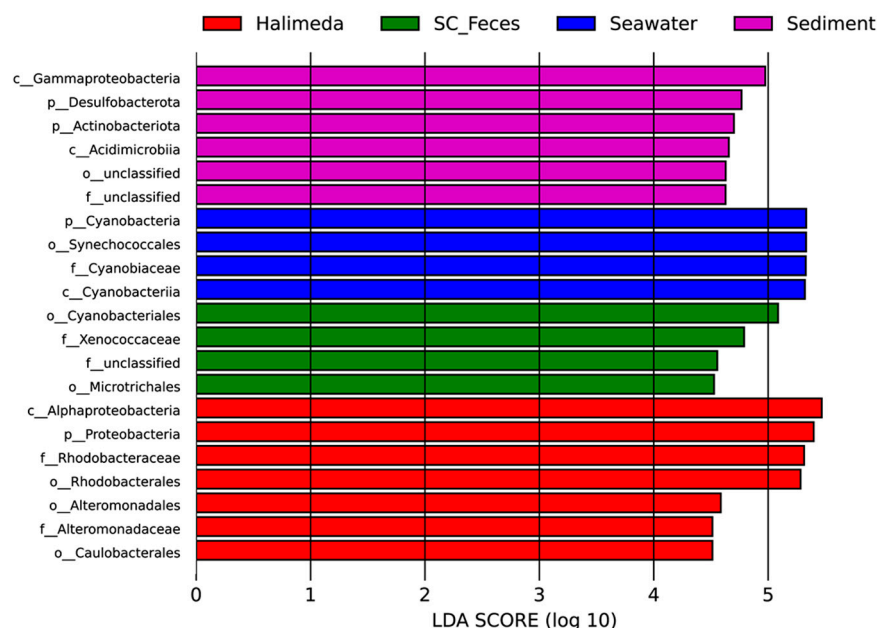


Figure 7. LEfSe analysis showing bacterial taxa that differed significantly among the four sample types. The histogram presents the linear discriminant analysis (LDA) scores of differentially abundant bacterial taxa. Taxa with an LDA score > 4.5 and $p < 0.05$ were considered significantly discriminative.

4. Discussion

This study demonstrated that bacterial communities differed markedly among *Halimeda macroloba* thalli, seawater, sediment, and *Holothuria atra* feces, indicating strong habitat-specific microbial assembly within a tropical *Halimeda*-dominated ecosystem. The pronounced differentiation among these benthic microenvironments suggests that the bacterial community structure is shaped by a combination of environmental filtering, substrate characteristics, resource availability, and host-associated biological processes. Similar patterns of microbial differentiation among microenvironments have been reported in other tropical benthic ecosystems, including macroalgal habitats, seagrass meadows, coral reefs, and mangrove forests, where distinct physicochemical conditions and host-specific traits promote the establishment of specialized microbial assemblages [30,39–44]. Our findings extend this concept to *Halimeda* habitats and suggest that interactions among macroalgal surfaces, sediments, seawater, and benthic detritivores contribute to the development of ecologically distinct yet interconnected bacterial communities. These results highlight the potential importance of microbial niche partitioning in maintaining ecosystem functioning within tropical macroalgal habitats.

The bacterial community associated with *Halimeda* thalli was markedly different from that associated with the other microenvironments, accounting for more than 85% of the total bacterial community. Proteobacteria was the dominant bacterial phylum, with *Alphaproteobacteria* showing a significantly higher relative abundance on *Halimeda* thalli than in the other microenvironments, a result similar to that of Kuba et al. [30,41]. This may be because the surface of *Halimeda* can serve as a specialized niche, selecting for specific taxa. This pattern is consistent with that in previous studies reporting that this group is a common and often dominant component of the bacterial community of photosynthetic marine organisms, particularly marine macroalgae [45–47], especially *Halimeda*, which show a high abundance of *Alphaproteobacteria* [30,41,48]. LEfSe analysis revealed that the families *Caulobacteriales* and *Rhodobacteraceae* were significantly more abundant in the *Halimeda* phycosphere than in the surrounding microenvironment. These two groups have been reported as core macroalgal surface-associated bacteria [48–50]. They perform a range

