

Bacterial shifts associated with coral–macroalgal competition in the Caribbean Sea

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ABSTRACT: Present-day coral reefs are impacted by frequent disturbance and increasing global stressors, which provide an environment that supports the growth of alternative dominants, such as macroalgae, sponges, and tunicates, rather than stony corals. Competitively damaging interactions arise between macroalgae and reef-building corals that can involve chemical, physical, and microbial mechanisms. Using 16S rRNA denaturing gradient gel electrophoresis (DGGE), amplicon sequencing, and multivariate analyses, we examined coral- and algae-associated bacterial assemblages along interaction gradients between the common Caribbean stony corals *Montastraea faveolata* and *Porites astreoides* and the benthic macroalgae *Halimeda opuntia* and *Dictyota menstrualis*. Benthic surveys were conducted in 3 Caribbean locations, and interactions were sampled for bacterial analyses. Both macroalgae were capable of inducing shifts in coral-associated bacteria to an assemblage that more closely resembled macroalgal-associated bacteria. Overall, *M. faveolata*-associated bacteria were more significantly affected by macroalgal competitors than were *P. astreoides*-associated bacteria at all sites. Bacteria associated with *M. faveolata* corals changed during >83% of interactions, even when samples were taken 5 cm away from competing macroalgae. Detectable shifts in coral-associated bacteria at all sampling sites suggest that macroalgae play a large role in coral holobiont health. The degree to which coral species maintain stable microbial assemblages in the face of environmental fluctuations and competitive stress may indicate specific species and coral reefs that have the capacity to resist perturbations and those that may need additional protection in the future.

KEY WORDS: *Montastraea faveolata* · *Porites astreoides* · Macroalgae · Microbial community · Allelopathy · Benthic survey · *Halimeda* · *Dictyota*

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INTRODUCTION

Coral reefs currently are undergoing global degradation due to natural and anthropogenic impacts that are increasing in frequency and intensity, including impacts from climate change (Hughes & Connell 1999, Hoegh-Guldberg et al. 2007, Pandolfi et al. 2011). Frequent disturbances, such as tropical storms, predation outbreaks, diseases and mass bleaching events, reduce the percentage of cover of

living coral on reefs, and without recovery, the available substratum is then colonized by sponges, soft corals, and macroalgae (reviewed by Chadwick & Morrow 2011). These competitors often become alternative dominants on stressed coral reefs and prevent the recovery of corals following disturbances (Norström et al. 2009). Phase-shifts from coral-dominated reefs to those dominated by other benthic organisms may be facilitated by reduced rates of herbivory (due to disease and/or overfishing) and by

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nutrient enrichment (from run-off and coastal eutrophication; see relative dominance model in Littler et al. 2006). The ability of particular coral holobionts to resist the effects of disturbances and phase-shifts may indicate species resilience. In the present study, we define resilience as the capacity to absorb, reorganize, and adapt to change resulting from stressors or disturbances (Nyström & Folke 2001, Mumby et al. 2007).

Over the past several decades, the incidence of coral disease has increased worldwide, in parallel with rising macroalgal cover on coral reefs (Goreau et al. 1998, Harvell et al. 1999, Weil et al. 2006). An enhanced emphasis on coral disease research, coupled with recent advances in molecular techniques, has highlighted the significant role that microorganisms play in the physiology of both healthy and diseased corals. Corals associate with diverse assemblages of microorganisms that are distinct from those in the water column and that may play a dominant role in host health and metabolism (reviewed by Ritchie & Smith 1997, Rohwer et al. 2001, Rosenberg et al. 2007a, Bourne et al. 2009, Sunagawa et al. 2010). Of the relatively modest number of coral species whose microbial communities have been assessed (~25; see references in Morrow et al. 2012a), many demonstrate some level of microbial specificity (Rohwer et al. 2002, Sunagawa et al. 2010). The composition of these healthy bacterial assemblages and of opportunistic infections can vary depending on geographic location (Littman et al. 2009, Sunagawa et al. 2009, Morrow et al. 2012a), the occurrence of bleaching events (Bourne et al. 2008), and fluctuations in environmental parameters such as temperature, pH, and nutrients (Vega Thurber et al. 2009). Despite the many important roles that coral microbes play in host physiology and health through the production of antibiotics (Ritchie 2006) and regulation of biogeochemical cycling (Lesser et al. 2004, Wegley et al. 2007, Raina et al. 2009, Kimes et al. 2010, Lema et al. 2012), it remains unclear whether coral hosts or external factors most influence the microbial community. Coral holobionts may adapt to changing environmental conditions by shifting their resident microbial assemblages, a concept first termed the coral probiotic hypothesis (Reshef et al. 2006) and subsequently the hologenome theory (Rosenberg et al. 2007b). However, the microorganisms that coexist in various compartments of the coral holobiont, including the surface mucopolysaccharide layer (SML), tissues, gut, and skeleton, may also vary in their maintenance of resident or transient microbial associates (Sweet et al. 2010).

Macroalgae likely mediate coral reef microorganisms through several mechanisms: (1) release of dissolved organic carbon (DOC) compounds (Smith et al. 2006, Haas et al. 2011), (2) production of antibacterial secondary metabolites (i.e. allelochemicals; Ballantine et al. 1987, Morrow et al. 2011), and (3) support of a diverse reservoir of potentially pathogenic microorganisms (Nugues et al. 2004, Barott et al. 2011). Elevated levels of DOC can cause coral mortality (Kuntz et al. 2005) and increased microbial activity (Kline et al. 2006). Direct coral–macroalgal contact may be a prerequisite to microbial-driven hypoxia (Barott et al. 2009, Vu et al. 2009) or the transfer of pathogens (Nugues et al. 2004). Labile carbon excreted by benthic macroalgae can also stimulate planktonic microbial activity (Haas et al. 2010). Thus, reefs dominated by macroalgae have lower levels of O₂ in the overlying water column (Dinsdale & Rohwer 2008, Haas et al. 2010). Algal-produced allelochemicals may cause oxidative imbalance and subsequent protein degradation in corals, in some cases leading to apoptosis and/or necrosis of coral tissues (Shearer et al. 2012). High macroalgal abundance, particularly when coupled with low water flow and reduced herbivory, appears to suppress the health of corals and alter their resident microbiota (Vega Thurber et al. 2012), leading to reduced reef resilience and potentially reef-wide effects on coral disease and mortality.

