RESEARCH ARTICLE

The green macroalga *Dictyosphaeria ocellata* influences the structure of the bacterioplankton community through differential effects on individual bacterial phylotypes

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Abstract

Marine macroalgae are subjected to large numbers of bacteria in their environment. These bacteria have the potential to affect the health and ecology of algae in a variety of ways and can be both beneficial and harmful to the algae. Therefore, algae have likely evolved mechanisms to differentially regulate the growth of bacterial species. In this study, we examined the effects of the green alga *Dictyosphaeria ocellata* on the bacterioplankton community in field enclosure experiments and on individual, naturally co-occurring bacterial strains in laboratory co-culture experiments. In field experiments, we compared the bacterioplankton communities of enclosures with and without *D. ocellata* using denaturing gradient gel electrophoresis and found that the alga significantly changed the bacterial community composition. Seven bacterial phylotypes were eliminated in the presence of the alga and five were found exclusively with the alga. We also examined the effects of algal-treated water on the development of the bacterial community within enclosures and found no change in the community composition. Laboratory co-culture experiments revealed that *D. ocellata* and *D. ocellata* extracts affect the growth of individual bacterial strains in a species-specific manner and that the mechanisms responsible for these effects also differed by bacterial species.

Introduction

Bacteria play important roles in the health and ecological interactions of sessile marine organisms. They can act as pathogens, nutrient sources and sinks, mediators of biofouling, and producers of defensive metabolites (Littler & Littler, 1995; Lopanik *et al*., 2004; Rao *et al*., 2007; Raina *et al*., 2009). Although bacteria are clearly important and abundant [as many as $2.5 \times 10^8$ bacterial cells mL$^{-1}$ seawater (Ducklow & Shiah, 1993)], investigation of the effects of benthic organisms on the microbial community has only begun relatively recently, driven largely by advances in technology (see reviews by Engel *et al*., 2002; Paul & Ritson-Williams, 2008).

Molecular investigations of the bacterial communities on the surfaces of and in the water surrounding macroalgae indicate that these organisms have evolved mechanisms to regulate their surrounding microbial environment (Dobretsoy *et al*., 2006a; Lam *et al*., 2008; Lachnit *et al*., 2009; Nylund *et al*., 2010). These studies show that the profile of the bacterial community associated with a macroalgal species, as examined using molecular fingerprinting techniques, is unique. Lam & Harder (2007) also determined that in some cases, the change in the bacterial community could be attributed to compounds found in algal-treated water and Nylund *et al*., (2010) attributed surface bacterial community regulation to algal surface-associated polyhalogenated compounds. Although these studies indicate that macroalgae regulate their associated bacterial community, the details of these interactions remain largely unknown.

Studies of the effects of macroalgae on the growth of individual bacterial species have mainly focused on the search for highly potent antibacterial compounds, in most cases for pharmaceutical use or as antifouling agents (Reichert & Borowitzka, 1984; Freile-Pelegrin & Morales, 2004; Salvador *et al*., 2007; Shanmughapriya *et al*., 2008).
However, examination of the activity of algal metabolites against ecologically relevant bacterial species is increasing. Engel et al. (2006) and Puglisi et al. (2007) determined that organic extracts from 55 out of 103 algal species tested inhibited the growth of a known algal pathogen, *Pseudoalteromonas bacteriolytica*. Further, in a study of the bacteriostatic and bacteriolytic effects of organic extracts from the red algae *Mastocarpus stellatus* and *Ceramium rubrum* and the brown alga *Laminaria digitata* on a wide variety of marine bacteria, Dubber & Harder (2008) found that bacterial strains were differentially susceptible to the algal extracts. There is also a growing body of evidence that several macroalgae produce compounds that interfere with quorum sensing (e.g. Borchardt et al., 2001; Skindersoe et al., 2008). In one of the most well-investigated macroalgal–bacterial interactions, it has been discovered that the red alga *Delisea pulchra* produces halogenated furanones that are secreted onto the surface of the alga by specialized gland cells and that mediate the bacterial colonization of the algal surface by interfering with quorum sensing (Maximilien et al., 1998; Dworjanyn et al., 1999; Manefield et al., 2002). Continued investigation of the effects of macroalgae on the bacterial community along with more nuanced studies of the mechanisms involved may lead to the discovery of important interactions influencing macroalgal community dynamics.

*Dictyosphaeria ocellata* is a tropical green alga that lives in the littoral zone attached to hard substrates. Like most benthic macroalgae, it is subjected to a high abundance of bacteria, but is rarely diseased. It also remains clean of macrofouling organisms, the growth and settlement of which are often controlled by biofilm bacteria (Wahl, 1989). *Dictyosphaeria ocellata* is not known to produce broad-spectrum antibiotic compounds and is therefore a good model organism for the investigation of subtle algae–bacteria interactions. In this study, we examined the ability of *D. ocellata* to regulate the composition of its surrounding bacterioplankton community by comparing the molecular profiles of the bacterial communities in field enclosures with and without algae as well as examining the effects of the alga on individual bacterial strains in laboratory co-culture experiments. We also investigated the role of algal extracts in this interaction.

