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The coral ecosphere: A unique coral reef habitat that fosters coral–microbial interactions

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Abstract

Scleractinian corals are bathed in a sea of planktonic and particle-associated microorganisms. The metabolic products of corals influence the growth and composition of microorganisms, but interactions between corals and seawater microorganisms are underexplored. We conducted a field-based survey to compare the biomass, diversity, composition, and functional capacity of microorganisms in small-volume seawater samples collected adjacent to five coral species with seawater collected > 1 m away from the reef substrate on the same reefs. Seawater collected close to corals generally harbored copiotrophic-type bacteria and its bacterial and archaeal composition was influenced by coral species as well as the local reef environment. Trends in picoplankton abundances were variable and either increased or decreased away from coral colonies based on coral species and picoplankton functional group. Genes characteristic of surface-attached and potentially virulent microbial lifestyles were enriched in near-coral seawater compared to reef seawater. There was a prominent association between the coral *Porites astreoides* and the coral symbiont *Endozoicomonas*, suggesting recruitment and/or shedding of these cells into the surrounding seawater. This evidence extends our understanding of potential species-specific and reef site-influenced microbial interactions that occur between corals and microorganisms within this near-coral seawater environment that we propose to call the "coral ecosphere." Microbial interactions that occur within the coral ecosphere could influence recruitment of coral-associated microorganisms and facilitate the transfer of coral metabolites into the microbial food web, thus fostering reef biogeochemical cycling and a linkage between corals and the water column.

Marine organisms are bathed in seawater that is densely populated by protists, bacteria, archaea, and viruses. This continuous contact likely facilitates interactions between marine bacteria and archaea and single-celled or multicellular organisms. For example, heterotrophic bacteria residing within the microenvironment surrounding and directly attached to eukaryotic phytoplankton cells can interact on a cellular level with the host eukaryote. These heterotrophic cells respire the dissolved organic matter (DOM) released by the eukaryote, synthesize and transfer essential vitamins to the host, and/or engage with the host using infochemicals (Seymour et al. 2017). These interactions can be beneficial, neutral, and/or exploitative and may impact productivity, growth rates, and life cycles of specific phytoplankton, potentially influencing the primary productivity of the ecosystem (Seymour et al. 2017). We hypothesize that these interactions may be present and even more pronounced for much larger sessile organisms such as kelp, corals, and sponges, as their fixed location on the seafloor provides the opportunity to foster specific host–microbial interactions.

The microbiomes of scleractinian corals are some of the most well-characterized host-associated communities in the marine environment (reviewed by Thompson et al. [2014] and Bourne et al. [2016]), but much less is known about how corals interact with surrounding seawater microbial communities. Previous investigations of reef water microbial community dynamics have revealed relationships among the composition of reef macrofauna, the composition and metabolism of bacteria and archaea in reef seawater (RSW), the abundances of heterotrophic bacteria and virulence genes, and coral health (Dinsdale et al. 2008; Kelly et al. 2012; Kelly et al. 2014; Haas et al. 2016). In addition, recent studies have suggested the existence of a previously unrecognized coral-associated microbial environment: the seawater adjacent to

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Additional Supporting Information may be found in the online version of this article.

corals (Tout et al. 2014; Silveira et al. 2017; Walsh et al. 2017; Ochsenkuhn et al. 2018).

Corals may indeed influence the composition, structure, and function of these surrounding planktonic microbial communities. For instance, corals secrete DOM that can be degraded by and even serve as chemical cues for motile marine bacteria (Nelson et al. 2013; Garren et al. 2014; Tout et al. 2015). Additionally, some corals graze on picoplankton and remove cells from the water column (Houlbrèque et al. 2006; McNally et al. 2017). Physically, coral colonies interrupt water flow at different scales and form centimeter-scale momentum boundary layers surrounding individual colonies as well as microvortices closer to the coral surface (Chamberlain and Graus 1975; Shashar et al. 1996; Kaandorp et al. 2003; Shapiro et al. 2014). Together, these factors suggest that distinct microbial communities may form surrounding corals within the coral momentum boundary layer. Furthermore, microbial interactions that occur within this environment could influence microbial symbiont acquisition and pathogen recruitment to the coral surface.

In a preliminary investigation of two coral colonies, Tout et al. (2014) detected genomic differences between seawater collected above corals and surface RSW. Despite collecting large volumes (10 liters) of seawater that may have integrated the microbial heterogeneity that exists at smaller scales, Tout et al. (2014) found enrichment of copiotrophic bacteria near the corals, as well as genes used for bacterial motility, chemotaxis, membrane transport, iron acquisition, and metabolism of aromatic compounds in addition to other pathways. In contrast, Silveira et al. (2017) did not detect any significant differences in the functional or taxonomic microbial composition between large (80 liters) volume samples of near-coral seawater (CSW) collected surrounding patches of the coral *Mussismilia braziliensis* and the water column. A study by Walsh et al. (2017) detected differences in the microbial communities of seawater within 5 cm of reef macro-organisms compared to seawater sampled 3 m off of the reef except for the coral *M. braziliensis*, similar to the study conducted by Silveira et al. (2017). Finally, a recent study used syringes (50 mL volume) to sample seawater 0, 5, and 50 cm away from individual colonies of *Acropora* and *Platygyra* spp. and reported that specific coral-associated bacteria were more abundant closer to *Acropora* (0 and 5 cm) compared to *Platygyra* colonies, attributing this to morphological differences between the corals that could impact momentum boundary layer dynamics and mixing processes (Ochsenkuhn et al. 2018).

In all cases, these investigations targeted their sampling within the diffusive or momentum boundary layer, the area in which microbial dynamics may be distinct from the overlying water column. However, most of the previous studies (Tout et al. 2014; Silveira et al. 2017; Walsh et al. 2017) did not sample smaller volumes of seawater (< 10 liters) that may be more relevant for examining the momentum boundary layer surrounding corals, which has an approximate thickness of a few centimeters (Shashar et al. 1996). Additionally, these previous studies collected samples surrounding one or two different coral species at a single reef site and could not investigate if local environmental conditions influenced the microbial community composition of this seawater. Although these efforts have brought attention to the importance of this CSW environment, there is still a need to examine the microbial interactions at a higher resolution by collecting smaller volumes of seawater as well as by investigating the influences that different coral species or reef locations impart on CSW microbial communities.

We designed this study to explore the hypotheses that (1) CSW environments harbor taxonomically and functionally distinct microbial communities compared to the overlying water column and (2) CSW is also distinct by coral species. To test these hypotheses, we examined microbial communities collected using small (1 and 60 mL) volume seawater samples from distances generally thought to include the momentum boundary layer surrounding individual coral colonies (Shashar et al. 1996; Barott and Rohwer 2012). We compared these microbial communities to RSW microbial communities collected from within the benthic boundary layer across multiple reefs.

Experimental procedures

Sampling design and sample collection

Seawater was collected near corals (≤ 30 cm away) as well as farther from corals (> 1 m off the reef) at 10 reefs during two separate field expeditions to the Cuban reef-systems of Jardines de la Reina (JR) and Los Canarreos (CAN) in February and April/May of 2015 (Supporting Information Fig. S1). Most reefs within JR lie within a marine protected area and they are some of the most protected and preserved reefs in the Caribbean. The surveyed reefs in JR included forereefs (JR 1 and 2) that are located on the southern side of the reef tract as well as back reefs (JR 3, 4, 5, and 6) that are located within the gulf of Ana Maria, lying between the island of Cuba and the reef tract (Supporting Information Fig. S1). JR reefs include a variety of habitats and hydrodynamic regimes (i.e., tidal currents and wave exposure), contributing to microbiological differences between these reefs (L. Weber et al., unpubl.). Reefs within CAN were hydrogeographically similar to each other and did not span distinct environmental gradients (Supporting Information Fig. S1).

Seawater samples were collected near five species of coral (CSW) within distances thought to comprise the lower (< 10 cm) and upper (30 cm) bounds of the momentum boundary layer surrounding individual coral colonies (Shashar et al. 1996; Barott and Rohwer 2012). The corals *Orbicella faveolata* (Ellis and Solander, 1786), *Montastraea cavernosa* (Linnaeus, 1767), *Pseudodiploria strigosa* (Dana, 1846; formerly known as *Diploria strigosa*), and *Porites astreoides* (Lamark, 1816) were chosen because they are commonly observed on Cuban reefs and the first three species are major reef builders in the Caribbean. *Acropora cervicornis* (Lamarck, 1816) was selected because this species was historically

Table 1. Description of reef locations and number and types of samples collected.

