RESEARCH ARTICLE

Marine sponges maintain stable bacterial communities between reef sites with different coral to algae cover ratios

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One sentence summary: Analysis of four different sponge holobionts reveals that their associated bacterial communities are host-specific and remain stable across habitats with different coral to algae cover ratios.

ABSTRACT

Marine sponges play a major ecological role in recycling resources on coral reef ecosystems. The cycling of resources may largely depend on the stability of the host–microbiome interactions and their susceptibility to altered environmental conditions. Given the current coral to algal phase shift on coral reefs, we investigated whether the sponge-associated bacterial communities of four sponge species, with either high or low microbial abundances (HMA and LMA), remain stable at two reefs sites with different coral to algae cover ratios. Additionally, we assessed the bacterial community composition of two of these sponge species before and after a reciprocal transplantation experiment between the sites. An overall stable bacterial community composition was maintained across the two sites in all sponge species, with a high degree of host-specificity. Furthermore, the core bacterial communities of the sponges remained stable also after a 21-day transplantation period, although a minor shift was observed in less abundant taxa (< 1%). Our findings support the conclusion that host identity and HMA–LMA status are stronger traits in shaping bacterial community composition than habitat. Nevertheless, long-term microbial monitoring of sponges along with benthic biomass and water quality assessments are needed for identifying ecosystem tolerance ranges and tipping points in ongoing coral reef phase shifts.

INTRODUCTION

Coral reefs are among the most productive ecosystems on Earth while residing under oligotrophic conditions owing to the efficient cycling of nutrients (Done *et al.* 1996). On coral reefs, benthic communities are fueled by primary producers, such as macroalgae and dinoflagellates in corals, which in turn release a significant part (up to 50%) of their photosynthetic "over" production as dissolved organic matter (DOM; Wild *et al.* 2004; Naumann *et al.* 2010; Tanaka *et al.* 2011; Hansell and Carlson 2015). Sponges are important nutrient cyclers within coral reef benthic communities (Diaz and Rützler 2001; de Goeij *et al.* 2013; Pawlik and McMurray 2020). Through their high filtering capacity and their close association with diverse symbiotic microorganisms, they play a key role in the uptake, processing and release of (in)organic nutrients within their ecosystems (Maldonado *et al.* 2012; de Goeij *et al.* 2017; Pita *et al.* 2018; Zhang *et al.* 2019). Specifically, the processing of DOM by sponges puts them into a unique position with regard to the cycling of resources within oligotrophic ecosystems. Sponges can turnover DOM—by far the largest potential organic resource on reefs, but unavailable as food source to most other reef heterotrophs—at rates close to the daily gross primary production rates of an entire reef (de Goeij *et al.* 2013).

Over the past decades, climatic events in combination with direct anthropogenic disturbances have resulted in considerable changes in benthic community structures worldwide, including a shift from coral to algal dominance on many coral reefs (Hughes 1994; McManus and Polsenberg 2004; Hoegh-Guldberg *et al.* 2007; Mumby and Steneck 2008; Conversi *et al.* 2015). Filamentous turf- and macroalgae are found to release higher quantities of bioavailable DOM than corals, which causes higher ambient bacterioplankton production, including the growth of potential pathogens, described as the microbialization of coral reefs (Haas *et al.* 2011, 2016; ; Morrow *et al.* 2013; Nelson *et al.* 2013; Cárdenas *et al.* 2018). Increased microbial respiration and pathogenic mechanisms at the coral–algae interface can weaken or even kill corals, which frees space for further algal growth, especially under warmer conditions (Kline *et al.* 2006; Smith *et al.* 2006; Rohwer *et al.* 2010; Silva *et al.* 2021). This mechanism, referred to as the DDAM (dissolved organic carbon, disease, algae and microorganisms) negative feedback loop, can catalyze reef destruction, such as dominance shifts from reef-building coral to non-reef-building fleshy algae (Dinsdale and Rohwer 2011; Barott and Rohwer 2012).

An increase of sponge biomass due to changing nutrients conditions and the subsequent increased release of inorganic nutrients have both been hypothesized to further exacerbate algal growth at the expenses of corals (Bell *et al.* 2013; Pawlik *et al.* 2016; Lesser and Slattery 2020). However, experimental and field data to corroborate these hypotheses are very limited. *Ex-situ* experiments suggest that sponges may cope better than other reef organisms with the effects of future higher temperatures and lower seawater pH (Fang *et al.* 2013; Bell *et al.* 2018), but expected changes in biomass and composition have, to our knowledge, not been reported for sponges at the ecosystem level. A total of two *ex-situ* studies have observed differential processing of naturally sourced coral and macroalgal-DOM by sponges, showing that macroalgal-DOM is more rapidly assimilated and recycled by the sponge holobiont, which suggests that

macroalgae release a more labile food source for sponges than corals (Rix *et al.* 2017; Campana *et al.* 2021a). However, little is known about the role of the sponge microbial symbionts in the differential recycling of coral- and macroalgal-DOM by sponges.

Recent nano-scale secondary ion mass spectrometry (NanoSIMS) studies have tremendously boosted our understanding of host versus symbiont processing of DOM within sponge holobionts. Both sponge cells and symbionts are found to rapidly take up carbon and nitrogen derived from DOM (Achlatis *et al.* 2019; Rix *et al.* 2020; Hudspith *et al.* 2021a) and were found to translocate these nutrients from sponge host to the microbial symbionts (Hudspith *et al.* 2021a). Moreover, sponges that host high microbial abundances (HMA) within their tissue were shown to rely much more on symbiont-processing of DOM (∼ 60% of total) than low microbial abundance (LMA) species (< 0.7% of total; Rix *et al.* 2020; Hudspith *et al.* 2021b). In HMA sponges, the associated bacteria can constitute up to 40% of their biomass and have a strong sponge-specific community composition, while LMA sponges generally possess a bacterial community more similar to that of the ambient seawater in abundance and composition (Taylor *et al.* 2007; Easson and Thacker 2014; Gantt *et al.* 2019). Seawater bacterioplankton adopts different metabolic strategies when exposed to coral-versus algal-DOM and its community composition becomes dominated by *Alphaproteobacteria* with coral-DOM, while *Bacteroidetes* and *Gammaproteobacteria* become more abundant with algal-DOM (Nelson *et al.* 2013; Haas *et al.* 2016). These bacterial lineages are also found in association with sponges, but it is unknown whether similar, or any, shifts in sponges-associated bacterial communities are found when exposed to different relative contributions of ambient coral and algae communities.

We compared if the sponge-associated bacterial communities change when exposed to different relative contributions of corals and macroalgae. Therefore, we sampled four different sponge species (representing different morphology, phylogeny and abundances of associated microbes) *in situ* at two reef sites that were characterized by different benthic communities (i.e., a site with high-coral/low-macroalgal relative cover and *vice versa*) on the Caribbean reefs of Curaçao. Additionally, we performed a reciprocal transplantation experiment of two sponge species between the two reef sites to assess sponge-associated bacterial community composition changes after three weeks of reallocation.

