Effects of the green alga *Dictyosphaeria ocellata* on its surrounding bacterial community

Dissertation

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List of abbreviations

16S rDNA	16S ribosomal deoxyribonucleic acid
16S rRNA	16S ribosomal ribonucleic acid
ANOSIM	analysis of similarity
ANOVA	analysis of variance
ASW	artificial seawater
bp	base pair
BDA	BioDoc Analyze
BSA	bovine serum albumin
BH	Bahia Honda
CO_2	carbon dioxide
DBL	diffusion boundary layer
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EtOAc	ethyl acetate
FSW	filtered seawater
HCl	hydrochloric acid
^{1}H NMR	proton nuclear magnetic resonance
GC-MS	gas chromatography-mass spectrometry
LK	Long Key
MeOH	methanol
NaCl	sodium chloride
NMDS	non-metric multidimensional scaling
NMR	nuclear magnetic resonance
PETG	polyethylene terephthalate copolyester
OD	optical density
oTOF	orthogonal time-of-flight
PCR	polymerase chain reaction
PERMANOVA	permutation multivariate analysis of variance
PVDF	polyvinylidene fluoride
RM ANOVA	repeated measures ANOVA
RNA	ribonucleic acid
ROS	reactive oxygen species
SC	solvent control
SDS	sodium dodecyl sulfate
SK	Summerland Key
SPE	solid phase extraction
TE	Tris-EDTA
THF	tetrahydrofuran
T-RFLP	terminal restriction fragment length polymorphism
Tris	tris(hydroxymethyl)aminomethane

UPGMA	unweighted pair-group average using arithmetic averages

Zusammenfassung

Marine Makroalgen sind ubiquitär an den Küstenzonen der Ozeane verbreitet. Makroalgen sind wichtige Primärproduzenten und werden seit Jahrtausenden von den Menschen als Nahrungsquelle genutzt. Die physiologische und ökologische Leistungsfähigkeit dieser Organismen wird grundlegend von der umliegenden bakteriellen Gemeinschaft beeinflusst. Aufgrund der hohen bakteriellen Abundanz von bis zu 2.5 x 10^8 Bakterienzellen pro Milliliter Seewassser ist es durchaus wahrscheinlich, dass Makroalgen Mechanismen entwickelt haben, um die Beschaffenheit der bakteriellen Gemeinschaft zu regulieren. Neueste Untersuchungen weisen tatsächlich auf eine gewisse Wirt-Spezifität der bakteriellen Gemeinschaften auf der Oberfläche von Algen hin. Weiterhin ist es seit geraumer Zeit bekannt, dass Algen Substanzen mit bakteriostatischen und bakteriolytischen Eigenschaften produzieren. Umfassende Untersuchungen der ökologischen Funktion dieser Substanzen sind jedoch bisher nicht bekannt. Um ein besseres Verständnis der Effekte von Makroalgen auf ihre umliegende bakterielle Gemeinschaft zu generieren, habe ich eine Reihe von Feld- und Laborversuchen mit der siphonalen Grünalge *Dictyosphaeria ocellata* als Modelorganismus durchgeführt.

Zunächst habe ich gezeigt, dass *D. ocellata* im Vergleich zu einer anderen Grünalge und abiotischen Oberflächen eine einzigartige bakterielle Biofilm-Gemeinschaft beherbergt. Allerdings variiert diese Gemeinschaft zwischen verschiedenen Standorten, was möglicherweise eine Folge der großen Unterschiede der gesamten bakteriellen Gemeinschaft an den jeweiligen Standorten ist. Diese Wirt-Spezifität legt nahe, dass *D. ocellata* Mechanismen besitzt, um die Zusammensetzung der umliegenden bakteriellen Gemeinschaft zu beeinflussen. Daher habe ich anschließend den Effekt von organischen Extrakten der Alge auf die Biofilmbildung untersucht. Ich konnte zeigen, dass mit Methanol extrahierte Substanzen die Zellkonzentration der Bakterien in dem Biofilm verringerte und auch die Zusammensetzung der bakteriellen Gemeinschaft veränderte.

Zusätzlich habe ich auch die Effekte auf die planktonisch lebende Bakteriengemeinschaft untersucht. Dabei fand ich, dass auch diese bakterielle Gemeinschaft signifikant von der Alge

verändert wird. Es war mir allerdings nicht möglich diesen Effekt einer spezifischen im Wasser vorkommenden Substanz zuzuordnen. Es scheint daher möglich, dass ein direkter Zellkontakt mit der Alge nötig ist oder die verantwortlichen Substanzen instabil sind. Um ein besseres Verständnis über die in dieser Interaktion involvierten Mechanismen zu erhalten, habe ich weitere Versuche im Labor durchgeführt. Hier habe ich den Effekt der Alge und deren Extrakte auf das Wachstum von individuellen, natürlich vorkommenden Bakterienstämmen durchgeführt. Ich zeigte, dass verschiedene Bakterien unterschiedlich auf die Alge reagierten und verschiedene Faktoren für den Effekt verantwortlich waren. Zum Beispiel haben einige Bakterienstämme auf organische Zellextrakte reagiert, während andere Stämme nur auf im Meerwasser vorliegende Substanzen eine Reaktion zeigten.

Zum Schluss habe ich eine Übersichtsanalyse der Aktivität von *D. ocellata* Extrakten gegenüber einer Reihe von natürlich koexistierenden Bakterien durchgeführt. Dabei stellte ich überraschenderweise fest, dass durch die Extrakte die Zelldichte der Bakterien in der stationären Phase entweder erhöht wurde oder kein Effekt festgestellt werden konnte. Mit Hilfe einer Bioassay geleiteten Fraktionierung konnte ich zeigen, dass sich die aktive Substanz in der Hexan und Chloroform Fraktion des Algen-Rohextraktes befand. Weitere Untersuchungen dieser Fraktionen zeigten die Präsenz einer Reihe von freien Fettsäuren, diese sind aber vermutlich nicht die aktive Komponente. Deshalb sind weitere Untersuchungen notwendig, um aufzuklären, welche Substanzen für die Aktivität verantwortlich sind.

Diese Ergebnisse zeigen, dass *D. ocellata* die bakterielle Gemeinschaft sowohl auf ihrer Oberfläche als auch im umliegenden Seewasser reguliert. Diese Alge produziert organische Substanzen, welche an der Regulation der bakteriellen Gemeinschaft involviert sind.

Abstract

Marine macroalgae are widespread throughout the world's oceans. They are important primary producers and have been used for millennia as food products for humans. The health and ecology of these organisms are greatly affected by the bacterial communities surrounding them. Because bacteria are so abundant in the oceans, as many as 2.5×10^8 bacterial cells per milliliter of seawater, it is likely that macroalgae have evolved mechanisms to regulate the composition of the bacterial community surrounding them. In fact, recent studies indicate that a certain degree of host specificity exists among bacterial communities on the surface of algae. In addition, it has been known for some time that algae produce compounds with bacteriostatic or bacteriolytic properties. However, a good understanding of the ecological roles of these compounds has yet to be established. In order to better understand the effects of macroalgae on their surrounding bacterial community, I performed a series of field and laboratory experiments using a green siphonous alga, *Dictyosphaeria ocellata,* as a model organism.

Initially, I determined that *D. ocellata* harbors a unique bacterial biofilm community in comparison to another green alga and an inanimate surface taken from the same location. However, the bacterial community on the surface of *D. ocellata* is not consistent across different locations, which is likely due to the large differences in the overall bacterial communities present in these locations. This host specificity suggested that *D. ocellata* has some mechanisms for regulating the composition of the bacterial community surrounding it, and I therefore examined the effects of organic extracts of the algae on biofilm formation. I found that compounds extracted in methanol significantly decreased the abundance of bacteria present in the biofilm and also changed the structure of the bacterial community.

In addition to examining the effect of the alga on biofilm bacteria, I also looked at its effect on the bacterioplankton community and found that this is also significantly altered by the presence of the alga. However, I could not attribute this alteration to stable waterborne compounds released by the alga, and it therefore appears that these effects may require direct

contact with the alga or that the compounds responsible may not be stable. In order to better understand the mechanisms involved in these interactions, I took the system into the laboratory setting and examined the effects of the alga and its extracts on the growth of individual, naturally co-occurring bacterial strains. I found that different bacterial strains reacted differently to the presence of the alga, and that the factors eliciting a response differed between bacterial strains. For example, some strains responded to the organic whole-cell extracts of the alga, while others responded to waterborne compounds.

Finally, I performed a screening of the activity of *D. ocellata* extracts against a suite of naturally co-occurring bacteria and found, surprisingly, that the extracts either increased the stationary phase abundance of these bacteria or had no effect on them. Based on bioassay guided fractionation, I determined that the active compounds were found in the hexane and chloroform fractions of the crude extract. Further examination of these extracts revealed a diversity of free fatty acids, however these are not likely the active compounds and further investigation is necessary to elucidate what compounds are responsible for the activity.

These results indicate that *D. ocellata* regulates the composition of the bacterial community on its surface and in the closely surrounding seawater. Furthermore, that the alga produces organic compounds that are involved in the bacterial community regulation.

1 Introduction

1.1 Marine macroalgae: characteristics and importance

Marine macroalgae, also known as seaweeds, are macroscopic, eukaryotic, photosynthetic organisms that live at least partially submerged in water. They are nonvascular and therefore depend on the absorbance of nutrients directly from the surrounding seawater. They include members of the green algae (Chlorophyta), brown algae (Phaeophyta), and red algae (Rhodophyta). The prokaryotic blue-green algae (Cyanophyta) are also sometimes included in this group when they form large macroscopic mats. Macroalgae vary greatly in their morphologies, pigments, and carbon storage products, but are grouped together based on their similar ecological habitats and roles (van den Hoek et al. 1995, Paul et al. 2001).

Macroalgae are important economically because of their use in food products, medicine and pharmaceuticals, as well as biofilters in aquaculture. Seaweeds constitute an important part of the diet in many Asian cultures and the commercial cultivation of seaweeds has become a major factor in the economies of these countries (Nisizawa et al. 1987, Tseng and Fei 1987). Additionally, red and brown macroalgae are harvested for their polysaccharides (agars, algins, carrageenans) which are used throughout the world in a variety of commercial products as gelling, stabilizing, or texturizing agents (Renn 1997). Because of their efficient uptake of nutrients, economically important macroalgae are also being paired with other aquaculture organisms to cleanse waste water of excess nitrogen and phosphorous (Marinho-Soriano et al. 2009). In addition to these qualities, it has also been reported that macroalgae are a rich source of bioactive compounds and some of these compounds show promising potential as pharmaceutical agents including kahalalide F, which is currently in phase II anti-cancer trials and also shows promise as an HIV drug (Smit 2004, Martin-Algarra et al. 2009).

In addition to their economic importance, macroalgae are also ecologically important, because they act as the main primary producers in many estuarine environments. However, algal biomass is often not readily consumed by herbivores and Smith (1981) postulated that macroalgae therefore serve as carbon sinks by removing CO_2 from the atmosphere and trapping it in the algal biomass until it is released by detritivores. This trapping could have

important implications as we face increasing global CO_2 levels in the future. Additionally, the ability of algae to act as biofilters also performs the ecological function of cleaning run-off water of pollutants as it passes through estuaries. Furthermore, algae can have a wide variety of roles within the ecosystems in which they live, including providing habitat for other organisms and producing chemical cues for the settlement of invertebrate larvae (Chemello and Milazzo 2002, Steinberg and de Nys 2002, Christie et al. 2009).

As with most organisms, marine macroalgae are constantly challenged by predators, competitors and disease. However, being marine organisms, they are subjected to the extra stress of living in an environment with a high density of bacteria, some of which have the potential to be highly detrimental to the health of the alga.

1.2 Importance of bacteria in algal ecology

Bacteria play important roles in the health and ecological interactions of marine macroalgae and are highly abundant in estuarine environments, with estimates of as many as 2.5×10^8 bacterial cells per milliliter of seawater (Ducklow and Shiah 1993). Bacteria can not only affect algae directly by acting as pathogens or nutrient sources, but can also have indirect effects by impacting other organisms in ways that affect the algae.

Direct effects

The most direct way that bacteria affect macroalgae is through pathogenesis, the occurrence of which is surprisingly rare considering the high density of bacteria in the seawater surrounding algae. In a recent review of macroalgal-bacterial interactions, Goecke et al. (2010) identified 14 examples of bacteria-elicited algal diseases and an additional five instances in which algae were affected detrimentally by bacteria but a disease was not specified. One of these examples is the red spot disease found in the economically important brown alga *Laminaria japonica* which has been attributed to the bacterium *Pseudoalteromonas bacteriolytica* (Sawabe et al. 1998). Another well known example is the coralline lethal orange disease (CLOD) which is caused by a conglomerate of five bacteria and affects a large variety of coralline red algae (Littler and Littler 1995, Cervino et al. 2005).

In addition to causing diseases, bacteria can also directly benefit macroalgae by providing them with necessary nutrients and growth factors. Several species of green macroalgae in the genus *Codium* obtain nitrogen from nitrogen fixing cyanobacteria that live either within the algal thallus or on the surface of the algae (Dromgoole et al. 1978, Rosenberg and Paerl 1981). In another example, Chisholm et al. (1996) identified a heterotrophic bacterium within the rhizoids of *Caulerpa taxifolia* that contains a gene encoding a nitrogenase and suggested that this endosymbiont may transform inorganic nitrogen into ammonia for use by the alga. Bacteria have also been shown to be necessary for the normal morphogenetic development of some macroalgae. When grown in axenic cultures, these algae lose their natural morphology and this can be recovered by the addition of bacteria into the culture (Nakanishi et al. 1999). Matsuo et al. (2005) found that a marine bacterium in the Cytophaga-Flavobacterium-Bacteroides (CFB) group produces a compound called thallusin that induces normal morphogenesis in *Monotstroma oxyspermum* grown under axenic conditions and suggest that similar phenomena may be widespread among Ulvacean and Monostromanacean green algae.

Indirect effects

Bacteria can benefit algae indirectly by reducing the settlement of macrofouling organisms on their surfaces. For example, the inhibition of larval attachment and metamorphosis caused by waterborne compounds originally thought to be produced by the green alga *Ulva reticulata* was later attributed to an associated bacterium in the genus *Vibrio* (Harder and Qian 2000, Dobretsov and Qian 2002). In the same study, waterborne compounds from five out of seven bacterial strains isolated from the surface of *U. reticulata* inhibited the growth of the diatom *Nitzschia paleacea* (Dobretsov and Qian 2002). Rao et al. (2007) determined that biofilms of *Pseudoalteromonas tunicata*, which grows on the surface of many sessile marine organisms, inhibited the settlement of spores of the red alga *Polysiphonia* sp. by 90% at ecologically relevant bacterial densities. Additionally biofilms of *Phaeobacter* sp. isolated from the surface of *Ulva australis* inhibited settlement of the bryazoan *Bugula neritina* (Rao et al. 2007).

Bacteria associated with macroalgae also affect the settlement and growth of other bacteria and fungi. Extracts of the brown alga *Lobophora variegata* contain a potent antifungal compound, lobophorolide, which is active against saprotrophic and pathogenic

marine fungi (Kubanek et al. 2003). The structure of lobophorolide is similar to compounds known to be produced by cyanobacteria and heterotrophic bacteria. The authors therefore speculate a bacterial origin for this compound. Numerous studies have also found that *Pseudoalteromonas* spp. isolated from marine macroalgae possess antibacterial and antifungal activities against ecologically relevant organisms (Egan et al. 2000, Holmström et al. 2002, Bowman 2007). Additionally, large screenings of bacterial strains isolated from the surfaces of macroalgae and marine invertebrates identified more than 150 bacterial strains with antimicrobial activity towards human pathogens (Burgess et al. 1999, Penesyan et al. 2009).

In addition to regulating the biofouling of algal surfaces, bacteria can also provide protection from predation. Although there are no examples to date of feeding deterrent compounds from algal associated bacteria, such phenomena have been observed in other marine organisms. For example, larvae of the bryazoan *Bugula neritina* harbor bacterial endosymbionts that produce bryostatins which deter feeding by pinfish (Lopanik et al. 2004). Additionally, Lindquist et al. (2005) discovered that some marine isopods harbor cyanobacteria that deter feeding by reef fishes. Given the large number of feeding deterrent compounds currently known from marine macroalgae, it is likely that future research will discover that at least some of these compounds are actually produced by associated bacteria (Paul et al. 2001).

1.3 Algal associated bacterial communities

Because bacteria play such important roles in the ecology of marine macroalgae and because their abundance is so high in seawater, it is likely that macroalgae have evolved mechanisms to regulate the bacterial community surrounding them. In fact, there is growing evidence that the composition of bacterial communities on the surfaces of macroalgae is different from that of non-living substrates and in the surrounding seawater. For example, Dobretsov et al. (2006) found that the bacterial community on the surface of *Caulerpa racemosa* differed from that found on rocks collected from the same area. Additionally, some macroalgal species maintain a unique bacterial community across varying geographical locations. Lachnit et al. (2009) found that the surface bacterial communities of six species of macroalgae (three brown, two red and one green) differed significantly from each other and that the composition of the bacterial community was affected more by the host species than by

the location from which the algae were collected (North Sea vs. Baltic Sea). Nylund et al. (2010) likewise found that algae collected from two locations within the North Sea maintained a specific surface bacterial community regardless of geography. Similar patterns of bacterial-host specificity have also been described for other sessile marine organisms, including sponges and corals (Rohwer et al. 2002, Taylor et al. 2004). Although these few examples indicate that algae may harbor unique bacterial communities, the prevalence of this among different algal species and in different geographical locations remains to be determined. Moreover, the mechanisms governing in this type of regulation are still mostly unknown, although, macroalgae are known producers of a wide variety of secondary metabolites and it is likely that these small molecules are involved in such regulation.

To date, studies on the effects of macroalgal secondary metabolites 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 (e.g. Reichelt and Borowitzka 1984, Freile-Pelegrin and Morales 2004, Salvador et al. 2007, Shanmughapriya et al. 2008). However, the 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 in their studies inhibited the growth of a known algal pathogen, *Pseudoalteromonas bacteriolytica*. Furthermore, 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 and Tilmann (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 the process of communication among bacterial cells known as quorum sensing (e.g. Borchardt et al. 2001, Skindersoe et al. 2008). During the process of quorum sensing bacterial cells release signaling molecules that bind to special receptors on the surface of other bacterial cells and induce the activation of certain genes, including those responsible for the production of more signaling molecules. The likelihood of a signaling molecule reaching the surface receptor of another bacteria increases with increasing bacterial density. Consequently, a response is only produced once the bacteria have reached a minimum density threshold (Nealson 1977, Visick and Fuqua 2005). In one of the most well investigated macroalgal-bacterial interactions, it

has been discovered that the red alga *Delisea pulchra* produces halogenated furanones which are secreted onto the surface of the alga by specialized gland cells and 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). While these studies indicate that macroalgae host a unique bacterial community and that they are capable of producing compounds that inhibit the growth of some bacterial strains, they have yet to fully characterize the mechanisms involved in regulating the bacterial community on and around the algae. Additionally, the bioassays traditionally used to assess the activity of algal extracts against bacteria were adapted from the pharmaceutical industry and are biased toward the discovery of inhibitory compounds. It is likely, however, that the growth of some bacterial species is selectively promoted by algae, but this has been largely uninvestigated.

Investigation of the influences of algae and their organic extracts on the naturally associated bacterial assemblage, combined with studies of their effects on the growth of individual bacterial strains, is key to understanding the ways that algae shape the bacterial community surrounding them. Such in-depth analysis requires the selection of a model algal species. Within the Bioorganic Analytics lab, we focus on studies of siphonous green macroalgae that grow in tropical and subtropical areas. *Dictyosphaeria ocellata* (M.A. Howe) J.L. Olsen-Stojkovich is a tropical green alga that lives in the intertidal zone up to a depth of 1 m, attached to hard substrates (Littler and Littler 2000). It is a siphonous alga that occurs in clusters of macroscopic cells (~ 1-2 mm in diameter) (**Figure 1**). Little research has been published about *D. ocellata* and, to the best of my knowledge, nothing is known about the secondary metabolism of this alga or about its associated bacterial community. However, a few studies have been reported on other members of this genus. For example, *Dictyosphaeria cavernosa* has been extensively studied because it is a problematic species on Hawaiian reefs (Szmant 2002) and a pair of novel polyketides were isolated from a fungus associated with *Dictyosphaeria versluyii* collected in Fiji (Bugni et al. 2004).

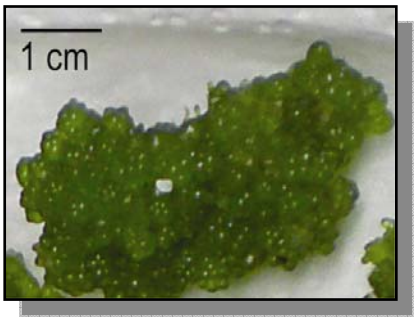

Figure 1: ***Dictyosphaeria ocellata***

Figure 2: Field site at Summerland Key, FL, USA

Although *D. ocellata* will grow in a variety of shallow, hard substrate, tropical environments, the main field site used in these experiments was a near shore area along the coast of Summerland Key, FL (**Figure 2**). At this site the algae were found attached to rocks covered by a thin layer of sediment. Like most benthic macroalgae, *D. ocellata* 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). This suggests that *D. ocellata* may have evolved mechanisms to regulate the bacterial community associated with it and it is therefore a good model organism for investigating algal-bacterial interactions.

In addition to the selection of a model algal species, investigation of the effects of algae on the structure of the bacterial community surrounding them requires the use of special techniques that provide information on the bacterial species composition of a given sample. Traditionally the study of bacterial communities has been limited by the methods available to identify the bacterial strains present within a sample. Microbiologists identified bacterial species based on their physiological and biochemical traits, which required the cultivation of the organism in the laboratory. However, it has since been discovered that the bacterial

strains which are cultivable using current techniques represent only a small fraction (< 1 %) of the total number of bacterial species present within a given sample (Amann et al. 1995). This highlights the need for culture-independent methods for determining bacterial species richness and diversity and has led to the development of a number of DNA and RNA sequence based methods.

Perhaps the most in-depth method currently used to investigate the species composition of bacterial communities within environmental samples is metagenomics (Xu 2006). This approach involves sequencing genomic DNA within an environmental sample and using this information to determine not only which organisms are present, but also what their metabolic capabilities are by analyzing genes with known functions. However, metagenomics is expensive and the analysis is time consuming. Alternatively, there are a variety of methods that examine bacterial species in an environmental sample based on ribosomal RNA (rRNA) gene sequences as opposed to investigating entire genomes. 16S rDNA is often used for the phylogenetic comparison of bacterial strains because it contains both highly conserved and highly variable regions (Olsen et al. 1986). This allows for the universal amplification of nearly all bacterial strains by using primers that anneal to highly conserved regions. Furthermore, primers can be chosen that amplify a highly variable region of the gene and these sequences can then be used for phylogenetic differentiation. Amplified 16S rDNA can be used to create clone libraries which can be sequenced and used to identify the source organism. Unfortunately, this approach is still relatively expensive and time consuming. Bacterial fingerprinting techniques, however, can be useful in cases where it is more important to know if the bacterial community is changing as opposed to what species are in the community.

The two most commonly used bacterial community fingerprinting techniques are terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) (Felske and Osborn 2005). For T-RFLP analysis, DNA is amplified using fluorescently labeled primers. The amplified DNA is then digested using one or more restriction enzymes resulting in a number of DNA fragments. The terminal fragments retain the fluorescent label from the primers and are separated using capillary electrophoresis. This is generally carried out on an automated DNA sequencer and an electropherogram is produced

in which the position of the peak relates to the fragment size and the intensity of the peak corresponds to the number of fragments present. Restriction enzymes are chosen so that the size of the resulting terminal fragments can be used to distinguish between phylogenetically different organisms, each peak representing one individual bacterial phylotype (Liu et al. 1997).

Similarly, DGGE produces a fingerprint of the bacterial community present in a sample, but does so based on the melting behavior of the DNA. With this technique, the 16s rRNA gene is amplified from environmental DNA using universal bacterial primers that produce fragments with the same length. The DNA is then separated on a vertical acrylamide gel containing denaturing agents in an increasing concentration gradient from top to bottom. As the DNA moves through the gel the double stranded conformation will begin to denature. Generally, primers are designed to add a 40 bp section of guanine and cytosine nucleotides known as a "GC clamp" to one end of the sequence. The GC clamp inhibits the DNA from completely denaturing into two single strands, which would move easily through the gel. Instead, an inverted Y shape is formed which retards the DNA's movement through the gel (**Figure 3**) (Muyzer et al. 1993).

The melting behavior of the DNA is dictated by its sequence data. Guanine and cytosine are bound by three hydrogen bonds as opposed to adenine and thymine which are bound together by only two hydrogen bonds. Consequently, bonds between quanine and cytosine (G-C bonds) are more resistant to denaturing than bonds between adenine and thymine (A-T bonds). Therefore, sequences with a higher G-C content will move further through the gel compared to those with a higher A-T content. This results in a community fingerprint in which each band represents a fragment of DNA with a unique sequence and is therefore considered as an individual bacterial phylotype.

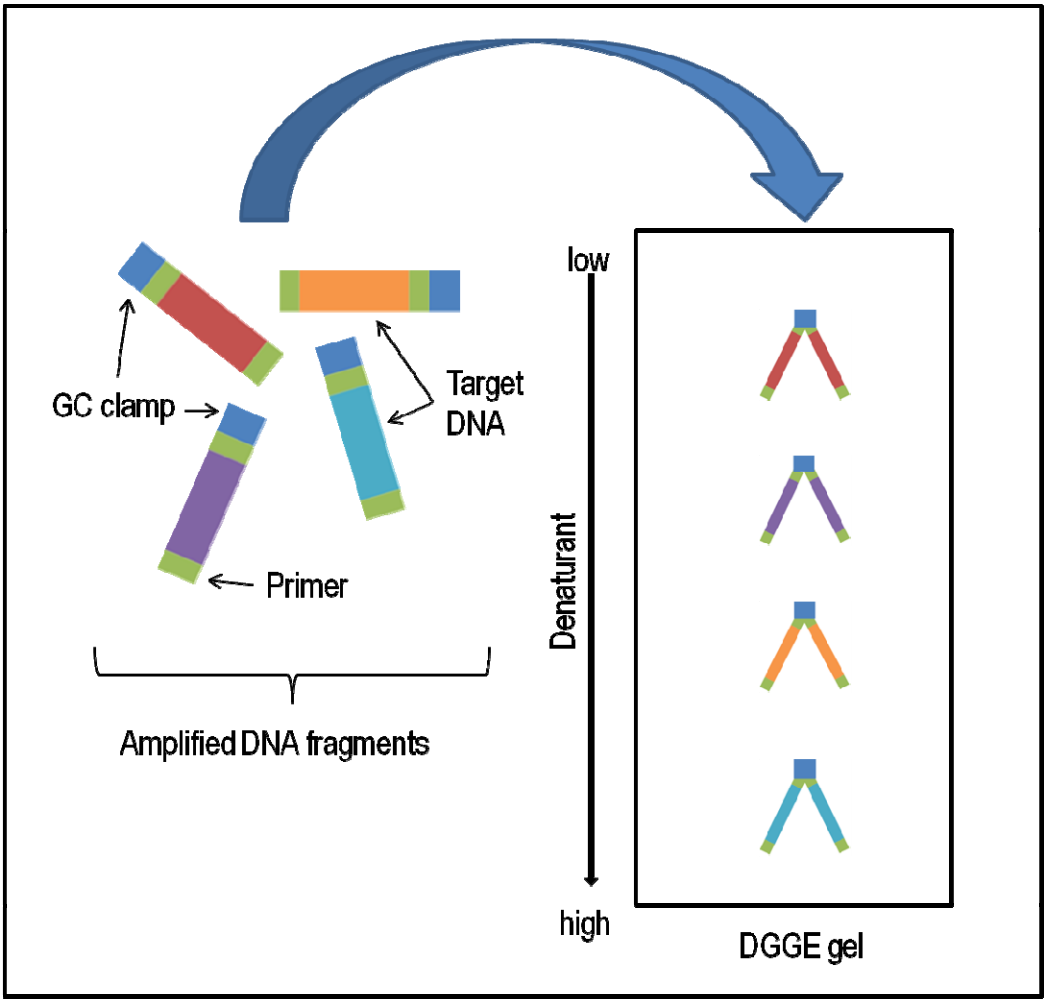

Figure 3: Schematic representation of the principle behind the separation of DNA fragments using DGGE.

Unfortunately, with both of these methods, bacterial strains with highly similar DNA sequences are unlikely to be distinguished from one another. Additionally, due to sampling and PCR bias, bacterial strains that make up < 1 % of the total community are unlikely to be detected (Felske and Osborn 2005). However, both methods offer a relatively quick and inexpensive means for comparing the major components of bacterial communities. DGGE has the advantage that bands can be excised and sequenced for identification, but databases are developing that aid in the identification of bacterial strains based on T-RFLP fragmentation patterns, as well (see review by Schutte et al. 2008). T-RFLP, on the other hand, has been reported in some cases to be more sensitive than DGGE, identifying a higher

number of bacterial phylotypes from the same sample (Moeseneder et al. 1999), but Felske and Osborn (2005) suggest that this may be dependent on the skill and experience of the researcher. T-RFLP also has the advantage of being highly reproducible whereas DGGE profiles are dependent on the casting and staining of the gel and may vary slightly between gels. Although including known standards and adjusting band position and intensity in relationship to these can accommodate for this variation, it is still often difficult to compare samples across multiple gels. As a result, this limits the number of samples that can be compared at one time.

The objective of my PhD research was to determine if macroalgae affect their surrounding bacterial community and whether these effects could be attributed to organic compounds extracted from or released by the algae. To determine this, I performed a series of field experiments in which I examined the effects of a model alga, *D. ocellata* and its organic extracts on the composition of natural biofilm and planktonic bacterial assemblages. In these experiments I used bacterial community fingerprinting techniques to profile the bacterial communities under different conditions. I also investigated the effects of *D. ocellata* and its organic extracts on the growth of ecologically relevant marine bacteria in a unique bioassay that allowed for the observation of both growth promoting and growth inhibiting effects. Furthermore, the effects of direct contact with the live algae on the growth of individual bacterial cultures were determined with this assay and this has rarely been looked at in the past.

2 *Results and Discussion: Effects of* D. ocellata *and its extracts on natural bacterial assemblages in the field*

A series of field experiments were performed using bacterial fingerprinting to determine if and how green algae affect the bacterial community surrounding them using the green alga *Dictyosphaeria ocellata* as a model species (see Introduction Section 1.3).

2.1 Bacterial community analysis

For the purposes of this study, it was important to understand if bacterial communities were different under different conditions, but not what bacteria were present in the communities. I therefore chose to use a bacterial community fingerprinting technique, DGGE (see Introduction, Section 1.3). DGGE was chosen because of its relative quickness, ease, and inexpensiveness compared to other techniques and because interesting bands could be excised and identified as necessary. Additionally, the most common alternative to DGGE, T-RFLP, requires the use of an automated DNA sequencer, which was not easily available to me. In brief, bacterial DNA was amplified using universal bacterial primers 357f and 907rM, which amplify a 550 bp section of the 16S rRNA gene. The 357f-907rM primer set was first described by Muyzer et al. (1995) for use in DGGE analysis, and was later identified as the preferential primer set for the routine DGGE analysis of marine bacteria (Sanchez et al. 2007). This primer set straddles a highly variable region of the 16S rRNA gene and produces DNA fragments small enough to run on a DGGE gel but large enough to contain a sufficient number of base pair differences to separate the DNA fragments (Muyzer et al. 1995). As is common practice in DGGE analysis, a 40 bp GC clamp was added to the 357f primer so that the resulting DNA fragments would not completely denature during the DGGE. The PCR protocol was optimized based on the protocol from Kirchman et al. (2001) using a mixture of known bacterial strains as well as samples of algal culture water.

The PCR protocol was optimized for the amplification of bacterial DNA from polycarbonate membrane filters. The optimized method was subsequently determined to be applicable to samples of bacterial DNA isolated from cotton swabs as well. To generate the greatest amount of amplified product without unspecific amplification of non target areas of

the DNA, different concentrations of the PCR reagents and different thermal cycling conditions were tested (**Table 1**, **Appendix I**). Reaction master mix #3 from **Table 1** was chosen because it consistently resulted in a high concentration of DNA and had a small amount of unspecific product, determined after separation on an agarose gel (**Appendix I**).

Table 1: PCR reaction mixtures tested for optimal amplification of marine bacterial DNA. Blue shading indicates the mix that produced the largest amount of DNA with a minimum amount of unspecific product. Bold numbers indicate differences between the reaction mixtures.