**Materials and methods**

**Field enclosure experiments**

**Experiment 1: Effect of *D. ocellata* on the natural bacterial assemblage**

Field enclosure experiments were performed in December, 2009 in a shallow, near-shore area off of Summerland Key, FL (GPS: 24°41.043′N, 081°26.654′W). Approximately 700 g of *D. ocellata* was collected from the surface of rocks at the field site and placed into a 5 gallon (18.9 L) bucket filled with water from the site. Within 30 min, 10 L Nalgene PETG sterile square media bottles with septum closures were filled with water from the bucket. Approximately 100 g of algae was added to five of the bottles to serve as the experimental treatments, while the other five were used as controls. Dive weights (∼1.8–2.3 kg) were used to hold bottles in place in the field. One treatment bottle and one control bottle were tied to each weight so that the bottles lay on the bottom, ∼70 cm deep, at high tide and were constantly submerged.

Bottles were brought into the laboratory after 48 h and 30-mL water samples were taken from each bottle using a sterile syringe under sterile conditions. Samples were immediately filtered through a 3-μm polycarbonate membrane filter (Millipore®), followed by a 0.2-μm polycarbonate membrane filter (Millipore®) that had been placed in Whatman® plastic filter holders and autoclaved at 121 °C for 15 min before use. Filters were rinsed by pushing 10 mL of autoclave sterilized distilled water through both filters using a sterile syringe. The 0.2-μm filters were removed from the filter holders, cut in half using sterile scissors, placed in sterile microcentrifuge tubes, and stored at −20 °C.

Bacterial DNA was amplified using the filter PCR method described by Kirchman et al. (2001). Filters were cut into sixths using sterile scissors and placed individually into sterile 0.2-μL PCR tubes. Filter pieces were subjected directly to PCR amplifications using 16S rRNA gene bacterial primers 358FGC (cgc cg ccg ccg ccg ggc ggc ggg ggc gcc gca ggg ggc gcc ggc gcc gcc ggc gcc gcc ggc gcc ggc gcc ggc gcc gcc ggc gcc) and 907rM (ccg tca att cmt tgt agt tt) (Muyzer et al., 1995). Each 50 μL PCR reaction contained 1.5 μL bovine serum albumin (BSA) (20 mg mL⁻¹), 1 μL dNTP mix (10 μM), 2.5 U DreamTaq® DNA Polymerase, 5 μL DreamTaq® Buffer (10×), and 2 μL of each primer (10 μM). All reagents came from Fermentas, with the exception of the BSA (A7030), from Sigma Aldrich. Amplification was performed using a Bio-Rad TGradient thermocycler beginning with an initial denaturation step (5 min at 95 °C), followed by 10 touchdown cycles, lowering the annealing temperature by 1 °C each cycle beginning at 65 °C (1 min at 95 °C, 1 min at 65 °C, 2.5 min at 72 °C) and then 25 cycles with an annealing temperature of 55 °C (1 min at 95 °C, 1 min at 55 °C, 2.5 min at 72 °C). A final extension step at 72 °C for 3 min completed the amplification. PCR products were quantified by comparison with the GeneRuler® Express DNA Ladder (Fermentas) run on a 1% agarose gel. Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode™ Universal Mutation Detection System (Bio-Rad). Two hundred nanograms of DNA was loaded onto an 8% acrylamide...
gel with a denaturant gradient from 20% to 70% denaturant (100% denaturant contained 7 M urea and 40% formamide). Standards were produced by combining the PCR products of the direct amplification of three known bacterial strains (Cytophaga sp., Micrococcus sp., and Pseudoalteromonas tetraodonis) so that each standard lane contained 200 ng of DNA from each bacterial strain. These were loaded at the sides and the center of each gel to control for gel smilling. Electrophoresis was run for 12 h at 100 V and 60 °C. Gels were stained with SybrGold (InvitrogenTM). Imaging was performed using the BIODOCANALYZE (BDA) digital system (Biometra).

Band position and volume (area × intensity) were determined for all bands present using BDA digital image analysis software (Biometra). The volume of each band was normalized to the total volume of all bands within one lane to determine the percent contribution of each band (bacterial phylotype) to the total species abundance within each sample. Treatments were compared using one-way ANOSIM based on the Bray–Curtis distance measure. Data were also subjected to nonmetric multidimensional scaling using the Bray–Curtis measure of similarity for graphical representation. Statistical analyses were performed using the statistics program PALEONTOLOGICAL STATISTICS (PAST) v. 1.99 (Hammer et al., 2001).