MATERIAL AND METHODS

Study sites and benthic community composition

The study was performed on two fringing coral reef sites on the leeward side of the Caribbean Island Curaçao, between June and August 2018. The two reef sites were selected based on their observed difference in relative projected reef surface cover of scleractinian corals and macroalgae. The reef in front of Playa Wachi (Site 1; Snake Bay; 12°08'20.9" N 68°59'54.0" W) was selected as the relative high-coral-to-low-algae site and the reef in front of Dive Wederfoort (Site 2; Boca Sami; 12°08'49.7" N 69°00'01.0" W) was selected as the relative low-coral-to-high-algae site (Fig. 1, from here on referred to as "high-coral-low-algae" and "low-coral-high-algae" for readability). The benthic cover was estimated at both sites using the software program
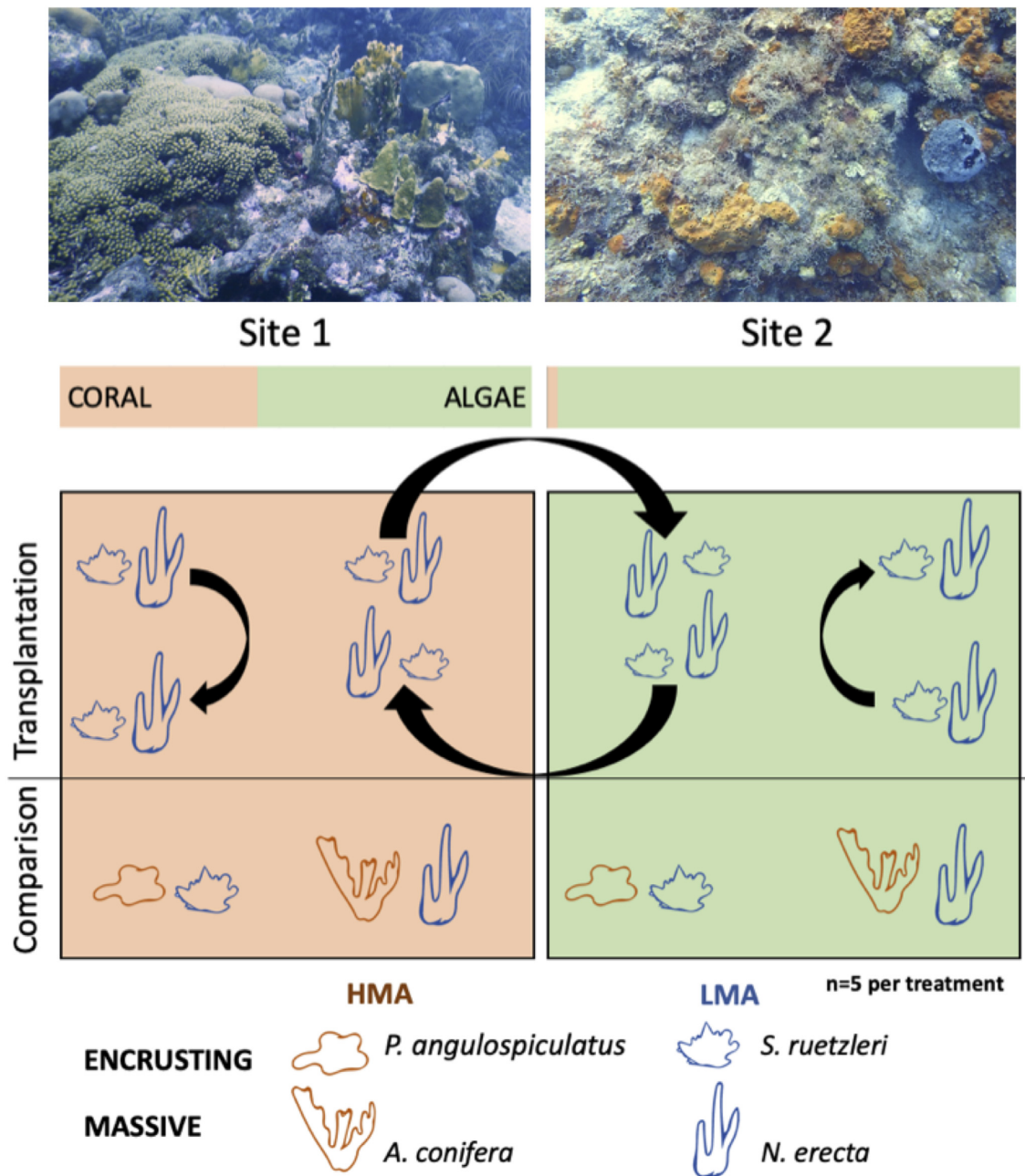
Figure 1. Experimental setup of the site comparison and the reciprocal transplantation experiments and *in-situ* aspects of the sampling sites. Site 1 was characterized by relatively high coral and low algae cover and Site 2 by relatively low coral and high algae cover. The selected sponges were two encrusting species *Plakortis angulospiculatus* (high microbial abundance; HMA) and *Scopalina ruetzleri* (low microbial abundance; LMA) and two massive species *Agelas conifera* (HMA) and *Niphates erecta* (LMA). In the comparison experiment five specimens of each sponge species were sampled at each site. In the reciprocal transplantation experiment five specimens of *N. erecta* and *S. ruetzleri* were transplanted within the same site and five specimens were transplanted between the two sites.

Coral Point Count with Excel extensions (CPCe, v 4.1; Kohler and Gill 2006). A total of 10 reef benthos areas of 1×1 m were chosen haphazardly per site along a stretch of 30 m reef between 12 and 20 m depth and the photos were taken from 2 m above the substrate. Each photo was analysed by determining the benthos under 100 random stratified points. A total of four main groups of benthic communities were assessed: scleractinian corals, sponges (encrusting and massive), macroalgae (*Dictyota* spp. and *Lobophora* spp.) and "other" (all remaining benthos). The differences between the benthic community composition at Site 1 and Site 2 were assessed with Mann–Whitney–Wilcoxon tests (data not normally distributed).

Site comparison experiment

A site comparison experiment was performed to assess baseline differences in community composition of both seawater and sponge-associated bacteria between the two sites (Fig. 1). Tissue samples (± 1 cm^3, containing both pinacoderm and mesohyl from $n = 5$ individuals) of four abundant sponge species

were sampled from both sites between 12 and 20 m water depth: two HMA species *Plakortis angulospiculatus* (*Homosclero-morpha*; encrusting lobate/ficiform) and *Agelas conifera* (*Demospongiae*; massive branching) and two LMA species *Scopalina ruetzleri* (*Demospongiae*; encrusting conulose) and *Niphates erecta* (*Demospongiae*; massive tubular/branching; Figure S1, Supporting Information). Additionally, during the same dive, an ambient seawater sample was collected at each site at 15 m depth using a 20 L collapsible low-density polyethylene carboy (Haas *et al.* 2014) to analyze the bacterial community in the seawater. All samples were stored in the dark on ice until further processing within 1 h. Upon returning to the laboratory, the tissue samples were rinsed with filtered seawater (FSW; pore-size: 0.7 μm) and subsequently with sterile ultra-pure (18.2 MΩ-cm type I) water. The tissue was cut into pieces of equal size using sterilized scalpels, cleared from any foreign tissue/substrate and stored at -80°C until DNA extraction. The seawater sample was filtered over three Sterivex filters (GP 0.22 μm; approximately 3 L per Sterivex) using a peristaltic pump. The Sterivex filters were stored at -80°C until DNA extraction.

