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REPORT

Allelochemicals produced by Caribbean macroalgae and cyanobacteria have species-specific effects on reef coral microorganisms

K. M. Morrow · V. J. Paul · M. R. Liles · N. E. Chadwick

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Abstract Coral populations have precipitously declined on Caribbean reefs while algal abundance has increased, leading to enhanced competitive damage to corals, which likely is mediated by the potent allelochemicals produced by both macroalgae and benthic cyanobacteria. Allelochemicals may affect the composition and abundance of coral-associated microorganisms that control host responses and adaptations to environmental change, including susceptibility to bacterial diseases. Here, we demonstrate that extracts of six Caribbean macroalgae and two benthic cyanobacteria have both inhibitory and stimulatory effects on bacterial taxa cultured from the surfaces of Caribbean corals, macroalgae, and corals exposed to macroalgal extracts. The growth of 54 bacterial isolates was monitored in the presence of lipophilic and hydrophilic crude extracts derived from Caribbean macroalgae and cyanobacteria using 96-well plate bioassays. All 54 bacterial cultures were identified by ribotyping. Lipophilic extracts from two species of *Dictyota* brown algae inhibited >50% of the reef coral bacteria assayed, and hydrophilic compounds from *Dictyota menstrualis* particularly inhibited *Vibrio* bacteria, a genus associated with several coral diseases. In contrast, both lipo- and hydrophilic extracts from 2 species of *Lyngbya* cyanobacteria strongly stimulated bacterial growth. The brown alga *Lobophora variegata* produced hydrophilic compounds with broad-spectrum antibacterial effects, which inhibited 93% of the bacterial cultures. Furthermore, bacteria cultured from different locations (corals vs. macroalgae vs. coral surfaces exposed to macroalgal extracts) responded differently to algal extracts. These results reveal that extracts from macroalgae and cyanobacteria have species-specific effects on the composition of coral-microbial assemblages, which in turn may increase coral host susceptibility to disease and result in coral mortality.

Keywords Antimicrobial · Bacteria · Competition · Coral–algal interaction · Phase-shift

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Introduction

Macroalgae are major competitors with corals and other benthic organisms on tropical reefs, especially where rates of herbivory are low and nutrient enrichment is high (Fong and Paul 2011; Rasher and Hay 2010). Competition can occur through several direct and indirect physical and chemical (allelopathic) mechanisms (reviewed in Chadwick and Morrow 2011). Therefore, benthic community structure on coral reefs can be strongly influenced by allelopathy (chemically mediated competition). A well-known function of macroalgal compounds is to provide chemical protection from abundant and diverse herbivores (Paul and Hay 1986; Nagle and Paul 1999; Paul et al. 2001). Previous studies suggest that as herbivorous fishes and invertebrates selectively remove palatable species of macroalgae, they are replaced by unpalatable, chemically defended seaweeds (Tsuda and Kami 1973). The results of this selective herbivory are commonly seen on many coral reefs that have undergone phase-shifts to increasing

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dominance by chemically defended seaweeds, including species of *Halimeda*, *Dictyota*, *Lobophora*, and cyanobacteria of the genus *Lyngbya* (Rogers et al. 1997; McClanahan et al. 1999, 2000; Fong and Paul 2011). Members of these 3 chemically defended genera are now the most abundant macroalgae on many reefs in Florida and the Caribbean (Rogers et al. 1997; McClanahan et al. 1999, 2000; Kuffner et al. 2006), reaching 7–17% cover at some sites (K. M. Morrow pers. obs.).

Seaweeds further utilize physical and chemical regulatory strategies to prevent bacterial and fungal colonization (Mann 1973; Nylund et al. 2005; Engel et al. 2006; Lane et al. 2009). The microbial diversity and abundance within the marine environment varies in response to changing environmental conditions (e.g., temperature, pH, nutrients, pollutants) (Wommack et al. 1999), and bacterial symbionts may allow host organisms to respond more efficiently to these changes (Reshef et al. 2006; Rosenberg et al. 2007). Specifically, corals are believed to harbor an abundant and diverse, species-specific assemblage of microorganisms (Rohwer et al. 2002). It has been suggested that the coral host regulates and selects for the most advantageous microbial symbionts in the context of prevailing environmental conditions (Reshef et al. 2006; Zilber-Rosenberg and Rosenberg 2008). Thus, variation in microbial symbionts can support or hinder adaptation of the holobiont (coral host and associated microorganisms). In the absence of microorganisms, many eukaryotic organisms may be less able to fight infections from pathogenic bacteria and viruses (Shanmugam et al. 2005). Microorganisms likely prevent infection by producing antibacterial and antifungal compounds in addition to occupying niches that would otherwise be available to opportunistic pathogens (Koh 1997; Castillo et al. 2001; Ritchie 2006). Corals also may rely on microbial symbionts for nutritional requirements and efficient adaptation to environmental change (Shashar et al. 1994; Ducklow and Mitchell 1979; Lesser et al. 2004; Croft et al. 2005). Thus, it is critical to understand the extent to which chemically defended competitors, such as macroalgae and cyanobacteria, can affect coral-associated microbial assemblages.