Experiment 2: Effect of algal-treated seawater on the natural bacterial assemblage

Two 5 gallon (18.9 L) buckets were half filled with water from the field site described above. Approximately 800 g wet weight of algae was collected at the field site and added to one bucket. The buckets were taken to Mote Marine Laboratory, Summerland Key, FL, and 7 L of water from each bucket was transferred into clean 10-gallon (37.9 L) aquariums. Algae (700 g wet weight) was added to the aquarium containing the water from the bucket that originally contained the algae. Aquariums were aerated with airstones and kept under artificial light in a 12:12 light/dark cycle for 24 h at ambient room temperature. Water from the tanks was then gravity filtered through coffee filters, followed by vacuum filtration through 0.22-μm GSWP, 47-mm filters (MilliporeTM) into autoclaved 500- or 1000-mL vacuum flasks using autoclaved MilliporeTM glass filter holders. Algal-treated and non-algal-treated filter-sterilized seawater was transferred to 1-L Nalgene PETG sterile square media bottles with septum closures (n = 3). Bottles were inoculated with 30 mL of natural seawater by injection through the septum cap and placed in the water at the field site as above. Bottles remained in the field for 24 h, after which they were sampled and the bacterial communities were analyzed as in the previous experiment.

Laboratory co-culture experiments

Experiment 3: Effect of D. ocellata on the growth of marine bacteria in co-culture

Dictyosphaeria ocellata was collected in October, 2009, from the surface of rocks in a shallow, near-shore area off of Summerland Key, FL (GPS: 24°41.043′N, 081°26.654′W). The algae were placed in a cooler filled with seawater from the site and transported directly to the Smithsonian Marine Station in Fort Pierce, FL. Here, they were kept indoors in aquariums placed near a window to allow natural light to reach the algae. Aquariums were filled with natural seawater, aerated with airstones, and covered with Plexiglas to reduce evaporation. Algae were cleaned before use by rinsing them under a tap of natural seawater and then removing macroscopic organisms using forceps.

Planktonic bacteria were isolated from seawater that was collected at the same place and time that the algae were collected. Seawater was collected in three sterile 50-mL BD FalconTM tubes. The tubes were placed in a cooler with collected algae and transported to the laboratory. Upon arrival, 100 μL of seawater was transferred to a DifcoTM Marine Broth 2216 1% agar plate and spread using a sterile spreader. This was repeated for each tube. Plates were kept at room temperature.

Surface-associated bacteria were isolated from glass slides that had been incubated in seawater from the collection site. Three glass microscope slides were sterilized with 70% ethanol and then placed into the cooler with the algae. Upon arrival in the laboratory, slides were transferred separately into sterile 50-mL BD FalconTM tubes filled with seawater from the cooler. Within 24 h, slides were rinsed with 10 mL of autoclaved sterile seawater using a 10-mL sterile syringe to remove loosely associated bacteria and placed onto DifcoTM Marine Broth 2216 1% agar plates. Each plate contained one slide. Plates were kept at room temperature until bacterial colonies could be seen growing (∼24 h).

Individual bacterial colonies were picked from the agar plates using a sterile inoculating loop and streaked onto new plates. This was repeated until only one colony form was seen on a plate. Bacterial isolates were then transferred to 25 mL of DifcoTM Marine Broth 2216 using a sterile inoculating loop and grown into a dense culture at room temperature with constant shaking. Liquid cultures were diluted by a factor of 1 × 104 using filter-sterilized seawater and 100-μL samples were spread onto clean agar plates as above. Individual colonies were again picked from the plates using a sterile inoculating loop and transferred to 30 mL of DifcoTM Marine Broth 2216 and allowed to grow for 48 h. Eight hundred and fifty microliters of each bacterial culture was added to 150 μL of autoclave sterilized glycerol and stored at −80 °C. The remaining liquid bacterial cultures...
were kept at room temperature on a shaker until further use in co-culture experiments.

For strains KSW2, KSW3, and S3, DNA was extracted from dense liquid bacterial cultures (started from glycerol stocks) using the UltraClean™ Microbial DNA Isolation Kit from MO BIO Laboratories Inc. The DNA extracts were then subjected to PCR amplification of a 1363 bp section of the 16S rRNA gene using the 27f (ggg ttt gat cct ggc tca g) forward primer and the 1390r (acg ggc ggt gtg trc aa) reverse primer following the same protocol as in the bacterial community profiling. KSW1 was amplified directly from the glycerol stock. PCR products were purified using the QIAquick™ PCR Purification Kit from Qiagen. The purified PCR products were then submitted to GATC Biotech for sequencing. The positions of the isolated bacterial strains within the genus *Pseudoalteromonas* were determined by aligning a 1286 bp section of the 16S rRNA gene of these strains with that of known *Pseudoalteromonas* sp. as in Holmström & Kjelleberg (1999) (Fig. 1). Sequences of known *Pseudoalteromonas* sp. were obtained from GenBank and the alignment was performed with CLUSTALW. Sequences obtained from bacterial isolates are available at GenBank under accession numbers HQ164445–HQ164448.