Reciprocal transplantation experiments

In addition to the site comparison experiment, a reciprocal transplantation experiment was conducted between the sites to determine whether the sponge bacterial community change during a 21-d exposure to environments characterized by different benthic communities (Fig. 1). For the reciprocal transplantation experiment only the two LMA sponges, *Scopalina ruetzleri* and *Niphates erecta,* were used as they were abundant enough at both research sites and were small enough to transplant whole individuals. The individuals were chosen so that they could be removed from the reef with their substrate still attached and without damaging the sponge tissue. A total of 20 individuals (10 per species) were collected per site, of which five individuals per species were placed back within the same site (coded as transplantation within site) and the remaining five individuals of each species were transplanted to the other site (coded as transplantation between site). In order to reduce artefacts that could influence the bacterial community within and around the sponge, wounding or cutting of the sponge individuals was avoided, as well as the use of artificial materials to secure the sponges onto the reef. After 21 days, the transplanted sponges were sampled following the same procedure as described above. A total of six out of the 40 individuals (but leaving at least $n = 3$ per species per treatment) could not be recovered at the end of the transplantation experiment due to disappearance/ displacement.

Water quality assessment: bacterioplankton abundance, organic and inorganic nutrient concentrations

During the entire duration of the experiments, weekly water samples were collected at both sites with acid-washed (0.4 mol/L HCl) 100 mL polycarbonate syringes to analyze the seawater bacterioplankton abundance, dissolved organic carbon and nitrogen (DOC and DON), and inorganic nutrient (ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate PO_4^{3-}) concentrations. The differences between the seawater quality measurements at Site 1 and 2 were tested with paired two-sample t-tests.

For bacterioplankton abundance, 10 mL of the sample water was fixed with 0.57 mL 37% (v/v) formaldehyde solution (final concentration $\sim$ 2% v/v) in the dark at 4°C for 2 h, and subsequently filtered on 0.2 μm polycarbonate filters (25 mm, Nuclepore Track-Etch; Whatman, Kent, UK) with a 0.45 μm cellulose nitrate support filters (25 mm; Sartorius Stedim Biotech GmbH, Goettingen, Germany). The polycarbonate filters were air-dried, stained with 100 μL of 2 x SYBR Gold solution (Invitrogen, Thermo Fisher Scientific, Waltham, MA), mounted on a microscopy slide and stored at -20°C in the dark until further analysis. Per slide, 10 fields were counted or up to a minimum of 200 bacteria using an epifluorescence microscope (1250x) and recalculated per L seawater.

Duplicate 8 mL inorganic nutrient samples were filtered through a 0.2 μm Fisherbrand Sterile PES Syringe Filter, collected in HDPE vials (Midi-Vial, PerkinElmer; Waltham, MA) and stored frozen at -20°C until further analysis. The concentrations of dissolved inorganic nutrients were measured with an automated Wet Chemistry Analyzer (SAN^{++}, Skalar Analytical, Breda, NL). Nitrate concentrations were derived as: $[NO_3^-] = [NO_x^-] - [NO_2^-]$.

Prior to DOC sample filtration, the filter setup was washed consecutively with 20 mL of 0.4 mol/L HCl, 20 mL of Milli-Q water and 20 mL of sample water. Duplicate 20 mL samples for DOC and total dissolved nitrogen (TDN) were gently (max 10 kPa pressure) filtered through a pre-combusted (4 h at 450°C) 0.7 μm GF/F glass microfiber filter (25 mm, Nuclepore Track-Etch), collected in pre-combusted amber glass vials (22 mL, Agilent Technologies, Santa Clara, CA) and acidified by adding seven drops of fuming 37% (v/v) HCl with a pre-combusted glass pipette. Clean acid-washed vials caps were rinsed in filtered sample water two times before closing the vials, which were stored in the dark at 4°C until analysis. DOC and TDN concentrations were measured using a high-temperature combustion total organic carbon analyzer (TOC-Vcph-TNM-1 autoanalyzer; Shimadzu, Kyoto, Japan). The instrument was calibrated with a standard addition curve of potassium hydrogen phthalate (0; 25; 50; 100; 200 μmol C/L) and potassium nitrate (0, 2.5, 5, 10, 20 μmol N/L). TOC and TDN analysis was validated with: Low Carbon Water (LCW), Deep Seawater (DSR), Surface Seawater (SSR) and Consensus Reference Materials (CRMs) provided by Hansell Laboratory of the University of Miami. Analytical variation of the instrument was < 3% coefficient of variation (CV; 5$-$8 injections per sample). Dissolved organic nitrogen (DON) concentrations were calculated as: $[DON] = [TDN] - [DIN]$, with dissolved inorganic nitrogen (DIN) as: $[DIN] = [NO_x^-] + [NH_4^+]$.

DNA extraction of sponge and seawater samples

DNA was extracted from about 0.5 cm^3 of finely cut sponge tissue samples using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA). Different tissue homogenization steps were employed depending on the structure of the sponge tissue. Tougher *Niphates erecta* tissue was homogenized in Powerbead tubes (garnet 0.70 mm, Qiagen) with 180 μL Buffer ATL and 20 μL proteinase K by vortexing for 15 min. For the other sponges, the tissue was homogenized in 180 μL Buffer ATL and 20 μL proteinase K using a small sterile pestle that fits in a 1.5 mL microcentrifuge tube. All samples were incubated overnight at 56°C. After the incubation, 200 μL of Buffer AL were added to each sample, followed by 200 μL of ethanol (100% v/v) and mix thoroughly by vortexing. All samples were centrifuged at 6000 x g for 2 min to precipitate unlysed material, and supernatant loaded onto DNeasy mini-spin columns and further treated according

to manufacturer protocol. DNA from the Sterivex filters (seawater samples) was also extracted using DNeasy® Blood and Tissue Kit (Qiagen). The volumes of Buffer ATL and proteinase K were doubled as deviation from the original manufactory protocol and pipetted into the filter cartridge after which the whole filter was incubated in an oven at 55°C with a sample rocker. After the incubation, 400 μL Buffer AL was pipetted into the filter cartridge through the luer-lock side and the filter was incubated for another 20 min at 70°C to deactivate the proteinase K. The entire volume was extracted from the cartridge using a sterile 3 mL syringe. Then, 400 μL of 100% (v/v) ethanol was added and the entire sample was loaded onto the spin column. The protocol was further followed as described by Qiagen. The concentration and purity of the extracted DNA were checked with a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific).