Master mix:	**1**	**2**	**3**	**4**
Buffer (10x)	5 µl	5 µl	5 µl	5 µl
dNTP mix (10mM)	1 µl	1 µl	1 µl	1 µl
357GC (10 µM)	2 µl	**1 µl**	2 µl	2 µl
907rM (10 µM)	2 µl	**1 µl**	2 µl	2 µl
BSA (20mg/ml)	1.5 µl	1.5 µl	1.5 µl	1.5 µl
DreamTaq (5 units/µl)	0.25 µl	0.25 µl	**0.5 µl**	0.25 µl
$MgCl_2$ (25 mM)	-	-	-	**5 µl**

The DNA concentration was determined by comparison to the GeneRuler™ Express DNA Ladder (Fermentas) run on a 1% agarose gel. Because all target DNA had the same molecular weight, any extra bands seen on the agarose gel were the result of unspecific amplification and the amount of unspecific product was determined visually based on the number and intensity of extra bands. As described by Muyzer et al. (1993) a touchdown PCR method was used starting at an annealing temperature of 65 °C and cycling down to 55 °C, which is the expected annealing temperature for these primers. The efficacy of these annealing temperatures was verified by performing gradient PCR using the reaction master mix #3 at temperatures ranging from 54 °C – 66 °C (**Appendix II**). Furthermore, the optimal total number of PCR cycles was determined for reaction mixture #3. A total of 35 cycles (10

touchdown cycles followed by 25 cycles at an annealing temperature of 55 °C) produced the highest DNA concentration with the lowest amount of unspecific product (**Appendix I**).

Amplified DNA was separated on a denaturing gel to produce a profile within each lane in which each band represented an individual bacterial phylotype. The DGGE conditions (time, voltage, acrylamide concentration, and denaturant concentration) were optimized based on the protocol outlined by Muyzer et al. (1993) using the PCR amplified DNA of known bacterial strains to produce a profile containing bands that were well separated and well defined. In order to analyze the profiles, gels were stained and digitally photographed. The digital photographs can be analyzed using a number of different gel analysis programs. For these experiments I used either GelCompar II from Applied Maths or BioDoc Analyze from Biometra. Both of these programs generate a densitograph for each lane that plots the average gray value (U) against the position as defined by the pixel number resulting in a densitometric curve (**Figure 4**). For every pixel, a gray value between 0 and 255 is assigned with 0 representing true black and 255 representing true white. In my experiments, the grey value was a measure of the fluorescence because the gels were stained with a fluorescent pigment. The height of the peak is related to the amount of DNA found in each band, and because each band represents one bacterial phylotype, peak height can be used as proxy for bacterial species abundance. Additionally, the volume of each band can be determined as sum of the gray values within a given area, and these values can also be used for the comparison of relative species abundance between samples.

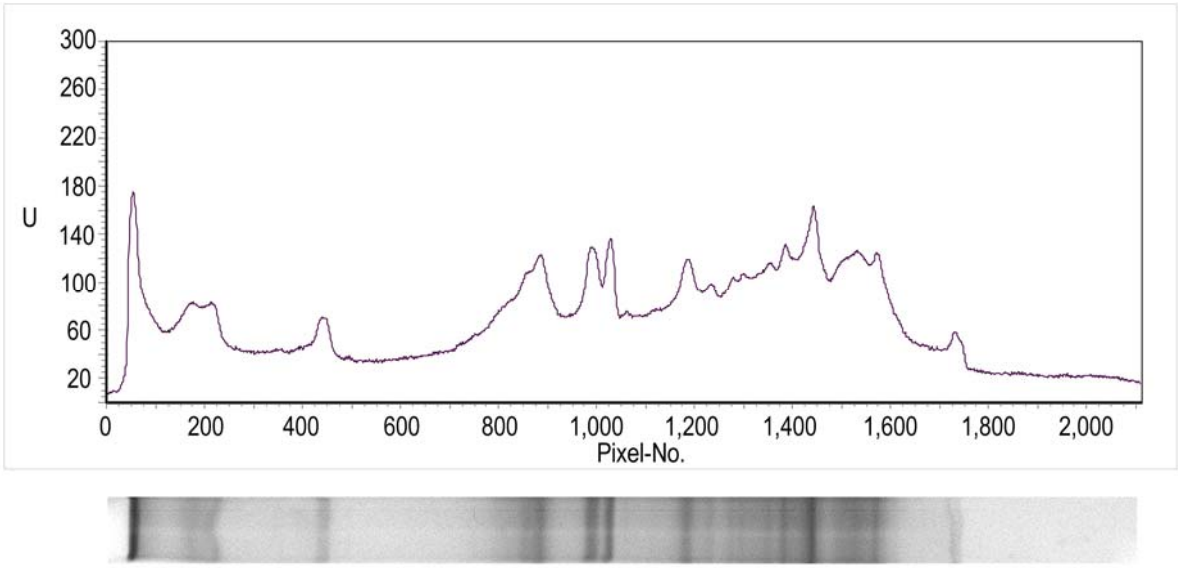

Figure 4: Example densitograph with the associated DGGE lane shown beneath.

Fingerprints (banding patterns or densitometric curves) produced as a result of DGGE can be compared either based on the presence/absence of individual bands or on the relative intensities of the bands present. The latter includes quantitative information about the relative abundance of species within the community, but it should be noted that this is only a semi-quantitative technique because PCR bias inhibits us from determining absolute values. However, this information is still useful because differences in the relative intensity of a band between samples indicate differences in the amount of the corresponding bacterial strain.

During the analysis of fingerprint profiles, the initial step is to generate a similarity or dissimilarity matrix based on the original fingerprint. For a review of commonly used similarity and dissimilarity coefficients see Rademaker & de Bruijn (2004) or Quinn & Keough (2002). The choice of a similarity/dissimilarity coefficient is based on the quality of the original data, design of the experiment and hypotheses being tested. They can be divided into two groups, those that compare presence/absence data (binary coefficients) and those that measure continuous variables and incorporate abundance data. The most commonly used binary coefficients used in the analysis of bacterial community fingerprints are the Jaccard and the Dice coefficients which differ only in the amount of weight given to presence values (Rademaker and Bruijn 2004). The Bray-Curtis coefficient was developed for use in plant ecology but has been adopted by microbial ecologists because it is a good measure of species

abundance data (Quinn and Keough 2002). In the case of community fingerprints, each band represents a species and the intensity of the band is a measure of abundance. Another commonly used coefficient for the analysis of fingerprint data is the Pearson correlation (Rademaker and Bruijn 2004). While the previously described coefficients require the prior assignment of bands, which can be difficult in complex communities, the Pearson correlation takes into account the entire densitometric curve. Therefore, this correlation reduces subjective bias that is introduced when the selection of bands is made (Rademaker and Bruijn 2004). Rademaker & Bruijn (2004) suggest that this is preferential method, however, not all gel analysis software provide this option.

After a coefficient has been selected and a similarity/dissimilarity matrix has been generated, the data can be analyzed in a number of ways depending on the goals of the study. For hypothesis testing, analysis of similarity (ANOSIM) is most commonly used, but non-parametric or permutation multivariate analysis of variance (PERMANOVA) is gaining popularity. ANOSIM is a statistical test often applied to ecological studies that compares species composition between different communities (Rademaker and Bruijn 2004). It has been adopted into the field of microbial ecology as a method to compare bacterial community profiles produced from such techniques as DGGE and TRFLP. ANOSIM is a test that ranks the dissimilarities between samples and compares the average rank dissimilarities between objects within a group (r_w) to the average rank dissimilarities between objects in different groups (r_b)(Quinn and Keough 2002).

$$R = \frac{r_b - r_w}{N(N\text{-}1)/4}$$

The resulting *R* value can theoretically range from –1 to 1, but most ecological studies do not result in an *R* value of less than 0. An *R* value of 0 indicates that objects within a group are as similar to objects in other groups as they are to each other. An *R* value > 0 indicates that objects within a group are more similar to objects from the same group than they are to individuals from other groups.

The significance of the *R* value can be determined by calculating a given number of random permutations (usually at least 999) and comparing the observed *R* value to the

permuted *R* values. A significant result is determined by the probability of getting the observed outcome in relation to the random permutations calculated (**Figure 5**). A drawback of permutation tests is that, because the random permutation values are calculated by all possible combinations of objects, the number of possible permutations is determined by the number of objects in the study. In principle, this means that studies with a low number of replicates are less likely to produce significant results.

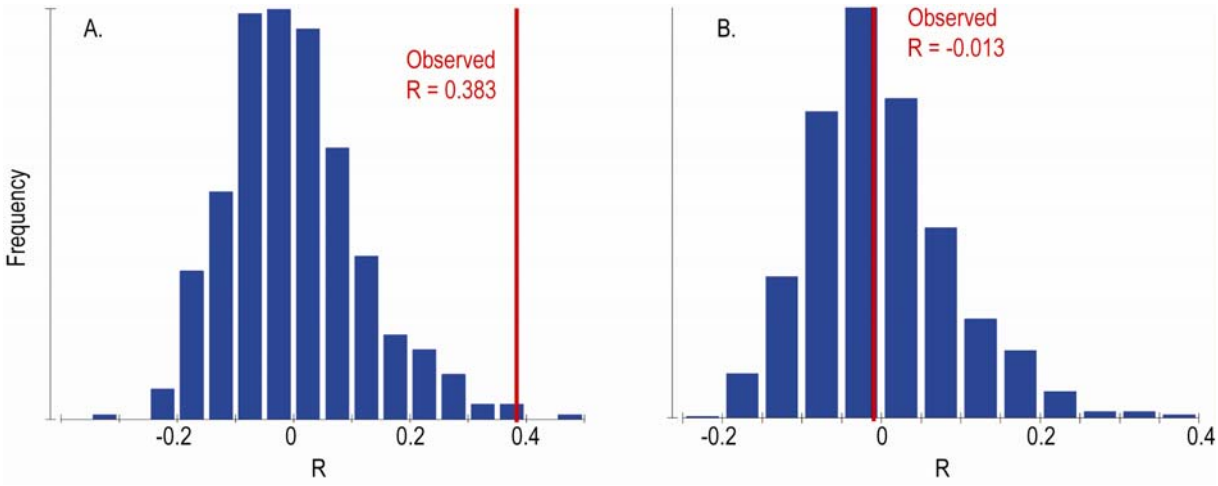

Figure 5: Histograms of random *R* value permutations where the observed *R* value is significantly different than that expected by random permutation (A) and where the observed *R* value is not significantly different than that expected by random permutations (B).

PERMANOVA is similar to ANOSIM although, it does not require the ranking of dissimilarities prior to analysis. PERMANOVA calculates an *F* value analogous to that of analysis of variance (ANOVA), but the data is not restricted by the same assumptions. Essentially, this test compares the variance among objects within a group (SS_W) and compares that to the variance between groups (SS_A) when *a* is the number of groups and *N* is the total number of observations (Anderson 2001).

$$F = \frac{SS_A/(a - 1)}{SS_W/(N - a)}$$

As with ANOSIM, significance of the *F* value must be determined by calculating permutations (Anderson 2001). Quinn and Keough (2002) suggest that this is a promising method for ecological studies because it is widely applicable to complex experimental designs but requires the careful choice of an appropriate randomization test.

In addition to hypothesis testing, classification methods can be used to group similar samples together based on their banding patterns (Quinn and Keough 2002). Fingerprint profiles are most often analyzed using cluster analysis which is a technique that forms groupings of samples based on their degree of similarity to each other and represents them in a hierarchical tree. The formation of clusters are commonly determined by one of three methods: single linkage (nearest neighbor), complete linkage (furthest neighbor), or unweighted pair-group average using arithmetic averages (UPGMA) although other less commonly used methods exist.

The single linkage method calculates the distance between clusters based on the two most similar samples in different clusters. Alternatively, the complete linkage method calculates the distance between clusters as the largest difference between individuals in different clusters. The UPGMA method determines the distance between clusters as the average distance between all pairs of individuals in two different clusters. This last method is robust to instances when objects form distinct clusters as well as instances when longer chain-like trees are formed.

In addition to cluster analysis, another common technique used to find patterns in the similarity data of DGGE profiles is non-metric multidimensional scaling (NMDS). NMDS plots objects in multidimensional space so that the distances between objects in space closely represent the distances calculated in the similarity or dissimilarity matrix. It provides a good graphical representation of the relationships among individuals based on the chosen similarity/dissimilarity matrix.

In the experiments presented here, I created similarity matrices based on either the Pearson correlation or the Bray-Curtis measure of dissimilarity because these two methods are useful when comparing relative abundances of each species (band intensity) within a sample. Although DGGE is not a quantitative method, differences in the relative intensities

of individual bands between samples indicate differences in the abundance of these bacterial phylotypes and I believe this information should also be taken into account during the comparison of bacterial communities. The choice of similarity/dissimilarity coefficient was based on the software available. Early experiments were analyzed with the GelCompar II software using the Pearson correlation, but later this software was no longer available to me and the remaining experiments were analyzed with the BioDoc Analyze software using the Bray-Curtis measure of dissimilarity. The resultant similarity matrices were then subjected to multivariate statistical analysis. For my experiments I used either cluster analysis or NMDS to graphically represent the data obtained from the DGGE profiles because these are the most commonly used classification methods for these types of studies. I also performed statistical comparisons of treatments using analysis of similarity (ANOSIM).

2.2 Comparison of surface-associated bacterial communities within and between locations.

In order to determine if *D. ocellata* influences the species composition of bacteria on its surface, I compared the bacterial community profiles of biofilms taken from *D. ocellata* to those from another species of green alga and from a reference surface, a rock, at three locations along the Florida Keys (**Figure 6**). I also compared the biofilms on *D. ocellata* individuals from different locations to determine if they retain a similar bacterial community regardless of geography.

2.2.1 Experimental design

I chose to compare the bacterial community on the surface of *D. ocellata* to that of another green macroalgae in order to determine if there was a difference in the bacterial communities associated with similar organisms growing in the same location. A difference in the communities would indicate a high degree of host specificity and the likelihood of factors present within the algae that regulate the structure of the bacterial community. Within each location, I chose a second green alga that was abundant and similar in size to *D. ocellata*. For the Summerland Key and Long Key locations, the second alga was *Batophora oerstedii* and at Bahia Honda it was *Cladophoropsis macromeres*. I also compared the bacterial communities associated with these algae to those taken from a rock at the same location. The rock was

used as a reference surface which should have a limited influence on the settlement and growth of biofilm bacteria and therefore represent the pool of bacteria available to form biofilms on surfaces in the area.

In addition to comparing the bacterial communities on different objects taken from within one location, I compared the bacterial communities of *D. ocellata* individuals across all three locations. I hypothesized that if the alga regulates the composition of its associated bacterial community, then the bacterial communities should be consistent between different locations. I also compared the bacterial communities on rocks taken from all three locations to determine the baseline amount of variation in the composition of biofilm communities.

The three objects were collected from three locations along the Florida Keys (**Figure 6**). I chose sites that had different environmental conditions in order to reduce the likelihood of obtaining similar bacterial communities on algae from different locations by chance alone. The Summerland Key site was a shallow, low wave action, subtidal zone fringed by mangroves. The substrate was rocky and covered with a thin layer of sediment. *D. ocellata* was abundant in this location and grew in clusters attached to rocks and mangrove prop roots The clusters ranged in size from ~1 – 3 cm in diameter. This site was used as the main field site throughout the course of these experiments due to the ease of access, abundance of *D. ocellata* and its close proximity to the Mote Tropical Research Laboratory on Summerland Key, which was the base from which all field studies were performed. On Bahia Honda I chose a high wave action, rocky shore with tide pools. The algae were growing in very small clusters, sometime as few as two or three cells, within the tide pools. At Long Key the objects were collected from the bank of a mangrove canal. The water here was low wave action as in Summerland Key, but based on the color of the water, contained an obviously high amount of tannins. *D. ocellata* also grew here in very small clumps in the crevices of rocks along the bank.

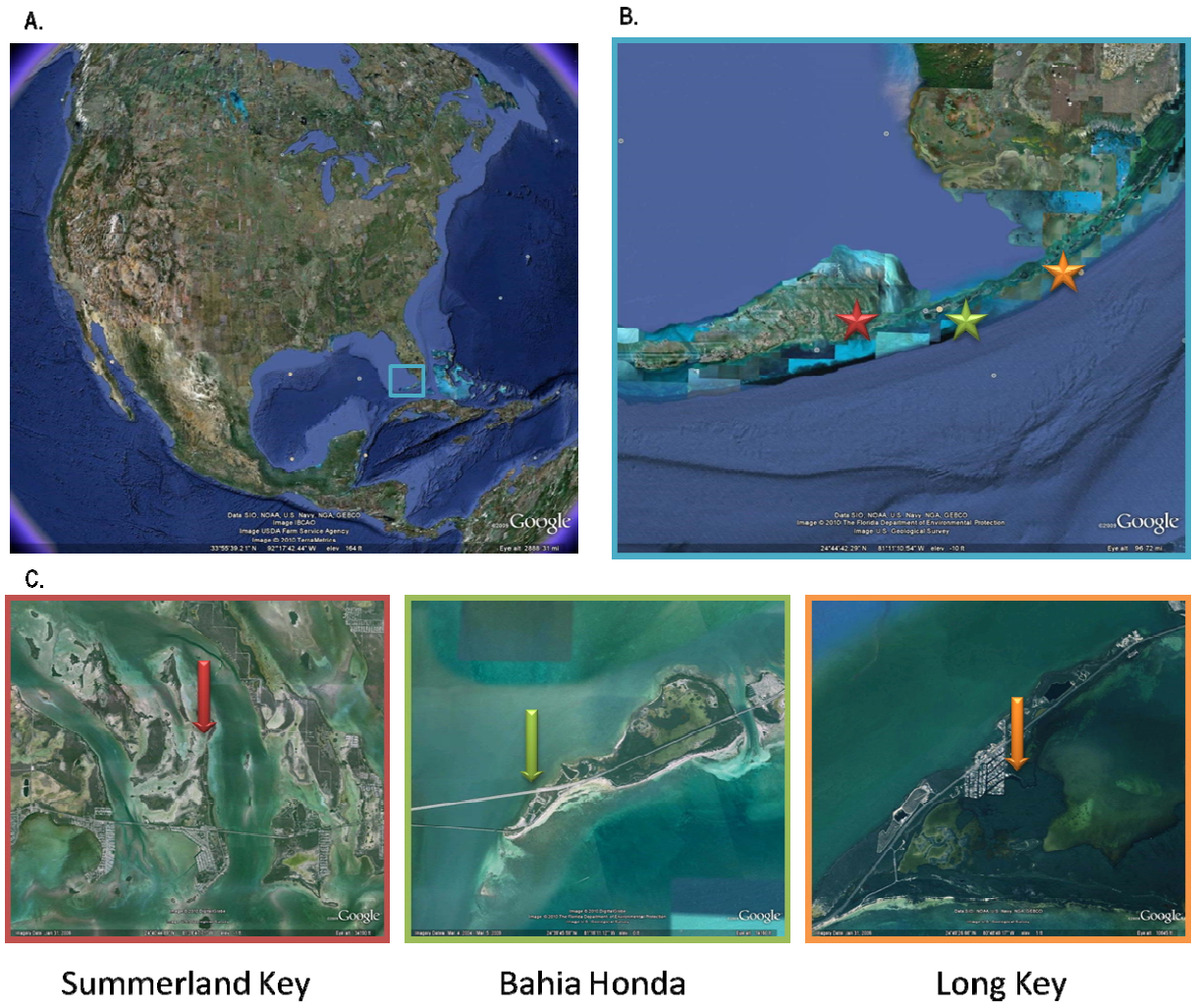

Figure 6: Maps showing the location of field sites. A: North America with the Florida Keys indicated by the blue box, B: Florida Keys with stars indication the location of the field sites, C: field sites along the Florida Keys with arrows indicating the collection sites. Pictures are from Google Earth.

2.2.2 Within-site comparisons

DGGE profiles revealed between 54 and 63 total bacterial phylotypes present on objects collected from each site. Cluster analysis of the bacterial communities on the surfaces of rocks, *D. ocellata*, and *Batophora oerstedii* within Summerland Key and Long Key, showed that the three objects formed distinct clusters in both locations, with the exception of one *D. ocellata* replicate from Summerland Key that clustered with *B. oerstedii* (**Figure 7**).

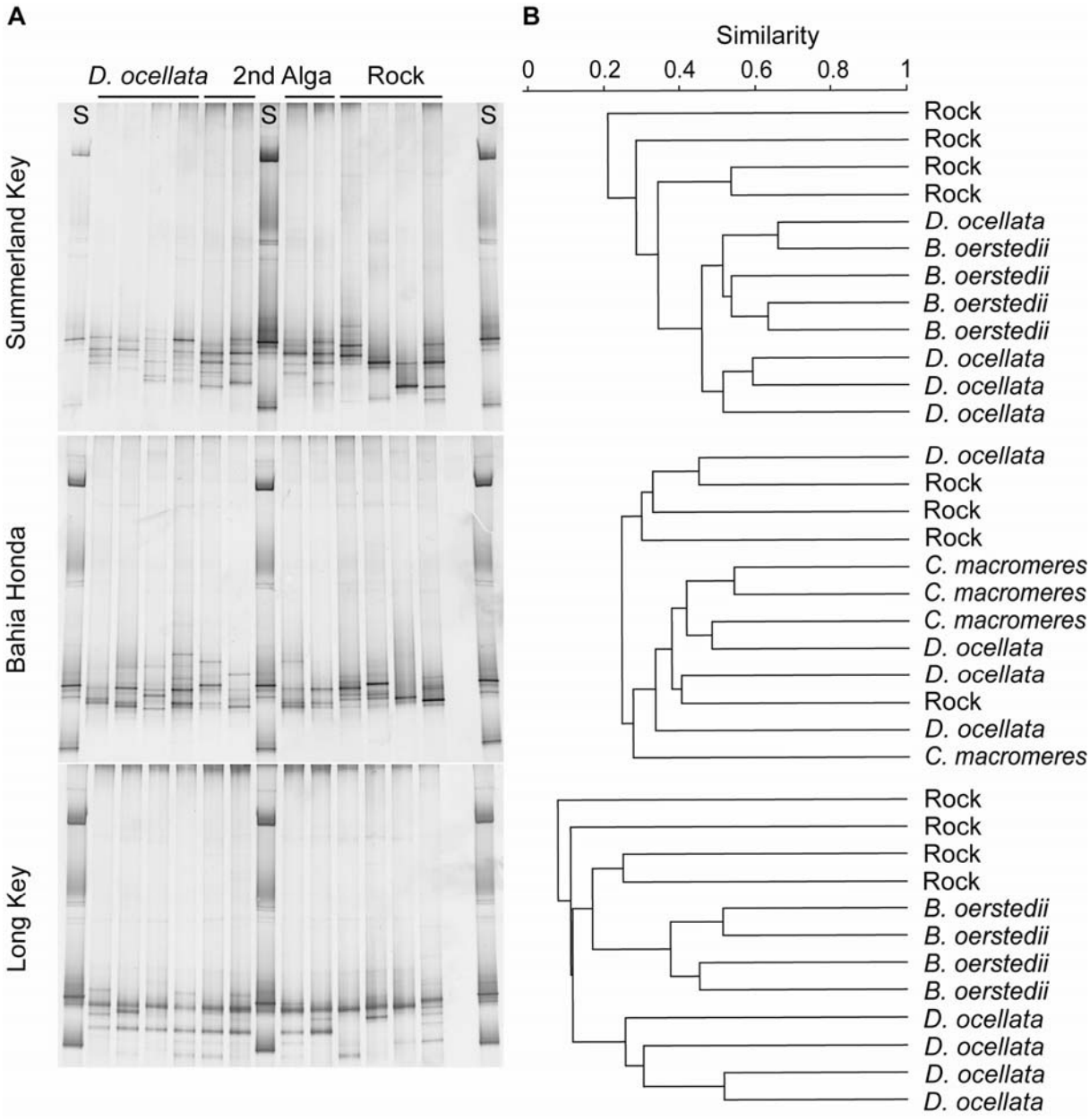

Figure 7: Comparison of the bacterial communities on the surface of *D. ocellata*, a second alga, and rocks taken from Summerland Key, Bahia Honda, and Long Key. In Summerland Key and Long Key the second alga is *B. oerstedii*, in Bahia Honda it is *C. macromeres*. A) DGGE profiles, S = standard. B) UPGMA cluster analysis of DGGE banding patterns based on the Bray Curtis distance measure.

Table 2: Analysis of similarity (ANOSIM) comparison of bacterial communities on the surfaces of *D. ocellata*, a second alga, and rocks taken from Summerland Key, Bahia Honda, and Long Key. R values are given for pairwise comparisons and the global R with p values in parentheses. Bold numbers indicate significance at $p < 0.05$. Significance levels for pairwise comparisons were not subjected to Bonferroni corrections. n = 4.

	Summerland Key	Bahia Honda	Long Key
Global R	**0.5162 (0.0015)**	0.1700 (0.1201)	**0.3727 (0.0011)**
Pairwise comparisons			
D. ocellata vs. 2nd Alga	**0.9271 (0.0276)**		0.3646 (0.0559)
D. ocellata vs. Rock	0.2917 (0.0553)		0.3646 (0.0856)
2nd Alga vs. Rock	0.3229 (0.0575)		**0.4271 (0.0277)**

The differences between these groups were significant according to ANOSIM (R = 0.5162, p = 0.0015 and R = 0.3727, p = 0.0011 respectively, **Table 2**), but pairwise comparisons showed significant differences only between *D. ocellata* and *B. oerstedii* in Summerland Key (R = 0.9271, p = 0.0276) and between *B. oerstedii* and rocks in Long Key (R = 0.4271, p = 0.0277, **Table 2**). However, the R values for all pairwise comparisons are relatively high and this indicates that the groups may be different but fail to meet the significance levels of this test due to low numbers of replicates (**Table 2**). As discussed in the in Section 2.1, the significance level is limited by the number of permutations that can be calculated and this is determined by the number of samples in each group. Therefore, in some cases you can obtain a relatively high R value that indicates separation of the groups from one another, but because the number of replicates is low, the value fails to be significant. Because the R values are relatively high for all pairwise comparisons and cluster analysis shows separate clusters for each group, it is likely that these indeed form separate groups, but failed to meet the test for significance due to the low number of replicates (n = 4).

The host specificity of bacterial communities on the surface of algae from Summerland Key and Long Key supported my hypothesis that green algae are capable of regulating their surface associated bacterial communities. Similar results were found by Lachnit et al. (2009) and Nylund et al. (2010) who both determined that bacterial communities varied among different species of temperate macroalgae collected from one location. Furthermore, my

results concur with those of Dobretsov et al. (2006), who found that the bacterial community on the surface of the green alga *Caulerpa racemosa* was different than that found on rocks.

Contrary to the results from Summerland Key and Long Key, both cluster analysis and ANOSIM show no distinct grouping of the bacterial communities on the surface of different objects within Bahia Honda (R = 0.1700, p = 0.1201, **Figure 7**, **Table 2).** There was no *B. oerstedii* present at the Bahia Honda site, and *Cladophoropsis macromeres* was therefore used as the second green alga at this site. The lack of significant differences in bacterial community composition between the three objects from this site may be related to the usage of *C. macromeres* instead of *B. oerstedii*. Lachnit et al. (2009) found that individuals within the same genus, but different species, had similar surface-associated bacterial communities. While *C. macromeres* and *D. ocellata* are not in the same genus, they are in the same order (Siphonocladales) as opposed to *B. oerstedii* which is in the order Dasycladales. Perhaps the closer phylogenetic relationship resulted in the decreased difference in the surface-associated bacterial communities of these two algal species.

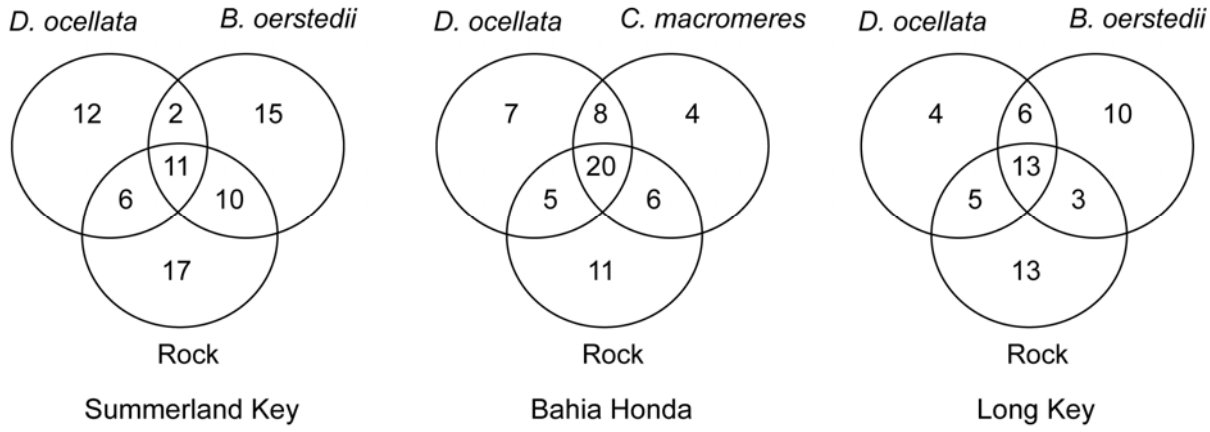

Figure 8: Venn diagram comparison of the number of bacterial phylotypes present on the surface of *D. ocellata*, a second alga, and rocks taken from Summerland Key, Bahia Honda, and Long Key. Numbers within the area where circles cross indicate the number of phylotypes shared by these objects.

Examination of the presence of individual bacterial phylotypes showed that 29 of the 63 different phylotypes found on the surfaces of objects from Summerland Key are specific to the algae (**Figure 8**). Of these, only two are shared by the two algal species, leaving twelve that are unique to *D. ocellata* and fifteen that are unique to *B. oerstedii* (**Figure 8**). Of the 44

phylotypes found on the surfaces of rocks, 10 were eliminated specifically by *D. ocellata*, 6 by *B. oerstedii*, and 17 by both algae (**Figure 8**). In Bahia Honda, 19 out of 61 phylotypes were found only in algae, 8 of which were found on both algae, 7 only on *D. ocellata* and 4 only on *Cladophoropsis macromeres* (**Figure 8**). Forty-two phylotypes were found on the surfaces of rocks, eleven of which were eliminated on the surfaces of both algae (Figure 8). An additional six phylotypes were eliminated by *D. ocellata* and five by *C. macromeres* specifically (**Figure 8**). Of the 54 phylotypes found in Long Key, 20 were found only on algae, 4 specific to *D. ocellata*, 10 specific to *B. oerstedii*, and 6 present on both algae. Of the 34 phylotypes found on the surfaces of rocks, 3 were eliminated by *D. ocellata*, 5 by *B. oerstedii*, and 13 by both (**Figure 8**). These numbers represent phylotypes found in at least one replicate within each group, but not necessarily common to all replicates within a group. This information serves as simplified overview of the data present within the bacterial community fingerprints and is useful in examining patterns, in addition to the statistical testing and cluster analysis.

Algae release dissolved organic carbon and other nutrients into the surrounding seawater. It is therefore not surprising that the surfaces of algae would be colonized by bacteria not found on inanimate surfaces (Cole et al. 1982, Jensen 1985, Smith et al. 2006, Wada et al. 2007). However, the fact that some bacteria are found on only one species of alga and not the other indicates the presence of some regulatory process, which acts to selectively attract or eliminate specific phylotypes (**Figure 8**). Between 11 and 17 bacterial phylotypes were found only on the surfaces of rocks (**Figure 8**). If we assume that the rock provides a neutral surface, lacking in chemical deterrents or attractants to bacterial growth or settlement, then the algae are actively eliminating these bacterial phylotypes. In addition, there were between three and ten phylotypes that were found on the surfaces of both rocks and one algal species, but were specifically eliminated by the other alga (**Figure 8**). These data provide evidence that the algae are selectively promoting and deterring the growth of bacterial species on their surfaces. Dobretsov et al. (2006) found similarly that some bacterial phylotypes found on stones were absent on the surface of *C. racemosa* while others were found only on the alga. Studies of the effects of macroalgae on the planktonic bacterial community have also found that individual bacterial phylotypes were eliminated in the presence of the algae and that these

results could in some cases be attributed to compounds released from the algae into the seawater (Lam et al. 2008).