The effect of *D. ocellata* on the growth of three planktonic (KSW1, KSW2, and KSW3) and one glass slide surface-associated (S3) bacterial isolate was investigated by co-culturing the algae with the bacteria. Because the microbial communities naturally associated with algae may play a role in interactions with other bacteria, the algae were not sterilized before use. Therefore, any reference here to *D. ocellata* includes both the algae and its naturally associated microbial community. The bacterial isolates were cultured overnight in Difco™ Marine Broth 2216 so that they were in the stationary phase before use. For each isolate, 15 mL of

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Fig. 1. Phylogenetic affiliation of isolated bacterial strains within the genus *Pseudoalteromonas*. The tree is a modification of that seen in Holmström & Kjelleberg (1999). Isolated strains are shown in bold with GenBank accession numbers in parentheses.
culture was added to 135 mL of filter-sterilized natural seawater in a 200-mL Erlenmeyer flask. *Dictyosphaeria ocellata* was rinsed with 20 mL of autoclaved seawater using a 60-mL sterile syringe. Five grams (wet weight) of algae was added to the flask (bacteria + algae) and the growth of the bacteria was monitored by measuring the OD_{550 nm} (Shi et al., 1940; Quinn & Keough, 2002). Bacterial growth in the presence of *D. ocellata* was compared with that in controls prepared without the addition of algae (bacteria alone). An additional control was prepared to ensure that the contribution of algal-associated bacteria to the overall abundance in the co-cultures was negligible (algae alone). Five grams of algae (prepared as above) was added to 135 mL of filter-sterilized seawater. Fifteen milliliters of each respective bacterial culture was sterile filtered to remove bacteria and added to the algae-alone treatment to control for the effects of added nutrients on the growth of the naturally associated bacterial community. Co-cultures and controls were continually shaken at room temperature under natural light. All treatments contained five replicates unless otherwise stated.

Bacterial cultures (abundance measured as OD) were compared in terms of the maximum growth rate (exponential phase) and the similarity of overall growth across time. The maximum growth rate was designated as the largest slope (μ) between two consecutive points on the growth curve:

\[ \mu = \frac{(\ln \text{OD}_2 - \ln \text{OD}_1)}{(t_2 - t_1)} \]

The maximum growth rates of different treatments were compared using Student’s *t*-test when two treatments were compared and using one-way ANOVA, followed by the Bonferroni multiple comparisons test to compare three or more treatments. All *P* values for results of the Bonferroni Multiple Comparisons are given as either *P* < 0.05 or *P* > 0.05. Growth rates are given as the mean ± SE. The similarities of the overall growth across time were examined using two-way repeated measures (RM) ANOVA. Before two-way RM ANOVA analysis, data were tested for sphericity using Mauchly’s test and were corrected when the assumption of sphericity was not met using the Greenhouse–Geisser correction (Mauchly, 1940; Quinn & Keough, 2002). Post hoc comparisons were made using a Bonferroni post-test where required (Neter et al., 1990). All analyses were performed using GRAPHPAD PRISM 5, except for tests and corrections of sphericity, which were performed using SYSTAT 9. Differences were considered significant when *P* ≤ 0.05 for all tests.

**Experiment 4: Effect of co-culture system components (media filtrate, media extract, algal extract) on the growth of marine bacteria**

Directly following the last measurement of the co-culture experiments, the medium from each replicate was vacuum-filtered through a 0.22-μm GSWP, 47-mm filter (Millipore ™) into an autoclaved 500- or 1000-mL vacuum flask using a Millipore ™ glass filter holder. Sterile filtrate (36 mL) was transferred to a sterile 50-mL BD Falcon ™ tube and frozen at −20 °C until further use. Organic compounds were extracted from the remaining filtrate using a Varian MegaBond Elut C18 cartridge (6 cc). The filtrate was passed through the cartridge, the cartridge was allowed to dry for 5 min, and organic compounds were eluted sequentially with 2 mL of methanol and 2 mL ethyl acetate, all under vacuum. The methanol and ethyl acetate eluates were combined, dried in a speed vac at 37 °C (SPD SpeedVac ThermoSavant), and stored at −20 °C. Cartridges were rinsed with 6 mL of distilled water, followed by 6 mL of methanol and then another 6 mL of distilled water and allowed to dry for 5 min under vacuum before the first use and after each subsequent use. They were reused a maximum of five times.

Algae from the co-culture experiments were rinsed in distilled water, patted dry with paper towels, and freeze-dried (Labconco® Freezone 6). Dried algae were ground to a fine powder using a mortar and pestle. The dry powder was weighed and extracted in 4 mL of methanol : dichloromethane (1 : 1) for 30 min. The extracts were centrifuged to remove cell debris and the supernatant was transferred to 6-mL glass vials. Solvents were allowed to evaporate from the extracts under the fume hood at room temperature. Dry extracts were stored at −20 °C.

In addition, 100 g (wet weight) of *D. ocellata* that had not been used in the co-culture experiment was extracted in order to determine whether the coculturing affected the activity of the algae against the bacteria. The alga was rinsed, dried, and ground as above. It was extracted in 80 mL of methanol : dichloromethane (1 : 1) for 30 min and then processed as above.