Reef system* and site	Date	Coral species for CSW†	RSW‡	Sand seawater controls§	Seawater experiment samples‖	Latitude	Longitude
JR 1	08 Feb 2015	*M. cavernosa* (3/3) *P. astreoides* (3/3) *P. strigosa* (3/3)	Surface (1/0) Reef depth (1/2)			20.77453	−78.91517
JR 2	09 Feb 2015	*M. cavernosa* (3/2) *P. astreoides* (3/3) *P. strigosa* (3/1)	Surface (1/0) Reef depth (1/2)			20.82598	−78.97931
JR 3	08 Feb 2015		Reef depth (0/2)			20.81478	−78.88320
JR 4	10 Feb 2015		Reef depth (0/2)			20.87765	−78.97028
JR 5	11 Feb 2015	*O. faveolata* (3/2) *P. astreoides* (3/3) *P. strigosa* (3/2)	Reef depth (1/2)			21.09232	−78.73354
JR 6	12 Feb 2015	*O. faveolata* (3/3) *P. astreoides* (3/3) *A. cervicornis* (3/3)	Reef depth (1/2)	Sand (1)		21.10845	−78.72080
CAN 12	28 Apr 2015 (CSW) 30 Apr 2015 (RSW)	*P. astreoides* (4/3)	Reef depth (1/1)	Sand (1)		21.58387	−81.62795
CAN 14	30 Apr 2015	*O. faveolata* (1/1) *P. strigosa* (2/1)	Surface (1/0) Reef depth (1/1)	Sand (1)		21.56893	−81.63820
CAN 15	06 May 2015	*P. astreoides* (4/3)	Surface (1/0) Reef depth (1)	Sand (1)		21.55521	−81.76323
CAN 17	04 May 2015 (CSW) 05 Apr 2015 (RSW)	*P. astreoides* (4/4)	Surface (1/0) Reef depth (1/0)	Sand (1)		21.60200	−81.93400
USVI—Tektite	29 Oct 2016				7	18.3095	−64.7219
USVI—Dock	29 Oct 2016				5	18.3182	−64.7241

*JR, Jardines de la Reina, Cuba; CAN, Los Canarreos, Cuba; USVI, St. John, USVI.
†Number of colonies sampled for CSW; CSW samples for microbial cell counts were taken at 0 and 5 cm (JR) or 0, 2, and 30 cm (CAN) away from the colony, and this number is the first number in parentheses; community DNA samples were taken 30 cm away from all JR and CAN coral colonies. The second number in parentheses reflects the number of samples that made it past sequence quality filtering and that were used in amplicon analysis.
‡RSW, number of RSW samples that were collected. Surface RSW samples were collected 1 m from the surface of the seawater. RSW (reef depth) samples were collected > 1 m off of the reef. The first number in parantheses indicates the number of samples collected for cell counts. The second number in parentheses indicates the number of samples that made it past sequence quality filtering and/or that were used in amplicon analysis.
§Number of seawater samples taken over sand for microbial cell counts. In JR, samples were taken at 5 cm, and in CAN, samples were taken at 0, 2, and 30 cm away from the sand.
‖ Seawater experiment samples = number of samples for seawater volume experiment.

a major reef builder on Caribbean reefs. However, disease outbreaks have decimated *Acropora* populations, and this species is now listed as threatened under the U.S. Endangered Species Act.

To sample the CSW for genomic analyses, a scuba diver used sterile 60 mL syringes to collect seawater 30 cm away from at least three colonies of at least three species on each reef (Table 1). Colonies were generally isolated from other corals and colony replicates were separated by more than 2 m across each reef. We did not collect CSW from colonies that were actively shedding their mucus in order to avoid potential mucus contamination. The natural distribution of coral species varied between reefs therefore some species (e.g., *P. astreoides*) were sampled more than others (e.g., *A. cervicornis*). In total, 49 CSW samples were collected across the two reef-systems, but six were removed from the final analysis because they had low numbers of sequences.

Additionally, smaller volume (1 mL) seawater samples were collected via syringe to examine general trends in microbial abundances along a gradient toward coral colonies and to complement the genomic analyses. Two distances (0 and 5 cm) were sampled around each colony in JR, and three distances (0, 2, and 30 cm) were sampled around each colony in CAN. We increased the number of sampling distances per colony for corals sampled in CAN so that we could more comprehensively evaluate how microbial abundance changed over a small-scale distance gradient from each colony. Additionally, control seawater samples were collected over sand patches (ranging from 0 to 30 cm away) at JR 6 and CAN 12, 14, 15, and 17. We compared cell abundances in these control samples with cell abundances in CSW to investigate if the presence of corals influenced the abundance of cells. Sampling distances were

measured by using the length of a custom syringe sampling device holder. Each sample was preserved with 1% paraformaldehyde (Electron Microscopy Sciences) (final concentration), and flow cytometry was used to quantify picoeukaryotes, *Prochlorococcus*, *Synechococcus*, and unpigmented (heterotrophic) cells (Supporting Information).

RSW samples were collected in duplicate from > 1 m above each reef at approximately the same time as when the CSW samples were collected (Table 1). While collecting paired RSW and CSW samples would have been ideal, we had limited bottom time to collect paired samples using our syringe sampling approach. Instead, we opted to integrate the RSW samples per reef based on previous observations of relatively high similarity in RSW microbial communities across individual reefs (Apprill et al. 2016). To collect RSW samples, seawater was pumped to the surface from reef depth (> 1 m off of the reef substrate) with a groundwater pump (Mini-monsoon sampling pump, Proactive Environmental Products). We rinsed the acid-cleaned plastic tubing with reef-depth seawater for 30 s and then collected 4.2 liters of the seawater into acid-cleaned plastic bottles (for amplicon sequencing) or duplicate 10-liter acid-washed bottles (for metagenome sequencing). All samples were kept cold in a cooler filled with ice until they were processed.

To filter RSW, the acid-cleaned tubing was rinsed with seawater and then duplicate 2 liter samples of seawater were filtered onto 0.22 μm, 25 mm Supor® filters (Pall Corporation) using peristalsis. Hand filtration was used to filter the CSW samples using the same filters. Additionally, 20 liters of seawater from sites JR 2, 4, 5, and 6 were each filtered onto 0.22 μm, 142 mm Supor® filters (Pall Corporation) in order to concentrate microbial biomass for shotgun metagenomic sequencing (Table 2). This seawater was not prefiltered. All filters were flash frozen in liquid nitrogen, shipped back to the United States in a charged dry shipper, and then stored at −80°C until DNA was extracted.

Amplicon sequencing of CSW and RSW DNA

DNA was extracted from RSW and CSW filters using two extraction methods that were performed sequentially, a modified sucrose–lysis extraction protocol (Santoro et al. 2010) as well as a simplified phenol–chloroform extraction (Urakawa et al. 2010), to increase cellular lysis efficiency and DNA yield (Supporting Information). The Genomic DNA Clean and Concentrator kit (Zymo Research Corporation) was used to combine the purified DNA extracts yielded from both methods. Additionally, DNA extraction ($n = 2$) and DNA pooling ($n = 2$) controls as well as a microbial mock community (HM-278D, BEI Resources) were prepared to account for potential DNA extraction contamination as well as amplification and sequencing error.

The nucleic acids were submitted to the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois, Urbana, IL) where V4 region SSU rRNA genes from bacteria and archaea were amplified using the Fluidigm® microfluidics quantitative polymerase chain reaction (PCR) platform and prepared for 2 × 250 bp paired-end Illumina MiSeq sequencing (Supporting Information). These primers were chosen to specifically amplify 16S rRNA genes from bacteria and archaea based on their fairly comprehensive coverage of prokaryotes, their frequent use in marine studies, and their size for sequencing on an Illumina MiSeq (Kozich et al. 2013). Furthermore, we used 515F-Y and 806R-B primers with degeneracies to minimize known amplification biases against Crenarachaeota/Thaumarchaeota (Parada et al. 2016) and the SAR 11 clade (Apprill et al. 2015), taxa that are both found in marine microbial communities. The primer-sorted and demultiplexed reads were screened for quality using mothur v.1.36.1 (Schloss et al. 2009; Supporting Information). The sequences were then subsampled to 8500 reads per sample in order to minimize the impacts of uneven sequence coverage across samples, but retain as many samples within the dataset as possible. All of the raw sequences used for this analysis were deposited into the NCBI Sequence Read Archive (SRA) under BioProject PRJNA422534.