Many allelochemicals can inhibit microbial growth, but these compounds rarely have been tested against ecologically relevant microorganisms. For example, the red alga *Delisea pulchra* produces halogenated furanones that resemble acylated-homoserine lactones (AHL) and are used in bacterial signaling (Kjelleberg et al. 1997). These halogenated furanones function by inhibiting bacterial quorum sensing, through disruption of a transcription regulator, LuxR, which regulates bacterial colonization and biofilm formation (Maximilien et al. 1998; Manfield et al. 1999). Thus, *D. pulchra* can prevent tissue damage by harboring compounds that inhibit the survival, virulence, and reproduction of fouling organisms. Another well-studied red alga, *Asparagopsis armata,* produces bromoform and dibromacetic acid, both of which are active against marine *Vibrio* bacteria that often are pathogenic (Paul et al. 2006). When halogenated metabolites were experimentally removed from *A. armata,* the thallus became fouled with significantly higher densities of epiphytic bacteria (Paul et al. 2006). Finally, another red macroalga, *Callophycus serratus,* was shown to produce bromophycolides and callophycoic acids that inhibit the growth of *Lindra thalassiae*, a marine fungal pathogen (Lane et al. 2009). Therefore, macroalgae such as *D. pulchra, A. armata,* and *C. serratus* employ species-specific chemical metabolites and harbor microbial assemblages that are resistant to compounds produced by the alga. Marine plants and animals frequently harbor species-specific microbial assemblages that are distinct from the assemblages found in the surrounding environment and are important to host defenses (Baker and Orr 1986; Wahl and Hay 1995; Rohwer et al. 2002). Several other studies have surveyed both tropical (Ballantine et al. 1987; Engel et al. 2006) and temperate macroalgae (Cetrulo and Hay 2000) and illustrate the ubiquitous nature of marine chemical defenses.

Macroalgal compounds not only inhibit but also can stimulate microbial growth, which may have an equally rapid and detrimental effect on organisms living in close proximity to the algal thalli. Little is known about the effects of stimulatory chemicals or primary metabolites on specific microorganisms, but several studies have examined the general effects on in situ microbial populations. In an aquarium study, Smith et al. (2006) showed that the release of high levels of dissolved organic carbon (DOC) by macroalgae caused explosive bacterial growth on adjacent coral surfaces and led to a zone of hypoxia that induced coral tissue death. High levels of DOC in the water column also led to coral mortality and increased microbial growth rates by an order of magnitude within the coral mucus layer (Kline et al. 2006). Elevated microbial growth rates appear to cause coral mortality through depletion of oxygen, accumulation of poisons (e.g., hydrogen sulfide, secondary metabolites), and/or microbial predation on weakened coral polyps (Segel and Ducklow 1982; Smith et al. 2006). However, high concentrations of DOC and metabolites may cause elevated and detrimental microbial growth only under certain conditions, including direct coral–algal contact, low water flow in interstitial microenvironments, and/or high temperature stress (Smith et al. 2006; Bruno et al. 2007; Vu et al. 2009).

Here, we report the results of bacterial growth assays using crude extracts from six common Caribbean macroalgae and two benthic cyanobacteria for inhibitory and stimulatory activity on coral- and algal-associated bacteria.

We screened 16 crude extracts, eight lipophilic (non-polar), and eight hydrophilic (polar), from each algal and cyanobacterial species against 54 bacterial cultures that were isolated from Caribbean reef corals and macroalgae to identify the specific response of each bacterial culture to these algal extracts. We hypothesized that the macroalgae and cyanobacteria produce both inhibitory and stimulatory compounds that may alternatively affect both beneficial and detrimental coral reef microorganisms. As macroalgae become more prolific on present-day coral reefs, this information is needed to understand the mechanisms of competition among reef organisms on both macro- and microscopic scales. Particular species of encroaching macroalgae may have more or less severe effects on corals and their associated microorganisms, and it is important to determine how these interactions alter the ability of reef-building corals to respond and adapt to changing environmental conditions.

Methods

Sample collection and preservation

Macroalgae and cyanobacteria were collected from coral reefs adjacent to the Mote Marine Laboratory, Summerland Key, Florida (the brown alga *Dictyota menstrualis* and the green alga *Halimeda tuna*), the University of the Virgin Islands MacLean Marine Science Center, St. Thomas (the red alga *Acanthophora spicifera,* the brown alga *Lobophora variegata,* and the cyanobacterium *Lyngbya majuscula*), and the Smithsonian's Carrie Bow Cay Field Station in Belize (the brown algae *Dictyota sp.* and *Dictyota pulchella,* and the cyanobacterium *Lyngbya polychroa*). Collections were made by hand on SCUBA at depths of 8–15 m in April 2003 (*D. pulchella*), July 2005 (*D. menstrualis*), and the remainder between May and August 2008 (*A. spicifera, Dictyota sp., H. tuna, L. majuscula, L. polychroa, L. variegata).* All samples were placed in plastic zip-lock bags at depth and brought to the surface then placed in seawater-filled coolers and transported back to the laboratory (<3 h). Clean plants, free of substantial epiphyte growth or other macroscopic material, were frozen at −20°C. Samples of the green alga *H. tuna* were flash-frozen in liquid nitrogen to prevent degradation of the diterpenoid compounds (Paul and Van Alstyne 1992). All frozen algal samples were transported on ice to the Smithsonian Marine Station in Fort Pierce, FL for further chemical extraction and analysis. *A. spicifera* was selected because it does not contain known chemical defenses and was not expected to inhibit marine bacteria in these assays. All other species of macroalgae and cyanobacteria were selected because they are suspected to produce potent allelochemicals.

Microbial samples were collected with 5-ml plastic-tipped syringes on SCUBA from coral mucus and algal surfaces on the above reefs in the Florida Keys, St. Thomas, and Belize at 8–15 m during May–August 2008. Two common species of Caribbean reef-building corals (*Montastraea faveolata* and *Porites astreoides*) and 2 common macroalgae (*D. menstrualis* and *Halimeda opuntia*) were sampled for associated microbes. Samples were collected from a 5×5 cm^2 surface area on all corals and macroalgae after gentle agitation, which encourages sloughing of the viscous mucus and reduces aspiration of seawater into the syringe (Ritchie 2006). Samples were collected from 3 locations along a gradient of coral–algal interaction: (A) algal surfaces interacting with corals, (B) coral mucus touching the algal thalli, and (C) coral mucus 5 cm from the algal thalli. They also were collected from two control areas: (X) coral mucus not in contact with algae or any other sessile organisms and (Y) algae not in contact with corals or any other sessile organisms (Fig. 1). Extracts from the brown alga *Dictyota* sp. and the green alga *H. tuna* were incorporated into non-toxic Phytagel and placed on separate corals from those sampled for the coral–algal gradient during a 3-day concurrent field experiment in Belize in October 2008. Coral mucus samples were collected from underneath experimental gels and also 5 cm away from the gels. Syringe samples were brought to the surface, placed in seawater-filled coolers, and transported back to the laboratory where they were immediately processed (<1 h). At the field station, a subsample (100 µl) of each microbial sample was spread-plated onto artificial seawater agar (FSWA; 1 l sterile seawater, 0.43 g beef extract, 0.64 g NaCl, 0.43 g peptone, and 15 g agar). After 24–48 h of growth at room temperature,