Filtered culture media, media extracts, and algal extracts were tested for activity against the same bacterial strain that they were originally exposed to in the co-culture experiment. Filtered culture media samples were allowed to thaw at room temperature before use. Seawater controls were prepared by adding 36 mL of filter-sterilized natural seawater to five 50-mL BD Falcon ™ tubes. Four milliliters of stationary-phase bacterial culture was added to each replicate.

Both media and algal extracts from co-cultures were dissolved in dimethyl sulfoxide (DMSO, 300 and 500 μL, respectively). The fresh algal extract, not exposed previously to the bacterial cultures, was dissolved in 10 mL DMSO.
Extracts (100 μL) were added to 50-mL BD Falcon™ tubes containing 27 mL of sterile-filtered natural seawater. The solutions were shaken and inoculated with 3 mL of stationary-phase bacteria culture. The final concentrations of the extracts in solution were volumetrically equivalent to those of the original co-culture experiments. Solvent controls were prepared by adding 100 μL of DMSO to 27 mL of sterile-filtered natural seawater before inoculation with 3 mL of bacterial culture. Tubes were shaken continually at room temperature. Bacterial growth was monitored and analyzed as above.

**Results**

**Field enclosure experiments**

**Experiments 1 and 2: Effect of *D. ocellata* and algal-treated seawater on the natural bacterial assemblage**

Bacterioplankton communities were significantly different in enclosures with algae and those without algae ($R = 0.988, P = 0.0073$, Fig. 2). DGGE revealed 24 total bacterial phytypes, seven found exclusively in the absence of algae (bands #7, 14, 15, 16, 18, 22, and 24), five found exclusively in the presence of algae (bands #4, 9, 12, 21, and 17), and 12 shared by both (Fig. 2). There was no difference in the bacterioplankton communities grown in algal-treated seawater as compared with non-algal-treated seawater ($R = −0.03704, P = 0.5012$, Fig. 3).

**Laboratory co-culture experiments**

**Experiment 3: Effect of *D. ocellata* on the growth of marine bacteria in co-culture**

To test the influence of *D. ocellata* on bacteria that are found in its natural environment, we isolated and cultured three planktonic and one surface-associated strain. Sequencing of the 16S rRNA gene and alignment indicated that all belong to the genus *Pseudoalteromonas* (Fig. 1). *Dictyosphaeria ocellata* significantly affected all three bacteria strains isolated from seawater (Fig. 4), but had no effect on the one glass slide surface-associated isolate tested ($P = 0.6477$ and $P = 0.0073$, Fig. 2). DGGE revealed 24 total bacterial phytypes, seven found exclusively in the absence of algae (bands #7, 14, 15, 16, 18, 22, and 24), five found exclusively in the presence of algae (bands #4, 9, 12, 21, and 17), and 12 shared by both (Fig. 2). There was no difference in the bacterioplankton communities grown in algal-treated seawater as compared with non-algal-treated seawater ($R = −0.03704, P = 0.5012$, Fig. 3).
0.6493 for overall growth and maximum growth rate of S3, respectively). In all cases, the alga had inhibitory effects on planktonic bacteria; however, the effects were seen at different growth stages in different bacterial strains. The maximum growth rate of bacterial strain KSW1 was significantly inhibited in the presence of the algae ($P = 0.0004$, Fig. 4d). KSW1 cultures with and without algae both began declining in abundance after 24 h; however, those with algae declined more rapidly, causing a significant difference in the bacterial abundance at these later time points ($P < 0.0001$, Fig. 4a).

In contrast to KSW1, algae had no effect on the maximum growth rate of either KSW2 or KSW3 ($P = 0.3635$ and $P = 0.9166$, respectively, Fig. 4d). However, the overall growth curves of both strains were affected by *Dictyosphaeria ocellata* ($P < 0.0001$ and $P = 0.0464$, respectively, Fig. 4b and c). The abundance of bacteria in KSW2 cultures with algae was significantly lower than in treatments without algae 8 h after inoculation and again 48 h after inoculation, continuing until the end of the experiment ($P < 0.0001$, Fig. 4b). While there was a significant effect of algae on the overall growth curve of KSW3 cultures, the abundance was only significantly different at 24 and 72 h ($P = 0.0464$, Fig. 4c). In KSW1 and KSW2 co-cultures, the algae-alone control exhibited minimal bacterial growth, reaching a maximum average absorbance of $0.074 \pm 0.012$ OD$_{550\text{nm}}$ and $0.058 \pm 0.010$ OD$_{550\text{nm}}$, respectively. Therefore, the contribution of algal-associated bacteria to the overall bacterial abundance in the algal–bacterial co-cultures was negligible. In both KSW3 and S3 co-cultures, the algae-alone treatment was obviously contaminated, having the characteristic colors associated with these bacteria (pink and yellow, respectively). The bacterial abundances in these treatments were therefore a result of contamination and not that of the growth of the alga’s naturally associated bacterial community and were excluded from further analysis.

Algae appeared to remain healthy throughout this experiment, retaining their normal color and rigidity.