2.2.3 Between-site comparisons

To determine if *D. ocellata* maintains the same bacterial community across locations, I reanalyzed the samples from the previous experiments, loading samples taken from the surfaces of *D. ocellata* from all three locations onto one gel. I did the same for the samples taken from the surfaces of rocks. This was necessary because the comparison of samples across different gels was found to be unreliable in other experiments (see Section 2.3.1). The bacterial communities on the surface of the green alga *D. ocellata* collected at three sites along the Florida Keys differed significantly according to analysis of similarity (ANOSIM) ($R = 0.7449$, $p = 0.0060$, **Figure 9**, **Table 3**). Although cluster analysis showed distinct clusters for all three locations, pairwise comparisons failed to reveal any significant differences among pairs of locations (**Table 3**). However, as in the previous experiment, the R values for all pairwise comparisons are large and likely indicated differences between these samples. This is contrary to my hypothesis that *D. ocellata* regulates it associated bacterial biofilm community and should therefore have a similar community on its surface regardless of location. However, the surfaces of rocks taken from different locations also harbored different microbial communities indicating that the pool of bacteria available to settle on the surface of the alga differed among locations ($R = 0.2755$, $p = 0.0076$, **Figure 9**, **Table 3**). Therefore, it was impossible for these algae to have the same surface associated bacterial communities at these locations.

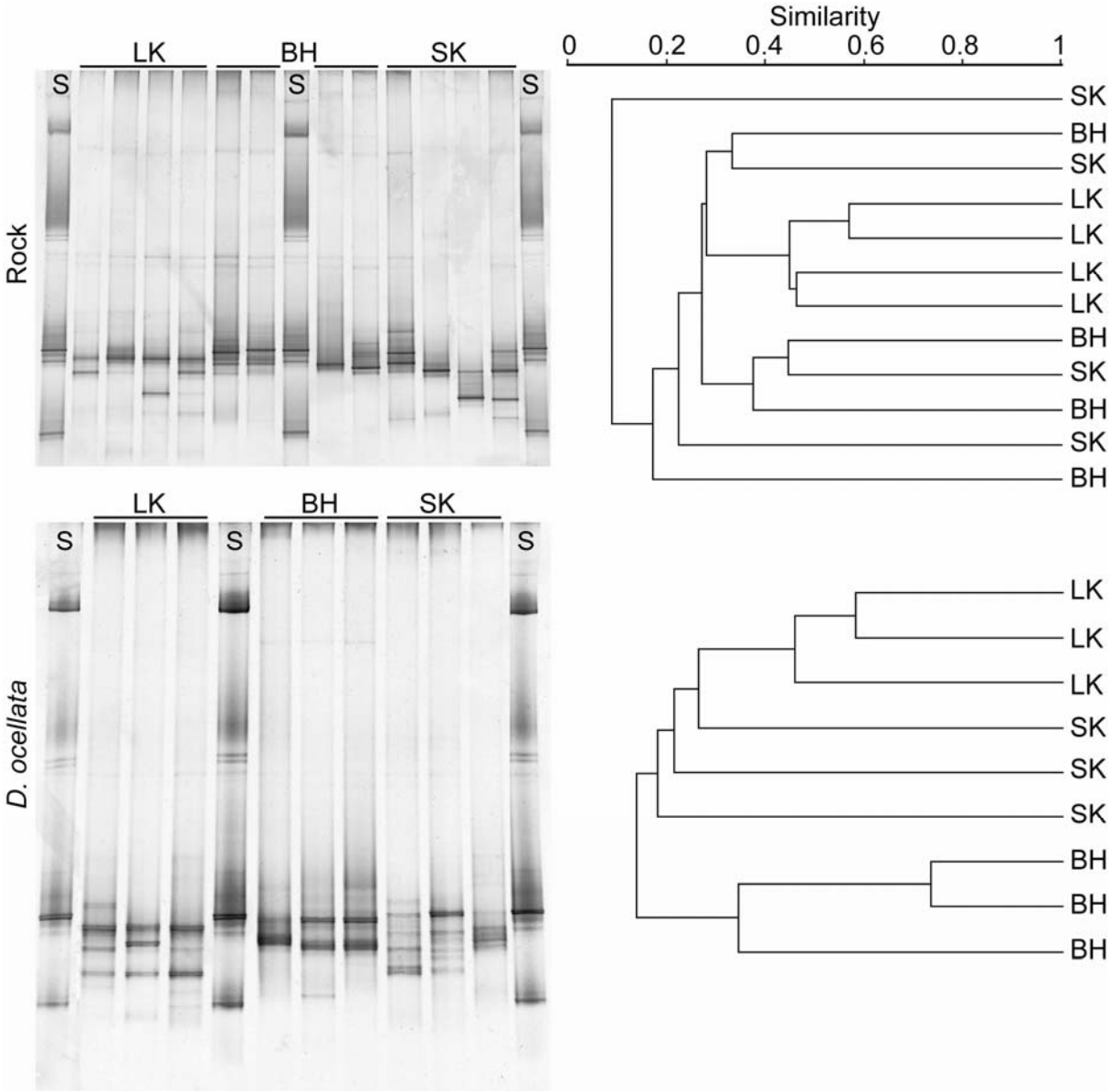

Figure 9: Comparison of the bacterial communities on the surface of rocks and *D. ocellata* from Long Key (LK), Bahia Honda (BH), and Summerland Key (SK). A) DGGE profiles S = standard. B) UPGMA cluster analysis of DGGE banding patterns based on the Bray Curtis distance measure.

Table 3: Comparison of bacterial communities on the surfaces of rocks and *D. ocellata* collected from three locations; Bahia Honda (BH), Long Key (LK), and Summerland Key (SK). R values are given for the global R and pairwise comparisons with p values in parentheses. Bold numbers indicate significant differences between locations ($p < 0.05$). Significance levels for pairwise comparisons were not subjected to Bonferroni corrections. n = 4 for rocks and n = 3 for *D. ocellata.*

	Rock	*D. ocellata*
Global R	**0.2755 (0.0076)**	**0.7449 (0.0060)**
Pairwise comparisons		
BH vs. LK	**0.4688 (0.0283)**	1.000 (0.1021)
LK vs. SK	**0.3021 (0.0264)**	0.4074 (0.1971)
BH vs. SK	0.1250 (0.2828)	0.8519 (0.1044)

Pairwise comparisons of the different locations revealed a significant difference in the bacterial community compositions of rocks from Bahia Honda and Long Key (R = 0.4688, p = 0.0283), as well as between Summerland Key and Long Key (R = 0.3021, p = 0.0264, **Table 3**). However, there was no significant difference in the communities found on the surfaces of rocks from Bahia Honda and Summerland Key (R = 0.1250, p = 0.2828, **Table 3**). The differences seen in the bacterial communities among locations is congruent with the finding that sediment bacterial communities in Florida Bay, which is bounded by the Florida Keys, differ between the eastern-central regions and the western region (Ikenaga et al. 2010). Ikenaga et al. (2010) attributed this difference to variations in salinity and nutrients based on the source and amount of run-off from the Florida Everglades that the area was exposed to. In my case, the difference in bacterial communities among sites was likely due to the vastly different environment conditions at each location (see Section 2.2.1).

It has been shown that some corals and sponge species harbor similar bacterial communities in different locations. However, these studies observed communities either within the coral mucus or inside the sponges, but not from the biofilm on the surfaces of these organisms (Rohwer et al. 2002, Taylor et al. 2005). It has also been shown that some temperate macroalgae retain a specific surface-associated bacterial community regardless of geographic location (Lachnit et al. 2009). Lachnit et al. (2009) examined the bacterial communities on the surfaces of a variety of macroalgae collected from the Baltic Sea and compared them to individuals of the same species collected from the North Sea. They found

that the phylogeny of the algae had a greater affect on the structure of the bacterial community than the location from which the algae were collected. This is not the case for *D. ocellata*, at least amongst the sites that we surveyed. Compared to the study by Lachnit et al. (2009), in which algae maintained a similar bacterial community even in such diverse environments as those in the North Sea vs. the Baltic Sea, it is surprising that the biofilm community of *D. ocellata* varied within a relatively close geographical area (**Figure 6**). However, my research was performed in a tropical environment as opposed to the temperate environment studied by Lachnit et al. (2009). To my knowledge this is the first study to compare the bacterial communities on the surfaces of tropical macroalgae collected from different locations. Further investigation is necessary to determine if there are different trends in the host specificity of macroalgal biofilms between tropical and temperate environments. It would also be interesting to examine the bacterial community in terms of function as opposed to phylogeny because it is possible that algae from different environments would harbor bacterial species that fill similar functional niches even when the pool of available bacterial phylotypes differs.

2.3 Effects of algal extracts on the surface-associated bacterial community

In order to determine if the observed differences in bacterial community composition could be attributed to organic compounds found on the surface of or within *D. ocellata,* I tested the effects of algal extracts on bacterial biofilm formation. In February 2008, I collected *D. ocellata* from my field site on Summerland Key. I took both surface and whole-cell extracts of the alga and tested these extracts for their effects on the formation of bacterial biofilms on test surfaces.

2.3.1 Surface extracts

Surface extracts were obtained using the hexane dipping method described by de Nys et al. (1998). Briefly, this method involves dipping the algae in solvent for a minimal amount of time (~ 30 s) so as to avoid rupturing the cells and which would result in the extraction of compounds from within the algae in addition to those on the surface. This method is limited to the use of highly nonpolar solvents because more polar solvents tend to disrupt the algal

cells. Therefore, it limits the range of compounds that can be extracted. However, there is a paucity of methods available for the extraction of compounds from the surface of algae, and the hexane dipping method remains the most commonly used despite its limitations.

The extracts were coated onto the surface of polystyrene Petri dishes so that the amount of extract was equivalent to that found on the same surface area of the alga. Control surfaces were prepared by coating Petri dishes with hexane. The solvent was allowed to evaporate from the surface and the coated Petri dishes were placed into the field. Six control and 6 treatment dishes were collected every day for five days. These were swabbed and the bacterial community profiles were analyzed as previously described (Section 2.1). According to two-way analysis of similarity (2-way ANOSIM) there was no effect of treatment on the bacterial community profiles ($R = 0.064$, $p = 0.181$), however there was a significant effect of time ($R = 0.134$, $p = 0.009$). When analyzing these data, samples were randomly distributed among DGGE gels and then gels were aligned using internal standards. However, I noticed that cluster analysis revealed that samples from the same gel tended to cluster together, separate from samples in other gels. I therefore reanalyzed the samples loading all extracts and controls onto one gel. Because one gel has space for only 13 samples I could only compare one set of samples per gel. I chose to analyze the samples taken after 48 hours because this was the minimum amount of time necessary to produce a difference between treatments in enclosure experiments described below (Section 2.4).

The reanalysis confirmed the results of my initial analysis. Surface extracts of *D. ocellata* had no significant effect on the composition of the bacterial community (**Figure 10**). Banding patterns of samples treated with extracts showed no groupings in cluster analysis and were not significantly different from controls according to ANOSIM ($R = 0.1907$, $p = 0.0932$, **Figure 10**). The lack of activity exhibited here might indicate that there are no active compounds on the surface of the alga, but might also result from incomplete extraction of the compounds from the algal surface or loss of compounds from the surface of the Petri dishes in the water. The surface of the alga was extracted in hexane and this method excludes the extraction of very polar compounds. Furthermore, in order to avoid disrupting the cells, the algae were only subjected to the solvent for 30 s which may result in a lower concentration of metabolites in the extract than are actually present on the surface of the alga. In addition to the limitations

of the extractions procedure, compounds extracted may have different affinities for the surface of the Petri dish. Because I am dealing with crude extracts here, it was not possible to determine the amount of compound remaining on the dishes after 48 hours in the field and the lack of activity may be due to a loss of compounds from the surfaces of the Petri dishes. Because the algae may contain active compounds that cannot be adequately extracted by surface extractions methods, I also performed a more extensive extraction using hexane, ethyl acetate and methanol. This cannot be done without disrupting the algal cells; therefore these extracts were taken from ground algae to produce whole-cell extracts.

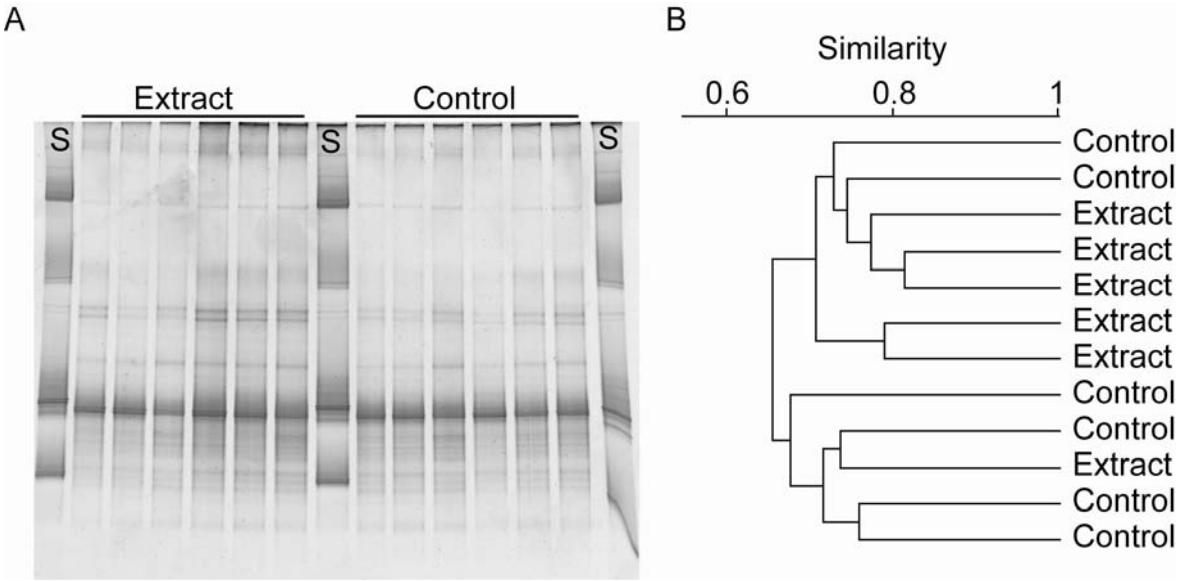

Figure 10: Comparison of bacterial communities on artificial surfaces treated with surface extracts of *D. ocellata.* A) DGGE profiles S = standard. B) UPGMA cluster analysis of DGGE banding patterns based on the Bray Curtis distance measure.

2.3.2 Whole-cell extracts

I extracted the algae sequentially in hexane and ethyl acetate by placing whole algal cells in hexane and blending with a commercial blender, removing the hexane layer, and then extracting the remaining cell debris in ethyl acetate. These extracts were incorporated into Phytagel™ plates at concentration that were volumetrically equal to the alga. This method was originally described for testing the antifouling activity of sponge extracts (Henrikson and Pawlik 1995). Briefly, extracts are incorporated into a hard gel matrix (Phytagel™) that is capable of remaining stable while submerged in seawater for a prolonged period of time. The

incorporation of extracts in such a matrix allows for the continuous diffusion of compounds into the surrounding seawater without affecting the physical properties of the surface (Henrikson and Pawlik 1995). Because the gel remains stable under water, this method can be used to test the effects of extracts in the field. The Phytagel™ method has been used most often for testing effects of extracts on the settlement of macrofouling organisms, but has also recently been coupled with bacterial fingerprinting techniques to monitor the effects of extracts on the formation of bacterial biofilms (Dobretsov et al. 2005, Lachnit et al. 2010).

Phytagel™ plates incorporating hexane and ethyl acetate extracts of *D. ocellata* were placed underwater at the field site and bacterial biofilms were allowed to form for 2, 4, 7, or 10 days. The biofilms were then harvested using sterile swabs and the bacterial communities analyzed as previously described (Section 2.1).

The bacterial communities on the surface of plates containing hexane extracts were compared to solvent control plates containing hexane alone. According to two-way analysis of similarity (2-way ANOSIM), there was no effect of either treatment or time on the bacterial community composition ($p = 0.075$ and 0.171, respectively). Solvent was removed from ethyl acetate extracts under vacuum and the resultant solid material was dissolved in methanol prior to addition to Phytagel™ plates. These ethyl acetate extracts were compared to solvent controls containing only methanol. The Phytagel™ mixtures containing methanol (ethyl acetate extract and methanol solvent control) failed to gel properly and produced clumps of Phytagel™ in the plates as opposed to a smooth layer. In several of these samples the Phytagel™ had floated out of the plates before the plates were brought into the lab for analysis. The remaining plates and any pieces of Phytagel™ remaining were swabbed and included in the analysis. The results indicated that there was no effect of either ethyl acetate extract treatment or time on the structure of the bacterial community, however due to the problem with incorporating methanol into the Phytagel™ these experiments were repeated in December, 2009 with some alterations.

Algae were collected from the same location as in February, 2008. This time the algae were extracted in both ethyl acetate and methanol. The extracts were dried and dissolved in dimethyl sulfoxide (DMSO) prior to incorporation into Phytagel™ plates. Extracts were again incorporated at levels volumetrically equivalent to the algae. Solvent controls were

prepared with an equal amount of DMSO alone. The bacterial community was sampled and analyzed as in 2008 except that the Phytagel™ plates were sampled only once, after 48 hours in the field. Additionally, cores were taken from the center of each plate and the number of bacterial cells present was determined using fluorescence microscopy.

There was a significant treatment effect on the banding patterns of samples taken from Phytagel™ plates containing whole cell extracts of *D. ocellata* (R = 0.2130, p = 0.0446, **Figure 11**, **Table 4**).

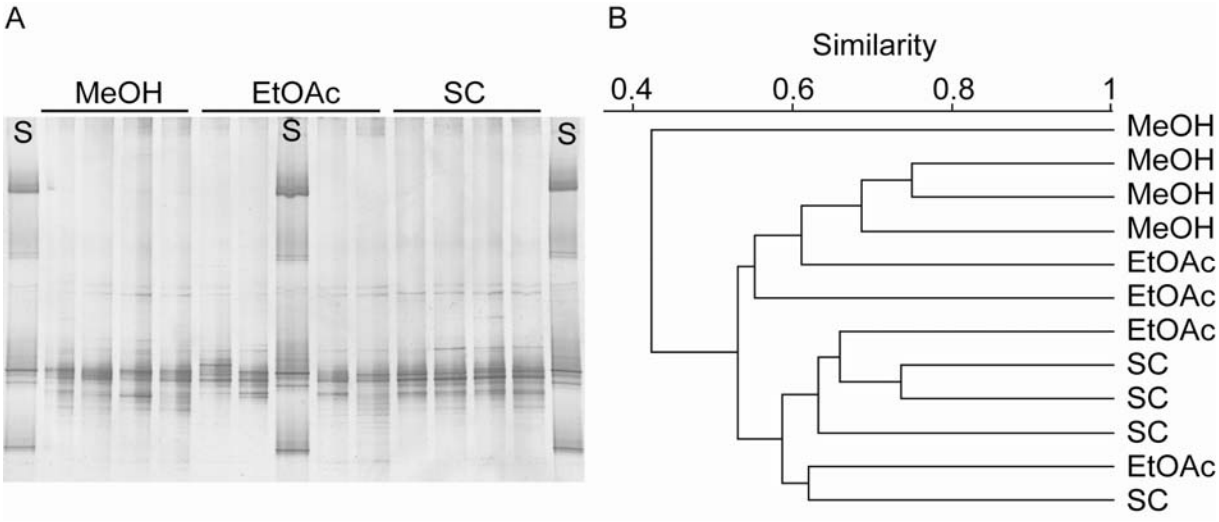

Figure 11: Comparison of bacterial communities on artificial surfaces treated with whole cell methanol (MEOH) and ethyl acetate (EtOAc) extracts of *D. ocellata* after 48 hours in the field. A) DGGE profiles, S = standard. B) UPGMA cluster analysis of DGGE banding patterns based on the Bray Curtis distance measure.

With the exception of one sample, bacterial communities on the surface of plates containing the methanol extract of *D. ocellata* formed a separate cluster from the solvent control (**Figure 11**). Ethyl acetate extracts clustered with both methanol extracts and solvent controls (**Figure 11**). Pairwise comparisons showed a significant difference between methanol extracts and solvent controls (R = 0.5000, p = 0.0304, **Table 4**). There was no significant difference between the ethyl acetate extract and solvent control or methanol extract (R = 0.1458, p = 0.1434 and R = 0.0000, p = 0.4860 respectively, **Table 4**).

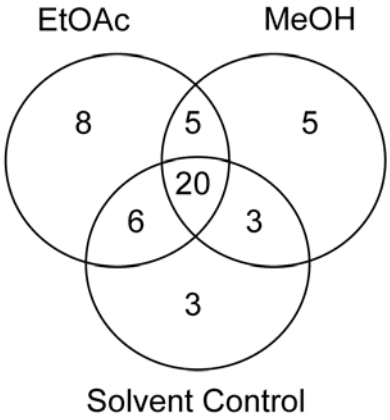

Figure 12: Venn diagram comparison of the number of bacterial phylotypes present on artificial surfaces treated with whole cell methanol (MeOH) and ethyl acetate (EtOAc) extracts of *D. ocellata* and the solvent control (SC). Numbers within the area where circles cross indicate the number of phylotypes shared by these surfaces.

Table 4: Analysis of similarity (ANOSIM) comparison of bacterial communities on artificial surfaces treated with whole cell methanol (MeOH) and ethyl acetate (EtOAc) extracts of *D. ocellata* and a solvent control (SC). R values are given for the global R and pairwise comparisons, p values are given in parentheses. Bold numbers indicate significant differences between treatments ($p < 0.05$). Significance levels for pairwise comparisons were not subjected to Bonferroni corrections. n = 4.

Global R	**0.2130 (0.0446)**
Pairwise comparisons	
EtOAc vs. SC	0.1458 (0.1434)
MeOH vs. SC	**0.5000 (0.0304)**
EtOAc vs. MeOH	0.0000 (0.4860)

Eighteen of the 50 bacterial phylotypes found on the Phytagel™ plates were found exclusively on plates treated with algal extracts (**Figure 12**). Five were found on both ethyl acetate extract and methanol extract plates, 8 only on ethyl acetate extract plates, and 5 only on methanol extract plates (**Figure 12**). Of the 32 phylotypes present on solvent control plates, 3 were eliminated by both algal extracts, 6 by only the methanol extract, and 3 by only the ethyl acetate extract (**Figure 12**).

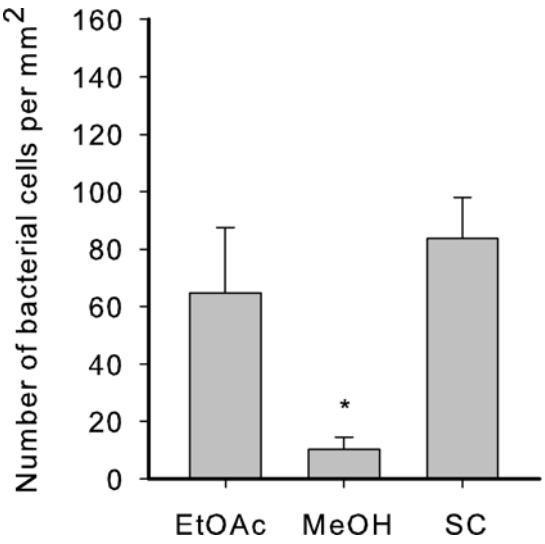

Figure 13: Bacterial cell counts on artificial surfaces treated with whole cell ethyl acetate (EtOAc) and methanol (MeOH) extracts of *D. ocellata* and solvent controls (SC). Bars indicate mean ± standard error, asterisks indicates significant differences between treatments according to one-way ANOVA ($p < 0.05$), n = 5.

The methanol extract of *D. ocellata* also caused a decrease in the number of bacterial cells found on the surface of the Phytagel™ when compared to solvent controls ($p = 0.012$, **Figure 13**). However, there was no significant difference in the number of bacteria present on the surface of Phytagel™ plates containing ethyl acetate extract and the solvent control ($p = 0.430$, **Figure 13**).

These results indicate that there are organic compounds that are extractable by methanol, but less so by ethyl acetate, and not at all by hexane, that effect the composition of the bacterial community on the surface of *D. ocellata*. It is likely that these compounds are at least slightly polar and are therefore extracted by a more polar solvent like methanol than a highly non-polar solvent like hexane. The polarity of ethyl acetate is between methanol and hexane and this may explain why there is slight activity seen in the ethyl acetate extracts.

Researchers have been investigating the antibacterial properties of macroalgal extracts for nearly a century (see review by Goecke et al. 2010). The majority of these investigations focused on the antibacterial activity of algal extracts on individual bacterial strains, often important human or marine pathogens. For example, Engel et al. (2006) and Puglisi et al. (2007) performed a large survey of the activity of algal extracts against a marine pathogenic

bacterium, *Pseudoalteromonas bacteriolytica*. In these studies they found that extracts of over half of the algal species tested inhibited the growth of *P. bacteriolytica* at ecologically relevant concentrations. They tested both lipophilic and hydrophilic extracts and found that for each algal species the bacteria most often responded to one fraction or the other, but not both. This indicates that some algal species produce highly polar compounds that have antibacterial properties, while others rely on more non-polar compounds. The methanol extract of *D. ocellata* reduced the overall bacterial abundance on the surface of Phytagel™ plates indicating that antibacterial compounds from *D. ocellata* are polar (**Figure 13**). However, the compounds found in the methanol extract of *D. ocellata* did not affect all bacterial strains equally as evidenced by the presence of some bacterial phylotypes exclusively on plates containing this extract (**Figure 12**).

Unlike traditional assays that examine the inhibitory effects of algal extracts on the growth of bacteria, this experiment looked at the alteration of the bacterial community composition caused by algal extracts. My results indicate that *D. ocellata* contains compounds that promote the growth of some bacteria while inhibiting the growth of others. Similar results were found in a study of the effects of crude sponge extracts on bacterial biofilm formation (Dobretsov et al. 2005). Additionally, Lachnit et al. (2010) recently demonstrated that the incorporation of lipophilic algal extracts into Phytagel™ along with the continuous release of hydrophilic compounds resulted in the formation of a bacterial community highly similar to that found on the surface of the alga. These studies support the idea that algae regulate the composition of the bacterial communities on their surfaces through the production of organic compounds.

It should be noted that it has been suggested that using whole-cell extracts may lead to misleading results because the compounds within the alga may not be present at the surface where they would affect the formation of biofilms (Nylund et al. 2006). However, the methods for extracting compounds from the surfaces of algae are limited to the use of highly non-polar solvents such as hexane (de Nys et al. 1998). As there is greater activity in the methanol extract compared to the ethyl acetate extract it is likely that the active compounds may be too polar to be quantitatively extracted by the hexane dipping method used for the surface extraction (de Nys et al. 1998). This could explain the lack of activity seen in analysis

of surface extract (Section 2.3.1). Here whole-cell extracts were incorporated into Phytagel™ so as to be volumetrically equal to the alga. Therefore, if the compounds were found ubiquitously throughout the algal cells, this method will represent the natural condition. Furthermore, if the compounds are enriched on the surface, then the natural activity would even be more pronounced than the one observed here. However, the degree to which the extracts leached from the Phytagel™ into the surrounding seawater was not determined and therefore, the concentration of compounds encountered by biofilm bacteria cannot be quantified. Further identification of active compounds and elucidation of their concentrations and location within the alga are necessary to determine if these compounds are responsible for structuring the bacterial community on the surface of *D. ocellata*.

There are few examples in which the role of algal secondary metabolism in structuring the surface-associated bacterial community has been well investigated. In the last section (2.2), I presented evidence that *D. ocellata* harbors a distinct bacterial community when compared to other objects in the same environment. Here I also determined that organic compounds extracted from the alga affect the formation of bacterial biofilms on treated surfaces. This indicates that the regulation of the species composition and bacterial abundance on the surface of *D. ocellata* may be, at least partially, due to compounds produced by the alga. As the bacterial biofilms present on the surfaces of benthic marine organisms are important determinants of further macrofouling, continued investigation of the factors that shape the surface-associated bacterial communities of these organisms is essential to understanding their relationships within their communities.

2.4 Effects of D. ocellata *on the natural planktonic bacterial assemblage*

In order to further investigate how *D. ocellata* affects the species composition of the bacterial community surrounding it, I performed a series of enclosure experiments. In these experiments, I compared the bacterial community in seawater from enclosures containing *D. ocellata* to those with no algae. The experiments were developed over the course of three field trips to Mote Tropical Research Laboratory in Summerland Key, FL. The protocol was altered each time in order to optimize the experimental design.

2.4.1 Field trip #1: February, 2008

In February 2008, the initial field enclosure experiments were performed in Summerland Key, FL. These experiments were designed to monitor the effects of *D. ocellata* on the planktonic bacterial community in a setting as close to its natural environment as possible. To accomplish this, algae were placed in sterile one L square bottles made from polyethylene terephthalate copolyester (PETG) which has a transparency of ~ 80 % (Tsai et al. 2008). The bottles were filled with seawater from the field site and placed under the water at the same site. This allowed enclosures to be exposed to light and temperature conditions nearly identical to those of the alga's natural environment. A relatively small amount of algae was added to each bottle (~ 5 - 15 g wet weight) in order to prevent the water from becoming hypoxic during the time of the experiment. This small amount was also used to prevent the algae from becoming stressed due to self-shading. As the algae appeared healthy at the end of the experiment, this amount was increased in subsequent experiments. The lids of the bottles contained septa which allowed for sterile sampling. In order to determine the time it takes for an effect to be seen, samples were taken every 24 hours over the course of six days. At each sampling time, 1 ml samples were filtered onto 0.2 µm membranes which were subjected directly to PCR according to the method described by Kirchman et al. (2001). The bacterial community present in each sample was profiled using DGGE (see Section 2.1). Because the number of samples that can be loaded on a DGGE gel is limited, banding patterns were compared across different gels. I found that this experimental set-up resulted in very low amounts of DNA after PCR amplification in some of the samples. I had to exclude these samples from DGGE analysis because this method requires a minimum of 200 ng DNA for reliable analysis, which in my DGGE set-up was equivalent to a minimum of 10 ng DNA μl^{-1}. Although Kirchman et al. (2001) found positive amplification with samples as small as 25 µl of coastal seawater, 1 ml appeared to be too small of a sample volume for my system.

After analyzing the samples that contained at least 10 ng DNA μl^{-1}, I found that the bacterial community composition was significantly affected by both treatment and time according to ANOSIM ($p = 0.038$ and $p = 0.001$, respectively, **Table 5**). However, when compared at individual time points, the bacterial communities were significantly different only after 144 hours ($p = 0.003$, **Table 5**). Additionally, I performed technical replicates by analyzing three sections from each filter in order to examine variations within samples.

Cluster analysis revealed that these technical replicates sometimes produced very different bacterial community profiles (e.g. **Figure 14**). It appears that the samples were not evenly distributed across the surfaces of the filters, and this might be due to the low sample volume.

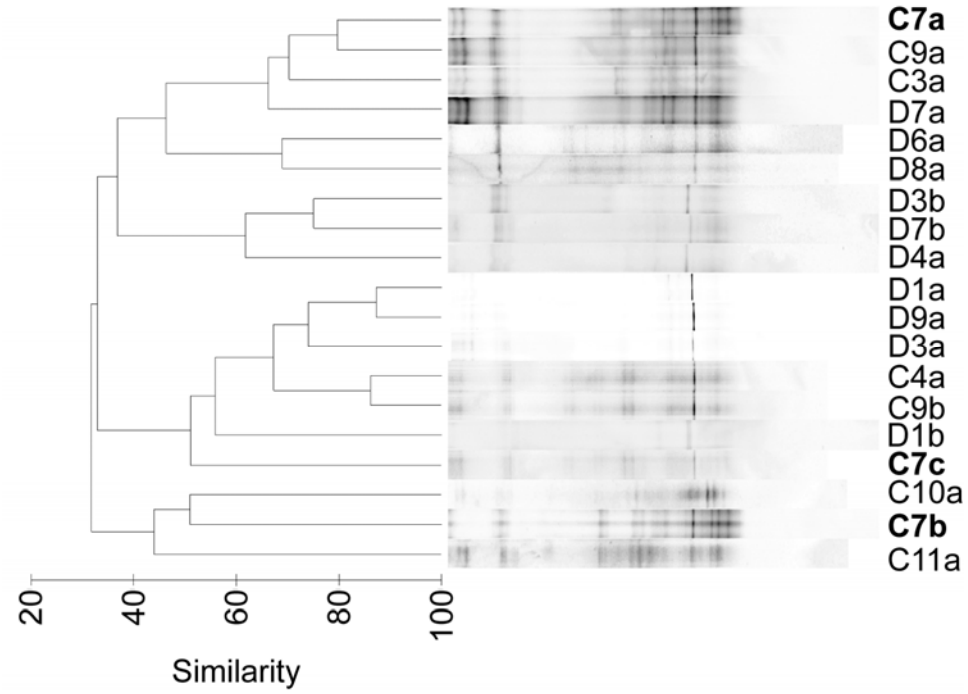

Figure 14: UPGMA cluster analysis of DGGE bacterial community profiles of water samples from enclosure with (D) and without (C) *D. ocellata* based on the Pearson Correlation measure of similarity. Numbers indicate replicates, lower case letters indicate technical replicates. Labels in bold indicate an example of three technical replicates which did not cluster together using this method.