In the present study, we examined whether coral competition with macroalgae correlates with shifts in coral-associated bacterial assemblages at both the coral–algal interface and on a larger colony-wide scale at several sites in the Caribbean Sea. Denaturing gradient gel electrophoresis (DGGE) analyses of 16S rRNA gene profiles isolated from naturally occurring coral–algal interaction gradients revealed shifts to algae-dominated assemblages. Coral colonies free of encroaching macroalgae or benthic invertebrates were sampled as controls at each site. SML bacterial samples were collected in St. Thomas, U.S. Virgin Islands (USVI), the Florida Keys, and Belize from 2 ubiquitous Caribbean corals (*Montastraea faveolata* and *Porites astreoides*) and adjacent foliose brown macroalgae (*Dictyota menstrualis*) and calcareous green macroalgae (*Halimeda opuntia*). Both macroalgae are common on Caribbean coral reefs even when grazing is intense (Littler & Littler 1994); they also reduce coral growth rates, cause coral tissue mortality, and produce potent allelochemicals that are active against coral reef microorganisms (Lirman 2001, Beach et al. 2003, Rasher & Hay 2010, Morrow et al. 2011). At all sites in the

study described here, *M. faveolata* were associated with stronger shifts in the coral microbiome than were *P. astreoides*.

MATERIALS AND METHODS

Coral-algal diversity surveys

Surveys were conducted at 3 sites: Flat Cay Reef (18° 19.04' N, 64° 59.26' W), adjacent to the University of the Virgin Islands MacLean Marine Science Center, St. Thomas, USVI (July 2008 and 2009); the Florida Keys reef tract, stretching from the Dry Tortugas National Park in the south (24° 28.10' N, 82° 35.09' W) north to Carysfort Reef (25° 16.21' N, 80° 12.46' W) near Key Largo, Florida (September 2009); and South Water Cay Reef (16° 48.24' N, 88° 04.42' W), adjacent to the Smithsonian's Carrie Bow Cay Field Station in Belize (October 2008 and August 2009). Surveys in Florida were conducted aboard the National Oceanic and Atmospheric Administration (NOAA) RV 'Nancy Foster' in conjunction with the Florida Keys National Marine Sanctuary (FKNMS) Coral Health and Diversity Cruise under direction of the NOAA. Corals were identified to species (Humann & Deloach 2002) and macroalgae identified to genus at each site (Littler & Littler 2000).

NOAA-FKNMS surveys were conducted using a radial arc method developed for coral disease studies (Santavy et al. 2001, 2005). Divers deployed a stainless steel rod at each previously defined site (see Santavy et al. 2001) and fastened a 12 m line to the rod by a carabiner that rotated freely. One diver pulled the line taut and slowly moved the line in an arc around the fixed central point. Two additional divers surveyed the circular band transect between the 8 and 10 m mark, which encompassed an area of 113 m². Previous studies determined that the 2 m band was sufficient to obtain a reliable estimate of coral reef disease prevalence (Santavy et al. 1999, 2001). One diver recorded the number of colonies of each coral species and whether signs of bleaching or disease were present, including Black Band, Dark Spot, Yellow Band, White Plague, Pox, and Band, while the second diver measured the length, width, and height of the first 10 colonies of each species encountered within the arc. Corals were included in the survey if at least half the coral colony was within the 8 to 10 m belt. The second diver also determined whether the first 10 encountered colonies of each species were interacting (i.e. in direct tissue contact along ≥ 1 edges) with *Dictyota* spp., *Halimeda* spp.,

Lobophora variegata, sponges, tunicates, invertebrate predators (e.g. corallivorous snails), and/or other benthic organisms.

The radial arc method required a large number of divers available from the NOAA ship and predrilled installation sites for the central rod; thus, we adopted a simpler but similar method for the surveys conducted in Belize and St. Thomas, where fewer divers were available. A 25 m linear band transect was established parallel to the reef crest with a randomly selected starting location. One diver swam each band transect in both directions, recording the number of colonies of each coral species within 1 m on either side of the transect tape, which encompassed a total area of 50 m². The first diver also recorded colony appearance, including the presence of bleaching, disease, or benthic interactions (as described above). A second diver swam the same band transect and placed a 0.25 m² divided quadrat at 10 locations, approximately every other meter and alternating sides of each transect. The quadrats were subdivided into 25 squares (each representing 4% of the quadrat), and the benthic component dominating each subdivision was recorded (after Carpenter & Edmunds 2006).

Bacterial sample collection

Bacterial samples were collected from the SML of apparently healthy *Montastraea faveolata* and *Porites astreoides* coral colonies at the above sites in Belize and St. Thomas, USVI. Microbial samples in Florida were collected from Looe Key Reef (24° 32.53' N, 81° 24.22' W) in the Florida Keys (September 2009). Collections were made via SCUBA at 5 to 10 m depth using sterile 5 ml syringes that were capped before and after sample collection. At each collection site, a 5 × 5 cm area was gently agitated on the surface of each coral using the plastic tip of the syringe, which encouraged sloughing of the viscous coral surface mucus (Ritchie 2006) (Fig. 1c). Sterile nitrile gloves were worn during collection to reduce human or seawater bacterial contamination.

Samples were collected from the SML ~5 to 10 cm from the colony edge of coral controls (CC), colonies of *Montastraea faveolata* and *Porites asteroides* species that were >1 m apart and not obviously in contact with other corals, macroalgae, or benthic invertebrates (n = 3 Belize and Florida, n = 5 St. Thomas; Fig. 1). Samples were also collected from corals that contacted the macroalgae *Dictyota menstrualis* (brown fleshy alga; Fig. 1a) and *Halimeda opuntia*

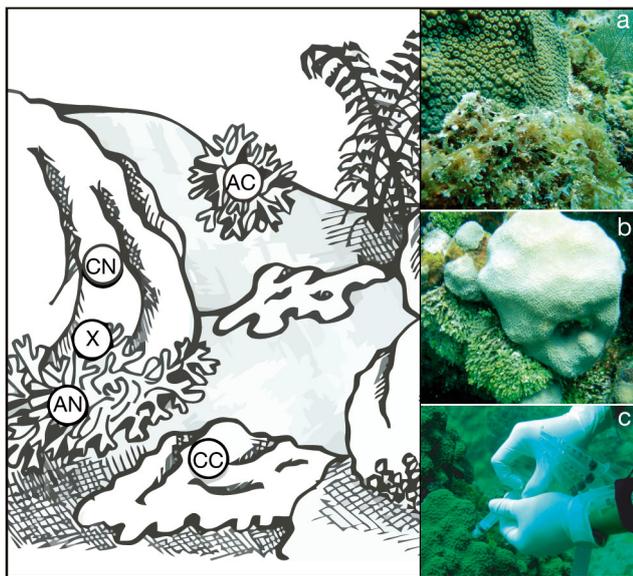


Fig. 1. Microbial sampling regime used to examine natural coral–algal interaction gradients. Plastic-tipped syringes (5 ml) were used to sample a 5×5 cm area of the coral surface mucopolysaccharide layer (SML) and macroalgal surface for molecular analysis from Coral Controls (CC; *Montastraea faveolata* or *Porites astreoides*) not interacting with (i.e. contacting) macroalgae or other benthic invertebrates; Coral Near (CN): coral SML near the coral–algal interface (5 cm away); Interaction (X): coral SML in direct contact with macroalgae; Algae Near (AN): the macroalgal surface near the coral–algal interface (5 cm away); and Algal Controls (AC) (*Dictyota menstrualis* or *Halimeda opuntia*) not interacting with corals or other benthic invertebrates. Photos depict (a) *Dictyota menstrualis* interacting with *Montastraea* sp., (b) *Halimeda opuntia* interacting with *Montastraea* sp., and (c) the method for collecting coral mucus with a plastic-tipped syringe. Photo credit: (a,b) K. M. Morrow, (c) J. Voss