**Experiment 4: Effect of co-culture system components (media filtrate, media extract, algal extract) on the growth of marine bacteria**

During the test of the activity of media filtrates on the growth of KSW1, there was no bacterial growth in any of the treatments and these data were therefore excluded from further analysis. Filtrate from the bacteria + algae treatment drastically inhibited the growth of KSW2, having both a lower maximum growth rate than the seawater control ($P < 0.05$, Fig. 5c) and lower abundances than all other treatments for the first 24 h, at which time the other
treatments had declined in abundance \((P < 0.0001, \text{Fig. 5a})\). Filtrates from the bacteria-alone and algae-alone treatments did not affect the maximum growth rate or the overall growth of KSW2 \((P > 0.05\) for both, Fig. 5a). In contrast to KSW2, media filtrates of bacteria+algae cultures significantly increased the maximum growth rate of KSW3 \((P < 0.05, \text{Fig. 5c})\). Media filtrates from the bacteria+algae treatment also increased the overall abundance of KSW3 compared with the seawater control and compared with the filtrate from bacteria alone starting 2 and 4 h after inoculation, respectively \((P < 0.0001, \text{Fig. 5b})\). Filtrates from the bacteria-alone treatment also increased the abundance of KSW3 compared with the seawater control starting 4 h after inoculation, but to a lesser extent than the bacteria+algae filtrate \((P < 0.0001, \text{Fig. 5b})\).

Media extracts showed no activity either on the maximum growth rate \((P = 0.3252, \text{Fig. 6d})\) or on the overall growth of KSW1 \((P = 0.5321, \text{Fig. 6a})\). Media extracts of both bacteria+algae and algae alone significantly inhibited the maximum growth rate of KSW2, but had no effect on the overall growth \((P < 0.05 \text{ and } P = 0.1299, \text{respectively, Fig. 6b and d})\). Bacteria-alone media extracts had no effect on either the maximum growth rate or the overall growth of KSW2 \((P < 0.05 \text{ and } P = 0.1299, \text{respectively, Fig. 6b and d})\). While there was a significant effect of media extract on the overall growth of KSW3, a comparison of individual treatments revealed a significant difference only at 24 h post-inoculation, at which time the bacterial abundance was higher in both treatments compared with the solvent control \((P = 0.0121, \text{Fig. 6c})\). Neither the bacteria alone nor the bacteria+algae media extracts affected the maximum growth rate of KSW3 \((P > 0.05, \text{Fig. 6d})\).

Extracts of algae from the bacteria+algae and algae-alone treatment inhibited the maximum growth rate of all three bacterial strains \((P < 0.05, \text{Fig. 7d})\). Extracts of the algae-alone treatment also inhibited the maximum growth rate of KSW1 and KSW2 \((P < 0.05, \text{Fig. 7d})\), but were not tested for KSW3 due to the contamination described in Materials and methods. Algal extracts had no effect on the overall growth of KSW1 \((P = 0.4749, \text{Fig. 7a})\); however, the OD of algal extract treatments was higher than the solvent controls during the first 6–24 h for KSW2 and KSW3 \((P < 0.0001, \text{for both, Fig. 7b and c})\).

There were no differences in the activity of algal extracts taken from co-cultures and those of algae that were not exposed previously to the bacterial cultures.
Discussion

The bacteria associated with a sessile marine organism considerably affect its interactions within the community in a variety of ways including mediating the settlement of fouling organisms, enhancing competitive fitness for space, and deterring predation (Lopanik et al., 2004; Dobretsov et al., 2006b; Smith et al., 2006; Rao et al., 2007). It is therefore important to understand the processes involved in regulating the growth and abundance of these bacteria in order to understand the relationships within the larger context. As opposed to studies of the broad-spectrum antibacterial activity of algal extracts (e.g. Engel et al., 2006; Puglisi et al., 2007), this study focused on the subtle effects of algae on the surrounding bacterial community, both inhibitory and stimulatory. As some bacteria are beneficial to sessile marine organisms while others are harmful, algae have likely evolved mechanisms to selectively control the growth of bacteria (Littler & Littler, 1995; Sawabe et al., 1998; Dobretsov & Qian, 2002; Rao et al., 2007).

Here, we show that the tropical alga *D. ocellata* has the ability to influence the composition of the surrounding bacterioplankton community within a closed environment. In this study, we designed closed enclosure experiments that mimicked the limited molecular exchange that occurs between the thin boundary layer of water that surrounds a macroalga and the ambient seawater (Hurd, 2000). In the case of *D. ocellata*, this exchange is particularly limited due to the structure of the alga. The siphonous alga is a composite of bubble-shaped cells, each reaching several millimeters in diameter. Between these cells, exchange with the external seawater is minimal. Interactions between macroalgae and the bacterioplankton will likely occur within diffusion boundary layers and it is therefore important to monitor the bacterioplankton community in a similarly stagnant environment. Enclosures containing *D. ocellata* had significantly different bacterial community compositions compared with those without the algae (Fig. 2). Seven bacterial phylotypes were eliminated in the presence of the algae while the growth of five others was promoted (Fig. 2). The selective elimination and promotion of bacterial phylotypes by *D. ocellata* supports the idea that algae are able to selectively control the growth of bacteria. These results are similar to those found by Lam & Harder (2007), who determined that several species of temperate macroalgae affected the richness of the surrounding bacterioplankton community by promoting the growth of some phylotypes and inhibiting the growth of others. However, algal-treated