2.4.2 Field trip #2: February, 2009

In an attempt to improve the experiment and address the problems discussed above in Section 2.4.1, a second enclosure experiment, with several adjustments to the experimental design, was performed at the same field site in February 2009.

Algae are surrounded by a thin boundary layer known as the diffusion boundary layer (DBL) which limits molecular exchange with the surrounding seawater (Hurd 2000). In the case of *D. ocellata*, this exchange is particularly limited due to the structure of the alga. As

described in the introduction, this 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 most likely occur within the DBL, however sampling seawater from within the DBL of an alga is technically difficult. In the previous study (Section 2.4.1), enclosure experiments were designed to mimic the limited molecular exchange that occurs between the thin boundary layer of water surrounding the macroalga and the ambient seawater. The water contained in the bottles had no molecular exchange with the outside seawater and thereby acted to concentrate compounds released from the alga, similar to the condition within the DBL. Sampling the water within the enclosures allowed for the investigation of the bacterial community within a stagnant environment similar to that of the DBL, while avoiding the technical difficulties involved in sampling the actual DBL. In order to better represent the conditions in the thin boundary layer surrounding *D. ocellata*, the algae to seawater ratio within the bottles was increased. Since the previous experiment indicated that the algae were not obviously stressed by enclosure within the bottles, the amount of algae in the enclosures was increased from ~5 - 15 g wet weight to ~ 100 g wet weight, in order to increase the concentration of any effects and further approximate the conditions within the DBL.

The results from the 2008 experiment (Section 2.4.1) also indicated that the sample volume was too small. I therefore increased the sample volume to 10 ml per sampling event, and took fewer samples over a shorter period of time in order to reduce the effect of sampling on the community within the bottle. Again, I found that both treatment and time significantly affected the bacterial community compositions of samples ($p < 0.001$ for both, **Table 5**). There was also a significant difference between treatments and controls after 48 and 96 hours ($p < 0.001$ for both, **Table 5**).

Table 5: Effects of *D. ocellata* on bacterial communities within enclosure experiments conducted in two consecutive winters according to 2-way ANOSIM. Pairwise comparisons of enclosures with and without algae at each sampling time. R values are given with p values in parentheses. Significant differences are indicated in bold.

	Treatment	Time	Pairwise comparisons				
			24 hrs	48 hrs	96 hrs	120 hrs	144 hrs
2008	**0.084**	**0.639**	-0.102	0.083	0.004	0.012	**0.452**
	(p = 0.038)	**(p = 0.001)**	(p = 0.883)	(p = 0.160)	(p = 0.387)	(p = 0.387)	**(p = 0.003)**
			0 hrs	7 hrs	24hrs	48 hrs	96 hrs
2009	**0.431**	**0.278**	0.519	0.315	0.019	**0.606**	**0.553**
	(p = 0.001)	**(p = 0.001)**	(p = 0.086)	(p = 0.131)	(p = 0.385)	**(p = 0.002)**	**(p 0.002)**

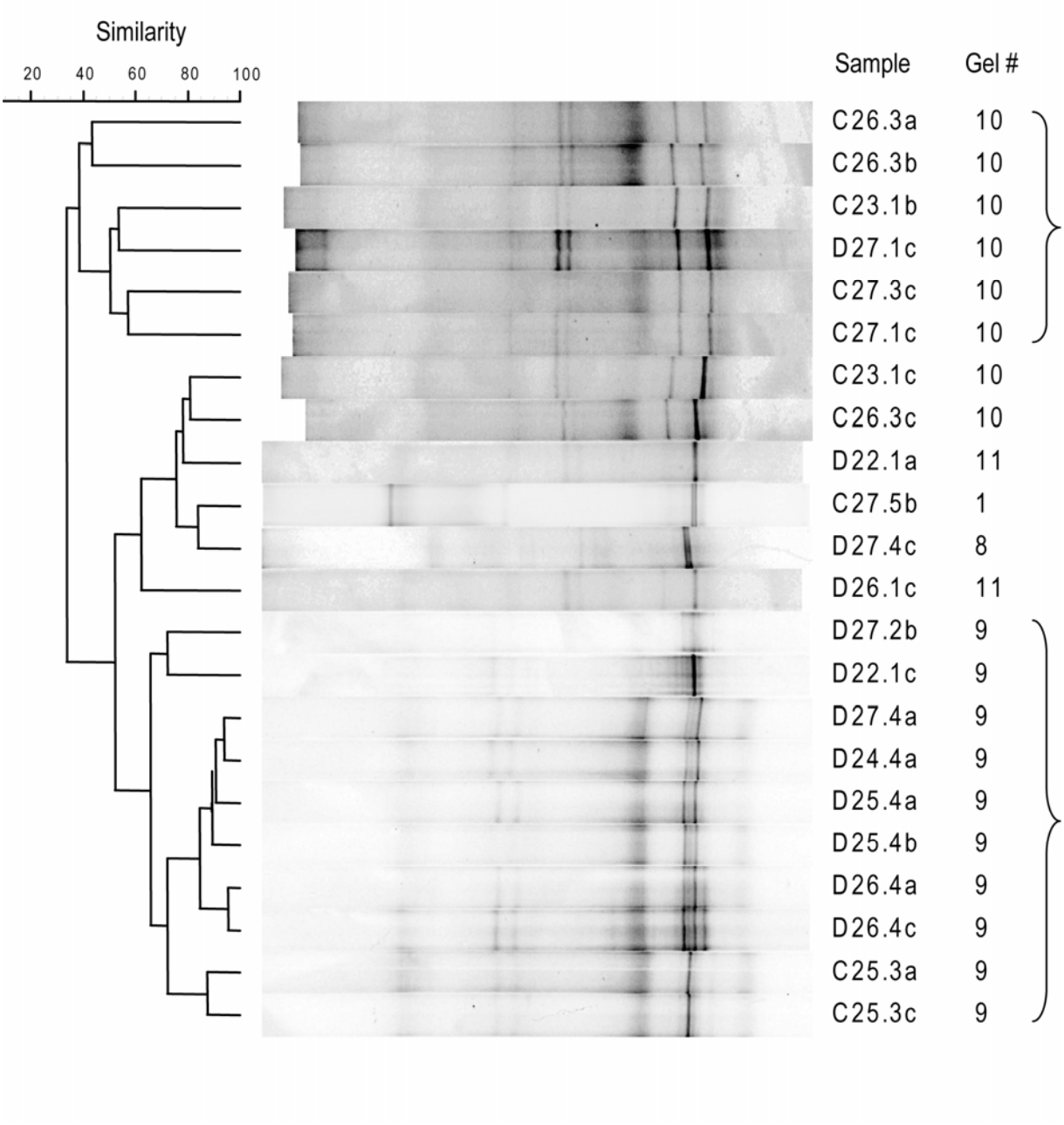

Figure 15: UPGMA cluster analysis of DGGE bacterial community profiles of water samples from enclosure with (D) and without (C) *D. ocellata* based on the Pearson Correlation measure of similarity. Numbers indicate replicate number and the day the sample was taken, lower case letters indicate technical replicates. Gel # indicates the DGGE gel on which the samples were run.

Because I was comparing samples on multiple DGGE gels, I examined how the gel affected the clustering of the samples and noticed that samples from the same gel often clustered together (e.g. **Figure 15**). Additionally, technical replicates failed to cluster together in some cases, similar to the data described in Section 2.4.1 (**Figure 15**). Although, when loaded onto the same gel technical replicates tended to cluster together indicating that the increased sample volume resulted in a better distribution of the sample on the filter. In order to verify that the differences observed between treatments and controls were actually a result of differences between samples and not an artifact of comparing samples across gels, I performed a third enclosure experiment in December, 2009.

2.4.3 Field trip #3: December, 2009

The experiment from February 2009 was repeated in December 2009, but with a few changes. One hundred grams of algae was again added to each treatment bottle and all bottles were filled with seawater from the site. However, the sampling volume was again increased from 10 ml to 30 ml in an attempt to get better PCR amplification. It was determined in the previous experiment that within enclosures containing 100 g of algae, 48 hours was sufficient time to see a change in the bacterial community (**Table 5**). Because increasing the sample volume was likely to affect the community remaining in the enclosure, sampling was performed at only one time point (48 hours). Comparing the treatments at only one time point also allowed me to analyze the communities within one gel instead of across gels, and this eliminated biases due differences in the gels.

In a second experiment, the effects of algal treated water on the bacterioplankton community within the bottles were examined to determine if the changes seen in the bacterial communities could be attributed to waterborne compounds released from the algae. Natural seawater collected from the field site was conditioned either with or without algae for 24 hours in glass aquariums. The algae to water ratio was the same as that used for the enclosure experiment (100 g algae L^{-1}). The water was then sterile-filtered and used to fill sterile one L bottles. The sterile algal- and non algal-treated water was inoculated with 30 ml of natural seawater and the bacterial communities within the bottles were compared after 24 hours. This set-up allowed me to test the effects of waterborne compounds released by the alga into the

seawater while avoiding changes in factors such as dissolved oxygen and pH that can result from the normal metabolism of the alga (Irwin and Davenport 2002, Larkum et al. 2003).

Bacterioplankton communities were significantly different in enclosures with algae compared to those without algae (R = 0.988, p = 0.0073, **Figure 16**). DGGE revealed 24 total bacterial phylotypes, 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 (**Figure 16**). As in the Venn diagrams from Sections 2.2 and 2.3, these numbers represent the presence of a bacterial phylotype in at least one replicate, and should be considered in addition to the statistical significance determined by ANOSIM. However, it should be noted that band #17 was found in all algal containing enclosures and was absent from all control replicates. Further investigation of this bacterial phylotype and its relationship to *D. ocellata* would be interesting. The selective elimination and promotion of bacterial phylotypes by *D. ocellata* supports my previous findings (Section 2.2) that the alga harbors a unique bacterial community on its surface and further supports the idea that algae are able to selectively control the growth of bacteria.

These results are similar to those found by Lam et al. (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 seawater had no affect on the composition of the bacterioplankton community (R = -0.03704, p = 0.5012, **Figure 17**). This indicates that waterborne compounds released from the algae are not responsible for the change in bacterial community or that these compounds are volatile or unstable (e.g. reactive oxygen species) and were therefore lost during the vacuum filtration of the algal-treated seawater (Potin et al. 1999, Küpper et al. 2002). Macroalgae are known to release volatile compounds into seawater. Some of these volatiles exhibit antibacterial activity and may therefore play a role in regulating the bacterial community (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.

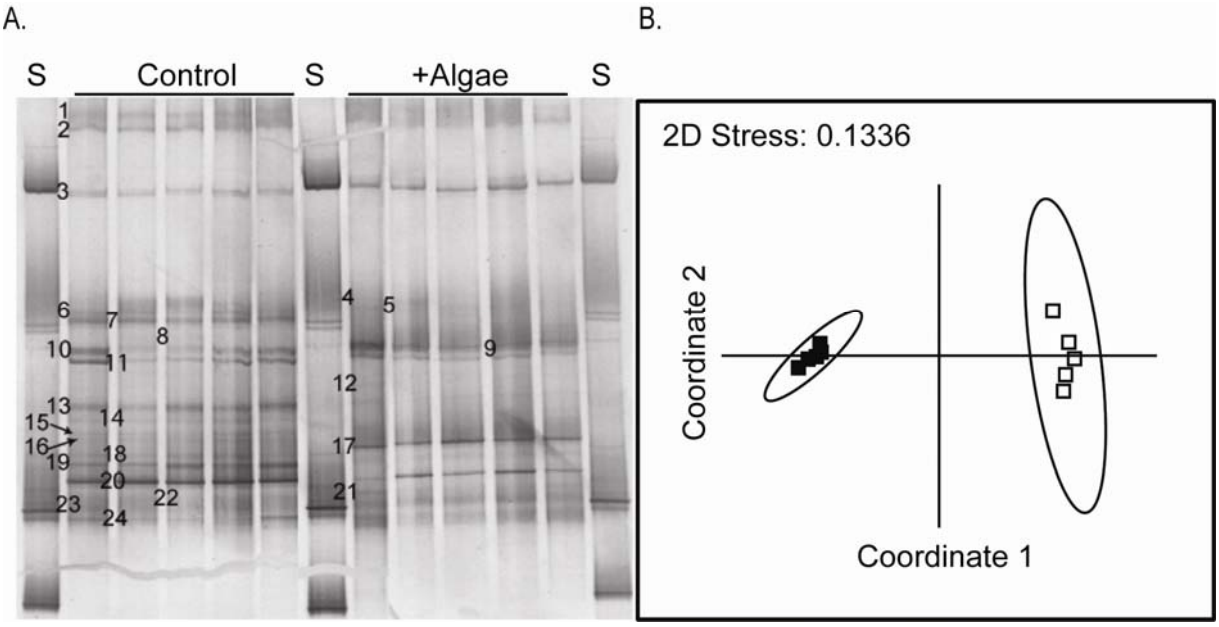

Figure 16: DGGE bacterial community profile (A) and non-metric multidimensional scaling (NMDS) plot (B) of enclosures containing *D. ocellata* (+Algae, open squares) and those without *D. ocellata* (Control, filled squares). Standard lanes are marked with an S and numbers indicate bands identified and used in analysis. NMDS was performed using the Bray-Curtis similarity measure with 95% confidence ellipses. Two dimensional stress = 0.1336.

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. Lam et al. (2008) found that in some cases alterations in the bacterioplankton community could be attributed to waterborne compounds while in others, direct contact with the alga was necessary to elicit an effect. In a study of the red macroalga *Bonnemaisonia hamifera*, Nylund et al. (2008) demonstrated that compounds on the surface of the alga inhibited the growth of marine bacteria at natural concentrations. Alternatively, the observed changes in the bacterial communities of algae containing enclosures may be the result of factors other than algal metabolites. For example, 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 and Davenport 2002, Larkum et al. 2003). The algae may also compete with bacteria for limiting nutrients, therefore shifting the bacterial community to one dominated by those strains best able to compete. Further investigation of the physical environment surrounding *D. ocellata* will aid in the understanding of the complex processes affecting the bacterial community surrounding this alga.

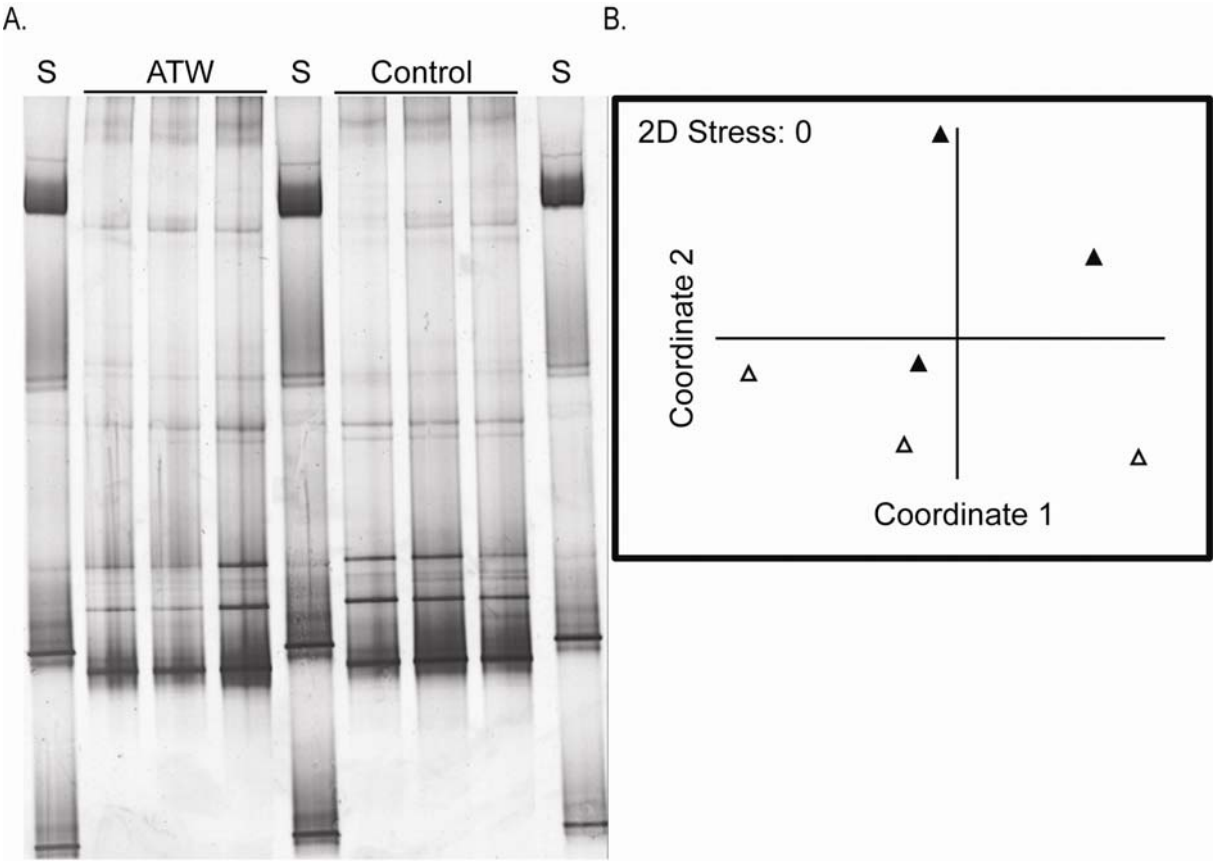

Figure 17: DGGE bacterial community profile (A) and non-metric multidimensional scaling (NMDS) plot (B) of enclosures containing algal-treated seawater (ATW, open triangles) and non algal-treated seawater (Control, filled triangles). Standard lanes are indicated with an S. NMDS was performed using the Bray-Curtis similarity measure. Two dimensional stress = 0.0000.

3 *Results and Discussion: Effects of* D. ocellata *and its organic extracts on the growth of marine bacteria in co-culture*

In field experiments it was determined that *D. ocellata* harbors a distinct bacterial community on its surface and that the composition and abundance of bacteria can be affected by organic extracts from the algae. Furthermore, the bacterioplankton community is also altered by the presence of *D. ocellata*. Based on these findings, a series of laboratory co-culture experiments were performed in order to further understand the mechanisms behind the regulation of the bacterial community by *D. ocellata*. In these experiments a number of individual bacterial isolates were grown in direct co-culture with *D. ocellata* and the effects of the algae on the growth of the bacteria were monitored. Additionally, the effects of organic extracts from *D. ocellata* on the growth of individual bacterial species were also examined.

3.1 Development of co-culture experiment

Co-culture experiments were optimized using readily available bacteria provided by Gunnar Gerdts and Antje Wichels from the Alfred-Wegner Institute for Polar and Marine Research prior to testing the effects of *D. ocellata* on naturally co-occurring bacteria from the Florida Keys. Few studies have investigated the effects of algae on bacterial growth in direct contact; most have focused on the effects of algal extracts on bacterial growth (see review by Goecke et al. 2010). Here bacterial cultures were grown in either sterile-filtered natural seawater (FSW) or sterile artificial seawater (ASW) in 200 ml Erlenmeyer flasks. Algae (~ 5 g) were added to half of the flasks and the effects of the algae on bacterial growth were monitored by spectrophotometric analysis of samples taken over the course of seven days. The algae in these experiments were not sterilized; therefore any reference here to *D. ocellata* includes the alga and its associated microflora. Most studies to date have designated activity as a difference in bacterial abundance at one time point post inoculation (Kubanek et al. 2003, Engel et al. 2006, Puglisi et al. 2007). During my initial studies, the efficacy of measuring inhibition at one time point as opposed to continuous monitoring of the growth curve when elucidating subtle algal-bacterial interactions was examined. To do this, the bacterial abundance over a long period of time (up to seven days) was monitored and the growth of

different treatments was compared by designating growth as either change in bacterial abundance 24 hours post inoculation, largest slope (μ) between two points on the growth curve (maximum growth rate), or overall growth curve.

When activity was measured as a change in bacterial density 24 hours after inoculation, all three species were significantly reduced in the presence of *D. ocellata*. *Micrococcus sp.* was reduced by 29% ($t = 13.81$, $p = 0.002$), *Pseudoalteromonas tetraodonis* by 57% ($t = 7.794$, $p = 0.0015$) and *Cytophaga sp.* by 22% ($t = 3.592$, $p = 0.0229$, **Figure 18**).

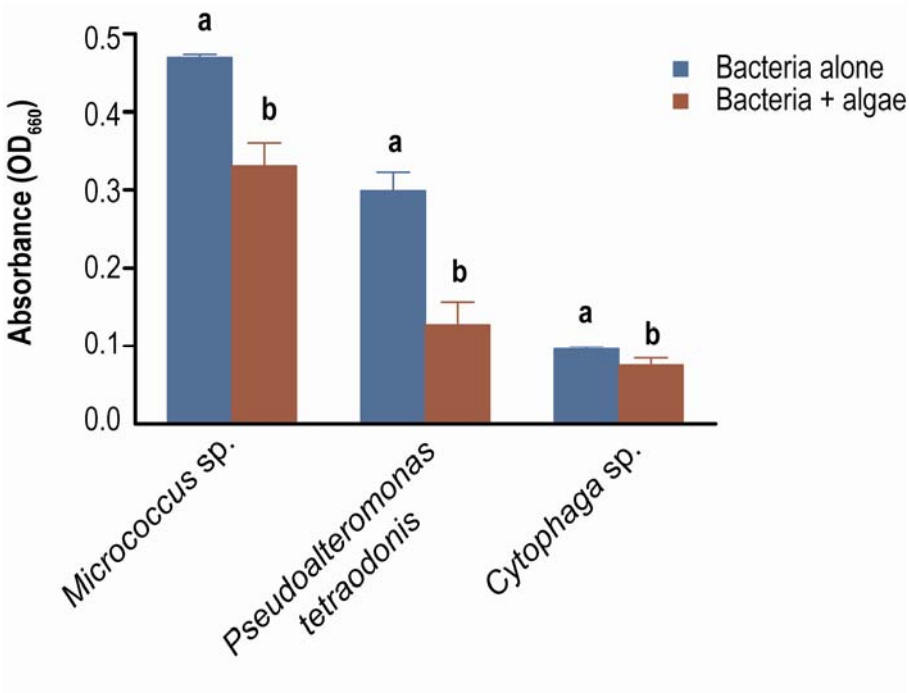

Figure 18: Bacterial abundance of *Micrococcus* sp., *Pseudoalteromonas tetraodonis*, and *Cytophaga* sp. 24 hours post inoculation in culture with (red bars) and without (blue bars) *D. ocellata*. Bars indicate mean ± standard error, different letters indicate significant differences between treatments within one bacterial strain according to the Student's t-test. $P < 0.05$, $n = 3$.

However, when activity was measured as a change in maximum growth rate there was no significant effect on either *Micrococcus sp.* ($p = 0.1362$, **Figure 19**) or *Pseudoalteromonas tetraodonis* ($p = 0.2952$, **Figure 19**). In contrast, the growth of *Cytophaga sp.* was completely inhibited for the first 10 hours, after which the culture began to grow but at a reduced rate ($\mu = 1.577 \times 10^{-2} \pm 1.891 \times 10^{-3}$) compared to the initial growth rate of the control ($\mu = 1.392 \times 10^{-1} \pm 4.4421 \times 10^{-3}$, $p < 0.001$, **Figure 19 andFigure** 20**C**).

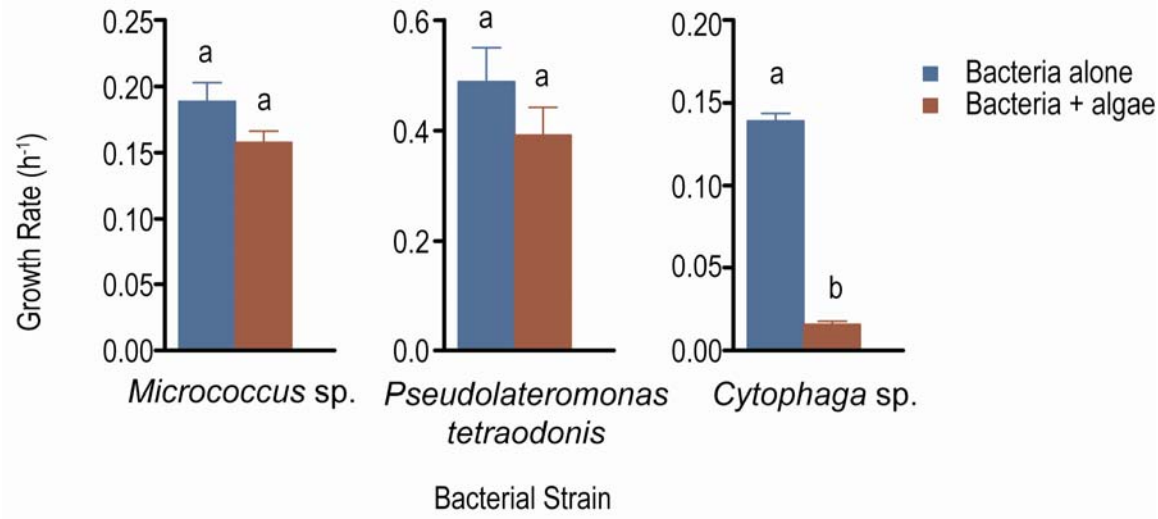

Figure 19: Maximum growth rates of bacterial strains grown in cultures with (red bars) and without (blue bars) *D. ocellata*. Bars indicate mean ± standard error, different letters indicate significant differences between treatments within one bacterial strain according to the Student's t-test. P < 0.05, n = 3.

Comparisons of the overall growth curves showed that there was a significant effect of *D. ocellata* on the bacterial growth for all three bacterial strains (**Figure 20**). *Micrococcus* sp. grown in the presence of the alga had a consistently lower abundance compared to controls without algae ($p < 0.0001$, **Figure 20**). The differences were significant starting 6 hours after inoculation and continuing until the end of the experiment ($p < 0.001$, **Figure 20**). Although the bacteria never reached the same abundance in the presence of the alga as it did in control treatments, it did follow a similar growth pattern for the first 36 hours after which there was a sharp decline in the abundance. No such decline was seen in the bacteria grown alone during the time period of the experiment (156 hours). While the relevance and cause of this early decline remain unknown, it is a pattern that is worth further investigation and would not have been detected using a single time point method of detecting differences.

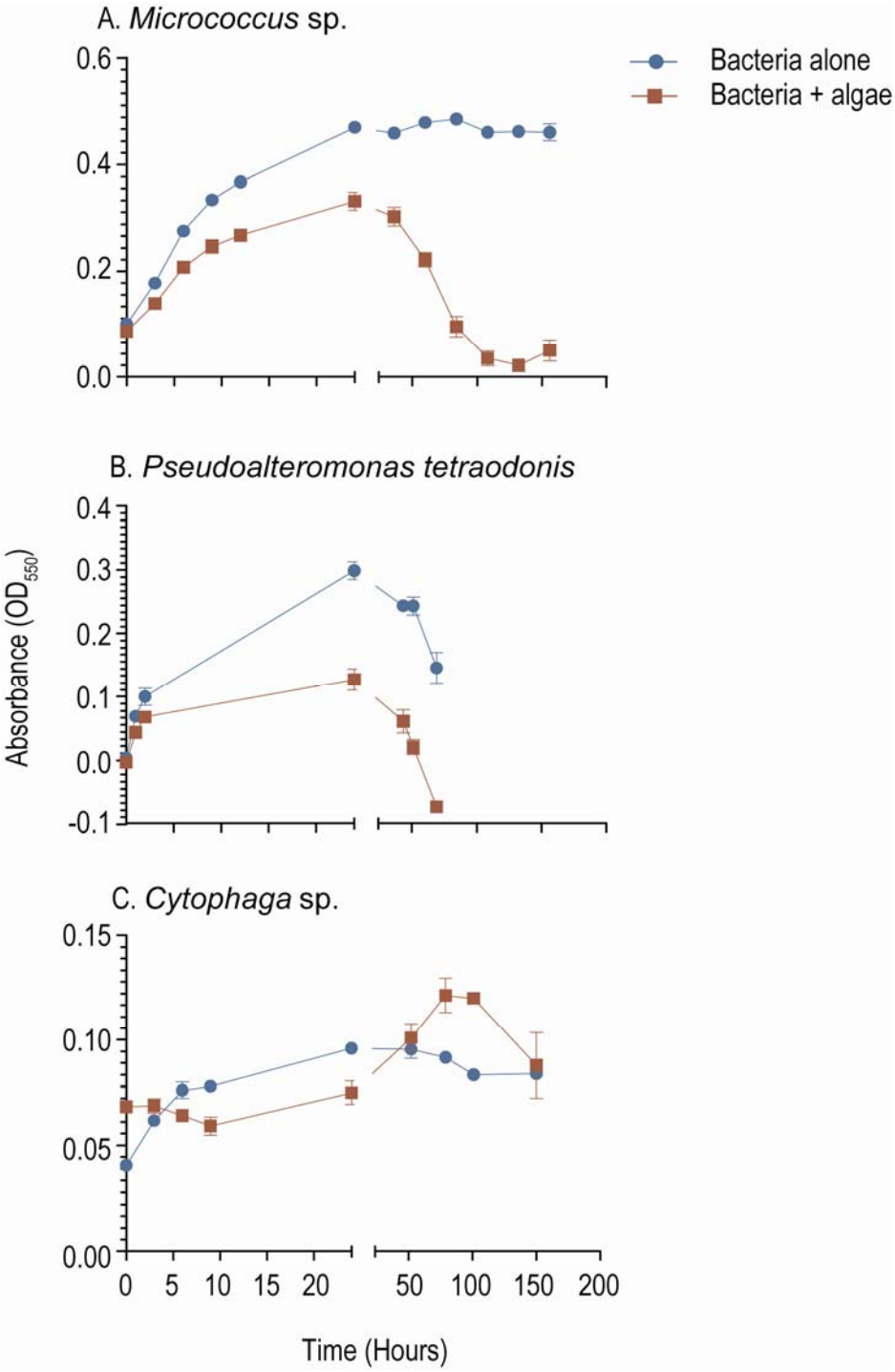

Figure 20: Growth curves of *Micrococcus* sp., *P. tetraodonis*, and *Cytophaga* sp. in cultures with (red) and without (blue) *D. ocellata*. Symbols = mean absorbance, error bars = standard error, n = 3.

Similar to *Micrococcus* sp., *P. tetraodonis* grown in the presence of algae had lower abundances than controls throughout the experiment (**Figure 20B**). These differences were significant starting 23 hours after inoculation and continuing to the end of the experiment (69 hours) ($p < 0.001$, **Figure 20B**). However, unlike *Micrococcus* sp., the general shape of the growth curve of *P. tetraodonis* in co-cultures with algae was similar to cultures without algae (**Figure 20B**). Both reached a peak absorbance after 23 hours and then began declining (**Figure 20B**).

While there was a significant difference between the growth curves of *Cytophaga* sp. grown with and without algae, the difference was less pronounced than that seen in *Micrococcus* sp. and *P. tetraodonis* ($p < 0.0001$, **Figure 20C**). As discussed above, the growth of *Cytophaga* sp. was completely inhibited for the first 10 hours, at which time it began to grow and eventually surpassed the controls in terms of abundance (**Figure 20C**). Comparison of treatments and controls at individual time points reveals a significant difference at the initial time point and at 79 and 101 hours post inoculation ($p < 0.01$, $p < 0.01$, and $p < 0.001$, respectively, **Figure 20C**). Additionally, the abundance of *Cytophaga* sp. was generally lower than that of the other two bacterial strains tested here throughout its growth curve. This may indicate that the conditions used here were not optimal for the growth of this strain. Since *D. ocellata* strongly inhibited the growth of this bacterial strain at early time points, it would be interesting to determine if this effect remains when the strain is grown in more conducive conditions.

Although the designation of growth as the abundance 24 hours post inoculation indicated an effect of *D. ocellata* on the growth of all three bacterial strains, it failed to reveal the differences in the effects among bacteria. Neither *Micrococcus* sp. or *P. tetraodonis* exhibited a reduced maximum growth rate in the presence of *D. ocellata*, however *D. ocellata* caused a reduction in the abundance of both bacteria throughout their respective growth curves. This is quite different from the response of *Cytophaga* sp. to the alga. The growth of *Cytophaga* sp. was completely inhibited for the first 10 hours in the presence of the alga, after which time the bacteria recovered and surpassed the controls in terms of abundance. It appears that the alga elicits a species-specific effect that would not have been discernable by measuring the growth at a single time point.