(green calcareous alga; Fig. 1b), along the interaction zones where their tissues were in contact. Samples were collected as follows—coral near (CN): coral SML 5 cm from the coral–algal interaction zone ($n = 3$); interaction (X): coral SML in direct contact with macroalgae ($n = 3$); and algae near (AN): the surface of macroalgae adjacent to coral–algal interaction zones ($n = 3$, Fig. 1). Finally, an algal control (AC) sample was collected from the surfaces of *D. menstrualis* and *H. opuntia* thalli that were not obviously in contact with corals, macroalgae, or other benthic invertebrates ($n = 3$ Belize and Florida, $n = 5$ St. Thomas; Fig. 1).

After collection, the syringes were placed in seawater-filled coolers and transported back to the laboratory (<3 h), where they were immediately processed for transport and subsequent culture-independent analyses. The syringes were placed tip down in test-tube racks for ~15 min to allow the

mucus to settle to the bottom, then 2 ml of the concentrated mucus were aspirated into cryovials and centrifuged at $10\,000 \times g$ for 10 min. The seawater supernatant was decanted, and the remaining mucus pellet was frozen at -20°C . Bacterial samples were transported to Auburn University on ice and thawed at 4°C prior to DNA extraction using the MOBIO Ultraclean® Microbial DNA Isolation kit, according to the manufacturer's instructions, with the recommended (10 min) heating step to 64°C to increase DNA yield. Extracted genomic DNA was stored at -80°C until PCR amplification.

PCR amplification and DGGE protocol

Universal bacterial primers 27F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG CAG AGT TTG ATC MTG GCT CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') were used to amplify the 16S rRNA gene from isolated bacterial genomic DNA from coral mucus. The forward primer was modified to incorporate a 40 bp GC clamp (underlined above) for resolution on a DGGE system (Muyzer et al. 1993, Ferris et al. 1996). These primers amplified a 491 bp section of the 16S rRNA gene of members of the domain *Bacteria*, including the highly variable V1 to V3 regions (Ashelford et al. 2005, Huse et al. 2008). All PCR were performed on a thermal-cycler (model: Master cycler eppgradient, Eppendorf) as follows: 12.5 μl EconoTaq PLUS GREEN 2X Master Mix (Lucigen) and 0.5 μl of each 20 μM primer, adjusted to a final volume of 25 μl with nuclease-free water. Strip tubes, master mix, and nuclease-free water were UV-irradiated for 20 min prior to the addition of primers (Millar et al. 2002). DNA template was amplified following a 'touchdown' PCR protocol, in which the annealing temperature was decreased from 65°C by 1°C every cycle until reaching a touchdown temperature of 54°C , at which temperature 35 additional cycles were performed as follows: 94°C for 45 s, 54°C for 45 s, and 72°C for 1.5 min, with a single final cycle at 94°C for 45 s, 54°C for 45 s, and 72°C for 7 min followed by cooling to 4°C .

Samples displaying effective amplification of 16S products were separated using a conventional vertical gel electrophoresis apparatus (model Hoefer SE 600) warmed with a tank heater (Lauda model M6a; Brinkmann Instruments) modified for use as a DGGE system (as in Nübel et al. 2001, Casamayor et al. 2002). PCR products and reference standards were loaded onto an 8% acrylamide gel and run with 0.5 TAE buffer (Tris base, acetic acid, and EDTA)

and a 35 to 60% linear denaturing gradient of formamide and urea. Reference standards were composed of PCR-amplified and pooled bacterial isolates that produced bands that spanned the gel gradient. Gels were first electrophoresed at 60°C for 15 min at 50 V and subsequently for 10 h at 100 V (or 1000 Vh) in the DGGE system. After electrophoresis, the gels were stained for 30 min with SYBR-Gold nucleic acid stain at a 1:10 000 dilution (Invitrogen) in TAE buffer, rinsed, and imaged using an AlphaImager HP gel documentation system (ProteinSimple).

Band excision and sequencing

Uniquely dominant and distinct bands were dabbed with a sterile pipette tip and placed directly into PCR strip tubes containing UV-sterilized nuclease-free water. The bands were re-amplified with the previously described touchdown protocol using the 27F/518R primer set without the GC clamp. The PCR products were checked with agarose gel electrophoresis (1% w/v agarose) stained with ethidium bromide and visualized using a UV transilluminator. An ammonium acetate-ethanol precipitation was performed by freezing the sample for 1 h at –20°C, followed by centrifugation at 4°C and a 70% ethanol wash. Genomic DNA was resuspended in sterile molecular-grade water and amplified using the BigDye® sequencing reaction: 1.0 µl of BigDye®, 1.5 µl of 5× Buffer, 0.5 µl of 10 µM 27F, 4 µl of nuclease-free water, and 3 µl of template. The following thermocycler conditions were used: 95°C for 30 s, 50°C for 30 s, and 60°C for 4 min, at which temperature 30 additional cycles were performed. The PCR products were purified using the BigDye® XTerminator Purification Kit (Applied Biosciences) and shipped to the Smithsonian Institution Laboratories of Analytical Biology (Suitland, MD, USA) for sequencing. The Smithsonian Institution performed high-throughput (96-well) sequencing on an ABI sequencer. Sequences were trimmed using CLC Genomics Workbench (CLC Bio), and the resulting sequences were compared to the NCBI nr/nt database using the BLASTn analysis tool. Sequences that displayed >96% identity and expected values $< 1 \times 10^{-20}$ were accepted for downstream analysis.

DGGE image analysis

Gel images were imported into Bionumerics V 5.0 (Applied Maths). To ensure that multiple gel images

could be reliably compared, they were subjected to the following series of steps: identify sample lanes, apply background subtraction, normalize to reference standards (described above), and identify bands. Sample comparison and band matching was initially conducted in Bionumerics, where band classes were constructed based on optimal position tolerance and optimization settings. A DGGE fingerprint for each coral–algal species pair for each site was converted to a binary matrix based on band presence/absence (1/0) and was exported from Bionumerics for multivariate analysis in the R statistical package and PRIMER.