Reads were clustered into homogenous groups using minimum entropy decomposition (MED), a program that selects specific information-rich nucleotide positions in the reads using Shannon entropy and decomposes these sequences into different groups referred to as MED nodes (Eren et al. 2015). The MED algorithm identifies closely related but biologically distinct organisms (MED nodes) using marker gene information and is valuable for examining patterns in microbial diversity that could be overlooked if sequences are grouped based on a lower level of

Table 2. Number of DNA samples collected from JR by reef location and by the coral species (i.e., CSW) used to create the pooled samples for metagenomic sequencing.

	Site						
Metagenome sample	**JR1**	**JR2**	**JR3**	**JR4**	**JR5**	**JR6**	**Pooled samples**
M. cavernosa CSW	3	2					5
P. astreoides CSW		2			1	2	5
P. strigosa CSW	3				1		4
O. faveolata CSW					2	3	5
A. cervicornis CSW						3	3
RSW*		1		1	1	1	—

*RSW samples were not pooled.

similarity (Eren et al. 2015). We chose to use the MED algorithm because it has been used to examine microbial community diversity in coral tissue and seawater environments and can discern between closely related, but potentially ecologically distinct microorganisms (Neave et al. 2017; Ward et al. 2017; Weber et al. 2017). Sequences representing each MED node were classified in mothur using the Silva v119 database ("knn" method; Pruesse et al. 2007). All MED node representative sequences were also realigned using the SINA alignment and taxonomic service (Quast et al. 2013) to verify taxonomic assignment of the reads (SILVA reference database v. 128). Sequences representing *Endozoicomonas* MED nodes were compared to each other and aligned using the NCBI BLASTN 2.8.0+ algorithm (Zhang et al. 2000) in order to investigate their similarity to each other as well as their similarity to other reported *Endozoicomonas* sequences.

Statistically significant enrichment comparisons of MED nodes between CSW and RSW were made using the differential expression package "DESeq2" (Love et al. 2014), following previous methods (McMurdie and Holmes 2014; Neave et al. 2017; Supporting Information). Within-site enrichment comparisons were conducted at each reef location in order to minimize geographic and depth-related variability. Enrichment tests were only completed for samples collected at JR reefs 1, 2, 5, and 6 because a majority of the CAN RSW samples were removed due to low sequence quality.

Microbial community visualization and statistical analyses were accomplished using several R packages (R Core Development Team 2017). To examine the similarity between RSW communities sampled across JR, we completed a cluster analysis (method = "average linkage") on the Bray–Curtis dissimilarity matrix using the "Pvclust" R package (Suzuki and Shimodaira 2006). We also examined the similarity between CSW communities separately using the same method. Nonmetric multidimensional scaling (NMDS) analysis was completed with the "vegan" package (Oksanen et al. 2018) using the square-root transformed Bray–Curtis dissimilarity matrix to visually compare the degree of similarity between the CSW and RSW bacterial and archaeal communities. Nested permutational multivariate analysis of variance (PERMANOVA) tests using distance matrices (PERMANOVA/Adonis; Oksanen et al. 2018) were performed on the Bray–Curtis dissimilarity index using 999 permutations to determine the degree to which the different factors explained the microbial community composition of the samples ($p < 0.05$; Supporting Information). Most of the nested PERMANOVA comparisons were completed using the CSW and RSW collected within JR. However, *P. astreoides* CSW was also sampled across three sites in CAN (12, 15, and 17), so we included these samples and one RSW sample (collected from CAN 15) in the NMDS and PERMANOVA tests for this species. We also collected CSW samples from *P. strigosa* and *O. faveolata* in CAN, but were unable to use these samples in the NMDS and PERMANOVA tests because too many sequences were removed during quality filtering.

Shotgun metagenomic sequencing

We combined three to five CSW DNA extracts per species across samples collected within JR and prepared the pooled mixtures for shotgun metagenomic sequencing (Table 2). Samples were pooled in order to increase the total concentration of DNA in each CSW sample. We recognize that this is not an ideal approach, but were concerned that the separate extracts were too diluted to be sequenced individually. DNA extractions were also performed on one half of each of four RSW metagenome filters (representing 10 liters of reef-depth seawater sampled at JR sites 2, 4, 5, and 6) using a modified cetyltrimethylammonium bromide-phenol : chloroform : isoamyl alcohol extraction and isopropanol precipitation (Table 2; Supporting Information).

Library preparation and sequencing of the pooled CSW and RSW DNA samples were completed at the W. M. Keck Center. Libraries were prepared using the Hyper Library construction kit (Kapa Biosystems) and sequenced using 2 × 150 bp paired-end Illumina HiSeq 4000 sequencing. The raw sequences used for this analysis were deposited into the NCBI SRA under BioProject PRJNA422534. Fastq files were demultiplexed and library adaptors were trimmed from the 3′ ends of the reads (Supporting Information). BBTools (Bushnell 2016) was used to quality filter and prepare the raw metagenomic reads for functional analysis (Supporting Information).

The functional mapping and analysis pipeline (FMAP) for metagenomics and metatranscriptomics (Kim et al. 2016) was used to annotate genes with the mapping program DIAMOND (Buchfink et al. 2014) against the UniRef100 database (uniprot.org), calculate Kyoto Encyclopedia of genes and genomes (KEGG) gene abundances (Kanehisa et al. 2016), and identify significantly differentially abundant KEGG orthologs (KOs), pathways, and modules between CSW and RSW (Kruskal–Wallis test, Fisher's exact test, p value < 0.05, False Discovery Rate adjusted to control for false positives; Supporting Information).

Sampling volume comparisons

Because we collected seawater samples for genomic analysis over a range of volumes (60 mL to 2 liters), we conducted a separate experiment to test if initial seawater sampling volume influenced alpha- and beta-diversity comparisons in seawater microbial communities. To do this, we collected replicate 60 mL, 1.5 liters, or 2 liters samples from surface RSW at two different sites in St. John, U.S. Virgin Islands (Table 1). We sequenced and analyzed these samples independently to validate our analysis of the CSW and RSW samples collected in Cuba (Supporting Information).

Results

Sample volume comparisons

Analyses of the SSU rRNA gene amplicon sequences from the seawater volume experiment showed that samples of larger volume (1.5 or 2 liters) had greater microbial species

richness compared to smaller volume (60 mL) samples (Supporting Information Figs. S7–S11). However, sampling volume was not found to impact comparisons of beta-diversity or enrichment analysis (Supporting Information Figs. S12, S13). Based on these results, further comparisons of alpha diversity were not made between Cuban CSW and RSW.

CSW microbial communities are influenced by reef and coral species

Analysis of SSU rRNA gene sequences showed that RSW microbial communities from JR were 37–84% similar in terms of bacterial and archaeal community composition, whereas CSW microbial communities from JR were more similar to each other (51–84% similarity; see Supporting Information Fig. S2 for class-level relative abundances). Individual NMDS analyses of amplicon sequences by coral species demonstrated that CSW communities generally separated from the RSW communities, although there was a degree of overlap with RSW especially for *P. astreoides* CSW (Fig. 1A–E). Nested PERMANOVA (Adonis) tests on the amplicon sequence data confirmed that both sample type (CSW vs. RSW) and reef site were significant determinants of community similarity (Fig. 1A–E). A NMDS including all CSW and RSW microbial communities revealed overlapping community composition between RSW and CSW, with some distinction by species as indicated by the covariance ellipses (Fig. 1F). Additionally, a nested PERMANOVA (Adonis) test completed on all CSW and RSW communities within JR demonstrated that both reef location and coral species significantly influenced microbial community structure (Fig. 1F).