16S amplicon sequencing and data analysis

The V3–V4 region of the 16S rRNA gene sequences was amplified using the primer pair 341f/806r (primer sequences: 5'-CCTACGGGAGGCAGCAG-3' and 5'-GGACTACHVGGGTWTCTAAT-3'), dual-barcoding approach (Kozich *et al.* 2013). PCR conditions were as follows: initial denaturation step for 30 s at 98°C, 30 cycles of denaturation for 9 s at 98°C, annealing for 30 s at 55°C and extension for 30 s at 72°C, followed by a final extension step of 10 min at 72°C. The quality and quantity of the PCR product were checked using gel electrophoresis. PCR products were normalized (SequalPrep normalization plate kit; Thermo Fisher Scientific) and pooled. Amplicon libraries were sequenced using MiSeq v3 chemistry sequencing kit (2×300 bp) on an Illumina MiSeq platform (MiSeq FGx; Illumina, San Diego, CA) at IKMB sequencing facilities (Kiel, Germany). Raw DNA sequences were quality-filtered and trimmed based on quality scores. Amplicon Sequence Variants (ASVs) were computed with the DADA2 algorithm within QIIME2 (version 2018.8). To train the error model, one million reads were used. Chloroplasts and mitochondrial sequences were removed from further analyses. Phylogenetic ASV trees were generated with the FastTree2 plugin. A primer-specific trained Naive Bayes taxonomic classifier was used to classify representative ASVs based on the Silva 132 small subunit rRNA database with a 99% identity criterion. Alpha and beta diversity indices (i.e., Shannon diversity and weighted UniFrac distances, respectively) were calculated within QIIME2 and sample separation in ordination space was visualized by non-metric multidimensional scaling (nMDS). A two-factors permutational multivariate analyses of variance (PERMANOVAs) was run in R (version 3.6.1), with 999 permutations and additional pairwise comparisons, to examine the effects of the sponge species or seawater bacterioplankton identity ("Species"), the sampling site ("Site") and their combination ("Species_Site") on the alpha diversity index. Similarly PERMANOVAs and pairwise comparisons were run on the beta diversity indices in QIIME2 to assess if the seawater bacterioplankton or sponge-associated bacterial communities sampled in the comparison and transplantation experiments differed significantly among the species, the sites and the combination of species and site. When a significant difference was found in the pairwise PERMANOVAs, specific taxa enrichment was determined and ranked using the linear discriminant analysis effect size (LEfSe) algorithm, with an LDA threshold of 2 (Segata *et al.* 2011). A significance level α of 0.05 was applied for all statistical analyses. The sequences generated in this study

can be downloaded from the NCBI database under BioProject ID PRJNA698450.

RESULTS

Site comparison of benthic community composition and water quality

The benthic community cover estimates confirmed our observation of Site 1 (Snake Bay) as the high-coral-low-algae site compared to Site 2 (Boca Sami), observed as the low-coral-high-algae site (Fig. 2 and Table 1). At Site 1, the relative coral cover was 22 times higher and the relative macroalage cover was more than twice lower than at Site 2. Sponge relative cover was 3-fold lower in Site 1 compared to Site 2. Among the measured organic and inorganic nutrients, there was a significant difference in DOC concentrations, on average 20 μmol/L higher at Site 1 compared to Site 2 (Table 1). The bacterioplankton abundances did not differ significantly between the two sites. The average concentrations of dissolved organic nitrogen and inorganic nutrients were all higher (note specifically the twice higher average nitrate [NO^{3-}] concentration) at the Site 1 compared to Site 2. However, these differences were not found significant with the relatively low sample size.

Site comparison of sponge and seawater bacterial community composition

After filtering and quality control, 609777 bacterial sequences were obtained from 75 samples, resulting in an average frequency of 8130 sequence reads per sample. We identified 7940 bacterial ASVs affiliated to 36 bacterial phyla. Taxonomic assignment revealed distinct bacterial community compositions among the four sponge species and the seawater bacterioplankton within the comparison experiment (Fig. 3). All relative abundances at the phylum level are depicted in Table S1 (Supporting Information). Bacterial communities were more conserved in HMA than in LMA species. The community composition of the LMA species *S. ruetzleri* was more similar to that of the seawater bacterioplankton than to the other LMA species *N. erecta* (Fig. 3). The bacterial communities of the HMA sponges were dominated by *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Nitrospirae* and *Spirochaetes*. LMA species were dominated by *Proteobacteria* and *Cyanobacteria*, but with clear differences in relative abundance for *N. erecta* (90.7 and 5.4%; average between sites) and *S. ruetzleri* (34.1 and 53.5%). *Scopalina ruetzleri* also showed a dominance in *Actinobacteria* (6.7%) closely resembling the relative abundances of *Proteobacteria*, *Cyanobacteria* and *Actinobacteria* (44.4, 29.7 and 7.2%, respectively) in the seawater bacterioplankton. The bacterioplankton samples further showed dominance in *Bacteroidetes* (12.6%), which distinguished them from HMA and LMA sponges (0.7 and 1.5%, respectively).

The Shannon diversity index indicated that the bacterial community richness was equal between the two sites (Site 1 = 4.60 ± 1.32 and Site 2 = 4.62 ± 1.31 average mean ± SD), but significantly different between sponge species and seawater bacterioplankton (PERMANOVA, Pseudo-F = 45.67, P = 0.001 and Table S1, Supporting Information). *Niphates erecta* showed the lowest diversity index, being significantly lower in all comparisons, while the seawater and *A. conifera* were characterized by higher diversity, followed by *P. angulospiculatus* and *S. ruetzleri* (Figure S2 and Table S1, Supporting Information). On average, the bacterial communities of HMA sponges (5.62 ± 0.29) were more
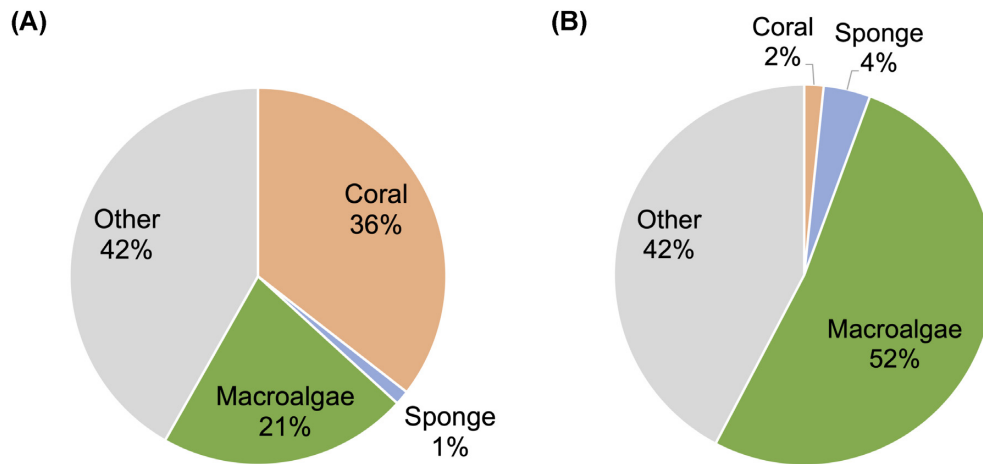
(A)

(B)

Figure 2. Cover estimation at **(A)** the high-coral-low-algae Site 1 and **(B)** the low-coral-high-algae Site 2 of the four major benthic cover categories. Percentage cover was estimated using CPCe (Coral Point Count with Excel extensions, 10 quadrants per site with 100 datapoints per quadrant).

Table 1. Average concentrations and statistical outputs testing the differences between the benthic community composition and the seawater quality measurements at the high-coral-low-algae Site 1 and the low-coral-high-algae Site 2. When data were normally distributed a paired two sample t-test was performed, if assumptions of normality were violated a Mann–Whitney–Wilcoxon test was performed. Significant *P-values* are written in **bold**.