The experimental design consisted of 1 factor: Treatment, a fixed factor with 5 levels (coral control [CC], coral near algae [CN], coral–algal interaction zone [X], algae near coral [AN], and algal control [AC]). Of particular interest was the contrast between CC and X groups, i.e. CC vs. CN, X, and AN. Multivariate analyses were performed on the basis of Jaccard distance measures for each coral–algal species pair for each site. The rank dissimilarities in composition among the bacterial assemblages in different treatments were visualized using Kruskal's nonmetric multidimensional scaling (nMDS) on the distances among centroids from the replicates per treatment. Coral–algal interactions were analyzed by site because spatial differences may occur among bacterial assemblages associated with the same coral species (Morrow et al. 2012a). Multivariate analyses were performed using the metaMDS utility within the Vegan package in R (Oksanen et al. 2009) and the nMDS function within PRIMER v6 (PRIMER E). metaMDS was unique in that it called on isoMDS to perform nMDS but also searched for the most stable solution by performing several random starts (we used 20; R Development Core Team 2012). The relationship among samples was represented in a plot of the first 2 nMDS dimensions from PRIMER v6. Hierarchical cluster analyses (complete linkage) of band patterns were constructed based on Jaccard distances, and similarity contours (25 and 50% similarity) were added to each corresponding nMDS plot.

Permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was used to analyze each resemblance matrix based on Jaccard distances and was performed using PERMANOVA+ for PRIMER v6. Post-hoc contrasts examined whether the bacterial community composition from corals interacting with macroalgae was significantly different from the composition of assemblages associated with coral controls, similar to the procedure applied by Martin et al. (2011). Specific sets of treatment combinations

were tested (see Fig. 1). The terms 'winner' and 'loser' were employed to clarify a complex set of data, after the use of these terms in other coral reef studies (Loya et al. 2001, Barott et al. 2012).

RESULTS

Coral–algal survey results

We surveyed a total of 2807 m² and recorded ~2.4 corals m⁻² within 29 species (Table 1). The percentage of corals interacting with other benthic organisms was highest on Florida reefs, followed by Belize and St. Thomas (Fig. 2b). A particularly high number of coral–algal interactions occurred on Florida reefs, especially involving macroalgae of the genera *Dictyota* (70% of corals) and *Halimeda* (40% of corals) (Fig. 2b). *Dictyota* was the most abundant macroalgae (18 ± 6% cover, mean ± SD), particularly in Belize, where it composed 25 ± 4% of all algae, followed by *Halimeda* spp. (10 ± 11%), also more abundant in Belize than the other 2 sites (18 ± 17%) (Table 2). Disease (Black Band, White Syndrome, Dark Spot, etc.) was observed in <2% of corals at all sites (Fig. 2b).

Analysis of the NOAA-FKNMS survey data also demonstrated that *Dictyota* spp. (73 ± 14%) and *Halimeda* spp. (35 ± 15%) macroalgae most frequently interacted with corals (Fig. 3b). Of the most common corals recorded, those that most frequently interacted with the surveyed macroalgae belonged to the genera *Siderastrea* (82%), followed by *Diploria* (69%) and *Montastraea* (54%; Fig. 3a). Several coral taxa interacted with the brown algae *Dictyota* in >80% of examined colonies: *Siderastrea*, *Diploria*, and *Meandrina meandrites* (Fig. 3a). Coral taxa that interacted with the green alga *Halimeda* in >50% of colonies were *Siderastrea*, *Madracis*, and *Diploria*. The studied corals *Montastraea faveolata* and *Porites astreoides*

Table 1. Coral diversity surveys in 2008 and 2009; shown are means ± SD between sampling years

	Corals per m ²	Species counted	Area surveyed (m ²)
St. Thomas	3.3 ± 0.11	21	350
Florida ^a	0.9	29	2147
Belize	3.0 ± 1.24	17	310

^aData were collected during the 2009 NOAA Florida Keys National Marine Sanctuary Coral Health and Diversity Survey Cruise

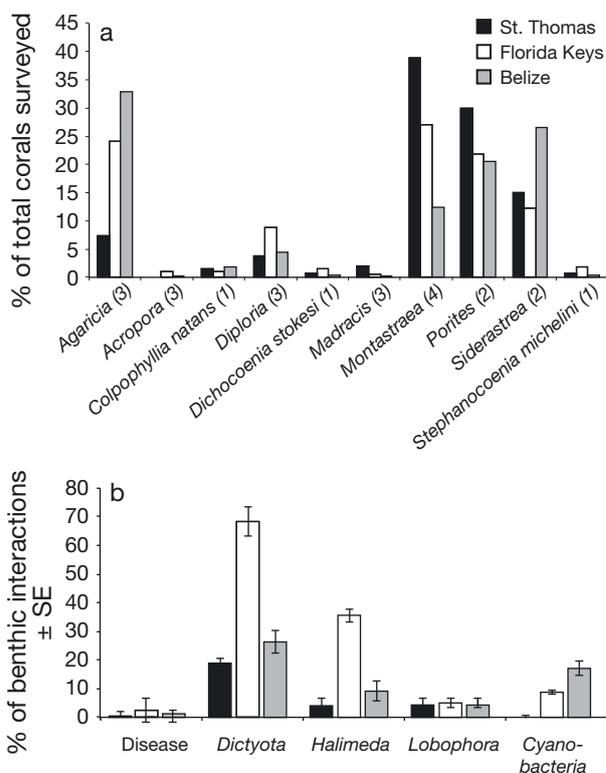


Fig. 2. (a) Coral diversity at all 3 sites (percentage of total corals surveyed at each site). The number in parentheses corresponds to the number of species surveyed within each genus. (b) Mean percentage of total corals (±1 SE) that were diseased and/or interacting with macroalgae (*Dictyota* spp., *Halimeda* spp., or *Lobophora variegata*) or *Cyanobacteria*

interacted with *Dictyota* in >70% of colonies and with *Halimeda* in >25% of colonies. However, colonies of *M. faveolata* interacted with both macroalgae more frequently than did those of *P. astreoides* (Fig. 3a).

DGGE results

DGGE community profiles based on 16S rRNA revealed diverse bacterial assemblages in all SML samples of *Montastraea faveolata* and *Porites astreoides* coral colonies that engaged in natural interactions with *Dictyota menstrualis* and *Halimeda opuntia* macroalgae. Hierarchical cluster analyses and nMDS plots of DGGE profiles indicated that the coral control samples grouped closely together at all 3 sites (bolded contours, Fig. 4). Algal control samples did not group as closely or consistently as the coral controls, except for *H. opuntia* controls in St. Thomas, suggesting less bacterial specificity generally associated with the surfaces of algal thalli in comparison to coral mucus layers.