Furthermore, understanding the response of bacteria to algae throughout their growth curve may be important in understanding the complex interactions occurring within the bacterial community. These co-culture experiments examine the effects of *D. ocellata* on only one bacterial strain at a time, however in the natural setting there are a vast number of other players involved including competitors, predators, and alternate nutrient sources. One might imagine that a bacterial strain that has a higher growth rate during the exponential phase could out-compete those with lower growth rates. Additionally, a strain that is slower growing, but is able to maintain higher stationary phase abundance may have other competitive advantages. The full understanding of how these factors play out in nature is extremely complex and cannot be determined by the results found here. It is mentioned merely to point out the importance of understanding the effects of algae on bacterial growth in a more holistic manner than is traditionally used.

In order to determine if the growth inhibition of *Cytophaga* sp. resulted from the production of active metabolites, the growth was monitored in the presence of both media extracts and whole cell algal extracts. Organic compounds from the media of the co-culture experiments were extracted using solid phase extraction and eluted using methanol/THF. Algae were flash frozen in liquid nitrogen and extracted using methanol/THF. Solvents were removed from all extracts and the remaining residue was dissolved in DMSO, and incorporated into ASW. Extracts were concentrated by a factor of five in order to accommodate for possible loss of compounds during the extraction process. Solvent controls were prepared by the addition of only DMSO. This new media incorporating either extracts or solvent was inoculated with *Cytophaga* sp. and the growth was monitored as before. Because DMSO was used as a carrier solvent, the effects of DMSO at concentrations ranging from 1% to 10 % (v/v) were tested on the growth of marine bacteria. It was found that while DMSO inhibited the growth of bacteria at high concentrations, it had a minimal effect at 1 % (v/v) (**Appendix III**). Extracts were therefore always added to co-cultures so that the amount of DMSO did not exceed 1% (v/v).

Media extracts had no effect on the maximum growth rate of *Cytophaga* sp. ($p = 0.1162$, **Figure 21A**). However, there was a significant effect on the overall growth curve ($p < 0.0001$, **Figure 21A**).

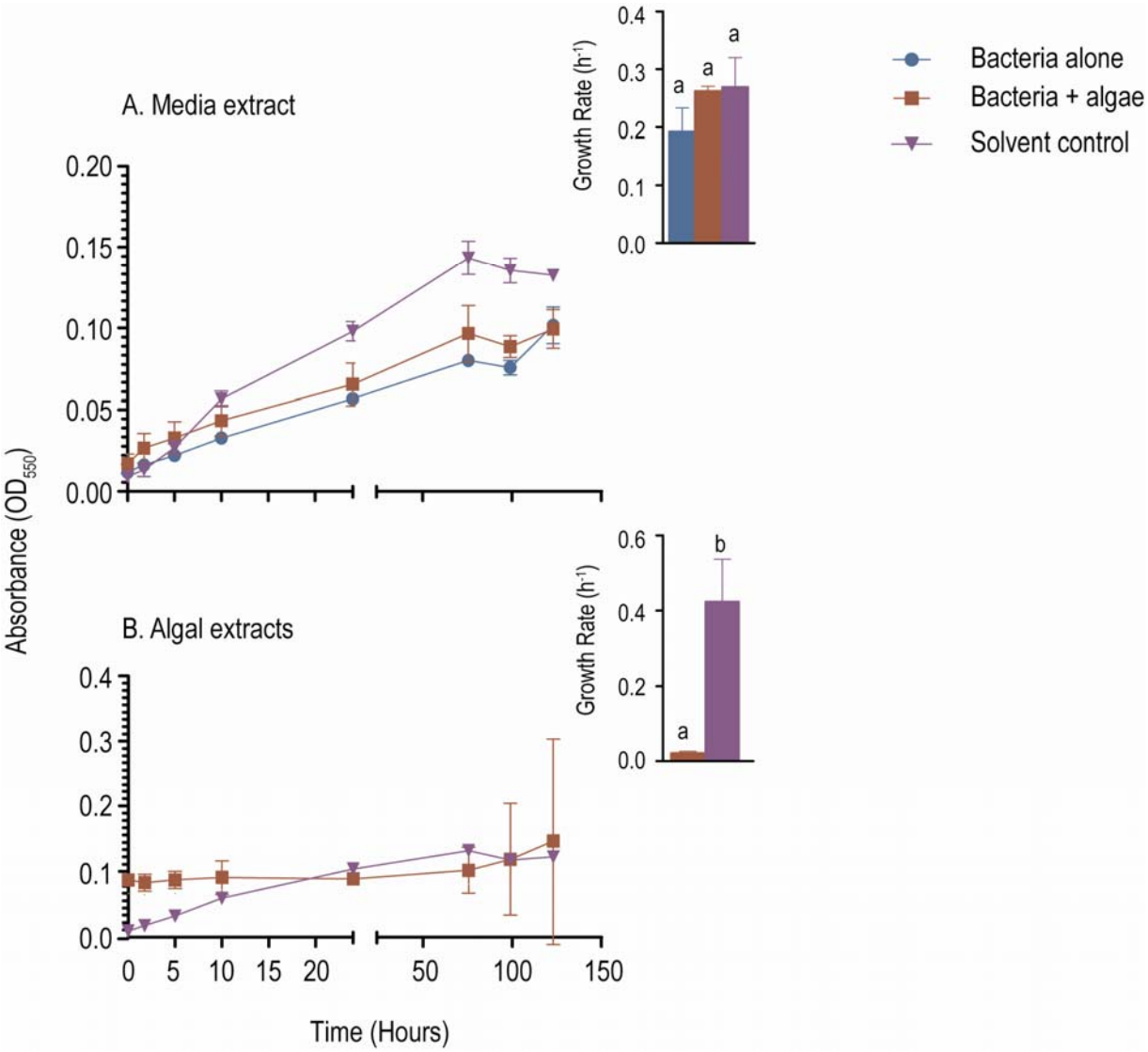

Figure 21: Growth curves and initial growth rates (insets) of *Cytophaga* sp. in response to extracts of the culture media (A) and of the algae used during the co-culture (B). Symbols = mean absorbance, bars = mean growth rate, error bars = standard error, n = 3. Different letters above the bars indicate significant differences between treatments ($p < 0.05$).

The abundance of *Cytophaga* sp. exposed to media extracts of cultures with and without algae were significantly lower than solvent controls starting at 24 hours and continuing to the end of the experiment ($p < 0.001$ for all, **Figure 21A**). Whole cell extracts of algae previously exposed to *Cytophaga* sp. completely inhibited the growth of the bacteria in terms

of maximum growth rate ($p = 0.0069$, **Figure 21B**). However, there was no difference in the overall growth curves of the different treatments ($p = 0.7990$). As in the co-culture experiment, the major effect of *D. ocellata* on the growth of *Cytophaga* sp. was seen in the first 10 hours. Because no similar inhibition was seen in bacteria exposed to media extracts, it is likely that growth inhibition seen in co-cultures is caused by an organic compound found within the alga that is not released into the surrounding media. The compound may be found at the surface of the alga and requires direct contact for activity, however I did not test surface extracts. Further studies are necessary to characterize the active compound or compounds and to determine their location within the alga.

After determining that my experimental design was sufficient for examining the effects of *D. ocellata* on the growth of individual bacterial strains, I began a set of experiments to determine the effects of *D. ocellata* on bacterial strains that naturally occur in the environment of the alga.

3.2 Effects of D. ocellata *and its organic extracts on the growth of naturally co-occurring marine bacteria*

To test the influence of *Dictyosphaeria ocellata* on bacteria that are found in its natural environment, I isolated and cultured three planktonic and one surface-associated strain from the field site at Summerland Key. Planktonic strains (KSW1, KSW2, and KSW3) were isolated directly from seawater samples and the surface associated strain (S3) was isolated from the surface of a glass microscope slide that had been placed in the water at the field site. Sequencing and alignment of the16S rDNA indicated that all isolated bacteria belong to the genus *Pseudoalteromonas* (**Figure 22**). Briefly, 16S rDNA was amplified from liquid cultures of the bacterial strains using universal bacterial primers 27F and 1390R. The amplified DNA was sent to GATC Biotech for sequencing and the resulting sequences were compared to known bacterial sequences using the GenBank database. Since comparisons with the GenBank database indicated that all four strains belonged to the genus *Pseudoalteromonas*, their positions within this genus were determined by the alignment of their sequences to other strains within the genus based on the tree presented in Holmström and Kjelleberg (1999).

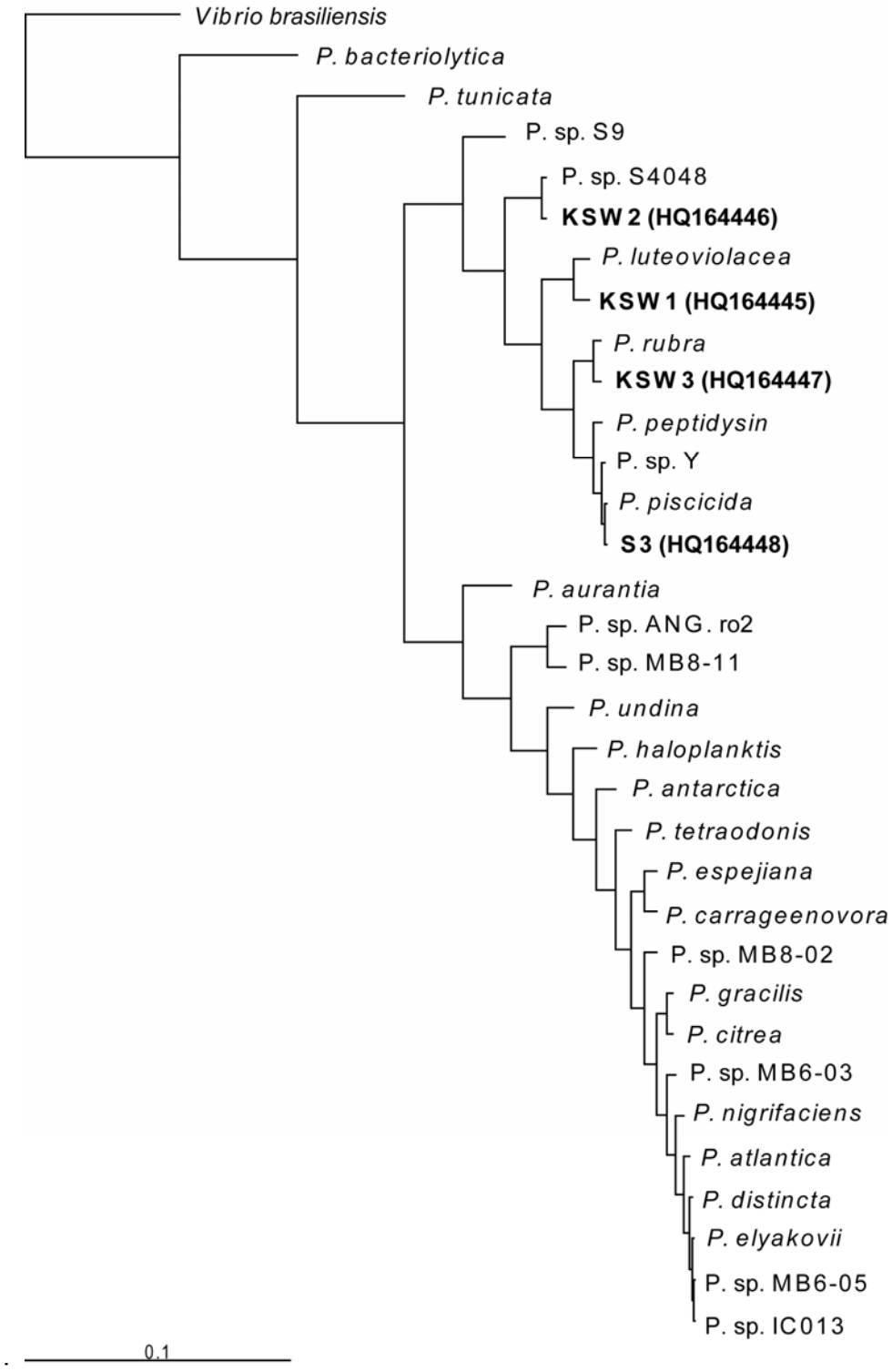

Figure 22: Phylogenetic affiliation of isolated bacterial strains within the genus *Pseudoalteromonas*. Tree is a modification of that seen in Holmström and Kjelleberg (1999). Isolated strains are shown in bold with GenBank accession numbers in parentheses.

The isolated bacteria were used to challenge *D. ocellata* in five-day co-culture experiments using maximum growth rate and the overall growth curve as measurements of growth because the previous experiment demonstrated the value of such detailed examination of bacterial responses to *D. ocellata*. The experimental set-up was the same as described in the previous section with the addition of a control containing algae, but no bacteria to examine the contribution of algal associated bacteria to the overall bacterial abundance in co-cultures. In KSW1 and KSW2 co-cultures, the algae alone control exhibited minimal bacterial growth, reaching a maximum average absorbance of 0.074 ± 0.012 OD_{550} and 0.058 ± 0.010 OD_{550}, 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. Despite the fact that high cell counts were reached in the co-culture experiments, none of the bacteria exhibited any obvious algicidal effects. *D. ocellata* exhibited its natural shape and texture after the co-culturing

Following co-culturing, the bacterial strains that were affected by the presence of *D. ocellata* were also tested against different parts of the co-culture system (media filtrates, media extracts, and algal extracts) to elucidate where the active properties lay. Briefly, co-culture media was sterile filtered to remove bacteria and divided in half. One half was reinoculated with the same bacterial culture, and the bacterial growth was monitored as before to determine if the algae released active properties into the surrounding medium or caused a change in the metabolites excreted by the bacteria. To investigate the activity of extractable waterborne compounds released by the algae, organic compounds were extracted from the other half using solid phase extraction followed by elution with methanol/ethyl acetate. Extracts were incorporated into FSW so that the concentration was volumetrically equal to the co-culture experiment and this medium was inoculated with the same bacterial culture from which the extracts were taken. Algae used in the co-cultures were freeze dried and extracted with methanol/dichloromethane, and algal extracts were investigated in the same manner as media extracts to determine if there were active compounds within the algae that were not

released into the media. Additionally, extracts of algae that had not been previously used in the co-culture experiments were also tested to determine if subjecting algae to the co-culture set-up altered their production of active metabolites. In all cases the extracts of non co-culture algae caused similar affects to those elicited by extracts of algae previously used in the co-culture experiments.

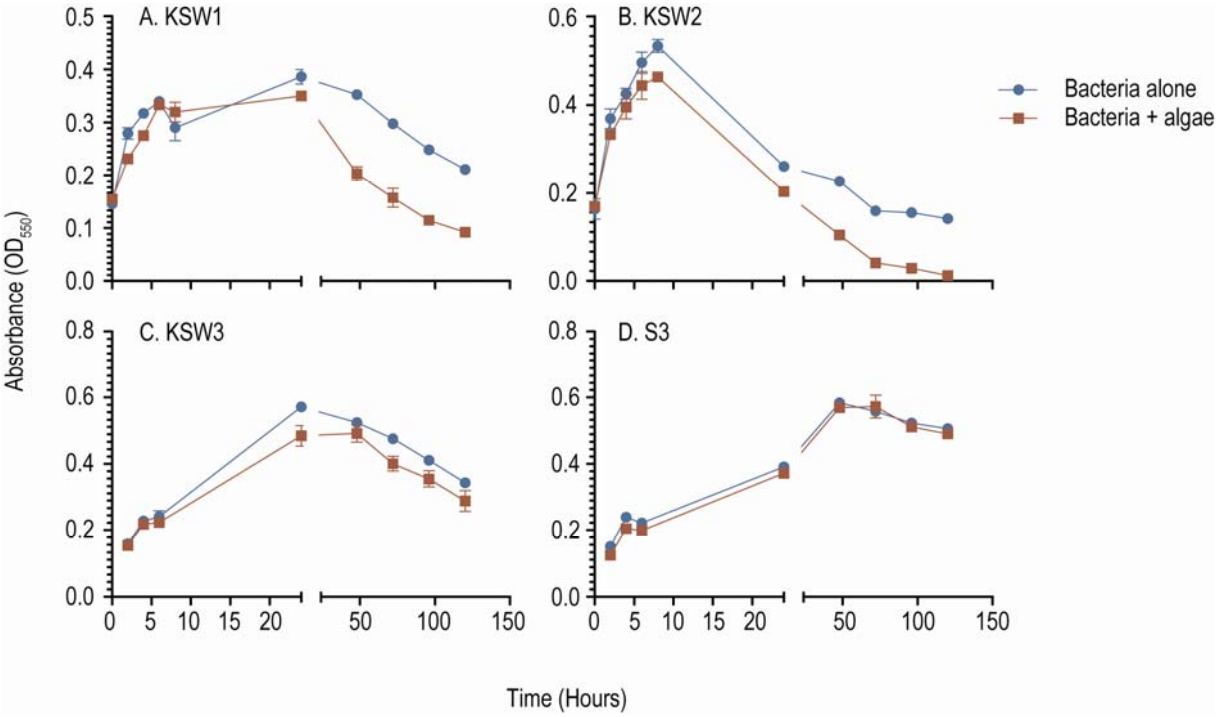

Figure 23: Growth curves of bacterial strains KSW1, KSW2, KSW3, and S3 in co-cultures with (red) and without (blue) *D. ocellata.* Symbols = mean absorbance, error bars = standard error, n = 5.

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* (**Figure 23**), while that of the one surface-associated isolate (S3) was not ($p = 0.6477$ and 0.6493 for overall growth and maximum growth rate of S3 respectively). S3 was therefore excluded from further analysis.

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 ($p = 0.0004$, **Figure 24**). A similar

inhibition was seen in KSW1 cultures exposed to algal extracts ($p < 0.0500$), but not media extracts ($p = 0.3252$), indicating that the alga is producing a growth inhibiting compound (or compounds) that is either not released into the water or is not stable (**Figure 25**). 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 prior to breaking down. Additionally, organic compounds have varying levels of affinity to solid phase extraction matrices and the compounds that affect the growth of KSW1 may not be extractable with the material used in this experiment. However, this lack of active waterborne compounds was also seen in the field enclosure experiments discussed above (Section 2.4). As discussed there, activity of volatile compounds that would be lost during the vacuum filtration process could not be excluded.

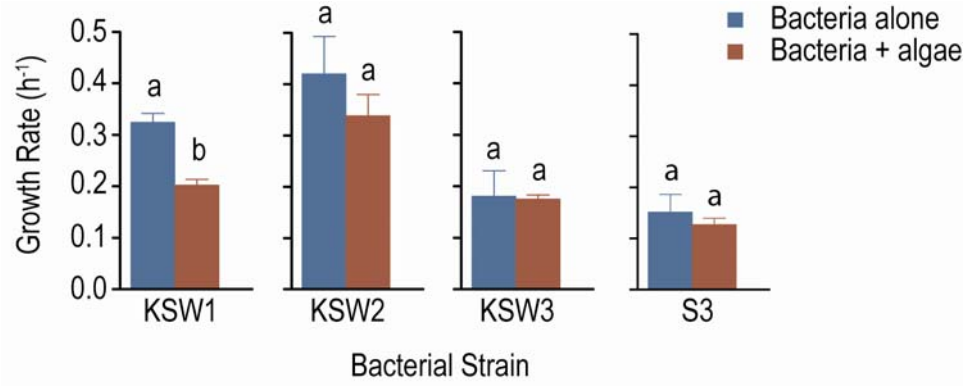

Figure 24: Maximum growth rates of bacterial strains KSW1, KSW2, KSW3, and S3 in co-cultures with (red) and without (blue) *D. ocellata*. Bars = mean growth rate, error bars = standard error, n = 5. Different letters above the bars indicate a significant difference between the two treatments ($p < 0.05$).

Co-culture with *D. ocellata* also initiated an earlier and more rapid decline in KSW1 abundance compared to bacteria alone controls ($p < 0.0001$, **Figure 23A**). 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 2-way RM ANOVA ($p = 0.5321$, **Figure 25A**). These results suggest that the causative agents of this early decline are compounds that cannot be quantitatively extracted by the method used here.

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, **Figure 24**). 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 hours after inoculation similar to that seen in KSW1 ($p < 0.0001$, **Figure 23A 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 dramatically inhibited the growth of the bacteria throughout the growth curve ($p < 0.0001$, **Figure 26**). Interestingly, there was no effect of the algae alone or bacteria alone media filtrates on the growth curve of KSW2 ($p > 0.0500$ for both), indicating a production of compounds in response to the interaction between algae and bacteria (**Figure 26A**).

It appears that the alga or algal associated microflora is producing defensive compounds in response to the presence of the bacteria, however I cannot currently rule out the possibility that the bacteria are producing an autotoxic compound in response to the presence of the alga. Studies of brown algae (Laminariales) and red algae in the genus *Gracilaria* have shown that these algae respond to the degradation products of their own cell walls by producing reactive oxygen species (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 my 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 hours), indicating something other than ROS as a causative agent (**Figure 26A**). While several studies have shown the production of antibiotic compounds by macroalgae, none to my knowledge have demonstrated the production or activation of these compounds in response to the presence of individual bacterial species (Engel et al. 2006, Paul et al. 2006, Nylund et al. 2008)

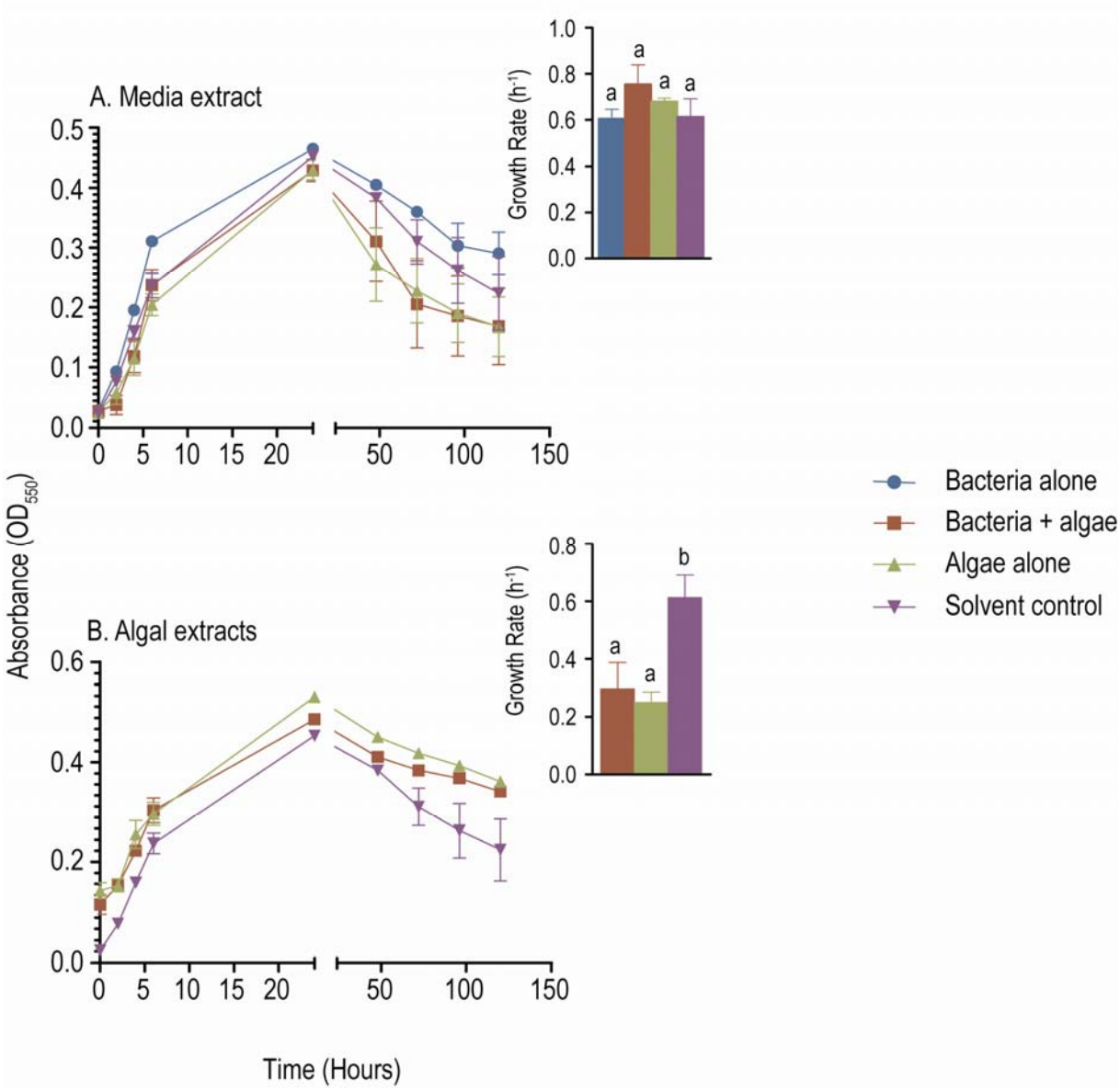

Figure 25: Growth curves and initial growth rates (inset) of bacterial strain KSW1 in response to extracts of the culture media (A) and of the algae used during the co-culture (B). Symbols = mean absorbance, bars = mean growth rate, error bars = standard error, n = 5. Different letters above the bars indicate significant differences between treatments ($p < 0.05$).

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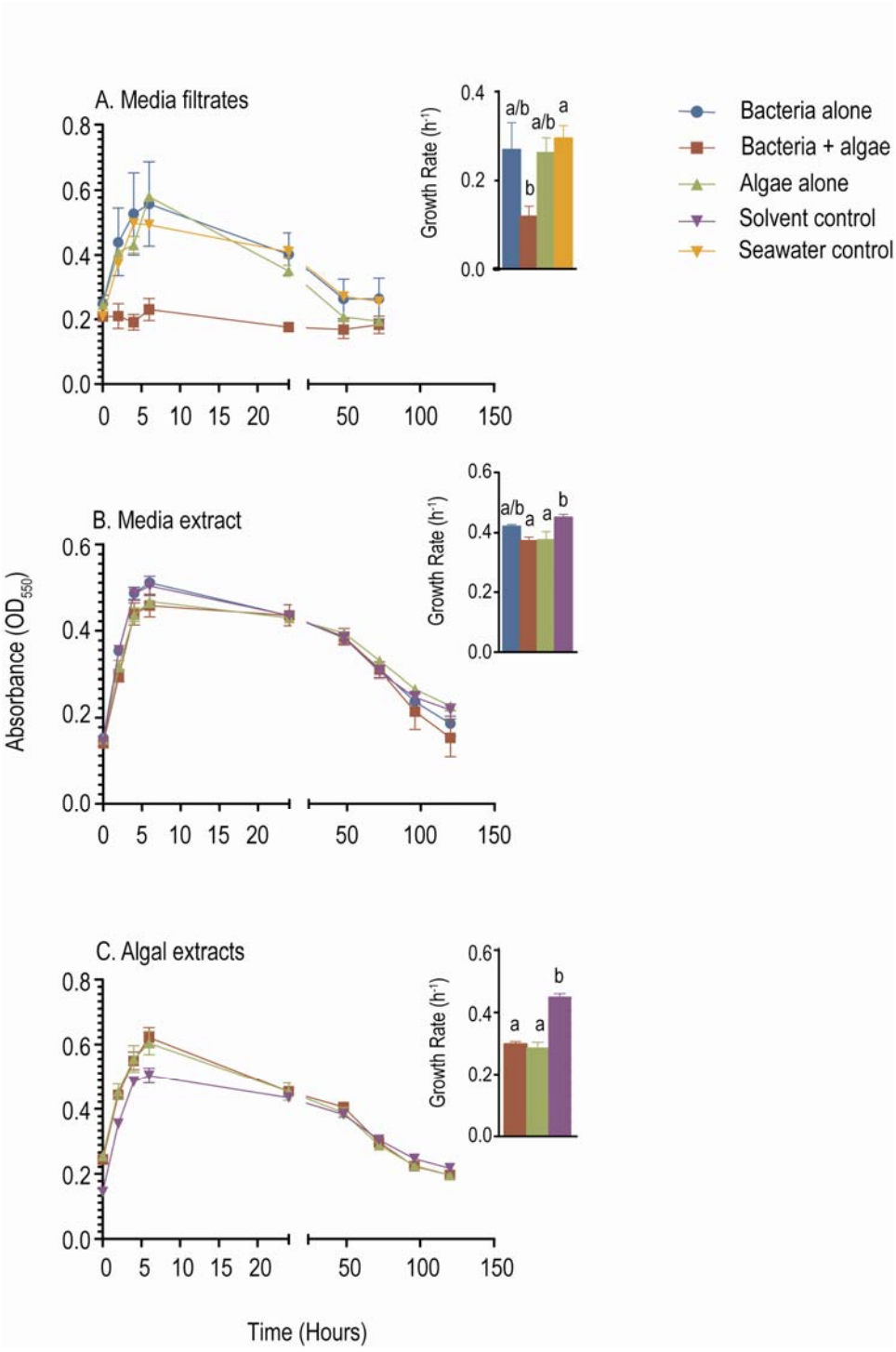

Figure 26: Growth curves and initial growth rates (inset) of bacterial strain KSW2 in response to sterile filtrates (A) and extracts of the culture media (B) and of the algae used during the co-culture (C). Symbols = mean absorbance, bars = mean growth rate, error bars = standard error, n = 5. Different letters above the bars indicate significant differences between treatments ($p < 0.05$).

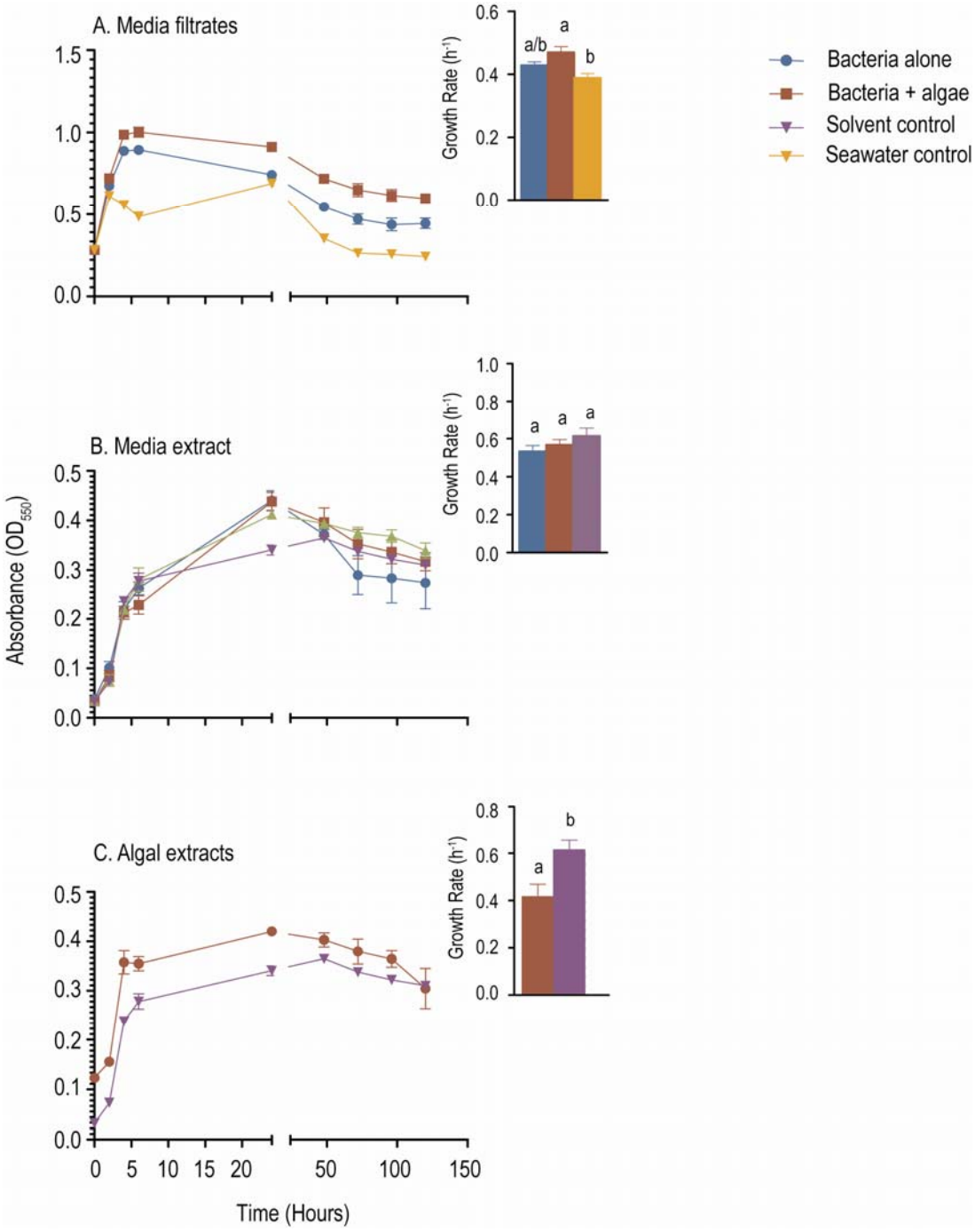

Figure 27: Growth curves and initial growth rates (inset) of bacterial strain KSW3 in response to sterile filtrates (A) and extracts of the culture media (B) and of the algae used during the co-culture (C). Symbols = mean absorbance, bars = mean growth rate, error bars = standard error, n = 5. Different letters above the bars indicate significant differences between treatments ($p < 0.05$).

