UNIVERSITY OF CALIFORNIA, SAN DIEGO

Bacterial Influence on the Bloom Dynamics of the Dinoflagellate *Lingulodinium polyedrum*

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Oceanography

by

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2007

The Dissertation of Xavier Mayali is approved, and it is acceptable in quality and form for publication on microfilm

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Co-chair

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2007

DEDICATION

To my family

EPIGRAPH

The process of finding the pathogen responsible for an outbreak was "…you put up a candidate and then try to tear it down. And, if you can't tear it down, it's probably bona fide. That's how we do science"

Dr. Ian Lipkin, quoted by *Elizabeth Kolbert*, The New Yorker Magazine, August 6, 2007

I think it is really important to think of all of what we do as hypothesis-driven, it's just that the hypothesis arrives at a different time.

Dr. Jo Handelsman

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ACKNOWLEDGEMENTS

I would like to profoundly thank my dissertation co-chairs, Peter J. S. Franks and Farooq Azam, for their invaluable assistance, for allowing me the freedom to follow my interests, and for teaching me how to be an independent scientist. Peter brought me to SIO and gave me "carte blanche" during my first two years, a tremendously generous act. Farooq allowed me to join his laboratory and provided guidance during my early years as a PhD student, fostering creativity and inspiring me to continue my work on algicidal bacteria. I learned more than he will ever know from our interactions, but I am also proud to have produced an independent body of work that I can truly call my own. The rest of my committee, Douglass Bartlett, William Fenical, and Milton Saier, have been supportive throughout this long process and I appreciate their input.

I have been fortunate to interact with many faculty members at SIO who have shaped my growth as a scientist through their exceptional teaching. Those that have made a lasting impression on me (other than my co-chairs who have each won teaching awards at SIO) include Doug Bartlett, Bill Fenical, Brian Palenik, Mark Ohman, and Bill Newman.

Thanks to the Azam lab (Steve, Francesca, Johnny, Ty, Christine, Melissa, Jessica), the Franks lab (Erdem, Yuji, Drew), and undergraduate assistants Ellie Wallner and Ann Sugiura for assistance over the years. I have also enjoyed interacting with many SIO graduate students who are already world experts in their fields and I am honored to be part of their group. Of particular note are my close friends Ryan Mueller and Federico Lauro, with whom I have spent many enjoyable hours; I look forward to many more years of their friendship.

Finally, I thank my family for tremendous support. My parents Laurent and Chantal have always been fully encouraging in all my endeavors. My children Max and Emma have provided perspective during scientifically frustrating times. Their mother and my better half, Michelle, has sacrificed a great deal for me to produce this dissertation and I am eternally grateful.

Chapter 1, in full, is a reprint of the material as it appears in: Mayali X, Azam F (2004) Algicidal bacteria in the sea and their impact on algal blooms. Journal of Eukaryotic Microbiology 51: 139-144.

Chapter 2, in part, has been accepted for publication: Mayali X, Franks PJS, Azam F, Bacterial induction of ecdysis in the dinoflagellate *Lingulodinium polyedrum*, accepted to Aquatic Microbial Ecology.

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Mayali X, Doucette GJ (2002) Microbial community interactions and population dynamics of an algicidal bacterium active against *Karenia brevis* (Dinophyceae). Harmful Algae 1: 277-293

Mayali X, Franks PJS, Azam F, Bacterial induction of ecdysis in the dinoflagellate *Lingulodinium polyedrum*, accepted to Aquatic Microbial Ecology

ABSTRACT OF THE DISSERTATION

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Algal blooms, particularly those dominated by dinoflagellates, are widespread in marine ecosystems and can have devastating ocean and human health impacts. The factors that lead to their demise are not well characterized but are thought to include a combination of physical, chemical, and biological factors. Previous studies have isolated bacteria that kill dinoflagellates in laboratory experiments, but little is known about the impact that such algicidal bacteria exert on natural blooms. In this dissertation, I investigated the possibility that algicidal bacteria can impact the population dynamics of the dinoflagellate *Lingulodinium polyedrum*, a species that causes large and long-lived

blooms off the coast of Southern California. I used a combination of laboratory experiments and field sampling to try to link knowledge about model systems in controlled experiments with the complex world of natural blooms in the environment. Chapter one is a review of the current knowledge about the impact of algicidal bacteria in the sea. In chapter two, I report the isolation of algicidal bacteria that induced the formation of temporary cysts in *L. polyedrum* laboratory cultures as a probable defense mechanism against bacterial colonization. In chapter three, I report bacterial colonization intensities in various *L. polyedrum* cultures and in two natural blooms, suggesting a relationship between algal population dynamics and bacterial colonization intensity. In chapter four, I report a novel interaction of algicidal bacteria on *L. polyedrum* motility mediated by excreted putative proteases, and describe decreased motility at the end of an *L. polyedrum* bloom, suggesting algal cells were unhealthy before bloom decline. In chapter five, I report the novel cultivation of a globally widespread and abundant marine bacterium from the *Roseobacter* group that killed *L. polyedrum* cultures by attachment. Using species-specific DNA probes, I studied the colonization of this bacterium on *L. polyedrum* cells in a natural bloom and found increased colonization during the bloom decline phase, suggesting a direct effect of this algicidal bacterium on bloom dynamics.

I

Introduction: Algicidal bacteria in the sea and their impact on algal blooms

ABSTRACT

Over the past two decades, many reports have revealed the existence of bacteria capable of killing phytoplankton. These algicidal bacteria sometimes increase in abundance concurrently with the decline of algal blooms, suggesting that they may affect algal bloom dynamics. Here, we synthesize the existing knowledge on algicidal bacteria interactions with marine eukaryotic microalgae. We discuss the effectiveness of the current methods to characterize the algicidal phenotype in an ecosystem context. We briefly consider the literature on the phylogenetic identification of algicidal bacteria, their interaction with their algal prey, the characterization of algicidal molecules, and the enumeration of algicidal bacteria during algal blooms. We conclude that, due to limitations of current methods, the evidence for algicidal bacteria causing algal bloom decline is circumstantial. New methods and an ecosystem approach are needed to test hypotheses on the impact of algicidal bacteria in algal bloom dynamics. This will require enlarging the scope of inquiry from its current focus on the potential utility of algicidal bacteria in the control of harmful algal blooms. We suggest conceptualizing bacterial algicidy within the general problem of bacterial regulation of algal community structure in the ocean.

The ecological importance of heterotrophic bacteria and archaea in the ocean is well established, from their utilization of dissolved organic matter (DOM) to their contribution of energy to higher trophic levels through the microbial loop (Pomeroy 1974, Hagström et al. 1979, Fuhrman & Azam 1980, King et al. 1980, Azam et al. 1983). Incorporating these findings into marine ecosystem models requires elucidating the mechanisms that underlie the variability in organic matter fluxes from phytoplankton to bacteria. How is a large fraction of the carbon fixed by phytoplankton converted to DOM, thus becoming accessible mainly to bacteria? Do bacteria depend on DOM being produced by other organisms, e.g. phytoplankton exudation and "sloppy feeding" on them by herbivores (Hellebust 1974)? Rather, do bacteria exert their own biochemical activities on the particulate phase, including live algal cells, to generate DOM? An emerging view (e.g. Azam & Smith 1991, Guerrini et al. 1998) is that nutrient and energy flows between algae and bacteria involve dynamic ecological relationships. Algae and bacteria may establish commensalisms that, under nutrient stress, shift to competition and eventually lead to killing and lysis of algae by bacteria. This dynamic scenario is consistent with reports, over the last two decades, of the occurrence of algicidal bacteria that kill marine microalgae. Furthermore, some of these bacteria may specialize in and even require an algicidal lifestyle (rather than being opportunistically algicidal), as the bacteria-killing *Bdellovibrio* genus and other predatory bacteria (Martin 2002).

Here, we synthesize the knowledge on algicidal marine bacteria and the algae susceptible to them. We also direct the reader to general reviews of bacterial-algal interactions (Cole 1982, Doucette 1995, Doucette et al. 1998). Our emphasis is to assess the significance of algicidal bacteria for marine ecosystems in general and for algal

blooms in particular. Further, we stress the need to develop a conceptual framework and quantitative methods for considering algicidal bacteria in an ecosystem context.

Definition. The literature lacks a clear definition of algicidal bacteria. One might argue that many, if not all, heterotrophic bacteria in the sea have the biochemical potential to kill algae. These bacteria would turn algicidal unless, or as long as, the algae can defend themselves against bacterial attack. However, there is evidence that certain bacteria specialize in an algicidal life style. One might define them as true algicidal bacteria (and possibly archaea as well) as algal pathogens that satisfy Koch's postulates in an environmental context. The requirement of an environmental context poses conceptual and technical challenges. For example, most marine bacteria are as-yet uncultured, making it challenging to demonstrate their algicidal activity in mixed natural assemblages. A direct test would be to observe individual algal cells "under attack" in seawater and determine the identity of the bacterium by culture-independent, molecular techniques. If the alga is in axenic culture, one might inoculate it into seawater, retrieve it (e.g. by micromanipulation or flow-cytometry) to test for algicidal attack, and identify the associated bacteria. As in animal hosts where the virulence of a bacterial pathogen depends on host susceptibility (e.g. Salyers & Whitt 2001), the alga's physiological state might be an important variable in algicidal interaction. Thus, only some individuals of an algal species in a seawater sample might be susceptible, complicating interpretation. Physiological state of the algicidal bacteria may also be a variable in "virulence". All these variables must be considered in determining whether a marine bacterium, culturable or not, satisfies the criteria of a pathogen of algae in an ecosystem context.

The current literature, based on both algal and bacterial culture-dependent methods, does provide important insights and poses new questions. Nevertheless, these culture systems are not so simple either and create a different set of difficulties than the highly complex ocean microbial ecosystem. Furthermore, various authors have used different protocols, making results difficult to interpret and inter-compare. Some studies have emphasized the need to wash the bacterial culture before addition to the algae (Doucette et al. 1999, Furusawa et al. 2003), presumably to remove waste products of bacterial metabolism in organically rich media. Others have considered bacterial culture filtrate as the source of algicidal activity (Lovejoy et al. 1998, Skerratt et al. 2002), although it remains unknown if adding washed bacterial cells would also result in algal death. Still others did not specify whether bacterial additions to the algae contained the spent medium, and it is unknown whether the bacterial medium played a role in algicidal activity in those studies. Interestingly, the time to algal culture death is drastically different whether a washed bacterial culture or a culture filtrate is added: days (Doucette et al. 1999) versus minutes (Skerratt et al. 2002). The reports using algicidal bacteria culture medium and algicidal bacteria cells are not necessarily comparable: the former examines the production of fast-acting anti-algal compounds in a bacterial monoculture, whereas the latter assays the ability of bacterial cells to kill algae in co-culture over longer time scales. Further, an algicidal bacterium might kill algae in one assay but not the other. Although both types of studies provide valuable insights, the ecological and evolutionary significance of these phenomena should be recognized as distinct.

Taxonomy. Before the advent of ribosomal DNA sequencing for microbial identification (Woese et al. 1985), biochemical and morphological methods defined

bacterial taxa and also provided physiological information about the organism, e.g. carbon source utilization and antibiotic resistance profiles. Although such methods may not be optimal for phylogenetic taxonomy, they provide information with potential ecological relevance. Today, sequencing of the 16S rDNA gene is the standard for phylogenetic analysis of bacteria, but it provides little insight into the organism's physiological ecology. Nonetheless, several studies have used 16S rDNA data to design molecular probes able to detect algicidal bacteria in the environment (Maeda et al. 1998, Kondo et al. 1999, Iwamoto et al. 2001, Kondo & Imai 2001), although field studies utilizing such probes are still lacking. Also, since marine algicidal bacteria belong to different taxa, these authors could design probes specific to a limited number of groups of algicidal bacteria, often only one strain.

To date, there has not been a comprehensive phylogenetic analysis of all known marine algicidal bacteria. Thus, many fascinating questions on the evolution of algal hosts and their bacterial pathogens remain unanswered. For example, are there metabolic properties common to broad bacterial taxa that allowed specific subgroups of bacteria to become algicidal? Are specific phytoplankton species more susceptible to certain types of algicidal bacteria? Studies specifically addressing such evolutionary hypotheses will be needed in order to begin to understand how algicidal bacteria have become adapted to their environment.

With morphological, biochemical, and molecular methods of taxonomic analyses, studies have recognized four major groups of algicidal bacteria and many strains, as well as some less common groups (Table 1). The most common groups include members of the genus *Cytophaga* (renamed *Cellulophaga* by Skerratt et al. 2002) and *Saprospira*

(phylum Bacteroidetes), and the genera *Pseudoalteromonas* and *Alteromonas* (phylum γ-Proteobacteria). Algicidal bacteria kill their prey by two main mechanisms: direct contact or algicide release (discussed below). *Saprospira* are generalist predators of bacteria (Sangkhobol & Skerman 1981) as well as algae (Sakata 1990) and require attachment to their prey (Lewin 1997). Most algicidal *Cytophaga* also require attachment, although there are some exceptions (Table 1.1). This is consistent with the finding that marine Cytophagales are often particle-associated (reviewed by Kirchman 2002). Further, their ability to degrade high molecular weight organic matter (also present on phytoplankton cell surfaces) supports this hypothesis. Attachment to algae and the ability to degrade cell surface macromolecules make *Cytophaga* well-suited for an algicidal lifestyle. In contrast, all *Alteromonas* and *Pseudoalteromonas* algicidal bacteria tested killed by releasing dissolved substances (Table 1.1). This is consistent with the finding that many *Pseudoalteromonas* produce extracellular bioactive molecules (Holmström & Kjelleberg 1999).

Mode of action. Release of a freely diffusible algicide is unlikely to be an energetically efficient strategy for killing algal cells suspended in seawater, based on the calculated volume/volume ratio of bacterial cells/seawater of 10^{-7} (Azam et al. 1992). However, this approach may be efficient in low diffusion microhabitats, such as marine snow. A different strategy would be to express cell-surface-bound algicides acting through physical contact with the prey alga. Three experimental techniques have distinguished between these two general strategies. The first tests for the presence of algicidal activity in cell-free spent media from algicidal bacteria by its effect on the target

algae (Baker & Herson 1978 and others). However, the algicide may be a by-product of the metabolism of a media component, and not produced under environmental conditions. Further, the production of an algicide might occur only in response to the presence of the algal prey (Yoshinaga et al. 1995a). A second technique tests for dissolved algicide production in cell free filtrate of an algal culture killed by a bacterium by adding the filtrate to a healthy algal culture (Imai et al. 1993 and others). If the filtrate kills the algae, it may be that a dissolved compound from algicidal bacteria is responsible. However, the dissolved compound may in fact have been derived post-mortem from the algal cells killed by bacterial attachment. Algae contain vacuoles replete with acids and hydrolytic enzymes that might kill a fresh culture, but this hypothesis remains untested. Further, a toxic metabolite from bacterial degradation of algal organic matter might be produced following cell lysis caused by attached bacteria. In this case, the metabolite would not be originally responsible for the algal lysis. A less ambiguous third method to test for dissolved algicides is to co-incubate the target algae and algicidal bacteria physically separated by a dialysis membrane or fine pore size filter allowing dissolved compounds to diffuse across (Yoshinaga et al. 1995a and others). Use of commercially-available tissue culture inserts (Nalge Nunc International, Rochester, NY, USA) as in Kim et al. (1999b), allows such a protocol to be carried out in 24-well plates. Microscopic examination of the killing event also provides information on whether attachment is necessary for algal lysis to occur.

Characterization of algicidal compounds. The compounds used by algicidal bacteria that require prey contact to kill (e.g. *Cytophaga* and *Saprospira*) remain uncharacterized, although they may be similar to those from bacteria that kill through

dissolved algicides (e.g. *Alteromonas* and *Pseudoalteromonas*). Ectoenzymes, particularly ectoproteases, are among the likely candidates. Lee et al. (2000) were the first to document a dissolved algicidal protease using a combination of genetics and biochemistry. They found that the culture filtrate from algicidal *Pseudoalteromonas* strain A28 exhibited high protease activity, whereas that of non-algicidal mutants did not. They subsequently isolated a 50 kDa serine protease from the wild-type strain filtrate that displayed algicidal activity. Further, Mitsutani et al. (2001) found that a stationary culture cell extract of *Pseudoalteromonas* strain A25 showed both algicidal and high protease activities while exponential phase (as well as both growth phases of a non-algicidal mutant) did not have either of the activities. These results support that at least some algicidal bacteria kill their algal prey using proteases. If proteases are indeed involved in algicidal activity, we hypothesize that phytoplankton cell-surface polysaccharides play a role in defense against algicidal bacteria by protecting the cell against proteolytic attack (see also: Azam & Smith 1991, Guerrini et al. 1998). Some putative algicides are resistant to autoclaving and thus unlikely to be enzymes (Skerratt et al. 2002), but their chemical structures remain uncharacterized.

Prey specificity and preference. Various studies have found different levels of prey specificity with no clear pattern: some bacteria lyse only one algal species, others multiple species within a given taxon, and still others can lyse cells of different species from several groups (Table 1.1). Algal taxa known to be affected by algicidal bacteria include members of the Chlorophyceae, Rhodophyceae, Bacillariophyceae, Dinophyceae, Haptophyceae, and Raphidophyceae. Algae forming harmful algal blooms (HABS) have

been studied because of the interest in using algicidal bacteria for biological control of the blooms. A few studies have also examined prey specificity of algicidal bacteria on benign phytoplankton species used in aquaculture (Baker & Herson 1978, Sakata et al. 1991).

While most algae studied thus far are large flagellates and diatoms that form harmful blooms, it is not known whether algicidal bacteria also kill the more abundant <5 μm algae that are major players in global ocean biogeochemical cycles. Indeed, one might speculate that the mortality of smaller algae creates conditions for harmful algal blooms to occur. In any event, it is important to test whether bacteria killing non-bloom-forming algae, irrespective of the alga's size, are more widespread and significant than currently recognized (but see Toncheva-Panova & Ivanova 1997). If so, then bacteria may simply be another common cause of algal mortality (versus being a rare event), along with virus infection (Bratbak et al. 1993), eukaryotic pathogenesis (Coats et al. 1996), grazing (Nakamura et al. 1996), and nutrient stress (Brussaard et al. 1997).

Prey preference in algal lysis by algicidal bacteria is not conclusively known. Studies have not examined the consequence of incubating an algicidal bacterium in the presence of two or more algal prey species together. A bacterium might lyse two prey species presented individually, but kill only one when the two preys are offered together. Conversely, an alga in monoculture may be resistant to an algicidal bacterium but be susceptible in the presence of another susceptible prey alga. For example, an algal species may release a substance that induces bacterial virulence against one or more other algal species. Such prey preference hypotheses need to be tested if we are to eventually predict the effect of algicidal bacteria on phytoplankton community structure in the ocean.

Ecosystem considerations. As mentioned, the studies of distribution and abundance of algicidal bacteria have been motivated by, and related to, algal bloom cycles in the coastal ocean. Quantitative studies of algicidal activity in an ecosystem context have not been done for lack of a suitable method. Algicidal bacteria are generally enumerated by their ability to kill a specific algal prey in the MPN (most-probable number) format (Imai et al. 1998a). This method showed the abundance of algicidal bacteria increased during the decline of several algal blooms (Fukami et al. 1991, Yoshinaga et al. 1995b, Imai et al. 1998b, Kim et al. 1998, Kim et al. 1999b), consistent with their involvement in bloom decline. In a culture-independent, fluorescent antibody based approach, Imai et al. (2001) quantified the abundance of an algicidal *Cytophaga* sp. over the course of a *Chattonella* sp. bloom. They also found an increase in the abundance of the specific bacterium following the bloom peak. Despite such documentation of population increases of algicidal bacteria at bloom's end, there remains no conclusive causal evidence for the killing of algae by algicidal bacteria in the ocean! Perhaps the situation is no different than for virus-induced mortality of algae, where laboratory demonstration of the phenomenon still needs to be followed up with conclusive and quantitative field measurements.

Whether algae constitute the sole prey of algicidal bacteria in the ocean is largely unstudied. Some algicidal bacteria may conceivably be generalist microbial predators, and kill bacteria and heterotrophic protists as well. The only published accounts of algicidal bacteria interactions with organisms other than their prey algal species involve other bacteria preventing the algicidal bacteria from killing their prey. Nagasaki et al. (2000) found that a bacterium isolated from an algal culture decreased the algicidal

activity of a *Cytophaga* (strain AA8-2) against its dinoflagellate prey *Heterocapsa circularisquama*, although it did not completely prevent it. Mayali and Doucette (2002) found that bacterial communities associated with cultures of the dinoflagellate *Karenia brevis* fully prevented algicidal activity by *Cytophaga* (strain 41-DBG2), while communities from other *K. brevis* cultures did not. Although the exchange of these communities among different dinoflagellate cultures reversed susceptibility to the algicidal bacterium, no cultured bacteria were identified as the cause of inhibition. Interestingly, in the co-cultures where *Cytophaga* strain 41-DBG2 did not cause algal lysis, this bacterium grew following inoculation but never to abundances greater than 10^6 cells ml^{-1}. In all other incubations, algal lysis was noticeable only after the bacterium reached concentrations > 10^6 cells ml^{-1}. Other studies have observed a similar threshold abundance of algicidal bacteria before algal lysis is manifested (Fukami et al. 1992, Mitsutani et al. 1992, Imai et al. 1993, Yoshinaga et al. 1995a).

The reason for this apparent threshold remains unknown, but it raises the question of whether a single species of algicidal bacteria can attain such high abundance in the ocean. The typical abundance of the entire diverse assemblage of bacteria in seawater is 10^6 ml^{-1}. However, bacterial abundances can reach over 10^7 ml^{-1} (Kamiyama et al. 2000, Heidelberg et al. 2002). Further, even a small number of algicidal bacteria cells, if aggregated around a single phytoplankton cell or concentrated in marine snow, can create an algicidal hot spot. Such microscale patchiness in bacterial communities has been documented on marine snow (Rath et al. 1998) and in seawater (Long & Azam 2001). These hot spots may be important in the ecology of algicidal bacteria and their significance for carbon cycling and sequestration in the ocean.

Another unstudied trophic interaction is that between algicidal bacteria and viruses. The abundance of viruses in seawater is usually one order of magnitude greater than bacteria, and phages are thought to keep bacterial communities diverse (Thingstad & Lignell 1997). Algicidal bacteria are likely to be subject to phage lysis and this may regulate their populations and therefore their algicidal activity. This potentially important interaction should be explored in order to place the impact of algicidal bacteria on phytoplankton community structure in an ecosystem context.

An additional component of the microbial loop that directly affects the activity of algicidal bacteria is the heterotrophic protists. They prey on algae and in this way compete with algicidal bacteria. In addition, protists might accidentally ingest algicidal bacteria caught in the process of killing or consuming an algal prey cell. Studying the time it takes for an algicidal bacterium to kill an algal prey and produce progeny may clarify this interaction. Does this process occur fast enough to be successful before an algal cell is grazed? If not, what is the fate of an algicidal bacterium accidentally ingested by a protist grazing on an algal cell? Further, smaller bacterivorous protists may control the populations of algicidal bacteria through direct grazing. On the other hand, bacterial attachment to algal cells may be a refuge against predation by these smaller protists, but they subsequently become subject to grazing by larger protists and herbivorous zooplankton. We need to clarify the interactions between algicidal bacteria, algal prey, and protist grazers in order to understand the ecological role of algicidal bacteria in marine ecosystems.

Conclusions. Although studies of marine algicidal bacteria continue to be published, more research is necessary to assess their impact in shaping phytoplankton community structure. Previous studies have thoroughly demonstrated the existence of bacteria that can kill phytoplankton cultures. Subsequent studies have demonstrated that the abundance of such bacteria increases following the peak of some algal blooms. However, it is possible that algicidal bacteria do not affect phytoplankton mortality in nature, even taking into account the available evidence. Phytoplankton bloom declines may be initially due to another cause, such as nutrient limitation, viral lysis, eukaryotic pathogens, or a combination thereof. The detritus resulting from the cell lysis increases bacterial community metabolism, and studies have documented increased bacterial abundances following algal blooms (Romalde et al. 1990). The bacterial bloom may be associated with an increase in algicidal bacteria, causing a secondary infection of the remaining moribund algal cells as well as the high abundances of algicidal bacteria found by the studies mentioned above.

The next step in algicidal bacteria research will be to document the phenomenon of bacteria killing phytoplankton in nature. A crucial step toward that goal is to gain a mechanistic understanding of how algicidal bacteria kill their phytoplankton prey. Several studies have indicated that proteases may be involved in the killing activity, but genetic and biochemical studies need to test this hypothesis. Once signature DNA sequences or proteins for the process of algicidal activity have been discovered, they can be used to develop molecular probes specific to those signatures to search for the algicidal process in nature. Until this is accomplished, phytoplankton-killing bacteria will

merely be considered an interesting scientific phenomenon (as they are today) but not a potentially ecologically significant process.

Future directions. An essential goal for the future is to conclusively demonstrate that the phenomenon of phytoplankton death due to bacteria does indeed occur in the ocean and that it is a significant process in the marine assemblages under natural conditions. This will require a mechanistic understanding of how algicidal bacteria kill their phytoplankton prey as well as in situ process rate measurements. Identification of the biochemical bases of the phenomenon, and the identification of the responsible molecules, should help constrain the phenomenon in an ecosystem context. It is also of interest whether algicidal bacteria are obligate or facultative in their life style of killing algae, and whether the relevant phenotype is expressed only during algal blooms. This is important in understanding whether there are energetic restrictions on the proliferation of algicidal bacteria. Finally, a goal for the future is to determine the significance of trophic interactions for the population dynamics of the algicidal bacteria in various ecosystem scenarios. These are challenging problems but their resolution is important in view of the considerable interest in understanding the potential role of bacteria in the decline of harmful algal blooms. The knowledge gained will also be important in incorporating the consequences of bacteria-algae interactions in our concepts and models of the oceanic carbon cycle.

Acknowledgement. This chapter is a reprint of: Mayali X, Azam F. Algicidal bacteria in the sea and their impact on algal blooms (2004) J. Eukar. Microbiol. 51(2): 139-144. The dissertation author was the primary investigator and author of this work.