Table 2. Composition of reef benthos in percentage cover as an average (Total AVG) among 3 sites (St. Thomas, Belize, and the Florida Keys) and at each site separately. Benthic components are listed by decreasing average percentage cover across sites. The percentage of cover of total macroalgae and the studied macroalgae (*Dictyota* and *Halimeda*) are in **bold**

Taxon	Total AVG	St. Thomas	Belize	Florida
Macroalgae	48	37	80	28
<i>Cyanobacteria</i>	41	44	73	6
Turf algae	23	17	15	36
Sand	22	22	34	11
<i>Dictyota</i> spp.	18	14	25	15
Sponge	14	17	17	8
Coral	12	22	7	8
<i>Palythoa</i>	11	0	6	26
<i>Halimeda</i> spp.	10	5	18	6
Gorgonian	9	6	2	19
<i>Lobophora variegata</i>	6	15	4	0
Crustose coralline algae	6	0	5	12
<i>Millepora</i>	5	2	2	11
<i>Peyssonnelia</i>	2	3	3	0
<i>Turbinaria</i> spp.	2	0	5	0
<i>Ventricaria</i>	2	0	5	0
<i>Jania</i> spp.	2	0	5	0
<i>Padina</i>	1	0	4	0
<i>Laurencia</i>	1	2	1	1
<i>Polysiphonia</i>	1	0	0	4
<i>Styopodium</i>	1	0	4	0

Montastraea faveolata-associated bacteria were susceptible to bacterial shifts in 100% (6 out of 6) of the coral–algal interaction contrasts tested, while *Porites astreoides* was affected in 67% (4 out of 6) of interaction types (PERMANOVA; Table 3). Furthermore, a shift was detected in *M. faveolata* SML samples collected at a 5 cm distance from the interaction zone with macroalgae during the majority of interactions (83%, Table 3). Significant differences occurred between the microbial profiles associated with coral controls and those associated with corals interacting with macroalgae, indicated as ‘algal winners’ (Fig. 4, Table 3). In contrast, when the microbial profiles associated with coral controls and with corals near macroalgae (5 cm away) were more similar to one another than they were to profiles on corals directly interacting with macroalgae, this limited macroalgal effect was termed a ‘stand-off’ (Fig. 4, Table 3). For example, macroalgae were the ‘winners’ and shifted the bacterial community composition associated with *M. faveolata* to one more similar to those found on macroalgae, and less similar to those associated with coral controls, in 83% of the interactions (Fig. 4, Table 3). Both macroalgae were associated with a bacterial shift in *M. faveolata* bacterial assemblages, but *Halimeda*

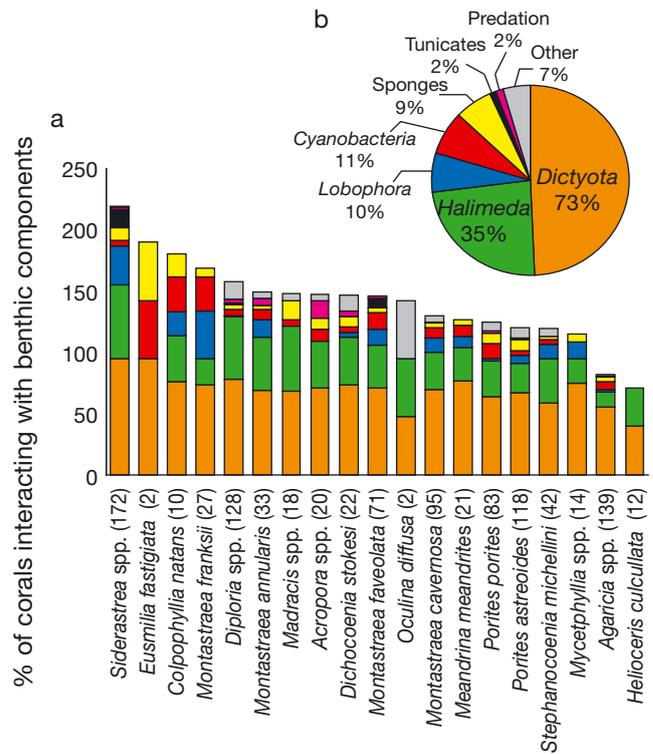


Fig. 3. (a) Top 19 corals surveyed at 21 sites (no. surveyed in parentheses) during the September 2009 NOAA FKNMS Coral Health and Diversity Cruise. Bar graph represents the percentage of corals that were interacting with 8 different benthic components: macroalgae within the genera *Dictyota*, *Halimeda*, or *Lobophora*; benthic *Cyanobacteria*; sponges; tunicates; predation such as from corallivorous snails or fire worms; and any other interacting benthic organisms. Corals are in descending order (left to right) from the species that interacted with the greatest percentage of benthic components to the species that interacted with the least (e.g. nearly 100% of *Siderastrea* colonies surveyed were interacting with *Dictyota* spp. macroalgae). (b) Average percentages of interactions for each benthic component across all 20 corals

opuntia was associated with more significant changes than *Dictyota menstrualis* (Fig. 4, Table 3).

The results for *Porites astreoides* coral–algal interactions revealed less variation than for *Montastraea faveolata*. Samples collected from the coral colony SML at 5 cm distant from coral–algal interaction zones differed significantly from coral controls in only 1 instance, during interaction with *Dictyota menstrualis* in St. Thomas ($p < 0.01$, Table 3b). All other significant bacterial shifts were ‘stand-offs’, with changes limited to the interaction zone between *P. astreoides* SML and macroalgal thalli (50% of interactions; Fig. 4, Table 3b). Also, no change was detected for *P. astreoides* corals interacting with *Halimeda opuntia* in St. Thomas or with *D. menstrualis* in the Florida Keys, indicating ‘coral winners’ (Fig. 4, Table 3).