Differential enrichment analyses of the MED clustered amplicon sequences revealed that CSW microbial communities were distinct from RSW microbial communities with regard to specific bacterial taxa. Broadly, CSW was mostly enriched with copiotrophic lineages of Gammaproteobacteria when compared to RSW collected within JR (Table 3). MED enrichment in *P. astreoides* CSW compared to RSW was attributed to the Gammaproteobacteria genera *Alteromonas*, *Endozoicomonas*, and *Bermanella* (Supporting Information Table S1). *Endozoicomonas* MEDs were significantly enriched in *P. astreoides* CSW at reefs JR 2, 5, and 6. *Endozoicomonas* and *Alteromonas* were also enriched in *P. strigosa* CSW (Supporting Information Table S1). Similarly, *O. faveolata* CSW was enriched with *Alteromonas* as well as two MED nodes identifying as *Pyschrobacter* (Supporting Information Table S1). *Marinobacter* was enriched in CSW from corals collected from JR 1, but not enriched at the other reefs (Supporting Information Table S1). Additionally, non-Gammaproteobacteria taxa frequently identified in nutrient-rich or sediment environments were significantly enriched in CSW, including *Propionigenium*, unclassified Bacillales, *Chitinophagaceae*, Deltaproteobacterial OM27 clade, *Owenweeksia*, and *Erythrobacter* (Supporting Information Table S1). RSW from JR was generally significantly enriched with MED nodes classifying as microbial taxa that are found within free-living seawater microbial communities, including Rhodobacteraceae, the ultra-small "*Candidatus* Actinomarina," SAR11, SAR86, and SAR116 clades, and AEGEAN-169 and NS5 marine groups (Supporting Information Table S1).

Diverse *Endozoicomonas* bacteria associate with *P. astreoides* seawater

Using the amplicon sequence data, we detected seven *Endozoicomonas* MED nodes in CSW, demonstrating *Endozoicomonas* genotype diversity within the CSW (Fig. 2). Two *Endozoicomonas* MED nodes, MED3416 and 798, had the highest relative abundance in *P. astreoides* CSW across JR and CAN (Fig. 2). We compared the 16S rRNA gene sequence similarity for these two MED nodes with other 16S rRNA genes in NCBI and found that the MED3416 sequence was 98% similar to *Parendozoicomonas haliclonae*, a bacterial isolate from a marine sponge (NCBI sequence ID: NR_157681.1) and 96% similar to *Endozoicomonas euniceicola* (NCBI sequence ID: NR_109684.2), *E. numazuensis* (NCBI sequence ID: NR_114318.1), and *E. montiporae* (NCBI sequence ID: NR_116609.1). The MED798 representative amplicon sequence was 96% similar to *Endozoicomonas* cultures isolated from gorgonians and *E. montiporae*, as well as an isolate from the sea slug, *E. elysicola* (NCBI sequence ID: NR_041264.1). We also compared the representative *Endozoicomonas* sequences to each other and found that some of the most abundant MED nodes detected in *P. astreoides* CSW, MED3416, 798, 810, and 832, were 99% similar to each other whereas MED nodes detected at lower relative abundances in *P. strigosa* and/or *O. faveolata* CSW were less similar (MED3145, 98%; MED2581, 96%; MED1451, 95% similar). In general, the relative abundances of *Endozoicomonas* MED nodes were low in RSW (ranging from no detection to 1.7% relative abundance).

Genomic evidence of surface-attached and dynamic microbial communities within CSW

Comparisons between the pooled CSW and reef-depth RSW metagenomes revealed 1058 differentially abundant genes (Fig. 3). CSW metagenomes were significantly enriched in genes involved in 15 KEGG pathways (Table 3) and 6 KEGG modules (Table 4). The two-component system was the most significantly enriched pathway in CSW (Table 3) and included genes involved in cell-cycle and biofilm response regulation, signal transduction (histidine kinases), as well as chemotaxis (Supporting Information Table S2). The other enriched pathways within CSW metagenomes included bacterial chemotaxis, flagellar assembly, biofilm formation (in *Pseudomonas aeruginosa* and *Vibrio cholera*), bacterial secretion systems, ABC transporters, the *Caulobacter* cell cycle, lipopolysaccharide biosynthesis, nitrogen metabolism, pentose and glucuronate interconversions, cationic antimicrobial peptide resistance, geraniol degradation, and glycan degradation (Table 3).

The KEGG modules enriched within CSW included type II and IV secretion systems, denitrification, the dipeptide transport system, and the CheA-CheYBV chemotaxis and PleC-PleD cell fate control two-component regulatory systems (Table 4). The

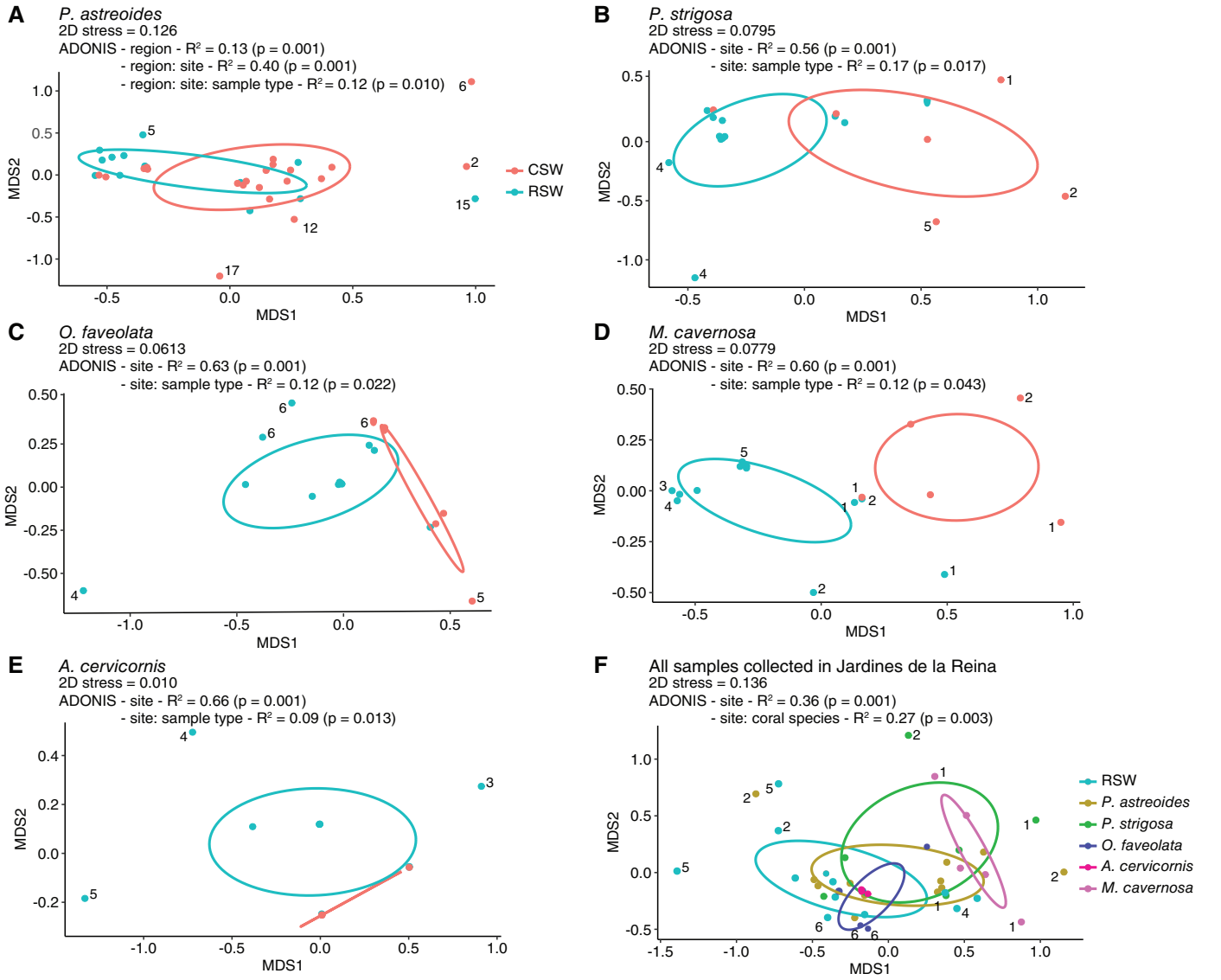

Fig. 1. NMDS analyses performed on Bray–Curtis dissimilarity indices that were obtained from square-root transformed relative abundances of 16S rRNA amplicon sequences grouped into MED nodes and results of the nested PERMANOVA (Adonis) tests. Ellipses are drawn using the group mean and covariance for each species. (**A**) *P. astreoides* CSW compared to RSW in JR and CAN, (**B**) *P. strigosa* CSW compared to RSW in JR, (**C**) *O. faveolata* CSW compared to RSW in JR, (**D**) *M. cavernosa* CSW compared to RSW in JR, (**E**) *A. cervicornis* CSW compared to RSW in JR, and (**F**) all JR samples. The number next to each symbol indicates the reef location of collection, and only the symbols outside of the covariance ellipses are labeled. The NMDS ordination stress and results of the nested PERMANOVA (Adonis) tests (factors, R^2 value, p value) are included for each comparison (**A**–**E**). The colon between factors indicates the nested structure of each PERMANOVA (Adonis) test; for example, "site: sample type" indicates that the factor "sample type" is nested within the factor "site." CSW = coral seawater; RSW = reef seawater.