of beneficial functions for their host, including cellulose decomposition, stabilization of the surface bacterial community, carbon and sulfur cycling, and synthesis of vitamins, all of which may enhance macroalgal growth, photosynthesis, health, pathogen resistance, and environmental adaptation [51–53]. The occurrence of *Alteromonadaceae* on *Halimeda* thalli may likewise reflect a close association between this bacterial family and marine macrophytes, as previously reported [54]. This bacterial group was highly abundant on the surface of *Halimeda*, likely because carbon compounds released by the macroalga can stimulate chemotaxis and support the growth of bacteria possessing genes involved in carbon utilization [54]. Furthermore, this bacterial group can form biofilms that may protect macroalgae from the settlement of algal spores, pathogenic bacteria, fungi, and invertebrate fouling organisms [55,56]. At the genus level, *Parvularcula*, *Kordiimonas*, *Agarilytica*, *Nisaea*, and *Aestuuriibacter* were dominant on *Halimeda*. *Parvularcula* and *Kordiimonas* have been described as aerobic bacteria commonly associated with marine environments [57–60], whereas *Aestuuriibacter* and *Nisaea* have previously been reported in seawater and sediment and may contribute to nutrient cycling, including nitrogen [61,62] and carbon cycling [63], and other ecological processes in coastal habitats [64–68]. *Agarilytica* has been reported in association with macrophytes [60]. One possible explanation for this is that the macroalgal surface is rich in polysaccharides, such as cellulose and xylans, which can serve as substrates for bacteria harboring genes involved in algal polysaccharide degradation [69]. The phylum *Planctomycetota* also showed the highest relative abundance on *Halimeda* thalli, consistent with previous studies reporting members of this phylum as common constituents of macroalgal surface biofilms [70,71]. Several macroalgae-associated *Planctomycetota* genera, including *Algisphaera* and *Rhodopirellula*, have been reported to possess sulfatases and other carbohydrate-active enzymes involved in the degradation of sulfated polysaccharides derived from algal tissues [72–74]. Furthermore, some *Planctomycetota* lineages have been implicated in carbon and nitrogen cycling in marine ecosystems [31,33]. These findings suggest that the enrichment of *Planctomycetota* on *Halimeda* thalli may be linked to the utilization and turnover of algal-derived organic matter. However, such functional roles should be considered tentative because they cannot be directly inferred from the 16S rRNA gene sequencing data generated in this study. This interpretation is supported by the UPGMA clustering results, which clearly separated the *Halimeda* samples from the other habitat microenvironment samples. In addition, alpha diversity was relatively low in the *Halimeda* samples, suggesting that *Halimeda* may structure its bacterial community in a manner similar to other host-associated systems. This pattern may reflect the influence of chemical compounds on the algal surface, which create a selective niche for particular microbial groups [75,76].

Seawater harbored a distinct bacterial community compared with the other sample types. At the genus level, *Synechococcus* CC9902, *Cyanobium* PCC-6307, and *Candidatus Actinomarina* were among the dominant taxa. The findings of the high relative abundance of *Synechococcus* CC9902 and *Cyanobium* PCC-6307, members of the class *Cyanobacteriia*, are consistent with those of previous reports showing that this taxon is widely distributed throughout seawater columns worldwide because of its ability to adapt to a broad range of temperature, salinity, and nutrient conditions [77–79]. *Synechococcus* plays a crucial role as a primary producer and contributes substantially to marine biogeochemical cycles [32,80]. *Candidatus Actinomarina* is also a typical pelagic bacterium. *Candidatus Actinomarina* is commonly distributed in the photic zone and oligotrophic seawater [81,82]. Together, these patterns may suggest that the seawater column within the *Halimeda* meadow is well lit and relatively low in nutrients. The SAR324 clade (Marine Group B) and *Marinimicrobia* (SAR406 clade) were significantly more abundant in seawater than in the other sample types. Both groups have frequently been reported in oxygen minimum zone environments [83,84].

Their presence in the present study may indicate intense microbial respiration within *Halimeda* patches, including in the sediment and on macroalgal surfaces. LEfSe analysis further identified *Cyanobiaceae* (a member of the order *Synechococcales*, class *Cyanobacteriia*) as a taxon enriched in seawater. *Cyanobiaceae* has been reported to be widely distributed across natural ecosystems [80,85]. This bacterial group is also considered a key contributor to nitrogen fixation in marine ecosystems [86]. Species diversity in seawater was the lowest among all sample types. This result is consistent with that of Mancuso et al. [87], who reported lower microbial diversity in seawater than in algal-associated habitats, which tend to be more stable over time. One possible explanation for this is that the shallow water column is frequently mixed, resulting in relatively homogeneous physical and chemical conditions. Such homogeneity may favor bacterial groups with broad environmental tolerance and a strong adaptive capacity [88,89]. The low overlap in bacterial genera between *Halimeda* and seawater further suggests that the algal surface does not allow colonization by all bacteria present in the water column but instead selectively recruits particular taxa through surface-associated chemical properties [75,90].