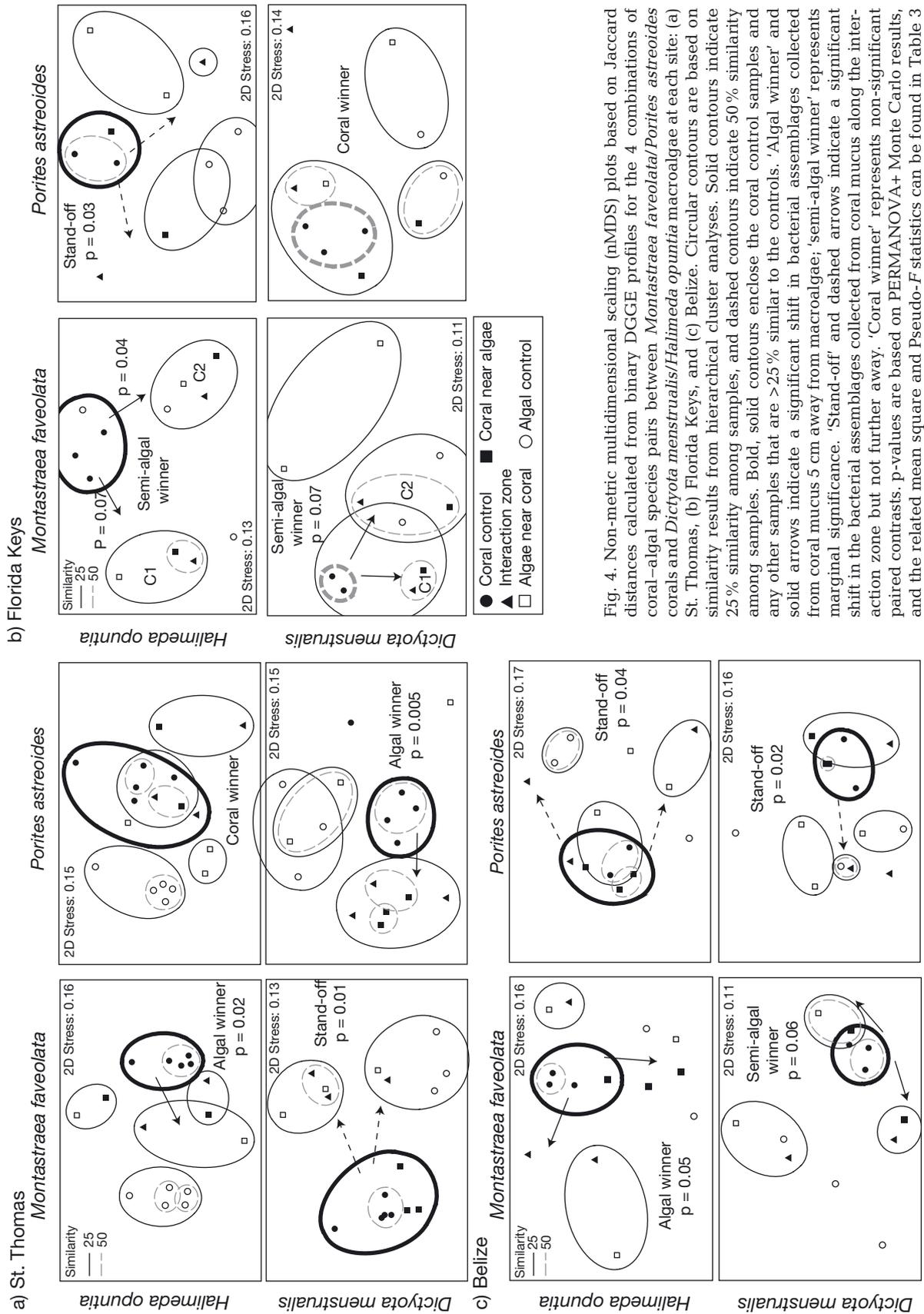


Fig. 4. Non-metric multidimensional scaling (nMDS) plots based on Jaccard distances calculated from binary DGGE profiles for the 4 combinations of coral-algal species pairs between *Montastraea faveolata*/*Porites astreoides* corals and *Diclyota menstrualis*/*Halimeda opuntia* macroalgae at each site: (a) St. Thomas, (b) Florida Keys, and (c) Belize. Circular contours are based on similarity results from hierarchical cluster analyses. Solid contours indicate 25% similarity among samples, and dashed contours indicate 50% similarity among samples. Bold, solid contours enclose the coral control samples and any other samples that are >25% similar to the controls. 'Algal winner' and solid arrows indicate a significant shift in bacterial assemblages collected from coral mucus 5 cm away from macroalgae; 'semi-algal winner' represents marginal significance. 'Stand-off' and dashed arrows indicate a significant shift in the bacterial assemblages collected from coral mucus along the interaction zone but not further away. 'Coral winner' represents non-significant paired contrasts. p-values are based on PERMANOVA+ Monte Carlo results, and the related mean square and Pseudo-F statistics can be found in Table 3

Table 3. PERMANOVA paired contrasts comparing coral and macroalgal controls against samples collected from corals competing with macroalgae at each of 3 reef sites. CC: coral control surface mucopolysaccharide layer (SML) not interacting with macroalgae or other benthic organisms; CN: coral SML 5 cm away from competing macroalgae; X: coral SML from interaction zone between coral and algae; AN: surface samples from macroalgae competing with the respective coral; AC: algal control, surface samples from macroalgae not interacting with corals or other benthic organisms; Group: pooled samples from CN, X, and AN into a single contrast

	Paired contrasts	MS	Pseudo- <i>F</i>	P (MC) ^a	Perceived shift	Winning species
(a) <i>Halimeda opuntia</i> interactions						
<i>M. faveolata</i>						
St. Thomas	CC × Group	6162.8	2.92	0.02	Colony	<i>Halimeda</i> algae
Florida Keys	CC × Group C1/ Group C2	–	–	0.07/0.04	Marginal colony	<i>Halimeda</i> algae
Belize	CC × Group	6359.4	2.08	0.05	Colony	<i>Halimeda</i> algae
<i>P. astreoides</i>						
St. Thomas	–	–	–	–	No change	<i>Porites</i> coral
Florida Keys	(CC + CN) × (AN + X)	5929.3	2.58	0.04	Interaction	Stand-off
Belize	(CC + CN) × (AN + X)	5332.3	1.76	0.04	Interaction	Stand-off
	Paired contrasts	MS	Pseudo- <i>F</i>	P (MC) ^a	Shift	Winning species
(b) <i>Dictyota menstrualis</i> interactions						
<i>M. faveolata</i>						
St. Thomas	(CC + CN) × (AN + X)	8491.9	5.08	0.001	Interaction	Stand-off
Florida Keys	CC × (CN + X)	3795.8	2.70	0.07	Marginal colony	<i>Dictyota</i> algae
Belize	CC × (CN + X)	5000.3	2.06	0.06	Marginal colony	<i>Dictyota</i> algae
<i>P. astreoides</i>						
St. Thomas	CC × (CN + X)	8370.9	4.90	0.005	Colony	<i>Dictyota</i> algae
Florida Keys	–	–	–	–	No change	Stand-off
Belize	(CC + CN) × (AN + X)	6970.7	2.55	0.02	Interaction	<i>Porites</i> coral