CSW metagenomes also differed from each other by coral species (Fig. 3), but the most abundant KEGG pathways were the same.

Trends in microbial cell abundance over a distance gradient from the corals

Microbial cell abundances sampled over a distance gradient from each colony were highly variable by coral species, microbial group, and reef (Fig. 4; Supporting Information Figs. S3–S6). For *P. astreoides* and *P. strigosa*, abundances of microorganisms generally increased away from the colonies (Fig. 4; Supporting Information Figs. S3–S6). This trend was also observed for picoeukaryotes and unpigmented cells surrounding *M. cavernosa* colonies (Fig. 4; Supporting Information Figs. S5, S6). For *A. cervicornis*, the abundance of picocyanobacteria (*Prochlorococcus* and *Synechococcus*) increased with distance from the colonies, but picoeukaryotes and unpigmented cells displayed the opposite trend (Fig. 4; Supporting Information Figs. S3, S4). For *O. faveolata*, cell abundances from all groups decreased with

Table 3. Significantly enriched KEGG pathways in microbial metagenomes from CSW compared to RSW in JR.

Number*	Pathway definition	Orthology count†	Coverage‡	*p* value§
02020	Two-component system	136	0.28	1.57E-12
02030	Bacterial chemotaxis	19	0.73	1.41E-10
02040	Flagellar assembly	23	0.58	2.29E-09
02025	Biofilm formation—*P. aeruginosa*	34	0.38	3.11E-07
03070	Bacterial secretion system	30	0.41	3.38E-07
02010	ABC transporters	107	0.22	1.00E-04
04112	Cell cycle—*Caulobacter*	14	0.45	1.19E-04
00540	Lipopolysaccharide biosynthesis	16	0.40	2.26E-04
02026	Biofilm formation—*Escherichia coli*	21	0.34	3.08E-04
00910	Nitrogen metabolism	19	0.32	1.84E-03
00040	Pentose and glucuronate interconversions	20	0.29	5.37E-03
01503	Cationic antimicrobial peptide resistance	16	0.30	8.34E-03
00281	Geraniol degradation	6	0.40	2.24E-02
00511	Other glycan degradation	7	0.37	2.24E-02
05111	Biofilm formation—*V. cholerae*	25	0.23	2.88E-02

*KEGG pathway map number.
†Number of individual KOs from this study that are included within this pathway.
‡Normalized coverage of orthologs within each pathway.
§*p* values were calculated using Fisher's exact test.

distance from the colonies, except for *Synechococcus* (Fig. 4; Supporting Information Figs. S3–S6). For the sand seawater controls in CAN, the increases and decreases within microbial groups generally followed the trends observed for the corals (Fig. 4; Supporting Information Figs. S3–S6). The overall cell abundance of the different microbial groups was related to reef location, with very strong site specificity for *Prochlorococcus* (Supporting Information Fig. S3).

Discussion

In this study, we used genomics to determine that CSW microbial communities are influenced by coral species and reef site. More specifically, we detected enrichment of copiotrophic bacterial taxa and genes indicative of potential mobile, surface attached, and virulent microbial lifestyles within CSW compared to reef-depth seawater. Microbial cell abundances collected along a gradient from coral surfaces were variable but influenced by coral species, reef site, and cell type. Overall, these results provide taxonomic and functional genomic support for the existence of an environment that we term the "coral ecosphere," a distinct and dynamic environment for microorganisms that forms surrounding individual coral colonies and that may serve as an interaction zone between the coral surface and the overlying seawater. In Fig. 5, we present a conceptual diagram of the microbial functions, potential interactions, and bacterial taxa that are enriched within the coral ecosphere compared to the surrounding seawater.

Enrichment of primary colonizers within the coral ecosphere may be influenced by coral-derived organic matter

We detected enrichment of several copiotrophic Gammaproteobacteria, including the genera *Endozoicomonas*, *Bermanella*, *Marinobacter*, and *Alteromonas*, within coral ecospheres. Several of these taxa have been commonly associated with corals and coral-derived organic matter (OM) (Nelson et al. 2013), and Gammaproteobacteria are typically early colonizers of marine surfaces (Dang and Lovell 2000; Sweet et al. 2011). *Endozoicomonas* are an established tissue and mucus symbiont of corals globally (Apprill et al. 2016; Glasl et al. 2016; Neave et al. 2017; Pollock et al. 2018), and our results extend the current knowledge of *Endozoicomonas* biogeography by indicating that *Endozoicomonas* may reside in the seawater surrounding corals (specifically *P. astreoides*). The other enriched bacteria, including members within the genus *Bermanella* and the order *Alteromonadales*, have previously been found in association with coral-derived particulate and dissolved OM including coral tissue homogenates (Randall et al. 2016), coral mucus (Sweet et al. 2011), the seawater close to corals (Tout et al. 2014), and within natural RSW cultures inoculated with coral mucus and exudates (Allers et al. 2008; Nelson et al. 2013).

The enrichment of copiotrophic groups in coral ecospheres compared to the RSW is also paired with enrichment of specific metabolic pathways involved in the cycling of OM. Genes used in the denitrification pathway were significantly enriched in coral ecospheres compared to RSW, possibly suggesting that the ecosphere environment is populated