![Graphs showing bacterial growth rates](image)
seawater had no effect on the composition of the bacterioplankton community (Fig. 3). This indicates that either waterborne compounds released from the algae are not responsible for the change in the bacterial community or that these compounds are volatile or unstable [e.g. reactive oxygen species (ROS)] and were therefore lost during the vacuum filtration of the algal-treated seawater (Potin et al., 1999; Kupper et al., 2002). Macroalgae are known to release volatile compounds into seawater and some of these volatiles exhibit antibacterial activity (Gschwend et al., 1985; Duque et al., 2001; Karabay-Yavasoglu et al., 2007). Nevertheless, the ecological role of these compounds in benthic marine interactions has been largely unexamined.

Lam et al. (2008) found that in some cases, alterations in the bacterioplankton community could be attributed to waterborne compounds, but in others, direct contact with the alga was necessary to elicit an effect. It is also possible that the compounds responsible for the alteration of the bacterioplankton community by D. ocellata are found at the surface of the alga as opposed to being released into the water. In a study of the red macroalga Bonnemaisonia hamifera, Nylund et al. (2008) demonstrated that surface compounds inhibited the growth of marine bacteria at natural concentrations. This possibility is lent further support by the effects of D. ocellata on the growth of KSW1, which can only be attributed to compounds found in the algal extract and not in the water (see discussion below, Figs 4d, 6d, and 7d).

Alternatively, the observed changes in the bacterial communities of algae containing enclosures may be the result of factors other than algal metabolites. The presence of algae will cause changes in many physical parameters of the closely surrounding seawater including an increase in dissolved oxygen and pH (Irwin & Davenport, 2002; Larkum et al., 2003). The algae may also compete with bacteria for limiting nutrients, shifting the bacterial community to one dominated by those strains best able to compete. Because of uncertainties involved in field enclosure experiments, we set out to further elucidate the mechanisms of algal–bacteria interactions in controlled laboratory experiments. In addition to the selective effects of D. ocellata on the bacterioplankton community found in enclosure experiments, laboratory co-cultures revealed differential effects of the alga on the growth of individual bacterial strains. We were able to take four bacterial isolates into culture that were obtained from the close environment of D. ocellata in the Florida Keys. Sequence alignment of the 16S rRNA gene suggested that all belong to the Pseudoalteromonas genus (Fig. 1). These bacteria were used to challenge D. ocellata in 5-day co-culture experiments. Despite the fact that high cell counts

Fig. 7. Overall growth and initial growth rates of bacterial strains KSW1, KSW2, and KSW3 in response to extracts of the algae used in the co-culture. Bars = mean absorbance, error bars = SE, n = 5 (except the KSW1 algae-alone treatment, n = 4). Letters above the bars indicate significant differences between treatments within each time point (a–c) or within each bacterial strain (d) (P ≤ 0.05). No letters above the bars indicates that there is no difference in the overall growth of different treatments. Algal extracts from: •, bacteria + algae; □, algae alone; △, solvent control.
were reached in the co-culture experiments, none of the bacteria exhibited algicidal effects. *Dictyosphaeria ocellata* exhibited its natural shape and texture after the co-culturing. Of the four bacterial isolates tested, the growth of the three planktonic isolates (KSW1, KSW2, and KSW3) was affected by the presence of *D. ocellata*, while that of the one surface-associated isolate (S3) was not. The three planktonic isolates studied were affected at different times in their respective growth curves and by different components of the co-culture system. The growth of KSW1 was inhibited by *D. ocellata* during its exponential phase (Fig. 4d). A similar inhibition was seen in KSW1 cultures exposed to algal extracts, but not media extracts, indicating that the alga is producing a growth inhibiting compound (or compounds) that is either not released into the water or is not stable (Figs 6d and 7d). Such compounds could affect the bacteria during co-culturing because the bacteria have direct contact with the surface of the alga. Also, unstable compounds may be continuously released by the alga and/or affect the bacteria before breaking down. This lack of active waterborne compounds was also seen in the field enclosure experiments discussed above. Again, the activity of volatile compounds that would be lost during the vacuum filtration process could not be excluded.

Co-culture with *D. ocellata* also initiated an earlier and more rapid decline in KSW1 abundance compared with bacteria-alone controls (Fig. 4a). A similar pattern was seen in media extracts of both bacteria + algae and algae-alone co-cultures, although there was no significant effect according to two-way RM ANOVA (Fig. 6a). This suggests that the causative agents of this early decline are compounds that cannot be quantitatively extracted by the method used here. It should be noted that changes seen during the declining phase of bacterial cultures grown in direct co-culture with the alga could be due to bottle effects that would not be relevant in ecological situations. However, when these effects are replicated by components of the co-culture system (e.g. algal extracts), it is likely that there is a true influence of the alga.