Mann–Whitney–Wilcoxon test		Site 1 (% ± SD)	Site 2 (% ± SD)	n		W	P-value
CPCe category	Coral	35.5 ± 14	1.6 ± 1.7	10		100	**0.0002**
	Macroalgae	21.5 ± 10.3	52.1 ± 22.3	10		6	**0.0003**
	Sponge	1.2 ± 1.4	4.0 ± 2.6	10		18	**0.0162**
	Other	41.8 ± 12.3	42.3 ± 23.7	10		46	0.7959
Paired two sample t-test		**Site 1 (μmol/L ± SD)**	**Site 2 (μmol/L ± SD)**	**n**	**t**	**df**	**P-value**
Bacterioplankton (cells/mL)		528284 ± 109804	542415 ± 135728	3	0.18	2	0.8689
NO_2^-		0.05 ± 0.01	0.04 ± 0.02	4	1.09	3	0.3534
NO_3^-		0.31 ± 0.11	0.15 ± 0.08	3	3.77	2	0.0637
NH_4^+		1.48 ± 0.66	1.39 ± 0.57	4	0.36	3	0.7434
PO_4^{3-}		0.06 ± 0.01	0.04 ± 0.01	4	1.58	3	0.2126
DOC		123.0 ± 10.8	102.8 ± 12.5	4	3.72	3	**0.0338**
DON		5.4 ± 1.6	4.3 ± 0.8	4	1.13	3	0.3388

CPCe = Coral Point Count with Excel extensions.

diverse than of LMA sponges (3.87 ± 1.08). A non-metric multidimensional scaling (nMDS) analysis based on weighted UniFrac distances was calculated to compare the bacterial communities sampled in the site comparison experiment (Fig. 4). All bacterial communities clustered per sponge species or as seawater bacterioplankton, with highest dispersion for *S. ruetzleri*. The seawater bacterioplankton community clustered closest to that of LMA sponge species but was distinct from all sponges. Accordingly, statistical analyses revealed that there was a significant difference among the bacterial community composition of the four sponge species and that of the seawater bacterioplankton (PERMANOVA, Pseudo-F = 37, P = 0.001 and Table S2, Supporting Information). Sample ordination did not show a distinctive separation between the sites, although a slight clustering was visible along the first axis in some species. This was confirmed by the PERMANOVAs comparisons which did not show a significant difference between sites overall (PERMANOVA, Pseudo-F = 0.580, P = 0.677), but only in the pairwise comparison in *A. conifera* (pairwise PERMANOVA, Pseudo-F = 2.354, P = 0.037) and in the seawater bacterioplankton (pairwise PERMANOVA, Pseudo-F = 2.668, P

= 0.039; Table S2, Supporting Information). The bacterial phyla driving these differences were *Nitrospirae* in *A. conifera* (LEfSe, P = 0.014) and *Cyanobacteria* in the seawater bacterioplankton (LEfSe, P = 0.037), which were present at higher abundance at Site 1 compared to Site 2 (Table S3, Supporting Information).

Although not statistically significant, some additional changes in the relative abundance of specific phyla were observable between the two sites (Table S3, Supporting Information). In the bacterial community of *P. angulospiculatus* the phyla *Actinobacteria*, *Nitrospirae* and *Proteobacteria* were more abundant at Site 2, while *Chloroflexi* showed the highest difference in relative abundance being 15% more abundant at Site 1. The opposite pattern was visible in the bacterial community associated with *A. conifera*, where *Actinobacteria*, *Nitrospirae* and *Proteobacteria* were more abundant at Site 1, while *Chloroflexi*, together with *Acidobacteria* and *Spirochaetes* at Site 2. There was no noticeable change in the bacterial community of *N. erecta* between the two sites, whereas *S. ruetzleri* showed a variation similar to that of the seawater bacterioplankton with *Cyanobacteria* having a higher abundance (14–15% difference) at
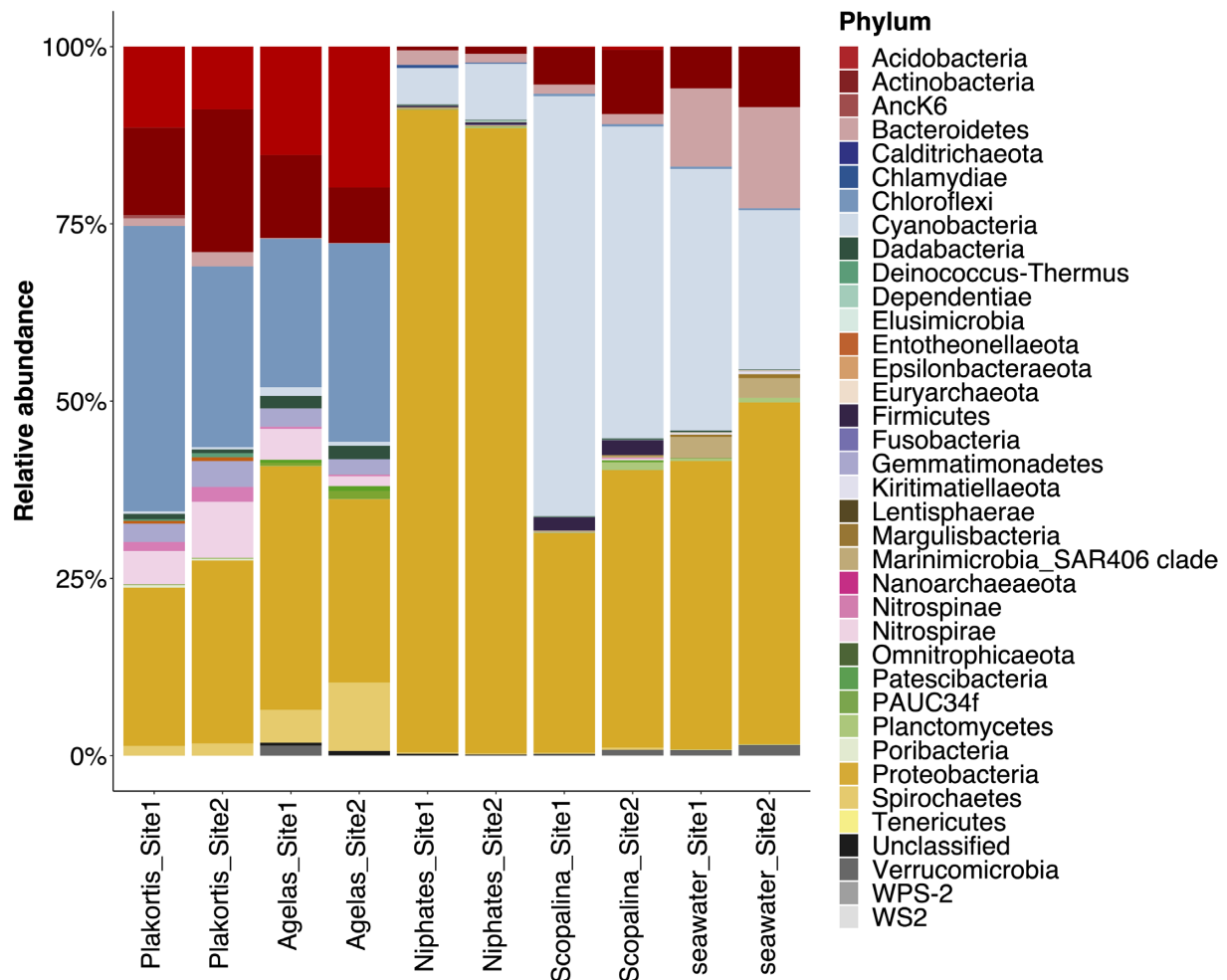
Figure 3. Relative abundance of bacterial communities at the phylum level among the four sponge species (*Plakortis angulospiculatus, Agelas conifera, Niphates erecta* and *Scopalina ruetzleri*) and the seawater bacterioplankton sampled in the comparison experiment at the high-coral-low-algae Site 1 and the low-coral-high-algae Site 2.

Site 1 and *Proteobacteria* (7–8% difference) at Site 2 along with *Actinobacteria* in *S. ruetzleri* and *Bacteroidetes* in the seawater bacterioplankton.