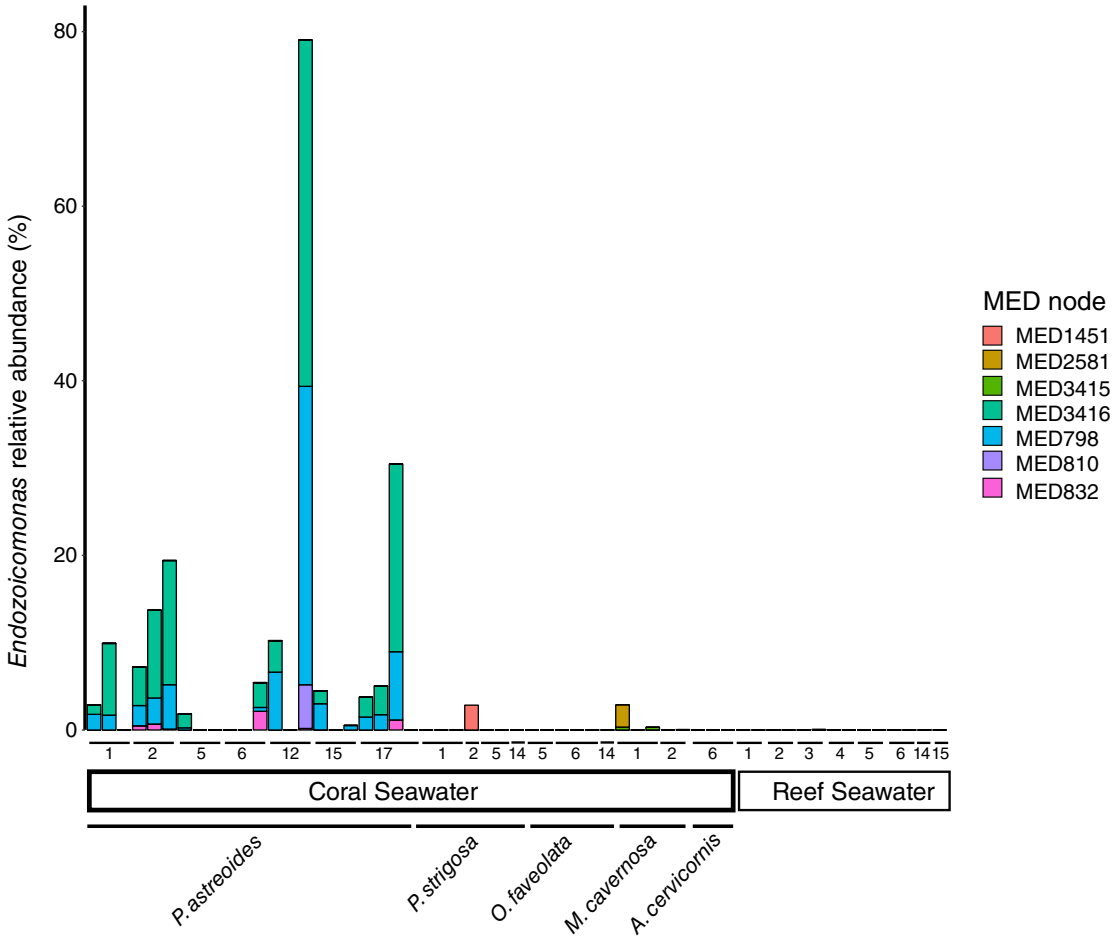

Fig. 2. Relative abundances of *Endozoicomonas* MED nodes identified in CSW and RSW samples. The numbers immediately underneath the bars indicate the reef site of collection. The black lines underneath the "coral seawater" or "reef seawater" rectangles indicate the coral species from which the surrounding seawater was sampled.

by anoxic or microaerobic regions where denitrification occurs, aligning with results of other studies that have investigated oxygen dynamics close to corals (Barott and Rohwer 2012; Wangpraseurt et al. 2012; Haas et al. 2013). In addition, corals exude amino acids and other dissolved organic nitrogen into the water column (Schlichter and Liebezeit 1991; Tanaka et al. 2009), and we detected enrichment of dipeptide transport system genes (ABC transporter) within ecospheres, suggesting that amino acid uptake could be an important source of nitrogen for microorganisms surrounding corals. *Alteromonas*, a genus shown to dominate natural seawater assemblages after the addition of dissolved OM produced by microbial communities fueled with nitrate and ammonium (Goldberg et al. 2017), was also enriched in a majority of the ecospheres, further suggesting that coral-derived OM may be influencing community composition within the ecosphere. Our genomic evidence suggests that ecosphere microbial composition may be influenced by the input of coral-derived OM and that microbial metabolisms within the ecosphere may be important for recycling and transferring this OM into the water column (Fig. 5).

Furthermore, the variability and lack of consistent trends in cell abundance suggest that interactions between multiple processes (including grazing or advection of cells) may mask influences of coral-derived OM on overall growth of planktonic microorganisms surrounding corals. It could also be that specific taxa, rather than the cell types we counted, respond to these coral-derived exudates and that these subtle responses cannot be detected using more coarse changes in microbial cell abundances. However, we did observe that microbial abundance was influenced by both coral species and reef location, reflecting the factors that influenced microbial community composition and suggesting that coral species influence these cell populations.

Microbes within the coral ecosphere are specialized for colonization and interaction with hosts

Coral ecospheres were enriched with microbial pathways characteristic of biofilm-forming, surface-attached, and potentially virulent microbial communities (Fig. 5). The two-component system pathway was the most significantly enriched

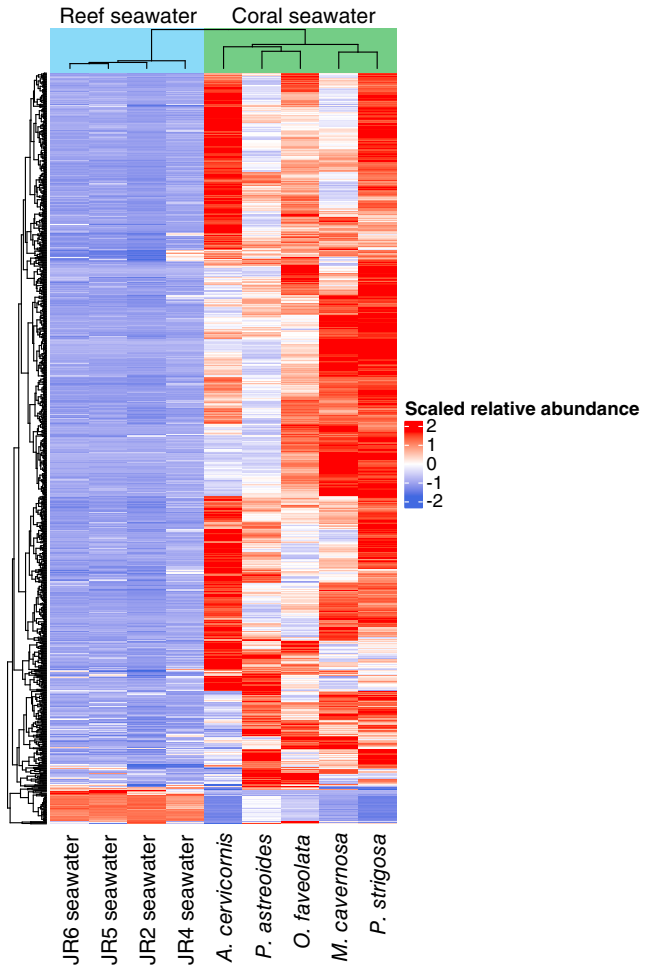

Fig. 3. Heatmap displaying the relative abundance of 1058 significantly different KO gene identifiers that were detected between CSW and RSW from JR. Relative abundances were calculated by dividing the KO counts for each gene by the total number of significantly different KO counts for each sample. The relative abundances of these KOs were scaled using the 10th and 90th quantiles of the data for visualization. The dendrogram reflects hierarchical clustering of the samples using the "hclust" function in R.

pathway within coral ecospheres and included genes involved in response regulation, cell-cycles, signal transduction, and chemotaxis. Genes used in the two-component system are found in bacteria, archaea, and eukarya but are most abundant in gram-negative bacteria and cyanobacteria (Capra and Laub 2012). The two-component signal transduction system permits bacteria to sense and respond to external stimuli (Capra and Laub 2012) and is also involved in the development of virulence and antimicrobial resistance (Gooderham and Hancock 2009). Enrichment of the two-component system suggests that cells within the coral ecosphere may be able to rapidly respond to changes in this fluctuating marine environment. For example, bacteria may respond to an environmental cue by transcribing virulence genes that enable them to colonize a host and potentially cause disease (Ribet and Cossart 2015). Furthermore, the type II and IV bacterial secretion systems were also enriched within coral ecosphere metagenomes and these systems are typically used by bacteria to colonize surfaces, transport and secrete molecules, induce endocytosis within the host cell, acquire virulence genes, and disrupt host cell defenses (Kohler and Roy 2015; Green and Mecsas 2016). The enrichment of secretion systems near corals suggests that these infection strategies may be used by putative pathogens as well as symbionts residing within the ecosphere microbial community to colonize the coral host.

Additionally, the prevalent KEGG pathways and modules detected within coral ecospheres suggest that specific taxa within these microbial communities have the capacity to exhibit chemotaxis, transport solutes, as well as produce, secrete, and resist antibiotics (Fig. 5). Many of the enriched coral ecosphere genes are also classified as interaction genes (Torto-Alalibo et al. 2009; Cardenas et al. 2018), genes that permit microorganisms to colonize and interact with hosts (Dale and Moran 2006). In support of our hypothesis, Tout et al. (2014) detected elevated abundances of these interaction genes, including bacterial chemotaxis and motility, membrane transport, and cell signaling genes, within the seawater close to the corals *Acropora aspera* and *A. palifera* (Tout et al. 2014). Additionally, Walsh et al. (2017) detected enrichment of genes used for antibiotic resistance, resistance to toxic compounds (methicillin resistance), and motility and chemotaxis in the seawater adjacent to *M. braziliensis* (Walsh et al. 2017).