Although there was no effect of *D. ocellata* on the maximum growth rate of KSW2, the algae caused a rapid decline in KSW2 abundance starting 48 h after inoculation similar to that seen in KSW1 (Fig. 4a and b). There was no effect seen on the declining phase of KSW2 in response to any of the co-culture components tested; however, the media filtrate of the bacteria + algae obtained after prolonged co-culturing drastically inhibited the growth of the bacteria throughout the growth curve (Figs 5a, 6b, and 7b). Interestingly, there was no effect of the algae-alone or bacteria-alone media filtrates on the growth curve of KSW2, indicating the production of compounds in response to the interaction between algae and bacteria (Fig. 5a). It appears that the algal or algal-associated microbial community is producing defensive compounds in response to the presence of the bacteria; however, we cannot currently rule out the possibility that the bacteria are producing an autotoxic compound in response to the presence of the alga. Nutrient effects can be excluded because controls indicated no limitation. Studies of brown algae (*Laminariales*) and red algae in the genus *Gracilaria* have shown that these algae respond to the presence of their own cell walls by producing ROS, which in turn confer resistance to the algae against pathogenic bacteria (Küpper et al., 2002; Weinberger et al., 2005; Weinberger, 2007). However, this is a relatively fast and short-term response not comparable to the processes observed in our assays (Küpper et al., 2002; Weinberger et al., 2005). The inhibition of KSW2 growth by the media filtrate of the bacteria + algae co-culture persists for the entire period of the experiment (72 h), indicating something other than ROS as a causative agent (Fig. 5a). While several studies have shown the production of antibiotic compounds by macroalgae, none, to our knowledge, have demonstrated the production or the activation of these compounds in response to the presence of a bacterial species (Engel et al., 2006; Paul et al., 2006; Nylund et al., 2008).

The inhibition of KSW2 growth during the exponential phase in response to media extracts of algae containing co-cultures and all algal extracts was relatively short term compared with the effects of the media filtrate on this bacterial strain (Figs 5a, 6b, and 7b). This indicates that some active compounds were extracted from both the media and the algae. However, the major cause of activity in the media filtrate could not be extracted using these methods.

The inhibition of the maximum growth rate of KSW2 by algal extracts appears to be contradicted by the higher absorbance values of treatments containing algal extracts compared with solvent controls for the first 6 h (Fig. 7b and d). However, this increase in absorbance is present at the time of inoculation (time = 0) and is likely to be caused by compounds, such as pigments, found in the algal extract as opposed to actual differences in bacterial density. The maximum growth rate was determined as a change over time and is therefore not affected by the discrepancy in the starting values between treatments. A similar pattern was seen in the growth of KSW3 in response to algal extracts. As with KSW2, *D. ocellata* did not affect the growth of KSW3 in the exponential phase; however, it did affect the overall growth curve, causing a lower abundance at 24 and 72 h postinoculation (Fig. 4c and d). Although there was only a limited effect of *D. ocellata* on bacterial growth in the co-culture experiment, there were significant effects of media filtrates and algal extracts on growth in the exponential phase as well as the overall growth curve (Figs 5b, c and 7c, d). Interestingly, media filtrates of bacteria + algae promoted the growth of KSW3 in the exponential phase while algal extracts inhibited it (Figs 5c and 7d). It is likely that these opposing effects cancel each other out in the co-culture (Fig. 4d). It is known that algae release dissolved
organic carbon (DOC) into the water column and that this can be used as a nutrient source for bacteria (Cole et al., 1982; Jensen, 1985; Smith et al., 2006; Wada et al., 2007). It is possible that compounds (perhaps DOCs) released by \textit{D. ocellata} could promote the growth of KSW3 and that inhibitory compounds found in the algal extracts are necessary to maintain an acceptable abundance of KSW3.

The alteration of the bacterio-plankton community by \textit{D. ocellata} in field enclosure experiments and the species-specific effects of \textit{D. ocellata} on the growth of various bacterial isolates indicate that this macroalga can selectively regulate the bacterio-plankton community surrounding it. It does not revert to universally active antibiotics to eliminate the entire bacterial community, but rather influences the surrounding organisms specifically and with different modes of action. Although the mechanisms involved in mediating these interactions remain unclear, it appears that different bacterial isolates are differentially sensitive to various components of the co-culture system. The activity of organic extracts against the bacterial strains tested here indicates the possibility of active secondary metabolites; however, other physical properties must also be considered. While we focused here on determining the role of organic compounds, it is likely that a combination of factors, including the algal morphotype and the mechanical properties of the algal surface, act together to shape the bacterio-plankton community surrounding \textit{D. ocellata}. More studies of the subtle interactions taking place between algae and their associated microbial community and the chemical signals involved are needed to elucidate the complex processes occurring in this system.

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