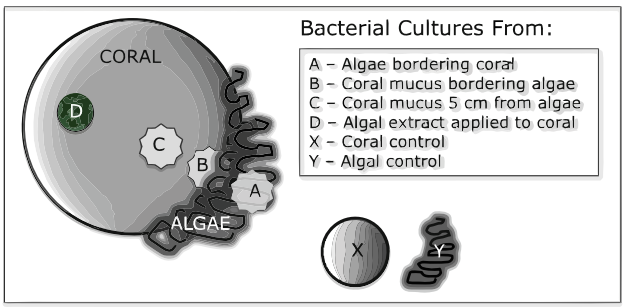

Fig. 1 Microbial samples were collected from 6 areas on stony corals and macroalgae in Belize, Florida, and the USVI during May–Aug 2008. Samples were collected from (*A*) macroalgal thalli (*Dictyota menstrualis* or *Halimeda opuntia*) in contact with corals (*Montastrea faveolata* or *Porites astreoides*), (*B*) coral mucus in contact with macroalgae, (*C*) coral mucus 5 cm away from coral–macroalgal interaction zones, (*D*) coral mucus interacting with crude algal extracts, (*X*) coral controls isolated from contact with macroalgae and other sessile organisms, and (*Y*) macroalgal controls isolated from contact with corals and other sessile organisms. Each sampled coral or alga was at least 3 m from other sampled individuals on the reefs. See text for collecting details

single colonies were picked and triple-streaked for isolation. Mixed and isolated cultured bacteria were transported back to Auburn University for further analysis. From ~250 isolated bacterial cultures, 54 unidentified strains were maintained consistently in culture. Thus, these 54 cultures were chosen for bacterial assays to test the effects of algal extracts.

16S rRNA gene sequencing

Genomic DNA was extracted from the above 54 bacterial isolates using the MOBIO UltraClean® Microbial DNA Isolation Kit. PCR was conducted using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCT CAG) and 1492R (5′-GGYTACCTTGTTACG ACTT) (Medlin et al. 1988). The thermal cycling conditions were as follows: initial denaturation (5 min at 95°C); 30 cycles of denaturation (30 s at 95°C), annealing (1 min at 55°C), and elongation (1 min at 72°C); then a final extension step (7 min at 72°C). The resulting amplicons were evaluated for yield and size by electrophoresis through a 1% (w/v) agarose gel and staining with ethidium bromide. PCR fragments of the correct size were sequenced in both directions by the Lucigen Corporation (Middleton, WI) using dideoxy sequencing chemistry via capillary electrophoresis. A consensus 16S rRNA sequence was determined for each cultured isolate using CromasPro v.1.42 (Technelysium Pty, Tewantin, Australia) and compared to the GenBank nr/nt database using BLASTn to identify the nearest neighbor for each respective bacterial isolate (http://www.ncbi.nlm.nih.gov/BLAST/).

Compound extraction

All biochemical extractions were conducted at the Smithsonian Marine Station in Fort Pierce, FL during September–December 2008. Crude extracts were obtained from *A. spicifera, D. menstrualis, D. pulchella, Dictyota* sp., *L. variegata*, *H. tuna, L. majuscula*, and *L. polychroa*. Frozen bulk samples of macroalgae and cyanobacteria were first wet-weighed and then lyophilized over several days. Then, freeze-dried samples were weighed and covered with a 1:1 ethyl acetate:methanol (lipophilic or non-polar extract) solvent solution over 3 consecutive 24-h periods, followed by three 24-h extractions in 1:1 ethanol:de-ionized water (hydrophilic or polar extract). Extracts were filtered to remove large fragments of organic material and the solvents removed via rotary evaporation at 35°C. Extracts then were dried overnight in a Thermo-Savant speed-vac concentrator and frozen (−20°C) for later use in the bacterial assays.

Extracts were tested at concentrations approximating those naturally found in macroalgal and cyanobacterial tissues based on algal wet weight (g), similar to methods used by Puglisi et al. (2007) (Table 1). Extract concentrations were also determined as g dried extract per g of sample tissue. Extracts were re-suspended to these estimated natural concentrations in artificial seawater (FSW) media with 2.5% ethanol added. Dissolved extracts were pre-filtered using a 0.8-µm (185 mm) circle filter (Whatman), followed by secondary filtration through a 0.22-µm filter (Millepore), attached to a vacuum pump. Using aseptic technique, filtered extracts were serially pipetted into a 96-well plate, including a blank for background absorbance of each extract, and FSW with and without ethanol for use as growth controls for each bacterial culture. Cultures were inoculated directly into extract-containing media and controls as described below.

Bacterial bioassays

Macroalgal and cyanobacterial hydrophilic and lipophilic crude extracts were tested for activity against coral reef

Table 1 The percent of bacterial isolates ($n = 54$) that were either inhibited, stimulated, or did not change ('0') their growth rates compared to those of control bacteria, in response to hydro- and lipophilic extracts from six species of Caribbean macroalgae and two species of cyanobacteria. Growth was classified as inhibited/stimulated if >25% different from control growth. Bolded percents indicate the main effects of each type of extract. The natural concentration of algal extract (mg) per algal wet weight (g) is listed below each species name