Furthermore, there are similarities between the potential microbial metabolic pathways detected within the coral ecosphere and coral tissue. Bacterial, archaeal, and fungal genes used to catalyze different conversions within the nitrogen cycle are commonly found in coral tissue metagenomes (Wegley et al. 2007; Vega Thurber et al. 2009; Kimes et al. 2010; Garcia et al. 2013), and we detected an enrichment of denitrification genes within the coral ecosphere. Metal tolerance and antimicrobial resistance genes as well as virulence genes have also been identified in coral tissue metagenomes (Vega Thurber et al. 2009; Kimes et al. 2010; Garcia et al. 2013) as well as in our study. In contrast, oxidative stress genes were more regularly detected in coral tissue metagenomes (Wegley et al. 2007; Vega Thurber et al. 2009), whereas motility and chemotaxis genes and genes used in the secretion of lipopolysaccharides and for biofilm formation were more commonly detected in coral ecosphere metagenomes.

The coral ecosphere may be a reservoir for potential coral symbionts and pathogens

Our results suggest that corals are bathed in microbial cells that are capable of colonizing and interacting with the coral surface. As such, the coral ecosphere may serve as a reservoir for coral symbionts or pathogens. In support of this hypothesis, we detected a prevalent association between the coral *P. astreoides* and *Endozoicomonas* bacteria within the coral ecosphere at sites JR 2, 5, and 6 using differential enrichment tests. *Endozoicomonas* MED nodes were also detected in

Table 4. Significantly enriched KEGG modules in microbial metagenomes from CSW compared to RSW in JR.

Module number*	Module definition	Orthology count†	Coverage‡	*p* value§
00333	Type IV secretion system	11	0.92	2.20E-03
00331	Type II general secretion pathway	13	0.76	1.73E-02
00529	Denitrification, nitrate≥nitrogen	9	0.82	2.51E-02
00324	Dipeptide transport system	5	1	2.62E-02≥
00506	CheA-CheYBV (chemotaxis) two-component regulatory system	5	1	2.62E-02
00511	PleC-PleD (cell fate control) two-component regulatory system	5	1	2.62E-02

*KEGG module map number.
†Number of individual KOs from this study that are included within this module.
‡Normalized coverage of orthologs within each module.
§*p* values were calculated using Fisher's exact test.

P. astreoides ecosphere samples at JR 1 but were not significantly enriched relative to RSW after *p*-value corrections for multiple testing were applied. Nevertheless, this association suggests that either *Endozoicomonas* cells reside in the seawater and are attracted (i.e., through some chemical cue) to the coral surface or *Endozoicomonas* cells are shed from the coral mucus or tissue. There is evidence supporting both of these hypotheses. *Endozoicomonas* genomes are fairly large (> 5 Mbp) and equipped with genes required for degrading amino and nucleic acids (Neave et al. 2014; Neave et al. 2017) as well as genes coding for enzymes that are used to degrade testosterone and glycosidic bonds (named Endo-AEmo) in glycoproteins (Ding et al. 2016). Ding et al. (2016) suggested that *Endozoicomonas* may be able to attach to the coral mucus layer, penetrate the mucus using the Endo-AEmo enzyme, and then enter the host tissue via endocytosis. Alternatively, *Endozoicomonas* genes may reside within the ecosphere because they have been shed from coral tissue and mucus. *Endozoicomonas* was identified as a dominant member of the newly formed communities in *P. astreoides* mucus (Glasl et al. 2016) and as cells die and mucus sloughs off into the water column, *Endozoicomonas* cells may be shed into the ecosphere environment. In addition to their putative roles as common coral symbionts, *Endozoicomonas* cells residing in the *P. astreoides* coral ecosphere may influence the ecosphere chemically through the production of extracellular superoxide. This ubiquitous molecule can be found in the coral ecosphere of *P. astreoides*, is produced by *Endozoicomonas* isolates, and likely plays important roles in bacterial interactions and coral health (Diaz et al. 2016; Zhang et al. 2016).

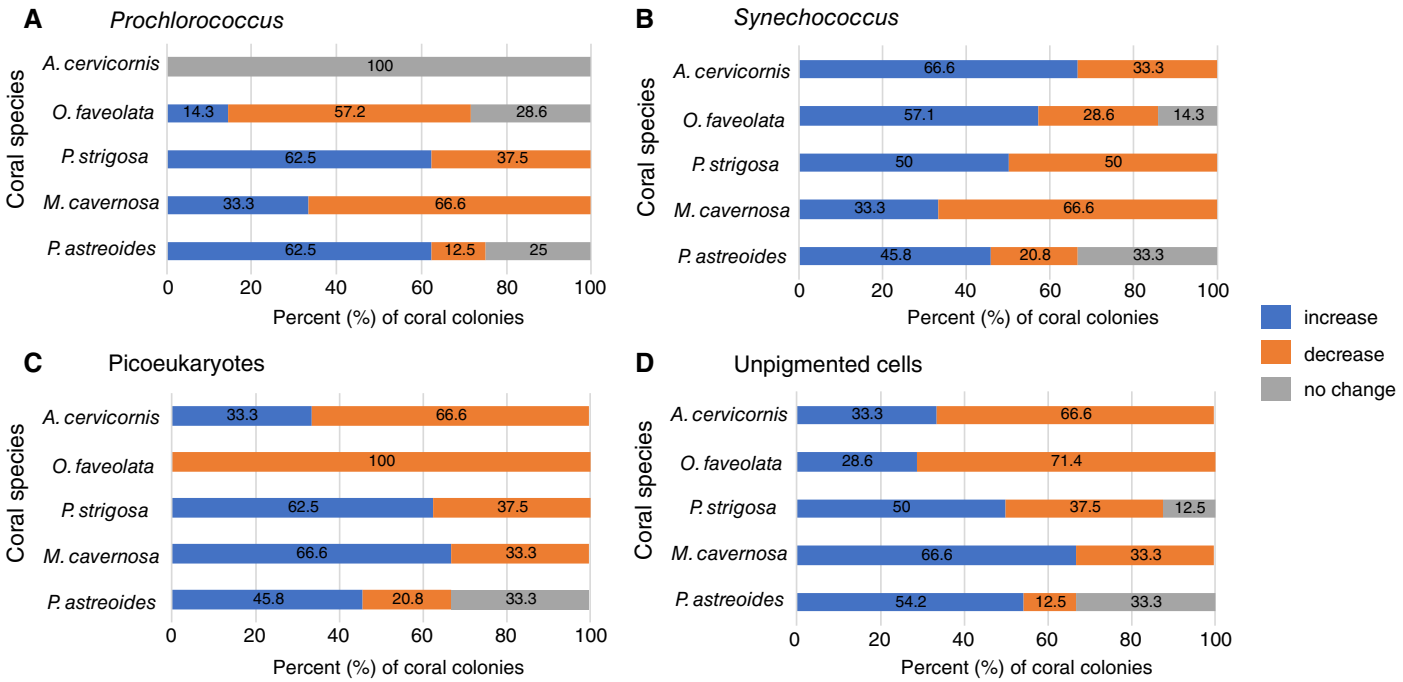

Fig. 4. Stacked bar graphs organized by picoplankton functional group in panels **A**–**D** depict the percent (%) of coral colonies by coral species where the abundance of cells increased (blue), decreased (orange), or where there was no change as the distance from the colonies increased. The numbers overlaid on the stacked bars reflect the percentage of each category.