Table 1.1: Summary data for marine algicidal bacteria active against microalgae

Strain name	Genus	Phylum[a]	Method of ID	# spp. tested	# spp. suscep.	Algal target(s)[b]	Type[c]	Reference
ACEM 21	*Cellulophaga*	Bacter.	16S	11	3	Raph., Din.	dissolved	(Skerratt et al. 2002)
A5Y	*Cytophaga*	Bacter.	morphology	6	4	Bacil, Raph.	attachment	(Mitsutani et al. 1992)
41-DBG2	*Cytophaga*	Bacter.	16S	6	3	Din.	dissolved	(Doucette, McGovern and Babinchak 1999)
Unknown	*Cytophaga*	Bacter.	biochemical	1	1	Raph.	attachment	(Furuki 1993)
MC8	*Cytophaga*	Bacter.	16S	1	1	Raph.	attachment	(Yoshinaga et al. 1998)
J18/M01	*Cytophaga*	Bacter.	16S	11	10	Raph., Din., Bacil.	attachment	(Imai et al. 1991)
LR2	*Cytophaga*	Bacter.	biochemical	6	3	Rhod., Chlor.	n/d	(Toncheva-Panova and Ivanova 1997)
AA8-2	*Cytophaga*	Bacter.	16S	1	1	Din.	attachment	(Nagasaki, Yamaguchi and Imai 2000)
5N-3	*Flavobacterium*	Bacter.	16S	4	1	Din.	dissolved	(Fukami et al. 1929)
SS98-5	*Saprospira*	Bacter.	16S	1	1	Bacil.	attachment	(Furusawa et al. 2003)
SS-K1	*Saprospira*	Bacter.	morphology	3	3	Bacil, Hapt.	attachment	(Sakata 1990)
Unknown	unknown	Bacter.	16S	10	1	Bacil.	n/d	(Chan, Kacsmarska and Suttle 1997)
C49	*Flavobacterium*	Bacter.	morphology	5	1	Raph.	n/d	(Yoshinaga, Kawai and Ishida 1997)
ACEM 20	*Zobellia*	Bacter.	16S	11	4	Raph., Din.	dissolved	(Skerratt et al. 2002)
K12	*Alteromonas*	γ-Proteo.	biochemical	11	10	Bacil.	dissolved	(Nagai and Imai 1998)
SR-14	*Alteromonas*	γ-Proteo.	biochemical	10	3	Bacil.	n/d	(Kim et al. 1999a)
ANSW2-2	*Alteromonas*	γ-Proteo.	16S	3	1	Din.	dissolved	(Doucette, McGovern and Babinchak 1999)
E401	*Alteromonas*	γ-Proteo.	16S	10	6	Din., Raph.	dissolved	(Yoshinaga, Kawai and Ishida 1995)
MC27	*Alteromonas*	γ-Proteo.	16S	1	1	Raph.	dissolved	(Yoshinaga et al. 1998)
GY21	*Alteromonas*	γ-Proteo.	16S	1	1	Raph.	dissolved	(Yoshinaga et al. 1998)
GY9501	*Alteromonas*	γ-Proteo.	16S	3	3	Raph., Din.	dissolved	(Kuroda, Yoshinaga and Uchida 2000)
S	*Alteromonas*	γ-Proteo.	16S	6	6	Raph., Din., Bacil.	dissolved	(Imai et al. 1995)
K	*Alteromonas*	γ-Proteo.	16S	6	4	Raph., Din., Bacil.	dissolved	(Imai et al. 1995)
D	*Alteromonas*	γ-Proteo.	16S	6	4	Raph., Din., Bacil.	dissolved	(Imai et al. 1995)
A25	*Pseudoalteromonas*	γ-Proteo.	16S	1	1	Bacil.	dissolved	(Mitsutani et al. 2001)
A28	*Pseudoalteromonas*	γ-Proteo.	16S	4	4	Bacil., Raph.	dissolved	(Lee et al. 2000)
Y	*Pseudoalteromonas*	γ-Proteo.	16S	9	4	Raph., Din.	dissolved	(Lovejoy, Bowman and Hallegraeff 1998)
ACEM 4	*Pseudoalteromonas*	γ-Proteo.	16S	11	3	Raph., Din.	dissolved	(Skerratt et al. 2002)
R	*Pseudoalteromonas*	γ-Proteo.	16S	6	6	Raph., Din., Bacil.	dissolved	(Imai et al. 1995)
T827/2B	*Pseudomonas*	γ-Proteo.	morphology	2	2	Bacil.	dissolved	(Baker and Herson 1978)
LG-2	*Pseudomonas*	γ-Proteo.	biochemical	5	2	Din.	dissolved	(Lee and Park 1998)
EHK-1	*Pseudomonas*	γ-Proteo.	16S	1	1	Din.	dissolved	(Kitaguchi et al. 2001)
T27	*Vibrio*	γ-Proteo.	morphology	18	18	Raph., Din.	n/d	(Ishio et al. 1989)
A47	*Vibrio*	γ-Proteo.	morphology	5	1	Raph.	n/d	(Yoshinaga, Kawai and Ishida 1997)
B42	*Vibrio*	γ-Proteo.	morphology	5	1	Bacil.	n/d	(Yoshinaga, Kawai and Ishida 1997)
C4	*Vibrio*	γ-Proteo.	morphology	5	2	Raph., Bacil.	n/d	(Yoshinaga, Kawai and Ishida 1997)
G42	*Pseudomonas*	γ-Proteo.	morphology	5	1	Raph.	n/d	(Yoshinaga, Kawai and Ishida 1997)
G62	*Vibrio*	γ-Proteo.	morphology	5	2	Raph., Din.	n/d	(Yoshinaga, Kawai and Ishida 1997)
ACEM 32	*Bacillus*	Gram +	16S	11	5	Raph., Din.	dissolved	(Skerratt et al. 2002)
ACEM 22	*Planomicrobium*	Gram +	16S	10	4	Raph., Din.	dissolved	(Skerratt et al. 2002)
LG-1	*Micrococcus*	Gram +	biochemical	5	1	Din.	dissolved	(Park et al. 1998)
KY1	n/d	n/d	n/d	1	1	Din.	dissolved	(Park, Kim and Kim 1999)

[a] Bacter. = Bacteroidetes, γ-Proteo = γ-Proteobacteria.
[b] Raph. = Raphidophyceae, Din. = Dinophyceae, Bacil. = Bacillariophyceae, Chlor. = Chlorophyceae, Rhod. = Rhodophyceae, Hapt. = Haptophyceae.
[c] Type of algicidal bacteria effect: by attachment or through the release of dissolved algicides.
n/d = not determined.

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II

Bacterial induction of temporary cyst formation by the dinoflagellate *Lingulodinium polyedrum*

ABSTRACT

We report the isolation of three novel bacterial strains from the Bacteroidetes group capable of inducing temporary cyst formation by ecdysis in the bloom-forming dinoflagellate *Lingulodinium polyedrum*. Phylogenetic analysis of 16S rRNA revealed that two of these strains are most closely related to previously identified algicidal bacteria, indicating potentially similar mechanisms of interaction. Long term (2 week) co-incubations of algae and bacteria under a 12/12 light/dark cycle resulted in decreased algal cell abundances (compared to bacteria-free controls) followed by cyst formation. Short term incubations in continuous light resulted in no apparent effects of the bacteria over 2 days, but incubations in continuous darkness resulted in algal ecdysis after 24 hours and significant decreases in total algal cell abundances after 52 hours compared to controls without algicidal bacteria. We also showed that ecdysis resulted in the removal of bacteria attached to the surface of the algal cells, demonstrating a potentially direct benefit to the algae if the bacteria are harmful. We further suggest that negative interactions of bacteria on phytoplankton may be enhanced in the absence of light.

INTRODUCTION

The flow of energy from phytoplankton to bacteria is a major but variable flux in pelagic marine ecosystems, and its mechanisms are not fully characterized (reviewed by Azam & Smith 1991). One critical factor controlling this interaction is whether bacteria are free-living or attached to particles, as particles (both living and dead) can provide microenvironments rich in organic matter and nutrients where bacterial activity and growth can be enhanced (Smith et al. 1992). While dead phytoplankton cells (algal detritus) are quickly and intensely colonized by bacteria (Bidle & Azam 1999), it appears that live phytoplankton cells remain relatively free of bacterial colonization (Kogure et al. 1982). The mechanisms that prevent bacterial colonization of phytoplankton cells have not been clearly identified but may include the production of antibiotics (Cembella 2003), mucus (Rosowski 1992), or simply a low encounter rate due to low bacterial abundance (Vaqué et al. 1989). Clearly, there is a need to describe specific mechanisms by which phytoplankton cells remain uncolonized by bacteria.

Bacterial attachment may also play a role in the population dynamics (growth and death) of phytoplankton in the sea. Bacteria are sometimes considered to be passive utilizers of phytoplankton organic matter, but many previous studies have discussed (Azam & Ammerman 1984, Azam & Smith 1991) and modeled (Jackson 1987, Bowen et al. 1993) the concept that bacteria can "attack" phytoplankton organic matter. Experimental evidence has also shown that bacteria can compete with phytoplankton for resources such as nitrogen (Caron et al. 1988) and phosphorus (Brussaard & Riegman 1998). There exist many examples of bacteria that directly affect phytoplankton

physiology, leading to increases in growth (Ferrier et al. 2002), inhibition of sexual reproduction (Adachi et al. 2001), and cell death (see review by Mayali & Azam 2004). The role of bacterial attachment in these various interactions has seldom been evaluated. Indeed, in the relatively dilute world of seawater, attachment of bacteria to phytoplankton will be crucial to mediate these interactions. For example, if bacteria release a chemical that is toxic to phytoplankton, the concentration of the chemical decreases dramatically away from the bacterium due to diffusion and advection. However, if the bacteria are attached to the algal cells, advection and diffusion are less significant, allowing persistent high concentrations of the molecules to contact the algal cell.

Research on negative bacterial effects on phytoplankton have focused on the mechanisms of algicidal bacteria attack, but surprisingly little is known about algal defense strategies against bacteria. Two previous studies noted that some dinoflagellates produced temporary cysts in response to incubations with algicidal bacteria (Lovejoy et al. 1998, Kitaguchi et al. 2001). Many species of armored dinoflagellates, in addition to having a complex sexual life cycle, have the ability to form such temporary (asexual) cysts through ecdysis, a process that involves shedding the theca and the outer plasma membrane (Morrill 1984). Temporary cysts are more resistant than vegetative cells to a variety of chemical treatments (Fensome et al. 1996), which has led to the hypothesis that cyst formation is a defense mechanism against unfavorable conditions. In the laboratory, temporary cyst formation has been induced by lowering temperature (Schmitter 1979), nutrient stress (Doucette et al. 1989), incubation in the presence of an allelopathic competitor (Fistarol et al. 2004). Temporary cysts are found in natural populations of dinoflagellates, but their ecological role remains unclear (Olli 2004). Confusing matters

further, some dinoflagellate species undergo asexual (Garcés et al. 1998) and sexual (Figueroa & Bravo 2005) division by ecdysis, forming cysts that closely resemble the temporary cysts caused by environmental disturbances. The function of temporary cysts as a short-term survival stage is still a hypothesis, based on laboratory findings that ecdysis is reversible: when the stress factor (nutrient, temperature, algicidal bacteria etc.) is removed, excystment into vegetative cells occurs.

In our search for bacteria that negatively influence the growth of the bloom-forming dinoflagellate *Lingulodinium polyedrum*, we have isolated several bacteria that induced ecdysis in *L. polyedrum* unialgal and previously axenic (bacteria-free) cultures. In order to help us understand why dinoflagellates would form temporary cysts in the presence of algicidal bacteria, we investigated several aspects of this interaction. Since ecdysis results in the removal of the outer components of the algal cell, we examined bacterial attachment dynamics in the context of ecdysis formation. Ecdysis may be an adaptation to remove of colonized bacteria. In addition, a previous study of a related dinoflagellate (*Alexandrium taylorii*), which undergoes ecdysis as its normal cell division mechanisms when nitrogen starved (Giacobbe & Yang 1999), found that temporary cysts increased in abundance during the night (Garcés et al. 1998). We tested whether bacteria-induced ecdysis also increases during periods of darkness. The major hypotheses tested here were 1) ecdysis-inducing bacteria are related to algicidal bacteria, 2) algicidal bacteria induce ecdysis faster in the dark, 3) ecdysis results in the removal of bacteria from the surface of the algal cells.

MATERIALS AND METHODS

Bacterial strain isolation and phylogenetic analysis. All incubations were performed at 18°C under 12/12 LD cycled cool white fluorescent tubes at 160 μE m^{-2} s^{-1} unless specified otherwise. Surface whole seawater was collected from Scripps pier (La Jolla, CA) on various dates, filtered through 0.6 μm polycarbonate filter to remove protists, and added (10 μl) to an axenic strain of *L. polyedrum* CCMP 1932 grown in 25 ml *f*/4 medium with 4X vitamin stock (Guillard 1975). This medium has been shown to be optimal for axenic growth of this algal strain (P. Von Dassow, personal communication). Such enrichments (mixed bacteria and viruses with *L. polyedrum*) as well as control (no addition) *L. polyedrum* cultures were monitored by *in vivo* fluorescence with a TD700 fluorometer (Turner Designs, Sunnyvale, CA). Enrichments that led to decreased fluorescence compared to controls were considered to contain putative algicidal agents, and this activity could be transferred to new axenic *L. polyedrum* cultures by additions of 1 μl of the "killed" culture. To isolate bacteria, these enrichment were spread on ZoBell agar (1.5%) plates (Oppenheimer & ZoBell 1952), and colonies picked with a sterile loop into new axenic *L. polyedrum* cultures. Three strains (ALC1, LPK13, LPK5) from three separate incubations exhibited putative algicidal activity. These isolates were PCR amplified (MasterTaq, Eppendorf, Germany) using 16S rRNA primers 27F and 1492R (manufactured by Invitrogen, Carlsbad, CA) and sequenced bidirectionally using internal primers (Giovannoni 1991). The full length (1488 bp) 16S sequences (deposited in Genbank under accession numbers EF527870, EF527871, EF527872) were inserted into ARB (Ludwig et al. 2004), aligned using the

internal aligner, and added to the global phylogenetic tree (Jan 04 ARB database) using parsimony. Closely related aligned sequences were exported into PAUP* v. 4.0b10 (Swofford 2002) and further analyzed using maximum likelihood with 100 bootstrap replicates, using a model of molecular evolution chosen with modeltest (Posada & Crandall 1998).

Effect of bacteria on algal growth. Once the bacteria causing a decrease in fluorescence of axenic *L. polyedrum* strain CCMP 1932 were isolated, the next step was to determine the specificity of this negative interaction. Here we again used *in vivo* fluorescence to quickly assertain whether the bacteria had any effect on the algal strains tested. A full list of tested algal cultures is presented in Table 1. These included seven other *L. polyedrum* cultures (all xenic), three other xenic dinoflagellates species previously isolated from Scripps pier, and two other flagellates. Since ALC1 was isolated two years before the other two strains, some of these growth experiments were performed at different times and are therefore plotted on separate diagrams. Bacteria were grown in ZoBell broth to log phase, washed in sterile *f*/4 medium, and added to triplicate exponentially growing algal cultures at 10^6 bacterial cells ml^{-1}. Controls included the same cultures with no added bacteria (also in triplicate). Subsequently, a more detailed population dynamics experiment was performed with ALC1 and the axenic *L. polyedrum* culture. Here, we performed cell counts of both *L. polyedrum* and ALC1 bacteria to determine if algal mortality occurred. Bacteria were inoculated at lower concentrations (10^5 cells ml^{-1}) than previous experiments to determine free-living bacterial growth dynamics. Triplicate 100 ml incubations in 250 ml Erlenmeyer flasks (both ALC1

additions and controls) were incubated as before and 1 ml samples were taken daily and fixed with 5% formalin. Samples were stained with DAPI, and filtered onto 0.22 μm polycarbonate filters (Millipore). Bacteria were counted under 1000X magnification and *L. polyedrum* cells under 10X magnification on an Olympus BX-51 microscope.

***Effect of darkness on ecdysis*.** To determine if bacteria-induced ecdysis was affected by light, the three algicidal strains were inoculated separately as above but with higher abundances (10^7 cells ml^{-1}) into triplicate axenic *L. polyedrum* cultures in continuous light or continuous darkness. We used higher bacterial numbers in order to induce an algal response faster, as we wanted to examine shorter time scales than previous experiments. As a control, we used a non-algicidal bacterium (*Flavobacterium* strain BBFL7) instead of no bacterial addition (also in triplicate). Samples were taken after 10, 24, and 52 hours for quantification of total algal cells (which included cysts and protoplasts), temporary cysts, and bacterial attachment. Culture vessels were gently but thoroughly mixed before sampling to collect both swimming and non-motile cells/cysts. We counted total algal cells (vegetative + cysts) using epifluorescence microscopy after post-fixation DNA staining with SYBRgreen II (Molecular Probes, Eugene OR). We examined the algal cells using light microscopy and counted rounded cells without thecae as cysts. In dark incubations with algicidal bacteria after 52 hours, cysts and other non-motile cells were embedded in a matrix of empty theca and bacterial biofilm, making them difficult to quantify and are not reported. Statistical comparisons for total algal cells and cysts were performed with a student's t-test, and comparisons of bacterial attachment with a non-parametric Wilcoxon test.

***CARD-FISH*.** In order to quantify the number of bacteria attached to dinoflagellate cells in the light/dark incubations, CARD-FISH (catalysed reporter deposition-fluorescent in situ hybridization) was used with a protocol slightly modified from Pernthaler et al. (2002). Samples were fixed with 5% unbuffered formalin (=1.8% formaldehyde) and gravity settled overnight (or gently centrifuged at 200 x *g* for 10 min). This procedure allowed separation of algal cells from free-living bacteria. The algal cell pellet was then washed in 1X PBS, and resuspended in 50% ethanol/PBS. Samples were spotted on Teflon-coated well slides and air dried. The slides were dipped in 0.1% low melting point agarose, air dried, and incubated in 1 mg ml^{-1} lysozyme (Sigma) in TE buffer at 37^{o}C for 1 hr. Slides were then washed 3X in MilliQ water, incubated for 10 min in 0.1 N HCl, washed again 3X in 1X PBS (3 min), and finally dehydrated in an ethanol series (50%, 80%, 95% for 3 min each). Slides were incubated for 2 hr at 35^{o}C in a hydrated chamber with hybridization buffer (35% formamide, 900mM NaCl, 20mM Tris, 0.01% SDS, 20% Roche Diagnostic Boehringer blocking reagent) containing 1 μl probe for every 25 μL buffer (final concentration = 2 ng μl^{-1}). We used eubacterial probe eub338 (Amann et al. 1995) conjugated with horseradish peroxidase (Eurogentec, San Diego, CA). Slides were subsequently washed for 20 minutes at 37^{o}C in wash buffer (70 mM NaCl, 5mM EDTA, 20 mM Tris, 0.01% SDS), rinsed in MilliQ, and overlaid with TNT buffer (0.1 M Tris–HCl pH 7.6, 0.15 M NaCl, 0.05% Tween-20) for 15 min. Wells were then incubated with 1 μl tyramide-Alexafluor488 (Molecular Probes, Eugene, OR) in 100 μl 1X PBS with 0.01% Boehringer blocking reagent, washed with TNT buffer at 55^{o}C, rinsed in water, air dried, stained with DAPI, and mounted with Vectashield (Vectorlabs, CA). Bacteria were visualized on an Olympus BX51 epifluorescence

microscope with a standard DAPI filter set and a FITC-Texas red dual band filter set (Chroma Technology Corp, VT). Up to 20 individual bacteria on an algal cell could be counted with minimal error, while higher numbers were categorized as >20 for statistical analyses. Our counts were most probably underestimates due to the inability to detect all probe-positive bacterial cells located behind the autofluorescent algal cells.

Anoxia and excystment experiments. Three additional experiments were performed to further constrain hypotheses about algicidal bacteria-induced temporary cyst formation in darkness. First, to determine the effect of continuous darkness on algal cell numbers and whether the presence of bacteria was a factor, we incubated axenic *L. polyedrum* cultures (5 ml) with the <0.6 μm and the <0.22 μm fractions from a freshly-collected seawater sample. The former contained most free-living bacteria and viruses and the latter only viruses. Triplicate incubations were performed in continuous darkness and samples were taken daily for three days. Total algal cell numbers were counted after staining with SYBRgreen II as above. We used a two-way ANOVA to determine statistical significance.

To exclude the hypothesis that ecdysis in darkness was caused by anoxia due to enhanced bacterial activity, axenic *L. polyedrum* cultures (triplicate 4 ml incubations in 16mm plastic tubes loosely capped) were incubated in the dark under both oxic and anoxic conditions for 24 hours. Anoxic conditions were achieved by placing the tubes in a BBL GasPak Plus anaerobic system (Becton Dickinson, Franklin Lakes, NJ). Temporary cysts were counted from samples collected after 24 hours.

To determine the rate of excystment of *L. polyedrum* temporary cysts incubated with the algicidal strains, we co-incubated log phase *L. polyedrum* with the three bacterial strains (separately) until temporary cyst formation was observed by microscopy. The algal cells were washed in sterile *f*/4 medium by gentle centrifugation (200 x *g*) to remove the free-living bacteria and resuspended into 3 ml *f*/4 (triplicate for each bacterial strain). After 24 hours, motile and non-motile cells were separately collected from the tubes: the former were in the supernatant, the latter in the pellet. Samples were fixed and counted as above. The percent excystment was calculated as the number of swimming cells divided by the total number of cells (swimming + pellet). We assumed no additional cell division in 24 hours from the swimming cells.

RESULTS

Bacterial strain isolation and phylogenetic analyses. Most 0.6 μm seawater filtrate additions to axenic *L. polyedrum* cultures did not lead to premature culture crashes (data not shown), but on three occasions, such enrichments led to putative algicidal activities that could be transferred indefinitely to new axenic algal cultures. From each of these algicidal enrichments, bacterial isolates were found to be responsible for the activity: strains ALC1, LPK5, and LPK13. Strain LPK5 has been subsequently isolated two more times from two xenic *L. polyedrum* isolates that crashed earlier than other *L. polyedrum* cultures. As with the other three bacterial strains, this activity could be transferred to the axenic *L. polyedrum* culture upon re-addition of the bacterial isolates (data not shown).

The bacterial isolates ALC1, LPK5, and LPK13, as expected from their yellow colony coloration and ability to grow on kanamycin-containing agar plates, were members of the Bacteroidetes as shown by their 16S sequence. Sequence comparisons of strain ALC1 revealed no close relatives among cultured isolates, with the closest cultured strain sharing 97% similarity. A maximum likelihood phylogenetic analysis with full-length 16S sequences placed ALC1, with moderate (72%) bootstrap support, in a group that included many bacteria associated with algae (Fig. 2.1a), including Chlorophyta and Phaeophyta.

Sequence analysis of strain LPK13 revealed that its closest relative, with 100% bootstrap support (Fig. 2.1b), is an algicidal bacterium active against a dinoflagellate (Doucette et al. 1999). A group of other algicidal bacteria (*Kordia* and relatives), appears to be a sister taxon, although 16S sequence similarity to this group is low (90.5%). The phylogenetic analysis of strain LPK5 revealed, with 100% bootstrap support (Fig. 2.1c), that its closest relative is another algicidal bacterium active against a dinoflagellate (Kondo et al. 1999).

Effect of bacteria on algal growth. Addition of bacterial strains ALC1, LPK5, and LPK13 to cultures of axenic *L. polyedrum* CCMP 1932 caused noticeable decreases in fluorescence compared to no addition cultures (Fig. 2.2a, b). The other *L. polyedrum* cultures, all xenic, responded differently to the addition of the algicidal bacteria. For example, some were affected by strains ALC1, LPK5, and LPK13 (Fig. 2.2c, d) similarly to the axenic strain. Others were not affected (Fig. 2.2e), and still others were affected by one strain but not the other(s) (Fig. 2.2f). We also tested three other species of

dinoflagellates (all isolates from Scripps pier) and two other flagellates and found that *Akashiwo sanguinea* was affected by strains LPK5 and LPK13 (Table 2.1). Moderate biomass decrease of *Isochrysis galbana* by strain LPK5 was also detected. For brevity, these experiments are not shown and the results are summarized in Table 2.1.

When putative algicidy was detected by a decrease in fluorescence, *L. polyedrum* cells formed a pellet at the bottom of the culture tubes. These pellets initially comprised vegetative cells that eventually formed temporary cysts if left undisturbed for ~ 1 week. To determine if algal mortality occurred, we quantified algal and bacterial numbers in *L. polyedrum* cultures incubated with strain ALC1 (Fig. 2.3). Following inoculation at 10^5 cells ml^{-1}, strain ALC1 grew over one order of magnitude over 24 hours ($\mu = 0.17\ hr^{-1}$) but thereafter never increased over 2×10^7 cells ml^{-1}. While algal cell numbers (including cysts and protoplasts) were significantly lower in the ALC1 incubations than the control after 6 days, they did not decrease below levels present at inoculation, demonstrating no net population mortality but rather population growth reduction under the conditions tested. Incubations of *L. polyedrum* with strains LPK5 and LPK13 revealed similar dynamics and are not shown.

***Effect of darkness on ecdysis*.** Incubation with non-algicidal bacterium BBFL7 that served as a control did not induce ecdysis either in the light or in the dark (Fig. 2.4a). Bacterial attachment was significantly higher in the dark than in the light except at the first time point sampled (10 hours), and showed temporal variability (both in light and darkness), with increased colonization after 24 hours compared to 10 and 52 hours (Fig. 2.4b). Total algal cell concentrations remained constant over 52 hours in the light.

Darkness increased net algal cell concentration at 24 hours (Fig. 2.4c), although the increased cell numbers were no longer significant at 52 hours, possibly due to increased variance of the 52 hour dark samples. No decrease in total algal cell numbers was detected, as measured by the difference between cell concentrations at 52 hours and 10 hours within a treatment.

Algicidal bacterium ALC1 induced ecdysis in the dark after 24 hours of incubation (Fig. 2.4d), while incubations in continuous light did not result in the formation of temporary cysts. Bacterial attachment was low in relation to the other strains but increased with time, and there was no significant difference between light and dark incubations (Fig. 2.4e). For this strain, ecdysis did not result in a detectable decrease in bacterial colonization on all cells. Total algal cell concentration in the light exhibited a small but significant decrease after 52 hours while the decrease in the dark was more pronounced (Fig. 2.4f), with a loss of 80% of the cells after 52 hours.

Algicidal bacterium LPK5 also induced ecdysis in the dark after 24 hours of incubation (Fig. 2.4g). Bacterial attachment in the light was very similar to the light incubations with non-algicidal strain BBFL7, showing an increase at 24 hours. Bacterial attachment in the dark was initially higher than in the light (Fig. 2.4h) but exhibited a gradual and eventually significant decrease. For this strain, ecdysis in the dark was correlated with a decrease in bacterial colonization. Total algal cell concentration in darkness was significantly lower than in the light after 52 hours (Fig. 2.4i), with dark-incubated treatments exhibiting a net population decrease of 84%.

Algicidal bacterium LPK13 caused a slight increase in the concentration of temporary cysts in the light after 24 hours but a dramatic and highly significant increase

in the dark (Fig. 2.4j). No further temporary cyst formation occurred in the light after 52 hours (data not shown). Bacterial attachment decreased over time, both in light and in darkness (Fig. 2.4k). Total algal cell concentrations significantly decreased only in darkness, with a net population change of 66% (Fig. 2.4l).

Anoxia and excystment experiments. One hypothesis to explain ecdysis in the presence of certain types of bacteria in the dark is anoxia due to the lack of photosynthesis by the algal cells and high respiration rates by the phytoplankton and bacteria. We tested this by incubating *L. polyedrum* cultures in anoxic and normal (oxic) conditions for 24 hours in darkness. Incubations were not performed in the light because we did not expect ecdysis to occur under those conditions. We did not detect a significant difference in the number of temporary cysts in anoxic versus oxic conditions (Fig. 2.5), rejecting the idea that anoxia alone caused ecdysis in the previous experiments.

To determine whether dark-induced algal mortality by bacteria was a general phenomenon, we incubated axenic *L. polyedrum* cells in continuous darkness with and without a mixed bacterial community and monitored cell abundances over three days. There was no significant effect of the presence of bacteria, while time in darkness was a significant factor (Fig. 2.6). Population decrease over two days was on the order of 25%, much lower than dark incubations with algicidal bacteria from the previous experiments.

Following the removal of free-living algicidal bacteria and re-inoculation into fresh algal medium, *L. polyedrum* temporary cysts formed swimming vegetative cells within 24 hours. Excystment rates were on the order of 15% for ALC1 and LPK5-incubated dinoflagellates, and around 2% for those from LPK13 incubations (Fig. 2.7).

We did not always detect a strong decrease in the attachment of algicidal bacteria concurrent with ecdysis as hypothesized, and we often observed cysts colonized by bacteria. However, recolonization after ecdysis was possible in our laboratory incubations where new ecdysed cysts and colonized theca were in physical contact. To directly determine if ecdysis physically removed bacteria attached to the dinoflagellate cells, we examined algal cells in the process of ecdysis sampled from the laboratory incubations. Vegetative cells without intact theca (Fig. 2.8a) could be easily distinguished from the cysts (Fig. 2.8c) that exhibited the characteristic temporary cyst morphology (round with thick pellicle membrane). Cells in the process of ecdysis still had thecae attached (Fig. 2.8b). A number of these cells incubated with strain LPK5 (Fig. 2.8d, e), and the other two strains (not shown) revealed thecae heavily colonized with bacteria while the newly formed cysts were uncolonized.

DISCUSSION

Two previous studies have reported dinoflagellate ecdysis in response to incubations with algicidal bacteria (Lovejoy et al. 1998, Nagasaki et al. 2000), but no knowledge existed on factors that influence bacteria-induced ecdysis and the potential benefits to the algal cells. Here, we have demonstrated that periods of darkness enhance bacterial induction of ecdysis in the dinoflagellate *L. polyedrum* and further result in decreases in total algal cell abundances. In addition, we provided direct evidence that ecdysis removed colonized bacteria from the surface of algal cells, potentially providing a benefit to the dinoflagellates.