Sediment harbored the highest microbial diversity among the four microenvironments. The heterogeneity of the sediment structure and chemical properties likely creates a wide range of microhabitats [4,91]. In particular, the vertical gradient within the upper few millimeters of sediment, from the surface to deeper layers, generates oxic, suboxic, and anoxic conditions that shape distinct bacterial communities associated with each microenvironment [4,92]. The bacterial community in the sediment samples reflected this geochemical complexity, with *Desulfobacterota*, *Actinobacteriota*, *Chloroflexi*, and *Acidobacteriota* being the dominant phyla and *Gammaproteobacteria*, *Acidimicrobiia*, and *Desulfobacteria* being the dominant classes. *Gammaproteobacteria* (a member of the phylum *Proteobacteria*) and *Acidimicrobiia* (a member of the phylum *Actinobacteriota*) have been frequently reported as dominant bacterial classes in marine sediments [43,93,94], and both groups play crucial roles in sediment biogeochemistry, including carbon, nitrogen, and sulfur cycling [94–96]. *Desulfobacteria* (a member of the phylum *Desulfobacterota*) have been reported at high abundance in organic-rich sediments, where sulfate in pore water serves as an essential electron acceptor for anaerobic respiration during organic matter degradation [97]. Sediments beneath *Halimeda* meadows may contain elevated levels of organic matter due to the high productivity and rapid turnover of *Halimeda* [20]. *Acidobacteriota* and *Chloroflexi* were also detected at relatively high abundance in the sediment samples. These groups have frequently been reported in organic-rich and anoxic sediments [98], suggesting that *Halimeda* patches may contribute substantial organic inputs to the underlying sediment. At the genus level, *Woeseia*, a member of *Gammaproteobacteria*, was among the most abundant taxa in sediment. This genus is commonly found in seafloor sediments [99,100] and exhibits considerable metabolic plasticity, enabling it to inhabit sediments across a broad redox gradient [101]. *Ilumatobacter* (a member of *Acidimicrobiia*) is a bacterial group that is present in high abundance in marine sediments and plays important roles in organic matter turnover, biogeochemical cycling, and sulfide detoxification [94,102]. LEfSe analysis confirmed that *Acidimicrobiia* (a member of the phylum *Actinobacteriota*), *Gammaproteobacteria*, and *Desulfobacterota* were the principal biomarkers for the sediment samples from *Halimeda* patches. These findings indicate that sediments beneath *Halimeda* patches harbor diverse bacterial communities with potential roles in organic matter remineralization and biogeochemical cycling, suggesting their importance in sediment ecosystem functioning.

The bacterial community of *Holothuria atra* feces exhibited an intermediate level of microbial diversity, being higher than that of seawater and *Halimeda* thalli but lower than that of sediment. A high overlap in bacterial genera between *H. atra* feces and sediment was expected, as sea cucumbers feed directly on sediment and therefore ingest sediment-

associated microorganisms into their digestive tract. However, once sediment passes through the gut, the bacterial community appears to be modified by gut physicochemical conditions, resulting in slightly lower diversity than that of the original sediment [103]. The enrichment of *Bacteroidota*, *Fusobacteriota*, and *Bdellovibrionota* in feces relative to that in seawater and *Halimeda* is particularly noteworthy. *Bacteroidota* and *Fusobacteriota* are commonly associated with the anaerobic fermentation of organic particles and are frequently found in animal gut environments, especially in the anterior intestine of sea cucumbers [104]. The hypoxic conditions within the sea cucumber gut may therefore provide a favorable environment for members of this phylum [104]. *Bdellovibrionota*, which are predatory bacteria that consume Gram-negative bacteria, were also relatively abundant in fecal samples. Their enrichment may indicate a high abundance of potential prey bacteria in the sea cucumber gut and active bacterial predation during gut transit [5,105]. At the class level, *Bacteroidia* was significantly enriched in feces. This group is commonly reported at high abundance in sea cucumber guts and is known to play an important role in the degradation of complex carbohydrates [106,107]. Because the sea cucumbers in this study inhabited dense *Halimeda* patches, where detrital material is likely rich in polysaccharides, the elevated abundance of *Bacteroidia* may reflect the digestion of polysaccharide-rich organic matter during sediment passage through the gut. *Pleurocapsa* PCC-7319 (family *Xenococcaceae*), *Lutibacter* (order *Flavobacteriales*), and *Vibrio* were identified as the dominant taxa in the sea cucumber fecal samples. The high relative abundances of *Pleurocapsa* PCC-7319 and *Lutibacter* in feces may reflect the capacity of these sediment-associated bacteria to survive and persist through gut transit conditions [108,109]. These findings are consistent with those of Yamazaki et al. [110], who reported that sea cucumber feces are enriched in *Flavobacteriales*—the order to which *Lutibacter* belongs—and suggested that fecal deposition contributes to the surrounding sediment bacterial community, potentially stimulating the mineralization of sedimentary organic matter [22,110]. The elevated abundance of *Vibrio* in excreted feces likely originates from the sea cucumber digestive tract, where this genus is commonly detected as a resident gut inhabitant [104,111]. *Vibrio* has been reported to play a probiotic role in sea cucumbers by enhancing the degradation of proteins and lipids [112,113]. Nevertheless, certain *Vibrio* species are recognized as opportunistic pathogens of sea cucumbers and other marine invertebrates, and their dispersal via fecal deposition may pose ecological risks to the surrounding benthic environment [114,115]. LEfSe analysis further identified *Microtrichales* (a member of the phylum *Actinobacteriota*) and *Xenococcaceae* (a member of the phylum *Cyanobacteria*) as differentially abundant taxa. *Microtrichales* has been reported in association with marine sediments [116], whereas *Xenococcaceae* has been found attached to macroalgal surfaces [117,118]. These two bacterial groups may be ingested by *H. atra* through feeding activity on sediment and macroalgae, subsequently passing through the gut where they may contribute to organic matter mineralization and carbon cycling in benthic ecosystems [116,118]. Collectively, these findings suggest that *H. atra* may play an important role in restructuring sediment-associated microbial communities [22,119,120]. This process may benefit *Halimeda* ecosystems by enhancing nutrient cycling, supporting more complete organic matter mineralization, and promoting nutrient regeneration.