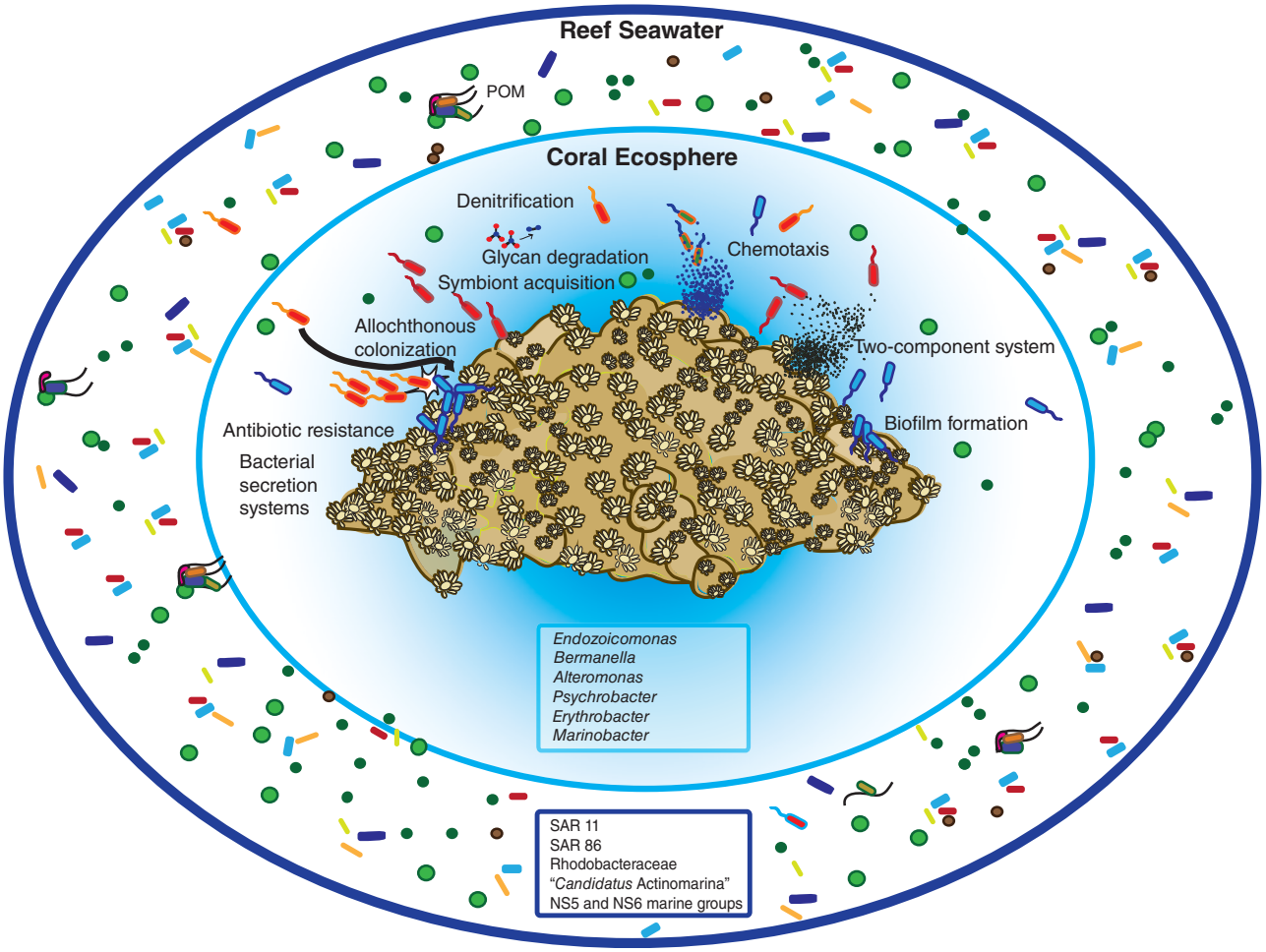

Fig. 5. Conceptual diagram of the microbial functions, potential interactions, and enriched bacterial taxa identified within the coral ecosphere. The coral ecosphere and reef-depth seawater hemispheres surround a representative *P. astreoides* coral colony. A light blue line highlights the coral ecosphere boundary and select microbial functions that emerged from this study are illustrated, with microbial cells depicted as colored circles or capsular objects. A darker blue line depicts the reef-depth seawater hemisphere. Enriched bacterial taxa are contained within the boxes within each hemisphere and are based on the within site coral and reef-depth seawater comparisons. The illustrations are not to scale and POM indicates particulate organic matter.

The exogenous recruitment of specific bacteria to corals has been investigated for larvae (Sharp et al. 2010; Apprill et al. 2012) but is still unresolved for adult colonies. Sweet et al. (2011) touched on this topic when they proposed that specific bacteria were recruited from the water column into the developing coral mucus biofilm through some selective process or direct contact with another surface. Our study extends this hypothesis by demonstrating that some of the primary mucus colonizers detected by Sweet et al. (2011) was also enriched within the coral ecospheres. Detailed exploration of microbial interactions within the coral ecosphere will deepen our understanding of which microbes are available to the corals to serve as potential symbionts and how the coral host, as well as the external environmental conditions, influence these microbial interactions.

Considerations for studying the coral ecosphere

In our comparisons, the reef of collection was also identified as an important predictor of microbial community composition. This finding suggests that local environmental conditions, like current direction and speed, temperature, light, and nutrient availability, may also influence microbial growth and community composition within the ecosphere. For example, seaward reef locations within JR are exposed to stronger currents (up to 40 cm s^{-1}) on average compared to locations within the Gulf of Ana Maria (13 cm s^{-1}; Arriaza et al. 2008), and these conditions likely influence the flux of cells and nutrients within the coral ecosphere. Future studies could investigate the connection between water flow and microbial dynamics within coral ecospheres. The variability in microbial community similarity between RSW samples collected within JR also corroborates the strong influence of reef-specific environmental conditions but is within the range of variability observed in seawater microbial communities at smaller geographic scales (Apprill et al. 2016). Future studies of the dynamic environment of the coral ecosphere should undoubtedly account for reef-specific variation by collecting

more ecosphere samples (biological replicates) at each site for every species surveyed. Additionally, although we were able to discern differences between CSW and RSW in this study, the magnitude of this distinction may be greater if paired CSW and RSW samples were collected at a variety of different reefs.

Continued research into microbial interactions within the coral ecosphere requires recognition of potential methodological biases and improvements to these methods, contingent upon the technology. We used 60 mL syringes to sample seawater from the coral ecosphere so that we could gently aspirate seawater close to each colony in a controlled manner. We then compared these ecosphere microbial communities to RSW communities that were sampled with larger volumes using a groundwater pump. We recognize that differences in sampling method could have led to potential biases in our analyses so we have made an effort to address the realized and potential impacts of these differences. First, we completed a field-based seawater volume experiment to understand how differences in sampling volume impacted microbial community analysis and found that sampling volume did not influence metrics of beta-diversity or enrichment analysis. Secondly, while we cannot directly examine how sampling method (syringe vs. groundwater pump) would influence our results, we postulate that cells could have been exposed to different physical stressors or grazing pressures when they were sampled with different techniques. That being said, we have used groundwater pumps to collect samples for flow cytometry and for microbial respiration experiments in the past and have no reason to believe that this method is shearing cells or collecting water in a manner that makes these collections incomparable to syringe-based collection methods. Furthermore, we stored the samples on ice immediately after they were harvested to reduce the influence of grazing or altered growth dynamics within the collection containers. Even if growth and grazing did occur, these processes would be unlikely to influence our results, because the average doubling time of microbial cells within RSW at ambient reef temperature is about a day (McNally et al. 2017) and zooplankton abundances are relatively low in RSW, present on the order of 0.06 zooplankter L^{-1} (0.004 zooplankton within 60 mL; Cox et al. 2006). Additionally, after sample collection, we also made efforts to standardize the concentrations of DNA used in PCR reactions in order to minimize the impact of sample volume and collection method. Lastly, we used conservative data analysis (subsampling and quality filtering) and multivariate statistical approaches (e.g., Bray–Curtis dissimilarity, NMDS, and PERMANOVA) to analyze the data.

Conclusions

We have shown that five reef-building coral species were surrounded by a distinct microbial environment, the coral ecosphere, which in turn was influenced by local environmental conditions at each reef. This coral ecosphere supports taxonomically and functionally distinct microbial communities and constitutes a dynamic seawater habitat harboring cells that seem capable of interacting with the coral surface. Recognition of the coral ecosphere provides new opportunities to study coral–microbial interactions within the water column and exogenous recruitment of microorganisms, including pathogens, to colonies. Future directions in coral ecosphere research include understanding the ecosphere microbial community variability in the context of changing environmental conditions, documenting how cells within the coral ecosphere use coral-derived OM, and exploring the significance and contribution of these interactions to biogeochemical cycling on coral reefs.

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Conflict of Interest

None declared.

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