Hydrophilic (Polar)	L. *variegata*	*H. tuna*	*Dictyota* sp.	*L. polychroa*	*D. pulchella*	*D. menstrualis*	*A. spicifera*	L. *majuscula*
Concentration (mg/g)	29.9	17.8	16.5	27.7	34.5	27.5	31.2	12.9
% Inhibited	**93**	14	6	29	13	**52**	4	4
0	6	4	6	10	15	7	0	4
% Stimulated	2	**82**	**88**	**61**	**72**	41	**96**	**93**
Lipophilic (Non-polar)								
Concentration (mg/g)	16.5	7.5	78.7	28.7	27.5	55.9	14.9	13.8
% Inhibited	2	35	**73**	24	19	7	6	17
0	4	**55**	6	24	19	33	7	4
% Stimulated	**94**	10	20	**51**	**63**	**59**	**87**	**80**

bacteria using a microdilution plate assay, a liquid culture method based on spectrophotometric readings of cellular growth developed by Gruppo et al. (2006). First, bacteria were re-streaked onto fresh FSWA 48 h prior to experimentation. From fresh cultures, a single colony was picked and placed in 2 ml of sterile liquid FSW in a 10-ml glass test tube. Test tubes were sealed and incubated at 33°C while shaking at 215 rpm overnight. After 24 h of growth, a 150-µl sample from each tube was sub-cultured into each 96-well plate containing algal extract media. Each 96-well plate tested 6 extracts and 2 controls (FSW media with and without ethanol) against 12 bacterial cultures. Five plates were used to test all 54 bacteria (12 cultures per plate × 5 plates). All extracts were plated in triplicate ($n = 3$) except for *Dictyota* sp., *H. tuna,* and *L. polychroa*, which were plated in duplicate ($n = 2$), due to limited extract availability. A single plate containing FSW media + extract was plated to control for background absorbance due to the dark coloration of some extracts. Culture wells were mixed by carefully pipetting their contents, then sealed with a breathable sealing film (Aeraseal^{TM}), and incubated at 33°C while shaking at 215 rpm for 48 h. Plates were transported to Harbor Branch Oceanographic Institute, and each well's absorbance at OD_{570} was determined using a BMG-labtech NOVOstar microplate reader. Prior to each reading, wells were shaken again to resuspend any settled material. A total of 20 OD_{570} readings were taken for each well and averaged for a final optical density for each well. The sterile cover had to be removed for accurate readings; thus, time series data were not taken to reduce the risk of contamination. However, because the initial bacterial inoculum was below the limit of detection, an initial spectrophotometric reading was unnecessary.

Data analysis

Initially, the background absorbance of each extract was subtracted from the optical density of respective culture wells to control for extract absorbance. To compare growth rates among bacterial cultures, the percent change in growth was calculated by subtracting the optical density (OD) for each treatment from the OD in the corresponding control well (FSW media + solvent), dividing by the control well OD, and multiplying by 100. Mean percent change ± SE of all duplicate and triplicate plate readings are reported for all bacterial culture treatments.

To compare levels of antibacterial or stimulatory activity among macroalgal or cyanobacterial extracts, the mean percent change of each cultured bacterium type was examined as a function of extract type (termed 'Extract'), source of bacterial culture ('Source', Fig. 1), species of coral, alga, or algal extract the bacteria were cultured from ('Species'), and bacterial taxon ('Bacteria', Table 3) using a 4-way analyses of variance (ANOVA) in the statistical program 'R' (R Development Core Team 2009). Tukey pair-wise comparisons were performed to evaluate growth and extract effects (Table 4).

Results

All extracts from six species of Caribbean macroalgae (6 hydrophilic extracts plus 6 lipophilic extracts) and two species of cyanobacteria (2 hydrophilic extracts plus 2 lipophilic extracts, $n = 16$ extracts total) exhibited inhibitory and/or stimulatory activity against one or more of the 54 assay bacteria (Table 1). Overall, three of the sixteen (19% overall) macroalgal and cyanobacterial extracts exhibited broad-spectrum antibacterial activity, and extracts from 10 macroalgae and two cyanobacteria (75% overall) exhibited broad-spectrum stimulatory activity, while the remaining one *H. tuna* extract exhibited little inhibitory or stimulatory activity. We arbitrarily defined broad-spectrum activity as causing 25% more or less growth in comparison to the control in >50% of the bacteria assayed. Specifically, two hydrophilic extracts from brown macroalgae demonstrated broad-spectrum antibacterial activity (*D. menstrualis, L. variegata*), and one lipophilic extract from a macroalga inhibited >50% of bacteria assayed (*Dictyota* sp. from Belize, Table 1). Conversely, of the 16 total extracts surveyed, six hydro- and six lipophilic extracts stimulated the growth of >50% of bacteria assayed. Two macroalgae (*D. pulchella, A. spicifera*), and both cyanobacteria (*L. majuscula, L. polychroa*), had both hydro- and lipophilic extracts that stimulated microbial growth (e.g., >50% growth, Table 1). The lipophilic extract from the green alga *Halimeda tuna* had the most limited effect on assay bacteria, with 56% of bacteria neither inhibited nor stimulated.

The 54 bacterial isolates were categorized based on the environmental culture source (i.e., coral mucus, algal surface, interaction zone, etc.) and included members of the alphaproteobacteria, gammaproteobacteria, firmicutes, and actinobacteria phyla (Table 2a). Both of the isolates most similar to the putative pathogen *V. shiloi* were cultured from algal thalli (Table 2a). On average, 21% ± 2.4 (mean ± SE) of bacteria from all environmental sources were inhibited by algal extracts, 11% ± 1.8 (mean ± SE) were not affected, and 68% ± 3.2 (mean ± SE) were stimulated. Bacteria cultured from coral mucus exposed to *Halimeda tuna* and *Dictyota* sp. lipophilic extracts exhibited the most growth (82%) and least inhibition (14%) in comparison to other culture sources (Table 2b).