^aP(MC) is based on the Monte Carlo test statistic and Jaccard distances

Sequencing results

Analysis of 16S rRNA genes via PCR-DGGE revealed significant variation of bacterial bands (a proxy for bacterial operational taxonomic units) (1) between coral species, (2) between coral species among sites, and (3) along coral–algal interaction gradients in both coral species at all sites. Of the 136 bands excised and sequenced from DGGE gels, only 11 sequences (>200 bp) were of acceptable quality (Table 4). Sequences from *Montastraea faveolata* colonies were affiliated with class γ -*Proteobacteria* ($n = 1$), phylum *Firmicutes* in the genus *Bacillus* ($n = 3$), and phylum *Actinobacteria* in the genus *Propionibacterium* ($n = 1$). The majority of sequences from *Porites astreoides* coral controls were all related to class γ -*Proteobacteria* ($n = 7$), with >90% identity to members of the family *Oceanospirillaceae*, a γ -*Proteobacteria* known to comprise >86% of the SML populations in healthy *P. astreoides* colonies (Morrow et al. 2012a). One sequence from *P. astreoides* was most similar to the class *Enterobacteriaceae* ($n = 1$). Collection details and accession numbers are listed in Table 4.

DISCUSSION

Most eukaryotes associate with assemblages of microbial symbionts that aid in their development, health, and adaptation to environmental conditions (Zilber-Rosenberg & Rosenberg 2008). Our study demonstrates that 2 common Caribbean macroalgae appear to drive changes in the composition of coral-associated bacterial assemblages, both (1) along coral–macroalgal interaction zones and (2) on corals at a 5 cm distance from interacting macroalgae. Bacterial assemblages associated with the coral *Porites astreoides* appear more resistant to change than those associated with *Montastraea faveolata*, in that they do not shift as much near areas of coral–algal contact and in most cases reveal ‘coral winners’ or ‘stand-offs’. The ability of a coral species to maintain a stable bacterial assemblage regardless of environmental fluctuations and ambient stress may indicate which species and reefs are most robust and resilient. The present data and previous results (Morrow et al. 2012a) imply that from a holobiont perspective, *P. astreoides* is more robust and resilient than *M. faveolata*, which was more frequently involved in

Table 4. GenBank similarity of 16S rRNA sequences from *Montastraea faveolata* and *Porites astreoides* surface mucus layers

Sampling zone	Collection site (Lat., Long.)	Collection date	Accession no.	Sequence length (bp)	Closest relative and accession no. (% similarity)
<i>M. faveolata</i>					
5 cm from <i>Dictyota</i>	St. Thomas (18° 19'N, 64° 59'W)	Jul 09	KC188057	489	<i>Gammaproteobacterium</i> DQ200474 (97)
Control	Belize (16° 48'N, 88° 04'W)	Aug 09	KC188066	252	<i>Bacillus amyloliquefaciens</i> HE610889 (100)
5 cm from <i>Halimeda</i>	Belize (16° 48'N, 88° 04'W)	Aug 09	KC188067	202	<i>Propionibacterium</i> GQ130087 (99)
Control	Florida Keys (24°32'N, 81°24'W)	Aug 09	KC188068	384	<i>Bacillus indicus</i> JX393081 (99)
Control	Florida Keys (24°32'N, 81°24'W)	Aug 09	KC188069	387	<i>Bacillus indicus</i> JX393081 (99)
<i>P. astreoides</i>					
Control	St. Thomas (18° 19'N, 64° 59'W)	Jul 09	KC188058	484	<i>Gammaproteobacterium</i> DQ200474 (96)
Control	St. Thomas (18° 19'N, 64° 59'W)	Jul 09	KC188059	496	<i>Gammaproteobacterium</i> DQ200474 (96)
Control	St. Thomas (18° 19'N, 64° 59'W)	Jul 09	KC188060	483	<i>Gammaproteobacterium</i> DQ200474 (97)
Control	Belize (16° 48'N, 88° 04'W)	Aug 09	KC188061	492	<i>Gammaproteobacterium</i> DQ200474 (99)
Control	Belize (16° 48'N, 88° 04'W)	Aug 09	KC188062	491	<i>Gammaproteobacterium</i> DQ200474 (99)
Control	Belize (16° 48'N, 88° 04'W)	Aug 09	KC188063	493	<i>Enterobacteriaceae</i> AB714458 (98)
Control	Belize (16° 48'N, 88° 04'W)	Aug 09	KC188064	491	<i>Gammaproteobacterium</i> DQ200474 (99)
Control	Belize (16° 48'N, 88° 04'W)	Aug 09	KC188065	488	<i>Gammaproteobacterium</i> DQ200474 (98)

interactions with an 'algal winner'. This pattern was consistent at all 3 sites examined (>1000 km apart) and may reveal in part why *P. astreoides* continues to proliferate on present-day coral reefs, whilst *Montastraea* corals frequently succumb to disease (Garzón-Ferreira et al. 2001, Lafferty et al. 2004).

Macroalgae are increasing in abundance on coral reefs, and their interactions with corals and other benthic organisms also are likely to increase. In the Florida Keys, coral cover declined by 53% from 1996 to 2010, leading to an absolute decline of ~6 to 12%, likely exacerbated by a prolonged thermal stress event during the 1997/1998 El Niño year (Ruzicka et al. 2011). Brown macroalgae of the genus *Dictyota* grew to cover nearly 56% of the Florida benthos during the summers from 1996 to 2000 and remain dominant members of most Caribbean reefs at 0 to 25 m depth (Lirman & Biber 2000, Edmunds 2002, Beach et al. 2003). Coral–macroalgal contact on Florida reefs is extremely high (>50% of corals), and corals most frequently interact with *Halimeda*, *Dictyota*, and turf algae (Lirman 2001). Our study similarly shows that both of these macroalgae frequently interact with

corals, particularly in Florida. Nearly a decade after Lirman's study, they were the 2 most abundant macroalgal species at our sites (with averages of 18 and 10% cover, respectively), with >70% of corals interacting with *Dictyota* and 40% with *Halimeda*. Similar surveys in the Pacific have shown that *Halimeda* macroalgae disproportionately interact with corals compared to their relative abundance (Tanner 1995, Barott et al. 2012). The high frequencies worldwide with which these 2 macroalgae interact with reef corals indicate extensive potential for disruptions of coral-associated microbial communities.