According to the 16S phylogenetic analysis, strains LPK5 and LPK13 are the closest known relatives to algicidal bacteria strains 5N-3 (Kondo et al. 1999) and 41-DBG2 (Doucette et al. 1999), respectively. The implication of this finding is that the strain pairs share recent common ancestors and they are likely to possess similar genomic architectures that enable them to display algicidal phenotypes. Algicidy and ecdysis induction are most likely related and may be regulated by the same biochemical processes. Indeed, under continuous darkness, algistatic bacteria ALC1, LPK5 and LPK13 appeared to become algicidal, as total algal cell numbers (including cysts and protoplasts) decreased significantly compared to incubations with non-algicidal bacterium BBFL7. This reinforces the notion that the definition of algicidy is ambiguous and interactions between potentially algicidal bacteria and their phytoplankton hosts are likely to vary with environmental conditions (Mayali & Azam 2004), in this case the presence of light. A further use of the phylogenetic analyses is the ability to identify environmental 16S sequences from uncultivated organisms as potentially algicidal if they are found to cluster in the groups discussed above. To date, no environmental sequences identical to those of strains LPK5 or LPK13 have been found, which may be caused by undersampling as only a handful of dinoflagellate blooms have been sampled for microbial community structure (reviewed by Garcés et al. 2007). In addition, these bacteria may be rare and found only attached to dinoflagellate cells, which would make them more difficult to detect in such studies. One study has found a sequence almost identical (1 base pair difference, band ATT5, 99.3% similar, GB acc. # AF125332), to that of ALC1 at the end of a 1997 *L. polyedrum* bloom at Scripps pier (Fandino et al. 2001), implying it was present in relatively high abundance in nature. While few colony-

forming bacteria are found to be numerically dominant in the environment (Bernard et al. 2000), we were successful in isolating strain ALC1 through enrichment with a culture of *L. polyedrum*. It remains unknown whether ALC1 exhibits algicidal activity in the environment.

Incubations of *L. polyedrum* cultures with the three algicidal strains in continuous light and continuous darkness convincingly revealed that darkness enhanced the ability of the bacteria to induce ecdysis. Non-algicidal strain BBFL7, another Bacteroidetes isolate, did not cause ecdysis under similar conditions. We used this isolate (16S Genbank acc. # AY028207) because an identical sequence was found during a 1997 *L. polyedrum* bloom (band ATT8, Genbank acc. # AF125335), suggesting that it is present in abundant numbers during blooms of this dinoflagellate. Further incubation in complete darkness (over two days), led to significant decreases in total algal cells (including cysts and protoplasts) compared to the controls, as measured by counting DNA-containing cells. This is most likely due to the algal cells that did not form temporary cysts (both vegetative cells and protoplasts) becoming non-viable. Why is darkness so important for algicidal bacteria to induce *L. polyedrum* to form temporary cysts? Our initial hypothesis of anoxia alone was rejected by our experiments performed in the anoxic chambers. The reason may be a combination of low oxygen and the production of algicidal agents. These molecules may be light sensitive, as proteins can be denatured by ultraviolet radiation for example. An alternative hypothesis, though less likely, could be that the algal cells produce antibiotic compounds while they are photosynthesizing but under extended darkness are no longer able to produce them in high quantities. A mechanistic understanding of algicidal bacteria killing phytoplankton is needed to clarify this issue.

However, our findings suggest that light is a factor in mediating the interactions between algicidal bacteria and their phytoplankton host. Light may also be critical to other types of bacterial interactions such as commensalisms and mutualism and merits further study.

The last major aspect of our work examined whether ecdysis resulted in a noticeable benefit to the algal cells. Temporary cysts with their still attached thecae revealed that newly formed cysts were not readily colonized, unlike the thecae. In terms of bacterial attachment in the culture incubations, the average number of bacteria per algal cell decreased significantly over time in incubations that were undergoing ecdysis (except for strain ALC1; Fig. 2.4). The case of strain ALC1 is a bit different because bacterial attachment did not start until after the 10 hr time point, and attachment intensity was low compared to the other strains. Sampling of ALC1 incubations farther in time might have revealed higher overall bacterial attachment but lower bacterial attachment in the dark compared to the light.

In dark incubations with algicidal bacteria, net algal population mortality occurred after the onset of ecdysis, contrary to what we would expect if ecdysis were a defense mechanism against bacterial attack. However, if ecdysis occurred in the water column, cysts would separate from empty thecae due to their different sinking velocities. In laboratory incubations, cysts were not separated from the empty thecae, allowing for subsequent bacterial colonization of the cysts and eventual cell mortality. We found that removing cysts from such incubations and washing them in clean media led to algal culture recovery of 20%, 20%, and 2% of the cells after 24 hours for strains ALC1, LPK5, and LPK13, respectively. We assume that if edysis occurred in the water column, attached bacteria would be removed, physically separated, and the cysts would have the

ability to resume vegetative growth. In that case, ecdysis would provide a benefit to algal cells colonized with algicidal bacteria.

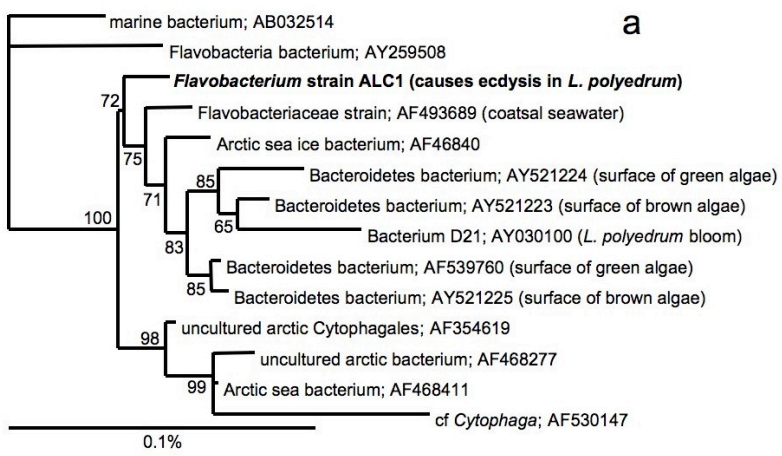
The potential significance of bacteria-induced ecdysis as compared to ecdysis caused by other environmental factors remains unknown. Temporary cysts are found in nature and there is evidence that some blooms can end through ecdysis (Marasovic 1989, Wang et al. 2007). Whether bacteria-induced ecdysis is significant in nature or is a laboratory artifact is a difficult question to answer, similar to the question about bacteria directly killing phytoplankton in nature. One strategy to corroborate these hypotheses is to quantify the abundance of algicidal (or ecdysis-causing) bacteria in nature in the context of bloom dynamics. Unfortunately, algicidal bacteria are phylogenetically very diverse, and different algicidal bacteria may occur in different blooms. Another strategy would be to study the molecular mechanism(s) of ecdysis in the laboratory (from the point of view of the algae) and find markers specific to bacteria-induced ecdysis. Searching for these markers in algal cells during natural blooms would further provide evidence that bacteria induce ecdysis in the ocean.

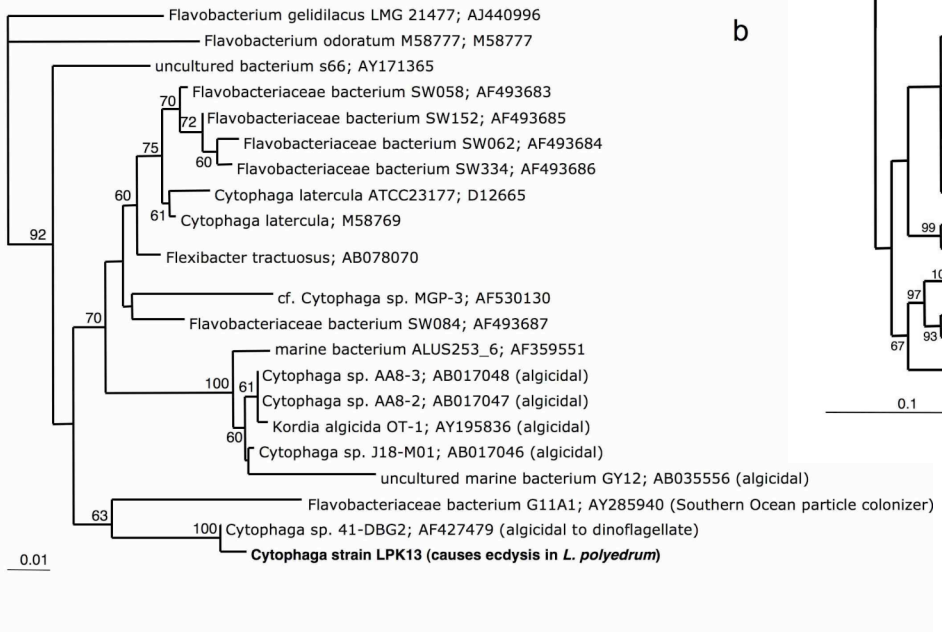
Acknowledgement. This chapter has been accepted for publication: Mayali X, Franks PJS, Azam F (in press) Bacterial induction of temporary cyst formation by the dinoflagellate *Lingulodinium polyedrum.* Aquat Microb Ecol. The dissertation author was the primary investigator and author of this work.

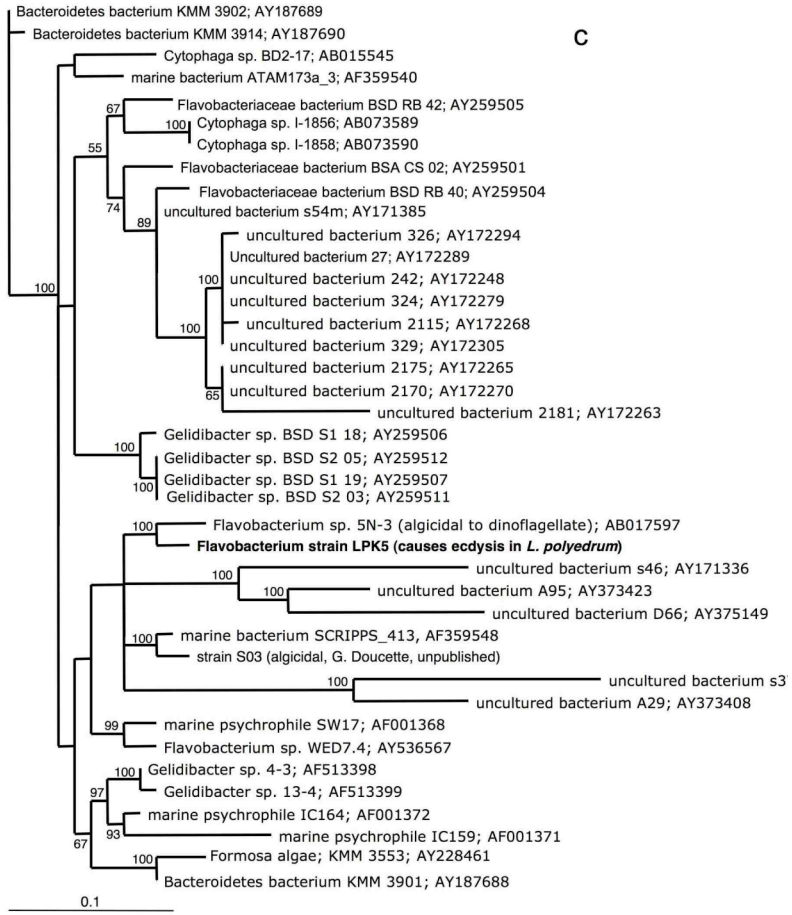
Table 2.1: Susceptibility of various *L. polyedrum* cultures and other phytoplankton species to incubations with 3 algicidal bacteria strains as monitored *by in vivo* fluorescence compared to no-addition control cultures. Decrease in fluorescence is denoted as +, no difference to control as -, blank as not tested. All cultures were xenic unless otherwise mentioned. Cultures without CCMP number were isolated from Scripps pier by various researchers.

Algal strain	ALC1	LPK5	LPK13
Axenic *L. polyedrum* CCMP 1932	+	+	+
L. polyedrum CCMP 1738	-	+	+
L. polyedrum CCMP 1933	+	-	-
L. polyedrum CCMP 1935	+		
L. polyedrum XM1020		+	+
L. polyedrum JF6		-	+
L. polyedrum JF2		-	-
L. polyedrum JF1		-	-
Axenic *Emiliania huxleyi* CCMP 374	-	-	-
Prorocentrum micans AS623	-	-	-
Scrippsiella trochoidea SIO strain	-	-	-
Akashiwo sanguinea SIO strain	-	-	-
Isochrysis galbana CCMP 462		+	-

Figure 2.1. Maximum likelihood phylogenetic analyses of strains ALC1 (A), LPK13 (B), and LPK5 (C) among full 16S sequences of closely related sequences. When useful, the origin of the sequence is indicated in parentheses. Numbers at the nodes indicate bootstrap values greater than 50% from full heuristic ML searches.


a
marine bacterium; AB032514
Flavobacteria bacterium; AY259508
Flavobacterium strain ALC1 (causes ecdysis in L. polyedrum)
Flavobacteriaceae strain; AF493689 (coatsal seawater)
Arctic sea ice bacterium; AF46840
Bacteroidetes bacterium; AY521224 (surface of green algae)
Bacteroidetes bacterium; AY521223 (surface of brown algae)
Bacterium D21; AY030100 (L. polyedrum bloom)
Bacteroidetes bacterium; AF539760 (surface of green algae)
Bacteroidetes bacterium; AY521225 (surface of brown algae)
uncultured arctic Cytophagales; AF354619
uncultured arctic bacterium; AF468277
Arctic sea bacterium; AF468411
cf Cytophaga; AF530147
0.1%


b
Flavobacterium gelidilacus LMG 21477; AJ440996
Flavobacterium odoratum M58777; M58777
uncultured bacterium s66; AY171365
Flavobacteriaceae bacterium SW058; AF493683
Flavobacteriaceae bacterium SW152; AF493685
Flavobacteriaceae bacterium SW062; AF493684
Flavobacteriaceae bacterium SW334; AF493686
Cytophaga latercula ATCC23177; D12665
Cytophaga latercula; M58769
Flexibacter tractuosus; AB078070
cf. Cytophaga sp. MGP-3; AF530130
Flavobacteriaceae bacterium SW084; AF493687
marine bacterium ALUS253_6; AF359551
Cytophaga sp. AA8-3; AB017048 (algicidal)
Cytophaga sp. AA8-2; AB017047 (algicidal)
Kordia algicida OT-1; AY195836 (algicidal)
Cytophaga sp. J18-M01; AB017046 (algicidal)
uncultured marine bacterium GY12; AB035556 (algicidal)
Flavobacteriaceae bacterium G11A1; AY285940 (Southern Ocean particle colonizer)
Cytophaga sp. 41-DBG2; AF427479 (algicidal to dinoflagellate)
Cytophaga strain LPK13 (causes ecdysis in L. polyedrum)
0.01


c
Bacteroidetes bacterium KMM 3902; AY187689
Bacteroidetes bacterium KMM 3914; AY187690
Cytophaga sp. BD2-17; AB015545
marine bacterium ATAM173a_3; AF359540
Flavobacteriaceae bacterium BSD RB 42; AY259505
Cytophaga sp. I-1856; AB073589
Cytophaga sp. I-1858; AB073590
Flavobacteriaceae bacterium BSA CS 02; AY259501
Flavobacteriaceae bacterium BSD RB 40; AY259504
uncultured bacterium s54m; AY171385
uncultured bacterium 326; AY172294
Uncultured bacterium 27; AY172289
uncultured bacterium 242; AY172248
uncultured bacterium 324; AY172279
uncultured bacterium 2115; AY172268
uncultured bacterium 329; AY172305
uncultured bacterium 2175; AY172265
uncultured bacterium 2170; AY172270
uncultured bacterium 2181; AY172263
Gelidibacter sp. BSD S1 18; AY259506
Gelidibacter sp. BSD S2 05; AY259512
Gelidibacter sp. BSD S1 19; AY259507
Gelidibacter sp. BSD S2 03; AY259511
Flavobacterium sp. 5N-3 (algicidal to dinoflagellate); AB017597
Flavobacterium strain LPK5 (causes ecdysis in L. polyedrum)
uncultured bacterium s46; AY171336
uncultured bacterium A95; AY373423
uncultured bacterium D66; AY375149
marine bacterium SCRIPPS_413, AF359548
strain S03 (algicidal, G. Doucette, unpublished)
uncultured bacterium s3
uncultured bacterium A29; AY373408
marine psychrophile SW17; AF001368
Flavobacterium sp. WED7.4; AY536567
Gelidibacter sp. 4-3; AF513398
Gelidibacter sp. 13-4; AF513399
marine psychrophile IC164; AF001372
marine psychrophile IC159; AF001371
Formosa algae; KMM 3553; AY228461
Bacteroidetes bacterium KMM 3901; AY187688
0.1

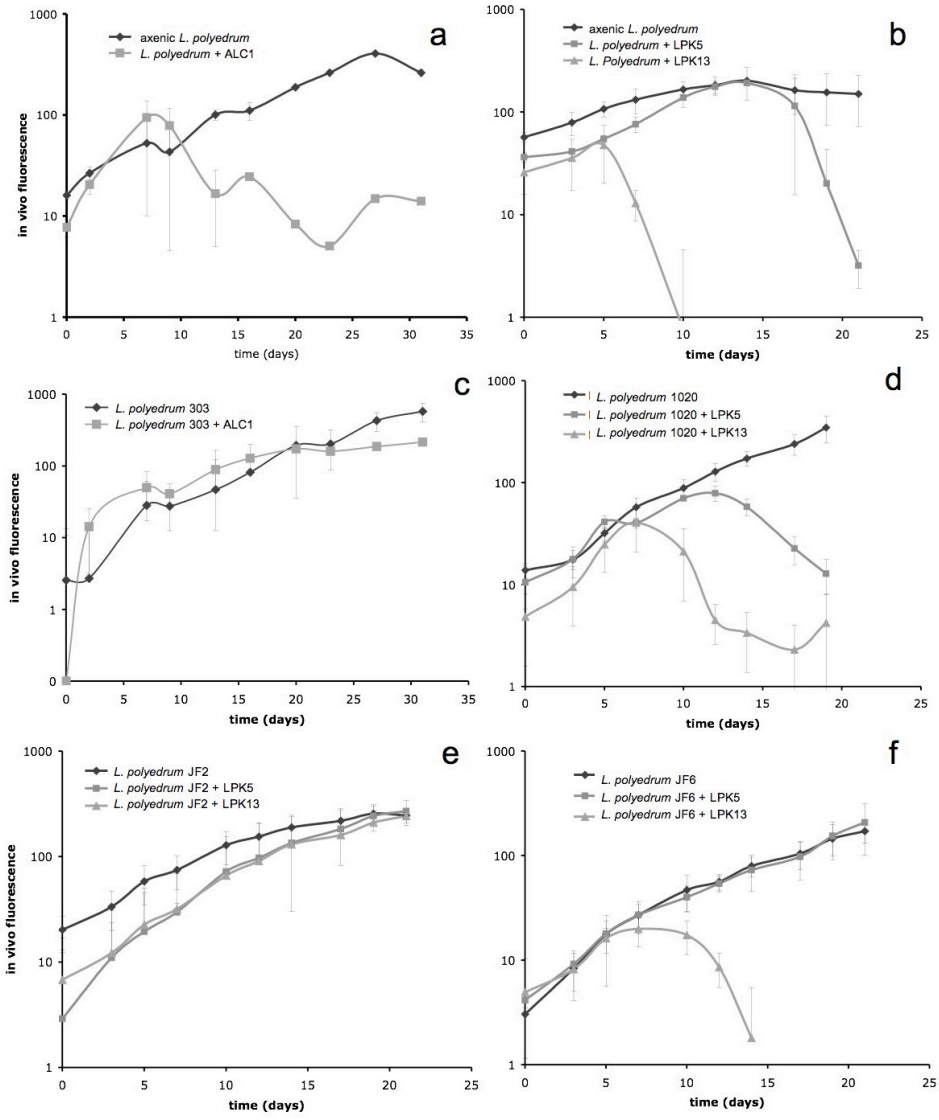


Figure 2.2. Growth of various *L. polyedrum* cultures incubated with algicidal bacteria strains versus no-addition controls as monitored by *in vivo* fluorescence. Bars indicate standard deviations of triplicate cultures.

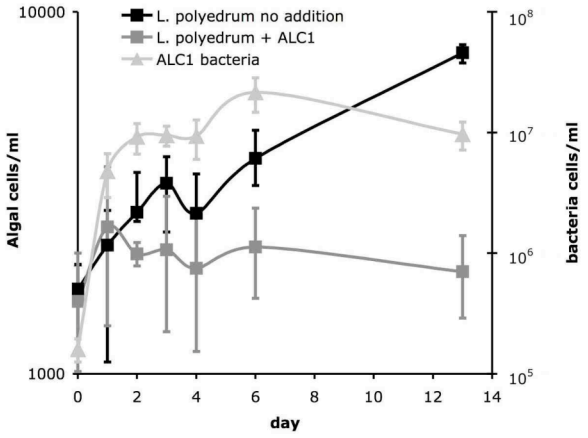
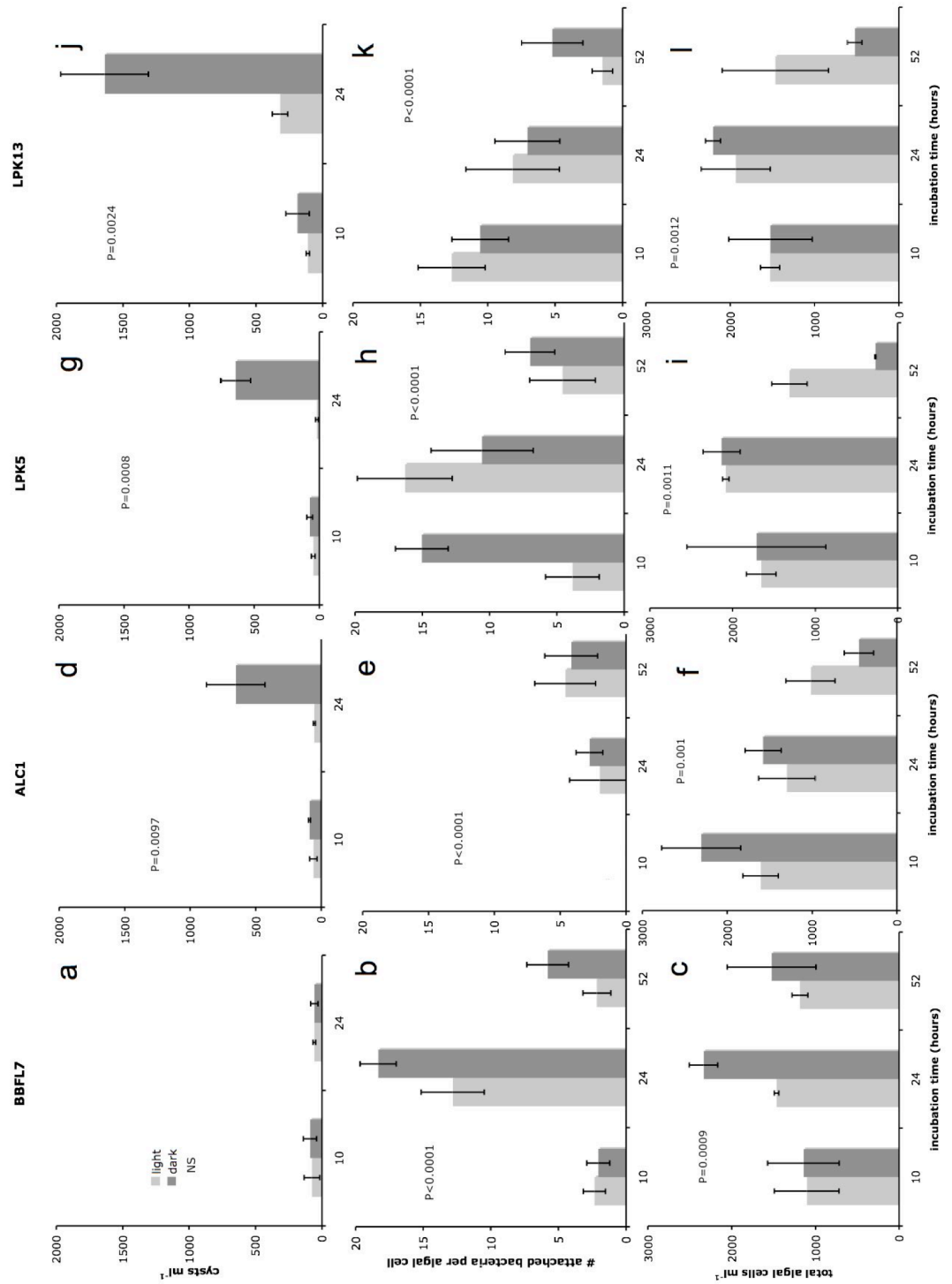


Figure 2.3. Growth of axenic *L. polyedrum* culture incubated with strain ALC1 compared to a no addition control, as monitored by cell counts. Bacterial numbers are also shown. Bars indicate standard deviations of triplicate cultures.

Figure 2.4. Quantification of *L. polyedrum* axenic strain CCMP1932 temporary cysts, total algal cells, and bacteria attached to algal cells during incubations with benign strain BBFL7 (a, b, c) and algicidal strains ALC1 (d, e, f), LPK5 (g, h, i) and LPK13 (j, k, l). Error bars indicate standard deviations (for the algal cells and cysts) or 95% C.I. (for attached bacteria).


BBFL7
ALC1
LPK5
LPK13
a
b
c
d
e
f
g
h
i
j
k
l
light
dark
NS
P=0.0097
P<0.0001
P=0.001
P=0.0008
P<0.0001
P=0.0011
P=0.0024
P<0.0001
P=0.0012
P=0.0009
cysts ml-1
attached bacteria per algal cell
total algal cells ml-1
incubation time (hours)

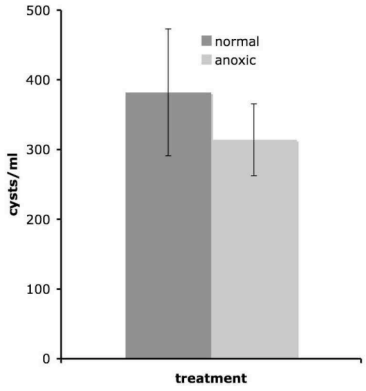


Figure 2.5. Quantification of temporary cysts of *L. polyedrum* incubated in the dark under anoxic and oxic (normal) conditions for 24 hours. Bars indicate standard deviations of triplicate cultures.

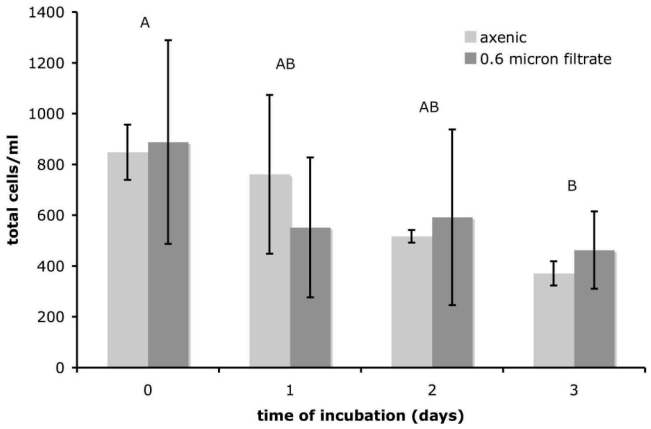


Figure 2.6. Changes in algal cell numbers over the course of three days in continuous darkness, both with no bacteria (0.22 μm filtrate) and with the natural bacterial community from seawater (0.6 μm filtrate). Bars indicate standard deviations of triplicate cultures.

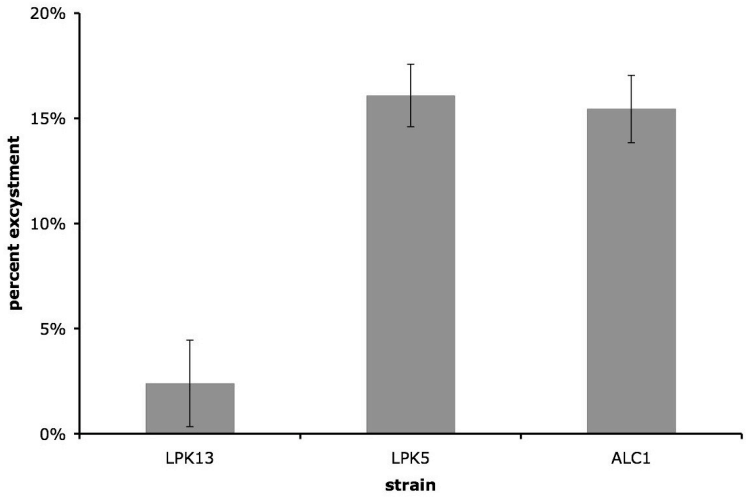


Figure 2.7. Percent excystment of *L. polyedrum* CCMP 1932 temporary cysts into motile vegetative cells previously incubated with algicidal strains LPK13, LPK5, and ALC1. Bars indicate standard deviations of triplicate incubations.