The 4-way ANOVA revealed significant variation in bacterial growth with all 4 main factors: Extract ($F_{15,384} = 30.147$, $P < 0.001$), Source ($F_{6,384} = 27.672$,

Table 2 (a) Types of examined bacteria and their environmental sources (b) mean percentage of bacteria from each environmental source that was inhibited, stimulated, or not affected by the 16 algal and cyanobacterial extracts. (1) Coral controls, coral mucus not in contact with macroalgae or other sessile organisms (corresponding to X in Fig. 1), (2) Near Algae, coral mucus collected 5 cm from interacting macroalgae (corresponding to C in Fig. 1), (3) Touching Algae, coral mucus collected from under macroalgae (see B in Fig. 1), (4) Algal Surfaces, bacteria cultured from the surface of algal thalli (see Y in Fig. 1), (5) Near Extract, coral mucus collected 5 cm from experimentally applied crude extracts (coral mucus 5 cm away from D in Fig. 1), and (6) Under Extract, coral mucus collected from under experimentally applied crude algal extracts (see D in Fig. 1). The italicized bacterial species are putative marine pathogens. The replicate number of coral colonies sampled and the corresponding number of bacterial cultures are listed in the first two rows

Culture source	Coral control	Near algae	Touching algae	Algal surface	Near extract	Under extract
n = # of coral colonies sampled	**8**	**5**	**6**	**8**	**4**	**4**
Total # of cultured isolates	**17**	**5**	**9**	**11**	**5**	**6**
Gamma Proteobacteria	1					
Order Alteromondales						
Alteromonas sp., A. macleodii	4				1	
Pseudoalteromonas sp., *P. prydzensis*	1					5
Shewanella sp.	1					
Order Vibrionales						
Photobacterium eurosenbergii				1		
Listonella pelagia			1			
V. sinaloensis	1					
V. harveyi	**7**		**1**	**3**	**1**	
V. brasiliensis, V. campbellii, V. charchariae, V. communis, V. fischeri, V. harveyi or *V. rotiferianus,*	1	2	5	5		
V. shiloi or ***V. mediterranei***			**1**	**1**		
V. parahaemolyticus or ***V. campbellii***		**1**				
Total *Vibrio* spp.	**9**	**3**	**7**	**9**	**1**	**0**
Alpha Proteobacteria				1		
Order Sphingomonadales; *Erythrobacter*		1			1	
Order Rhodobacterales; *Roseobacter*			**1**			
Firmicutes						
Exiguobacterium sp. or *E. profundum*					2	1
Salinicoccus roseus		1				
Actinobacter: *Micrococcus flavus*	1					
% Inhibited	26	22	19	27	21	14
% Not affected	13	10	13	8	16	4
% Stimulated	60	69	68	65	63	82

The bolded bacterial species are putative marine pathogens

$P < 0.001$), Species ($F_{7,384} = 13.584$, $P < 0.001$), Bacteria ($F_{11,384} = 10.599$, $P < 0.001$) and also revealed several interaction effects (Table 3). Tukey pair-wise comparisons of effects of the 16 extracts on bacterial growth indicated significant differences in the effects of *L. variegata* hydrophilic versus lipophilic extracts (lower CI = −1,042.28, upper CI = −285.47, $P < 0.001$, Fig. 2). All Tukey pair-wise comparisons among and between algal extracts are detailed in Table 4. The brown alga *L. variegata* (hydrophilic extract) mostly inhibited bacterial growth rates (mean −105% ± 8.8 SE), and the cyanobacterium *L. majuscula* (hydrophilic) stimulated the highest mean growth rates among all bacteria assayed (mean +759% ± 98.0 SE, Fig. 2). On average, bacteria in the genus *Pseudoalteromona*s and other members of the order Alteromonadales demonstrated the most growth across all algal extracts surveyed (>700%). Bacterial growth also varied with culture origin: the most rapid growth occurred in bacteria isolated from corals interacting with crude extracts of *H. tuna* lipophilic compounds (∼+800% growth), and the slowest growth in bacteria cultured from the surfaces of living *H. opuntia* green algae and *D. menstrualis* brown algae.

Table 3 4-way ANOVA of % change in bacterial growth with four factors: (1) extract, the type of macroalgal or cyanobacterial extract, (2) source, the location on the reef from where bacteria were originally cultured, (3) species, the species of coral or macroalga from where bacteria were originally cultured, (4) bacteria, the bacterial taxonomic grouping

4-way ANOVA	*df*	SS	MS	*F* value	Pr(>*F*)	Significance
Extract	15	63,252,552	4,216,837	30.1465	<0.001	***
Source	6	23,223,888	3,870,648	27.6716	<0.001	***
Species	7	13,300,811	1,900,116	13.5841	<0.001	***
Bacteria	11	16,309,686	1,482,699	10.5999	<0.001	***
Extract*Source	90	26,728,568	296,984	2.1232	0.001	***
Extract*Species	99	20,587,917	207,959	1.4867	0.004	**
Source*Species	5	4,351,113	870,222	62.212	<0.001	***
Extract*Bacteria	159	48,916,193	307,649	2.1994	<0.001	
Source*Bacteria	3	5,634,701	1,878,234	13.4276	<0.001	***
Species*Bacteria	3	490,769	163,590	1.1695	0.321	
Extract*Source*Species	69	75,152,538	1,089,167	7.7865	<0.001	***
Extract*Source*Bacteria	45	7,182,557	159,612	1.1411	0.255	
Extract*Species*Bacteria	45	2,255,647	50,125	0.3584	0.999	
Residuals	384	53,713,181	139,878			