According to the analysis of shared genera among the four sample types, only 60 genera (10.36%) were common across all compartments. This limited overlap suggests strong compartment-specific structuring of the bacterial communities. Such patterns may be influenced by differences in habitat characteristics among sediment, seawater, *Halimeda* thalli, and sea cucumber feces, including factors such as redox conditions, substrate properties, nutrient availability, and host-associated processes. However, these potential drivers were not measured in this study and should be investigated in future research. A comparison of genus-level overlap among sample types revealed the greatest overlap

between feces and sediment (47.28%), supporting a strong connection between these two microenvironments through the non-selective deposit-feeding strategy of holothurians via sediment ingestion, whereby material is processed and redeposited [110]. This situation may indicate that sea cucumber plays a key role as an active mediator of benthic microbial recycling, linking sediment and consumer compartments within the biogeochemical cycling of the *Halimeda*-dominated habitat [110]. In contrast, *Halimeda* and sediment shared 27.92% of genera, which may reflect the deposition of senescent *Halimeda* fragments into the sediment, as well as the attachment of suspended sediment particles to the surface of living thalli. Feces and *Halimeda* shared 22.20% of genera, suggesting that sea cucumber foraging on *Halimeda*-derived detritus may create microbial connectivity between the algal surface and the benthic fecal niche. The lowest overlap was observed between seawater and *Halimeda* (10.46%), supporting the idea of host-specific microbial selection on the *Halimeda* surface [121]. Taken together, these patterns suggest that microbial communities in *Halimeda* patches are not isolated but rather connected through the movement of organic matter and biological activity within the benthic habitat. By ingesting sediment and depositing feces beneath *Halimeda* patches, sea cucumbers may function as biological vectors linking otherwise distinct microbial niches. In this way, *Holothuria atra* may facilitate the dispersal of bacteria among microenvironments, particularly within the benthic community. Bioturbation resulting from the deposit-feeding behavior of sea cucumbers can disturb and restructure sediment, potentially influencing the distribution of the resident bacterial community [110]. As sea cucumbers feed, move, and excrete at different locations, sediment particles and their associated microbiota are relocated and redistributed, potentially establishing a mobile link between the sediment and *Halimeda*-associated microbial pools within the benthic community. Moreover, sea cucumber feces serve as a nutrient-enriched substrate that promotes bacterial growth and colonization at the deposition site [107]. This process may, in turn, drive bacterial community assembly and influence nutrient cycling within the habitat [22,110,122].

Synthesizing bacterial community patterns across the four microenvironments revealed strong habitat-specific community structuring within the *Halimeda*-dominated ecosystem. Seawater communities were characterized by relatively high abundances of picocyanobacteria and oligotrophic heterotrophs, whereas sediment communities were dominated by taxa commonly associated with organic matter degradation and anaerobic metabolism. In contrast, *Halimeda* thalli harbored distinct host-associated bacterial assemblages, while sea cucumber feces supported bacterial communities that differed from those in the surrounding sediment and seawater. These patterns suggest that each microenvironment provides unique ecological niches that select for different bacterial taxa. Although the functional roles of these communities were not directly assessed, the observed taxonomic differentiation indicates the potential for distinct microbial contributions to ecological processes within *Halimeda* patches. Further studies incorporating environmental measurements and functional analyses are needed to clarify the ecological roles and interactions of microbial communities in *Halimeda*-dominated ecosystems.