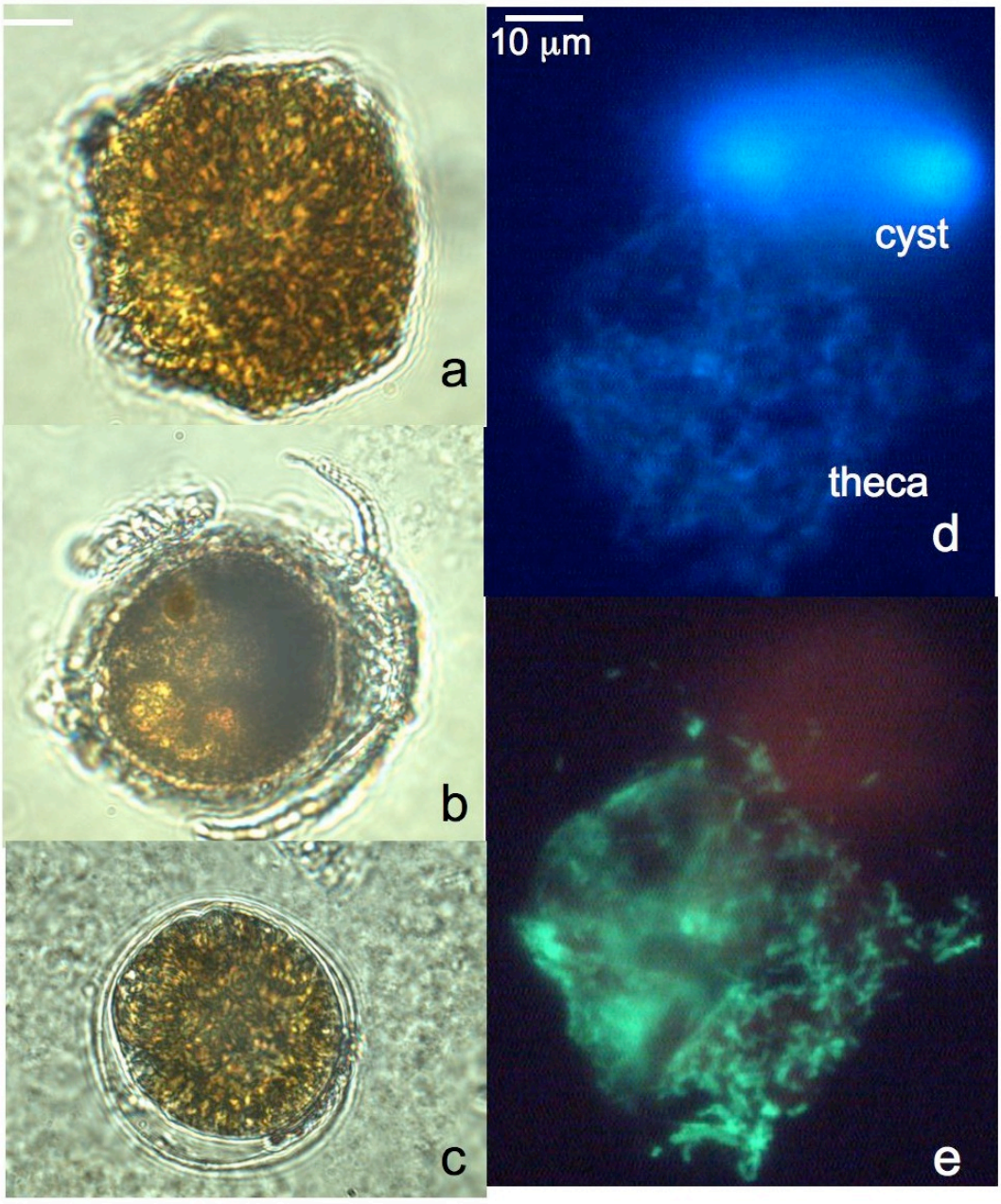


Figure 2.8. Light (a-c) and epifluorescent (d-e) micrographs of *L. polyedrum* CCMP 1932 cells incubated with algicidal bacteria; a) motile vegetative cell, b) vegetative cell undergoing ecdysis, c) ecdysed temporary cyst with characteristically thick pellicle membrane; d) DAPI-stained cell in the process of ecdysis, with brightly stained nucleus from the cyst (top) and colonized theca (bottom); e) same field of view as (d) under dual red and green fluorescence filter after CARD-FISH, showing chlorophyll-containing cyst (red) and bacteria-specific probe stained cells (green).

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III

Attachment and population dynamics of alga-associated bacteria during blooms of *Lingulodinium polyedrum* (Dinophyceae)

ABSTRACT

Little information exists on the physical interaction between bacteria and phytoplankton in pelagic aquatic environments. Here, we investigated the dynamics of bacterial attachment to cells of the red tide dinoflagellate *Lingulodinium polyedrum* in laboratory cultures and during two natural blooms in summer 2003 and 2005 off the coast of La Jolla, CA, USA. The number of attached bacteria on each algal cell ranged from 0-20, with an average of 1.9 in algal cultures and 1.8 in natural samples, and temporal variability was very high. Our results show that (1) contrary to prevalent ideas, bacterial colonization was greater in log phase than in stationary algal growth phase, (2) higher bacterial colonization in culture was associated with lower algal growth rate, (3) bacterial abundances and colonization intensities were closely linked to algal population dynamics during an algal bloom, and (4) algal cells at depth were more colonized than near the surface during bloom termination phases. We suggest that bacterial colonization intensity can be used as a marker for imminent bloom decline and provide evidence consistent with the hypothesis that bacterial attachment affects algal bloom dynamics in nature.

INTRODUCTION

A major portion (30-50%) of phytoplankton primary production is incorporated by bacteria (Cole et al. 1988), mostly through algal dissolved organic matter excretion followed by bacterial hydrolysis and uptake. Another strategy utilized by bacteria is attachment to particles (Paerl 1975) subsequently shown to be a major site of bacterial activity (Smith et al. 1992). Understanding the dynamics of bacterial attachment to particles is crucial for predicting carbon export from the surface ocean as the size and density of sinking particles is altered by the enzymatic activities of attached bacteria (Azam & Long 2001). Particles can be live or dead or a combination thereof, and while detritus is highly colonized by bacteria (Caron et al. 1982), some studies have suggested that live phytoplankton cells are relatively free from bacterial colonization (Droop & Elson 1966, Kogure et al. 1982). Surprisingly, no studies have directly quantified bacterial colonization on phytoplankton cells in nature. In one previous study, attachment of bacteria to particles (operationally defined by filter pore size) was negatively correlated to chlorophyll a and primary productivity (Albright et al. 1986), suggesting that growing (healthy) algal cells harbor fewer attached bacteria than senescent ones. Another study, examining a mesocosm algal bloom, further suggested that bacterial attachment to algal cells is a function of encounter probability (Vaqué et al. 1989): bacterial attachment increased when both free-living bacteria and algal cell numbers were high. These previous studies suggested that a dynamic physical interaction exists between

phytoplankton and bacteria, but a quantitative analysis of bacterial attachment to phytoplankton in nature is clearly warranted to further support this idea.

Blooms of dinoflagellates and other phytoplankton, toxic or otherwise, occur worldwide in both freshwater and marine environments (Granéli & Turner 2006). It is now well established that direct or indirect interactions between bacteria and phytoplankton have important consequences for both algae and bacteria (Cole 1982), and such interactions likely regulate algal bloom dynamics. Examples of bacterial-algal interactions are numerous and include vitamin production by bacteria and subsequent uptake by algae (Droop 2007), the acquisition of micronutrients by algae through putative bacterial siderophores (Keshtacher-Liebson et al. 1995), production of anti-algal compounds by bacteria (Lee et al. 2000) and many more (Doucette 1995). Attachment of bacteria to phytoplankton cells must play a role in such interactions, particularly because turbulence, algal motility, or diffusion can diminish indirect interactions that are occurring without attachment. Attachment will prolong the interaction, and dinoflagellate cells that are migrating up or down will retain their attached bacteria. Indeed, if bacterial attachment is damaging to motile algae, we would expect bacterial attachment to increase with depth, as unhealthy algal cells would sink faster than healthy, highly motile ones. The lack of empirical data on bacterial attachment to phytoplankton in nature has kept us from relating the above interactions, most of which have been documented only in laboratory cultures, to their probable activity in the field.

A potential reason for the shortage of information on bacterial attachment to phytoplankton may be methodological. Some studies have used DNA-binding dyes

combined with epifluorescence microscopy (Vaqué et al. 1989), but in our experience, it is difficult to differentiate attached bacteria from plastid DNA of the host dinoflagellate using such a method. Others have used scanning electron microscopy and transmission electron microscopy (Lewis et al. 2001), more accurate but time-consuming methods. A recent study has combined a fluorescent *in situ* hybridization technique (FISH) with laser confocal microscopy (Biegala et al. 2002) to enable the identification and localization of different types of bacteria attached to dinoflagellates. While nucleic acid stains bind to both bacterial and algal DNA including chloroplast and mitochondria, FISH allows the unambiguous detection of organisms to various taxonomic levels. Here, we employ CARD-FISH (Pernthaler et al. 2002) with a bacteria-specific probe and widefield epifluorescence microscopy to quantify bacteria attached to cells of the dinoflagellate *Lingulodinium polyedrum*, both in culture and during natural blooms of this red tide organism. The specific hypotheses addressed were (1) bacterial attachment to *L. polyedrum* is generally low, (2) bacterial attachment increases at stationary phase in batch cultures, (3) bacterial attachment increases at the end of blooms, when cells are senescent, and (4) bacterial attachment increases with depth.

MATERIALS AND METHODS

Culture conditions and field sampling. *Lingulodinium polyedrum* strains (Table 3.1) were grown in 25 ml *f*/4 medium batch cultures with 4X vitamin stock (Guillard 1975) at 18^{o}C under 16/8 LD cycled cool white fluorescent tubes at 160 μE m^{-2} s^{-1}.

Growth from triplicate cultures was monitored by *in vivo* fluorescence with a TD700 fluorometer (Turner Designs, Sunnyvale, CA). Samples for bacterial attachment were collected from duplicate cultures (and pooled) during log and early stationary phases and fixed as below. A first *L. polyedrum* bloom (summer of 2003) was sampled at the surface approximately every two days as below. For a subsequent *L. polyedrum* bloom (summer 2005), surface seawater samples were collected daily and depth samples (1 m increments from surface to 6 m depth) taken on seven different occasions, fixed in 5% unbuffered formalin (= 1.8% formaldehyde) and frozen at -20^{o}C. The 2005 bloom was interrupted by a ten-day period and a subsequent five-day period when algal numbers were low and ocean discoloration was not evident. We did not sample these periods as we had assumed the bloom was terminated. As the bloom reappeared, sampling resumed as before. For all field samples, algal cell numbers in fixed samples were enumerated in 24 well plates with an inverted microscope (Olympus IX-71) under brightfield illumination. Depth samples were collected from the Scripps pier with a peristaltic pump and a weighted tygon® tube to decrease the sampling variability of Niskin bottles that collect water both at the top and the bottom of the device. Bacterial attachment was quantified after CARD-FISH. Total bacteria from the 2003 bloom were enumerated by fluorescence microscopy after DAPI staining (Porter & Feig 1980). Total bacteria from the 2005 bloom were enumerated with a FACSort flow cytometer (BD Bioscience, NJ, USA) after fixation and staining cells with SYBRgreen I (Lebaron et al. 1998) (Invitrogen, CA, USA).

CARD-FISH. In order to quantify the number of bacteria attached to dinoflagellate cells, CARD-FISH (catalysed reporter deposition-fluorescent *in situ*

hybridization) was used with a protocol slightly modified from Pernthaler et al. (2002). Fixed culture and field samples were thawed (if previously frozen), gravity settled overnight (or gently centrifuged at 200 x *g* for 5 min), washed in 1X PBS, and resuspended in 50% ethanol. This protocol prevented free-living bacterial cells from becoming artificially attached to algal cells during filtration. Samples were spotted on Teflon-coated 10-well slides and air dried. The slides were dipped in 0.1% low melting point agarose, air dried, incubated in 1 mg ml^{-1} lysozyme (Sigma) in TE buffer at 37^{o}C for 1 hr, washed 3X in MilliQ water, incubated for 10 min in 0.1 N HCl, washed 3X in 1X PBS (3 min), and dehydrated in an ethanol series (50%, 80%, 95% for 3 min each). Slides were incubated for 2 hr at 35^{o}C in a hydrated chamber with hybridization buffer (35% formamide, 900mM NaCl, 20mM Tris, 0.01% SDS, 20% Roche Diagnostic Boehringer blocking reagent) containing 1 μl probe for every 25 μl buffer. We used eubacterial probe eub338 (Amann et al. 1995) conjugated with horseradish peroxidase (Eurogentec, San Diego, CA). Slides were subsequently washed for 20 minutes at 37^{o}C in wash buffer (70 mM NaCl, 5mM EDTA, 20 mM Tris, 0.01% SDS), rinsed in MilliQ, and overlaid with TNT buffer for 15 min. Wells were then incubated with 1 μl tyramide-Alexafluor488 (Molecular Probes, Eugene, OR) in 100 μl 1X PBS with 0.01% Boehringer blocking reagent, washed with TNT buffer at 55^{o}C, rinsed in water, air dried, stained with DAPI (Porter & Feig 1980), and mounted with Vectashield (Vectorlabs, CA, USA). Negative controls were always run simultaneously and included either no-probe or archaea-specific probe ARC915 5'–GTGCTCCCCCGCCAATTCCT–3' (Amann et al. 1990), which gave similar signals since the permeabilization protocol was not designed

for archaea. Bacteria attached to dinoflagellate cells were visualized on an Olympus BX51 epifluorescence microscope with a standard DAPI filter set and a FITC-Texas red dual band filter set (Chroma Technology Corp, VT). Up to 20 individual bacteria on an algal cell could be counted with minimal error, while higher numbers, which were rare, were categorized as 20 for statistical analyses. Our counts were most probably underestimates due to the inability to detect all probe-positive bacterial cells located behind the autofluorescent algal cells.

Statistical analyses. Algal growth rates in batch cultures (μ, day^{-1}) were calculated using *in vivo* fluorescence values taken at days 8 and 17 after inoculation, using the equation $\mu = \ln(T_{17}/T_8)/9$. Between 22 and 50 individual *L. polyedrum* cells were examined for bacterial attachment from each pooled culture and treatment (log or stationary phase). These data were normally distributed and a one-way ANOVA was performed to determine if growth phase affected bacterial attachment. For field samples, between 10 and 70 *L. polyedrum* cells were examined for each sample depth and day. These data were not normally distributed, and Kruskal-Wallis tests were performed to determine if depth affected bacterial attachment. For temporal cross-correlation analyses, data were log transformed to satisfy assumptions of normality and lagged manually (+/- 3 days) due to uneven sampling. Correlation analyses for each lag were performed in JMP v5.0.

RESULTS

Bacterial attachment in batch cultures. Batch cultures of *L. polyedrum* exhibited considerable differences in growth yield, measured by the maximum *in vivo* fluorescence (Fig. 3.1). We classified the cultures into high yield (solid lines on Fig. 3.1) and low yield (broken lines on Fig. 3.1). One low-biomass culture (Lp 303) did not reach stationary phase due to its low growth rate and might have been considered high yield if we had carried out the experiment longer. Growth rates were calculated as the slope of the measured fluorescence between days 8 and 17 (Fig. 3.2a, top panel). There was no correlation between growth rate and growth yield. Some cultures, such as JF3 and Lp1936, had relatively high growth rates but low growth yield: their populations started to decline relatively early (27 days after inoculation). Other cultures, such as JF5 and JF1, had medium growth rates but high growth yields: their populations remained stable (> 1,000 relative fluorescence units) for several weeks after day 27 (not shown). Bacterial attachment was variable and ranged from near zero to a mean of three bacteria on each algal cell (Fig. 3.2a, bottom panel). Bacterial attachment in log phase was greater than at stationary phase, with a p-value nearly significant at the 95% level (one-way ANOVA, $p = 0.0587$). Growth rates were lower in cultures with more bacteria attached during log phase, although the p-value was only significant at the 90% level (Fig. 3.2b). If we removed culture Lp303 that did not reach stationary phase, the regression analysis was significant at the 95% level (inset of Fig. 3.2b). Interestingly, cultures with higher growth

yield had higher bacterial attachment numbers in log phase, although this analysis was only statistically significant at the 90% level (one-way ANOVA; $R^2 = 0.31$, $p = 0.096$).

Bacterial attachment in natural blooms. The dynamics of algal blooms have been previously classified into stages of initiation, maintenance, and decline (Steidinger & Vargo 1988). The natural *L. polyedrum* blooms sampled here (summer 2003 and 2005) each exhibited two prominent peaks of algal abundances (Fig. 3.3, solid lines), and it was not possible to determine if the two peaks represented separate events or whether it was the same bloom that returned. Therefore, we considered each bloom as two separate (but related) events, each with their own initiation, maintenance, and decline phases.

Total bacterial abundances during both the 2003 and 2005 blooms were elevated compared to non-bloom conditions. Average bacterial abundances at Scripps pier, as in most coastal mesotrophic environments, are on the order of 10^6 cells ml^{-1} (Ammerman et al. 1984). Bacterial abundances during the *L. polyedrum* blooms ranged from 1×10^6 to 2×10^7 cells ml^{-1} (Fig. 3.3), at times 20 fold higher than average. Cross correlation between *L. polyedrum* abundances and total bacteria were not statistically significant for the 2003 bloom, but were significant (and positive) for the 2005 bloom with zero ($r = 0.57$, $p < 0.0001$), +2 days ($r = 0.16$, $p = 0.037$), and -2 days ($r = 0.29$, $p = 0.0028$) lags. The significant 2 d lag correlation is due to the highest peak in bacterial abundance (on July 4, 2005) coming 2 d before the algal peak prior to the first crash (on July 6, 2005). The positive zero lag correlation is due to the concurrent peaks in both *L. polyedrum* and bacterial abundances on August 9, 2005, prior to the final bloom crash. The position

minus 2 day correlation is due to the increased bacterial abundances following the final bloom peak on August 11, 2005.

The mean number of bacteria attached to each *L. polyedrum* cell in nature was quite variable, both in magnitude and how it changed temporally. In both the 2003 and 2005 blooms, sample means ranged from zero to 12 bacteria per cell (Fig. 3.4) and could go from one extreme to the other over the course of 3 d. Due to the low total number of samples and lower temporal resolution, the cross-correlation analysis of the 2003 bloom data was not statistically significant. The two peaks of algal biomass were followed by peaks of bacterial attachment (Fig. 3.4a), and the corresponding negative 1 d lag (bacteria peak after the algae) resulted in the highest correlation coefficient of all lags analyzed ($r = 0.75$, $p = 0.25$). The 2005 bloom, on the other hand, was much longer in duration and was sampled more frequently. Bacterial attachment was relatively low during the first maintenance phase and peaked on July 6 when algal numbers peaked before the first decline phase. During the second bloom phase, bacterial attachment gradually increased, peaked on August 8, 1 d before the peak in algal numbers preceding the last decline phase (Fig. 3.4b). Analysis of these data resulted in a significant negative cross-correlation with a positive 2 day lag (bacteria peak before an algal crash; $r = 0.13$, $p = 0.016$) and a negative 3 day lag (bacteria peak after an algal crash; $r = 0.11$, $p = 0.036$).

Another useful measure of bacterial colonization is the percentage of algal cells colonized by at least one bacterium, which ranged from 2-92% in 2003 and 6-100% in 2005 (Fig. 3.5). Again, this measure was highly variable in magnitude and time, as *L. polyedrum* cells could go from being nearly 100% colonized to less than 10% (and vice-

versa) in two days. Cross-correlation of the 2003 data was again not statistically significant but showed the same trend as the average colonization intensity. The 2005 data were statistically significant and negative at the 95% level, with a lag of 2 days (bacteria peaked 2 days before an algal crash; $r = 0.15$, $p = 0.031$).

Bacterial attachment by depth. We sampled the 2005 bloom at different depths on seven occasions to test hypotheses about the relationship between bacterial colonization and depth. A log scale plot of *L. polyedrum* abundances over that time period (Fig. 3.6) shows some population dynamics during low algal abundances that were not apparent on a linear scale, including a short bloom of lower abundances that occurred between the first and the second large peaks in abundances. This intermediate bloom was first sampled on July 20, peaked on July 22, and crashed on July 23.

The water samples for the colonization data over depth were collected during the first large bloom and the second small bloom. Samples comprised the maintenance phase of the first bloom (June 16, June 29), the peak before the first crash (July 5), the first decline phase (July 7), the first post-bloom phase (July 8), and the peak before the second crash (July 21 and 22). During the first maintenance phase, there were no significant differences in bacterial colonization intensity over depth (Fig. 3.7a, b). Two days before (July 5) and during (July 7) the first decline phase, algal cells at depth were significantly more colonized by bacteria than algal cells at the surface (Fig. 3.7c, d). During the first post-bloom phase (low algal abundances), there were again no significant differences in bacterial colonization (Fig. 3.7e). Finally, during the second minor bloom peak, two days

before the crash, the algal cells at the surface were more colonized (Figure 3.7f), and one day before the crash, the algal cells near the bottom were more colonized (Figure 3.7g). These data are consistent: samples during maintenance or post-bloom phases showed no difference in bacterial colonization over depth, whereas samples preceding or during the bloom decline phases showed significantly higher bacterial colonization at depth compared to the surface.

DISCUSSION

We found bacterial attachment in log phase was inversely related to algal culture growth rate (Fig. 3.2b): cultures with fewer attached bacteria grew faster. This is consistent with the idea that attached bacteria exert a negative influence on the algae, although the converse (slower-growing algal cells attract more bacteria) is also a possibility. Regardless of which is the cause and which the effect, colonization intensity may provide a marker for algal growth status if this trend holds true in nature.

Contrary to previous reports suggesting that bacterial attachment increases from log to stationary phase (Vaqué et al. 1990, Simon et al. 2002), we found lower bacterial attachment in stationary phase compared to log phase (Fig. 3.2a). Since no other study has examined a high number of cultured strains of the same species (ten, in our case), we cannot compare our results to previous ones. Stationary phase algal cells are less healthy than those in log phase (Veldhuis et al. 2001) and may produce more cell surface mucus as protection against bacteria (Azam & Smith 1991). Our data showing lower bacterial

attachment in stationary phase are consistent with the latter hypothesis. Although we did not sample the decline (late stationary) phase of the cultures, it is likely that bacterial colonization increased at that point, since dead cells (detritus) are known to be highly colonized by bacteria (Caron et al. 1982, Bidle & Azam 1999).

Unlike Vaqué and colleagues (1990), we found very high temporal variability in bacterial colonization during natural blooms, especially when examining the percentage of algal cells colonized on any given day (Fig. 3.5). For instance, over one night during the 2003 bloom, the *L. polyedrum* population went from 56% colonized down to 2% (between 9/21 and 9/22). Twenty days later (between 10/9 and 10/10), the population went from 4% colonized up to 76% over one night. The noted differences between our and the previous study could be due to several factors, including that we examined natural blooms (versus mesocosms), used a different method of quantification (CARD-FISH vs DNA staining with acridine orange), and examined a different algal class (dinoflagellates versus diatoms). A particularly relevant attribute of certain thecate dinoflagellates, including *L. polyedrum*, is their ability to form temporary cysts through ecdysis. The extensive temporal variability of bacterial attachment in nature is most easily explained by the finding that *L. polyedrum* cells remove attached bacteria by forming temporary cysts by ecdysis (Mayali et al. submitted). Temporary cysts of *L. polyedrum* have been found in nature at the end of a bloom (Marasovic 1989), and there is further evidence that some dinoflagellate blooms terminate via cyst formation (Wang et al. 2007). While the ecdysis hypothesis may explain how algal cells could quickly lose attached bacteria, it does not explain how they could gain them. Future work combining

bacterial attachment dynamics, measures of algal physiology (such as membrane integrity and surface properties), and spatial sampling to quantify horizontal patchiness will help constrain hypotheses about the causes of increased local bacterial attachment over time.

The temporal variability of total bacterial abundance in the context of algal bloom dynamics revealed some interesting patterns, particularly the close relationship between them as evidenced by significant cross correlation analyses. This finding is not surprising as phytoplankton-derived organic matter is thought to provide the majority of nutrition to bacteria (Cole 1982). These data show evidence of bottom-up control of bacterial abundance by the presence of the dominant algal species, presumably the major producer of organic matter during the bloom. We also documented a sudden 4-fold increase in bacterial abundances on July 4, 2005, when numbers increased from 6 to 23 x 10^6 cells ml^{-1} in one day. Such a high and fast increase has been documented previously in a dinoflagellate bloom (Gasol et al. 2005) and was explained by the vertical migration of the dinoflagellate cells. In our case, there did not appear to be a drastic change in algal cell abundances on July 4, and the bacterial bloom remains unexplained.

An examination of the temporal variability of bacterial attachment in the context of bloom dynamics revealed interesting patterns. No statistically significant patterns were detected with the 2003 bloom data, most likely due to the low sampling frequency. One pattern emerged, however, as peaks in bacterial attachment seemed to precede peaks in algal abundances (Fig. 3.4a). The 2005 bloom data also exhibited this trend with statistically significant results due to the higher sampling frequency and longer bloom length. Noteworthy is the high frequency of algal cells colonized by bacteria found on

July 5, just one day after the bloom of bacteria on July 4. This finding is in agreement with Vaqué et al. (1989) who found highest colonization when both bacterial and algal numbers were high. However, this relationship did not hold true for the rest of the bloom, as a multiple regression analysis of bacterial colonization with both bacterial and algal abundances as variables was not significant (data not shown).

Based on both field and culture data, the following is one interpretation of the observed 2005 bloom bacterial attachment dynamics. During the first maintenance phase (June 13 to July 5), bacterial attachment was relatively low, probably due to a combination of surface polysaccharide production and ecdysis. This is similar to batch cultures in log or early stationary phase. The day before the first bloom decline (July 6), bacterial attachment increased to an average of 3 bacteria per algal cell. It is tempting to hypothesize that increased bacterial attachment either caused the bloom decline directly or was in response to another factor that led to bloom decline, such as nutrient limitation or infection by viruses or eukaryotic pathogens. At this point, these hypotheses remain untested. After the algal death or ecdysis responsible for the first decline phase, algal cells were again not highly colonized. During the following weeks of low and intermediate algal numbers, which included a smaller bloom, bacterial attachment increased gradually, unlike the first maintenance phase after which bacterial attachment was abrupt. We cannot explain this difference but speculate that perhaps bacterial attachment stimulated growth during the second bloom phase rather than inhibited it. The other possibility was that the apparent algal population growth was not due to actual growth but rather advection, aggregation, or excystment of cells from the first bloom

phase. In that case, the high number of attached bacteria may still have been a sign of low growth rate and unhealthy cells. The second and highest (12 bacteria per algal cell) peak in bacterial attachment (August 8) came one day before the final peak in algal numbers. In that case, high bacterial attachment preceded and may have caused the bloom decline. Such a pattern, if repeatable, could be used in future studies to determine bloom phase and to predict bloom decline.

We further examined whether depth affected bacterial attachment to *L. polyedrum* during the 2005 bloom. Most bloom-forming dinoflagellates, including *L. polyedrum*, perform daily vertical migrations to absorb nutrients at depth and photosynthesize during the day (Eppley et al. 1968). We hypothesized that unhealthy algal cells swim more slowly, based on laboratory incubations of *L. polyedrum* with algicidal bacteria (unpublished data) and evidence that eukaryotic pathogenesis decreases swimming speed (Park et al. 2002). If increased bacterial attachment in natural blooms is a sign of unhealthy algal cells, then bacterial attachment should be higher at depth than at the surface. This would be due to the higher sinking rates of unhealthy cells that cannot swim upward as well as their healthy counterparts. In support of this hypothesis, we did find increased bacterial attachment at depth, but only during the decline phase of the bloom and not during the maintenance phase. These results are again consistent with the ideas that 1) increased bacterial attachment is a sign of unhealthy algal cells and 2) bacteria play a role in algal bloom decline, either directly or responding to other factors.