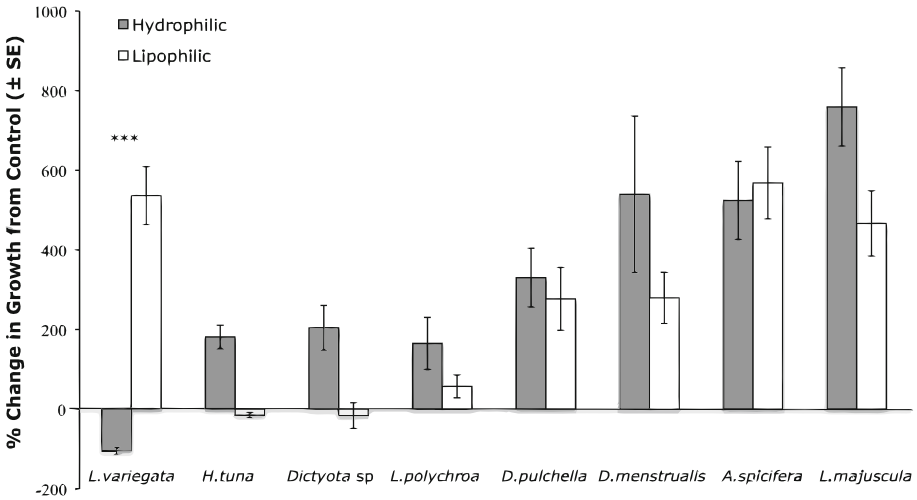

Fig. 2 Mean ± SE of the percent change in growth of 54 bacterial isolates from Caribbean stony corals and macroalgae, in response to crude hydro- and lipophilic extracts from macroalgae and cyanobacteria. Tukey pair-wise comparisons between hydro- and lipophilic extracts only revealed a significant difference for extracts from *L. variegata* (***$P < 0.001$). Cross comparisons for Tukey post hoc tests between and among algae and cyanobacteria are listed in Table 4

Hydrophilic extracts from the brown alga *D. menstrualis* preferentially inhibited *Vibrio* spp. (Fig. 3). This extract inhibited 30 bacteria (56%), of which 22 were *Vibrio* spp. The five *Vibrio* spp. isolates that demonstrated growth in response to *D. menstrualis* extracts had the following nearest neighbors based on 16S rRNA comparison: (a) *V. shiloi/V. mediterranei*, a putative coral pathogen (97.5% 16S rRNA gene identity), (b) *V. harveyi/V. rotiferianus* (99.4% 16S rRNA gene identity), (c) *V. harveyi/V. campbellii* (99.7% 16S rRNA gene identity), (d) *V. harveyi/V. communis* (99.4% 16S rRNA gene identity), and (e) *V. harveyi/V. rotiferianus* (99.7% 16S rRNA gene identity, Fig. 3). Additional analysis of cultures with nearest neighbors most similar to *V. shiloi* ($n = 2$) and *V. harveyi* ($n = 10$), two putative coral pathogens, indicated that twelve extracts (75%) stimulated *V. shiloi* and only two inhibited the growth of this bacterium (lipophilic *H. tuna* and *Dictyota* sp.). Ten extracts (63%) stimulated *V. harveyi* growth, three (19%) inhibited *V. harveyi* growth, and three had no effect on the putative pathogen. Lipophilic extracts from the Belize brown alga, *Dictyota* sp., were the only extracts that inhibited both putative *Vibrio* pathogens.

Discussion

Coral populations have declined precipitously on many tropical reefs over the past three decades while algal abundance has increased, exacerbating competitive interactions between corals and macroalgae at all life history stages. Phase-shifts to higher algal biomass on reefs result not only in stronger negative interactions with adult corals,

Table 4 Tukey post hoc pair-wise comparisons between the growth responses of 60 bacterial isolates to polar and non-polar macroalgal and cyanobacterial extracts

Extract name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. L. avariegata P																
2. L. variezata NP	***															
3. H. tuna P																
4. H. tuna NP		***														
5. Dictyota sp. *P*																
6. Dictyota sp. *NP*		***														
7. L. polychroa P		*														
8. L. polychroa NP																
9. D. pulchella P	**															
10. D. pulchella NP	*															
11. D. menstrualis P	***		*	***	*	***		***								
12. D. menstrualis NP	*															
13. A. spicifera P	***			***		***										
14. A. spicifera NP	***		***	***	*	***										
15. L. majuscula P	***		***	***	***	***	***	***	***	***		***				
16. L. majuscula NP	***			***		***		**								

* = $P < 0.05$, ** = $P < 0.001$, and *** = $P < 0.0001$

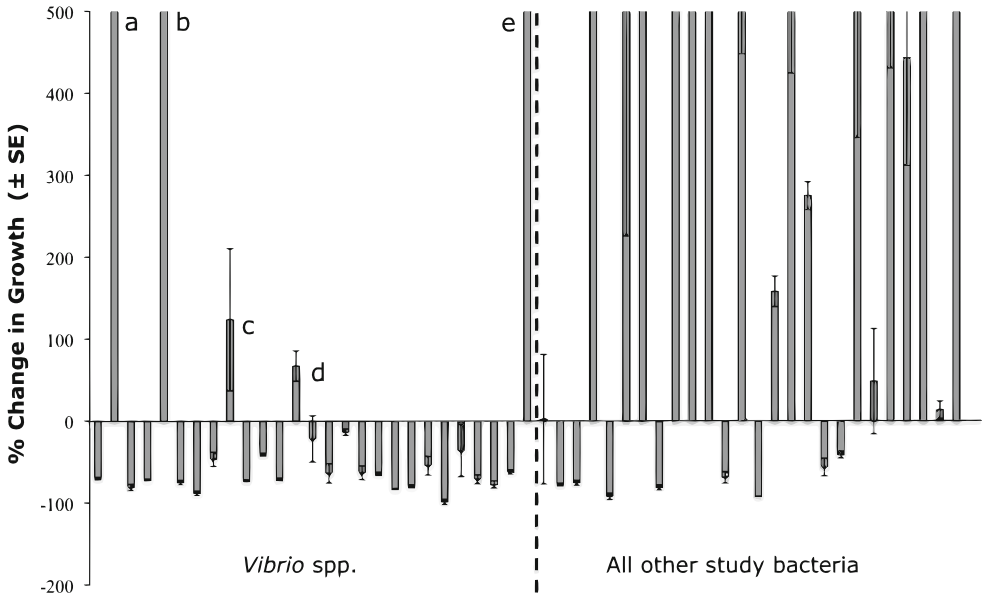

Fig. 3 Effects of *Dictyota menstrualis* (hydrophilic) extract on the growth of bacterial cultures in comparison to controls (no extract). Growth is illustrated only up to 500% to reveal the details of inhibitory effects. This extract inhibited 83% of 28 *Vibrio* spp. bacteria tested. The five *Vibrio* strains stimulated by *D. menstrualis* extracts were most closely related to: (*a*) *V. shiloi/ mediterranei*, (*b*) *V. harveyi/ rotiferianus*, (*c*) *V. harveyi/ campbellii,* (*d*) *V. harveyi/ communis,* and (*e*) *V. harveyi/ rotiferianus*