Sponge bacterial community composition after reciprocal transplantation

No clear pattern indicating a sponge-associated bacterial community shift was visible for both LMA sponge species *S. ruetzleri* and *N. erecta* after 21 days of reciprocal transplantation (Fig. 5). A nMDS analysis based on weighted UniFrac distances was also calculated to compare the bacterial communities sampled in the transplantation experiment. The nMDS showed no distinctive clustering of samples according to site nor treatment within any of the sponge species, but there was a separation according to host species (Fig. 6). The separation between sponge species was also supported also by the statistical analysis (PERMANOVA, Pseudo-F = 38.848, P = 0.001). For transplanted *S. ruetzleri* individuals, no significant differences in the bacterial communities were found at the end of the transplantation experiment compared to non-transplanted individuals (indicated as "no transplantation"; Table S2, Supporting Information). Conversely, a significant difference was found in the bacterial community composition of *N. erecta* sampled at the end of the transplantation experiment at both Site 1 and Site 2 (pairwise PERMANOVA, Site 1: Pseudo-F = 2.947, P = 0.035 and Site 2: Pseudo-F = 2.325, P =

0.004 and Table S2, Supporting Information) compared to non-transplanted individuals. At both sites there was a significant decrease in *Dadabacteria* (LEfSe, Site 1: P = 0.024 and Site 2: P = 0.018) and a significant increase in *Planctomycetes* (LEfSe, Site 1: P = 0.021 and Site 2: P = 0.027), along with *Verrucomicrobia* at Site 2 (LEfSe e, P = 0.008; Table S4, Supporting Information). These bacterial phyla however, constitute less than 1% of the overall community composition in *N. erecta* (Table S4, Supporting Information).

DISCUSSION

This study compared the *in-situ* bacterial community composition of four sponge species sampled at two reef sites with different relative coral to algae benthic cover composition and assessed the stability of two of these host-symbiont relationships through a reciprocal transplantation experiment between the two reef sites. We found that the sponge-associated bacterial communities were generally host-specific and no major bacterial community shifts were observed in the tested sponges between sites, nor after reciprocal transplantation.

Site comparison of benthic community composition and water quality

The two reef sites showed significant differences in the relative benthic cover composition, with higher coral and lower algae
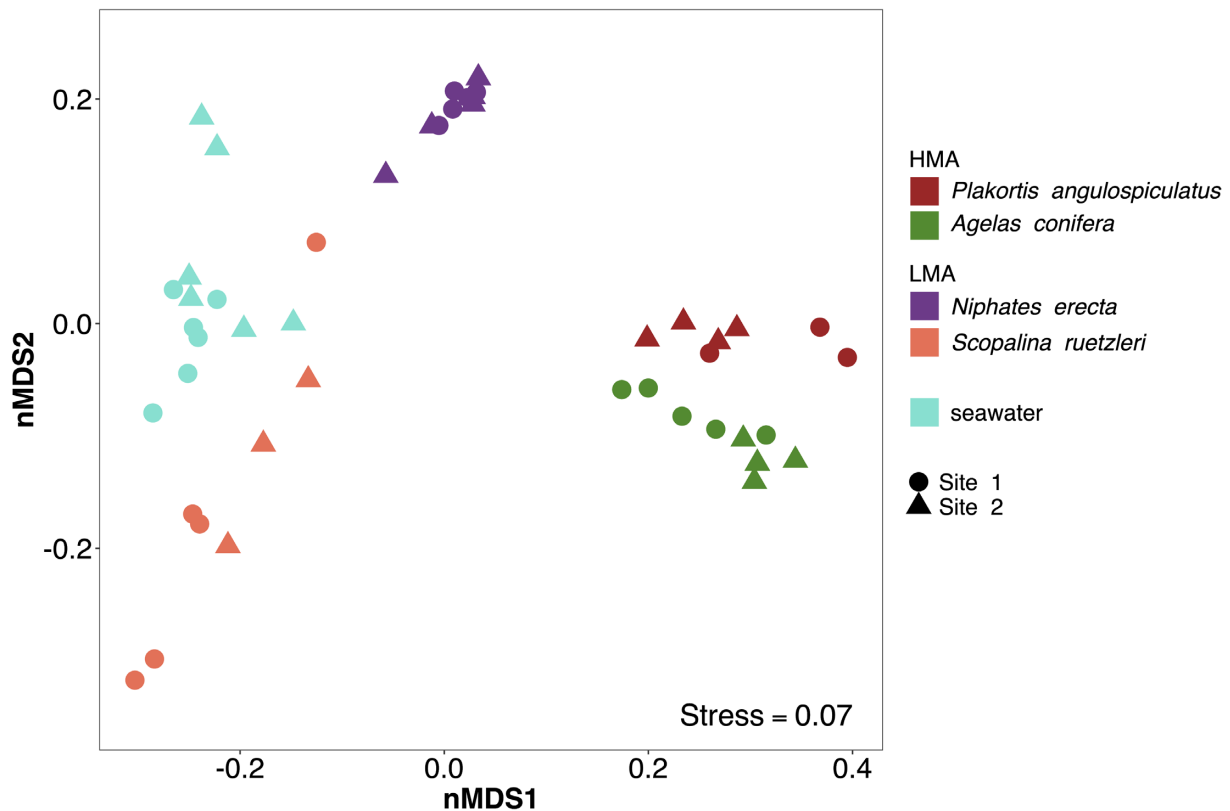
Figure 4. Bacterial community composition of the four sponge species and the seawater sampled in the comparison experiment visualized by a non-metric multidimensional scaling plot on weighted UniFrac distances at ASV level. Each marker is one bacterial community, with different symbols representing the sampling location (circles for high-coral-low-algae Site 1 and triangles for low-coral-high-algae Site 2) and different colors indicating the sample group (i.e. sponge species and seawater bacterioplankton) as indicated in the legend.

cover at Site 1 compared to Site 2, as predicted during initial site selection (based on visual observation; Fig. 1). The water quality assessment at the two sites shows bacterioplankton abundances and (in)organic nutrient concentrations that are well in the range of previously measured concentrations using similar methods in Curaçao reef waters (e.g., van Duyl and Gast 2001; Mueller *et al*. 2014; Campana *et al*. 2021a). It may seem contradictory that the high-coral-to-low-algae Site 1 was characterized by significantly higher dissolved organic carbon (DOC) concentrations, as macroalgae are known to release higher quantities of DOC than corals (Haas *et al*. 2011; Mueller *et al*. 2014). However, DOC released by macroalgae can stimulate bacterioplankton production (Nelson *et al*. 2013), causing a decrease in DOC standing stock and increase in ambient bacterioplankton abundance as found for reefs worldwide (Haas *et al*. 2016). Strangely, bacterioplankton abundances were not found to be significantly higher in Site 2 and that could be due to the higher abundance of sponges at that site, which efficiently feed on bacterioplankton, but this needs to be tested in future studies. Also, the bioavailability of DOM does not necessarily depends on its concentration, but on its composition. The interaction between bacterioplankton and sponge holobionts in the cycling of DOM (components), referred to as "the battle for sugar" on reefs (de Goeij *et al*. 2017) is still largely unknown.