The work presented here strongly suggests that bacteria play a major role in the dynamics of algal blooms, particularly through attachment to algal cells. This idea is not

novel (Cole 1982, Doucette 1995, Doucette et al. 1998) but surprisingly little data from natural blooms exist to support it. The long list of studies demonstrating changes in bacterial community structure during algal bloom progression (reviewed by Garcés et al. 2007) has been explained by the idea that the organic matter released by the algae changes throughout the bloom, affecting the next trophic level (the bacteria). How the new microbial community then turns around and affects the phytoplankton cells has largely been ignored. Recently, a novel study has documented increased bacterial numbers (on a daily basis) associated with vertically migrating dinoflagellate cells (Gasol et al. 2005), again suggesting a bottom-up stimulation of bacterial abundance due to exuded organic matter from the dinoflagellates. Future work needs to address the physiological changes in the algal cells that allow increased bacterial colonization as well as species-specific effects of attached bacteria on algal physiology. Our present inability to predict the dynamics of algal blooms, particularly those harmful to people and ecosystems, merits further research in this area.

Table 3.1. List of clonal, xenic *Lingulodinium polyedrum* strains used in this study; all cultures were isolated from the coast of La Jolla, CA, USA.

Strain name	Alternate name	Date of isolation	Isolated by
JF6	1708A5m#4	Fall 2004	J. Frommlet
JF4	1708#7	Fall 2004	J. Frommlet
JF2	1708#8	Fall 2004	J. Frommlet
LP1020	XM1020	Fall 2003	X. Mayali
CCMP1932	Lp 104	Spring 1998	A. Shankle
CCMP1936	Lp 703	Fall 1998	A. Shankle
JF3	1708#13	Fall 2004	J. Frommlet
JF1	1708#9	Fall 2004	J. Frommlet
JF5	1708#10	Fall 2004	J. Frommlet
CCMP1933	Lp 303	Summer 1998	A. Shankle

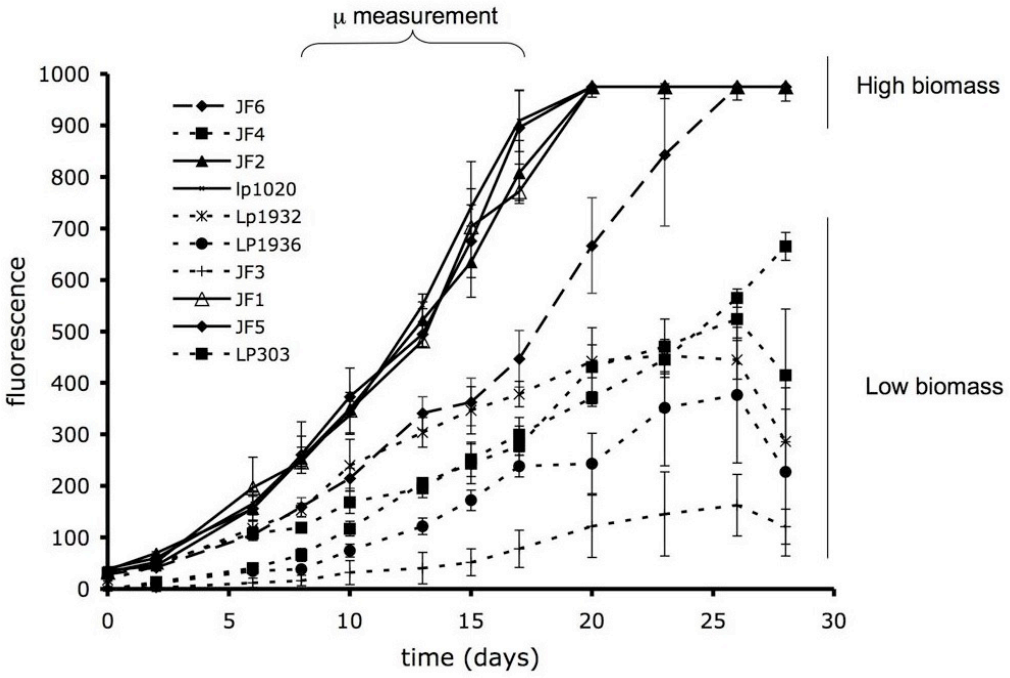

Figure 3.1. Growth of triplicate batch cultures of ten *L. polyedrum* strains (see Table 3.1) monitored by *in vivo* fluorescence (+/- standard deviation). The strains are separated among high and low biomass producing cultures (solid and broken lines, respectively). Growth rates (in Fig. 3.2) were calculated between the points indicated.

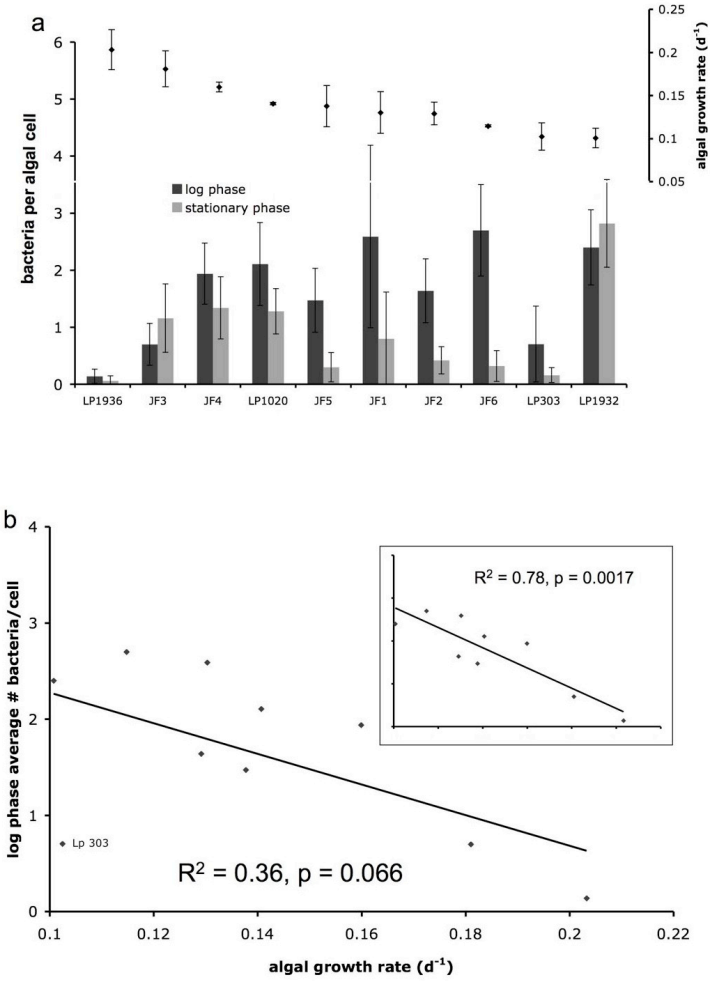

Figure 3.2. a. Bacterial attachment (enumerated after CARD-FISH) in log and early stationary phases of *L. polyedrum* cultures (bottom panel) and algal growth rates (top panel). Bacterial numbers represent means and 95% confidence intervals, and growth rates are means and standard deviations. A one-way ANOVA of growth stage effect on bacterial attachment was not significant but detected a trend ($p = 0.0587$); b. Linear regression of growth rate and bacterial attachment during log phase, showing a trend of lower bacterial attachment at higher growth rate. The model was significant if culture Lp303, which did not reach stationary phase, was removed (inset).

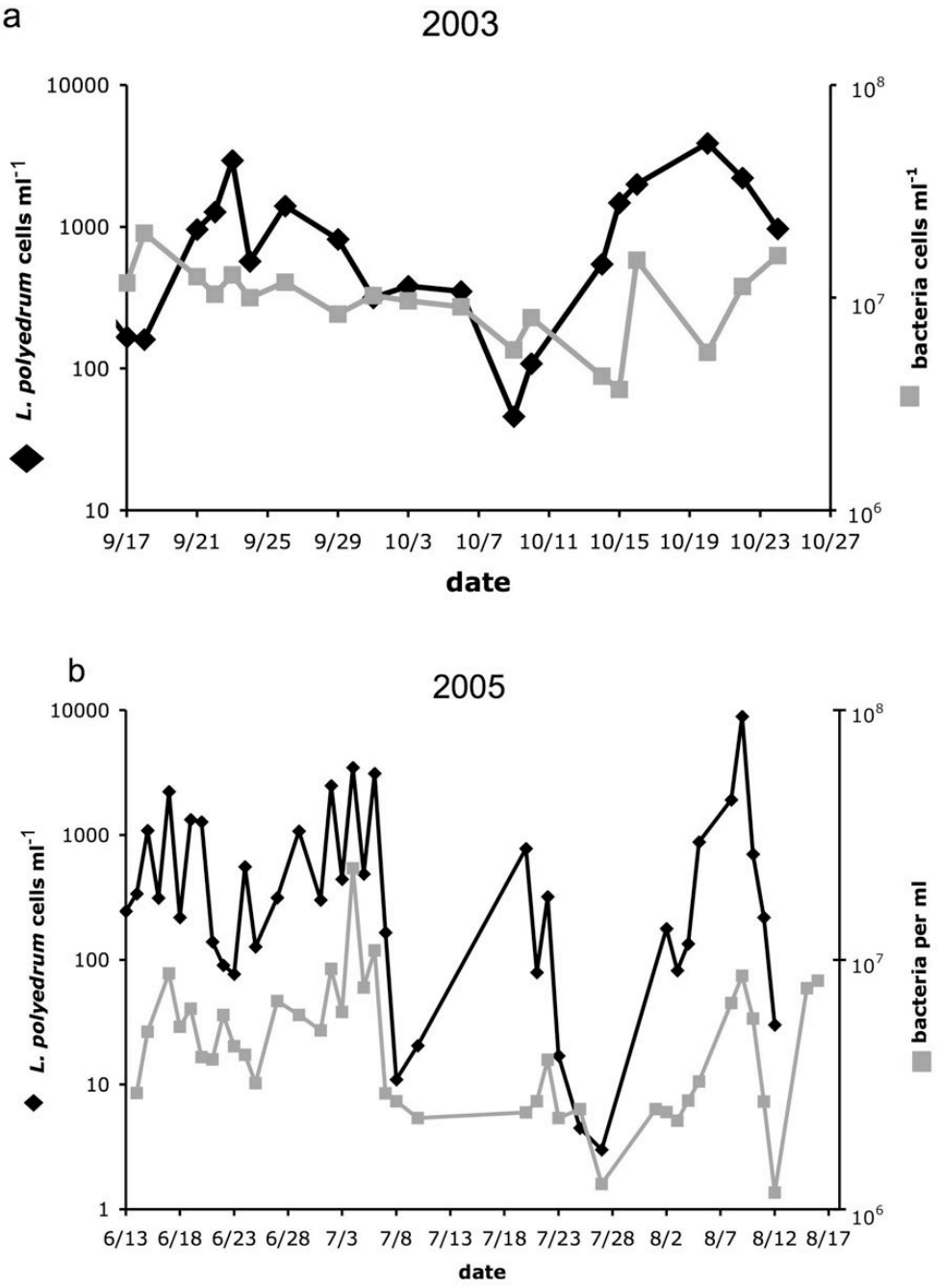

Figure 3.3. *L. polyedrum* abundances (black) and total bacterial counts (gray) for summer 2003 bloom (a) and summer 2005 bloom (b). Cross-correlation analysis was not significant for the 2003 data but was significant for the 2005 data with zero and both positive and negative two-day lags.

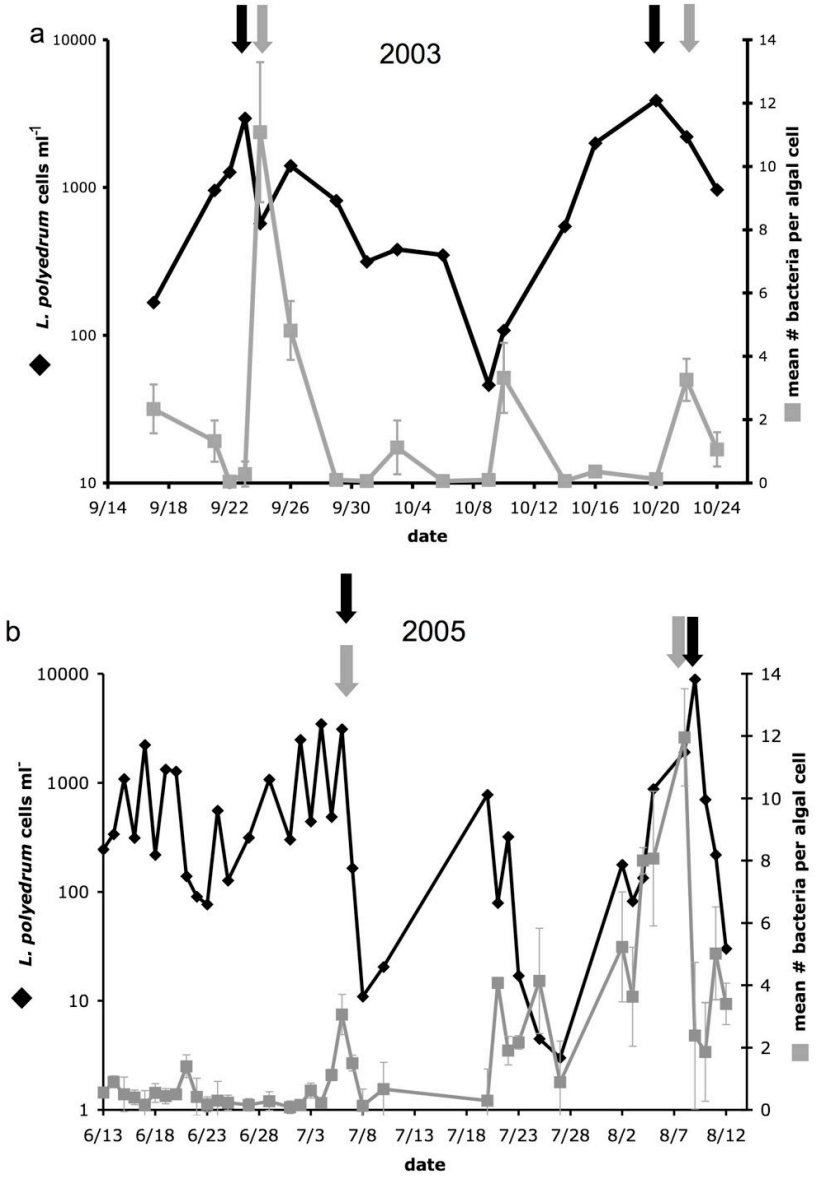

Figure 3.4. *L. polyedrum* abundances (cells ml^{-1}) and bacterial attachment (# bacteria per algal cell; means +/- 95% confidence intervals) for summer 2003 bloom (a) and summer 2005 bloom (b). Black and gray arrows above the plots indicate peaks in algal abundances and bacterial attachment, respectively. Cross-correlation analysis was not significant for the 2003 data but was significant for the 2005 data with a 1-day lag.

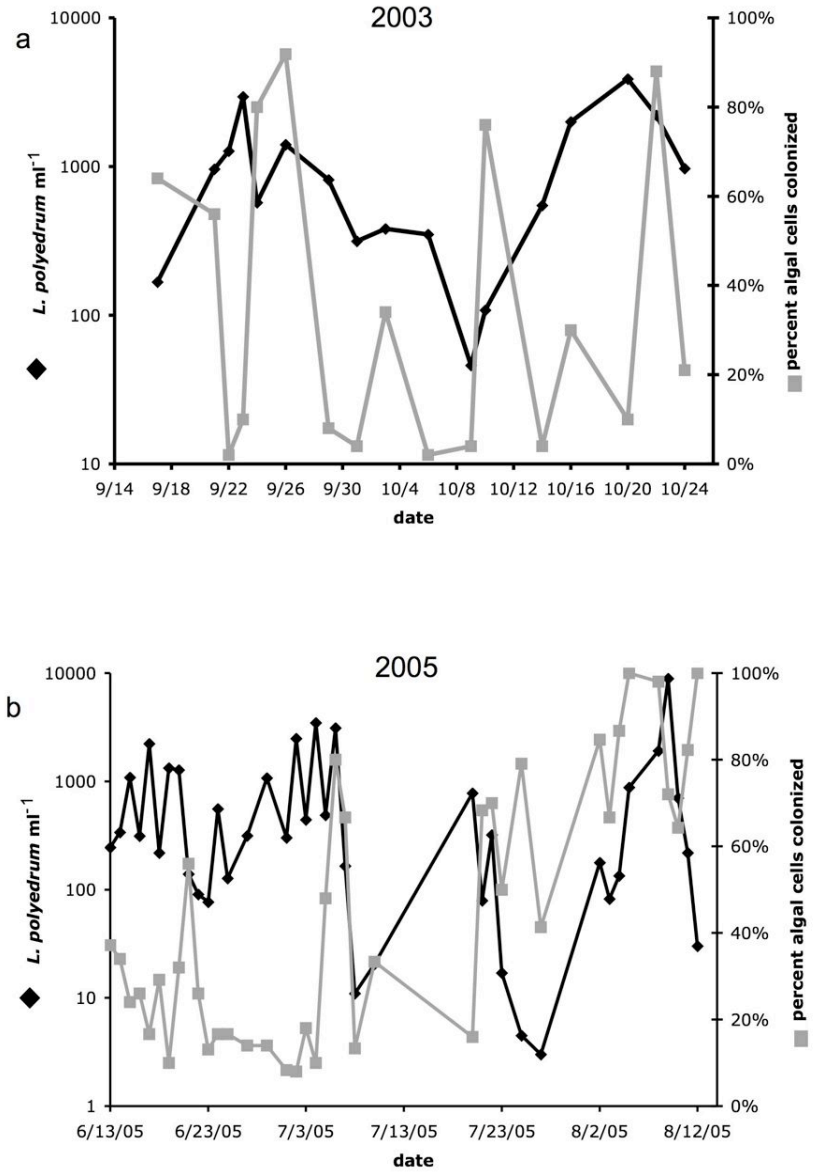

Figure 3.5. *L. polyedrum* abundances (black) and percent algal cells colonized by bacteria (gray) for summer 2003 bloom (a) and summer 2005 bloom (b).

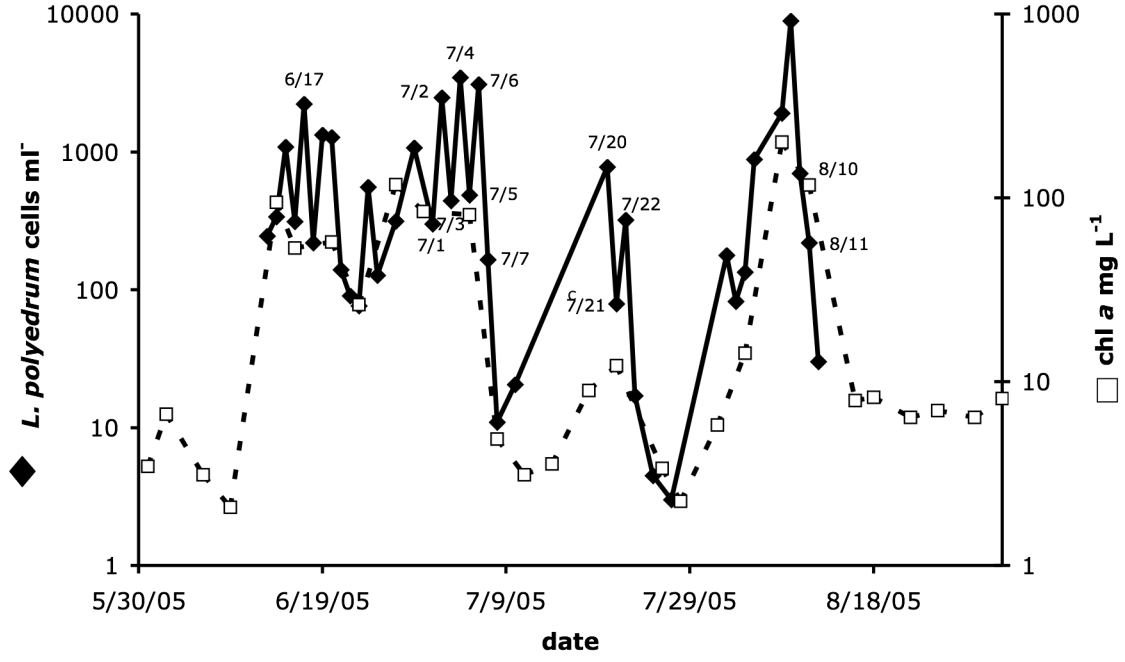

Figure 3.6. *L. polyedrum* abundances during the 2005 bloom (black solid line). Points labeled with the date were sampled for bacterial attachment at different depth (plotted on Fig. 3.7).. Also shown are extracted chlorophyll a data from the Scripps pier Chlorophyll Program (broken line).

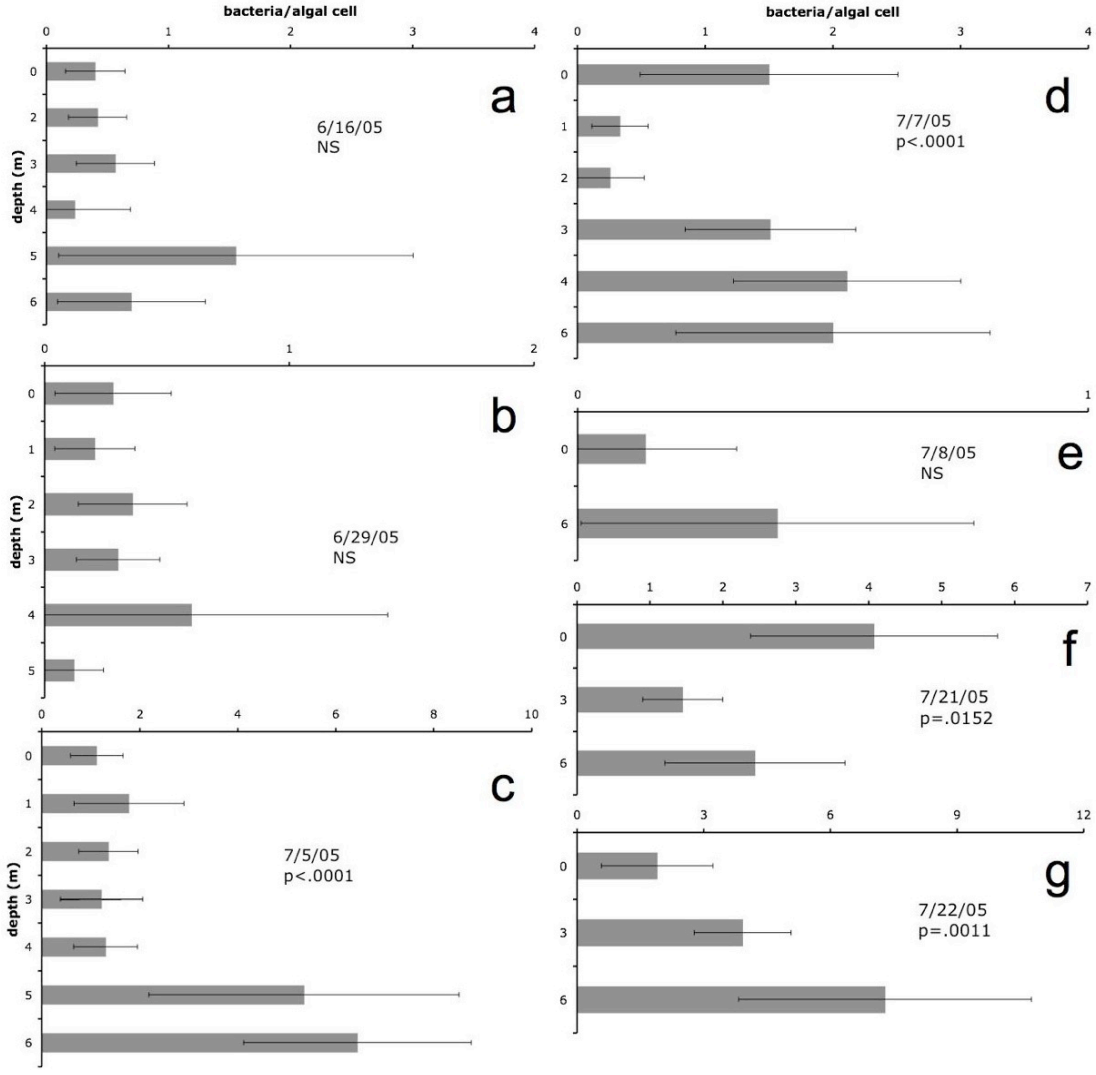

Figure 3.7. Bacterial colonization (mean number of bacteria per algal cell +/- 95% C.I.) as a function of depth. Dates with a p-value indicate bacterial attachment was significantly affected by depth as measured by a Krustal-Wallis test.

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IV

Bacteria-induced motility reduction in a marine dinoflagellate

ABSTRACT

Interactions among heterotrophic bacteria and phytoplankton have been identified to be central to the understanding of marine food web dynamics. Here we describe, for the first time, evidence that certain marine bacteria decrease the swimming speed of motile phytoplankton through the release of putative protease(s). Using the dinoflagellate *Lingulodinium polyedrum* as a model system, we show that the motility-reducing components of bacterial-algal cocultures were mostly heat-labile, of high molecular weight (>50kD), and could be partially neutralized by incubations with protease inhibitors. We further show that additions of purified protease decreased dinoflagellate swimming speed in a concentration-dependent manner. We then describe the first quantification of dinoflagellate swimming speeds from natural populations, finding the lowest motility during the bloom decline phase. Lower algal swimming speeds were correlated with higher proportions of algal cells colonized by bacteria, suggesting a relationship between bacterial attachment and algal motility. We propose that motility can be used as a marker for dinoflagellate stress or general unhealthy status and may be useful to predict imminent bloom decline.

INTRODUCTION

Blooms of dinoflagellates and other phytoplankton, many of which produce potent toxins that adversely affect wildlife and human health, appear to be increasing in incidence worldwide (Van Dolah 2000). While there is currently no way to predict bloom formation or decline, certain environmental conditions are known to be optimal for dinoflagellate blooms, including water stratification and a subsurface source of nutrients (Paerl 1988). Motility is a key element that allows dinoflagellates to outcompete other phytoplankton in those conditions, as they can perform diel vertical migration (DVM) to uptake nutrients below the thermocline at night and photosynthesize at the surface during the day (Smayda 1997). The factors that trigger or prevent DVM are not well characterized and are sometimes contradictory. The dinoflagellate *Lingulodinium polyedrum*, the subject of this study, has been shown to vertically migrate both in nature and in deep tanks, but stopped migrating under nitrogen stress (Eppley et al. 1968). Conversely, experiments with a closely-related organism, *Alexandrium tamarense*, showed that DVM in tanks was triggered to begin following nitrogen stress: as nitrate became limiting, the cells migrated progressively deeper (MacIntyre et al. 1997). Whichever the cue, it is clear that dinoflagellates possess sensory mechanisms to direct swimming as they exhibit both phototaxis and geotaxis (Kamykowski et al. 1998).

Besides sensing their environment, the other factor critical for dinoflagellates to successfully carry out DVM is the ability to swim fast. Instantaneous speeds of *L. polyedrum* have been measured between 250 and 400 μm s^{-1} (reviewed by Lewis &

Hallett 1997), which is roughly 10 body lengths s^{-1}. Due to the helical swimming pattern (they do not swim in straight lines), the overall migration rate of *L. polyedrum* has been empirically measured to be between 1-2 m hr^{-1} (Eppley et al. 1968). Factors that influence both instantaneous swimming speed and trajectory will therefore have a significant effect on DVM. Some experiments have demonstrated an effect of shear on dinoflagellate trajectories (Karp-Boss et al. 2000). Instantaneous speed of *L. polyedrum* is affected both positively and negatively by salinity and temperature (Hand et al. 1965), and ultraviolet radiation decreased the percentage of motile cells of the dinoflagellate *Gymnodinium* sp. (Tirlapur et al. 1993). In addition, various studies have described biologically-mediated motility reduction of flagellates, though the actual swimming speeds of the organisms were not directly quantified. For example, toxins from *Anabaena* inhibit the motility of *Chlamydomonas* (Kearns & Hunter 2001), potentially explaining their negatively correlated abundances in nature. Both viral infection (Lewis & Hallett 1997) and programmed cell death mediated by inorganic carbon limitation (Vardi et al. 1999) have been shown to increase sinking rates and decrease flagellate motility, respectively. No studies to date have examined the effect of heterotrophic bacteria on the motility of eukaryotic phytoplankton.