but also a decrease in coral recruitment by reducing larval settlement and post-settlement survival (reviewed in Birrell et al. 2008; Ritson-Williams et al. 2009), indicating that macroalgae alter the overall structure of reef communities (Hughes and Tanner 2000; Kuffner et al. 2006). The results of the present study reveal another potential mechanism by which macroalgae may impact coral reef community structure. We show here that algal and cyanobacterial crude extracts both positively and negatively impact coral reef-associated bacteria and likely affect natural assemblages of coral-associated bacteria. Several macroalgae exhibited broad-spectrum activity, while others had species-specific effects on particular taxa or groups of bacteria (e.g., *Vibrio* spp.).

Two of the macroalgae produced hydrophilic compounds that exhibited broad-spectrum antibacterial activity (*D. menstrualis, L. variegata*). However, previous studies have suggested that inhibitory compounds are primarily lipophilic (Ballantine et al. 1987; Steinberg et al. 2001), but we found that 25% of the examined hydrophilic extracts

inhibited the majority of bacteria assayed, and all hydrophilic extracts inhibited one or more bacteria (Table 1). Hydrophilic (polar) compounds are more readily solubilized in the water column than are lipophilic compounds. Thus, these macroalgal-derived metabolites have a greater potential than lipophilic compounds to inhibit microbial symbionts on organisms in contact with or downstream from algal thalli. These inhibitory effects are determined in part by the location of compound production, in that surface-emitted compounds are further mediated by water flow. Low-flow and interstitial microenvironments thus could experience a build-up of compounds leading to rapid changes in microbial assemblages on interacting organisms.

Antibacterial activity is exhibited by metabolites produced by many marine plants from temperate and tropical locations around the world (reviewed in Goecke et al. 2010). An antibacterial disk diffusion assay of 102 Puerto Rican macroalgae demonstrated at least some antibacterial activity in 64% of lipophilic extracts examined. The distribution of activity was relatively even (63–71%) among the major algal divisions, but was consistently higher in several orders (83% in Dictyotales, 76% in Caulerpales; Ballantine et al. 1987). In another extensive study of 54 species of marine algae and 2 species of seagrasses collected from Indo-Pacific coral reefs, 95% of the extracts demonstrated antimicrobial activity; however, broad-spectrum activity was demonstrated in <50% of samples. In particular, extracts from the green alga *Bryopsis pennata* and the red alga *Portieria hornemannii* inhibited all assay microorganisms, which included one pathogenic bacterium (*Pseudoalteromonas bacteriolytica*), 2 saprophytic stramenopiles (e.g., oomycete water molds), and 2 fungi (Engel et al. 2006; Puglisi et al. 2007). Thus, antimicrobial chemical defense is widespread among marine plants and algae and may be species-specific to both algal and microbial taxa.

Most previous studies on the microbial impacts of algal compounds have focused on their antibacterial activity; however, the majority of algal extracts surveyed in this study stimulated bacterial growth, which also has the potential to cause significant and detrimental effects on reef corals and thus should not be overlooked. Cole (1982) hypothesized that cyanobacteria within the phytoplankton stimulate bacterial growth via three mechanisms for transfer of organic material from algae to bacteria: (1) microbes may parasitize algal cells, (2) microbes may obtain nutrition from the decomposition of dead cells, and (3) dissolved organic carbon (DOC) released from algae during cell growth (lysis, excretion, autolysis) may also be available to bacteria. That study also intuitively predicted that productive aquatic habitats likely support pathogenic and enteric microorganisms that can grow using algal organic matter (Cole 1982). Recent research has confirmed these early theories and demonstrated that high levels of DOC can cause coral mortality and increase microbial growth rates by an order of magnitude within the coral mucus layer (Kline et al. 2006). Smith et al. (2006) showed that macroalgal-induced microbial growth on coral surfaces could lead to a zone of hypoxia that induces coral tissue death. Thus, many coral-associated microbes likely are carbon-limited, and if encroaching macroalgae increase the amount of labile DOC present, microbes may break down complex and previously unavailable carbon sources via co-metabolism, leading to uncontrolled microbial growth. Two macroalgae and two cyanobacteria in the present study produced both hydro- and lipophilic compounds that stimulated the growth of the majority of bacteria assayed. Microbial growth can cause coral mortality through oxygen depletion, accumulation of poisons, and/or microbial predation on weakened coral polyps (Cole 1982; Segel and Ducklow 1982; Smith et al. 2006). However, macroalgae may need to be in direct contact with coral tissues to result in high enough DOC and other metabolite levels to influence microbial proliferation and/or coral bleaching and subsequent tissue death (Vu et al. 2009; Rasher and Hay 2010). Antibacterial and/or stimulatory effects of coral-algal contact on associated microbes likely would be exacerbated in low flow and high-temperature microclimates (Smith et al. 2006; Bruno et al. 2007). This study may have selected for bacteria that can take advantage of allelochemicals and primary metabolites, such as DOC, when we applied crude extracts from *Halimeda tuna* and *Dictyota* sp. directly to coral surfaces in Belize. The bacteria cultured from under these extracts exhibited the highest growth and least inhibition in comparison to all other cultures sources, including surfaces of algal thalli.