Both macroalgae in the present study have been shown experimentally to damage competing corals (Rasher & Hay 2010) and/or their beneficial microbial symbionts (Morrow et al. 2012b, Shearer et al. 2012). Vega Thurber et al. (2012) conducted a manipulative experiment to examine competition between *Porites astreoides* and macroalgae in the Florida Keys and found that 4 out of 5 macroalgae altered the coral microbiome, including *Halimeda tuna*. As described previously, macroalgae also may drive shifts in microbial composition and dissolved oxygen at coral–

algal interfaces through the release of DOC and potent allelochemicals (e.g. secondary metabolites). A recent rapid and acute white plague-like disease outbreak in Dry Tortugas National Park in Florida was associated with significant changes in the abundance of *Dictyota* algae (Brandt et al. 2012), possibly due to synergistic interactions in which the presence of one exacerbated the other or due to the macroalga acting as a reservoir for the disease pathogen(s). The changes to coral SML bacterial assemblages along natural gradients of coral–algal interaction revealed here, and in previous studies, indicate that macroalgal impacts are complex and variable among coral and algal species.

Several factors may explain why *Montastraea faveolata*-associated bacteria were more susceptible to the effects of competing macroalgae than those of *Porites astreoides*. These 2 coral genera differ in reproductive strategies (broadcast spawner vs. brooder, respectively) that likely influence microbial acquisition (Sunagawa et al. 2010). The presence of bacterial cells in newly released larvae from *P. astreoides* colonies (Sharp et al. 2012) suggests that microbial symbionts are vertically transmitted and indicates that a stable relationship exists between host and symbiont. In contrast, broadcast spawning corals such as *M. faveolata* likely rely on environmental recruitment (e.g. horizontal transmission; Sharp et al. 2012) and phagocytosis to supply microbial symbionts (Apprill et al. 2009). Therefore, *M. faveolata* may be at a disadvantage to *P. astreoides* in terms of its ability to maintain specific and stable bacterial assemblages across generations and environmental gradients.

Another indication regarding why the stony corals *Montastraea faveolata* and *Porites astreoides* differ in their reactions to competing macroalgae may be variation in their disease susceptibility. *M. faveolata* is susceptible to a wide range of bacterial diseases, including but not limited to White Plague, Dark Spot, Black Band, Yellow Blotch/Band, and Red Band; *P. astreoides* has been associated with only 2 diseases, White Plague and Yellow Blotch/Band (Garzón-Ferreira et al. 2001). In general, massive reef building corals such as *Montastraea* tend to be more susceptible to diseases than are many other types of corals (Lafferty et al. 2004). *Porites* corals can fight fungal invasion by laying impenetrable walls of calcium carbonate (Le Campion-Alsumard et al. 1995, Ravindran et al. 2001), and their tissues may be completely devoid of adhering microbes, suggesting strong mechanisms for microbial mediation (Johnston & Rohwer 2007). Thus, colonies of *Porites* may

possess more host factors that permit the manipulation of microbial symbionts than do those of *M. faveolata*.

As with most molecular techniques, technical errors are possible when using DGGE. However, prior to recent reductions in cost for high-throughput sequencing, DGGE was the most appropriate and affordable option for interrogating large datasets of complex microbial communities. Several factors must be considered when using DGGE for the evaluation of large and complex datasets, and additional sequencing should be utilized to confirm patterns whenever feasible. Stringent standardization of primers, internal standards, quantity of PCR product, gel composition, run conditions, gel staining, and imaging all are required for reliable and consistent gel-to-gel comparisons (Ferrari & Hollibaugh 1999, Sánchez et al. 2007). Additionally, the use of powerful software for gel analysis, such as Bionumerics (see 'Materials and methods'), is necessary to reliably compare multiple gel images and create similarity matrices for robust statistical analyses.

Following standardization of the above technical parameters, caution must be applied when making assumptions about community profiles obtained from DGGE analysis. Band intensity does not necessarily indicate relative bacterial abundance due to the restricted resolution of sequences with similar mobility characteristics (Kirk et al. 2004), PCR bias toward high-GC bacterial taxa (Chakrabarti & Schutt 2001), and the occurrence of multiple bands per organism. Multiple bands are often observed for a single bacterium due to the existence of >1 16S rRNA gene in some taxa (e.g. *Escherichia coli* has 7 copies). Thus, DGGE can reliably identify community members but not determine which are dominant (Calábria de Araújo & Schneider 2008). Further, the number of bands per taxa may vary with the amount of DNA per sample (Calábria de Araújo & Schneider 2008). We took care during the analysis and interpretation of our results to address the above biases associated with DGGE. Variation in the concentration of DNA for each organism in our samples likely caused some variation in our banding patterns; however, without sequencing every band, it is difficult to control for this type of error in mixed consortia of unknown microorganisms.

Given the above variables, our DGGE-based diversity analyses are most likely to be conservative because they are based on field surveys of natural variation in our study organisms and inherently underestimate bacterial diversity compared to next-generation sequencing techniques. Even so, our

methods detected differences between the specific assemblages of bacteria associated with isolated coral controls and those associated with corals competing with macroalgae, suggesting ecological relevance. Future studies are needed to examine how coral species vary in the mechanisms they employ to maintain their microbial symbionts. This is a very interesting area of study, and previous researchers have suggested that antimicrobial compounds may be released by the host and/or microbial symbionts in addition to holobiont excreted nutrients and possibly growth factors (Crossland et al. 1980, Brown & Bythel 2005, Raina et al. 2010).

Because corals contact diverse benthic organisms, the influence of competitors must be considered when drawing conclusions about coral–microbial relationships. Previous evidence that bacterial assemblages from the same coral host species vary when collected from different locations on the colony (Daniels et al. 2011) and/or from different geographic locations (Littman et al. 2009) may at least in part be due to unreported or unidentified environmental perturbations and/or interactions with adjacent organisms. A recent study identified 4 types of alterations to the taxonomic stability of coral-associated microbial communities when in direct competition with macroalgae, including an increase or decrease in the abundance of microbial taxa previously present on the coral colony, the establishment of new microbial taxa, and the vectoring or growth of new microbial taxa onto the coral colony from the alga (Vega Thurber et al. 2012). These results are similar to those reported in the present study and confirm the important and often overlooked role that macroalgae play in coral health.

Assessment of the microbial consequences of coral–macroalgal phase-shifts is another essential step toward determining which coral species are the most stable when exposed to stress. Because significant shifts in coral-associated bacterial community structure away from the norm are likely to be detrimental to the host, corals that experience such changes due to competing macroalgae (e.g. *Montastraea faveolata*) appear to be ‘losers’ during macroalgal competition. Thus, these species may require enhanced protection from anthropogenic stressors relative to more robust coral holobionts (e.g. *Porites astreoides*) that are apt to be ‘winners’ (Table 3). We predict that differential survival of coral species through impacts on their associated bacteria from macroalgal encroachment will contribute to significant changes in species composition and reef community structure.

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