The results of this study demonstrate that each microenvironment harbors unique functional bacteria that play important roles within the *Halimeda*-dominated habitat, implying that the loss of any single component could influence bacterial community structure and associated biogeochemical functions. In particular, sea cucumbers play a key role in shaping sediment-associated bacterial communities through feeding activity and feces deposition. Given this key ecological role, the conservation of sea cucumbers should be prioritized. These findings support the use of microbiome information as an indicator for monitoring and management frameworks in tropical *Halimeda*-dominated coastal ecosystems and potentially in other marine ecosystems. Such information could also inform

marine protected area (MPA) zoning and restoration efforts aimed at preserving microbial connectivity among microenvironments, habitats, and overall ecosystem resilience.

However, several limitations of this study should be acknowledged. First, 16S rRNA amplicon sequencing targeting the V3–V4 hypervariable region was used to characterize the bacterial community composition based on the SILVA reference database. Although this approach provides valuable taxonomic information, it does not directly reflect the functional potential or metabolic activity of microbial communities in each sample type. Future studies should therefore incorporate shotgun metagenomics and metatranscriptomics to better resolve functional gene profiles and actively expressed metabolic pathways, thereby providing deeper insight into the mechanisms underlying bacterial community structure and function. Second, only three biological replicates were collected for each microenvironment, which may not fully capture the spatial heterogeneity of the bacterial communities within *Halimeda*-dominated habitats. Although clear differences among sample types were detected and replicate samples showed generally consistent community patterns, the limited replication reduces statistical power and may underestimate within-habitat variability. Therefore, the results should be interpreted as an initial characterization of bacterial communities associated with *Halimeda* surfaces, seawater, sediment, and sea cucumber feces. Third, this study represents only a single spatial and temporal snapshot. Microbial communities have been reported to vary markedly across seasons and geographic locations [123,124]. Consequently, the observed community patterns may not fully represent temporal or spatial variability within *Halimeda*-dominated ecosystems. Future studies incorporating larger sample sizes, multiple locations, environmental measurements, and seasonal sampling would provide a more comprehensive understanding of microbial dynamics and ecological processes in these habitats. Finally, dedicated PCR-negative controls, extraction blanks, and positive mock community controls were not included in this study. As a result, potential low-level reagent or laboratory contamination could not be directly identified or removed using control samples. However, the clear separation of microbial communities among sample types and the dominance of ecologically relevant taxa suggest that the major community patterns observed in this study were primarily associated with habitat-specific differences rather than contamination.

5. Conclusions

This study demonstrated clear differences in the bacterial community structure among ambient seawater, sediment, *Halimeda* thalli, and *Holothuria atra* feces within a tropical *Halimeda*-dominated benthic habitat. Sediment showed the highest microbial diversity, whereas seawater showed the lowest. Distinct bacterial assemblages characterized each microenvironment, with macroalgal-associated taxa dominating *Halimeda* thalli, remineralization-related taxa dominating sediment, *Synechococcus* CC9902 dominating seawater, and fecal samples enriched in taxa associated with gut processing and microbial restructuring. Limited overlap among microenvironments, together with the strong similarity between feces and sediment communities, indicates a close microbial relationship between these habitats and suggests that *H. atra* may influence bacterial community composition within the benthic environment. These findings improve our understanding of habitat-specific microbial assemblages and ecosystem functioning within *Halimeda* patches. The high proportion of bacterial overlap between *H. atra* feces and sediments suggests that sediment processing by sea cucumbers may influence benthic bacterial community dynamics. Such insights contribute to our understanding of microbial ecology in tropical coastal ecosystems and may support coastal management and blue carbon habitat conservation. Future studies using shotgun metagenomics and metatranscriptomics are needed to clarify the functional roles and activities of these microbial communities under natural conditions. Additionally,

carbon connectivity across these four microenvironments should be investigated using stable isotope probing (SIP) to observe the flow through the loop of *Halimeda*–sediment–sea cucumber–feces.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ecologies7030069/s1>, Table S1: Sample-wise sequencing and DADA2 processing statistics; Table S2: Results of permutational multivariate analysis of variance (PERMANOVA) testing for differences in the bacterial community composition among the four microenvironments; Table S3: Relative abundance (%) of the top 30 bacterial genera detected across the four microenvironments.

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