Media extracts of both bacteria + algae and algae alone significantly inhibited the maximum growth rate of KSW2, but had no effect on overall growth curve ($p < 0.0500$ and $p = 0.1299$ respectively, **Figure 26B**). The algal extracts of all treatments also significantly inhibited the maximum growth rate of KSW2 ($p < 0.0500$, **Figure 26C**). While the growth of KSW2 was inhibited during the exponential phase by media extracts of algae containing co-cultures and all algal extracts, this inhibition was relatively short-term compared to the effects of the media filtrate on this bacterial strain (**Figure 26**). This indicates that, while some active compounds were extracted from both the media and the algae, 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 to solvent controls for the first 6 hours (**Figure 26C**). During this time the optical density was significantly higher in all algal extract treatments than the solvent control ($p < 0.0001$, **Figure 26C**). However, this increase in absorbance is present at the time of inoculation (time = 0) and is likely to be caused by colored compounds 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 starting values between treatments. A similar pattern was seen in the growth of KSW3 in response to algal extracts (**Figure 27C**).

As with KSW2, *D. ocellata* did not affect the growth of KSW3 in the exponential phase ($p = 0.9166$), however, it did affect the overall growth curve causing a lower abundance at 24 and 72 hours post inoculation ($p = 0.0464$, **Figure 23C and Figure** 24). 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 exponential phase as well as the overall growth curve (**Figure 27**). Contrary to KSW2, media filtrates of bacteria + algae cultures significantly increased the maximum growth rate of KSW3 ($p < 0.0500$, **Figure 27A**). Media filtrates from the bacteria + algae treatment also increased the overall abundance of KSW3 compared to the seawater control and compared to filtrate from bacteria alone starting two and four hours after inoculation respectively ($p < 0.0001$, **Figure 27A**).

Filtrates from the bacteria alone treatment also increased the abundance of KSW3 compared to the seawater control starting four hours after inoculation but to a lesser extent than the bacteria + algae filtrate ($p < 0.0001$, **Figure 27A**). While there was a significant effect of media extract on the overall growth of KSW3, comparison of individual treatments revealed a significant difference only at 24 hours post inoculation, at which time the bacterial abundance was higher in both treatments compared to the solvent control ($p = 0.0121$, **Figure 27B**). Neither the bacteria alone nor bacteria + algae media extracts affected the maximum growth rate of KSW3 ($p > 0.0500$, **Figure 27B**). Extracts of algae from the bacteria + algae and algae alone treatment inhibited the maximum growth rate of all KSW3 ($p < 0.0500$, **Figure 27C**), but the optical density of algal extract treatments was higher than solvent controls during the first 6-24 hours ($p < 0.0001$, **Figure 27C**). This increase in optical density is likely due to colored compounds in the algal extract as discussed with KSW2.

It is interesting that media filtrates of bacteria + algae promoted the growth of KSW3 in exponential phase while algal extracts inhibited it (**Figure 27**). It is likely that these opposing effects cancel each other out in the co-culture (**Figure 23C**). 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 *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.

These results demonstrate that individual bacterial strains respond differentially to the presence of *D. ocellata* and that different factors are responsible for eliciting these responses. Although studies of the differential effects of algal extracts on the growth of individual bacterial strains are rare, there are a couple of examples. For instance, similar to the results presented here, Ribalet et al. (2008) found that bacterial strains responded differentially to polyunsaturated aldehydes produced by diatoms. Additionally, Maximilien et al. (1998) determined that furanones extracted for the red alga *Delisea pulchra* elicited species-specific responses from individual bacterial strains. The differential effects of *D. ocellata* and its extracts on the growth of individual bacterial strains demonstrated here provide a possible

means by which the alga is able to selective regulate the composition of the bacterial community surrounding it.

While it is clear that individual bacterial strains are differentially affected by *D. ocellata,* and that these effects are brought about by different components of the co-culture system, the exact mechanisms involved remain unknown. The activity of organic extracts of the alga and the media indicate that secondary metabolites may be involved in these interactions, but other factors must also be considered. Further studies are necessary to fully characterize the mechanisms by which algae affect the surrounding bacterial community.

3.3 Bioassay guided fractionation of whole-cell D. ocellata extracts.

3.3.1 Crude extracts

The co-culture experiments discussed above indicate that there are compounds within *D. ocellata* that affect the growth of marine bacteria. In an attempt to characterize any active metabolites, a screening assay of the activity of algal extracts at different concentrations was performed against 13 bacterial strains that were isolated from the Florida Keys, including KSW2, KSW3, and S3. KSW1 was not tested here because the culture failed to grow. These experiments were performed in 96-well plates, which allowed me to include treatments and controls within one plate with a high number of replicates (n = 8-12). The use of 96-well plates also allowed for rapid absorbance measurements using a microplate reader, which enabled me to measure more variables and more replicates than could be done in larger volumes. Treatments were set up as described in the previous section, but at a volume of 200 µl. Extract concentrations were chosen to reflect the amount of algae used during the co-culture experiments (see Section 3.1). The 1x concentration was equivalent to the amount of extract obtained from 5 g wet weight of *D. ocellata* dissolved in a total of 150 ml media. Hereafter, I will refer to this as the co-culture concentration. A range of relative concentrations spanning three orders of magnitude were tested to determine the minimum active concentrations, as well as to accommodate for loss of compound during the extraction and handling processes. In addition, a treatment containing antibiotics (+) was added to each trial as a positive control. In all trials the bacterial abundance was significantly reduced in

antibiotic treatments (**Figure 28**). These bacterial cultures were compared in terms of their stationary phase abundance, which was measured as optical density 24 hours after inoculation. Although, as discussed in Section 3.1, a more detailed examination of the growth in terms of maximum growth rate and overall growth curve would be preferable, this was not possible here due to high fluctuations in abundance during the exponential phase. This fluctuation may be a result of the experimental set-up using 96-well plates. Further optimization is necessary to correct this problem. However, the stationary phase abundances were stable and we therefore used these to measure the effects of algal extracts on the growth of bacterial cultures. All absorbance values were transformed to give the relative percent increase or decrease compared to the solvent control.

Surprisingly I found that the algal extracts either promoted bacterial growth or had no effect. In no case did they inhibit the growth of bacteria as was seen during the co-culture experiments (see Section 3.2). This finding could be a result of the change in analysis. In this set of experiments I often saw erratic fluctuations in bacterial abundances during the exponential phase and therefore based my analysis on differences during the stationary phase. Previously, I measured growth as either maximum growth rate during the exponential phase or overall growth curve. However, absorbance was either greater than or equal to solvent controls in bacterial cultures treated with algal extracts after 24 hours in the previous experiments as well (**Figure 25B, Figure** 26**C, and Figure** 27**C**). I attributed this increased absorbance to the presence of colored compounds in the algal extracts that were not present in the solvent controls. The presence of colored compounds was adjusted for in the current experiments by subtracting the absorbance of blanks containing algal extracts from the absorbance of treatments containing algal extracts and bacteria. In this way I was confident that the absorbance value related to the density of the bacteria within the solution. I considered cases in which the bacterial strain exhibited a dose dependant response to the algal extracts as indicative of chemical cues and eliminated those that did not exhibit such a pattern from further analysis.

Of the 13 strains tested, eight were significantly affected by *D. ocellata* extracts in a dose dependant manner (**Figure 28**). Bacterial strain KSW3 exhibited a significant increase in abundance at half the co-culture concentration with an abundance that was 163 ± 7.19 %

higher than solvent controls ($p < 0.001$, **Figure 28A**). The abundance continued to increase with increasing extract concentration reaching a maximum of 232 ± 6.28 % at 5x co-culture concentration ($p < 0.0001$, **Figure 28A**). At 10x co-culture concentration the abundance was not significantly different from the solvent control. Algal extracts significantly promoted the growth of KSW4 at one tenth of the co-culture concentration with an increase of 10.5 ± 8.79 % over solvent controls ($p < 0.0001$, **Figure 28B**). This effect increased with extract concentration to a maximum of 129 ± 6.44 % at 5x co-culture concentration ($p < 0.0001$, **Figure 28B**). Although the 10x co-culture concentration treatment also significantly increased the abundance of KSW4, it did so to a lesser extent than the 5x treatment (69.6 ± 4.99 %, $p < 0.0001$, **Figure 28B**). KSW5, S3, and S4 all responded similarly to the algal extracts. They began to exhibit a response at one-tenth the co-culture concentration with increases in abundance of 48.4 ± 7.81 %, 47.4 ± 9.18 %, and 47.0 ± 5.23 %, respectively ($p < 0.0001$, **Figure 28C-E**). The response peaked at 5x co-culture concentration (142 ± 11.1 %, 161 ± 10.8 %, and 152 ± 4.36 %, respectively, $p < 0.0001$) and then disappeared at 10x co-culture concentration (**Figure 28C-E**). The decline or disappearance of any effect at the highest extract concentration for KSW3, KSW4, KSW5, S3, and S4 is puzzling. It could be that there are both growth promoting and growth inhibiting compounds in the extracts and that the effects of inhibitory compounds are masked at low concentration. The concentrations used in this experiment are conservative compared to other studies that have investigated the effects of algal extracts on bacteria. Most studies test extracts at a range of concentrations around that which is volumetrically equal to or greater than algal volume (Kubanek et al. 2003, Engel et al. 2006, Puglisi et al. 2007). In an attempt to determine the causes of activity in previous co-culture experiments that examined the effects of *D. ocellata* on bacterial growth in the surrounding media, I tested concentrations that would be relevant to those experiments. Due to potential loss of compounds during the extraction and experimental set-up, I may be working in a concentration range that is below the effective concentration of any inhibitory compounds.

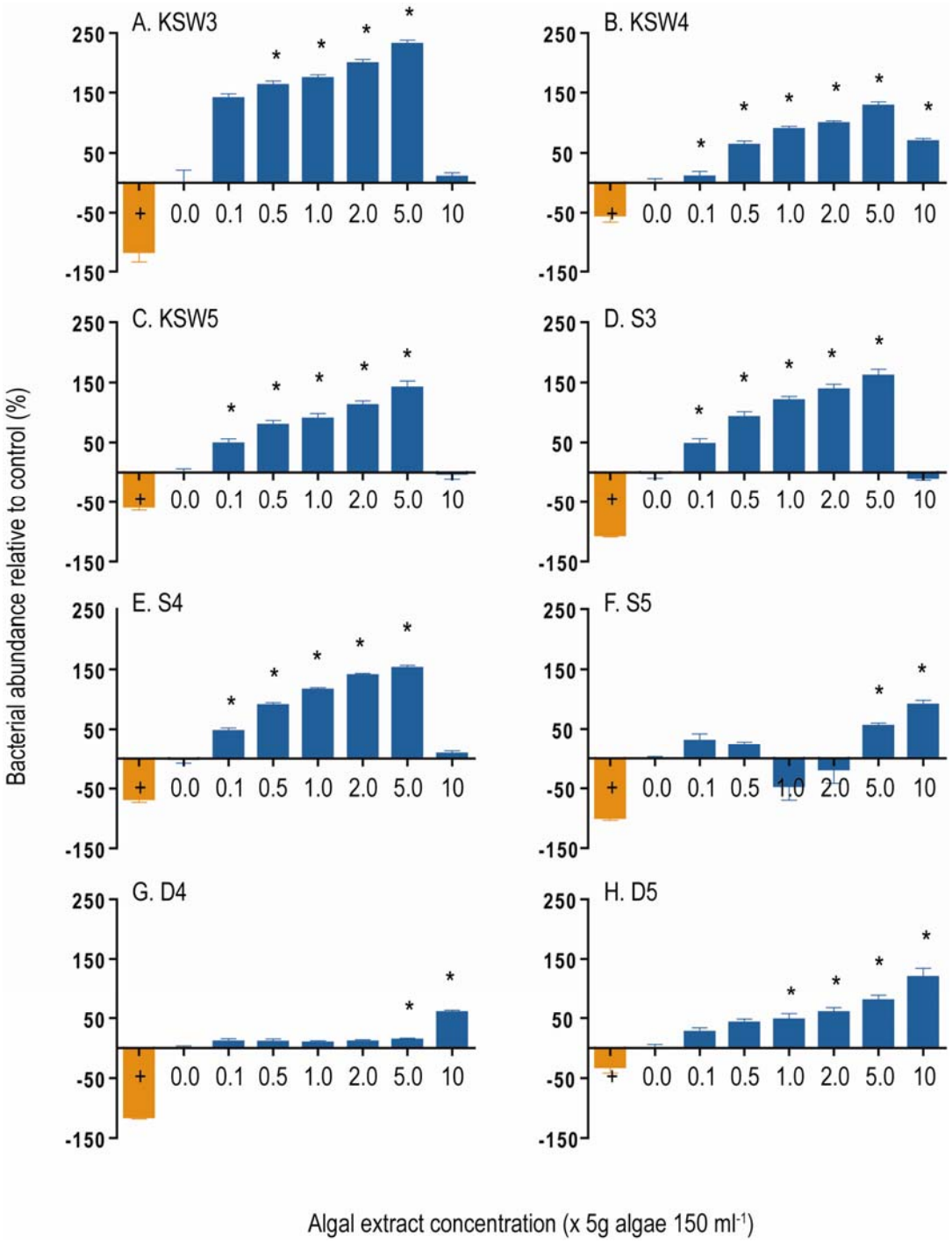

Figure 28: Percentage density relative to solvent controls 24 hours post inoculation of bacteria exposed to different concentrations of *D. ocellata* extracts. Bars indicate mean percentage ± standard error during stationary phase (n = 12). Asterisks indicate significant differences from the solvent control ($p < 0.05$). Orange bars represent the positive control and numbers along the x-axis represent the concentrations of extracts tested as a multiple of the co-culture concentration.

Bacterial strains S5, D4, and D5 were less sensitive to the algal extracts than other strains, requiring higher concentrations for the production of an effect and lacking any change in effect at the 10x co-culture concentration level. They were affected at minimum concentrations of one (D5) to five (S5 and D4) times co-culture concentrations (**Figure 28F-H**). At five times co-culture concentration S5 abundance increased by 55.6 ± 5.01 % ($p < 0.0001$) and D4 increased by 14.2 ± 2.60 % ($p < 0.0500$, **Figure 28F and G**). Both showed a maximum increase in abundance at 10x co-culture concentration (91.1 ± 7.36 % and 59.7 ± 3.57 %, respectively, **Figure 28F and G**). Growth of D5 was significantly promoted at 1x co-culture concentration (26.9 ± 7.04 %, $p < 0.05$, **Figure 28H**). Similar to S5 and D4, it also reached a maximum increase in abundance at 10x co-culture concentration (120 ± 14.9 %, $p < 0.0001$, **Figure 28H**). In all cases the antibiotics treatment reduced the stationary phase abundance of the bacteria relative to the solvent control (**Figure 28**).

3.3.2 Fractions

To further understand the nature of the compounds involved in the promotion of bacterial growth by algal extracts, I fractionated the crude extract between hexane, chloroform, ethyl acetate and water and tested each fraction for activity (**Figure 29**). I left the water fraction out of the initial analysis because it contained a high salt content and would have required further preparation. Because I found activity in the other solvent fractions, I did not further investigate the water fraction.

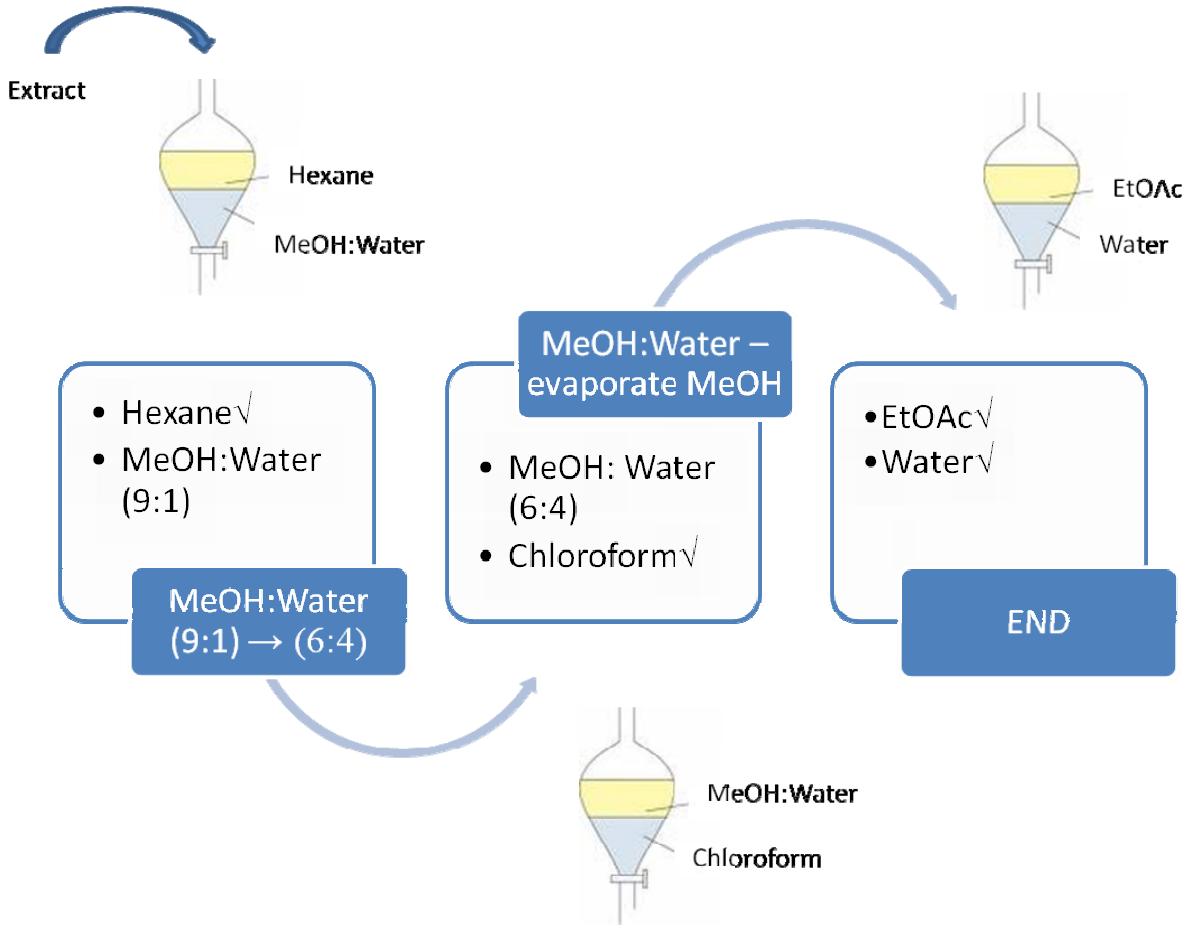

Figure 29: Scheme representing the liquid-liquid partitioning of algal extracts into hexane, chloroform, ethyl acetate, and water.

As with the crude extracts, I saw no inhibition of growth in any of the fractions, but saw promotion of growth in some cases. The chloroform and hexane fractions significantly promoted the growth of all the bacterial strains tested in response to at least one concentration, with the exception of S3, which did not respond to any of the fractions tested (**Figure 30D**).

The chloroform fraction caused a dose dependant increase in the bacterial abundance of strains KSW3 and D4, increasing from 20.4 ± 1.74 % to 46.1 ± 4.06 % and 13.1 ± 4.16 to 63.1 ± 4.25 %, respectively, although in both cases the increase was only statistically significant at 2x and 5x co-culture concentration ($p < 0.05$, **Figure 30A and G**). KSW4, KSW5, S4, and D5 had approximately the same level of response to the chloroform fraction regardless of extract concentration indicating that for these strains the compounds responsible

for the activity seen in the crude extracts are not present in the chloroform fraction (**Figure 30**). It should be noted that while I looked here for a concentration dependant response as an indication of the presence of active compounds, instances where the abundance was raised to the same level in response to all concentrations may indicated the presence of a growth promoting compound that is active at low concentrations but that the growth of the bacteria is limited by some other factor. Additionally, for all strains but S4, the recombination of all fractions at 2x co-culture concentration caused a higher increase in bacterial abundance than the chloroform fraction alone at the same concentration (**Figure 30**). Strain S4 was an exception to this pattern, exhibiting no significant response to the combined fractions ($p > 0.05$, **Figure 30E**). KSW3 abundance increased by 48.5 ± 3.98 % in response to the combined fractions as opposed to 31.7 ± 2.51 % from the chloroform fraction and D4 abundance by 41.5 ± 6.19 % versus 32.1 ± 3.43 %. The differences between the percent increase in abundance in response to 2x co-culture concentration of combined fraction and the chloroform fraction of KSW4, KSW5 and D5 were 64.8 ± 3.48 % vs. 34.7 ± 2.12 %, 127 ± 8.35 % vs. 103 ± 9.86 % and 46.6 ± 4.21 % vs. 33.4 ± 3.66 %, respectively.

Strains KSW3, KSW4, KSW5, S5, D4 and D5 responded to the hexane fraction in a dose dependant manner while S3 was not affected and S4 was affected only at 1x and 2x co-culture concentration (**Figure 30**). Strain KSW3 abundance was significantly higher than controls at 2x and 5x co-culture concentration ($p < 0.05$, 30.7 ± 4.87 % and 36.2± 2.66 %, respectively). KSW4, KSW5, S5, D4, and D5 all had significantly higher abundances than the solvent control starting at 1x co-culture concentration and increasing to 5x co-culture concentration. KSW4 increased by 35.1 ± 3.42 % to 53.8 ± 4.29 % (**Figure 30B**). The relative abundance of KSW5 ranged from a 101 ±6.88 to 146 ±9.85 % increase over solvent controls (**Figure 30C**). S5 abundance increased by 30.9 ± 3.08 % at 1x concentration up to 69.6 ± 25.8 % at 5x co-culture concentration (**Figure 30F**). D4 and D5 increased by 40.2 ± 6.89 % – 49.7 ± 6.75 % and 38.8 ± 4.57 % - 56.3 ± 4.51 %, respectively (**Figure 30G and H**).

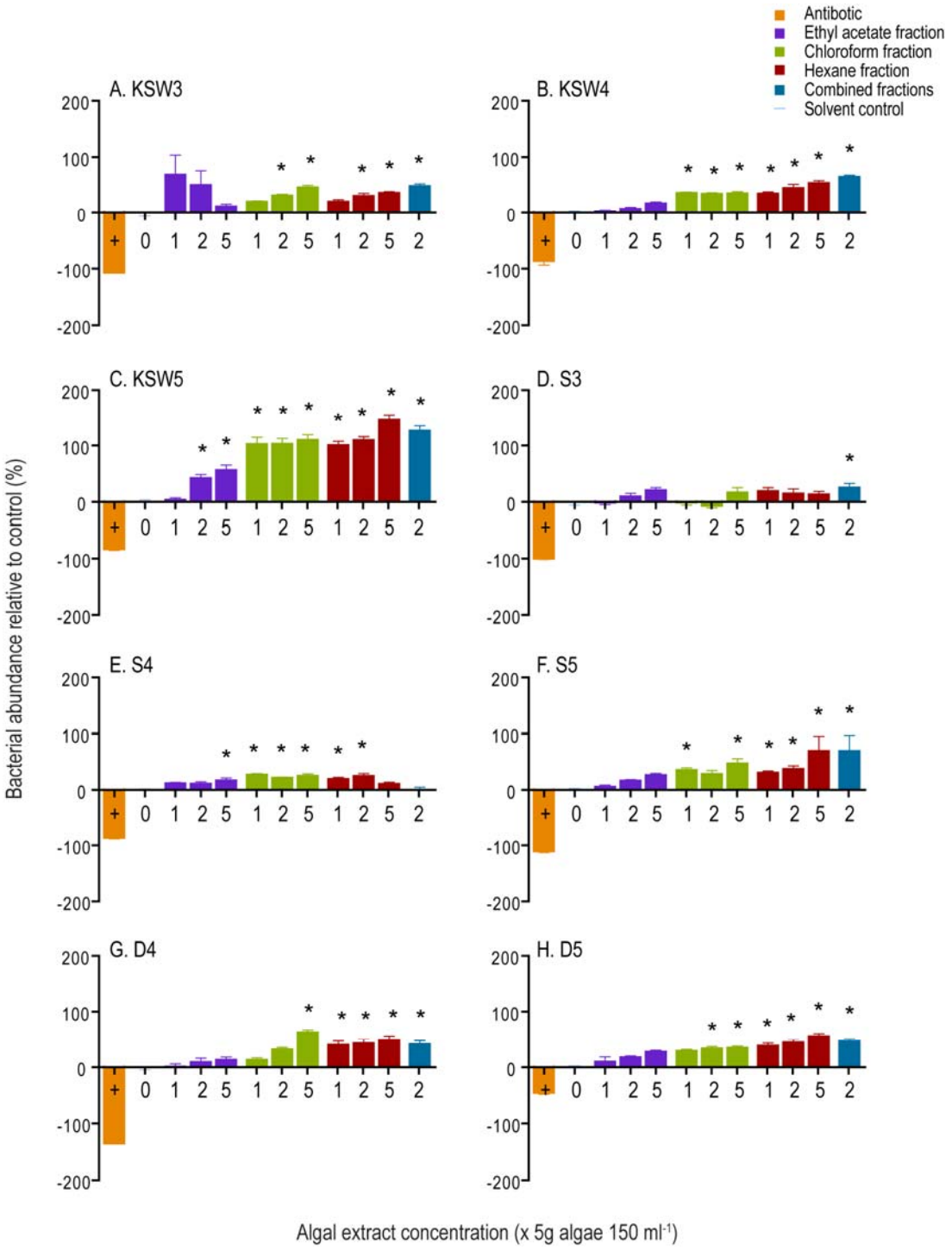

Figure 30: Percentage density relative to solvent controls 24 hours post inoculation of bacteria exposed to different fractions of *D. ocellata* extracts. Bars indicate mean percentage ± standard error during stationary phase (n = 8). Asterisks indicate significant differences from the solvent control ($p < 0.05$). Orange bars represent the positive control and numbers along the x-axis represent the concentrations of extracts tested as a multiple of the co-culture concentration.

As with the chloroform fraction, the response of strains KSW3, KSW4, KSW5 and S5 to the hexane fraction at 2x co-culture concentration was less than that induced by the combination of all three fractions at the same concentration **(Figure 30)**. For KSW3, the combined fractions caused a 48.5 ± 3.98 % increase in stationary phase abundance, but the hexane fraction caused only a 30.7 ± 2.66 % increase (**Figure 30A**). KSW4 abundance increased by 64.8 ± 3.48 % in response to combined fractions and 44.8 ±6.94 % in response to the hexane fraction (**Figure 30B**). KSW5 increased by 110 ± 6.43 % in response to the hexane fraction as opposed to 127 ± 8.35 % in response to the combined fractions (**Figure 30C**). And the increase in S5 abundance caused by combined fractions was 69.1 ± 2.8 % as opposed to 37.5 ± 5.28 % caused by the hexane fraction (**Figure 30F**).

The ethyl acetate fraction caused a significant increase in KSW5 abundance at 2x and 5x co-culture concentration (42.2 ± 6.29 % and 56.6 ± 8.79 %, respectively) and in S4 at 5x co-culture concentration (17.2 ± 4.44 %), but had no significant effect on any of the other bacterial strains ($p < 0.001$, **Figure 30**). Although the effect of the ethyl acetate extract on KSW5 was significant ($p < 0.001$), it was a weaker effect than that caused by the chloroform and hexane fractions and the combination of all three fractions (**Figure 30C**). It appears that there is a small amount of growth promoting compounds in the ethyl acetate fraction, but the majority of the activity is in the more non-polar hexane and chloroform fractions.

The presence of activity in both the hexane and chloroform fractions for strains KSW3, KSW4, KSW5, and S5 suggests that either more than one compound is active or that the compounds were not completely separated into one fraction or the other. The higher level of activity seen in the combination of fractions could be caused by an additive or synergistic effect of two or more compounds found within different fractions. It could also be the result of incomplete fractionation which would result in one compound being found in more than one fraction and the concentration of the compound, therefore increasing in the combined fractions.

However, for strains D4 and D5, the hexane fraction at 2x co-culture concentration had approximately the same impact on the bacterial abundance as the combined fractions (43.0 ± 7.20 % vs. 41.5 ± 6.19 % and 44.7 ± 5.07 % vs. 46.6 ± 4.21 %, respectively) (**Figure 30G**

and H). It therefore seems likely that the compounds responsible for the growth promotion of these two strains are found primarily in the hexane fraction.

S3 was not affected by any of the fractions tested individually, but was affected by the combination of the three fractions at 2x co-culture concentration (25.3 ± 7.43 %, **Figure 31D**). However, the response of S3 to the combined fractions was much lower than the response to the crude extract at the same concentration (139 ± 8.52 %, **Figure 28D**).

S4 was significantly affected by the hexane fraction, but only at 1x and 2x co-culture concentrations (19.6 ± 2.94 % and 24.9 ± 4.33 % increases, respectively, **Figure 31E**). These increases and the increases in response to the chloroform fraction (maximum increase of 27.4 ± 1.82 %) are relatively low in comparison to the increases seen in response to crude extracts at the same concentrations (141 ± 3.02 % and 152 ± 4.36 %, **Figure 28E**). Additionally, the combination of all three fractions had no effect on bacterial abundance ($p > 0.0500$, **Figure 31E**). These results indicate that compounds in the crude extract responsible for the growth promotion of bacterial strain S3 and S4 were not present in the fractions tested.

Overall, the results of the bioassay guided fractionation indicate that the metabolites responsible for the activity seen in crude extracts are somewhat nonpolar and can be extracted by both chloroform and hexane. Additionally, responses of individual bacterial strains to different fractions varied, suggesting that they may be responding to different compounds within *D. ocellata*. This further supports my previous findings that *D. ocellata* has species-specific effects on individual bacterial strains.

3.3.3 Sugars and fatty acids

It is known that heterotrophic bacteria feed on dissolved organic carbons such as sugars and lipids released from algae (Bell et al. 1974, Cole et al. 1982). However, the amount of sugar and lipids added to the medium from the algal extracts is minimal compared to the nutrients provided in the culture medium. To determine if the increase in bacterial growth seen here in response to algal extracts could be the result of increased nutrients, I added glucose and linolenic acid to bacterial cultures of strains KSW4 and KSW5 at various concentrations and monitored the growth. The concentrations of glucose and linolenic acid were set so that the mass added was approximately equal to the total amount of crude extract

in the treatments above. This amount is an over-estimation of the actual amount of glucose and linolenic acid present in the algal extracts and therefore is likely to produce a greater effect than that seen in the experiments above if these are the components responsible for the increased growth rate. However, it is possible that high concentration could result in an overdose of these compounds, although this is unlikely. I also included the hexane fraction tested above as a positive control.

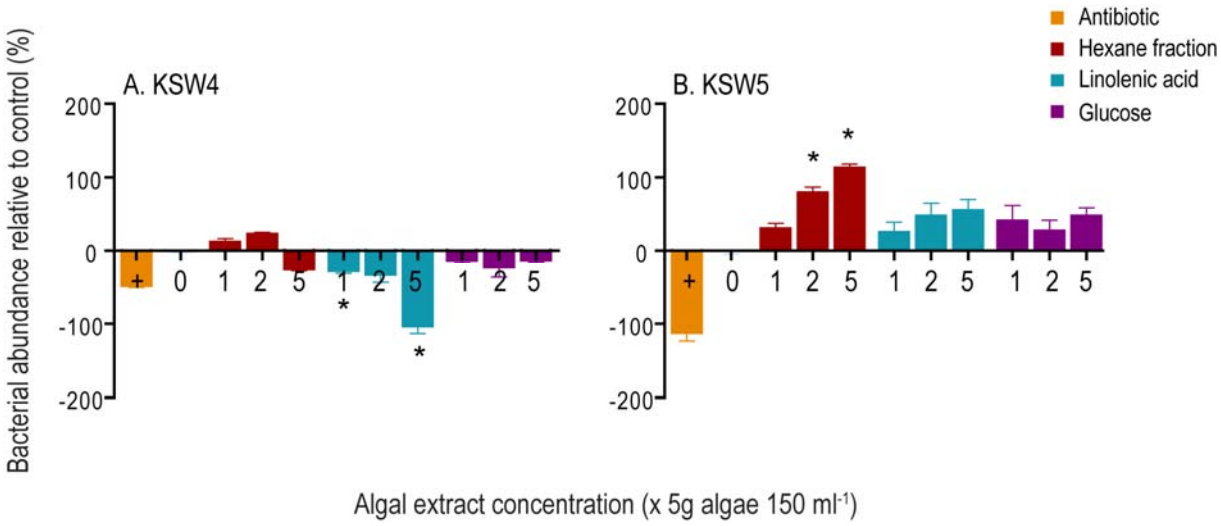

Figure 31: Percentage density relative to solvent controls 24 hours post inoculation of bacteria exposed to glucose, linolenic acid, and the hexane fraction of *D. ocellata* extract. Bars indicate mean percentage ± standard error during stationary phase (n = 8). Asterisks indicate significant differences from the solvent control ($p < 0.05$). Orange bars represent the positive control and numbers along the x-axis represent the concentrations of extracts tested as a multiple of the co-culture concentration.