Interactions between bacteria and phytoplankton play major roles in structuring marine pelagic ecosystem dynamics (Cole 1982, Azam 1998), yet it is probable that we have not yet identified many of these associations. There exist many examples of bacteria that directly affect phytoplankton physiology, leading to changes in growth (Ferrier et al. 2002), sexual reproduction (Adachi et al. 2001), and even cell death, the latter often

induced by algicidal bacteria from the Bacteroidetes division (see review by Mayali & Azam 2004). Although algal-bacterial interactions are likely occurring constantly, their magnitude and frequency may be increased during an algal bloom because there are more phytoplankton cells present in the water column, and there are sometimes more bacteria as well (Gasol et al. 2005). Such increased abundances of both algae and bacteria have two potential consequences: increased collision frequencies leading to increased bacterial colonization (Vaqué et al. 1989), and a greater potential for excreted substances (e.g. allelochemicals, antibiotics) to be effective in such a "crowded" environment.

We have recently isolated three bacterial strains, related to previously isolated algicidal bacteria, capable to decreasing net algal growth rates and inducing temporary cyst formation of the bloom-forming dinoflagellate *L. polyedrum* (Mayali et al. accepted). We observed that before cyst formation, the algal cells looked "sluggish" and appeared to swim more slowly than axenic (bacteria-free) control cultures. Since several studies (Lee et al. 2000a, Lee et al. 2000b) have implicated excreted enzymes as putative algicidal agent(s), we examined whether excreted enzymes were implicated in this motility reducing phenomenon. We also report directly measured motility of field populations of *L. polyedrum* in the context of an extensive and long-lasting bloom during the summer of 2005 on the coast of La Jolla, CA, USA. The hypotheses tested in this paper were the following: 1) our algicidal bacteria isolates excrete proteases that reduce *L. polyedrum* motility, 2) additions of purified protease similarly reduce *L. polyedrum* motility, 3) slower dinoflagellate motility is detected during the bloom termination phase when algal cells are unhealthy, and 4) *L. polyedrum* motility is not constant with depth.

MATERIALS AND METHODS

Laboratory incubations. Axenic *Lingulodinium polyedrum* strain CCMP 1932 batch cultures were grown in 25 ml *f*/4 medium with 4X vitamin stock (Guillard 1975) at 18^{o}C under 16/8 LD cycled cool white fluorescent tubes at 160 μE m^{-2} s^{-1}. Bacterial isolates ALC1, LPK5, and LPK13 were grown in ZoBell 2216 medium (5 g peptone, 0.5 g yeast extract in 1 L autoclaved seawater), washed in *f*/4, and added to algal cultures at final concentrations of 10^{6} bacterial cells ml^{-1}. Algal culture growth was monitored by *in vivo* fluorescence with a TD700 fluorometer (Turner Designs, Sunnyvale, CA). When fluorescence in bacterial-algal co-incubations decreased (compared to no addition controls) due to algal cells settling, cultures were filtered through a 0.22 μm acrodisc HT Tuffryn® filter (Pall Life Sciences) to remove both algal and bacterial cells. Filtrates were size fractionated using Centricon® filters (Millipore, Billerica, MA) and/or incubated at 80^{o}C for 10 minutes to denature proteins. To test whether the filtrates contained active proteases, we tested three irreversible protease inhibitors for their ability to suppress motility reduction. These included the cysteine-protease inhibitor E-64 (Sigma cat. # E3132; working stock 2.8 mM in distilled water) and the serine protease inhibitors PMSF (Sigma cat # P7626; working stock 100 mM in isopropanol) and Pefabloc®SC (Sigma cat # 76307; working stock 210 mM in distilled water ph = 5.2). Algal filtrates were incubated in 10, 100, and 100 μM (final concentration) E-64, PMSF, and Pefabloc®, respectively for 30 minutes at room temperature in the dark. Subsequently, these filtrates were washed through a Centricon®-50 to remove the unbound inhibitor. This step was

crucial as presence of the unbound protease inhibitors decreased dinoflagellate motility on their own (data not shown). We could not test reversible protease inhibitors such as amastatin or leupeptin as these also decreased dinoflagellate motility. Treated filtrates were resuspended in f/4 medium (4.5 ml final volume), and dinoflagellate cells from late log phase cultures (500 μl) were added and incubated for 16 hours in the dark. All experiments were performed in triplicate. Two sets of two experiments were performed, each set using the same batch of stock *L. polyedrum* cultures and motility-reducing filtrates. The first two experiments were completed within 24 hours of each other, and the second set one month later, also within 24 hours or each other.

Analysis of motility patterns. After incubations, algal cells in treated filtrates were mixed up and down with a pipette and placed in a plastic cuvette. Samples were illuminated from the side with a metal-halide cold spot light source PCS-UMX250 (Nippon-PI, Tokyo, Japan). Cells were video recorded (also from the side) for 15 sec using a FASTCAM-PCI R2 camera (512 x 480 pixel resolution; Photron Co. Ltd., Tokyo, Japan) with a BSL-Z50HD lens and an additional 0.5x front converter. The data were saved as .avi files at 30 fps. Each video file was compressed into 1 image using Matlab and brightness and contrast adjusted with ImageJ. Cell tracks were qualified as fast, slow, or sinking (Fig. 4.1). Fast cells showed the characteristic helical swimming pattern of dinoflagellates. Sinking cells did not show any lateral movement and were unidirectional (usually vertical, although convection inside the cuvette sometimes altered the direction of sinking cells slightly). Slow cells exhibited an intermediate wave-like motility pattern,

neither fully unidirectional yet not helical. The differences in the percentage of fast and/or slow cells among treatments were compared with nonparametric Kruskal-Wallis tests.

Protease activity measurements. To determine if motility-reducing filtrates possessed higher protease activities than control filtrates, we compared cell-free *L. polyedrum* filtrate to the filtrate from a *L. polyedrum* ALC1 co-culture. We used four different 7-amido-4-methylcoumarin (AMC) labeled substrates (Hoppe 1983) conjugated to the amino acids leucine, serine, threonine, and glycine (Sigma). Change in fluorescence over time was monitored with a TD700 fluorometer with a Hoechst dye filter set (Turner Designs, Sunnyvale, CA). Control incubations in autoclaved seawater were used as negative controls. Data were fitted to a standard curve of AMC and are reported as nmol subtrate hydrolyzed per volume filtrate per hour.

Pronase experiments. We previously observed that additions of relatively high (1 unit ml^{-1}) concentrations of pronase E to *L. polyedrum* cultures led to fast motility reduction (within minutes) whereas additions of equivalent amounts of cellulase, phosphatase, lipase, or glucosidase had no effects (data not shown). We therefore incubated duplicate log phase *L. polyedrum* batch cultures (all from the same mother culture) in lower concentrations of pronase E (Sigma cat # P6911) over time at final concentrations of 36, 9, and 1.8 $\mu g\ ml^{-1}$ (equivalent to 0.18, 0.045, and .009 units ml^{-1}).

Samples were taken after 5 minutes, 30 minutes, 8 hours, and 23 hours. Cultures were filmed as above. Motility quantification was performed as below.

Field sampling. During a bloom of *L. polyedrum* during the summer of 2005 off the coast of La Jolla, CA, we collected seawater from Scripps pier at the surface (on 13 dates) and at depth (on 6 dates) for motility analysis. Depth samples were collected with a peristaltic pump and a weighted tygon® tube to decrease the sampling variability of Niskin bottles that collect water both at the top and the bottom of the device. Samples were collected during the same 4-hour interval (11:00 am to 3:00 pm) to minimize diurnal effects on motility. Before motility measurements, all samples were acclimated to the same temperature (22^{o}C) and kept in the dark for 2 hours. Samples (in triplicate) were filmed as above. To determine if motility data were correlated with bacterial colonization, we performed a linear regression analysis using bacterial colonization data from another study (Mayali et al. submitted). Both motility and bacterial colonization frequency data were log transformed to satisfy the assumptions of normality.

Motility quantification. Videos from pronase E addition experiments and natural populations were analyzed as follows (with Matlab). Fifteen frames, corresponding to 0.5 sec of movie time, were combined and the length of the cell path (in pixels) was calculated. This was repeated for the entire 15 sec movie. Small objects (less than 10 pixels in area) were omitted, and each pixel was approximately 10.63 μm (1 mm scale = 94 pixels). The resulting dataset (for each movie) was a frequency distribution of path

lengths (in pixels). Path lengths greater than 35 pixels were not included in the analysis as they represent actual swimming speeds of 600 μm s^{-1} (see below), much greater than literature data for *L. polyedrum* motility. Laboratory cultures of *L. polyedrum* never exhibited path lengths greater than 35 pixels. In order to translate pixels to instantaneous swimming speeds, we used an average cell diameter of 45 μm (average diameter of cells from one frame = 4.26 pixels):

$$speed(\mu m/\text{sec}) = ((\#\ pixels \times 10.63 \mu m/pixels) - 45 \mu m/cell) \times 2\ frames/\text{sec}$$

For duplicate (pronase E experiment) or triplicate (field samples) incubations, frequency distributions were combined before statistical analyses comparing treatments (or sampled days). Data were not normally distributed and were analyzed with nonparametric Krukal-Wallis tests. Post-hoc tests of significance (if necessary) were performed with a Wilcoxon test after p-value modification with the Dunn-Sidak method.

RESULTS

Motility effects. In most cases, a comparison of the fraction of fast cells was sufficient to compare treatments, although the fraction of slow cells was sometimes informative as well. The results from all the experiments are summarized in Table 4.1. In the first experiment, we determined that the motility-reducing component of the ALC1 filtrate originated in the >50kD fraction and was not detectable in the <50kD fraction ($p = 0.028$). The motility-reducing component from LPK13 was found in both size fractions

(Fig. 4.2a; $p = 0.024$). Fractionated axenic *L. polyedrum* filtrates (<50kD and >50kD) were similar to the unfractionated control and are not reported.

In the second experiment, we found that E-64-treated axenic control filtrates had no effect on algal motility, while Pefabloc®SC-treated axenic control filtrates showed minor motility reduction, though not statistically significant (Fig. 4.2b). Comparing bacterial-filtrates to the controls, we determined that Pefabloc®SC neutralized the motility reduction in the ALC1 filtrate but not in the LPK13 filtrate ($p = 0.037$). The cysteine protease inhibitor E-64 did not have a dramatic effect on either ALC1 or LPK13 filtrate (both were statistically slower than the E-64-treated control filtrate; $p = 0.049$).

In the third experiment, we determined that as in ALC1, the motility-reducing component of the LPK5 filtrate originated in the >50kD fraction and was not detectable in the <50kD fraction (Fig. 4.2c; $p = 0.026$). Treatment of the filtrates from the three bacterial strains with the serine-protease inhibitor PMSF did not fully prevent motility reduction, although the treated LPK13 filtrate was significantly more motile than the untreated LPK13 filtrate based on the fraction of fast cells ($p = 0.037$).

In the fourth experiment, we determined that the motility reducing >50kD fraction from LPK13 could be denatured at 80°C and thus was likely a protein, while the motility-reducing <50kD fraction retained most of its activity after this treatment (Fig. 4.2d; $p = 0.039$). We also found that the motility-reducing activity from LPK5 was almost fully prevented upon treatment with the serine-protease inhibitor Pefabloc®SC. On the other hand, the cysteine protease inhibitor E-64 did not reduce the motility reduction from

LPK5 ($p = 0.049$). Also included in this experiment was a control incubation of PMSF with the bacteria-free *L. polyedrum* filtrate.

Protease activity. Comparison of protease activities from the ALC1 and axenic *L. polyedrum* filtrates did not show dramatic differences in any of the four substrates tested (Table 4.2). Leucine-AMC and threonine-AMC hydrolysis rates were similar between the two treatments. Serine-AMC and glycine-AMC were consistently higher in the ALC1 filtrate compared to the axenic *L. polyedrum* filtrates, but the magnitude of increase was not dramatic (16% for both serine-AMC and glycine-AMC).

Pronase experiments. Before analyzing the motility data from algal cultures incubated with pronase E, we tested whether our analysis method could easily differentiate swimming and sinking cells. Frequency distributions of live, log phase *L. polyedrum* cells and formalin-fixed cells from the same culture were significantly different ($p < 0.0001$; Fig. 4.3), thus we were confident that we could statistically detect noticeable differences between treatments.

The first motility evaluation was performed 5 minutes after addition of pronase E, and the cultures with the highest concentration added (36 $\mu g\ ml^{-1}$) were already swimming significantly slower than the controls (Fig. 4.4; $p < 0.0001$). This decrease was apparent throughout the 23-hour experiment. Additions of 4-fold less pronase E led to decreased swimming speeds after 30 minutes ($p < 0.0001$), lasting throughout the experiment. Interestingly, additions of the lowest concentrations of pronase (1.8 $\mu g\ ml^{-1}$)

led to an initial increase in motility, which lasted through the 30-minute sampling ($p < 0.0001$). At the 8 and 23-hour sampling, the algal cells in those incubations were swimming significantly slower than the controls, but significantly faster than the cells incubated with the higher pronase E concentrations ($p < 0.0001$).

Field samples. The *L. polyedrum* bloom sampled during summer 2005 has been described in more detail elsewhere. It comprised three distinct bloom phases, each with peaks in abundances followed by declines. *Lingulodinium* dominated the autotrophic microplankton community during that period, as evidenced by good agreement between *L. polyedrum* cell counts and extracted chlorophyll *a* concentrations (Mayali et al. submitted). The motility data from the thirteen surface samples collected over the two-month period ranged from means of 11.8 to 17.8 pixels, corresponding to 160 to 287 μm s^{-1}. The sample with the lowest measured motility originated from August 10, during the third (and final) decline phase of the bloom (Fig. 4.5; statistically slower than the closest sample, $p < 0.0001$). The highest motility was measured from the July 4 sample, during the first maintenance phase of the bloom. The remaining data, according to statistical groupings, could be divided into moderately fast (between 15 and 17 pixels) and moderately slow (between 12 and 15 pixels) samples. At first glance, there appeared to be no particular pattern of temporal distribution of these data. However, a regression analysis between algal motility and the percentage of *L. polyedrum* colonized by bacteria (data from Mayali et al. submitted) was statistically significant (Fig. 4.6): lower motility was correlated with higher bacterial colonization of the dinoflagellates.

The last analysis included an examination of motility across depth. In three out of six samples, there was no significant difference detected among depths (Fig. 4.7). These included water taken on July 7 (during the first crash), July 21 (two days before the second crash), and August 11 (after the final crash). We consider the July 21 sample to have similar motility across depth even though the 1m sample was significantly different from the others (the pattern did not appear related to depth). On two dates, algal cells were significantly more motile at the surface compared to deeper depth: on July 5 (two days before the first crash) and on July 22 (one day before the second crash). The earliest sample taken (June 17) showed higher motility at depth compared to the surface.

DISCUSSION

Previous studies of bacterial interactions with phytoplankton have focused on easily observable effects, such as morphological change (e.g. cyst formation) or changes in growth rates (both positive and negative). Here we have revealed that some marine bacteria can excrete dissolved proteases that significantly decrease the swimming speed of the dinoflagellate *L. polyedrum*. Cell counts or fluorescence could not detect such changes in motility, and microscopic observation could only detect differences if they were extreme, such as when the algal cells were barely motile. Subtle differences in motility could only be detected after careful analysis of video files, which enabled us to subsequently detect differences in motility among natural bloom samples. Even a small decrease (10-20%) in instantaneous motility may not seem of great consequence but

would either decrease the depth that a population of cells can migrate down, or increase the time it takes to reach a particular depth. Either outcome will have important implications for the dinoflagellates' ability to compete with other phytoplankton since DVM is so critical to connect spatially dispersed nutrients and light under stratified conditions. Our results exemplify the concept that many potentially important interactions between heterotrophic bacteria and phytoplankton remain unobserved.

Laboratory incubations. Our finding that the protease inhibitors Pefabloc®SC, PMSF, and E-64 differentially affected the motility-reducing filtrates suggests that the three bacterial strains tested did not excrete the same molecule(s). Strain LPK5 most likely excreted a serine protease, as Pefabloc®SC neutralized its motility-reducing component. Results of experiments with the other two bacterial strains were more ambiguous. ALC1 likely excreted one or more large proteins, possibly proteases as two protease inhibitors slightly counteracted motility reduction. LPK13 excreted both proteinaceous (>50kD, heat labile) and non-proteinaceous (<50kD, heat resistant) components, the former likely including a protease as PMSF significantly (but not fully) neutralized the motility reduction. Previous studies of algicidal bacteria have uncovered numerous types of algicidal agents, both proteinaceous (Baker & Herson 1978, Lee et al. 2000b) and non-proteinaceous (Dakhama et al. 1993, Skerratt et al. 2002), in accordance with our findings.

Unexpectedly, we could not detect any difference in the protease activities (using four different exopeptidase substrates) between control and motility-reducing filtrates.

We conclude that 1) axenic *L. polyedrum* cultures exhibit dissolved protease activity, and 2) the motility-reducing proteases do not break down the exopeptidase substrates tested. The first conclusion is in accordance with previous work demonstrating that dinoflagellates produce protease activity (Stoecker & Gustafson 2003), although that study found the protease activity associated with the cellular surface and not in the medium. Our second conclusion is not surprising as (Obayashi & Suzuki 2005) found that endopeptidase activity can be similar or higher than exopeptidase activity in surface seawater. Other protease substrates, particularly those specific for endopeptidases, might reveal strong differences between control and motility-reducing filtrates.

Additions of the purified serine-protease pronase E showed a concentration dependent effect on *L. polyedrum* motility, and greater additions decreased motility faster. Previous work has shown that additions of pronase E increased diatom frustule dissolution (Bidle & Azam 1999) and decreased diatom cell aggregation (Smith et al. 1995) at concentrations of 1 and 0.1 units ml^{-1}, respectively. Here we have observed another effect of pronase E on phytoplankton with lower concentrations of added protease (9 $\mu g\ ml^{-1}$ is equivalent to 0.045 units ml^{-1}). Since proteolytic activity is measurable in seawater (Obayashi & Suzuki 2005) and marine bacteria can have very high protease hydrolysis rates (Martinez et al. 1996), it is likely that motility reduction by bacteria occurs in nature. These effects would be more significant during algal blooms, as bacterial abundances (Gasol et al. 2005) and enzymatic activities of bacteria (Fandino et al. 2001) are elevated during such events..

Field samples. Our investigation of microplankton motility by video analysis from natural samples is, to our knowledge, novel. Due to logistic reasons, we were not able to collect more than 13 surface samples for motility analysis over the course of the 2005 *L. polyedrum* bloom, and cross-correlation analysis could not be performed to determine if the temporal dynamics of motility and other factors (cell counts, etc) were related. Nevertheless, it is crucial to note that the sample with the slowest motility was obtained from the final bloom crash on August 10, 2005. This is possible indication that the bloom ended due to pathogen infection or senescence, as viral infection (Lawrence & Suttle 2004) and programmed cell death (Vardi et al. 1999) both lead to motility inhibition. Our finding that bacterial attachment to algal cells is negatively correlated with algal motility raises some intriguing possibilities. Perhaps bacteria can sense that an algal population is in senescence or in the late stages of pathogen infection, and then are able to increase their colonization of the algal cells. Another possibility is that the algal cells can no longer prevent bacterial colonization when they are infected or running out of nutrients (ie. the bacteria don't change their behavior but the algae do). Yet a third possibility is that bacterial colonization increases first, thereby leading to motility reduction and potentially other factors that contribute to bloom termination. This idea that bacteria "attack" organic matter in the form of algal cells has been discussed previously (Azam & Smith 1991, Azam 1998) and our findings corroborate this hypothesis. Most likely, all three possibilities mentioned above occur simultaneously, and their interactions and feedbacks ultimately determine the fate of a bloom.

Our findings from the motility differences among depth are more difficult to interpret because we were not able to sample all the bloom phases. Since we sampled during the middle of the day, we expected cells at the surface to swim slower than cells at depth because the deep cells are presumably migrating up to the surface. We only found this case in one of our samples, taken during the first maintenance phase of the bloom. In three other sampled dates, we found no detectable differences of motility with depth, potentially demonstrating homogeneously distributed populations. In two samples from 1 and 2 days before a bloom decline phase, respectively, we detected significantly faster cell motility at the surface compared to deeper down. These findings corroborate the hypothesis that some of the *L. polyedrum* populations were unhealthy during the days before the decline phases: unhealthy cells swim more slowly and would not be able to reach the surface during the day (hence they would be sampled at depth). It is worthy of note that another sample taken 2 days before a bloom crash (on July 21), while not statistically significant, also showed a trend of decreased motility with depth (Fig. 4.7d).

Conclusions. In this report, we have demonstrated 1) dinoflagellate motility reduction by dissolved bacterial proteases in the laboratory and 2) decreased dinoflagellate motility at the end of a natural bloom. Although we detected an inverse relationship between bacterial colonization and algal motility in nature, we did not investigate the effect of bacterial attachment on algal motility in the laboratory. Thus, we cannot link our laboratory and field results directly, although bacteria attached to particles are hypothesized to produce dissolved enzymes (Azam & Smith 1991). To

corroborate the hypothesis that dissolved enzymes actively decrease algal motility in nature, future investigations might collect filtrate from field samples and test motility inhibition on laboratory cultures. In addition, a mechanistic understanding of proteolytic motility reduction on dinoflagellates would help elucidate whether this phenomenon occurs in the environment. Regardless of the mechanism causing motility reduction, we suggest that slow motility in dinoflagellates may be a useful marker for general unhealthy status imminent bloom decline.

Table 4.1. Summary of Figure 4.2 data, indicating whether incubation of algal cells in the filtrates strongly inhibited motility (+), had no effect (-), or weakly inhibited motility (+/-) compared to the control.

Strain\treatment	>50kD	<50kD	PMSF	Pefabloc®	E-64
ALC1	+	-	+	+/-	+/-
LPK13	+	+	+/-	+	+
LPK5	+	-	+	-	+

Table 4.2: Results of fluorescent protease activity assays (duplicates) from 0.22 μm filtrates of axenic *L. polyedrum* and *L. polyedrum* incubated with motility-inhibiting bacterial strain ALC1. Values are reported in nmol substrate L^{-1} min^{-1}.

substrate	axenic		ALC1	
Leucine-AMC	54.3	45.4	52.4	35.9
Serine-AMC	194.9	195.1	211.7	243.1
Glycine-AMC	9.42	10.04	11.27	11.26
Threonine-AMC	62.4	79.0	72.9	72.2

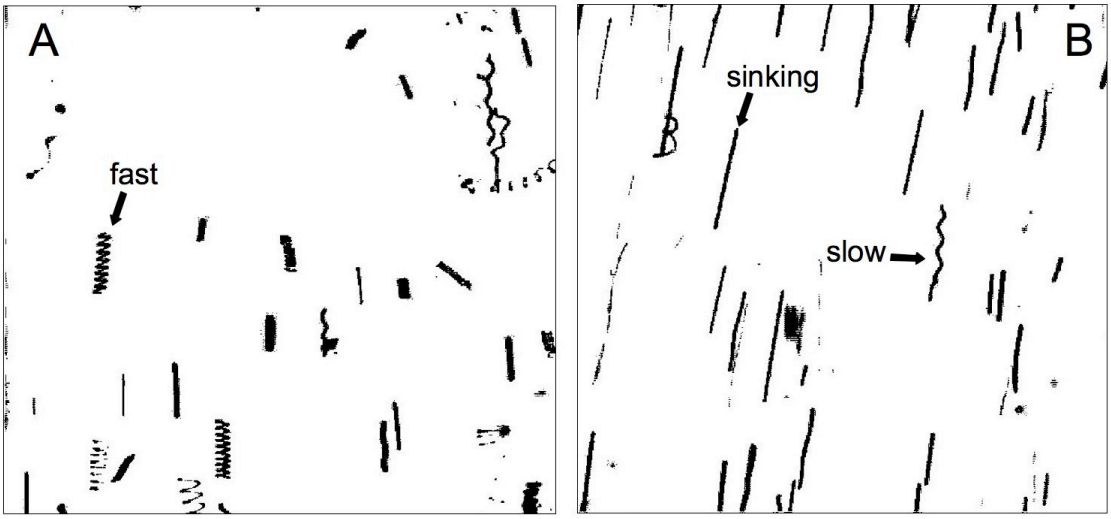

Figure 4.1. Images (5.0 x 5.4 mm) of two-dimensional cell tracks (total time =15 seconds) of axenic *L. polyedrum* (panel a) and *L. polyedrum* incubated in ALC1 filtrate (panel b) recorded while swimming in a cuvette. Tracks were considered fast, slow, and sinking according to criteria described in the text.

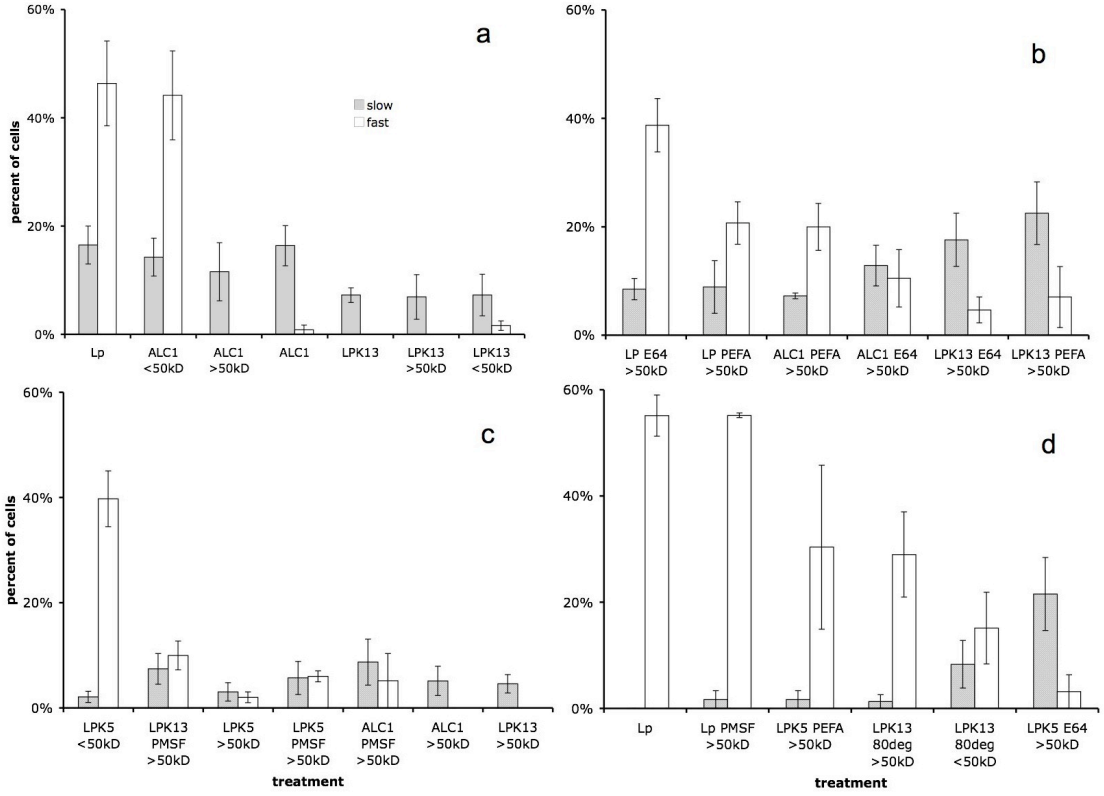

Figure 4.2. Motility (measured by percent of fast and slow cells) measured after image analysis of video-recorded *L. polyedrum* in cuvettes. Algal cells were incubated in filtrates from motility inhibited cocultures subsequently treated with protease inhibitors and size fractionated. Bars indicate means and standard deviations of triplicate incubations. Statistically different treatments are pointed out in the text. Abbreviations: PEFA = Pefabloc®SC, 80deg = 80 degree denaturation

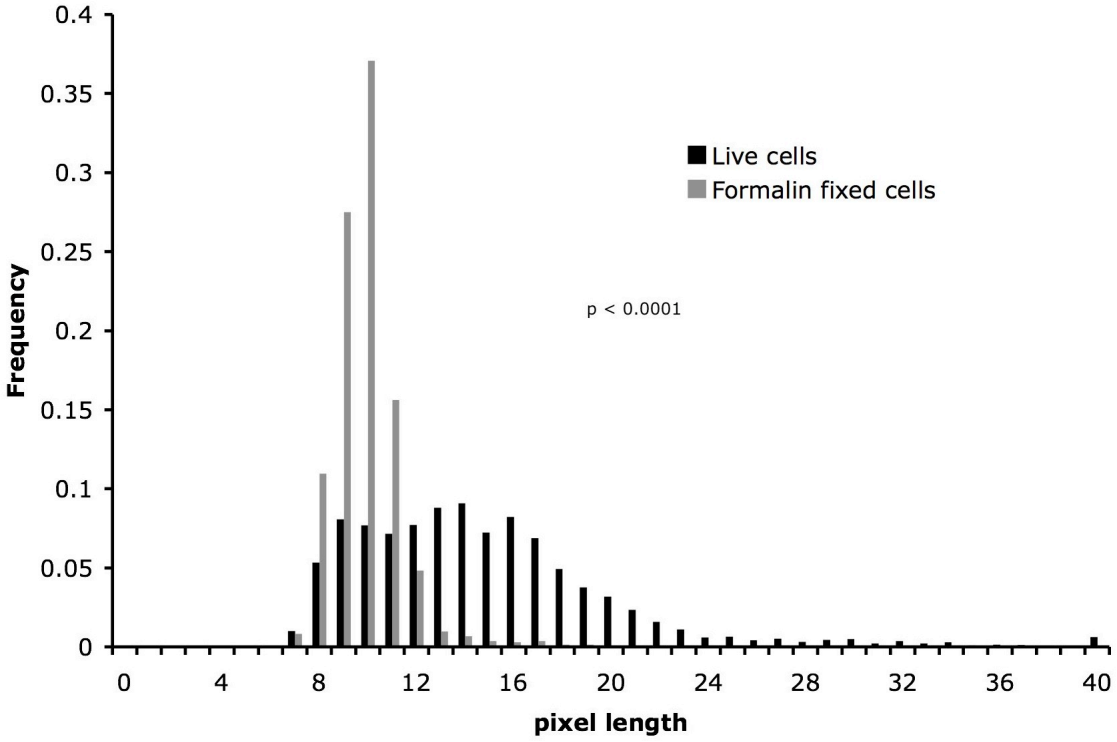

Figure 4.3. Frequency distribution of cell tracks (each for 0.5 sec over the entire 15 sec) from live and formalin-killed *L. polyedrum* cells video recorded inside a cuvette. A nonparametric Wilcoxin test was statistically significant.