This is the first study to determine the direct effects of compounds produced by macroalgae and cyanobacteria on a diversity of bacteria cultured directly from reef corals and macroalgae. Only about 0.1–1.0% of marine microbial taxa are estimated to be cultured on a laboratory medium such as seawater agar, but we hypothesize that this percentage is much higher for coral-associated bacteria due to the relatively nutrient-rich environment they inhabit (Amman et al. 1995). Future studies are needed that also incorporate culture-independent analyses to examine the effects of macroalgal compounds on a wider diversity of coral-associated microbes. Here, we documented exponential growth in bacterial taxa cultured from coral mucus that was exposed to *H. tuna* extracts. In contrast, bacteria cultured from the surfaces of *Halimeda* and *Dictyota* macroalgae exhibited the smallest amount of growth in comparison to bacteria cultured from un-manipulated coral mucus, indicating that algal-associated bacteria may be less stimulated by these extracts than are coral-associated bacteria. The highest overall growth rates were among bacterial taxa

within the order Alteromondales (Phylum gamma-Proteobacteria), some of which are thought to be resident bacteria of corals (Ritchie 2006). Within the Alteromondales, members of the genus *Pseudoalteromonas* had the highest growth in comparison to controls and are potential coral visitors and disease-causing pathogens (Ritchie 2006). Some members of the Pseudoalteromonadaceae, a small family within the gamma-Proteobacteria, produce algicidal compounds (Egan et al. 2001; Lovejoy et al. 1998; Ivanova and Mikhailov 2001; Mayali and Azam 2004) and may be associated with Yellow Blotch Disease (Cervino et al. 2004) and white plague-like diseases in corals (Sunagawa et al. 2009). Finally, some of the slowest growers are potential coral residents, including *Exiguobacteria* and *Roseobacter* spp. (Ritchie 2006). Coral reef-associated bacteria were strongly affected by macroalgal and cyanobacterial extracts during the short-time course of this experiment (48 h). Thus, reef seaweeds, although sometimes ephemeral, have the potential to rapidly alter coral-associated microbial assemblages and to potentially induce or exacerbate coral disease.

Approximately half the bacterial types that were cultured from corals and macroalgae were members of the genus *Vibrio* (Phylum gamma-Proteobacteria). Members of this genus often cause human diseases (e.g., *Vibrio cholerae*, *Vibrio parahaemlyticus*, and *Vibrio vulnificus*) and can be virulent marine pathogens. In stony corals, *Vibrio* spp. are associated with yellow blotch/band disease (*V. alginolyticus, V. rotiferianus, V. harveyi, and V. proteolyticus*; Cervino et al. 2004, 2008), white band Type II (*V. harveyi, V. charchariae*; Ritchie and Smith 1998), tissue necrosis, rapid tissue loss, and shut down reaction (*V. coralliilyticus, V. harveyi*; Antonius 1977; Jones et al. 2004; Luna et al. 2007; Anthony et al. 2008). *Vibrio* species also are affiliated with diseased *Porphyra* and *Laminaria* macroalgae (Wang et al. 2008). *Vibrio coralyticus* causes tissue lysis of the reef-building coral *Pocillopora damicornis*, spreading so rapidly in some cases that all coral tissue is destroyed in <2 weeks (Ben Haim and Rosenberg 2002). One of the bacteria isolated in the current study was most closely related to *V. shiloi/V. mediterranei,* a bacterium associated with *O. patagonica* bleaching in the Mediterranean Sea. A recent study identified several genes related to virulent functions in *V. shiloi* that were strongly induced by exposure to crushed coral tissue (e.g., Zot toxin, superoxide dismutase; Banin et al. 2003; Reshef et al. 2008). Ten other bacterial isolates examined in this study were strains of *V. harveyi,* which is implicated in yellow blotch/band disease, white band Type II, and rapid tissue disease. The majority of the macroalgal and cyanobacterial extracts stimulated both of these potential pathogens. Notably, hydrophilic compounds produced by *D. menstrualis* preferentially inhibited *Vibrio* spp. relative to other bacteria assayed. *D. menstrualis* extracts inhibited 83% of the 28 *Vibrio* spp. tested, but not the putative coral pathogen, *V. shiloi*. Individuals of *D. menstrualis* exhibit relatively less fouling than do other macroalgae, and their surface extracts include the terpenoid compounds pachydictyol A and dictyol E that deter settlement by the epiphytic bryozoan *Bugula neritina* (Schmitt et al. 1995). However, although *Dictyota* sp. and *D. menstrualis* exhibited natural antibacterial activity in this study, *D. pulchella* produced both hydro- and lipophilic compounds that stimulated bacterial growth, suggesting metabolite differences among species even within the same genus. Furthermore, *V. shiloi* was one of the only bacterial isolates that experienced exponential growth in the presence of *D. menstrualis* (hydrophilic) extracts which otherwise inhibited almost all other *Vibrio* spp. assayed. These results indicate specific effects of macroalgal compounds on particular taxonomic groups of bacteria. Coral microorganisms may be stimulated or inhibited depending on the type of macroalgae and associated metabolites they contact (reviewed in Goecke et al. 2010), but further study is needed to understand the dynamics of these relationships, using a combination of laboratory and in situ studies involving both culture-dependent and -independent methods.

Future research is needed to elucidate the mechanisms by which allelochemicals influence coral-microbial associations, aside from microbial growth inhibition and stimulation. Allelochemicals may regulate microbial composition and abundance by suppressing surface colonization (Chet and Mitchell 1976; Amsler et al. 2001) or attachment and colonization of microbial cells (Steinberg et al. 2001). Future studies also are needed to determine whether these compounds are released into the water column, and at what concentrations, as well as whether the compounds are sequestered near the surface and/or deep within the tissues of algal thalli where they would have more limited effects on interacting organisms. The present results provide evidence that common Caribbean macroalgae produce broad-spectrum as well as species-specific compounds with diverse impacts on a large variety of coral reef bacteria, including three putative coral pathogens. Because the diverse microbial assemblages associated with corals are thought to play an integral part in their innate immune responses (Rosenberg et al. 2007), any shifts in the natural microbial assemblages on corals will contribute to the dynamics of coral health and disease. We have shown here that blanket statements cannot be applied to describe the outcomes or mechanisms of competition among corals, macroalgae, and cyanobacteria on reefs, particularly in terms of the dynamic interactions among their associated microorganisms and allelochemicals.

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