Neither glucose nor linolenic acid had any effect on the growth of KSW5 at any of the concentrations tested ($p > 0.05$), but the hexane fraction retained its activity on KSW5, indicating that, for KSW5, the growth promoting activity of algal extracts is not likely due to the presence of excess sugars or fatty acids (**Figure 31A)**. Glucose also had no effect on the growth of KSW4, however linolenic acid significantly inhibited the growth at 1x and 5x co-culture concentration (-27.9 ± 3.15 % and -103 ± 9.96 %, respectively), but not at 2x co-culture concentration (**Figure 31B)**. In addition the growth promoting effect of the hexane fraction seen above was not replicated here for KSW4. This trial should be repeated to validate the results of KSW4, but at the very least I can say that glucose and linolenic acid did not promote the growth of KSW5.

Because most investigations of the effects of algal extracts on bacterial growth are designed with the aim of finding antibacterial compounds, examples of growth stimulating effects are rare. In general it is known that bacterial growth and production is enhanced by dissolved organic carbons (DOC) released from algae, but the growth promoting activity of algal extracts has been largely unexamined (Bell et al. 1974, Cole et al. 1982, Haas et al. 2010). However, the concentration of algal extracts added in the experiments presented here are low, and it seems unlikely that large increases in stationary phase abundance (nearly 250% in one case) could be attributed to the additional food source provided by DOC alone. In fact, KSW4 and KSW5 had no significant increase in abundance when glucose was added in concentrations that would represent the situation in which the entire algal extract was made up of only glucose. Recently, Ribalet et al. (2008) demonstrated that polyunsaturated aldehydes from marine diatoms have differential effects on marine bacteria. In a screening of 33 bacterial strains they found that the growth of some strains was inhibited by the compounds, some were stimulated and others were not affected at all (Ribalet et al. 2008). However, to my knowledge no studies have examined the growth promoting activity of marine macroalgae on individual bacterial strains.

Because the majority of the activity seen in this screening was found in the chloroform and hexane fractions, I subjected these fractions to proton nuclear magnetic resonance spectroscopy (H^1 NMR) and gas chromatography coupled with mass spectrometry (GC-MS) in an attempt to find any compounds that were likely to be responsible for the effects seen.

Extracts were derivitized using (trimethylsilyl)diazomethane and both derivatized and underivatized samples were run on the GC-MS. Derivatized samples contained a number of methyl esters of free fatty acids (FFA)(**Figure 32**). The underivitized samples did not contain fatty acid methyl esters (FAME), indicating that the fatty acids within the extracts are in the unmethylated form, but were methylated during the derivatization. Green algae are known to contain a number of FFAs, and all of the FFAs seen in *D. ocellata* extracts are known to occur in other green algal species (Khotimchenko et al. 2002). The hexane and chloroform fractions produced similar GC chromatograms, however, NMR data suggest that FFAs are the major component of the hexane fraction but are minor components of the chloroform fraction (**Appendix IV**).

Although these fractions contain a variety of FFAs, it is unlikely that the effects seen in these experiments are caused by these compounds. Most studies on the effects of FFAs on bacterial growth indicate that they are inhibitory as opposed to the growth stimulating effects seen here (Benkendorff et al. 2005, Desbois et al. 2009). Furthermore, linolenic acid, which was a dominant FFA within the algal extracts, did not increase the abundance of either KSW4 or KSW5 in the experiment described above (**Figure 31**). Continued investigation is necessary to characterize the active compounds found in *D. ocellata* extracts.

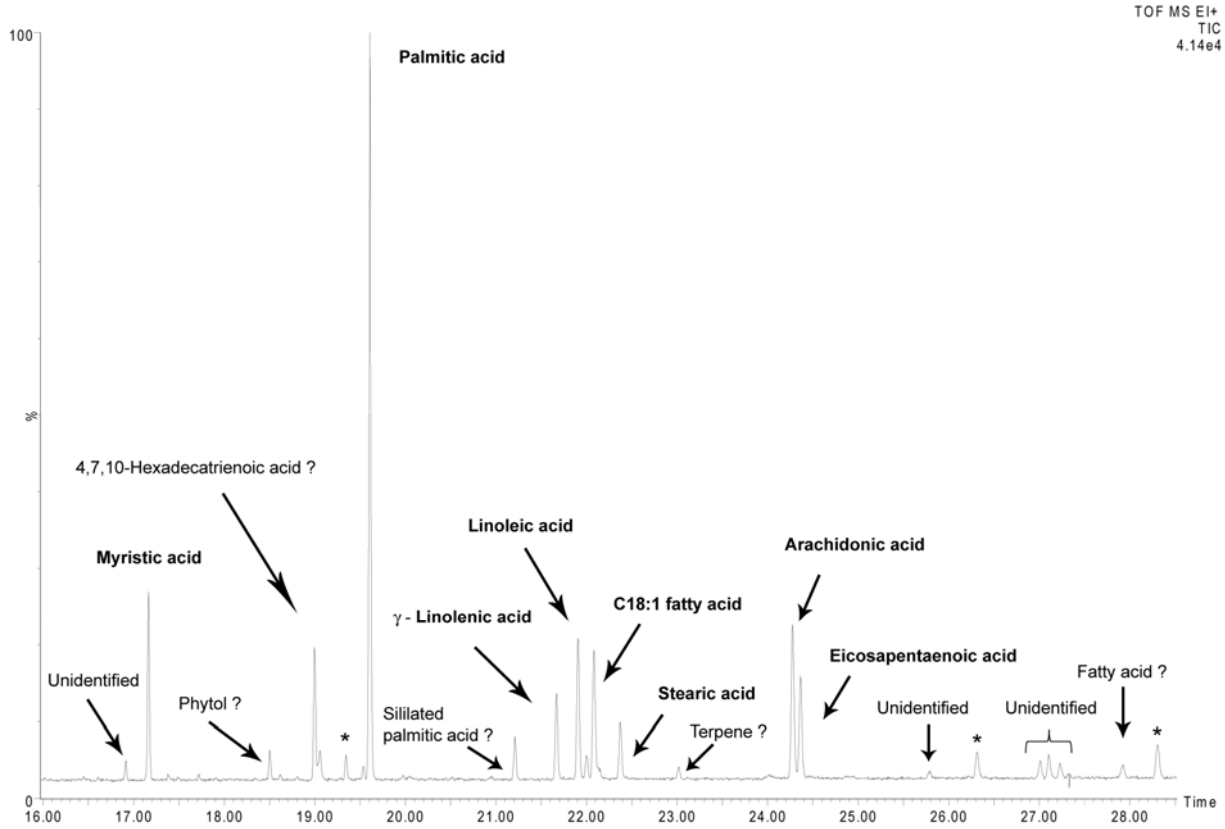

Figure 32: Gas chromatogram of the derivatized chloroform fraction of *D. ocellata* extracts. Fatty acid methyl ester (FAME) peaks identified by comparison to standards are in bold. All other identification is based on comparison to chromatogram libraries. Asterisks indicate peaks identified as contamination to the sample.

4 Conclusions

The purpose of my doctoral research was to examine if and how green macroalgae regulate the species composition and abundance of the bacterial community surrounding them. To accomplish this goal, I chose the siphonous green macroalga *Dictyosphaeria ocellata* as a model organism and performed a series of field and laboratory experiments monitoring the alga's effect on natural bacterial assemblages and individual bacterial isolates. Additionally, I examined the role of organic compounds in mediating the effects of the alga on the bacterial community.

Based on my results, I have concluded that *D. ocellata* is capable of regulating the bacterial community surrounding it through the production of a variety of organic compounds that differentially affect the growth of bacteria. *D. ocellata* harbors a unique bacterial community on its surface when compared to that of another green alga *Batophora oerstedii* and reference inanimate surfaces sampled from the same location. However, the community is not significantly different from another, more closely related, green alga *Cladophoropsis macromeres*. The degree of host specificity of bacterial assemblages on the surface of algae may be related to the genetic or morphological similarities of the hosts. Because *C. macromeres* is more closely related to *D. ocellata* than *B. oerstedii* is, it may harbor a more similar bacterial community. Nevertheless, there was an overall pattern of host specificity seen in the biofilm community of *D. ocellata* in comparison to other surfaces in the same area.

Although the biofilm bacterial community of *D. ocellata* is distinct from other surfaces within one location, the alga does not maintain the same bacterial community across different locations. Most of the previous studies indicating that bacterial communities are specific to their eukaryotic hosts were focused on endosymbionts or those that can be transferred along with the propagules of the host. Because I analyzed the surface biofilm community, the bacterial strains present are likely dependent on the available pool of bacteria within a location, and this varied across the locations studied. Therefore, the bacterial community on the surface of *D. ocellata* varied amongst locations, but within each location harbored a unique bacterial assemblage. Furthermore, methanol extracts of *D. ocellata* incorporated into

Phytagel™ plates altered the composition of the bacterial community and decreased the abundance of bacteria that settled and grew on the surface. This evidence suggests that the formation of the unique assemblage of bacteria on the surface of *D. ocellata* could be, at least in part, due to organic compounds found within the alga.

D. ocellata is also able to alter the composition of the bacterioplankton community surrounding it. However, this effect could not be attributed to waterborne compounds released from the alga, suggesting that the bacteria must be in direct contact with the alga in order to exhibit a response or that the active compounds are not stable under the conditions used here.

Furthermore, investigations of the effects of *D. ocellata* on the growth of individual bacterial strains in laboratory co-culture experiments revealed that bacterial strains respond differentially to the alga. Examination of the response of bacteria to the presence of the alga throughout their respective growth curves allowed me to distinguish that the alga caused changes in the maximum growth rate of some strains, but affected the stationary or declining phase in others. Additionally, bacterial strains responded differently to media filtrates and organic extracts of the media and alga. This difference in the responses of bacteria to the alga and its metabolites could have important implications when thought of in an ecological context in which competition between, and predation on bacterial species must also be considered.

Finally, crude extracts of *D. ocellata* caused an increase in stationary phase abundance in eight out of thirteen bacterial strains tested. The activity was narrowed to the chloroform and hexane fractions of the crude extract indicating that the active compound or compounds is not highly polar. GC-MS and H^1 NMR analysis of these fractions revealed a large diversity of free fatty acids; however, it is unlikely that these compounds would cause an increase in abundance at the low concentrations tested here. Furthermore, the increased abundance could not be triggered by the addition of glucose or linolenic acid to the bacterial cultures indicating that the mechanism responsible for this effect is something other than the simple addition of a food source for the bacteria.

The presence of a unique bacterial biofilm on the surface of *D. ocellata* in conjunction with the alteration of the bacterioplankton community by *D. ocellata* in field enclosure experiments indicates that this macroalga can selectively regulate the bacterial community surrounding it. This conclusion is further supported by the species-specific effects of *D. ocellata* on the growth of various bacterial isolates. This alga 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. In a screening of 33 marine bacterial strains, Ribalet et al. (2008) found similar differential responses of individual bacterial strains to polyunsaturated aldehydes that are known to be released from marine microalgae. Marine bacterial strains also responded to furanones extracted from the red alga *Delisea pulchra* in a species specific manner (Maximilien et al. 1998). These sorts of screening assays against ecologically relevant bacterial species are rare, but these examples in addition to the evidence provided here suggest that the differential response of various bacterial strains to marine algae may be common.

Although the mechanisms involved in mediating these interactions in our study remain unclear, it appears that different bacterial isolates are differentially sensitive to various components of the co-culture system. The activity of organic extracts tested in co-culture and Phytagel™ experiments indicates the possibility of active secondary metabolites, however, other physical properties must also be considered. While I focused here on determining the role of organic compounds, it is likely that a combination of factors, including the algal morphotype and mechanical properties of the algal surface act together to shape the bacterial community surrounding *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.

As discussed in the introduction, bacteria play important roles in the ecology of macroalgae and understanding the mechanisms involved in structuring an alga's associated bacterial community is essential to understanding its relationships within the larger community.

5 Materials and Methods

5.1 Field Experiments

5.1.1 Experimental Design

All field experiments were performed at a field site off the coast of Summerland Key, FL (N24°41.043' W081°26.654) unless otherwise specified. The site was a shallow subtidal area with low wave action that could be reached by wading from the shore. The area was bordered by red mangroves and had a rocky bottom covered in a thin layer of sediment.

5.1.1.1 Comparison of surface-associated bacterial communities within and between locations.

Algae and rocks were collected between February 27, 2009 and March 3, 2009 from three sites along the Florida Keys (Long Key: N24°49.518' W080°48.776', Bahia Honda: N24°37.43' W081°16.525, Summerland Key: N24°41.043' W081°26.654). At each site six clusters of *Dictyosphaeria ocellata* (M.A.Howe) J.L.Olsen-Stojkovich cells, six individual *Batophora oerstedii* J.Agardh, and six small rocks (diameter ~ 3 cm) were collected and transferred to the lab in a 5 gallon (18.9 L) bucket containing seawater from the collection site. At the Bahia Honda, site there were no *B. oerstedii* present and *Cladophoropsis macromeres* W.R.Taylor was collected in its place. Upon returning to the lab, the objects were rinsed twice with autoclave sterilized seawater, first dipped into the water then under a stream of water from a 20 ml sterile syringe. A ~ 1 cm^2 area of each sample was swabbed with a sterile swab which was immediately placed into 800 µl of lysis buffer (1% Triton X-100, 20 mM Tris HCl pH 8.2)(Dobretsov et al. 2006) in a sterile 2 ml centrifuge tube.

DNA was extracted from the cotton swabs and subjected to PCR amplification and DGGE separation (section 5.2). Four samples were chosen at random from each group of objects except for the comparison of bacterial communities of *D. ocellata* from different locations, from which three samples were chosen for each location. For each sample, 300 ng of DNA

was loaded onto the DGGE gel. Samples were only compared within one gel, not between different gels. Samples were analyzed according to the BDA method described below (section 5.2).

5.1.1.2 Effects of algal extracts on the surface-associated bacterial community

Surface Extracts

Dictyosphaeria ocellata was collected from the surfaces of rocks at the Summerland Key field site in February, 2008. Algae were weighed and then dipped in hexane for 30 s (211.60 g wet weight algae in 250 ml hexane)(de Nys et al. 1998). Surface area of the algae was determined by using a modified wetting method first described by Harrod and Hall (1962). Briefly, the algae blotted dry with a paper towel, weighed, dipped into a detergent/water solution, allowed to drip for 20 s, and then weighed again. The amount of weight gained was correlated to the surface area using a regression equation of weight gain to surface area of known objects. The inside bottom surfaces of 90 mm diameter sterile polystyrene Petri dishes (Roth) were coated with either 0.9 ml of algal surface extract or 0.9 ml of hexane, so that the amount of extract on the surface of the Petri dish was equivalent to that taken from the same surface area of algae. The solvents were allowed to evaporate and Petri dishes (30 extract and 30 control) were placed in the water at the same site from which the algae were collected. They were strung with clear monofilament line and hung from the prop roots of red mangroves (*Rhizophora mangle* L.) so that they hung parallel to the bottom and were constantly submerged approximately 2 cm above the bottom. They were weighted with 1.27 cm hex nuts to maintain their position. Six control and six extract dishes were brought into the lab each day for 5 days. Plates were swabbed with sterile cotton swabs and the swabs were frozen at -20 °C. DNA extraction, PCR amplification, DNA quantification, DGGE conditions, and gel imaging are described in Section 5.2. DGGE band pattern analysis was performed according to the GelCompar method described below (Section 5.2). Samples were compared between multiple DGGE gels using standard lanes for gel alignment. Each lane contained 300 ng DNA. Replicates within each treatment and time were distributed among the gels to reduce the probability of treatments grouping together because of a gel effect as opposed to true similarities in the bacterial communities. Reanalysis of amplified DNA from

48 hour samples was performed by running only these 12 samples on one DGGE gel and comparing the bacterial community profiles using the BDA method (Section 5.2).

Whole-cell Extracts

Phytagel™ assays were modified from the protocol described by Henrikson and Pawlick (1995). Algae were collected from the field site in February, 2008, rinsed with distilled water and 2.88 L (determined by volume displacement) was homogenized in a Waring commercial blender with an excess of hexane. The homogenized cell and hexane mixture was transferred into 50 ml Falcon™ tubes and centrifuged for 2 minutes at 3000 rpm in a IEC MultiRF centrifuge (ThermoIEC). The hexane layer was transferred to a glass beaker and the solvent allowed to evaporate off. The remaining cell parts and water were placed in a 2.8 L Erlenmeyer flask with 400 ml ethyl acetate and 200 ml methanol. This slurry was stirred for an hour at room temperature and then centrifuged as before. The ethyl acetate layer was transferred to a 500 ml round bottom flask and dried under vacuum.

The dried hexane and ethyl acetate extracts were dissolved in 16 ml of hexane and methanol, respectively. Extracts and solvent controls were incorporated into Phytagel™ plates to prepare four separate treatments: hexane extract, hexane control, ethyl acetate extract in methanol, and methanol control. For each treatment 31.2 g Phytagel™ was blended with 720 ml distilled water in a Waring commercial blender for 5 seconds. The mixture was transferred to a 1 L beaker and microwaved on high until boiling (4-5 min). The extract or solvent control (16 ml) was added to the liquid Phytagel™ and this mixture was immediately poured into twenty-four 90 mm diameter sterile polystyrene Petri dishes (Roth). The resulting solidified Phytagel™ plates were placed underwater at the field site as described above (Section 5.1.1.1). Six control and six treatment plates were brought into the lab every 3 days for 12 days and swabbed and analyzed as described above for surface extracts.

In October, 2009, *D. ocellata* were collected again from the field site on Summerland Key. The algae were placed in a cooler filled with seawater from the site and transported directly to the Smithsonian Marine Station in Ft. Pierce, FL. Here they were kept indoors in aquariums placed near a window to allow natural light to reach the algae until extraction.

Aquariums were filled with natural seawater, aerated with airstones, and covered with Plexiglass to reduce evaporation.

Two hundred forty ml of *D. ocellata* was freeze-dried (LABCONCO Freezone 6) and ground to a fine powder using a mortar and pestle. The dry powder was weighed and divided into two equal portions. One portion was extracted in ~ 250 ml methanol and the other in ~ 250 ml ethyl acetate overnight. The extracts were gravity filtered through Whatman no. 1 filter paper. Water was removed from the ethyl acetate extract with an excess of sodium sulfate, and solvents were removed from both extracts using rotary evaporation. The dried extracts were each dissolved in 15 ml dimethyl sulfoxide (DMSO). Phytagel™ plates were prepared by adding 5.2 g of Phytagel™ to 120 ml of distilled water and blending for 5 seconds in a blender. The mixture was then placed in the microwave until boiling. The entire extract (15 ml DMSO in the case of the solvent control) was added to the Phytagel™ solution just prior to pouring the solution into 55 mm sterile Petri dishes (n=6). This process was completed three times, once for each treatment: methanol extract, ethyl acetate extract, and solvent control.

The Petri dishes had been strung with monofilament line prior to the addition of Phytagel™ so that they could be hung with the exposed side of the Phytagel™ perpendicular to the water surface. Phytagel™ plates were placed in the field for 48 hours. Each plate was tied at the top to a *Rhizophora mangle* (red mangrove) prop root and weighted below using a 3/8" (9.5 mm) stainless steel hex nut. Plates were randomly distributed and tied so that only one plate was attached to each root. Each plate was approximately 4 cm from the bottom, constantly submerged.

Plates were sampled as above except that swabs were stored in 800 µl lysis buffer at room temperature as in section 5.1.1. Additionally, a 6 mm diameter core was removed for subsequent bacterial counts prior to swabbing the surface. Cores were taken from the center of each plate using a sterile Kimble borosilicate glass culture tube, placed in microcentrifuge tubes containing 1 ml of glutaraldehyde (2.5% in seawater), and kept at room temperature. Cores were stained with Sybr® Gold (25 x concentrate, Invitrogen™) for 15 minutes and then rinsed by dipping in sterile distilled water. Bacteria on the surfaces of cores were counted in 10 randomly chosen fields of view at 400x magnification using a Leica DM IL LED inverted

epifluorescence microscope with an excitation wavelength of 495 nm. The number of bacterial cells counted within one sample were combined and transformed to the number of cells per mm^2. Treatments were compared using a one-way analysis of variance (ANOVA) followed by multiple comparisons testing using the Holm-Sidak method (n = 5). Prior to analysis data were tested for the presence of outliers using Dixon's Q test (Dixon 1950). One sample of methanol extract treatment was determined to be an outlier and was removed from the data set. ANOVA was performed with SigmaPlot 11. Bacterial communities were analyzed using the BDA method (Section 5.2).

5.1.1.3 Field enclosure experiments

Field enclosure experiments were performed in February, 2008, February, 2009 and December, 2009.

February, 2008

Approximately 5 g of *D. ocellata* was added to ten 1 L Nalgene® PETG sterile square media bottles with septum closures and the bottles were filled with seawater from the field site. Ten control bottles were prepared with only seawater. Bottles were placed on their sides and held underwater at the field site with 5 cm mesh netting held down on the edges with rocks. One milliliter samples were taken every day for seven days using a sterile 2 ml syringe with a 21G needle. Samples were immediately filtered through a 5 µm PVDF 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 minutes prior to 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 sections were again cut into thirds using sterile scissors resulting in filter pieces that were 1/6 of the original filter. Filter pieces were placed individually into sterile 0.2 µl PCR tubes. Filter pieces were subjected directly to PCR amplification (see details in section 5.2). Bacterial community profiles were analyzed using the GelCompar method.

February, 2009

Approximately 100 g wet weight of *D. ocellata* was added to seven bottles and these plus seven control bottles were filled with seawater from the field site. Bottles were placed in the field as above and sampled after 0, 7, 24, 48, and 96 hours. Ten milliliter samples were taken using a 2.5 ml syringe with a 23G needle. Samples were prepared as above and bacterial communities were analyzed using the GelCompar method (Section 5.2).

December, 2009

Approximately 700 g wet weight of *D. ocellata* was collected from the surface of rocks at the field site and placed into a five gallon (18.9 L) bucket filled with water from the site. Within 30 minutes, ten 1 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 5 of the bottles to serve as the experimental treatments while the other 5 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 lab after 48 hours and 30 ml water samples were taken from each bottle using a sterile syringe under sterile conditions.

Two five gallon (18.9 L) buckets were half filled with water from the field site described above. Approximately 800 g wet weight of *D. ocellata* was collected at the field site and added to one bucket. The buckets were taken to Mote Tropical Research Laboratory, Summerland Key, FL, USA and 7 L of water from each bucket was transferred into clean 10-gallon (37.9 L) aquariums. Algae (700 g wet weight) were added to the aquarium containing the water from the bucket which originally contained the algae. Aquariums were aerated with airstones and kept under artificial light in a 12:12 light/dark cycle for 24 hours 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 (Millipore™) into autoclaved 500 ml or 1000 ml vacuum flasks using autoclaved Millipore™ glass filter holders. Algal-treated and non algal-treated filter-sterilized seawater was transferred to 1L

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 hours after which 30 ml samples were taken using a sterile syringe under sterile conditions.

Samples were prepared as in February, 2008, but were analyzed using the BDA method (Section 6.2).

5.1.2 Bacterial community profiling methodology

5.1.2.1 DNA Extraction of cotton swabs

DNA was extracted from swabs using a phenol:chloroform extraction protocol. Eighty-eight µl sucrose (67%), 1.6 µl EDTA (0.5 M), and 21 µl lysozyme were added to the swab/lysis buffer and shaken at 37 °C for 30 minutes. Then 83.8 µl TE and 50 µl SDS-TE were added and the mixture was shaken at 50 °C for one hour. The samples were then centrifuged for 10 min at 4 °C and 13,000 rpm (Hermile Z 383 K). The supernatant (~ 700 µl) was transferred to a clean sterile 2 ml centrifuge tube containing 70 µl NaCl (5 M) and 700 µl phenol: chloroform: isoamylalcohol (25:24:1). These tubes were mixed by inversion and centrifuged again as above. The supernatant was transferred to a clean sterile 2 ml centrifuge tube containing 700 µl isopropanol and kept overnight at -20 °C. Tubes were then centrifuged for 20 min at 4 °C and 13,000 rpm. The supernatant was removed and the remaining DNA pellet was allowed to air dry under sterile conditions overnight. The dry DNA pellet was dissolved in 30 µl filter-sterilized ChromoSolv water and kept at -20 °C until further use.

5.1.2.2 Polymerase chain reaction (PCR)

PCR amplification was performed using 16S rDNA bacterial primers 357fGC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG) and 907rM (CCG TCA ATT CMT TTG AGT TT) (Muyzer et al. 1995). The reagent volumes and concentrations are given in **Table 6**. For analysis of DNA extracted from cotton swabs, an additional 1 µl of DNA extract was added to the reagent solution. For analysis of

bacterial cells retained on 0.2 µm filters, the filters were placed into PCR tubes and subjected to direct filter PCR as described by Kirchman et al. (2001).

Amplification was performed using a Biometra® 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 to the GeneRuler™ Express DNA Ladder (Fermentas) run on a 1% agarose gel.

Table 6: Amounts and concentrations of reagents used in PCR amplification of bacterial DNA.

Amount	Reagent	Concentration	Supplier
1.5 µl	Bovine serum albumin (BSA) A7030	20 mg ml^{-1}	Sigma Aldrich
1 µl	dNTP mix	10 mM	Fermentas
5 µl	DreamTaq™ Buffer	10x	Fermentas
2 µl	Forward primer (357fGC)	10 µM	Biomers.net
2 µl	Reverse primer (907rM)	10 µM	Biomers.net
0.5 µl	DreamTaq™ DNA Polymerase	5 U $µl^{-1}$	Fermentas
to 50 µl	ChromoSolv® water (filter-sterilized)		Sigma Aldrich

5.1.2.3 DGGE

Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode™ Universal Mutation Detection System (Bio-Rad). Standards were prepared 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 standards were loaded at the sides and the

center of each gel to control for gel smiling and for comparison across gels when needed. Samples and standards were loaded onto an 8% acrylamide gel (acrylamide:bis-acrylamide ratio 37.5:1) with a denaturant gradient from 20 – 70% denaturant (100% denaturant contained 7 M urea and 40% formamide). Electrophoresis was run for 12 hours at 100 V and 60 °C. Gels were stained with Sybr® Gold (Invitrogen™). Imaging was performed using the BioDocAnalyze (BDA) digital system (Biometra®).

5.1.2.4 Statistical analysis

DGGE profiles were analyzed in one of two ways. For the GelCompar method multiple DGGE gels were aligned using the program GelCompar II from Applied Maths. After gels were aligned, banding patterns were compared and a similarity matrix formed based on the Pearson Correlation. Treatments were compared statistically with 2-way analysis of similarity (ANOSIM) followed by pairwise comparisons when appropriate. Data were also subjected to cluster analysis with unweighted pair-group average using arithmetic averages (UPGMA). ANOSIM and cluster analysis were performed using the Primer 6 statistical program. For the BDA method, band position and volume (area x 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 cluster analysis and non-metric multidimensional scaling (NMDS) using the Bray-Curtis measure of similarity for graphical representation. Statistical analyses were performed with the statistics program PAST v. 1.99 (Paleaontological Statistics)(Hammer et al. 2001).

5.2 Laboratory Experiments

5.2.1 Development of co-culture experiment

5.2.1.1 Algal and bacterial collection and culturing

Dictyosphaeria ocellata were collected in early February, 2008 from the field site at Summerland Key. The algae were attached to rocks in the intertidal zone and removed from

the rocks by hand. Samples were transported to Mote Tropical Research Laboratory field station where they were cleaned of mud and visible epiphytes and stored in flow-through tanks until transport to the laboratory in Jena, Germany. There, the algae were cultured in Instant Ocean® Sea Salt (30ppt) at 23°C with a 14:10 hr light: dark cycle until further use.

Three bacterial cultures, *Pseudoalteromonas tetraodonis*, *Micrococcus* sp., and *Cytophaga* sp., were obtained from the collection of Gunnar Gerdts and Antje Wichels (Wichels et al. 2004, Wichels et al. 2006). Cultures were transferred from plates into Difco™ Marine Broth 2216 and kept in continuous liquid culture at room temperature. Glycerol stocks of each strain were prepared and stored at -80 °C.

5.2.1.2 Co-culture set -up

The effect of *D. ocellata* on the growth of three species of marine bacteria was investigated by co-culturing the algae with the bacteria. The algae were not sterilized prior to use, therefore, any reference here to *D. ocellata* includes both the algae and its naturally associated microbial community. The bacteria *Cytophaga* sp., *Micrococcus* sp., and *Pseudoalteromonas tetraodonis* were cultured overnight in Difco™ Marine Broth 2216 so that they were in stationary phase prior to use. For each species, 15 ml of culture were added to 135 ml of autoclave-sterilized Instant Ocean® medium (salinity = 30 ppt) in a 200 ml Erlenmeyer flask. *D. ocellata* (~ 5 g) was added to the flask, and the growth of the bacteria was monitored every 2-3 hours for the first 9 hours then at 24 hours and once every 24 hours after that until the treatment exhibited an obvious decline in abundance. Flasks were kept at room temperature and constantly shaken. At each sampling time, 1 ml of media was transferred from the flask into a plastic cuvette using an Eppendorf® pipette under sterile conditions. The bacterial density was then measured spectrophotometrically as optical density (OD) at 660 nm (Carl Zeiss Jena Specord M42). The flasks were sealed with sterile cellucotton stoppers, kept at room temperature, and continuously shaken throughout the experiment. Bacterial growth in the presence of *D. ocellata* was compared to that in controls prepared without the addition of algae. Because the algae were not sterilized prior to the experiment, a second control was prepared with the addition of algae to 135 ml of autoclave-sterilized Instant Ocean® and 15 ml of uninoculated media to monitor the impact of algal-associated bacteria on the turbidity of the water.

After the end of the co-culture experiment, both the media and the algae from the co-culture experiment were extracted, and the effects of the extracts on bacterial growth were investigated. Media (~140 ml) were extracted using 3 ml solid phase extraction (SPE) cartridges (CHROMAbond® Easy, Macherey-Nagel). Media were vacuum pumped first through sand filters and then though the SPE cartridges. The cartridges were air dried by an additional five minutes under vacuum, rinsed with bidistilled water, and the organic compounds were eluted with 4 ml methanol/tetrahydrofuran (MeOH/THF) (1:1). Whole cell extracts of the algae employed in co-cultures (~ 5 g wet weight per sample) were prepared by grinding the fresh algae in liquid nitrogen and extracting the frozen powder with 4 ml of MeOH/THF (1:1). Solvents were removed from all extracts under a constant stream of nitrogen and dissolved in 100 µl of MeOH/THF (1:1). The 100 µl extracts were added to 27 ml of autoclave-sterilized Instant Ocean® medium (30 ppt) and inoculated with 3 ml of stationary phase bacterial culture in a 50 ml Falcon™ tube (BD Biosciences). The experimental volume was reduced from the 150 ml volumes used in the co-cultures to 30 ml in order to accommodate for possible loss of compounds during the extraction process. Solvent controls were prepared by adding 100 µl of MeOH/THF (1:1) to 27 ml of sterile Instant Ocean® medium (30 ppt) and were inoculated as above. The tubes were kept at room temperature and continuously shaken throughout the experiment.

5.2.1.3 Statistical analysis

Bacterial growth was examined in three ways. 1. Change in abundance 24 hours post inoculation, 2. Maximum growth rate, or 3. Overall growth curve. First, bacterial abundance 24 hours after inoculation was compared between treatments and controls using a two-tailed t-test. Second, maximum growth rate was designated as largest slope (µ) between two consecutive points on the growth curve.

$$\mu = (\ln OD_2 - \ln OD_1)/(t_2 - t_1)$$

Maximum growth rates of different treatments were compared using the Student's t-test when two treatments were compared and using one way analysis of variance (1-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 ± standard error. Similarities

of overall growth across time were examined using two-way repeated measures analysis of variance (2-way RM ANOVA). Prior to 2-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 and Keough 2002). Post-hoc comparisons were made using a Bonferroni posttest 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 (Systat® Software). For all tests, differences were considered significant when $p \leq 0.05$.