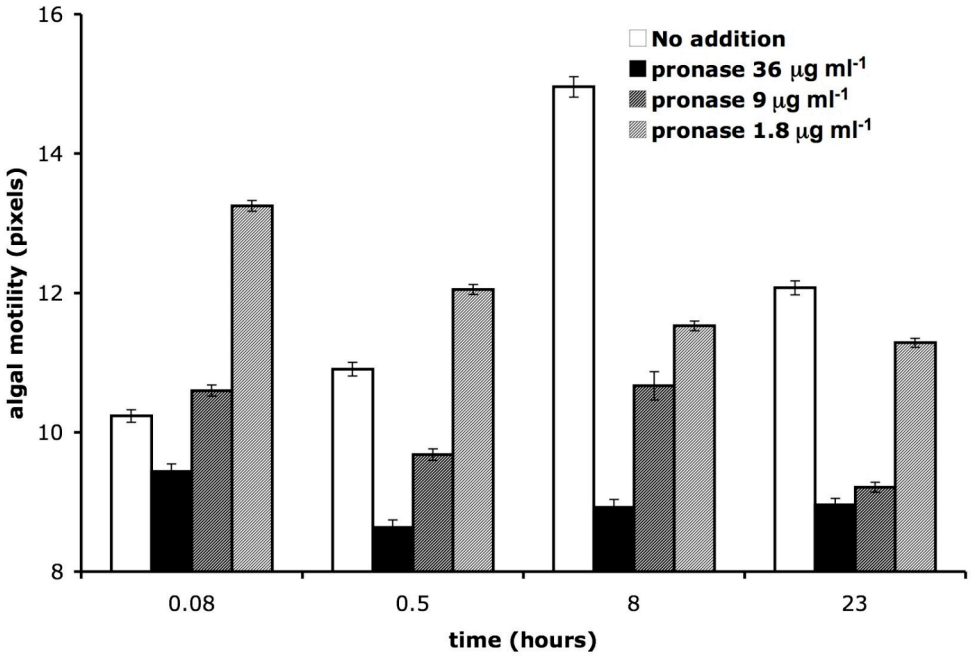

Figure 4.4. Average cell tracks (in pixels per half second) of duplicate treatments of *L. polyedrum* cells incubated with three different concentrations of pronase E, sampled at 4 different times after inoculation. Means and standard errors are reported (average N for each treatment, from 2 duplicate incubations, was about 4,000 measurements). Statistically different treatments are described in the text.

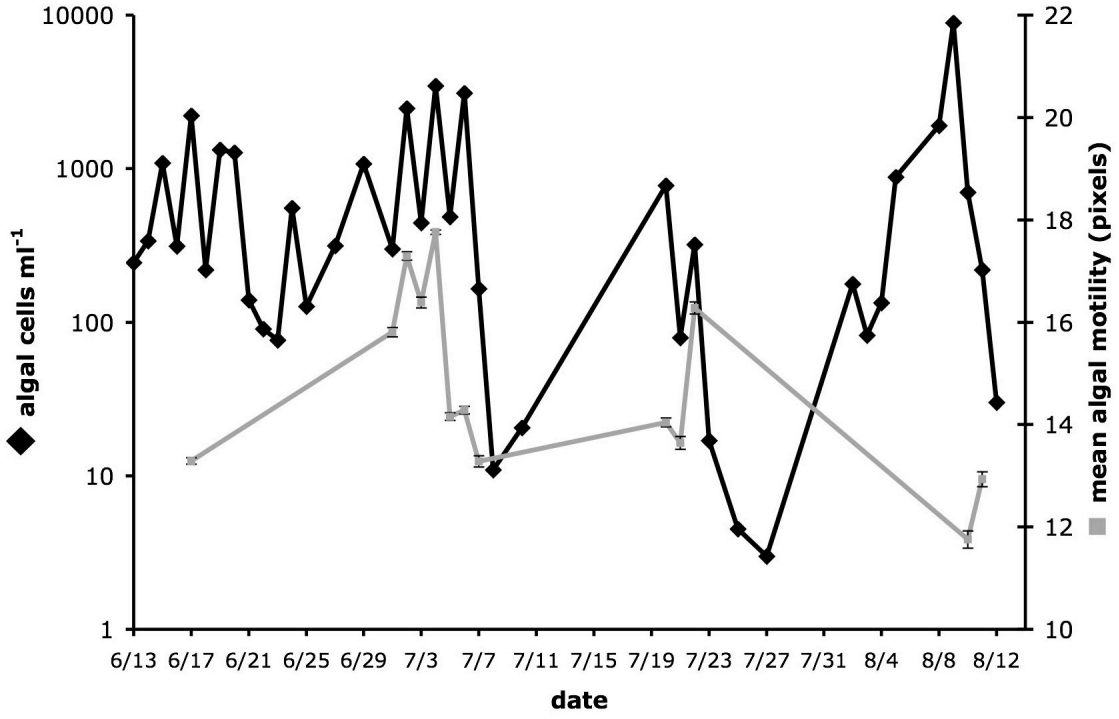

Figure 4.5. Total *L. polyedrum* cells ml^{-1} (black line) and *L. polyedrum* motility (gray line, in pixels per half second) plotted over time during a natural bloom in summer 2005. Motility data are means +/- standard error.

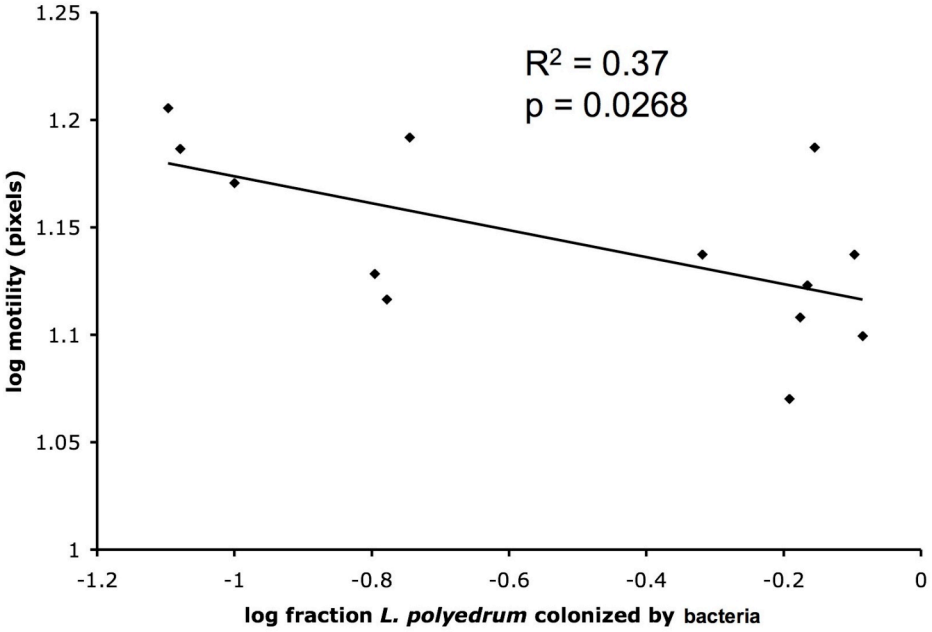

Figure 4.6. Regression analysis between the percentage of *L. polyedrum* cells colonized by bacteria, taken from (Mayali et al. submitted), and motility measured by video analysis from the same samples during a 2005 bloom.

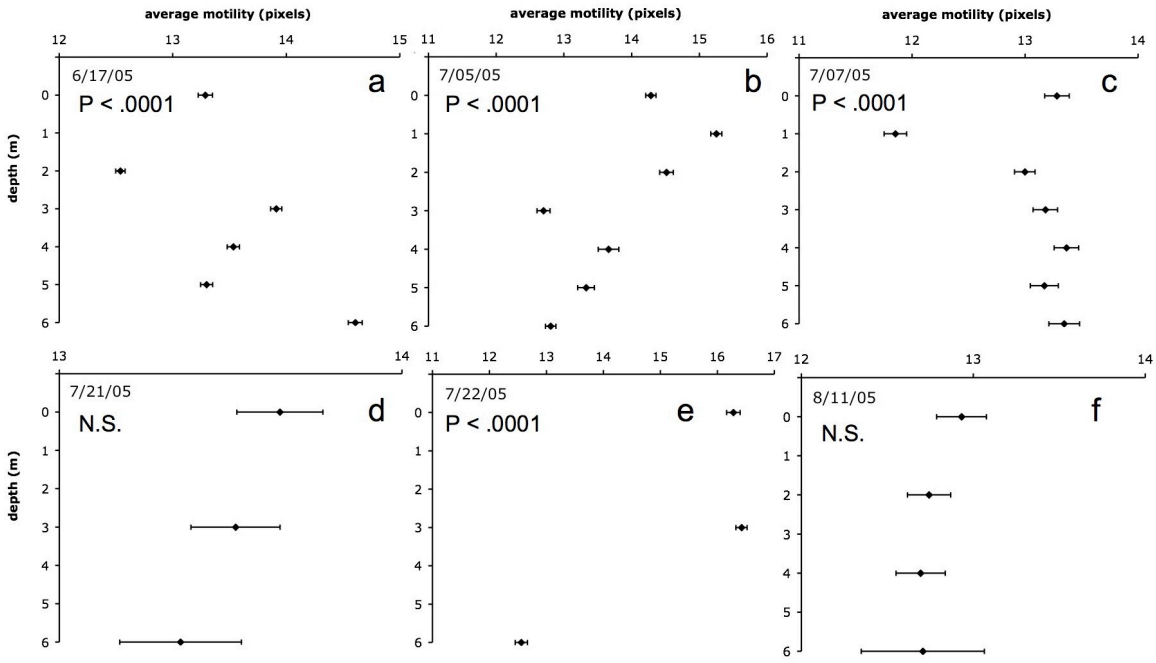

Figure 4.7. Average motility paths measured from six samples collected over different depths during the summer 2005 *L. polyedrum* bloom in La Jolla, CA. Points represent means and standard errors, and significance was tested with Kruskal-Wallis tests. Samples originated from a) the first maintenance phase, b) 2 days before the first crash, c) during the first crash, d) 2 days before the second crash, e) 1 day before the second crash, and f) after the third crash.

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V

First cultivation of algicidal RCA cluster bacteria and their role in algal bloom dynamics

ABSTRACT

Isolation and cultivation is a crucial step in elucidating the physiology, biogeochemistry, and ecosystem role of microorganisms. However, many abundant marine bacteria, including the widespread RCA cluster (Roseobacter-Clade Affiliated) group, cannot be cultured with traditional methods. Using novel techniques of co-cultivation with algal cultures, we have accomplished the first successful isolation of a strain of the RCA cluster. Our experiments revealed that, in addition to requiring algal-excreted organic matter for growth, the bacterium killed the dinoflagellate cultures through attachment to the algal cells. Using a phylotype-specific rRNA probe, we documented increasing attachment of these algicidal bacteria during a dinoflagellate bloom, with a maximum of 70% of the algal cells colonized just prior to bloom termination. Cross-correlation analyses between algal abundances and RCA bacterial attachment were statistically significant, in agreement with predator-prey models suggesting RCA cluster bacteria caused algal bloom decline. Further investigation revealed that RCA cluster bacteria are numerically enriched in algal blooms worldwide. Our findings suggest that RCA cluster bacteria exert a significant control over phytoplankton biomass and community structure in the oceans.

INTRODUCTION

It is now well established that most bacteria cannot grow as colonies on plates (Staley & Konopka 1985). Efforts to culture marine bacteria by dilution to extinction without solid substrates (Connon & Giovannoni 2002) have been quite successful, yielding, for example, the cultivation of the ubiquitous SAR11 group (Rappe et al. 2002). Many other abundant groups however, including the RCA (Roseobacter Clade Affiliated) cluster, are resistant to cultivation with such methods. The RCA cluster, discovered three years ago by Selje et al. (2004), contains closely related (>98.5% similarity by 16S sequence) and abundant bacteria found worldwide in temperate and polar waters. These bacteria have been found attached to particles as well as free-living and can comprise up to 10% of the coastal bacterial community (Selje et al. 2004). The ecosystem role of RCA cluster bacteria remains unknown, due in part to the unavailability of isolates from this group.

Based on the knowledge that most of the organic matter utilized by marine bacteria originates from phytoplankton, we investigated the possibility that novel, non-colony-forming bacteria such as the RCA cluster could be isolated from the surface and grown in the presence of algal cells. This type of algal-bacterial interaction would be expected to be dominant during algal blooms, when algal biomass is elevated, so we focussed our efforts on samples collected from a bloom of the dinoflagellate *Lingulodinium polyedrum*. We further tested the idea that such bacteria can affect the

growth of the algal cells (either positively or negatively), which would clarify their ecosystem role and biogeochemical consequences.

Bacteria that kill phytoplankton have been previously documented (see review by Mayali & Azam 2004), though algicidal activity has not been previously associated with numerically dominant alpha-Proteobacteria (such as the RCA cluster) or bacteria that do not form colonies. Surprisingly, there remains no conclusive evidence that algicidal bacteria kill phytoplankton in nature: all studies on this subject have been performed in the laboratory. The only attempt to directly enumerate algicidal bacteria in nature used a polyclonal antibody with unknown specificity (Imai et al. 2001) and only examined bacteria in the free-living fraction. Many algicidal bacteria require attachment to their host to kill it (Mayali & Azam 2004), and evidence of algicidal bacteria attachment in nature is lacking. Several studies have also indirectly suggested that algicidal bacteria may be involved in algal bloom decline, by utilizing a most-probable-number (MPN) analysis using laboratory algal cultures (Imai et al. 1998). Such studies add diluted seawater bacteria into algal cultures; the highest dilutions causing cell lysis allow indirect back-calculations of the number of putative algicidal bacteria in the original sample. Direct evidence of algicidal bacteria killing phytoplankton in nature requires quantification of infection rates in natural blooms. This has been done for eukaryotic pathogens (reviewed by Park et al. 2004) and algal viruses (reviewed by Brussaard 2004) but never with algicidal bacteria.

Using *L. polyedrum* as a model system, here we report evidence consistent with the following hypotheses: 1) RCA cluster bacteria can be isolated using algal cells and

successfully propagated with additions of algal-derived organic matter, 2) RCA cluster bacteria kill phytoplankton in the laboratory, and 3) the dynamics of RCA cluster attachment to dinoflagellates in nature suggest they are responsible for bloom decline. We also examined previous microbial community structure analyses of algal blooms and suggest that RCA cluster bacteria play a major role in regulating algal bloom dynamics worldwide.

MATERIALS AND METHODS

Culture conditions. Axenic (bacteria-free) *L. polyedrum* CCMP strain 1932 was grown in modified *f*/4 medium (Guillard 1975), with vitamin concentrations increased four fold, at 18 ^{o}C illuminated by cool white fluorescent tubes at 160 μE m^{-2} s^{-1}on a 12/12 light/dark cycle. Surface seawater was collected from Scripps pier, La Jolla, CA, USA during an intense *L. polyedrum* bloom in summer 2005 and immediately processed in the laboratory as described below. All cultures, enrichments, and single cell washes were carried out in borosilicate glass. For bacterial culture isolation, we incubated the *L. polyedrum* culture with the bacterial fraction (<0.6 μm) of seawater collected during the bloom. After allowing bacteria to colonize the algae for several days, single dinoflagellate cells were collected with a pipette and washed several times in sterilized seawater (to remove unattached bacteria) before being added to new *L. polyedrum*

cultures. Such enrichments were analyzed with bacteria-specific PCR-denaturing gradient gel electrophoresis (DGGE, Muyzer et al. 1993) to check for single bands indicating potentially pure bacterial isolates. Algal culture growth was monitored with a TD700 fluorometer (Turner Designs, CA, USA). Growth of bacterial isolates in various liquid media was monitored with a FACSort flow cytometer (BD Bioscience, NJ, USA) after staining cells with SYBRgreen II (Lebaron et al. 1998) (Invitrogen, CA, USA).

16S sequencing and phylogenetic analysis. One microliter enrichment samples were incubated with LYSE-N-GO (Pierce Biotechnology) and amplified directly by PCR with primers 27F and 1492R (Giovannoni 1991). PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany) and sequenced with internal primers on an ABI Megabase (Applied Biosystems Inc., CA, USA). The sequences were imported into the most current ARB database (Ludwig et al. 2004), aligned using the island hopping algorithm, checked manually, and added to the global tree using parsimony. Seventeen of the most closely related sequences (and two outgroups) were imported into PAUP*4.10b (Swofford 2002) and analyzed using maximum likelihood. Model parameters were optimized from the data using modeltest (Posada & Crandall 1998), and 100 bootstrap replicate heuristic searches were performed. The RCA cluster isolate 16S sequence has been deposited in Genbank under accession number EF661583.

Field sampling, probe design and enumeration of RCA cluster. Samples from Scripps pier were collected as above and immediately fixed in 2% final volume unbuffered formaldehyde. Within 24 hours, samples were filtered through polycarbonate

membranes and frozen at -20°C. For quantification of bacteria attached to *L. polyedrum* cells, water samples from the *L. polyedrum* bloom were collected, immediately fixed, and allowed to gravity settle overnight at 4 °C. *L. polyedrum* cells were washed in sterile PBS buffer and stored in 50% ethanol at -20°C until hybridization.

Quantification of RCA cluster bacteria was accomplished using CARD-FISH (catalysed reported deposition fluorescent in situ hybridization, Pernthaler et al. 2002) with a 16S rRNA probe. While previously published RCA cluster specific probe RCA-825 (Selje et al. 2004) hybridized successfully against our isolate, it also exhibited a positive signal against *Roseobacter* strain Y3F (GB accession # AF253467) which has a one base pair difference at the probe binding site and is not a member of the RCA cluster. Increasing the hybridization stringency was not successful in removing the positive signal in strain Y3F without also removing it in strain LE17. Thus, we designed another RCA specific probe, LE17-998 5' TCTCTGGTAGTAGCACAG with helper probes RCA-980H 5' GATGTCAAGGGTTGGTAA and RCA-1016H 5' CCCGAAGGGAACGTACCA. Using *Roseobacter* strain DG1128 (GB accession # AY258100), which has two base pair differences at the probe-binding site, we optimized hybridization conditions (35% formamide at 35°C hybridization temperature) to remove positive signal in strain DG1128 while retaining signal in strain LE17. We used the hybridization protocol for CARD-FISH from Pernthaler et al. (2002). Negative (no probe) and positive (LE17) controls were always performed concurrently with all hybridizations. Due to the high background fluorescence of chlorophyll, samples of probed bacteria attached to chlorophyll-containing particle were visualized with a dual

FITC-Texas Red® filter (part # 51006, Chroma Technology, VT, USA) on a widefield epifluorescence Olympus BX-51 microscope. To enumerate bacteria attached to *L. polyedrum* cells, individual algal cells were scanned manually in the z direction for the presence of probe-positive cells and those cells were counted. A total of 2179 algal cells from 22 field samples were examined. Our counts were most probably underestimates due to the inability to detect all probe-positive bacterial cells behind the autofluorescent algal cells. Total RCA cluster bacteria during the 2005 bloom were enumerated using probe RCA-825, while RCA cluster attached to *L. polyedrum* were enumerated using probe LE17-998. For cross-correlation analysis, all data were log-transformed to satisfy the assumptions of normality. Due to uneven temporal sampling, bacterial data were manually lagged in both directions (0-3 days) relative to algal abundance data and correlation analyses for each lag were performed with the statistical software JMP v.5. We examined three indices of RCA cluster abundance: 1) *total* cells, 2) *colonization frequency*, defined as the percentage of *L. polyedrum* cells colonized by at least one RCA cluster bacterium, and 3) *colonization intensity*, defined as the mean number of RCA cluster bacteria colonizing the algal cells. It is important to note that the latter index did not include algal cells with no detected RCA cluster colonizers, making it independent from the colonization frequency index.

RESULTS AND DISCUSSION

Incubation of seawater bacteria (0.6 μm filtrate) with *L. polyedrum* cultures followed by single algal cell micromanipulation into new algal cultures was successful in enriching for bacteria capable of attachment to algal cells. Several such enrichments displayed single bands (phylotypes) after DGGE (Muyzer et al. 1993) and were considered monospecific bacterial cultures (not shown). After sequencing the full 16S rRNA gene, one of these isolates (strain LE17) was revealed to be a member of the RCA cluster (99.9% sequence identity to clone NAC11-3; Fig. 5.1). Although a close relative (HTCC2150, 95.5% 16S sequence similarity) has been isolated and its genome partially sequenced (Genbank accession number NZ_AAXZ0000000), LE17 is the first successfully isolated and serially transferred RCA cluster bacterium, and remains in culture two years after isolation.

We investigated the growth characteristics of strain LE17 with various types of organic matter additions. LE17 grew to moderate abundances (3×10^7 cells ml^{-1}) in co-culture with *L. polyedrum* or in the presence of its filtrate. Incubations in diluted bacterial media (0.05 gpeptone and 0.01 g yeast extract in 1 L autoclaved seawater) resulted in slower growth; those in non-bloom seawater amended with inorganic nutrients showed no measurable growth (Fig. 5.2). LE17 never formed colonies on solid media (identical to the liquid media with 1.5% agar) yet required additions of organic matter to grow. This is contrary to the current paradigm that discriminates oligotrophic bacteria unable to form

colonies from copiotrophic bacteria that can (Simu et al. 2005). In addition, our finding that strain LE17 is unable to grow in non-bloom seawater explains the failure of previous attempts to successfully serially transfer RCA cluster isolates obtained by dilution-to-extinction in unamended seawater: these bacteria require the presence of other organisms or their excreted organic matter to grow.

To investigate the ecology of strain LE17, we chose a CARD-FISH ((Pernthaler et al. 2002) approach that enabled direct visualization of cells detected with a phylotype-specific 16S rRNA probe. Using probe RCA-998, we quantified abundances and micro-scale localization of these bacteria in monthly samples taken from the Scripps pier. Although they were detected in all samples, RCA cluster abundances ranged from 100 to 16,000 cells ml^{-1}, with the two highest abundances occurring during two dinoflagellate blooms (Fig. 5.3). These data complement the laboratory finding that LE17 grows well in the presence of dinoflagellate-derived organic matter but not in non-bloom seawater. Further, we documented RCA-cluster probe-positive cells attached to detritus and also to diatom and flagellate cells (including dinoflagellates), demonstrating that the surface of live phytoplankton can be a niche for these bacteria. This is consistent with our method of isolation using micro-manipulated single dinoflagellate cells to remove algal-attached bacteria from bacterial "contaminants" in the free-living phase.

Although RCA cluster bacteria were not always numerically dominant at our sampling location (particularly during non-bloom conditions), their *in situ* activity might be quite significant. Indeed, a recent study using a novel bromodeoxyuridine incorporation method to identify fast-growing uncultured bacteria (Hamasaki et al. 2007)

found that the RCA-cluster was part of the actively-growing microbial community off the coast of Japan, while some abundant bacteria, such as SAR11, were not. It has been shown that fast-growing bacteria are more susceptible to predation than slow-growing ones (Sherr et al. 1992), and while their total abundances may be kept relatively low, their per cell activity and biogeochemical role can be significant (Hamasaki et al. 2007).

Since RCA cluster strain LE17 required the presence of *L. polyedrum* (and presumably other algae) for growth, we tested whether algal growth was influenced by the bacteria to determine if strain LE17 maintains a mutualistic, parasitic, or commensal relationship with the algae. Axenic *L. polyedrum* cultures incubated with RCA cluster strain LE17 exhibited a dramatic and repeatable decrease in fluorescence compared to no-addition controls (Fig. 5.4, top panel), leading us to describe strain LE17 as parasitic to algae (algicidal). Algicidal activity required contact and attachment in laboratory cultures of *L. polyedrum*, with an average of 4 bacterial cells attached to each algal cell (Fig. 5.4, bottom panel). Cell-free filtrates never displayed algicidal activity (not shown).

In order to examine the population dynamics of RCA cluster bacteria during the summer 2005 *L. polyedrum* bloom, we first quantified total free-living counts with probe RCA-825 (before the realization that some cross-hybridization occurred with this probe). Number of RCA cluster bacteria ranged from 3×10^3 to 5×10^5 cells ml^{-1} (Figure 5.5). Cross-correlations analyses between free-living RCA cluster and *L. polyedrum* abundances were not statistically significant, indicating no temporal relationship between the two.

Based on the knowledge that RCA cluster strain LE17 required attachment to kill *L. polyedrum* in the laboratory, we proceeded to test the hypothesis that attachment of RCA cluster bacteria to *L. polyedrum* cells caused the termination of the bloom. In a Lotka-Volterra-type predator-prey system (here the bacteria are the predators and *L. polyedrum* the prey), peaks in the predator populations follow peaks in the prey. These peaks in predators cause subsequent decreases in the prey population, followed by decreases in the predator. Thus increases in LE17 just after peaks in *L. polyedrum*, and subsequent decreases in both species would be consistent with our hypothesis that LE17 is a factor in bloom termination. The *L. polyedrum* bloom we sampled consisted of two peaks in abundance separated by more than a month, interspersed with a period of low cell concentrations. Both *colonization frequency* (the percentage of *L. polyedrum* cells colonized by RCA cluster; Fig. 5.6) and the *colonization intensity* (measured by the average number of RCA cluster bacteria on each colonized algal cell; Fig. 5.7) increased with *L. polyedrum* abundances, in magnitudes similar to what we saw in laboratory cultures. These colonization intensities are consistent with previous work that showed that as few as two or three bacteria attached to a large cyanobacterial cell can affect its physiology (Paerl & Gallucci 1985). The most intense colonization occurred at the end of the second stage of the bloom, with 51-72% of the algal cells colonized by RCA cluster bacteria (Fig. 5.6). Cross-correlation of *L. polyedrum* abundances and RCA bacteria *colonization frequency* showed a significant positive correlation with a negative 2-day lag (dinoflagellate peaks leading the bacterial peaks), implying a response in bacterial colonization to high algal numbers. Conversely, the cross-correlation analysis of *L.*

polyedrum abundances and RCA cluster *colonization intensity* was statistically significant (and negative) with a positive 2-day lag (bacteria peak first). This implies that peaks in RCA cluster colonization intensities preceded crashes in *L. polyedrum* abundances, consistent with predator-prey or infection dynamics. These data strongly support our hypothesis that RCA cluster bacteria killed *L. polyedrum* cells during the summer 2005 bloom in La Jolla.