Stable sponge bacterial communities between sites and after transplantation

Overall, sponge-associated and seawater bacterial communities were conserved between the two sampling sites, with the exception of the phyla *Nitrospirae* in *A. conifera* and *Cyanobacteria* in the

seawater bacterioplankton. These differences could be driven by the differences in organic and inorganic nutrients availability at the two sites; indeed, *Nitrospirae* are well known nitrogen-oxidizing bacteria in sponges (Feng and Li 2019) and possibly also involved in organic carbon uptake (Campana *et al*. 2021b), while free-living *Cyanobacteria* are capable of both inorganic carbon and nitrogen fixation (Foster *et al*. 2013). Nonetheless, an opposite (but non-significant) change in the relative abundance of *Nitrospira* between the two sites was noticeable in the other HMA species *P. angulospiculatus*. Therefore, other environmental parameters, such as light conditions, turbidity, water flow or even sponge functional traits, such as morphology (massive versus encrusting growth forms) could be involved in these differences. Some differences were also found in the bacterial community composition of *N. erecta* after the transplantation experiment. However, it should be noted that the bacterial groups which abundance significantly changed after the transplantation accounted for less than 1% of the overall bacterial community of *N. erecta*. Furthermore, the change in relative abundances was similar at both sites (i.e., a decrease in *Dadabacteria* with an increase in *Planctomycetes*), therefore the shift seems to be an effect of the transplantation to a new site rather than the difference in environmental conditions present at the respective sites after transplantation.

The greatest separation was found between the bacterial communities of the seawater bacterioplankton and that of the sponges and between high versus low microbial abundance (HMA vs LMA) sponges. Based on community composition and diversity indices, the two HMA sponges, *P. angulospiculatus* and *A. conifera,* were clearly differentiated from the two LMA sponges, *S. ruetzleri* and *N. erecta*. While the HMA sponges hosted more
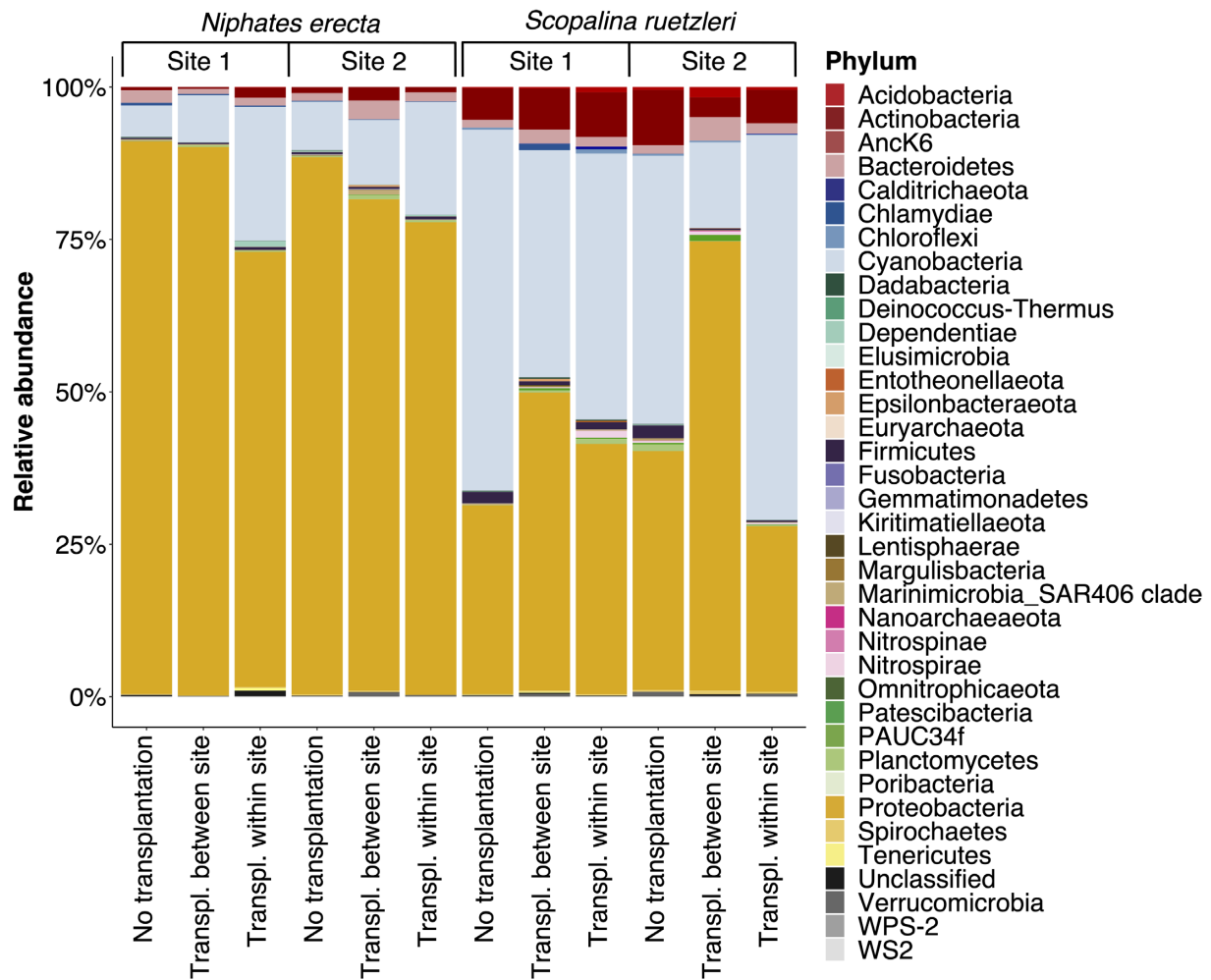
Figure 5. Relative abundance of bacterial communities at the phylum level in *N. erecta* and *S. ruetzleri* sampled before and after the transplantation experiment. Each column indicates a sample group: sponge species, sampling location and treatment (i.e., no transplantation, transplantation between sites and transplantation within the same site).

diverse but homogeneous symbiont communities, LMA sponges were characterized by few dominant groups and a community composition more similar to that of the seawater bacterioplankton, as reported in numerous LMA and HMA host species collected from diverse geographic regions (Schmitt *et al.* 2011; Bayer *et al.* 2014; Erwin *et al.* 2015; Moitinho-Silva *et al.* 2017; Gantt *et al.* 2019). Furthermore, the bacterial community composition of the species described in our study fits well within the groups of LMA and HMA indicator taxa identified by Moitinho-Silva *et al.* (2017). The LMA species *N. erecta* was dominated by the LMA indicator taxa *Proteobacteria* (mostly *Alphaproteobacteria*) with a lower contribution of *Cyanobacteria*, as already reported in Panama (Easson and Thacker 2014). Consistently with previous work, the LMA species *S. ruetzleri* was dominated by *Cyanobacteria*, *Proteobacteria* and *Actinobacteria*, but it was also the species with the most variable community composition among replicates (Rua *et al.* 2015). The HMA species *P. angulospiculatus* and *A. conifera*, were characterized by the HMA key indicator taxa *Chloroflexi*, *Acidobacteria* and *Actinobacteria* (Olson and Gao 2013), and by less abundant, but characteristic taxa such as *Gemmatimonadetes*, *Dadabacteria*, PAUC34f and *Poribacteria* (Erwin *et al.* 2015). In conclusion, our results support the role of the host identity and of the HMA-LMA status in shaping symbiont community composition, an emerging paradigm in sponge microbiology (Moitinho-Silva *et al.* 2017).