5.2.2 Co-culture experiment

5.2.2.1 Algal collection and culturing

Dictyosphaeria ocellata was collected in October, 2009 from the surface of rocks at the field site in Summerland Key. The algae were placed in a cooler filled with seawater from the site and transported directly to the Smithsonian Marine Station in Ft. 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.

5.2.2.2 Bacterial isolation and identification

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 Falcon™ tubes. The tubes were placed in a cooler with collected algae and transported to the lab (see above). Upon arrival, 100 µl of seawater was transferred to a Difco™ Marine Broth 2216 1% agar plate and spread using a sterile spreader. This process 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 and from the surface of *D. ocellata*. Three glass microscope slides were sterilized with 70% ethanol and then placed into the cooler with the algae (see above). Upon arrival in the lab, slides were transferred separately into sterile 50 ml BD Falcon™ tubes filled with seawater from the cooler. Within 24 hours, slides and algae were rinsed with 10 ml of autoclaved sterile seawater using a 10 ml sterile syringe to remove loosely associated bacteria and placed onto Difco™ Marine Broth 2216 1% agar plates. Each plate contained one slide or one cluster of algal cells. Plates were kept at room temperature until bacterial colonies could be seen growing (~ 24 hours).

Individual bacterial colonies were picked from the agar plates using a sterile inoculating loop and streaked onto new plates. This process was repeated until only one colony form was seen on a plate. Bacterial isolates were then transferred to 25 ml of Difco™ Marine Broth

2216 using a sterile inoculating loop and grown into dense cultures at room temperature with constant shaking. Liquid cultures were diluted by a factor of $1x10^4$ 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 Difco™ Marine Broth 2216 and allowed to grow for 48 hours. An aliquot of each bacterial culture (850 µl) 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 (see Section 5.2). 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 those of known *Pseudoalteromonas* spp. as in Holmström and Kjelleberg (1999) (**Figure 22**). Sequences of known *Pseudoalteromonas* spp. 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.

5.2.2.3 Experimental design

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 as in section 5.3, but with some changes to improve the setup. The bacterial isolates were cultured overnight in Difco™ Marine Broth 2216 so that they were in stationary phase prior to use. For each isolate, 15 ml of culture was added to 135 ml of filter-sterilized natural seawater in a sterile 200 ml Erlenmeyer flask. Flasks were sealed with

sterile stoppers made of cotton surrounded by cheese cloth. *D. 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 optical density at 550 nm (Shimadzu UV-Visible spectrophotometer) every 2 hours until the culture reached stationary phase then at 24 hour intervals after that for five days or until the treatment exhibited an obvious decline in bacterial abundance. Bacterial growth in the presence of *D. ocellata* was compared to 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.

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 ml or 1000 ml vacuum flask using a Millipore™ glass filter holder. Thirty-six ml of the sterile filtrate 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 minutes, 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 minutes under vacuum prior to 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 minutes. 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 if the co-culturing 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 minutes 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 (see above) 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 µl and 500 µl respectively). The fresh algal extract, not previously exposed 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 prior to inoculation with 3 ml of bacterial culture. Tubes were continually shaken at room temperature.

5.2.2.4 Statistical analysis

Bacterial cultures (abundance measured as OD) were compared in terms of maximum growth rate (exponential phase) and similarity of overall growth across time as described in Section 5.3.2.

5.2.3 Bioassay guided fractionation of D. ocellata *crude extracts*

5.2.3.1 Preparation of crude extract

D. ocellata (1195 g wet weight) was 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 methanol:dichloromethane (1:1) at a volumetric ratio of 2:1 solvent to algal powder overnight. The extracts were gravity filtered through Whatman® no. 1 filter paper. The solvent was removed from the filtrate using rotary evaporation. Dry extracts were stored at -20 °C.

Prior to use in the bioassay, the dry extract was dissolved in 20 ml methanol:ethyl acetate (1:1). One milliliter was transferred to a 4 ml vial and dried under a constant stream of nitrogen and then dissolved in 580.32 µl DMSO. This sample was designated as the 10x concentration and was diluted with DMSO to produce 5x, 2x, 1x, 0.5x, and 0.1x concentrations.

5.2.3.2 Bioassay set-up

Bioassays were performed in sterile 96-well cell culture plates (Cellstar®, Greiner Bio-one). Plates were sealed with Breathe-Easy™ gas permeable sealing film. Each plate was used for one bacterial strain. Thirteen bacterial strains isolated from the Florida Keys (see 5.4.2) were tested (KSW2, KSW3, KSW4, KSW5, KSW6, D1, D3, D3.2, D4, D5, S3, S4, and S5). Each of the eight rows contained one treatment and each treatment contained 12 replicates. In addition to the five concentrations listed above, there was a solvent control (0x) with only DMSO and a positive control (+) containing penicillin (10 mg/ml), gentamycin (2.5 mg/ml), and germanium dioxide (0.2 mg/ml). Each well contained 178 µl of sterile-filtered ASW, 2 µl of treatment (extract, solvent, or antibiotics), and 20 µl of stationary phase bacterial culture. An additional plate was prepared as a blank containing only ASW and the treatment, but no bacteria.

5.2.3.3 DMSO test

The effects of three concentrations (1%, 5%, and 10%) of DMSO on the growth of the same 13 bacterial strains were determined using the same set up as above (Section 5.5.2).

These treatments were compared to a control that contained no DMSO. Tests were performed in triplicate.

5.2.3.4 Bioassay analysis

Absorbance of bacterial cultures within 96-well plates was measured at 470 nm using a Mithras LB 940 microplate reader (Berthold Technologies). Absorbance was measured every hour for the first 8 hours and then at 24 hours post inoculation and every 24 hours after that. Absorbance values for the blank plate were averaged for each treatment and subtracted from the absorbance values of the respective treatments in plates containing bacteria. Significant effects of treatment on the absorbance 24 hours after inoculation were determined using one-way ANOVA or the Kruskal-Wallis test when data failed to meet the assumption of equal variance. These tests were followed by either Dunnett's or Dunn's Multiple Comparison Test, respectively. Differences were considered significant when $p < 0.05$. Statistical tests were performed using GraphPad Prism 5.

5.2.3.5 Fractionation

Crude extracts were divided into fractions using liquid-liquid partitioning according to the method described by Kupchan et al. (1975) (**Figure 29**). One milliliter of crude extract from above was dried under nitrogen and dissolved in 200 ml mixture of hexane:methanol:water (10:9:1). This mixture was shaken in a separatory funnel and the hexane fraction was removed. The remaining methanol:water mixture was adjusted to a ratio of 6:4, and 150 ml chloroform was added. This mixture was again shaken and the chloroform fraction was removed. The methanol was removed from the remaining methanol:water fraction using rotary evaporation. The remaining water and 75 ml of ethyl acetate were shaken in the separatory funnel and the two layers removed separately. Solvents were removed from the resulting four fractions (hexane, chloroform, ethyl acetate, and water) using rotary evaporation to produce a volume of approximately 2-4 ml. Each was divided equally into two 4 ml glass vials and the remaining solvents were removed under a constant stream of nitrogen. One vial was stored at -20 °C for later chemical analysis and the other was dissolved in 1 ml DMSO. The stock solutions were used to prepare 1x, 2x, and 5x concentrations as above (Section 5.5.1). The hexane, chloroform, and ethyl acetate fractions were tested against bacterial strains KSW3, KSW4, KSW5, S3, S4, S5, D4 and D5. Additionally, a combination

of the three fractions at 2x concentration, a solvent control (DMSO alone), and a positive control (antibiotic mix, see Section 5.5.2) were also tested. The bioassay set-up was the same as above with a few modifications. Each of the 12 columns contained one treatment, and each treatment contained 8 replicates. The bioassay was analyzed as above (Section 5.5.4).

5.2.3.6 Glucose and linolenic acid test

Solutions of glucose and linolenic acid were prepared in DMSO to a 10x concentrated solution (60 mg/ml). These were diluted in DMSO to 5x, 2x, and 1x concentrated solutions, which were tested against bacterial strains KSW4 and KSW5 as in Section 5.5.4.

5.2.3.7 Chemical analyses

GC-MS and ^{1}H NMR were performed on the hexane and chloroform fractions. Dried extracts were dissolved in deuterated chloroform and subjected to ^{1}H NMR using a Bunker 400 MHz Bruker Advance NMR spectrometer. Samples for GC-MS analysis were derivatized with (trimethylsilyl)diazomethane. Dried extracts were dissolved in ~ 200 µl methanol. Approximately 100 µl of (trimethylsilyl)diazomethane was added and the solution stood at room temperature for 1 hour. Solvents were removed from derivatized extracts under a constant stream of nitrogen and both derivatized and underivatized extracts were dissolved in hexane for GC-MS analysis.

Derivatized and underivatized samples were analyzed with GC-MS using an Agilent 6890N gas chromatograph (GC) with a DB-5ms column (Agilent) coupled to a Waters GCT Premier™ orthogonal time-of-flight (oTOF) mass spectrometer (MS). The GC column had an internal diameter of 0.25 mm and a film thickness of 0.25 µm. GC parameters used during these experiments are listed in **Table 7**. Mass spectra were produced using electron impact ionization (70 eV) in the positive mode. Samples were injected with an Agilent 7683B autosampler.

Table 7: GC parameters

Injection	10 µl		
Injector	280 °C		
Carrier gas	Helium		
Flow rate	1.0 ml/min		
Split ratio	1		
Oven program	Time (min)	Temp (°C)	Ramp rate (°C/min)
	0.0	60.0	Hold
	3.0	60.0 – 200.0	10.0
	17.0	200.0	Hold
	19.0	200.0 – 300.0	5.0
	39.0	300.0	End

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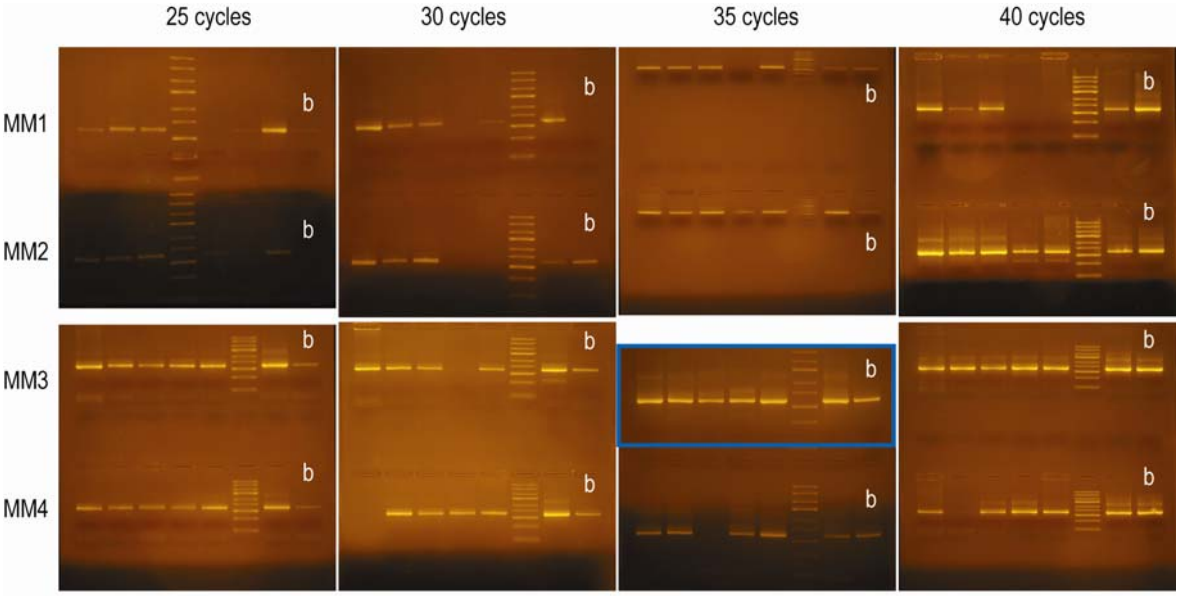
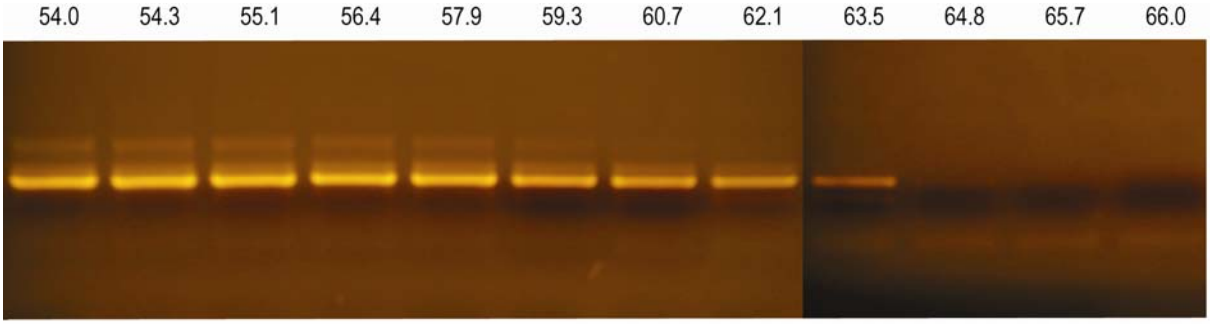
Appendices

Appendix I: Agarose gels of PCR amplified DNA fragments amplified with different reaction mixes and for different thermal cycling protocols. The number of PCR cycles is listed across the top, and the reaction mix used, down the left side. Unlabeled lanes contain amplified DNA from known bacteria, b indicates no bacteria added to the PCR mixture. Each treatment contains a GeneRuler Express DNA ladder. The protocol chosen for further use is outlined in blue. The reagents and concentrations used for different reaction mixtures are given in the table below.

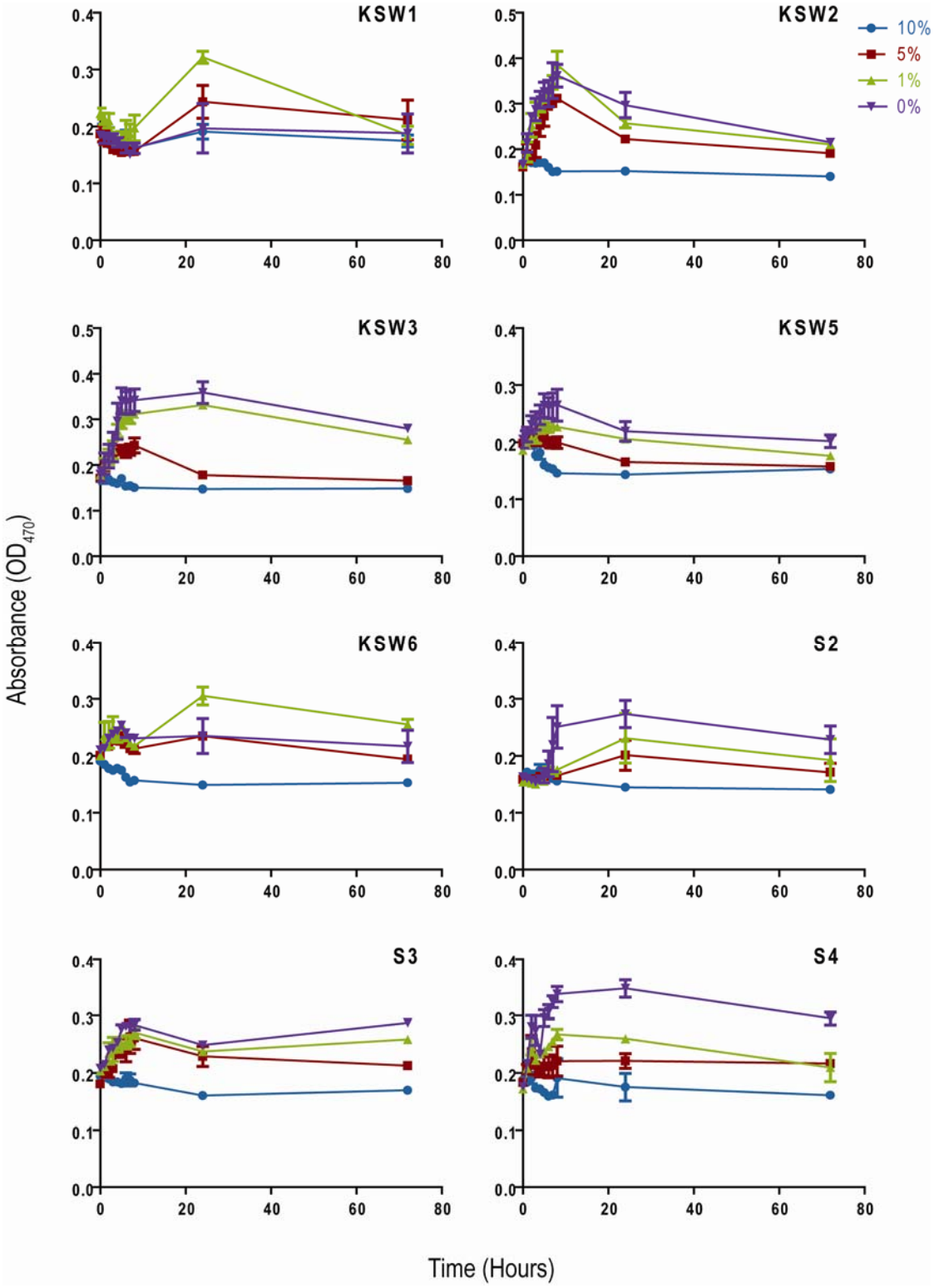


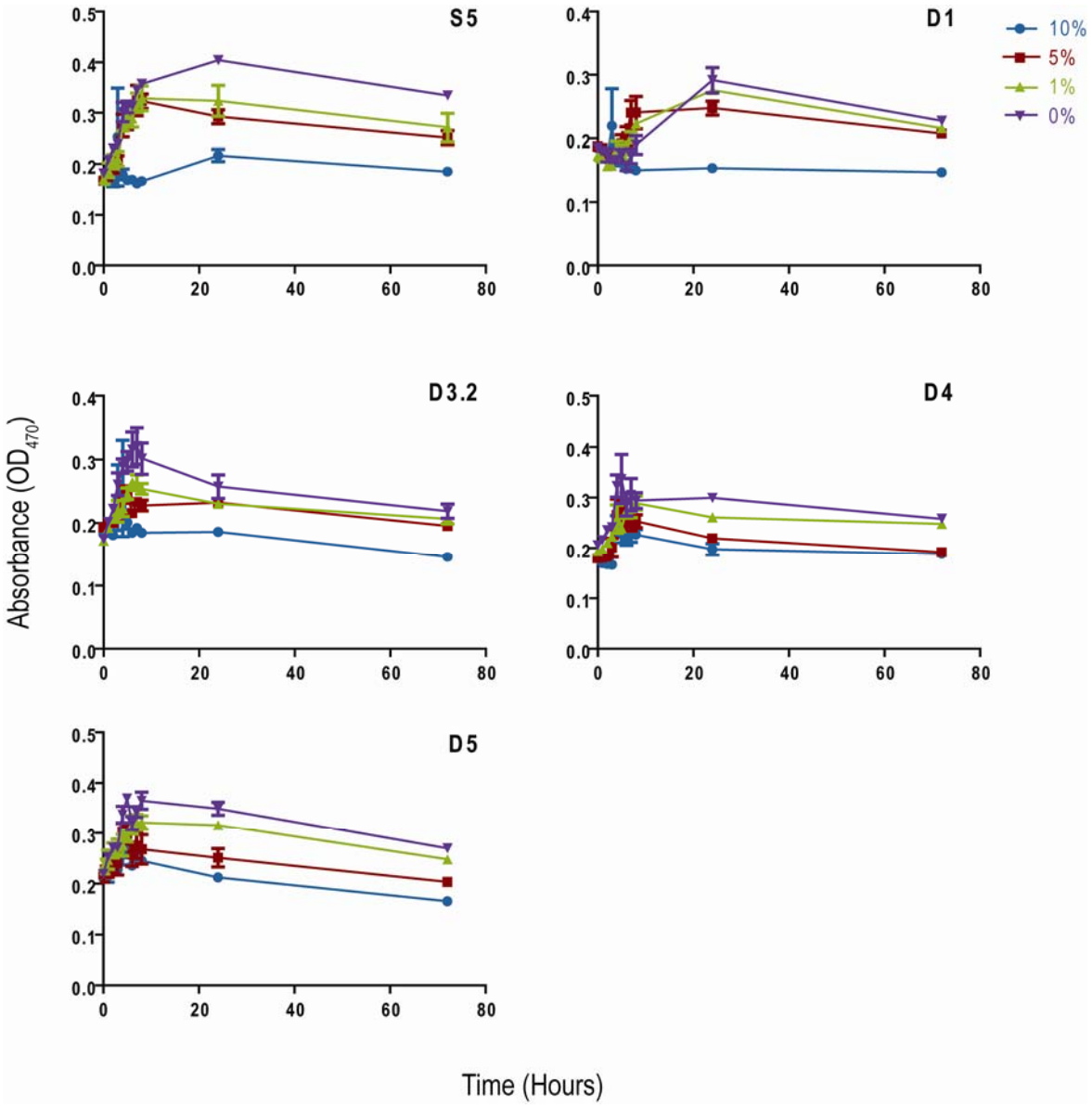
Master mix:	1	2	3	4
Buffer (10x)	5 µl	5 µl	5 µl	5 µl
dNTP mix (10mM)	1 µl	1 µl	1 µl	1 µl
357GC (10 µM)	2 µl	**1 µl**	2 µl	2 µl
907rM (10 µM)	2 µl	**1 µl**	2 µl	2 µl
BSA (20mg/ml)	1.5 µl	1.5 µl	1.5 µl	1.5 µl
DreamTaq (5 units/µl)	0.25 µl	0.25 µl	**0.5 µl**	0.25 µl
$MgCl_2$ (25 mM)	-	-	-	**5 µl**

Appendix II: Agarose gels of PCR amplified DNA fragments amplified using varying annealing temperatures (°C).

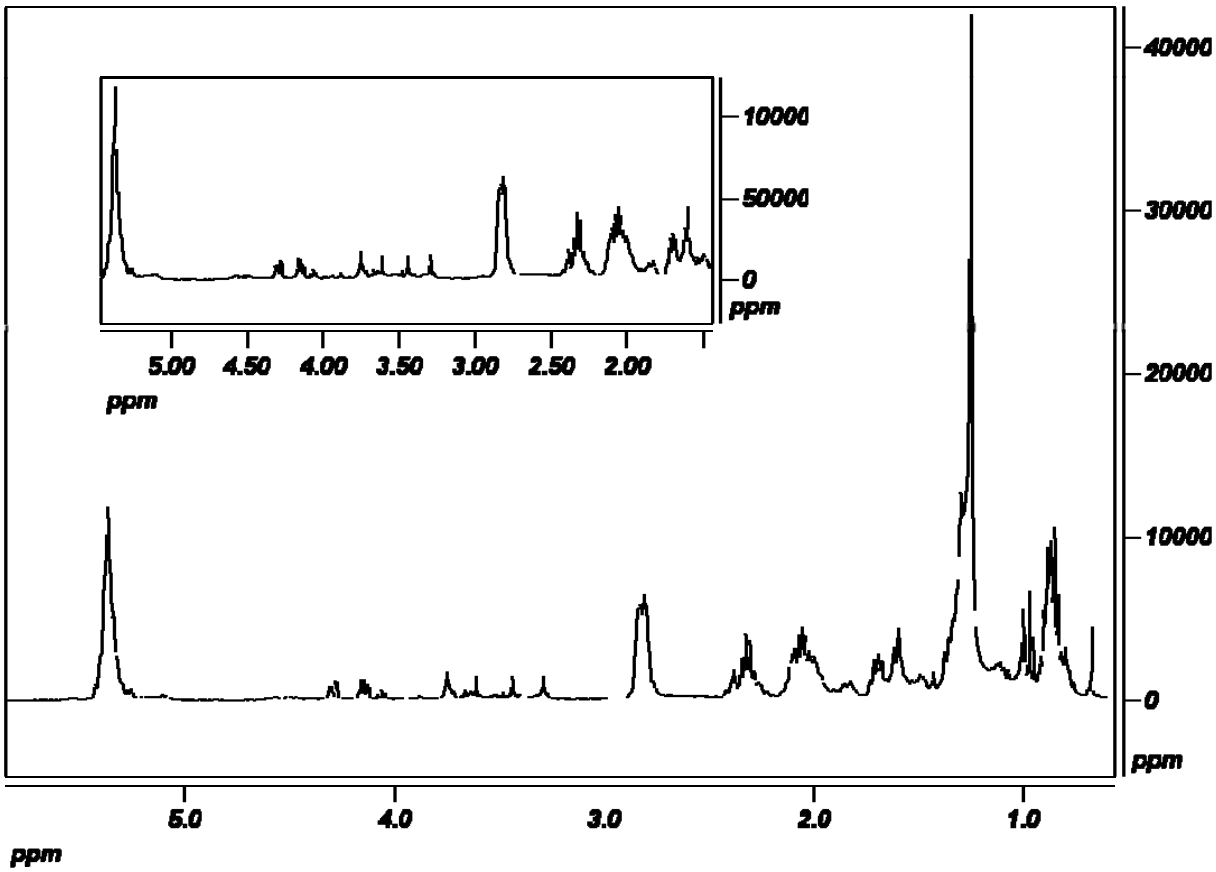


Appendix III: Effects of different concentrations of DMSO on the growth of marine bacterial strains. Values are mean ± standard error, n = 3.

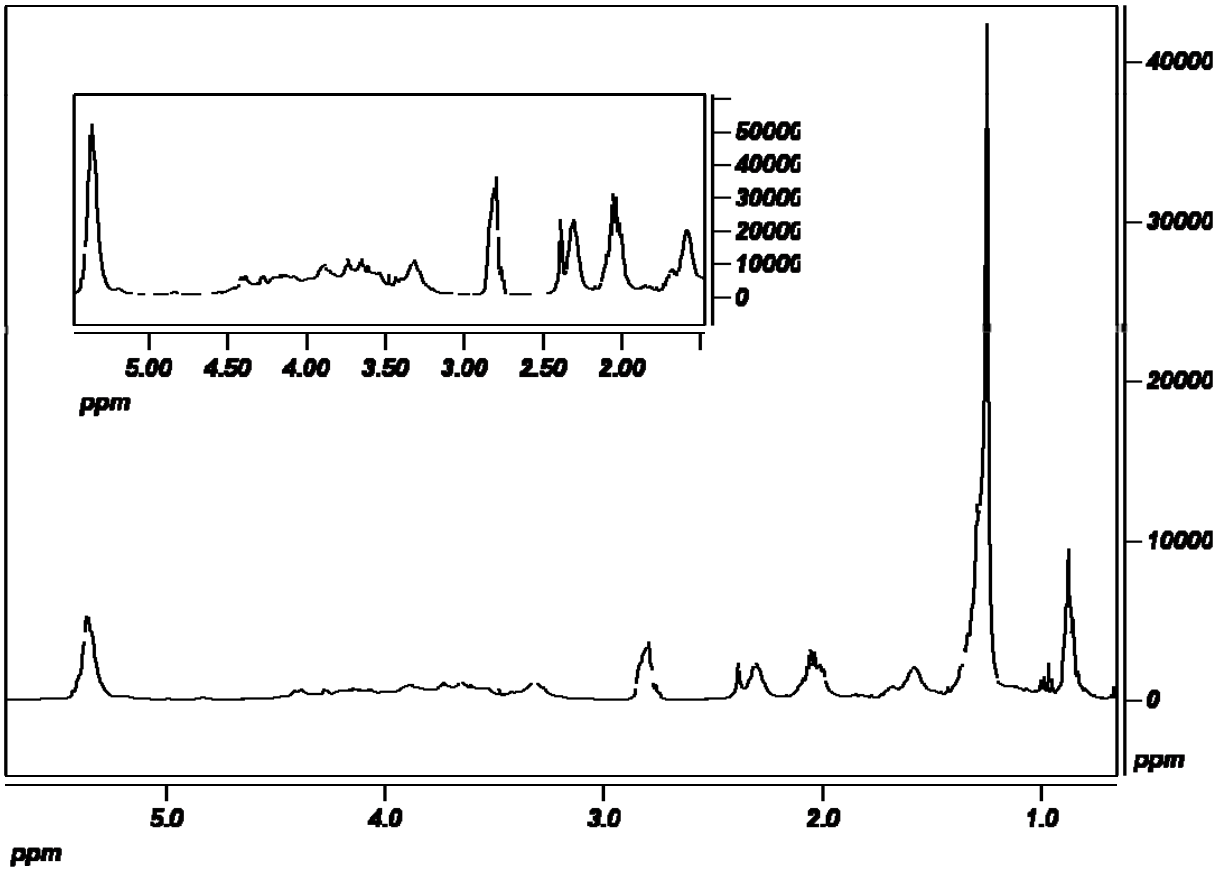



S5
D1
D3.2
D4
D5
10%
5%
1%
0%
Absorbance (OD470)
Time (Hours)

Appendix IV: H^1 NMR spectrum of the hexane fraction of *D. ocellata* crude extract



Appendix V: H^1 NMR spectrum of the chloroform fraction of *D. ocellata* crude extract.



Curriculum Vitae

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A. Professional Preparation

Friedrich Schiller University/International Leibniz Research School, Jena, Germany
Ph.D. in Chemistry, Area of Specialization: Chemical Ecology, *in progress*
Graduate Advisor: Georg Pohnert

University of South Florida, Tampa, Florida,
M.S. in Biology, Area of Specialization: Chemical Ecology, 2005
Graduate Advisor: John Romeo

Central Missouri State University, Warrensburg, Missouri,
B.S. in Biology with minors in Chemistry, Psychology and International Studies 2000

Katholiek Universiteit Brabant, Tilburg, The Netherlands, Study Abroad, 1999

Fatima High School, Westphalia, Missouri,
High School Diploma, 1996

B. Grants and Fellowships

- International Leibniz Research School, Graduate Student Fellowship (65,000€)
- Link Foundation Fellowship, Smithsonian Marine Station ($5,500)
- British Phycological Society Winter Meeting Student Travel Award (£350)
- University of South Florida Teaching Assistantship (80% tuition + $16,000/yr)
- Aylesworth Scholarship for the Advancement of Marine Science ($4,000)
- Old Salt Fishing Club Scholarship ($4,000)
- Fern Garden Club Scholarship ($500)
- Orange Blossom Garden Club Scholarship ($500)
- University of Central Missouri University Scholars Award (full tuition)
- Missouri Brightflight Scholarship ($8,000)
- University of Central Missouri Honors Award ($800)
- University of Central Missouri Biology Departmental Scholarship ($2,400)

C. Publications

Sneed JM, Pohnert G The green macroalga *Dictyosphaeria ocellata* influences the structure of the bacterioplankton community through differential effects on individual bacterial phylotypes **submitted to FEMS Microbiology Ecology**

Sneed JM, Pohnert G Investigating bacterial community diversity on the surface of green algae **in prep**

D. Presentations and Abstracts

Sneed JM, Pohnert G 2010 Effects of the green alga, *Dictyosphaeria ocellata* on the bacterial community (talk). European Student Conference on Microbial Communication: Jena, Germany ***session chair**

Sneed JM, Pohnert G 2010 Effects of the green alga, *Dictyosphaeria ocellata* on the bacterial community (talk). Benthic Ecology Meetings: Wilmington, NC. ***awarded best graduate student oral presentaiton**

Sneed JM, Pohnert G 2009 Growth inhibition of ecologically relevant bacterial species by the green alga *Dictyosphaeria ocellata* (poster). British Phycology Society Winter Meeting: London, England.

Sneed JM, Romeo J 2005 Effects of infection, salinity, and light on the production of phenolic compounds in *Thalassia testudinum* (poster). 21st Annual Meeting of the International Society of Chemical Ecology: Washington D.C.

E. Invited Seminars

- Smithsonian Marine Station Seminar Series – Ft. Pierce, FL (10/2009)
- International Leibniz Research School Symposium – Dornburg, Germany (9/2008)
- Bioorganic Analytics Seminar – Jena, Germany (4/2007)

F. Professional Service and Membership

- British Phycological Society (2009)
- International Society of Chemical Ecology (2005)
- Biology Graduate Student Organization, University of South Florida (8/2001 – 8/2005)
    - Instructor Search Committee Graduate Student Representative
    - Seminar Committee

Selbständigkeiterklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mittelungen und Quellen angefertigt habe.

Jena, den 05/11/2010 Jennifer M. Sneed