If RCA cluster bacteria kill *L. polyedrum* in the Southern California Bight, it is possible that they are killing phytoplankton in other ecosystems as well. We searched Genbank for previous studies that examined microbial diversity in marine phytoplankton blooms, both natural and mesocosm-induced. Over seventy percent (11/15) of these studies reported a sequence >99.5% similar to that of LE17 (Table 5.1), including blooms of dinoflagellates (Fandino et al. 2001, Rooney-Varga et al. 2005, Sala et al. 2005), prymnesiophytes (Gonzalez et al. 2000, Brussaard et al. 2005), a raphidophyte, diatoms (Riemann et al. 2000, Morris et al. 2006), and undescribed phytoplankton (Pinhassi et al. 2004, Sapp et al. 2007b). Although three of the sequences were relatively short (~150 bp), they spanned variable regions of the 16S rRNA, were 100% identical to the LE17 sequence, and failed to match with any other organisms in the database. Thus, we are confident that these short sequences represent the same 16S phylotype as LE17. Unfortunately, the majority of these studies did not include a temporal component and it is unknown whether RCA cluster colonization of algal cells increased at the end of the blooms. One exception is a study of a *L. polyedrum* bloom from 1997 in the same location as our study (Fandino et al. 2001), in which the RCA cluster sequence (clone

ATT9) was detected in the attached fraction at the end of the bloom and subsequently disappeared post-bloom. These findings indicate that RCA cluster bacteria are numerically enriched during algal blooms in general and potentially play a role in bloom dynamics worldwide through direct mortality. Isolation of additional RCA cluster bacteria causing mortality of a range of phytoplankton taxa will be crucial toward validating this hypothesis.

While episodic phytoplankton blooms exemplify ocean conditions dominated by high primary production, eutrophic marine waters contain persistently high phytoplankton biomass. Coastal waters, in particular, are characterized by relatively high concentrations of phytoplankton. Interestingly, 16S sequences >98.8% similar to that of isolate LE17 have been found in 36 previous molecular diversity studies of temperate coastal marine waters (Table 5.2), including the recent Global Ocean Sampling metagenomic analysis (Rusch et al. 2007). This finding suggests that the success of the RCA cluster transcends episodic phytoplankton blooms; it may become dominant under any eutrophic condition, perhaps making it the most successful marine copiotrophic bacterium in the oceans. In addition, the widespread distribution of the RCA cluster raises the possibility that these bacteria are also killing phytoplankton under non-bloom conditions, a hypothesis that remains to be tested.

Based on its widespread occurrence, its obligate requirement for algal derived organic matter, and its relatively fast growth in algal cultures, we might expect RCA cluster bacteria to be found among the bacterial flora of laboratory phytoplankton cultures derived from single algal cell isolates. Such algal cultures most often contain

bacteria and in fact, many algal taxa cannot grow without bacteria (Kodama et al. 2006). Sixty-five algal cultures from temperate oceans have been analysed by culture-independent methods (Table 5.3), and no RCA cluster sequences have been found. This finding again corroborates the hypothesis that RCA cluster bacteria kill phytoplankton cells, as single algal cells isolated in the presence of RCA cluster bacteria would not survive long enough to culture.

The first successful cultivation of a member of the RCA cluster and its recognition as an algicidal bacterium are noteworthy for several reasons. First, it demonstrates the usefulness of co-cultivation with other organism(s) to isolate bacteria that will not grow on their own. The discovery that a common marine bacterium that requires organic matter additions but cannot form colonies on solid media is also important in light of the continual search to explain "the Great Plate Count Anomaly" (Staley & Konopka 1985). While most efforts have focused on decreasing organic matter and using non-solid media (Connon & Giovannoni 2002), here we have shown that organic matter enrichments to single bacterial cells may be a fruitful approach to isolating previously uncultured organisms. Second, the isolation of a strain of the RCA cluster will allow future biochemical, physiological, and genomic analyses on one of the most common bacterial phylotypes in the oceans. Third, it lends support to the hypothesis that bacterial killing of phytoplankton cells is not an artefact of laboratory incubations and that this phenomenon is an important mechanism shaping phytoplankton community structure in aquatic environments.

Table 5.1: Characteristics of RCA cluster sequences from phytoplankton blooms

% similar to LE17	Source	Clone name	GB acc. #	Reference	# bp
99.5	*Chattonella* bloom, coastal Japan	N7	AB254272	unpublished	151
100	*L. polyedrum* bloom, California coast (USA)	ATT9[2]	AF125336	(9)	164
100	Mesocosm bloom, California (USA)	MBE14	AF191765	(45)	169
99.9	*Emiliania huxleyii* bloom, Georgia (USA)	NAC11-3	AF245632	(15)	1414
100	phytoplankton microcosm, Spain	ST-11	AY573528	(40)	492
100	Mesocosm *Phaeocystis* bloom, North Sea	band_4[1]	AY672827	(5)	505
100	Dinoflagellate bloom, Catalan coast (Spain)	BH7	DQ008454	(49)	512
100	spring phytoplankton bloom, Wadden Sea	GWS-a3-FL[1]	DQ080937	unpublished	498
100	spring phytoplankton bloom (North Sea)	F089[1]	DQ289544	(51)	562
99.9	diatom bloom, Oregon Coast (USA)	NH10_24	DQ372848	(34)	1426
99.6	Bay of Fundy *Alexandrium* bloom	AFB-2[1]	AY353557	(47)	475

[1]representative clone of several RCA cluster clones

[2]clone collected from the attached fraction

Table 5.2: Characteristics of RCA cluster sequences found in non-bloom temperate

% similar	Source	Clone name	GB acc. #	# bp	Ref.[1]
100	saline lake, Japan	1w	AB154432	524	(Koizumi et al. 2004)
100	Japan coast	HB02-8b	AB265989	169	(Hamasaki et al. 2007)
100	coast of Georgia, USA	GAI-36	AF007259	400	(Gonzalez & Moran 1997)
100	Long Island Sound, USA	pC2-12	AF055225	298	(Fuhrman & Overney 1998)
100	Coastal North Sea	OTU_E	AF207853	534	(Winter et al. 2001)
99	Coastal Georgia, USA	EC-I1	AF287022	392	(Covert & Moran 2001)
100	Weser estuary, Germany	WM11-37[2]	AF497861	523	(Selje & Simon 2003)
100	English Channel	DGGE band-5	AJ242822	515	(Fuchs et al. 2000)
99	Mediterranean	S1-090-F-C-Nr1	AJ508432	523	unpublished
99.5	North Sea, Germany	UNHYB_26[2]	AJ630678	450	(Sekar et al. 2004)
100	Oregon coast	HTCC152	AY102029	647	(Connon & Giovannoni
99.7	marine sediment	s29	AY171302	1018	(Kaeberlein et al. 2002)
100	coast of Skagerrak Sea	SKA55	AY317122	429	(Simu & Hagstrom 2004)
99.3	Black Sea oxycline	BSBd6-20/40m[2]	AY360519	829	(Vetriani et al. 2003)
98.8	Estuary, Portugal	RAN-63	AY499446	592	(Henriques et al. 2004)
99.1	North Sea, U.K.	PEL-52	AY550815	550	(Franklin et al. 2005)
100	estuary, MA, USA	PI_RT343[2]	AY580461	816	(Acinas et al. 2004)
100	coastal salt pond, USA	SP_C23	AY589480	783	(Simmons et al. 2004)
99.8	estuary, Oregon, USA	LS-F4	AY628657	608	(Bernhard et al. 2005)
100	Wadden sea, Germany	Flo-37[2]	AY684343	465	(Grossart et al. 2005)
100	salt marsh, Georgia, USA	SIMO-662[2]	AY712199	451	unpublished
100	coast of Scotland	FFW402	AY828410	719	unpublished
99.5	fjord, Norway	LUR12	AY960287	570	(Riemann et al. 2006)
99.7	California coast, USA	SPOTSAPR01_5m124[2]	DQ009296	1400	(Brown et al. 2005)
99.9	Eutrophic bay, WA, USA	PB1.27[2]	DQ071074	1386	(Lau & Armbrust 2006)
99.7	NE Pacific, 2500m depth	CTD005-37B-02	DQ513055	1427	(Huber et al. 2006)
99.2	Coast of Chile	Chili1-G4[2]	DQ669616	404	(Pommier et al. 2007)
99.6	O_2 min., Chilean coast	ESP10-K27II-52	DQ810325	1301	unpublished
99.6	North Sea, Denmark	NS5	DQ839250	539	unpublished
99.9	Coast of China	PV2-27	EF215774	918	unpublished
100	North Atlantic, 1700m	001733_3285_1268[2]	n/a	60	(Sogin et al. 2006)
99.5	Coast, NC, USA	OM65[2]	U70682	643	(Rappe et al. 1997)
99.6	Northwest Mediterranean	T41_191	DQ436604	751	unpublished
100	Plum island sound, USA	PIdgge30	AY308694	132	(Crump et al. 2004)
100	Eastern USA coast	various	n/a	1470	(Rusch et al. 2007)
99.6	Coastal Mediterranean	isolate a	EF018061	498	unpublished

[1]unpublished indicates that sequences found in Genbank are no yet associated with a publication.

[2]representative sequence among several RCA cluster clones

Table 5.3: 16S diversity studies of uncultured bacteria in 65 phytoplankton cultures from temperate environments; no RCA cluster sequences have been detected

Phytoplankton	# of cultures	Location	Ref.
diatoms	6	NY, USA and unknown locations	(Schäfer et al. 2002)
Flagellates and diatoms	8	Various locations in Europe	(Nicolas et al. 2004)
Dinoflagellate *H. circularisquama*	5	Coastal Japan	(Maki et al. 2004)
Diatom *P. multiseries*	2	Coastal Canada	(Kacsmarska et al. 2005)
Dinoflagellate *Alexandrium* spp. and various taxa	23	Gulf of Maine, USA (mostly)	(Jasti et al. 2005)
Dinoflagellates	4	U.K. and Canada	(Hold et al. 2001)
Dinoflagellates	2	Spain	(Sala et al. 2005)
Diatoms and dinoflagellates	7	North Sea	(Sapp et al. 2007a)
Dinoflagellate *G. catenatum*	8	worldwide	Genbank only

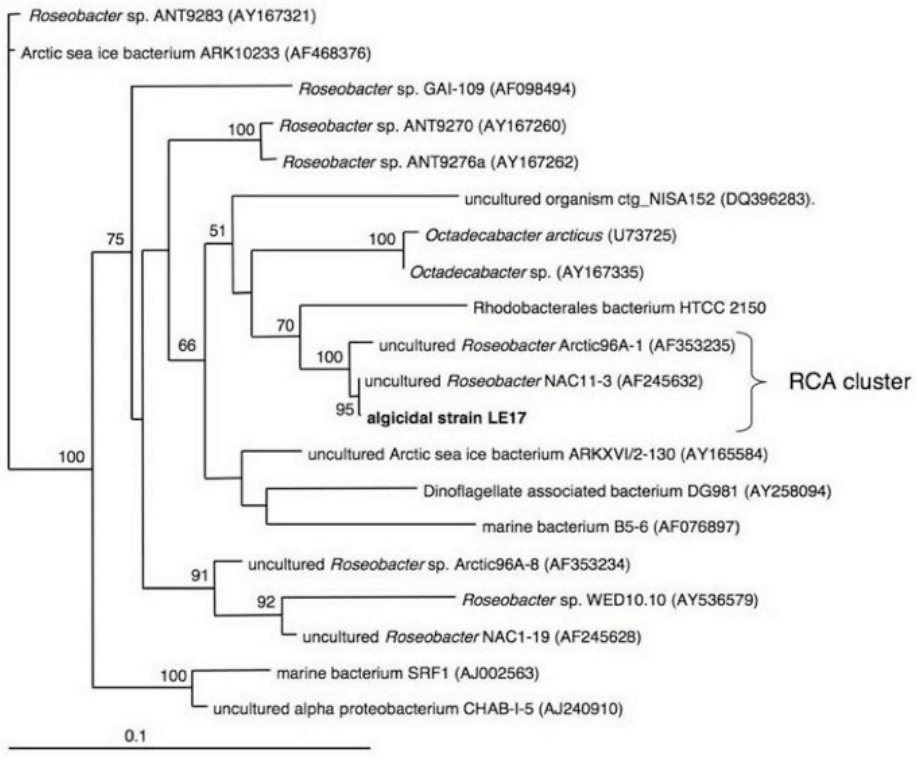

Figure 5.1. Maximum likelihood phylogenetic analysis of LE17 16S sequence within closely related *Roseobacter* sequences from the family Rhodobacteriaceae (GB accession numbers in parentheses). Numbers at nodes indicate full heuristic search ML bootstrap values (100 replicates). Scale bar indicates % sequence divergence. Model of molecular evolution (GTR+G+I) was chosen using MODELTEST(Posada & Crandall 1998).

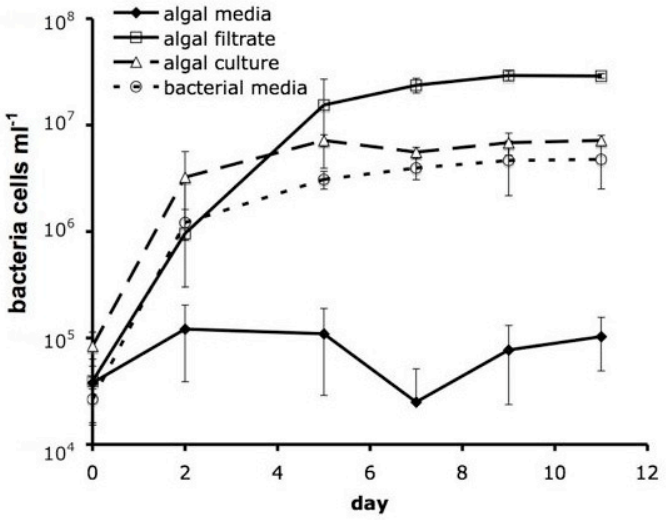

Figure 5.2. Dynamics of RCA cluster in the laboratory. Abundance of RCA cluster strain LE17 over time in seawater with inorganic nutrient (*f*/4 algal medium), in *L. polyedrum* stationary culture filtrate, in log phase *L. polyedrum* whole culture, and in 100X diluted ZoBell bacterial medium (seawater with .05 g peptone and 5 mg yeast extract L^{-1}) measured with flow cytometry. Error bars represent standard deviations of triplicate incubations.

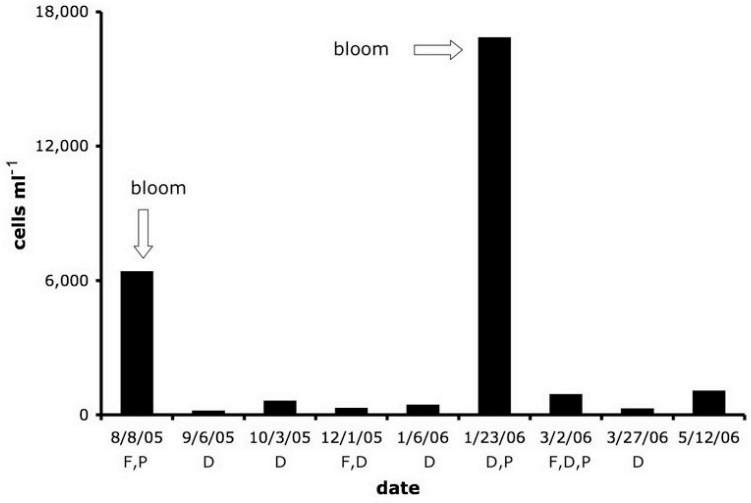

Figure 5.3. Dynamics of RCA cluster in nature. RCA cluster total cells ml^{-1} over the course of ten months at Scripps pier, CA, USA counted using probe RCA-998 and CARD-FISH. Letters below the dates indicate that some probe-positive cells were found attached to: F = flagellates, D = diatoms, P = detritus particles. Two arrows indicate dinoflagellate bloom samples.

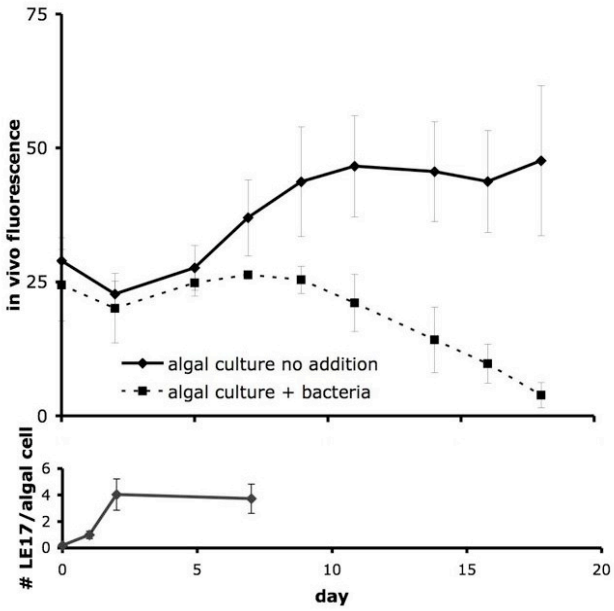

Figure 5.4**.** Algicidal activity and attachment of RCA cluster to *L. polyedrum*. Abundance of *L. polyedrum* cells over time monitored by *in vivo* fluorescence with and without RCA cluster strain LE17 +/- standard error of triplicate incubations (upper panel); colonization intensity quantified as number of RCA cluster bacteria per algal cell +/- 95% confidence intervals measured with CARD-FISH (lower panel).

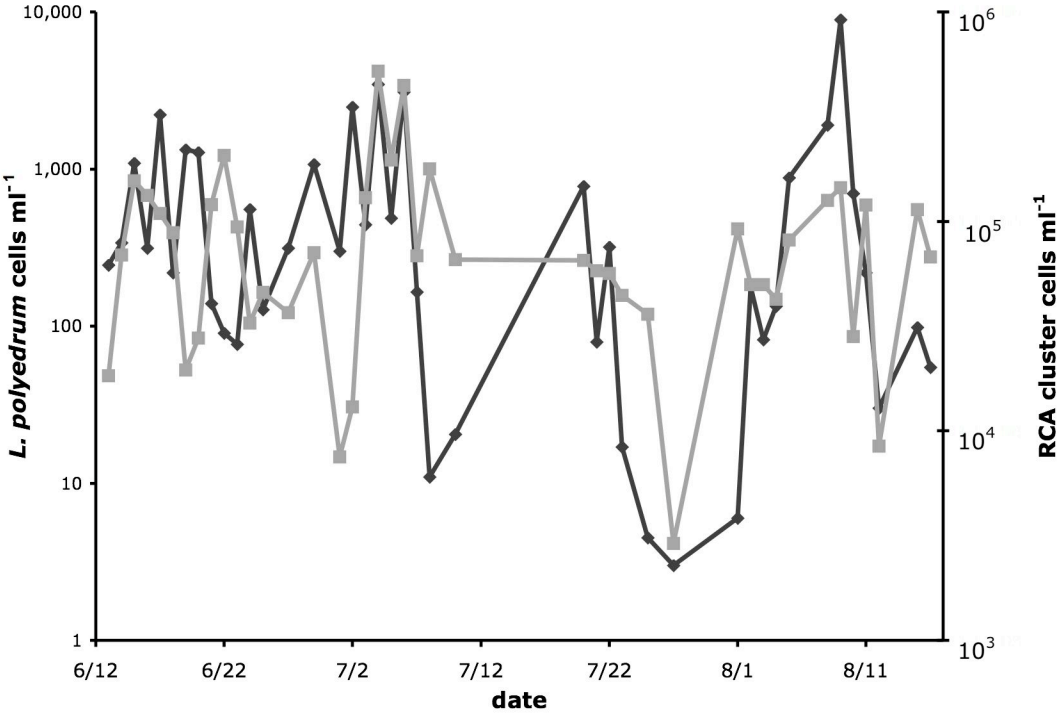

Figure 5.5. Population dynamics of *L. polyedrum* (black line) and total RCA cluster bacteria (gray line) during the summer 2005 bloom at Scripps pier, La Jolla, CA. Cross-correlation analyses were not statistically significant.

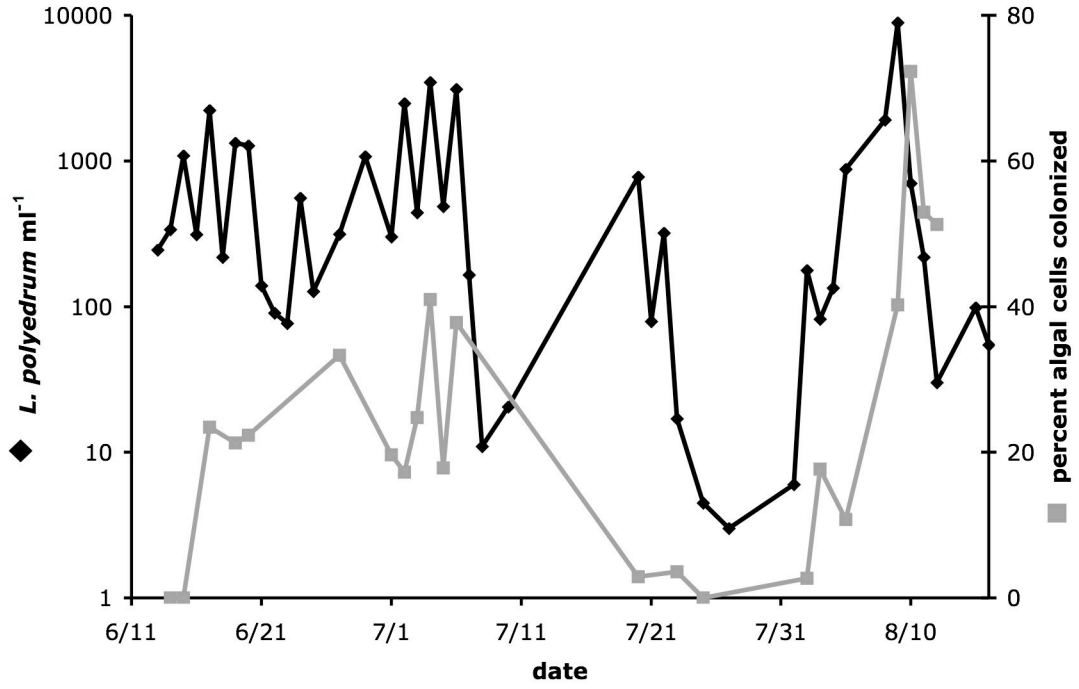

Figure 5.6. Temporal dynamics of *L. polyedrum* abundances (black line) and RCA cluster bacterial attachment frequency (gray line) during the summer 2005 bloom. Cross-correlation analyses were statistically significant (see text).

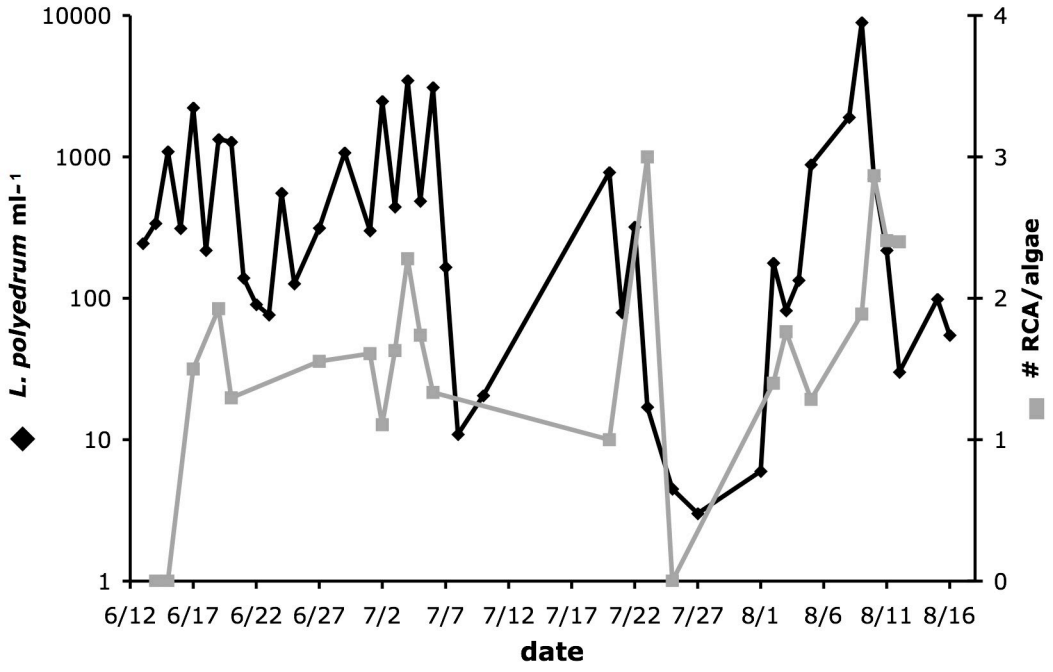

Figure 5.7. Dynamics of *L. polyedrum* abundances (black line) and RCA cluster attachment intensity (gray line) during the summer 2005 bloom. Cross-correlations analyses were statistically significant (see text).

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VI

Conclusion

Bacteria and phytoplankton dominate the biomass of the oceans (Sogin et al. 2006), and their interactions with one another are crucial to structuring marine ecosystems and controlling carbon flux (Azam 1998). This dissertation has focused on negative interactions of bacteria on one species of phytoplankton, the dinoflagellate *Lingulodinium polyedrum*, combining laboratory experiments and field data. In chapter 2, I reported that *L. polyedrum* forms temporary cysts through ecdysis when in the presence of algicidal bacteria and that this process is enhanced in the absence of light. Ecdysis involves shedding the cellulose theca and the plasma membrane and results in the removal of attached bacteria. In chapter 3, I documented a statistically significant correlation between *L. polyedrum* abundances and total bacterial counts during a bloom, as well as generally low and variable bacterial colonization of *L. polyedrum* by bacteria. Bacterial colonization increased at the end of the bloom, suggesting that either: 1) bacteria can sense that algal cells are unhealthy before the bloom decline and are able to colonize them, or 2) bacteria colonize the algal cells, leading to bloom decline directly. In chapter 4, I described a novel effect of motility reduction by algicidal bacteria on dinoflagellates through the release of putative proteases. I also documented slower dinoflagellate motility at the end of the bloom and detected a statistically significant inverse correlation between algal motility and bacterial colonization. This is further evidence to support the idea that bacteria either play a direct role in bloom termination or quickly react to the presence of unhealthy algal cells. In chapter 5, we documented the isolation of a well-known but previously uncultivated bacterium from the *Roseobacter* clade that killed *L. polyedrum* cultures through attachment. Species-specific ecology

using FISH probes revealed increased attachment of this bacterium to *L. polyedrum* cells at the end of the bloom (up to 70% of the algal cells colonized), suggesting a direct bloom termination mechanism. The global distribution of this *Roseobacter* phylotype, combined with evidence of high abundances during previously sampled algal blooms worldwide, suggest that bacteria of this type are a major force in controlling bloom dynamics in marine systems.

The data presented in this dissertation failed to reject the null hypothesis that bacteria play a passive role in their interactions with phytoplankton. While it is simple to conclude that bacteria directly control algal bloom dynamics though pathogenesis, several alternative hypotheses will need to be rejected in the future. It is entirely possible that the effects of algicidal bacteria on *L. polyedrum* batch cultures were laboratory artifacts. In addition, increased attachment of algicidal bacteria to algal cells at the end of the bloom may simply be a response to algal cells becoming unhealthy due to another factor. Algicidal bacteria may simply be secondary pathogens that kill off a bloom that was already going to end. Several future lines of inquiries would provide further evidence that bacteria can directly control algal blooms. First, for a given bloom, the possibility that other biological entities (viruses, fungi, protist pathogens or grazers) play a role in the bloom termination has to be rejected. Second, an understanding of the molecular mechanism of bacterial killing in the laboratory would be valuable to provide a marker for bacteria killing algae in nature. Third, studying the interaction between algicidal bacteria, their algal host, and environmental factors such as nutrients, light, and dissolved organic carbon concentrations will be critical to place this problem in an environmental

context. There are many biological and physical forces that work simultaneously to determine the fate of phytoplankton cells and thus the dynamics of carbon in the oceans, and only with a holistic perspective will this fascinating question be unraveled.

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