Although the relative abundance of some bacterial phyla varied between the two sites, sponge host identity remained the major factor shaping the associated bacterial community composition before and after reciprocal transplantation. A stable host-specific partnership between the sponge host and its microbial symbionts has been observed in several studies along different environmental gradients (Lee *et al.* 2010; Erwin *et al.* 2012; Reveillaud *et al.* 2014; Erwin *et al.* 2015; Souza *et al.* 2017). For example, the sponge *Aplysina cavernicola,* found in low-light habitats (deeper waters or in submarine caves), did not show a change in its microbial community composition following a three-month long transplantation from their original location to shallower and light-exposed sites (Thoms *et al.* 2003). The sponges *Cymbastela stipitata* and *Aplysina cauliformis* maintained conserved microbial communities despite exposure (5 days to 4 weeks, respectively) to high levels of nutrients (Gochfeld *et al.* 2012; Luter *et al.* 2014). The same was true for the symbiotic bacterial, archaeal and eukaryotic communities of the Great Barrier Reef sponge species *Rhopaloeides odorabile* under elevated inorganic nutrients levels and temperatures for a week (Simister *et al.* 2012). Across longer time spans, sponge-associated bacterial communities exhibited a high degree of seasonal stability especially in HMA sponge hosts, despite large fluctuations in temperature and irradiance (Erwin *et al.* 2012; Erwin *et al.* 2015).
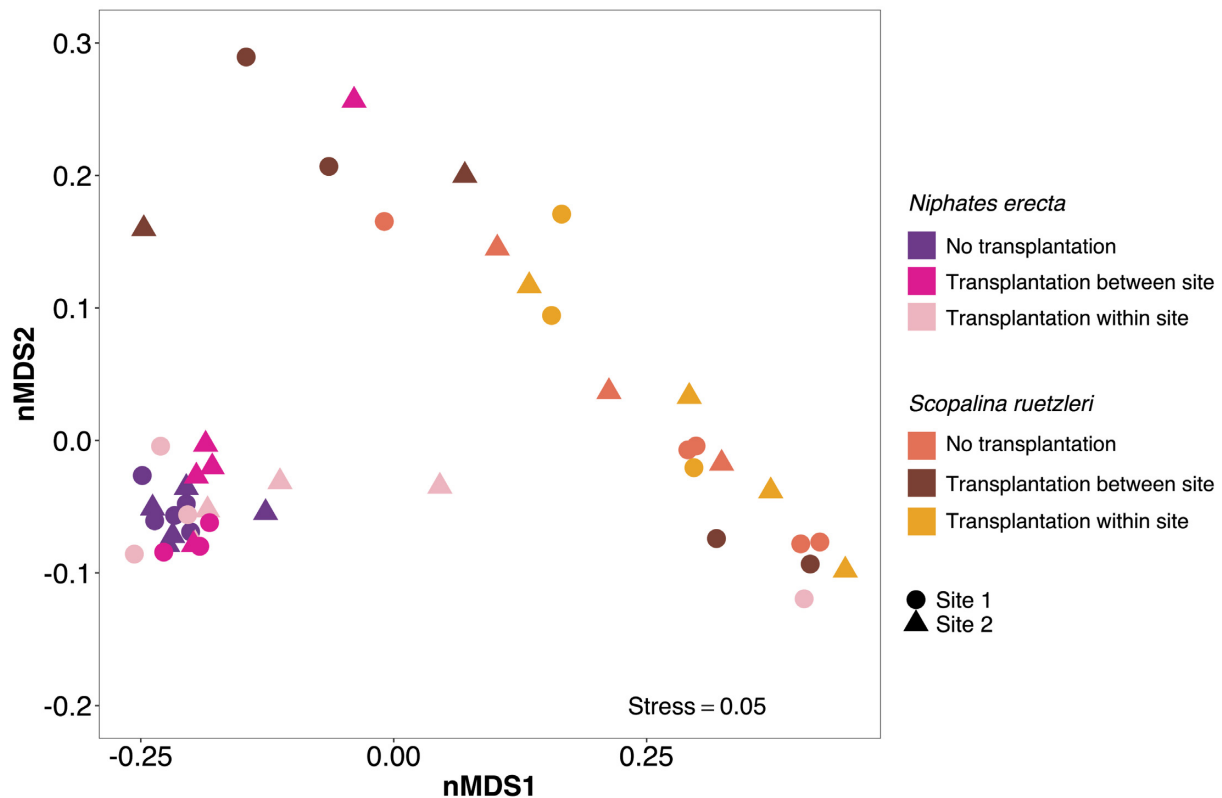
Figure 6. Sponge bacterial community composition of *N. erecta* and *S. ruetzleri* sampled before and after the transplantation experiment visualized by a non-metric multidimensional scaling plot on weighted UniFrac distances at the ASV level. Each marker is one bacterial community, with different symbols representing the sampling location (circles for high-coral-low-algae Site 1 and triangles for low-coral-high-algae Site 2) and different colors indicating the sample treatment (i.e., no transplantation, transplantation between sites and transplantation within the same site).

Nonetheless, some shifts in sponge microbial community composition were found under seasonal and temporal scales and after transplantation between intertidal and subtidal environments (Cao *et al.* 2012; White *et al.* 2012; Weigel and Erwin 2017).

The lack of a major shift in the sponge-associated bacterial communities between sites implies that the tolerance limits to changes in environmental conditions have not yet been met for these sponges. It does not imply that both the microbiome or the biomass of sponges are resilient to future reef regime shifts, but that the difference in benthic community composition and related environmental conditions observed were not yet strong enough to cause a significant change in the sponge-associate bacterial communities. Phase shifts or alternative states within ecosystems are usually triggered by a critical threshold or tipping point, which causes the ecosystem to change from one state to a new state. Reef environments can be seen as nested ecosystems because they are characterized by complex networks, which interact at different scales, from microbes to ecosystem level (Pita *et al.* 2018). In sponges, key functions carried out by the sponge microbiome, influence the whole sponge holobiont, which in turn interact with the surrounding holobionts, influencing community structure and ecosystem functioning (Pita *et al.* 2018). Vice versa environmental and biological stressor can act at multiple scales altering the various components of these nested ecosystems. Each ecosystem level can have a certain buffering capacity against perturbations. Therefore, the strength of these perturbations can be a major factor in determining the ability of the holobiont to maintain a stable state. Additionally, a possible response to change could be the acquisition of novel functions without shifts in

taxonomic composition, therefore we would need to improve our understanding not only of the composition but also of the functionality of the sponge-associated microbial communities under coral reefs phase shifts. To understand the role and the resilience of sponge holobionts under reef phase shifts, not only the microbial composition of sponges need to be assessed, but also their biomass and functional changes therein. A major part of sponge diversity (Vicente *et al.* 2021) and biomass (Kornder *et al.* 2021) on reefs can be "hidden" in so-called cryptic habitats (e.g., coral overhangs, crevices and cavities) and are usually missed in traditional surveys. Extensive baseline data on sponge biomass and microbiome composition accompanied by benthic biomass and water quality assessments are therefore needed to identify the ecosystem buffering capacity and tipping points in ongoing coral reef phase shifts. A more comprehensive approach can enable intervention before the key ecosystem functions provided by sponges are lost.

AUTHOR CONTRIBUTIONS

SC and JMG designed the experiments. SC and CD performed the experiments and analyzed the samples. SC and KB analyzed the data. SC wrote the manuscript and all authors reviewed and contributed to the manuscript writing.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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